MICROBIAL DEODOURISATION AND NEUTRALISATION OF PIT LATRINE ODOUR CAUSING COMPOUNDS

JOHN BRIGHT JOSEPH NJALAM'MANO

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ABSTRACT

Title: Microbial Deodourisation and Neutralisation of Pit Latrine Odour Causing Compounds

Candidate : John Bright Joseph Njalam'mano

Supervisor : Professor Evans M. N. Chirwa

Department : Chemical Engineering

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In this study, the degradation of one of the identified odour causing compounds from pit latrine feacal sludge, butyric acid, was determined to follow modified logistic, Gompertz and Richards models. The results revealed that the modified logistic model could be applied to adequately describe and predict *Bacillus cereus* and *Serattia.marcessens* growth under isothermal conditions from 25 to 45 ^{o}C. The maximum growth rates (μ_{max}) and lag times (λ) derived from the modified logistic model were fitted to Ratkowsky model and inverse Ratkowsky model to determine the effect of temperature on μ_{max} and λ, respectively. The equations to describe this relationship have been developed which may be valid to predict μ_{max} and λ at sub-optimal temperatures. In order to realistically model the system, the activity was limited to the performance of key players in the degradation processes in pits, i.e., *B.cereus* and *S.marcessens*, which were identified earlier in feacal sludge using 16S rRNA genotype fingerprinting.

Prior to the optimisation and modelling processes, the volatile organic compounds (VOCs) emitted from pit feacal sludge samples were identified using Gas Chromatography coupled with Time of Flight Mass Spectrometry (GC-ToF-MS) system and characterised. Nineteen VOCs including; alpha-pinene, butyric acid, dimethyldisulfide (DMDS), dimethyltrisulfide (DMTS), ethylacetate, ethylformate, indole, isobutyric acid, limonene, methyl thioacetate, methyl thiophene, p-Cresol, phenol, toluene, 1-propanol, 2-butanone and 2-methylbutyric acid were the most-frequently occurring compounds in the sampled pit latrines. The full 16S rRNA gene analysis yielded nine genotype homologies in the range 93-100% probability, i.e., *Alcaligenes* sp. strain SY1, *Achromobacter animicus, Pseudomonas aeruginosa, S. marcescens, Achromobacter xylosoxidans, B.cereus, Lysinibacillus fusiformis, Bacillus*

methylotrophicus and *Bacillus subtilis*. An elimination matrix was designed to select most influential parameters of model compounds for the experiment and representative cultures for evaluation of degradation and deodourisation of pit latrines.

Finally, based on degradation potential of butyric acid by different strains, six bacterial strains were selected for construction of bacterial consortia, which could be utilised in the formulation of bacterial cultures to be used in actual pit latrine biodeodourisation processes. Nineteen bacterial consortia were artificially prepared and consortium C3 that was composed of *B.cereus* and *S.marcescens*, resulted in remarkably higher butyric acid degradation efficiency. High Performance Liquid Chromatographic (HPLC) analysis showed 100% degradation of 1000 mg/L butyric acid after 16 h. The results indicated that some bacterial consortia may effectively degrade butyric acid, even though other bacterial consortia showed non-synergetic degradation of the compound despite high degradation activity in pure cultures. Clearly, the environmental conditions such as temperature, pH and inoculation size showed that they have an influence on butyric acid degradation of each of the members of the consortium C3.

Keywords: Pit latrine deodourisation; butyric acid biodegradation; odourants; response surface modelling; solid phase microextraction; volatile organic compounds.

DECLARATION

I, J**ohn Bright Joseph Njalam'mano**, declare that this thesis, which I hereby submit for a Doctor of Philosophy in Chemical Technology degree at the University of Pretoria, is my own work and has not been previously submitted by me for any degree at this or other institutions.

…… ……
John Bright Joseph Njalam'mano Date

DEDICATION

I affectionately dedicate this work to Kaduwa*, aka* KD*.*

… As, you are the 'real force' behind this academic journey

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NOMENCLATURE

Abbreviations

AIC	Akaike Information Criterion
AICc	Corrected Akaike Information Criterion
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical Oxygen Demand
bp	Boiling point
CPS	Conducting polymer sensor
DALYS	Disability-Adjusted Life Years
dH_2O	Deionised water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DVB	Divinylbenzene
GC	Gas chromatography
GC-O	Gas chromatography- Olfactometry
GHG	Greenhouse gas
HLC	Henry's law constant
HPLC	High-performance liquid chromatography
HRC	Human Rights Council
HRWS	Human rights to water and sanitation
HS	Head space
ID	Internal diameter
IS	Internal standard
ITC	Information Theory Criterion
LLE	Liquid-liquid extraction

LOD	Limit of Detection
LOQ	Limit of Quantification
MAFFT	Multiple Alignment using Fast Fourier Transform
MDG	Millennium Development Goal
MOS	Metal oxide sensor
MS	Mass spectrometry
MSM	Mineral salts medium
MΩ	Milliohm
m/z	Mass to charge ratio
NCBI	National Centre for Biotechnology Information
NCC	Nitrogen-containing compounds
NIST	National Institute of Standards and Technology
NNI	Nearest-Neighbour Interchange
OD	Optical density
OI	Odour index
O & M	Operation and maintenance
OU	Odour unit
OU_E	European odour unit
PA	Polyacrylate
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
QCM	Quartz crystal microbalance
PTFE	Polytetrafluoroethylene
RH	Relative humidity
RMSE	Root-mean-square error
rpm	Revolutions per minute

rRNA	Ribosomal ribonucleic acid
RSS	Residual sum of squares
R^2	Coefficient of determination
R^2_{adj}	Adjusted coefficient of determination
SAWS	Surface acoustic wave sensor
SCC	Sulfur-containing compound
SDG	Sustainable Development Goal
SPE	Solid Phase Extraction
SPME	Solid phase microextraction
SPR	Subtree-Pruning-Regrafting
SVOC	Semi-volatile organic compound
TIC	Total ion chromatogram
ToF	Time of Flight
U	Unit of enzyme's catalytic activity
UHP	Ultra-high purity
UN	United Nations
UNICEF	United Nations Children's Fund
UV	Ultraviolet
VIP	Ventilated improved pit
VOC	Volatile organic compound
VVOC	Very volatile organic compound
WASH	Water, sanitation and hygiene
WHO	World Health Organisation
WSSD	World Summit on Sustainable Development

Mathematical Symbols

$\binom{n}{r}$	combinatorial symbol, read as "n choose r",
Δ_i	difference between AICc values ($AICc_i$-$AICc_{min}$)
μ_{max}	maximum specific growth rate [h^{-1}]
σ^2	estimate of error variance from fitting the full model
C_a	apparent odour concentration
C_x	perceptibility threshold of an individual pure odourous compound (*x*)
C_{xy}	collective olfactory perception threshold of the mixture of two pure odourous compounds (*x* and *y*)
C_y	perceptibility threshold of an individual pure odourous compound (*y*)
F_{ij}	concentration capability factor of the fiber *j*.
H_{ij}`	peak height of i analyte with the use of j fibre coating.
$OT_{100\%}$	odour threshold at 100% [ppm]
P_{vap}	vapour tension of the odourous compound [ppm]
Q_f	reference non-odourous flow rate [m^3/s]
Q_o	flow rate of odourous sample [m^3/s]
T_{min}	conceptual minimum temperature for microbial growth [^oC]
y_i	*y* value of the i^{th} candidate sigmoidal model
y_{max}	maximum *y* value in all *M* candidate sigmoidal models,
y_{min}	minimum *y* value in all *M* candidate sigmoidal models
α_i	weighted mean of standardised indicators
β_i,	linear coefficient of the generalized response –surface model
β_{ii}	interaction coefficient of the generalized response –surface model
β_{ij}	interaction coefficient of the generalized response –surface model
β_o	constant of the generalized response –surface model
A_c	concentration of butyric acid in the abiotic culture [mg/L]
A_s	concentration of butyric acid in the biotic culture [mg/L]
b	an empirical parameter of Ratkowsky model [$h^{0.5}\,{}^oC^{-1}$]
C_{oct}	molar concentration of the organic compound in the octanol phase [mol/L]
C_{wat}	molar concentration of the organic compound in the water phase [mol/L]
D_e	degradation efficiency of butyric acid [%]

d_i	individual desirability of the i^{th} response,
e	Euler's constant, which is the base of the natural logarithm, equal to 2.718.
k	number of fibres
k	number of experimental responses
K_{ow}	octanol-water partition coefficient
L_i	Derringer desirability function lower specification limit
M	total number of candidate sigmoidal models
ϒ	mathematical parameter in Richards model
T	temperature [^oC]
U_i	Derringer desirability function upper specification limit
w	weight coefficient.
y_i	i^{th} response value
λ	lag phase duration [h]
ν	Richards model shape parameter
A	asymptotic value as time decreases indefinitely
C	number of central points in CCD
C	odourant concentration [mg/L]
I	odour intensity
N	number of experiments in CCD
a	constant in Weber-Fechner law
a	constants that relate to the use of mean odour intensity levels
a	mathematical parameter in logistic, Gompertz and Richards models,
b	constant in Weber-Fechner law
b	mathematical parameter in logistic, Gompertz and Richards models
b	constants that relate to the use of mean odour intensity levels
c	mathematical parameter in logistic, Gompertz and Richards models,
f	number of factors in CCD
k	constant that differs for each odour
k	number of parameters in sigmoidal model
n	number of experimental observations
n	constant that differs for each odour
n	total number of bacterial strain types

r	number of bacterial strains in each consortium
y	bacterial concentration

LIST OF PUBLICATIONS

Njalam'mano J. B.J., Chirwa E.M. N. and Seabi R. L. (2020) *In vitro* study of butyric acid deodourisation potential by indigenously constructed bacterial consortia and pure strains from pit latrine feacal sludge. Sustainability 12 (12): 5156 https://doi.org/10.3390/su12125156.

Njalam'mano J.B.J. and Chirwa E.M.N. (2019) Determination of growth parameters of butyric acid degrading bacterium, *A.xylosoxidans* as a function of constant temperature in batch system. Chemical Engineering Transactions 76: 1321-1326 https://doi: 10.3303/CET1976221.

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Njalam'mano J.B.J. and Chirwa E.M.N. (2018). Evaluation of butyric acid degrading bacteria isolated from pit latrine: Preliminary bacterial growth studies. Water Institute of South Africa Biennial Conference and Exhibition (WISA 2018). 24-27 June 2018, CTICC, Capetown, South Africa.

Njalam'mano J.B.J. and Chirwa E.M.N. (2018) Potential biodegradation of butyric acid by *L.fusiformis* isolated from a pit latrine in South Africa. WM Symposia – Nuclear and Industrial Robotics, Remote Systems and Other Emerging Technologies, (WM2018), March 18-22, 2018 in Phoenix, Arizona, USA.

Njalam'mano J.B.J. and Chirwa E.M.N. (2017) Isolation of high strength butyric acid-degrading bacterium from pit latrine feacal sludge. International IWA Conference on Sustainable Solutions for Small Water and Wastewater Treatment Systems (S2small2017), 22-26 October, 2017 in Nantes, France.

CHAPTER ONE

1 INTRODUCTION

1.1 Background

1.1.1 Definition of sanitation

Generally, sanitation is referred to as safe collection, disposal and management of human excreta (faeces and urine), solid wastes and sewage (WaterAid, 2011). In this thesis, the working definition of sanitation is looked at in its narrow sense as safe disposal of human excreta using facilities that ensure hygienic separation of human excreta from human contact (WHO and UNICEF, 2010). In addition to hygienic separation, safe disposal means that the human excreta must be properly contained or treated to prevent transmission of diseases agents (Mara, *et al.,* 2010).

1.1.2 Sanitation, health and human well-being

Every person has the right to be protected from the many health risks including sanitation related diseases and other serious infections caused by unsafe disposal of human excreta (UNICEF, 2000).

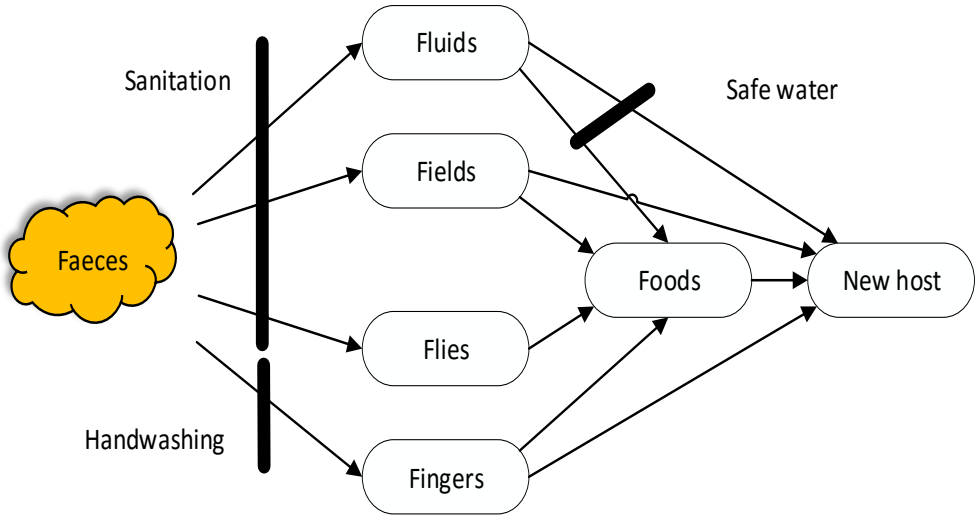


Figure 1.1: F-Diagram showing potential feacal-oral transmission pathways
(Adopted from Wegner and Lanoix, 1958)

The absence of improved sanitation facilities compels people to practice open defecation and this threatens people's health due to increased risk of transmission of feacal-to-oral related diseases (Koyra, *et al.*, 2017). The open defecation is considered as the riskiest sanitation

practice according to WHO (2013) and has contributed to 40 million disability-adjusted life years (DALYS) (Obeng, in press).

As demonstrated by Wagner and Lanoix (1958) in Figure 1.1, faeces of an infected individual, which are not safely disposed and contained, represent a risk to public health. The F-diagram graphically provides an explanation on how human-derived enteric pathogens can be transmitted by multiple inter-linked pathways i.e. fluids (or water), fields (or soils), flies and fingers, which allow feacal matter to enter the mouth; the feacal-oral disease transmission route (Mara, *et al.*, 2010). Thus, Figure 1.1 vividly demonstrates that a combination of improved sanitation facilities and hygiene practices are the primary barriers or blocks that prevent the transmission of diseases. Poor sanitation does not only have adverse effects on human health but also affect the environment through the contamination of water bodies, soils, and food sources. Moreover, adequate sanitation is not only important for improved health outcomes for users, but also has positive environmental effects, improved educational outcomes, greater convenience and comfortability, dignity and gender equality on the broader community (Hutton and Chase, 2016). This further suggests that sanitation has inherent potential to harness socio-economic development for the public at large. There are also higher returns of USD 5.5 for every dollar invested in sanitation as compared to USD 2.0 for every dollar invested in water (Hutton, 2012). Paradoxically, despite the numerous benefits of sanitation, investment in sanitation is seen as a large investment that is sidestepped and more efforts are directed towards water supply projects (Katukiza *et al.,* 2012).

1.1.3 Historical perspective of sanitation at international level

Over the years, sanitation has received international community attention. It all started over four decades ago, in their quest to protect the environment at Stockholm United Nations Conference on the Human Environment in 1972, United Nations (UN) member states were provided with guidelines and were encouraged to ‘protect and improve the human environment and to remedy and prevent its impairment’ (UN, 2012). This was followed by 1977 World Water Conference in Mar del Plata, Argentina at which the International Drinking Water Supply and Sanitation Decade (IDWSSD) of 1981–1990 was proclaimed. This was done with original intent of increasing water supply and sanitation services within the framework of the health-for-all strategy (Black, 1998). Hence, the global community committed itself to ensuring that globally people would have access to a minimum basic water and sanitation services at the end of the decade. Hitherto this target was not realised, with over 1.1 billion people still without access to improved water supplies, and at least 2.7 billion without access to sanitation services (Whittington *et al.,* 2009). Another decade after the closure of IDWSSD, about 1 billion people gained access to improved water and sanitation services. However, because the access rate

did not keep pace with population growth rate and a huge gap in financing sanitation, the number of those still unserved was approximately the same level as in 1990 (Whittington *et al*., 2009).

At the Johannesburg World Summit on Sustainable Development (WSSD) in September 2002, the international community endorsed a new set of Millennium Development Goals (MDGs), and which, *inter alia*, includes MDG7c that was addressing the environmental sustainability. Target 10 is one of the MDG7c targets that aimed at halving the number of people without access to improved water supply and without basic sanitation by 2015 based on the numbers estimated in 2000 (Hutton and Bartram, 2008). Further, efforts to meet the MDG target were renewed in 2005that marked the genesis of the International Decade of Action, Water for Life, 2005-2015 (Salman, 2005). Within the MDG period, in recognition of the fundamental importance of water and sanitation on undermining a wide array of other human rights, the UN General Assembly (UNGA) and the Human Rights Council (HRC) adopted a series of international treaties and declarations that led to resolution to explicitly recognise human rights to water and sanitation (HRWS) in 2010 (UN, 2010). In 2015, the resolution was supplemented with adoption of another resolution that elucidated the distinction between the human right to water and the human right to sanitation (UN, 2015). '*The human right to sanitation entitles everyone, without discrimination, to have physical and affordable access to sanitation, in all spheres of life, that is safe, hygienic, secure, socially and culturally acceptable and that provides privacy and ensures dignity'* (UN, 2015).

With the end of MDG era, notwithstanding the global MDG target for water being realised, the MDG assessment revealed that sanitation targets progress fell far short particularly in the low-income countries. There was still 780 milliom people without access to improved water sources and 2.5 billion people without access to improved sanitation (WHO and UNICEF, 2012; WHO and UNICEF, 2017). Building on the achievements of the MDGs, in 2015, the UNGA unanimously adopted 2030 Agenda of Sustainable Development with 17 Sustainable Development Goals (SDGs) with water, sanitation and hygiene (WASH) SDG6 targets 6.1 and 6.2 providing an opportunity to address the unfinished business and shortcomings of MDG 7c. This was with the aspirations to achieve universal and equitable access to safe and affordable drinking water, sanitation and hygiene by 2030 (Hutton and Chase, 2016). These targets are, by any standard, extremely ambitious. This is because the extent of these targets are unprecedently large, which entails that approximately, 10 million people per day will require to access safely-managed sanitation facilities and 240, 000 people per day to upgrade from open defecation to fixed-safely managed facilities by 2030 (Mara and Evans, 2017).

1.2 Statement of the problem

Pit latrines are basic minimum acceptable forms of improved on-site excreta disposal facilities for rural inhabitants and peri-urban dwellers in many rapidly growing developing cities, particularly for resource poor households throughout much of developing countries (Thye*, et al*., 2011, Jenkins *et al.,* 2015). It is crudely estimated that globally, 2.7 billion people use onsite sanitation system as means of human waste disposal (Strande, 2014). Approximately, 1.8 billion people around the world that use onsite sanitation are servedwith some types of pit latrine as their common mode of human excreta disposal in low income countries (Graham and Polizzotto, 2013). This number is anticipated to rise in response to SDG 6.2, which targeted universal access to safe sanitation by 2030 (Ravenscroft *et al.* 2017) under a plethora of WASH programmes. Low capital, operation and maintenance (O&M) costs, little to no water requirement for its functionality, construction with locally available materials and the ease for modification to meet the users' preference i.e. squatting, washing and wiping, are associated with pit latrines. These are some of the determinants that have led to high adoption of pit latrines relative to other alternatives such as bucket latrines, aqua privies, conventional sewerage and septic tank-soakaway systems (Katukiza *et al,* 2012; Graham and Polizotto, 2013; Nakagiri *et al.,* 2016).

However, despite their widespread application and use, there is a growing serious concern that pit latrines are used with their operational performance difficulties. Offensive smells-malodours that are associated with pit latrines, which elicit disgusting or repulsive response to pit latrine users, are one of the determinants that affect their performance (Nakagiri *et al*., 2016). These offensive smells offer an important but often overlooked main barrier that contributes to inconsistent use, non-adoption and negative reputation of pit latrines that consequently affect successful effective global sanitation promotion efforts (Rheinländer *et al.,* 2013). This is primarily because some people have a miasmic view that associates malodourous emissions with conditions of 'unhealthy' air that causes diseases (Jorgensen, 2013). Notwithstanding limited epidemiological evidence on the health threats associated with malodours, there have been established cases that malodours have significant effects on the physiological and psychological well-being of the inevitably exposed local inhabitants (Bullinger, 1989; Schiffman *et al*., 1995; Wrzesniewski *et al.,* 1999; Wang et al., 2002; Kadohisa, 2013; Hayes *et al*., 2017; Hirasawa *et al*., 2019). Malodours cause considerable annoyance and disturbance as well as physical symptoms such as nausea, headaches, loss of appetite, runny nose, shortness of breath, classical stress response and a range of other acute and chronic effects (Schiffman *et al.,* 1995; McGinley and McGinley, 1999; Shusterman, 2001).

Furthermore, in negative visceral reaction to these unpleasant odourous episodes, people are deterred from using the pit latrines thereby opting for open defecation out of a set of alternatives, if private and secluded spaces availability is not a constraint. Occasionally, this also takes place in the immediate precincts of the pit latrines. This 'open air' approach is considered healthier and more pleasant (Cavill, *et al.,* 2015) hence hampering access to adequate and equitable sanitation and hygiene for all and end open defecation pursed in Sustainable Development Goal 6 (UN, 2016). This consequently derails the attainment of the SDG 6 intrinsically interconnected SDGs *inter alia* include; ending poverty in all its forms everywhere (SDG 1), ending hunger, achieve food security and improved nutrition, and promote sustainable agriculture (SDG 2) and ensuring healthy lives and promoting well-being for all at all ages (SDG 3) among others (Osborn *et al.,* 2015).

Regrettably, hazards and squalor accompanied by open defecation in fields, bushes, or other open spaces have spillover effects, as they do not just put those who defecate in the open at risk as illustrated in Figure 1. Even in the absence of open defecation, pit latrine odour emissions are closely linked to insect nuisance including houseflies and blowflies (Lindsay *et al*., 2013; Nakagiri *et al*., 2016). The inextricable linkage arises for the reason that smells generated from the pits attract the flies (Emerson *et al.,* 2005; Irish *et al*., 2013). Moreover, studies have undoubtedly incriminated blowflies such as *Chysomya putoria* (Lindsay *et al*., 2013) and houseflies such as *Musca domestica* (Chavasse *et al.,* 1999) as common mechanical transmitters of pathogens. These pathogens cause diarrheal diseases, which are equally detrimental to both the rich and the poor – including those who use the latrines (Coffey *et al*., 2014).

1.3 Significance of the study

Previous studies have indicated that degradation processes in pit latrines are assumed predominantly anaerobic due to unfavorable redox conditions in pits even though aerobic degradation processes may occur (Buckley *et al*., 2008). That implies that a pit acts like anaerobic-aerobic digester. Fundamentally, the anaerobic biodegradability property of feacal sludge in the pit latrines is intrinsically concomitant with unpleasant odourous emissions. The human nose is exceptionally sensitive to odours even at very low concentrations of the odourants (Sela and Sobel, 2010).

Over the years, an array of odour treatment approaches have been used for odourous emissions in pit latrines. The approaches include use of water seal (Green and Ho, 2005), addition of carboneous material and wood ash (Awasighe *et al*., 2015), use of ventilation

systems (Ryan and Mara, 1983) and urine separation (Niwagaba *et al*., 2009). Currently, the existing odour control measures are insufficient as odourous emissions are still observed and they find it difficult to decrease smells below human odourant detection threshold.

Moreover, a number of shortfalls have been discovered with the current measures to address pit latrine olfactory nuisances. The shortfalls are mostly environmental, economic, technological and social constraints associated with the measures. For example, the ventilated improved Pit (VIP) latrines are designed to control some of the common operational difficulties such as offensive odours and fly nuisance that are associated with simple pit latrine designs.However, in terms of capital investment, without external financial assistance, VIP latrines may be expensive for low-income households. Furthermore, the effectiveness of the ventilation pipes depends on the local wind speed and direction. In densely populated informal settlements in which the height of the vent pipes is inadequate because of the height and location of neighboring buildings, the VIP latrine become ineffective in odour reduction (Obeng *et al*., 2019). These shortcomings have necessitated a search for alternative or complementary measures for pit latrine odour attenuation or elimination which are cost effective and ecofriendly.

Some researchers found that aerobic bacteria have the capacity to reduce offensive smells through active aerobic degradation of odourous compounds (Grubb, 1979; Bourque, 1987; Jolicoeur and Morin, 1987; Zho, 2000). A wide variety of microorganisms capable of degrading malodourous compounds may be an attractive alternative to the aforementioned existing odour control techniques currently used in low-income settings in developing countries. Thus, bioremediation provides an alternative tool to remove or degrade the malodourous compounds through utilisation of odourous compounds as carbon and energy sources by the microorganisms in aqueous solution to innocuous state end-products. Biological technologies that remove odour compounds have attracted increasing attention in recent years. This is because they have been proven to be cost-effective, preclude the use of chemicals (Charles and Ho, 2017), simple to operate and environmentally acceptable (Barbusinski *et al*., 2017). Biological odour removal treatments are recognized as the most economical and effective systems for the deodourisation of waste odours streams considered of high flow rates and low concentrations (Rappert and Müller, 2005). Hence, there is need to explore the potential biological deodourization of odourous compounds in the pit latrines at the point of emission in order to optimally increase the consistent use and uptake of pit latrines in developing countries. To the best of the researcher's knowledge and after thorough literature search, there is no research that has been devoted to the study of the biological treatment of odourous compounds in pit latrines. This is in spite of their widespread use throughout developing countries. This could be because sanitation related offensive odours

are intrinsically unappealing or not photogenic subject matter. Moreover, various techniques that researchers have devised, including biofilters, bioscrubbers, and biotrickling filters are applied in high-tech odour treatment.

1.4 Objectives of the study

In view of the importance of malodour emissions from pit latrine as discussed above, the current study **"Microbial Deodourisation and Neutralisation of Pit Latrine Odour Causing Compounds"** is undertaken with the aim of gaining more insight in the potential of microbial deodourisation or neutralisation of pit latrine odourants under laboratory batch experiments. To achieve the above aim, therefore, the specific objectives of the study are:

- To identify and characterise volatile organic compounds emitted from pit latrines by means of headspace microextraction (HS-SPME) and Gas Chromatography coupled with Time of Flight Mass Spectrometry (GC-ToF-MS);
- To develop a method for simultaneous determination of key pit latrine volatile organic odourous compounds below the human odourant detection threshold;
- To isolate and characterise butyric acid degrading bacterial strains from pit latrine sludge and to evaluate their potential application in biodeodourisation;
- To investigate the potential of the constructed aerobic bacterial consortia for butyric acid biodeodourization;
- To develop predictive models for the growth of *B.cereus* and *Serattia marcescens* in butyric acid liquid medium as a function of constant temperature.

1.5 Structure of thesis

This thesis is divided into nine chapters in essence reflecting the progression of the study objectives.

Chapter 1: This chapter covers an introduction, which provides the background to the study. Furthermore, the problem statement and need for the study are highlighted in this chapter outlining the significance and rationale of this study. The chapter also covers the aim and specific objectives of the study as well as structure of this thesis. The chapter also highlights the contribution of this study to the body of knowledge.

Chapter 2: This chapter is a compilation of a review of the current knowledge on the characteristics of odour, odour perception, odourous compounds, sources of odour, odour formation; microbial processes related to odour formation, human excreta related volatile compounds, odour measurement and an overview of biodegradation.

Chapter 3: This chapter focuses on the experimental design that provides details of materials and methods that have been used to achieve the objectives of the study. The key aspects presented in this chapter include; chemicals and reagents used in this study, collection of feacal sludge samples, biodegradation assays, analytical approaches and data analysis techniques.

Chapter 4: This chapter discusses the investigation in which headspace solid phase microextraction (HS-SPME) combined with Gas Chromatography Time of Flight Mass Spectrometry (GC-ToF-MS) system was used to identify the composition of volatile organic compounds (VOCs) and characterise the key odourants species emitted from pit latrine feacal sludge.

Chapter 5: This chapter concentrates on the second objective of the study; specifically, the chapter describes the multivariate optimisation of HSPME coupled with GC-ToF-MS for simultaneous determination of four key pit latrine odourants.

Chapter 6: This chapter focuses on isolation of butyric acid degrading bacteria from pit latrine feacal sludge and their identification using phylogenetic analysis. The main highlight of this chapter is the comparison between the bacterial strains' capability to degrade butyric acid.

Chapter 7: This chapter discusses the results of formulation of bacterial consortia that can be directly employed to deodourise butyric acid. The butyric acid biodegradation potential offered by different bacterial consortia were then analyzed against the biodegradation potential of the individual bacterial strains

Chapter 8: This chapter presents the results of the comparison of different growth models for *B.cereus* and *S.marcescens* in butyric acid liquid medium at constant temperatures.

Chapter 9: This chapter presents summary of the findings of this study, conclusions that are drawn and some recommendations based on the findings of the study for future research.

1.6 Contribution of the study

This study has contributed to improved understanding of the restricted knowledge of odourous emissions from on-site sanitation systems which are commonly used in developing countries. This study is anticipated to further support the development of pit latrine odour control in the rural areas and informal settlements of developing countries as far as olfactory nuisances are concerned. It is further envisaged that good understanding of odour emissions from pit latrines has provided helpful information that can be used to develop sustainable and effective pit

latrine odour emission reduction technologies that would be appropriate for the local and users' circumstances, affordable, environmentally acceptable and compatible with pit latrine feacal sludge management systems and their management capabilities of operation. This would consequently reduce the detrimental effects of odour nuisance on aesthetics, property values, and wellbeing of exposed adjacent sensitive receptors and pit latrine users.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 General basics of odour

2.1.1 Definition of odour

There is a consensus on what odour, which refers to smells, is though different authors have coined it differently. There is, therefore, no indistinctness in its usage as it appears in the literature. A selected few of these definitions are given below. For instance, Health Canada (1979) defined it as "*that sensation that is due to the presence of substances that have an appreciable vapour pressure and that stimulate the human sensory organs in the nasal and sinus cavities*". McGinley*, et al*. (2000) referred it to "*as the perception experience when one or more chemicals come in contact with the receptors on the olfactory nerves and stimulate the olfactory nerve".* Government of India (2008) defined odour as "perception of smell" or in scientific terms as *"a sensation resulting from the reception of stimulus by the olfactory sensory system".* Age, gender and occupation are some of the variables that affect the olfactory sensitivity, although none so much as individual variability (Mackie *et al*., 1998) and different groups of people have naturally different levels of acceptance for odours in their societies (McGinley, *et al*., 2000)**.**

On the other hand, it is important to distinguish terminology as words odourants (in this review referred to as odourous compounds) and odours whenever odour identification and quantification are being considered as are sometimes used interchangeably. Odourant and odour are terminologies that are related but have distinct definitions and should not be confused. In this review, an odourant is any chemical in the air that is part of the perception of odour by "stimulating the olfactory nerves" (McGinley, *et al.,* 2000). Odour perception may occur when one or a mixture of odourous chemical compounds, dependent upon their concentration, are present in a substance (Naddeo *et al.*, 2012). This fundamentally entails that an odour is the organoleptic perception while an odourant is the chemical or the substance (McGinley, *et al.,* 2000; Gostelow *et al.,* 2001). Odour is an unrestrained and complex array of a variety of organic and inorganic chemicals of dissimilar classes and quite dissimilar physical and chemical properties (Hudson and Ayoko, 2008). Odour can be generated from either an individual odourous compound or a mixture of odourous compounds (SEPA and Natural Scottish Scotland Government, 2010). However, seldom, in nature individual odourous compounds are encountered and each one has a characteristic smell (Mackie *et al.*, 1998; Marsili and Laskonis, 2014). As a result of interactions that take place between odourous components in a mixture, an olfactory perception of a mixture of odourous

compounds in the substance is not related to that they would be perceived independently (Mackie *et al*., 1998; Li, 2014; Marsili and Laskonis, 2014). This is so much so that the characteristic smells of the individual compounds cannot be recognised (SEPA and Natural Scottish Scotland Government, 2010). Furthermore, the mixture of odourous compounds may likely induce different correlations or effects such as (Baker, 1963; Naddeo *et al*., 2013):

(1) Independence, in which the resultant odour may be equal or less than the individual odourous compounds$\left(C_{xy} \leq C_x \text{or} C_y\right)$;

(2) Additivity, in which the resultant odour may be equal to the sum of the individual odourous compounds$\left(C_{xy} = C_x + C_y\right)$;

(3) Synergism, synonymous with 'intensification', in which the resultant odour may be equal to greater than the anticipated direct sum of the individual odourous compounds$\left(C_{xy} \geq C_x + C_y\right)$;

(4) Antagonisms, synonymous with 'suppression', in which the resultant odour may be less than the anticipated direct sum of the individual odourous compounds$\left(C_{xy} < C_x + C_y\right)$.

where C_x and C_y denote the perceptibility threshold of two individual pure odourous compounds, and

C_{xy} is the collective olfactory perception threshold of the mixture of two pure odourous compounds.

The above effects render the evaluation of odour perception problematic. This suggests that the identification and quantification of distinct odourous compounds in an odourous air samples are not sufficient to describe the odour strength and quality (Zhang *et al.,* 2002). However, Kim (2011) in a study to learn the effects of mixing different odourous compounds (e.g. in this case, four reduced sulphur compounds; hydrogen sulphide (H_2S), methanethiol (CH_3SH), dimethylsulfide $(CH_3)_2S$ and dimethyldisulfide $(CH_3)_2S_2$). It was indicated that the mixing phenomenon of the odourous compounds of the same family i.e.similar chemical properties could be characterised by the averaging effect of all individual odourous components in the mixture. This was as opposed to the previously mentioned common theoretical basis of the mixing effect. Hence, because of lack of detailed theory of olfaction the relationship between odourant properties and odour perception are not well-understood (Gostelow *et al*., 2001). However, Li (2014) and Marsili and Laskonis (2014) reported that the ratio of two odourous compounds in a mixture has impact on its odour property which is exclusively dissimilar to that of individual odourous compounds. Furthermore, one more cofounding aspect is that odourous compounds below threshold levels and elements, which have no odour activity as individuals may in fact, contribute odour activity when they are

blended. This therefore, makes it difficult to predict the organoleptic perception of the mixture of odourous compounds. (Marsili and Laskonis, 2014).

Odour can be either pleasant or unpleasant, but both are induced by breathing in air-borne volatile organics or inorganics. The odourants are the ones that induce the pleasant and unpleasant effects (Government of India, 2008). The unpleasant effects are the one that elicit olfactory response of abhorrence (Hammond, 2013). However, different people have dissimilar sensations (which can be found to be acceptable, objectionable or offensive) and emotional responses to the same odourous compounds because of biological olfactic dissimilarity and life experiences (Naddeo *et al.,* 2013; Bull *et al*., 2014). As a result, the same odourous compound that is perceived as pleasant to one person might be unpleasant to another (Naddeo *et al*., 2012).

2.1.2 Characteristics of odour

Odour is the most difficult of all the air pollution problems (Government of India, 2008). Odour has distinctly dissimilar characteristics in comparison to conventional air pollutants and this, therefore, makes the subject of odour to become more comprehensibly difficult, the thing that is further compounded by the individuals' subjectivity in their response to odour exposure (SEPA and Natural Scotland Scottish Government, 2010). Nuisance is the most important consequence of odour on the public. Some of the characteristics of odour may include (Dague, 1972; Government of India, 2008):

- Substances of same or different chemical composition may have same odour.
- Nature and strength of odour may vary on dilution.
- Weak odour is not perceived in presence of strong odour.
- Odours of similar strength combined, produce a mixture in which one or both may be undetectable.
- Persistent intensity of odour causes a person to quickly loose cognizance of the sensation and only perceived when it varies in intensity.
- Fatigue for one odour may not affect the perception of different odour but will affect the perception of same odour.
- An unaccustomed odour is more likely to cause complaint than an accustomed one.
- At least two odourous compounds may mask the smell of each other.
- Odour moves downwind.
- Person can smell at a distance.
- Many animals have stronger sense of smell than human beings do.

- Likes and dislikes often depend on association of the scent with pleasant or unpleasant experiences.

The above listed characteristics of odour and its associated highly inconstant impact and subjectivity can further make detection and assessment of pollution due to odourous compounds more difficult (SEPA and Natural Scotland Scottish Government, 2010).

2.1.3 Odour perception

Mammals, including humans, have the ability to detect and discriminate a large number and a complex combination of odourous compounds as having dissimilar odours encountered in nature as well as pure molecules synthesised in the laboratory. For instance, humans can perceive 10,000 to over 100,000 different volatile compounds as having distinct odours and even those with very low concentrations that are much less than detectable by gas chromatography (GC) as discussed in section 2.7.4.1, for instance, hydrogen sulphide (H_2S) (Mackie *et al*., 1998; Buck, 2004; SEPA and Natural Scottish Scotland Government, 2010; Agapaks and Tolaas, 2012). The human nose is capable of detecting even extremely small amount of odourant vapour with 10^8 and 10^9 molecules (Rappert and Müller, 2005) of an extensive variety of odourous compounds (Naddeo *et al*., 2012). The minimum concentrations required for the detection of odours are referred to as odour threshold values (OTV) (Mackie *et al*., 1998; Rappert and Müller, 2005).

However, the human sense of smell is regarded as the least developed of all the five (5) human senses as well as the inferior among other animals (Bratolli, *et al.,* 2011) as earlier indicated in section 2.1.2. The chemicals in the inhaled air, which are dependent on their water solubility and other mass transfer factors, are trapped and dissolved. The more water-soluble the chemical, the more easily it is dissolved into the olfactory epithelium where the odourous chemical compounds stimulate an electrical response of the olfactory nerves which eventually triggers the olfactory signal. The olfactory signal is transmitted to the olfactory bulb in the brain where the odour is perceived through a series of neural computations and then discriminated (Brattoli *et al*., 2011).

The odour intensities are recorded dependent upon their molecular concentrations (Dorling, 1977; Brattoli *et al.,* 2011). Both the type and intensity of odour are important to trigger the electrical impulse sent to the brain (Naddeo *et al.,* 2012). The human response to odour becomes progressively less sensitive with continuous exposure due to fatigue (Dorling, 1977) and it is subjective as people either adapt or become sensitised. This is why people who are exposed to persistent odourous environments usually do not experience this. On the other hand, acute exposure episodes or repeated exposure to annoyance levels of odours can result

in individuals becoming sensitised to olfactory stimulants (Ministry for Environment, NZ, 2003). Hence, the human nose cannot be trusted as a scientific instrument (Agapakis and Tolaas, 2012). This is because within a given population, individuals will have a range of different olfactic sensitivities to odours. This is due to such factors as genetics, the context in which it is experienced, earlier experiences, perception, coping mechanism, social convention, semantics, the socioeconomic structure of the area, dispersion situation, topography, weather and distance between the receptor and the odour source (Nimmermark, 2004; Aatamila *et al*., 2010; Hammond, 2013). In general, the high variability of human response to odours is a function of a combination of five interrelated factors, which are collectively known as **FIDOL** factors. The factors refer to the **F**requency (how often), **I**ntensity (the strength of the odour), **D**uration (the time length of a particular odour episode), **O**ffensiveness (pleasantness or unpleasantness i.e. mixture of odour character and hedonic tone) and **L**ocation (the type of land use and nature of human activities in the vicinity of an odour source) (Ministry for the Environment, NZ., 2003; Nicell, 2009; Ubeda *et al*., 2013). In addition, the human olfactory perception capacity in identifying odour is reported to be low with the increased complicated nature of the odourous mixture (Kim, 2011).

2.1.4 Odourous compounds

Wastewater or sludge odourous compounds are either organic or inorganic compounds, which are mainly produced by biological activity through either aerobic or anaerobic decomposition of proteins and carbohydrates in sludge or are presented in emissions as a result of chemical processes (Son and Striebig, 2003; Government of India, 2008). However, Dincer and Muezzinoglu (2008) and Dague (1972) reported that many of the odourous inorganic compounds that arise due to anaerobic decomposition have sulphur and nitrogen. Hydrogen sulphide (H_2S), ammonia (NH_3), carbon dioxide (CO_2) and methane (CH_4), nitrogen (N_2), oxygen (O_2) and hydrogen (H_2) are the principal volatile compounds that are highly associated with wastewater collection and treatment systems.

H_2S and NH_3 are the principal compounds that are malodourous while the rest are odourless. Other odourous compounds of high concentrations are organic sulphides, mercaptans, inorganic and organic amines, organic acids, aldehydes and ketones (Dague, 1972; Stuertz and Frenchen, 2001; Dincer and Muezzinoglu, 2008). Most intense odourous compounds have a substantial tendency to vaporise and as such, they are gaseous at room temperature and atmospheric pressure. This is usually influenced by their molecular weight. The higher the molecular weight, the lower the compound volatility and thus normally results into less odour and vice versa (Government of India, 2008; Smet *et al*., 1998; WEF, 2008). In essence, odourous compounds are volatile compounds, but not all volatile compounds found in the environment contribute to malodourous emissions (Rappert and Müller, 2005).

Characteristically, odourous compounds have molecular weight that varies between 30 and 300g/mole (Gardner and Bartlett, 1999). Grosch (2001) showed that odourous compounds with higher odour activity value (OAV), which measures the contribution of an individual odorant to the overall odour, are frequently correlated to smell. Compounds heavier than this are inherently not odourous with water vapour at room temperature (Gardner and Bartlett, 1999). The OAV is of an individual odourant is determined according to equation 2.1 (Yand *et al*., 2015):

$$OAV = C/SCOT \tag{2.1}$$

where OAV is the odour activity value for an individual odourant (dimensionless),

C is the concentration of the individual odorant (µg/m^3), and

$SCOT$ is the odour detection threshold for the individual odorant (µg/m^3).

Likewise, compounds with an Odour Index (OI) below 10^5, for instance, alkanes and alcohols of low molecular weight, are faintly odourous whereas those with higher than that up to 10^9, for instance, mercaptans and surfurs, are considerably odourous (Naddeo *et al*., 2013). The OI of an individual odourous compound is calculated according to equation 2.2(Naddeo *et al*., 2013):

$$OI = P_{vap}/OT_{100\%} \tag{2.2}$$

where P_{vap} is the vapour tension of the odourous compound (ppm) and

$OT_{100\%}$ is the odour threshold at 100%(ppm)

Moreover, most odourous compounds have a tendency to have not more than two polar functional groups. Volatile compounds with more than two functional groups are not odourous (Gardner and Bartlett, 1999). Furthermore, the odour threshold concentrations also influence the odourous of the compounds. The key odourous compounds are often present in very low concentrations of not more than a few µg/L or mg/L (Godayol *et al*., 2011). This is the reason the reduced sulphur compounds, such as mercaptans and organic sulphides, are odourous as their odour threshold concentrations are extremely low (Smet *et al*., 1998; Government of India, 2008). A selection of odourous compounds that may be associated with domestic wastewater and sludge and their respective odour threshold values and other information are presented in Table 2.1. It is worth noting that that the principal malodourous compounds in human faeces are fatty acids, aromatic hydrocarbon compounds, nitrogenous compounds (e.g. ammonia and amines) and sulphurous compounds (Sato *et al*., 2002).

Table 2.1: Odourous compounds associated with domestic wastewater and sludge

Chemical class	Compound	Formula	Odour threshold (ppm)	Characteristics
Sulfurous	Hydrogen sulfide	H_2S	0.0005	Rotten egg
	Dimethyl sulfide	$(CH_3)_2S$	0.001	Decayed vegetables ,garlic
	Diethyl sulfide	$(C_2H_5)_2S$	0.0045-0.31	Nauseating, ether
	Diphenyl sulfide	$(C_6H_5)_2S$	0.0001	Unpleasant , burnt rubber
	Diallyl sulphide	$(CH_2CHCH_2)_2S$	0.00014	Garlic
	Carbon disulfide	CS_2	0.001	Decayed vegetables
	Dimethyl disulfide*	$(CH_3)_2S_2$	0.003-0.014	Putrefaction
	Methyl mercaptan *	CH_3SH	0.001	Decayed cabbage, garlic
	Ethyl mercaptan*	C_2H_5SH	0.0003	Decayed cabbage
	Propyl mercaptan	C_3H_7SH	0.0005	Unpleasant
	Butyl mercaptan	C_4H_9SH	0.001	Unpleasant
	Tert-Butyl mercaptan	$CH_3)_3CSH$	0.00008	Unpleasant
	Allyl mercaptan	CH_2CHCH_2SH	0.0001	Garlic
	Crotyl mercaptan	$CH_3CHCHCH_2SH$	0.000029	Skunk, rancid
	Benzyl mercaptan	$C_6H_5CH_2SH$	0.002	Unpleasant
	Thiocresol	$CH_3C_6H_4SH$	0.0001	Skunk, rancid
	Thiophenol	C_6H_5SH	0.000062	Putrid, nauseating, decay
	Sulphur dioxide	SO_2	0.009	Sharp, pungent,
Nitrogenous	Ammonia	NH_3	47	Sharp, pungent
	Methylamine*	CH_3NH_2	4.7	Fishy
	Dimethylamine*	$(CH_3)_3N$	0.047	Fishy
	Trimethylamine	$(CH_3)_3N$	0.0002	Fishy, ammoniacal
	Ethylamine*	$C_2H_5NH_2$	0.83	Ammoniaca
	Diethylamine	$(C_2H_5)_2NH_2$	-	-
	Triethylamine	$(C_2H_5)_3N$	0.0004	-
	Diamines, i.e. Cadaverine*	$NH_2(CH_2)_5NH_2$	-	Decomposing meat
	Pyridine	C_6H_5N	0.66	Disagreeable, irritating
	Indole*	C_8H_6NH	0.0001	Feacal, nauseating
	Scatole or Skatole*	C_9H_8NH	0.0001	Feacal, nauseating
Acids	Acetic (ethanoic)*	CH_3COOH	1	Vinegar
	Butyric (butanoic)*	C_3H_7COOH	0.001	Rancid, sweaty
	Valeric (pentanoic)*	C_4H_9COOH	0.0006	Sweaty
Aldehydes	Formaldehyde *	HCHO	0.8	Acrid, suffocating

and ketones	Acetaldehyde*	CH_3CHO	0.067	Fruit, apple
	Butyraldehyde*	C_3H_7CHO	0.0046	Rancid, sweaty
	Isobutyraldehyde	$(CH_3)_2CHCHO$	-	Fruit
	Isovaleraldehyde	$(CH_3)_2CHCH_2CHO$	0.072	Fruit, apple
	Acetone*	CH_3COCH_3	1.1-240	Fruit, sweet
	Butanone	$C_2H_5COCH_3$		Green apple

Source: (Dague, 1972; Henry and Gehr, 1980; U.S. Department of Health and Human Services, 1988; Le Cloirec *et al.,* 1994; Mackie et al., 1998; Gostelow, *et al*., 2001; Boon and Vincent, 2003; Son and Striebig, 2003) *The main chemical compounds that are associated with malodours in sewage (Le Cloirec *et al*., 1994; Boon and Vincent, 2003)

2.2 Sources of odours

Odour can arise from various sources but most of the sources are man-made (Government of India, 2008). For an odour to be perceived, it must first be transferred to the gas phase from the liquid phase. The presence of odourous compound in the liquid phase is vital to qualify a unit as an odour source. The conditions that stimulate mass transfer will as well have significant effect (Gostelow, *et al.,* 2001). Odour sources can be generically classified as follows: point, area, volume and fugitive.

2.2.1 Point sources

Point sources are usually the confined emissions points at which canalised odours escape to the atmosphere. The common confined emission points are vents, stacks and exhausts. The measurement of emission rate of odour in these sources is reported to be much easier but care is also needed in order to measure the average flow rate accurately. This is because velocity profiles within canals (ducts) change (Gostelow, *et al*., 2003; Government of India, 2008).

2.2.2 Area sources

Area sources are sources that do not have a well-defined airflow associated with them. These include sources like sewage treatment plant, wastewater treatment plant, solid waste landfill, composting, household manure spreading, settling lagoons etc. Diffusion processes are the ones that govern the emissions from the source. Thus, the concentration gradient provides the driving force for the movement of the odourous compound from the emission sources to the air. To prevent difficulties associated with emission rate measurements in area sources, special methods are employed. The commonly used methods are:

1) Micrometeorological methods: This is the method whereby emission rate is indirectly measured by sampling wind velocity and downwind odour concentration of the emission source. It is usually used in analytical measurements due to the fact that dispersion often lead to low concentration downwind of the source.

2) Hood methods: This is the method in which a hood is positioned on the emission surface and air is blown through it. The emission rate is hence given by airflow through the hood and the odour concentration of the outgoing air (Gostelow, *et al*., 2003).

2.2.3 Volume sources

Volume sources are referred to as building sources of odour like pig sheds and hog confinement chicken. They are different from stacks because they are likely to be many inlets and outlets to the buildings that are relatively unrestrained. Determination of building emission rates is problematic. However, ventilation rates measurements are achieved by the use of either anamometers at building's inlets and outlets or by the use of tracer gases such as carbon monoxide (CO), carbon dioxide (CO_2) and Sulfur Fluorine (SF_2). The previously mentioned mesurement methods can be used for both force and naturally ventilated buildings and for force-ventilated building, respectively (Gostelow *et al*., 2003).

2.2.4 Fugitive sources

Fugitive sources are sources that are difficult or elusive to identify and they release undefined amount of odourous compounds. These sources include odour emissions from soil bed or bio-filter surface, passive ventilation apertures, valve and flange leakage (Gostelow *et al*., 2003; Government of India, 2008).

Besides, the above sources can be either active or passive. Active odour source that those sources where an outward airflow is present, for instance, point sources such as stacks (Bockreis and Steinberg, 2005) whereas in the passive odour source, for instance, landfill surface, compost heaps, the airflow is absent (Frenchen, 1997).

2.3 Odour formation

Several compounds have been identified as odourous compounds associated with domestic wastewater and sludge as indicated in Table 2.1 in section 2.1.4. Classically, these compounds are volatile fatty acids (VFAs), sulphurous or nitrogenous compounds, aldehydes and ketones. The production of odourous compounds is a complex process that involves different types of bacteria. The mechanisms in microbiology and biochemistry of producing key odourous components in domestic wastewater and sludge are discussed in section 2.4.

Generally, odourous chemical compounds in sludge are classified either as organic or inorganic (Henry and Gerh, 1980). There are mainly produced by biological activity through either aerobic or anaerobic decomposition of proteins and carbohydrates in sludge (Son and Striebig, 2003). But then again, according to Dincer and Muezzinoglu (2008) majority of the odourous compounds in wastewater, collecting systems and treatment facilities are because

of anaerobic decomposition of organic matter, which contains sulphur and nitrogen. Sewer gases are mainly comprised of H_2S, NH_3, CO_2 and CH_4. H_2S and NH_3 are the two main inorganic compounds that are strongly malodourous which arise from anaerobic decomposition of nitrogen and sulphur containing compounds. The remaining compounds are odourless (Gague, 1972; Devai and DeLaune, 1999; Dincer and Muezzinoglu, 2008) as earlier indicated in section 2.1.4.

However, other highly malodourous compounds such as mercaptans and other organic sulphides (Muezzinoglu, 2003) and amines such as indole and skatole (Young, 1984) are found at lower concentrations. The organic compounds include organic acids, aldehydes and ketones, which are metabolic products, might be in existence either as single or as a combination of compounds in a given collection of odourous organics (Dague, 1972; Henry and Gerh, 1980). The other organic compounds are ammonia derivatives, amines, sulphur compounds (mercaptans) and sulfur and nitrogen bearing compounds (indole, skatole) (Hartman and Long, 1975 in Henry and Gerh, 1980).

2.4 Microbial processes related to odour formation

Odour formation is a phenomenon in which microorganisms (bacteria) referred to as heterotrophic bacteria transform organic matter through biochemical processes. In these processes, the heterotrophic biomass uses organic matter as carbon source for development of cell materials and as an electron donor (i.e. energy source required for the growth process and maintenance) through anabolic and catabolic processes, respectively. The energy accumulated in the organic matter is provided to the microorganisms through catabolic process in which the organic matter is oxidised hence acting as the electron donor (Hvitved-Jacobsen and Volertsen, 2001). The corresponding electron acceptor compounds, which are reduced, (e.g. dissolved oxygen in an aerobic process or nitrate in an anoxic process or sulphate in an anaerobic process) determines the form of reactions that occur and the resultant products of the reaction (Hvitved-Jacobsen and Volertsen, 2001). In the presence of oxygen, the organic matter is oxidised to carbon dioxide and water as aerobic metabolism is promoted (Yang and Hobson, 2001). =). When oxygen is depleted, nitrate is utilised as an alternative electron acceptor through anoxic metabolism and the end-products are nitrogen, carbon dioxide as well as alkalinity. Sulphate and carbon can also be used as electron acceptors when both oxygen and nitrate are used up or absent giving rise to H_2S, mercaptans, volatile fatty acids (VFAs), and other reduced organic compounds. The products of this process are generally malodourous in nature (Yang and Hobson, 2001).

The intensity of odour of wastewater is dependent upon the presence or absence of dissolved oxygen. The presence of dissolved oxygen in the wastewater indicates that it is fresh. The absence of dissolved oxygen shows staleness of the wastewater. Wastewater becomes septic if the microbial activity takes place in the absence of molecular oxygen and nitrate. The septicity is the least desirable condition because it is the favourable for the production of sulfide and other malodourous compounds that give rise to odour problems (Boon and Vincent, 2003).. It is important to note that not all odours are produced in wastewater because of chemical and biological activity but may also be added from industrial or other sources. Alcohols, aldehydes, ethers, sulphides, amines may be some of the added chemicals or any other odourous compounds (Dague, 1972).

Odours are mainly derivatives of the anaerobic digestion of organic material not including the volatilisation of ammonia (Sohn, 2005). The microbial conversion of organic material into carbon dioxide and methane provides a source of energy and building materials to the bacterial cells (Cimochowicz-Rybicka, undated). Digestion of carbohydrates produces alcohols, VFAs, ketones and aldehydes whereas the digestion of fats and proteins produces NH_3. H_2S is the product of protein degradation (Fang *et al*., 2012). The production of much high concentration of odourous compounds are also highly associated with the depletion of oxygen and the activity of anaerobic bacteria in the wastewater treatment plants (WEF, 2008). In this review, therefore, anaerobic digestion of organic matter is discussed in order to understand how some odourous substances are generated in relation to anaerobic condition. Organic matter is heterogeneous in nature comprised of such compounds as carbohydrates and sugars, proteins, fats, hemicelluloses, celluloses, and lignin. These materials are elementary chemical building blocks (Nalivata, 2007).

Anaerobic digestion of organic matter process encompasses a number of conversions of the available macromolecules by a mixed consortium on different species of symbiotic microorganisms under oxygen-free conditions into biogas such methane (60%) and carbon dioxide (40%) (Gujer and Zehnder 1983; Pavlosthatis and Girado-Gomez, 1991; Wilkie, 2005) along with some odourous gases, NH_3 and H_2Sthat contribute minimally to the overall odour intensity (Nahm, 2003). The process has many environmental benefits including odour reduction.

However, this is not the case always because instead of methane and carbon dioxide, which are both odourless, as final products of microbial degradation of organic material, other odourous compounds are produced. This is because in stored organic matter the rate of methane production is low such that there is more accumulation of volatile malodourous

intermediates (phenols, indoles and volatile fatty acids). This is because of imbalance between acididogenesis and methanogenesis steps discussed below in the microbial degradation of stored organic matter. Therefore, the production of malodourous compounds is based on this imbalance. In other words, under balanced conditions, odourous compounds are not produced as the VOCs are converted to methane and carbon dioxide (Wilkie, 2005). Incomplete degradation of stored organic material in oxygen free condition are also reported by Nahm (2003) that are responsible for production and accumulation of malodourous intermediate compounds if the populations of bacteria that degrade these compounds are not sufficient. Six different reaction processes have been identified in the degradation of particulate organic material to methane. These six processes are (Doku, 2002; Koffour, 2010):

1) Hydrolysis of particulate organic matter.

a. Hydrolysis of proteins

b. Hydrolysis of carbohydrates

c. Hydrolysis of lipids

2) Fermentation of amino acids.

3) Anaerobic oxidation of long chain fatty acids (LCFAs) and alcohols.

4) Anaerobic oxidation of intermediate products such as volatile acids (excluding acetate).

5) Conversion of acetate to methane.

6) Conversion of hydrogen to methane.

The above distinct reaction processes are distinguished in four main sequential process phases which involve:

- Hydrolysis
- Acididogenesis
- Actogenesis
- Methanogenesis (Cimochowicz-Rybicka, undated; Vavilin *et al*., 2008; Kuffour, 2010)

and are described below in this section and Figure 2.1 shows the schematic representation of the anaerobic digestion.

Hydrolysis

Hydrolysis is broadly defined as a chemical compound decomposition in which an organic material, RX, reacts with water, resulting in the formation of a new covalent bond with OH and

cleavage of chemical bond with X (the leaving group) in the original material. The two products formed, one will have H and the other OH. The general formula of a hydrolysis reaction that follows is (Larson and Weber, 1994):

$RX + H_2O \rightarrow ROH + X^- + H^+$

Hydrolysis is the first stage in which microbial group composed of hydrolytic fermentative bacteria with the help of their excreted extracellular enzymes, breakdown solid complex organic materials, biopolymers such as protein, carbohydrates, lignins and lipids into monomers or dimeric compounds, which are dissolved compounds of a lower molecular weight. Proteins are converted into amino acids by exoenzymes, protease or peptidases (Suwannoppadol, 2012). Carbohydrates are degraded into sugars (mono and disaccharides) and lipids are transformed to organic long chain fatty acids (LCFAs) and glycerine. Other products are alcohols, CO_2 and NH_3 (Cimochowicz-Rybicka, undated; WEF, 2008; Kuffour, 2010). Acccording to Suertz and Frechen (2001) during hydrolysis, sulphur bearing amino acids, cysteine and methionine i.e. proteinaceous material, are converted to H_2S, organic sulphides and disulphides such as ethyl mercaptan (ethanethiol), dimethyl sulphide and methyl disulphide. The hydrolytic fermentative bacteria that are involved in the process are of the genera *Bacteroides, Clostridium, Butyrivibro, Eubacterium, Bifidobacterium, Lactobacilllus* (Suwannoppadol, 2012) and *Proteus* (Suertz and Frechen, 2001). Hydrolysis is considered the rate-limiting step in the anaerobic digestion process (BaniHani, 2009).

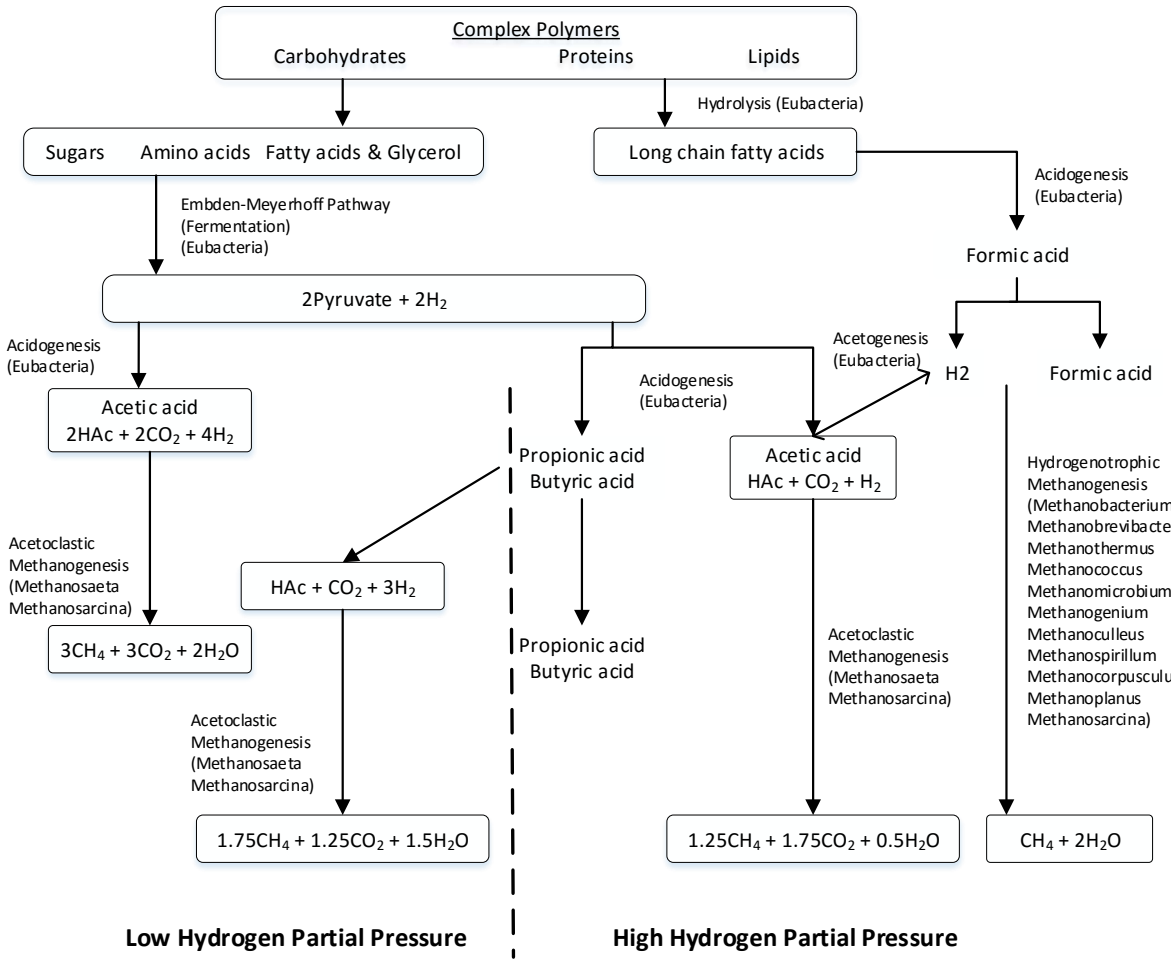


Figure 2.1: Schematic representation of anaerobic digestion process
(Adopted from Torondel, 2010)

Acididogenesis

Acididogenesis, also known as the acidification, due to a number of organic acids are produced (Kuffour, 2010), is the second stage in which another very diverse group of microorganisms (Santosh *et al*., 2004), fermentative bacteria, of which the majority are strict anaerobes are involved. These anaerobes can also use the dissolved oxygen to metabolise organic components down to acidic condition of pH of around 4 although the availability of oxygen or nitrate can be noxious (van Haandel and van der Lubbe, 2007). In this process, the hydrolysed products are converted to numerous short chain organic acids such as butyric acid, propionic acid, acetic acid (Buysman, 2009), lactic acid while alcohols (for instance, ethanol, methanol, and glycerol) and ketones (for instance, acetone), acetate (Kuffour, 2010) and aldehydes and gases such as CO_2, H_2, NH_3 (van Haandel and van der Lubbe, 2007) and H_2S (Kuffour, 2010) are also formed. Other volatile acids that are produced although they are in smaller concentrations include formic, valeric, isovaleric, and capronic acids (Lue-Hing *et al*., 1998).

Acetogenesis

Acetogenesis is the third stage in which the other products of acidogenesis i.e. the propionic acid, butyric acid and alcohols (for instance, ethanol) are converted to hydrogen, carbon dioxide and acetic acid (Mata-Alvarez, 2003) by related microorganisms, obligate H_2 – producing acetogenic bacteria such as *Syntrophobacter wolinii*, a propionate decomposer, *Sytrophomonos wolfei*, a butyrate decomposer etc. In this stage, ethanol and lactate ($CH_3CH(OH)COO^-$) are also transformed to acetate ($C_2H_3O_2^-$) and H_2 by an acetogen and *Clostirridium formicoaceticum*, respectively (FAO, 1997). The presence of hydrogen is of great significance in the conversion of propionic acid and butyric acid because the reactions involved are endothermic, therefore, at low partial pressure of hydrogen the thermodynamic feasibility of this reaction is ensured, of course, with the presence of hydrogen scavenging bacteria that consume hydrogen (Ostrem, 2004; Guevara, 2012;).

Methanogenesis

Methanogenesis is the last stage of the anaerobic digestion process in which the acid fermentation products are converted to methane and carbon dioxide (Guevara, 2012). About 70% of the total methane produced is derived from acetic acid or fermentation of alcohols such as methanol formed in the preceding stages. The reminder of the produced methane is mainly generated from the reduction of carbon dioxide by hydrogen. Methanogenesis of carbon dioxide and hydrogen is of critical importance because it keeps the hydrogen pressure low thereby supporting the anaerobic oxidation of VFAs to acetate and hydrogen. This is possible because of the symbiotic relationship that exists between the microorganisms in the acetogenesis and methanogenesis stages as the methanogens utilise the metabolic products of the actogenic stage hence lowering the hydrogen partial pressure in the media.

There are two different types of dominant microorganisms that are involved in this anaerobic digestion stage. These are the acetoclastic methanogens such as *Methanosarcina, Mathanosaeta* (*Methanothrix*) which convert acetate to methane while the hydrogenotrophic archea uses hydrogen as electron donor and carbon dioxide as the electron acceptor to produce methane (BaniHani, 2009; Arsova, 2010). Even though anaerobic digestion has four distinct process stages, all the processes take place concurrently and synergistically (Arsova, 2010). Generally, the ideal situations for anaerobic digestion of organic material to occur are pH close to 7, constant temperature, and a relatively steady feeding rate (CIWMB, 2008).

2.5 Human excreta odourous compounds

Human excreta, which are comprised of faeces and urine, are the main input of pit latrine contents. This is much as it is appreciated that other inputs may also contribute significantly depending on local practices (Graham and Polizzotto, 2013). A number of studies have been conducted to identify and quantify human excreta malodourous compounds. The types and quantities of odourous compounds in human excreta differ from individual to individual dependent upon their diets, health condition (Sato *et al*., 2002; Garner *et al*., 2007; De Preter *et al*., 2009), genetics, cultural dynamics and nature of gut flora (Hammond, 2013). In a study of analysis of volatile substances of human waste responsible for malodour in community wastewater treatment, Sato *et al.* (2001) found that around 90% of the volatile substances which included the fatty acids, acetic acid (65%), propionic acid (15%), butyric acid (6.5%), *iso*-valeric acid (2.3%), and *n*-valeric acid (1.4%). The other minor substances that were identified include nitrogen compounds such as indole, skatole, pyridine, and pyrrole and their contents were 0.31%, 0.55%, 0.14%, and 0.01%, respectively. The proportion of ammonia was 6.5% of malodourous compounds. The sulphur compounds such as hydrogen sulphide and methyl mercaptan were also identified and their contents were 1.6% and 0.62%, respectively. Sato *et al*., (2002) also analysed malodourous substances of human faeces immediately after the use of a western style toilet, flush toilet. The study investigated the types and concentrations malodorous compounds of individuals dependent upon the food they had consumed and their health status. The study revealed that acetic acid, propionic acid, butyric acid, iso-valeric acid, pyridine and pyrrole had average concentrations (ppb) of 3, 3, 0.16, 0.03, 0.02, 6 and 2, respectively, for health individuals. The average concentrations for individuals who had diarrhoea for acetic acid, propionic acid, butyric acid, iso-valeric acid, pyridine and pyrrole had concentrations (ppb) of 549, 3.1, 2.5, 0.32, 0.84, 0.15 and 0.02, respectively. Additionally, the study revealed that hydrogen sulphide and methyl mercaptan in human faeces of health individuals had concentrations 7.4 and 0.5, respectively while an individual with diarrhoea; the human faeces had hydrogen sulphide and methyl mercaptan with concentrations (ppb) of 25.5 and 14.6, respectively. The individuals with mild cold, the human faeces had hydrogen sulphide and methyl mercaptan with concentrations (ppb) of 6.0 and 2.7, respectively.

Lin *et al*. (2013) investigated the chemical composition of latrine malodours from human wastes in Kenya, Uganda, South Africa and India. The countries were of different climatic conditions and the toilet designs were also different. The results revealed that the climatic conditions have less influence on emitted odour composition than the toilet design. Sulphur compounds such as H_2S, methyl mercaptan and dimethyl-monosulfide were odourous compounds that were detected under anaerobic conditions whereas p-Cresol and indole along

with short chain fatty acids (SCFAs) such as butyric acid were detected under aerobic conditions. From the literature above the knowledge about changes over time and the composition of odours between pit latrines is still restricted.

2.6 Odour measurement

It is generally recognised that knowledge of the chemical composition of odours emanating from human waste is a foundation for the creation of appropriate technologies that can prevent, eliminate or offset the unpleasant odours (Sato *et al*., 2001; Lin *et al*, 2013). In order to measure odour there are four different dimensions, which characterise it that need to be considered. These include: character; intensity, concentration and hedonic tone. A clear understanding of these odour dimensions is required in order to understand odour measurements and to fully define an odour.

Character

Character is fundamentally what the odour smells like. It is generally subjective but it allows one to distinguish one odour from another. For instance, thiocresol ($CH_3C_6H_4.SH$) has a rancid, skunk-like odour (Gostelow *et al*., 2001; Department of Environmental Protection, Western Australia, 2002). However, the inherent subjectivity of the human sense of smell creates some challenges to draw a distinction between the specific odour characters (Naddeo *et al*, 2013). The character of an odour may change with concentration level through dilution (Gostelow *et al*., 2001; Department of Environmental Protection, Western Australia, 2002). However, each odourous compound has its distinctive odour character such that even if present at the same concentration may have significantly dissimilar odour impacts (Brewer *et al.,* undated). This attribute is helpful in establishing an odour source as well as providing the likelihood of recognising the core chemical constituents of the specific odour based on odour complainants' description (Naddeo *et al.,* 2013).

Intensity

Intensity is referred to as the perceived magnitude of odour above its detection threshold. It is determined by an odour panel (a group of indviduals that are appropiately screened and trained in odour testing) and is described in subjective category scale as the chemical concentration level increases from "faint", then "moderate", through to "strong". Alternatively, it can be assessed according to descriptor scales such as:

0 =not perceptible;

1 = very weak;

2 = weak;

3 = distinct;

4 =strong;

5 =very strong;

6 =extremely strong

Nevertheless, it is important to recognise that these scales are ordinal. This suggests that the differences between the values are not likely to be equal. For instance, an odour with an intensity of 4 is not necessarily two times as odourous as an odour of intensity of 2 (Gastelow *et. al*, 2001). The interdependence of this odour dimension (*I*) and odour concentration (*C*) is well described by Steven Power Law as psychophysical power function as shown in equation 2.3 (Lewis *et al*., undated):

$$I = kC^n \quad (2.3)$$

where:

I is the odour intensity;

C is the odourant concentration (empirically determined);

k and n are constants that differ for each odour;

The Stevens exponential 'n' usually lies between 0.3 and 0.8 and can be determined by measuring the perceived odour intensity of a sample under consideration as a function of the sample dilution factor or sample concentration. This law states that the apparent magnitude of intensity grows as a power function of stimulus magnitude. This means that equal ratio changes in sensation strength is directly proportional to equal changes in the stimulus strength (Brewer and Cadwallader, undated; Gostelow *et al*., 2001; Department of Environmental Protection, Western Australia, 2002). Odour intensity is the odour parameter that has received the most attention in quantification of odour nuisance problems for both researches as well as for regulatory purposes (Mackie *et al*., 1998).

Concentration

The concentration of odour generally relates to the numerical dimension of individual odourous material (Naddeo *et al*., 2013). This is usually quantified using either analytical or mixed or sensor- instrumental techniques (Zarra *et al*., 2013) which have been detailed in section 2.7. When the sensorial techniques of dynamic olfactometry is used the concentration is expressed in terms of the number of times the sample has to be diluted to reach its odour perceptibility or threshold of detection (OT) by at least 50% of pre-selected sensory panel members sniffing the sample (Misselbrook, *et al*., 1992; Naddeo *et al.,* 2013). This may be expressed as the number of European Odour Units (OU_E) in a cubic metre ($1OU_E/m^3$ or $1OU/m^3$) of gas at standard conditions with reference to the entire sample i.e. a mixture of odourous compounds

(EPA, 2001; Gostelow *et al*., 2001; Naddeo *et al*., 2013). The concentration is expressed in ppm or ppb or µg/m^3 with reference to individual odourous compounds in the odourous gas sample when the analytical technique is used for determination (Naddeo *et al*., 2013). In sensorial technique of olfactometry, threshold concentrations are normally delineated into three types (Naddeo *et al*., 2013):

- *Perceptibility or detection threshold concentration*: This is referred to as the lowest concentration of a certain odour compound at which a noticeable change in the odour system is perceived within the controlled conditions of an odour laboratory.
- *Recognition threshold concentration*: This is referred to as the minimum concentration of an odour compound at which the odour becomes recognisable.
- *Annoyance threshold concentration*: This is referred to as the concentration of odour compounds cause population annoyance

But two dissimilar odourous compounds with same perceptibility and be existent in an air sample at the same concentration will exhibit smells of very different intensities (Naddeo *et al.,* 2013).

There is no linear relationship between odour concentration and its consequential intensity. This relationship is well represented by Weber-Fechner law (Gostelow, 2001):

$$I = a \ logC \ \text{x} \ b$$

where

I is the intensity;

C is the odourant concentration;

a and b are the constants.

According to Misselbrook *et al*. (1992), in their study in which they examined the relationship between the intensity and concentration of an odour for pig slurry and broiler houses, they concluded that these relationships could be important in determining the required reduction of odour to acceptable levels and the effectiveness of odour control measures. The authors further concluded that the relationships could also be used to determine the minimum distances between the source of odour and potential complaints when used in combination with dispersion models.

Dravnieks and Prokop (1975) and Leonardos *et al*. (2012) indicated that the odour threshold measurement can be affected by six (6) variables. These variables include the method of presentation of the stimulus to the panellist, the effect of extraneous odourants in the

presentation system, the kind of panellist used, the description of the odour response requested, the treatment of the data obtained, and the chemical purity of the odourant.

Hedonic Tone

Hedonic tone is the extent to which an odour is perceived as either pleasant or unpleasant and hence it is a measure of acceptability (Naddeo *et al*., 2013). For instance, odours from perfumes are mostly regarded as pleasant while the odours from sewers are generally regarded as unpleasant. The hedonic odour tone of the perceived concentration uses a nine-level category scale representing most pleasant at one end and the most unpleasant at the other (Gostelow *et al*, 2001) ranging from low score, negative such as -4 (very unpleasant through zero (neutral odour or no odour) to high score, positive such as +4 (very pleasant) (Frechen, 1997) as presented in Table 2.2. Similar to other odour parameters such as odour character and odour intensity, it has a certain degree of subjectivity influenced, *inter alia,* by such factors as previous experience and circumstances of the individuals at the time of odour perception (Department of Environmental Protection, Western Australia, 2002; Naddeo *et al.,* 2013). Hedonic tone is an extremely important part of the olfactory experience because an inoffensive odour to one person may be offensive or nauseating to another (Feddes and Edeogu, undated).

Table 2.2: Qualitative category scale range used by panellist for an odour sample to an offensiveness scale

Score	Perceived hedonic tone
+4	Very pleasant
+3	Pleasant
+2	Moderately pleasant
+1	Mildly pleasant
0	Neutral odour/no odour
-1	Mildly unpleasant
-2	Moderately unpleasant
-3	Unpleasant
-4	Very unpleasant

Source: DEFRA, 2010

As is the case with relationship between odour concentration and the intensity of an odour, there are also a quite few relationships which exist between odour character, odour intensity and hedonic tone. The character of the odour influences the odour hedonic tone. The odour intensity influences the odour character as well as the hedonic tone of the odour (Gostelow, *et al.*, 2003) and overall, the degree of unpleasantness rises as intensity falls (Mackie *et al*., 1998; Nimmermark, 2004) and also increases with increasing concentrations of the odorants (Frenchen, 1997) in the same sample presented to the odour assessors.

Concentration is the only odour parameter that can be objectively determined whereas the others (intensity, hedonic tone and character) are determined in a highly subjective manner (Naddeo *et al*., 2013) as described in section 2.7.

2.7 Overview of odour measurement techniques

The techniques that are available for odour identification and quantification are fundamentally of three (3) different categories (Gostelow, *et al*., 2001):

- Sensorial: dynamic olfactometry;
- Analytical: chemical analyses; and
- Senso-instrumental: electronic nose

2.7.1 Sensory measurement techniques

Sensorial techniques use the human nose for detecting odour (Gostelow, *et al*., 2001), and generally, in form of a panel of trained assessors (Rappert and Müller, 2005). The techniques measure the perceived effect of the odourous compounds on the olfaction (Gostelow and Parsons, 2001). The perceptions of odour are influenced by a number of individual cognitive factors.Hence, human subjects as measuring instruments are;

(1) quite variable over time,

(2) variable among themselves in terms of sense of smell,

(3) highly subjective in odour perception,

(4) inurement, which causes a short-term reduction in olfactic sensitivity during exposure to a stimulus and

(5) variations in such climatic conditions as temperature, humidity and wind speed, when measuring under field conditions, as well as effects of age, gender, health and personal habits on individual panellist olfaction.

To account adequately for these shortcomings to a certain extent requires;

(1) Several panellists are used so that the results are representative and they are expressed by some measures of central tendency of the individual panellists,

(2) Measurements are repeated, but excessive care must be taken in the presentation of samples and

(3) Panellists should respect the rules and procedures that govern their attitudes as members of the panel (Mackie *et al*, 1998; Gostelow, *et al*., 2001; Davoli, *et al*., 2003; Meilgaard, *et al*., 2007). Above all, the complexity of odourant mixtures, the interactions among dissimilar odours, makes odour perception a difficult event to be understood (Davoli *et al.,* 2003) and odour monitoring of time-dependent processes is not feasible with human sensory system (Yuwono and Lammers, 2004).

The other factors with pronounced effects that also have to be significantly considered are the atmosphere in which the evaluations are undertaken, the order in which the samples are presented and the volume flow rate of the stimulus at the nose (Gostelow *et al.,* 2001). Duffee and Cha (1980) reported that a 100- times variation in volume flow rate of the stimulus can result into at least a 1000-times variation in the reported dilution ratio or suprathreshold intensity of the same odourous compound by the same panellists. Therefore, standardisation in relation to some stable, central measure of human odour perception, volume flow rate of stimulus at the nose and the order in which the sample is presented, are highly commended. This allows odour measurements of by one panellist to be comparable with those of any other (Koe *et al.,* 1986). Sensory measurement techniques can be categorised into two (Bratolli *et al*., 2011):

1) Parametric measurements
2) Quantitative measurements in which the nose is coupled with some forms of dilution instrument.

2.7.2 Parametric sensorial measurement technique

The nose is used without any other equipment in this technique. The human nose has the ability to detect very low concentrations of odourous compounds (Hwang, *et al.,* 1995). The advantage of this technique is that the measurements are quickly obtained at relatively low cost as no particular device is needed. However, a particular care has to be taken for results interpretation because sensitivity to odour between observers in a panel varies from one panellist to another, even for opportunely trained panellists. This, therefore, renders the interpretation of results difficult and subjective (Gostelow, *et al*., 2001; Bratolli *et al*., 2011). The dimensions of odour, which may be subjectively measured, as earlier discussed in section 2.6 include odour character, hedonic tone and perceived odour intensity. Certainly, there is no objective technique but electronic nose that is employed to measure odour character and hedonic tone (Gostelow *et al*., 2001).

2.7.3 Sensorial: Dynamic olfactometry technique

The technique is based on the sensory odour evaluation method that allows people to determine the odour concentration of an odourous air sample through a panel of appropriately screened and trained people (Capelli *et al.,* 2009; Baltrėnas *et al*., 2013). The odour concentration is expressed in OUE/m^3 of air, and it represents the number of dilutions with reference non-odourous air required to make the sample concentration equal to its perception threshold concentration, also termed odour threshold detectability (Rappert and Müller, 2005; Capelli *et al.,* 2009). This implies that the higher the concentration of the odourous compound,

the more the dilutions will be required to reach for the odourous compound to become undetectable. For single pure compounds in air, the concentration at which it becomes undetectable is expressed as in typical concentration units (e.g., mg/m^3), whereas the mixture of odourous compounds, which is odour is mostly made of, cannot be expressed in such units but expressed as odour units (OU) (Nicell, 2009).

The human nose does not do the measurement in isolation but with the help of a device called Olfactometer (Bratolli *et al*., 2011). Generally, there are two kinds of olfactometers that are commonly used. These include dynamic and static olfactometers. Dynamic olfactometer delivers to the panellist a prescribed amount of odourous compound in a less humid air background.In static system, the panellist is given a well-regulated gas flow of odourous compound mixed in humid air of approximately 100% relative humidity (Acree, 1997). Dynamic dilution is better than static dilution because the effects of odourous sample adsorption to the internal surface of the instruments used are insignificant (Gostelow *et al*., 2003). The static olfactometers are mostly used for research and commercial purposes, as they are simple, portable and standard (Acree, 1997). The Olfactometer monitors the progressive dilution of an odourous air sample with reference non-odourous air, according to precise ratio for presentation to a panel of odour observers at a controlled flow rate, in order to determine odour concentrations (Dravnieks and Jarke, 1980; AWMA EE-6 Subcommittee on the Standardization of Odour Measurement, 2002). The estimations are often done in a specially prepared atmosphere, which is carefully purified (Littarru, 2007). “The odour concentration is calculated as the geometric mean of the odour threshold values of each assessor in the panel, multiplied by a factor that depends on the olfactometer dilution step factor” (Capelli, *et al.,* 2009). The geometric mean is used because it takes into account the logarithmic relationship between odour intensity and odour concentration (Stevens, 1960). For any panelist, the apparent concentration of odour in an air sample is reflected by the number of dilutions with reference non-odourous air needed to render the odour perceivable. In a continuously diluted system, this is mathematically expressed as equation 2.4 (Koe *et al*., 1986):

$$C_a = (Q_o + Q_f)/Q_o \qquad (2.4)$$

where C_a is the apparent odour concentration,
Q_o (m^3/s) is the flow rate of odourous sample and
Q_f (m^3/s) is the reference non-odourous flow rate.

The equation 2.4 shows that the apparent concentration is dimensionless but it is expressed as apparent odour units per unit volume (aou/m^3). This is because this allows odour-emitting

sources to be measured in terms of emission rates expressed in odour units per unit time. However, for sources whose odour is emitted from either unknown odourous chemical compound or from mixture of odourous air, no other basis for such quantification is currently available (Koe *et al*., 1986). The olfactometry technique allows the assessment of a mixture of various odourous compounds and determination of the odour threshold of the single chemical compound (Litarru, 2007).

2.7.4 Analytical techniques

Analytical techniques, which deal with the properties of the odourous compounds, (Gostelow and Parsons, 2001) allow both the identification and quantification of odourous compounds responsible for odour emissions. The advantages of the techniques are that they are accurate, repeatable and objective and they relate directly to odour formation or emissions theoretical models (Gostelow *et al*., 2001; Mun˜oz *et al*., 2010). The drawbacks of the analytical measurements are that they are not sensitive enough to completely identify and quantify the odourous compounds that are present at concentrations too low to be analysed (Wolkoff and Nielsen, 2001). Moreover, the sampling methods greatly impact the analysis method. This can be subject to whether the source was areal or point, active or passive type (Gostelow *et al*., 2001; Naddeo *et al*., 2012) as discussed in section 2.2. The two most widely used techniques: gas chromatography (GC), and electrochemical sensors.

2.7.4.1 Gas chromatography

GC is a highly accurate separation method where individual odourous compounds of a mixture are readily separated and distinguished from each other in a chromatography column (Hu *et al*., 2014). This method coupled with various detectors, specifically Mass Spectrometry (MS), has been extensively used for characterisation of odour chemical compound or composition of odourous air quality (Dincer *et al*., 2006). The MS is the most effective method for the identification of unknown odour compounds of an odour (Hobbs, 2001) because most environmental odours are mixtures (Gostelow *et al*., 2003). The GC, a basic research tool for odour analysis (Rappert and Müller, 2005), is applied in order to rapidly separate the odourous components present in odourous air for qualitative or quantitative measurement of odour composition. If these odourous components are unknown or the mixture is complicated, this is followed by MS and which identifies and quantifies them (Gostelow *et al*., 2001) and electronic library of compounds is necessary (Rappert and Müller, 2005).

GC uses the principle of differential absorption in which different molecules of odourous air samples emerge from the end of the column at different times range. This, therefore, means that great care is needed when it comes to sample collection, choice of chromatography column and detector due to complexity of the technique (Gostelow *et al*., 2003). The technique

primarily makes use of a carrier gas that passes over a stationery phase for which the volatile components have differential affinities, which influence the separation of the odour components (Hobbs, 2001). The advantages of the technique are that (Gostelow *et al*., 2003):

- Can be used for qualitative as well as quantitative analysis of complex odour samples;
- Unknown odour components are identified with the use of appropriate detectors;
- It allows sensory measurements to be conducted by diverting the chromatography eluent to a sniffing port of olfactometer, otherwise well-known as Gas Chromatography- Olfactometry (GC-O).

The technique does, however, have a number of drawbacks which include:

- It is dependent on the complexity of odours that are often at lower concentrations beyond the instrumental detection limit.
- It is expensive and time consuming as there are many separate analytical steps that are involved i.e. sampling, sample transportation, pre-concentration process, separation, detection, data transmission and post data analysis (Bratolli *et al.,* 2011; Li, 2014).
- It is not possible to carry out the analysis on-site that is required for rapid detection in many real-world applications. This, therefore, demands sample collection and storage (; Gostelow *et al*., 2003; Li, 2014).
- It requires pre-concentration of odourous air samples (Gostelow *et al*, 2003).
- It does not represent the experiences of human odour perception that result from numerous odourous components (Gralapp *et al*., 2001; Bratolli *et al.,* 2011).

Nonetheless, some efforts have been made in order to investigate the behaviour of odour compounds in a mixture and the possible masking occurrences that may take place to overcome the above shortcomings as well as to evaluate the linkage between the instrumental and olfactometric techniques (Bratolli *et al.*, 2011).

2.7.4.2 Electronic nose

Electronic nose is the recently developed alternative technique of measuring odours, which is currently utilised in order to overcome the shortcomings associated with olfactometric, and other analytical techniques (Gostelow *et al*., 2001; Arshak, *et al*., 2004). This device is comprised of an electronic gas sensor array with different selectivities, a data analysis and signal-processing system for feature extraction and significant information and a pattern of recognition system. It is capable of measuring and characterizing the chemicals that humans perceive as odours and registers numerical results (Grallap *et al*., 2001; Pan and Yang, 2007;

Bratolli *et al*., 2011; Chen *et al*., 2013). The technology is the instrumental methodology being used in an attempt to mimic the mammalian sense of smell by using an array of sensors in order to get repeatable measurements, allowing identifications and discriminations of odour mixtures while eliminating the human odour fatigue (Arshak *et al*., 2004; Wilson and Baietto, 2009).

The instrument generates a distinct digital array of responses for dissimilar types of odour air samples that can be recognised by comparing it with previously recorded patterns in recognition system (Grallap *et al*., 2001; Chen *et al*., 2013). A range of sensor technologies have been employed in electronic noses but the most common are metal oxide sensors (MOS), conducting polymer sensors (CPS), quartz crystal microbalances (QCM) surface acoustic wave sensors (SAWS) and piezo-electric sensors (Gostelow, 2001; Stuetz and Fenner, 2001). The individual sensors have a specific characteristic response and some of the sensors overlap and are sensitive to chemicals that are alike, the same as the receptors of the mammalian nose. An individual sensor on its own is partially responsive to a wide variety of chemicals and more responsive to a narrow variety of compounds. A collection of sensors is responsive to numerous and different types of chemicals, with particular sensors in the array being moderately to extremely sensitive to particular compounds (Grallap *et al*., 2001). The distinctiveness of a simple or complex mixture represented by an electronic finger print pattern may be determined without having the mixture in its separate components before or during analysis (Wilson and Baietto, 2009).

The advantages of the use of an electronic nose over other odour measurement techniques are that (Littarru, 2006; Bratolli *et al.,* 2011):

- It is relatively cheaper;
- It is fast in implementation;
- It does not require extraction from absorbent support;
- The problems of synergic effects of mixed odourous components are circumvented;
- It can accommodate real-time performance *in situ* when executed in portable form.

On the contrary, the technique requires calibration against olfactometric measurements and it does not give results in terms of odour concentration, measurable through olfactometric analysis using mammalian nose (Gostelow *et al*., 2001; Littarru, 2006). Nevertheless, there is still need for further improvements of the technique's detection sensitivity and specificity in order to attain a level that is equal to the ability of the human nose (Li, 2014) for odour detection.

An electronic nose has been widely applied in many industries including; indoor air quality, health care, safety, security, quality control of food and beverages, disease diagnosis etc. However, in its application for environmental analysis, it has been used for odour monitoring in measurement of odours in factories, sewage, farms, waste sites, agriculture activities, inside buildings as well as in work places to preserve the health of workers (Bratolli et al., 2011).

2.8 Current odour control approaches in low-income countries

A number of prevention and treatment strategies are used to attenuate or eliminate pit latrine odour emissions in the low-income countries. These include:

2.8.1 Ventilated improved pit latrines

The VIP latrine is designed to control some of the common operational difficulties (ie odours and flies nuisance) with simple pit latrine designs (Mara, 1984). Unlike the simple pit latrine, the VIP latrine has a substantial superstructure and a tall vertical vent pipe of appropriate dimensions, fitted with a fly screen at its top, from the pit to the above roof of the latrine superstructure (Mara, 1984; Ryan and Mara, 1983 Otti, 2011; Obeg, 2015;). This, however, makes it more costly compared to the simple pit latrine due to additional cost of providing the vent pipe and the required full superstructure (Franceys *et al*., 1992)

The vent pipe, if properly designed and used, eliminates unpleasant smells and flies. This offers great improvement that makes the VIP latrine superior to traditional pit latrine (Ryan and Mara, 1983 b). The design of the VIP is in such a way that when air flows across the top of the vent pipe, it causes air to be continuously carried upward the pipe from the pit and fresh air is drawn into the pit through the latrine squat hole. The removal of odours from the latrine pit is by the chimney effect which aids adequate ventilation (Ryan and Mara, 1983a; Ryan and Mara, 1983b). Unpleasant odorous air from the pit thus pass through the vent pipe and do not enter the interior of the latrine superstructure (Mara, 1984). Inoder to take advantage of a draught passing across the vent pipe, the top of the vent pipe are extended about 500 mm above flat or sloping roofs or to the apex of conical roofs (Ryan and Mara, 1983). The location of VIP latrines is important: unless a clear flow of air is maintained across the top of the vent, otherwise the ventilation system may not be effective. VIP latrines should therefore be sited away from obstacles such as trees or high buildings that may limit airflow leading to unpleasant odours (Franceys *et al*, 1992).

Since the effectiveness of the ventilation pipes depends on the local wind speed and direction it is not known how effective are the VIP latrines in densely populated urban settings in which

the height of the vent pipes is inadequate. This usually because of the height and location of neighbouring buildings (Cotton *et al*., 1995). The obstruction of wind blowing incidence across the top of the vent and into the latrine superstructure reduces the chimney effect that is the dominant mechanism for reducing odour in VIP latrines.

VIP latrines may be very costly in particular for households with narrow financial resources base especially those at lowest sub-subsistence levels in terms of capital investment, without external financial assistance, (Pickford, 1994) because of a ventilation pipe and the full superstructure. The cost may be too high when set against their basic needs such as food, shelter and clothing for poor resource households (Franceys *et al*., 1992).

2.8.2 Addition of water

Addition of water in pit latrine maintains scrum layer, water seal of few millimetres, which is created on top of the human wastes liquid part of latrine. This safeguards odorous air from emitting into the atmosphere hence rendering the pit latrine odourless. However, odours are momentarily perceived from gases due to falling excreta. Also, this is not suitable in areas where the water seal might freeze due to cold weather (Mara, 1985; Chaggu, 2004).Further, the added water quite remarkably increases the filling up rate of the pit latrine and this also renders it difficult for the treatment of wastewater (Chaggu, 2004).

2.8.3 Addition of carboneous materials

Human faeces have comparatively small C/N ratio of 8 (Schönning and Stenström, 2004). This is much lower than the ideal ratio for compositing which is between 25 and 35(Bernal *et al*., 2009) Inorder to increase the C/N ratio the percentage of carbon has to be increased Schönning and Stenström, 2004). This is achieved by addition of carboneous materials in form of sawdust, organic household and garden wastes (Moe and Rheingans, 2006). The C/N ratio is the compositing process factor that defines the nutritional balance if aerobic decomposition is to be achieved (Mehl *et al*., 2011; Bernal *et al*., 2009).

Composting is a self-heating aerobic, biological process in which biodegradable organic wastes are decomposed or transformed to a humus-like product, which can be used as fertiliser, under the action of naturally occurring microorganisms. The process is effective in destroying microorganism due to increased temperatures, transforms nitrogen from unstable ammonia to stable organic forms, and decreases the waste volume (Zhu *et al*., 2004; Nigagaba *et al*., 2009).

The users are encouraged to add a mixture of desiccants after each use of the latrine. They usually add such dessicants as sawdust, wood ash, rice husks, dry leaves, dry grass, sand,

lime or dry dirt. These desicants apart from creating an interstitial air in the pit that facilitates a good compositing process/ aerobic digestion (Pranger *et al*., 2013). The carboneous materials also increase the pH levels that are the dorminant mechanism for destruction and inactivation (Mehl et al., 2011). Some dessicants as sand, ash and inert materials are non-degradable within the pit (Sugden, 2014) and as such, they present a number of problems to the households:

1. The frequency at which the biodegradable material over in the top layer and therefore deprived of oxygen resulting in deoxygenated environment that necessitates production of unpleasant smells due to reduced potential for aerobic digestion during the residence in the top layer.
2. The frequency of addition can also result in the pit to filling quicker than expected (Foxon, 2008).

Moreover, the desiccants reduce the moisture contents of the waste (Shönning and Stenström, 2004) but wastes retained in dry condition in which non-biodegradable anal cleansing materials are used tend to have high sludge accumulation rate of 90 litres per person per year. This implies that the pit fills too quickly. This is because the consolidation of the pit contents is generally poor (Franceys *et al*., 1992).

2.8.4 Urine separation

Urine- diverting toilet is a sanitation system that has two different outlets with two collection systems that are used for collecting and storing human waste fractions: one for urine, which is in front and another one, in the rear for feaces and anal cleansing material other than water (Münch and Dahm, 2009; Langergraber and Mullegger, 2012). The urine- diverting toilet can either be flushed, (these are known as UD flush toilets) or non-flushed (urine diverting dry toilets (UDDT)). These toilets can either be a mix of water and feaces or water and urine but not urine and feaces (Münch and Winker, 2009)

A substantial reduction of the offensive odours associated with ordinary pit latrines is achieved when the wastewater fractions of faeces and urine are collected and treated separately using a specially designed pedestal (Langergraber and Mullegger, 2012). This is the desiccation approach that is aimed at keeping the faecal storage vault and reducing the amount of dry absorbent material added to the vault after each time the toilet is used and reducing odour (Moe and Rheingans, 2006). However, Zhang and others, in their recent lab-scale study revealed that there is a strong potential for unpleasant odour emission during the urine storage as gases such as carbon dioxide and the major odorous compound, ammonia which are facilitated by the natural enzyme , urease. These compounds are released during the initial step of the hydrolysis process in the first few days of urine storage due to gradual

decomposition of urea found in urine. The study further revealed that more odorous emissions were apparently observed when urine hydrolysis was slower. Moreover, it was observed that storage of urine in the anaerobic environment in concert with slow ureolysis process volatile fatty acids gas streams such as acetic acid, propanoic acid and butyric acid in ionised forms were produced. However, other organic odorous compounds were reported to be responsible for persistent and strong odour that was emitted during the slow ureolysis process (Zhang *et al*., 2013).

Another drawback of urine diverting toilets is that there is user compliance problems because the sitting position during defecation and urination is a norm. This means that men must sit to urinate in the absence of the waterless urinal (which are principally used in public places such as schools, restraint, etc) (Kinstedt, 2012) of which they are often unwilling to comply. Unlike females who urinate in a squatting position, males prefer to urinate while standing. This is principally done in an attempt to avoid their sexual organs to touching the ground or the toilet surface (Drangert, 2004). This would lead to an unsolicited mixing of urine and feaces (Langergraber and Mullegger, 2012) which can result in odour (Münch and Winker, 2009). A review study in seven European countries by Lienert and Larsen (2009) also reported that less men (60%±11%) usually sit than women (75%±8%) when using UD toilets. The end application of urine diversirsion systems rests with users who need some behavioural change to shift away from their current practices and adopt these new systems of sanitation.
Urine diverting toilets are also difficult for small children because in some cases, particularly for faeces and urine depositing in the incorrect vault (Lienert and Larsen, 2009) therefore the system fails. Urine diverting toilets are not well suited in certain regions and with certain religions in which water is used for anal cleansing. This means that special arrangements have to be made for dealing with contaminated water (Canady, 2011; Kinstedt, 2012).

In recent years, bioremediation of organic pollutants including odour-causing compounds in the environment has gained great attention. The overview of bioremediation of organic compounds including odourants found in waste streams are discussed in sections 2.9 to 2.12.

2.9 Bioremediation of organic pollutants

Environmental organic pollutant contamination can be remediated by physical, chemical and biological (bioremediation) methods (Megharaj *et al*., 2011). Bioremediation is considered as the best treatment technique of the environmental organic pollutants. This is because it is cost effective, versatile, non-invasive, efficient, ease of implementation and environmentally benign compared to other remediation methods that are used for pollutant clean-up (Jain and Bajpai, 2012; Ji *et al*., 2012; Mishra and Maiti, 2018). Bioremediation is a technique that uses

microorganisms or microbial processes to convert, modify and utilise environmental organic pollutants for production of energy and biomass. This is achieved through a well organised biochemical activities in which the organic compounds are transformed to relatively more stable and less toxic daughter compounds than the parent compound and innocuous by-products such as carbon dioxide (CO_2) and water (H_2O) (Boopathy 2000, Abatenh *et al*., 2017; Rana *et al.*, 2017).

Bioremediation can be broadly either categorised as *ex situ* or *in situ*. The technique is referred to as *ex situ* bioremediation when the treatment of the contaminated material involves the physical removal from its place to another location for treatment biologically. In contrast, *in situ* bioremediation involves the treatment of the contaminated material onsite. The choice of the technique to adopt is dependent upon numerous factors *inter alia* include; cost, environmental (site) characteristics, type and concentration of pollutant (Boopathy, 2000; Azubuike *et al*., 2016). In situ bioremediation approaches are advantageous. This is because there are less destructive to the environment as there is no need to remove the contaminated matrix, cost effective and potentially complete removal of the pollutant (Kensa, 1970; Vidali, 2001; Farhadian *et al.*, 2008). The spreading of contaminant owing to the removal and transportation is evitable (Tomei and Daugulis, 2013). However, it requires relatively longer treatment time due to slow kinetics and less control over the variations in environmental conditions that lead to variations in microbial activities (Vidali, 2001). The advantage of *ex situ* techniques is that the remediation processes can be possibly better controlled (Aislabie *et al*., 2006) and the treatment process is more predictable (Tomei and Daugulis, 2013). The main limitation of *ex situ* bioremediation is that, it is generally costly because of extra costs associated with the removal and transportation of the contaminated matrices. It is also risky due to the potential dispersion of the contamination during removal and transportation of the contaminated matrices (Tomei and Daugulis, 2013). *In situ* bioremediation has more advantages than disadvantages hence it is a more preferred technique (Kumar *et al*., 2011).

2.9.1 Biodegradation

Biodegradation is the most important process for total contaminant mass reductions among the intrinsic biotic and abitic mechanisms employed by microorganisms (Lv *et al*., 2018). Biodegradation is the term given to microbial mediated chemical reactions that degrade organic pollutant by mainly bacteria, fungi, protozoa and other organisms (Ali, 2010; Das and Chandran, 2011). This process predominantly occurs in aqueous phase and microorganisms are the most important agents. Nonetheless, such physical and chemical processes as volatilisation, sorption, dispersion, sorption, dilution, dispersion and abiotic transformation are also important (Scow and Hicks, 2005).

Due to reliance on the microbial abilities to grow in specific environmental conditions, the pollutant can be biodegraded either aerobically, in the presence of oxygen or anaerobically, in the absence of oxygen (Al-Khalid and El-Naas, 2012). Aerobic biodegradation results into the production of carbon dioxide and water and in contrast, anaerobic biodegradation results in the formation of carbon dioxide, water and methane as end products (Gu, 2003; Shah *et al*., 2008). However, studies have shown that biodegradation efficiencies differ dependent upon the physical and chemical properties of the contaminant as well as the operating environmental conditions. For instance, on one hand in their studies, (Lomans *et al*., 1999; Patureau and Trably, 2006; Nyholm *et al*., 2010) found that aerobic biodegradation efficiencies were better compared to anaerobic biodegradation efficiencies in the removal of the pollutant of their concern. On the other hand, (Bollag and Russel 1976; Shinoda *et al*., 2004; Beltran *et al*., 2008; Mohee *et al*., 2008; Vasquez *et al*., 2011) found that anaerobic biodegradation of the pollutant of their concern was comparatively more effective than aerobic degradation. Even though, aerobic and anaerobic microbes have the capability to biodegrade organic compounds, conventionally, aerobic biodegradation has been studied as a preferred one. This is due to relatively low cost associated with the aerobic processes, faster growth of aerobic microorganisms and its end products are usually inorganic compounds i.e. carbon dioxide and water (Al-Khalid and El-Naas, 2012; Rücker and Kümmerer, 2012). Besides under uncontrolled anaerobic conditions, a variety of odourous compounds are produced as intermediate products. However, under aerobic condition, the organic compounds are degraded without apparent production of odourous compounds (Zhang *et al*., 2004). For these reasons, the focus will be on aerobic biodegradation.

Fundamentally, there are three approaches of *in situ* biodegradation with microorganisms to enhance the biodegradation efficiency namely, natural attenuation, biostimulation and bioaugmentation.

2.9.2 Natural attenuation

Natural attenuation is a passive reduction in toxicity, mass and/or mobility of a contaminant in the environment that depends on natural mechanisms. This is possible due to the involvement of both physical processes e.g. dilution, sorption, volatilisation, ion exchange and precipitation and biological processes of autochthonous microflora such as degradation and transformation (Röling and van Verseveld, 2002; Scow and Hicks, 2005). The advantage of this approach is that it avoids damaging of the ecologically sensitive environments. Nonetheless, the biodegradation process takes long time to complete due to low microbial population of the indigenous degrading microorganisms (Yu *et al*., 2005).

2.9.3 Biostimulation

Biostimulation is the bioremediation approach in which the naturally occurring contaminant-degrading microorganisms are stimulated by the addition one or more rate-limiting nutrients (nitrogen (N) and phosphorous (P)) or substrates, vitamins, oxygen and other compounds to the contaminated matrices to enhance their scant biodegradative capacity (Tyagi *et al*., 2011; Agamuthu *et al*., 2013). For instance, the addition of fertilizer that contain nitrogen and phosphorous to a contaminated matrix. The addition of nutrients, which are limiting the growth of the microorganism that degrade the organic contaminant, to the contaminated matrix can degrade the contaminant rapidly. This works when the contaminant is utilised as a carbon source and fertiliser as a source of nitrogen (N) and phosphorous (P) (Agamuthu *et al*., 2013). Liebeg and Cutright (1999) and Sutherland *et al.*,(2000) demonstrated that the approach is more effective when a combination of inorganic nutrients is added compared to individual nutrients in case where a low quantity of macronutrients and a high quantity of micronutrients are required to enhance the biodegradation activities of the indigenous microbial community. However, the performance of this approach varies from environments to environments and pollutant-to-pollutant (Balba, *et al*., 1998).

2.9.4 Bioagumentation

Bioaugmentation approach involves the inoculation of high biomass and specialised pre-adapted seeded cultures (indigenous or exogenous) of catabolically relevant microorganisms into a contaminated matrix to hasten the effectiveness of the remediation of the contaminant in the affected environment (Thompson *et al*., 2005; Nna Orji, 2018). The approach is applied when the natural attenuation and biostimulation have proved unsuccessful (Mrozik and Piotrowska-Seget 2010). According to Forsyth *et al.*, (1995) and Stocking *et al*., (2000), the approach would be warranted in contaminated environments where;

(a) the population of indigenous microflora existing in the contaminated environment is small and/or undetectable to be effective and efficient in the degradation of the pollutant,

(b) the need to accelerate biodegradation of the target contaminant by minimizing the acclamation or adaptation period of time

(c) the cost of bioaugmentation does not exceed the cost of alternative biological and non-biological methods.

Additionally, the exogenously introduced microorganisms have the capacity to survive and degrade the mixed contaminant stream including multiple compounds that could be detrimental or toxic to inhabitant microorganisms. This is due to lack of or undeveloped mechanisms to degrade the compounds by the indigenous microbial population. Previous studies (Roane *et al*., 2001; Dams *et al*., 2007; Niu *et al.*, 2009) demonstrated that

bioaugementation successfully accelerated biodegradation of pollutant in comparison to natural attenuation. On the other hand, some different workers (Bouchez *et al.*, 2000; Thompson *et al*., 2005) demonstrated bioagumentation failures to enhance appropriate intrinsic biodegradation potential of the target contaminant. One of the drawbacks of using bioagumentation as remediation strategy is to ensure that survival and prolonged activity of the exogenous microbial population in the contaminated environment. pH, redox, toxicity of other pollutant, concentration of pollutant and their availability to microorganisms, or the absence of key co-substrates are some of the critical factors that can inhibit bioagumentation (Perelo, 2010). Additionally, competition or inhibition that changes indigenous microbial community may have either positive or negative effects. However, the positive effects are commonly sustained for a short period after inoculation (Herrero and Stuckey, 2015). The best strategy to overcome bioargumentation failures due to ecological barriers is to use the microorganisms isolated from ecological niche the same as the contaminated environment or from the contaminated environment itself (El Fantroussi and Agathos, 2005). This is because it is evident that the microorganisms, which had been exposed to the pollutant, respond to the presence of the pollutant more expeditiously and have higher biodegradation potency as compared to the microorganisms that had not been exposed to the pollutant (Adams *et al.*, 2015).

2.10 Biodegradation mechanisms

As can be seen in section 2.1.4, most of the odour-causing compounds are organic in nature, hence in this review the focus is on biodegradation of organic compounds under aerobic conditions. In general, biodegradation is an electron transfer process (Eskander and Saleh 2017). Microorganisms obtain energy from the organic pollutant through the oxidation of the contaminant. Microbial enzymes, which are specific for each type of reaction, break the chemical bonds and transfer electrons away from the contaminant to the terminal electron accepter, which in itself is reduced (Eskander and Saleh, 2017). Under aerobic conditions, the microorganisms use molecular oxygen as the terminal electron accepter and this is referred to as aerobic respiration (Kyrikou and Briassoulis, 2007). In this process the reactions are catalysed by oxygenases, are oxidoreductases, which use molecular oxygen to incorporate oxygen into the contaminant as a substrate (Fritsche and Hofrichter, 2000).

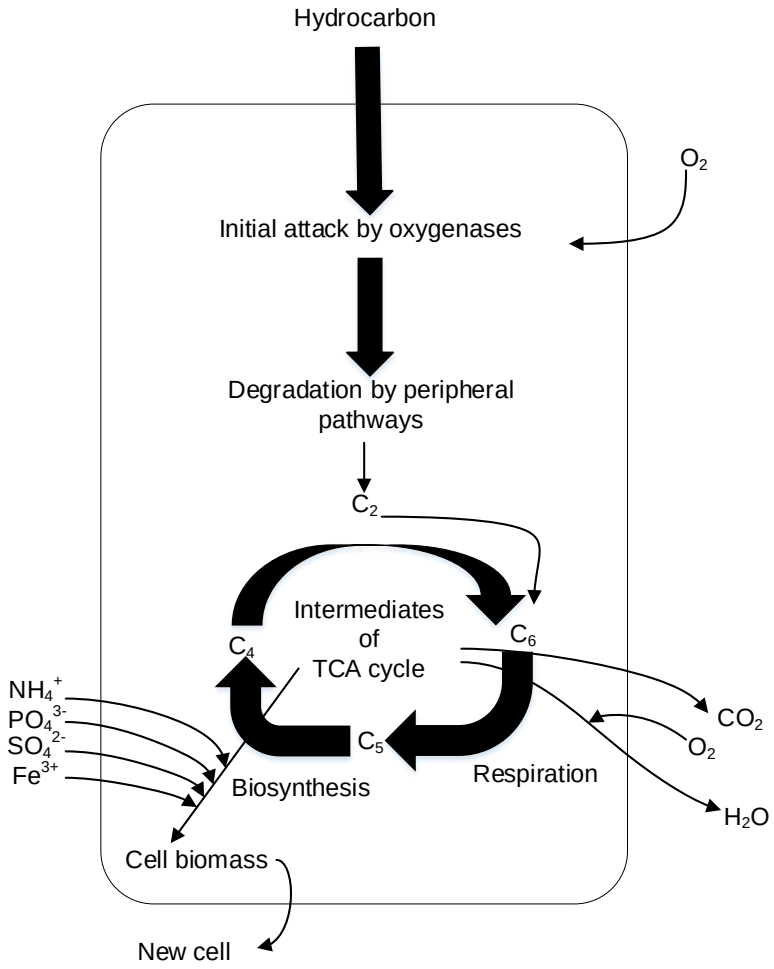


Figure 2.2: Main principle of aerobic degradation of organic compounds
(Adopted from Sonawdekar, 2012)

There are typically two metabolic sites at which the contaminant-degrading microorganisms need oxygen. These metabolic sites are at the initial enzymatic attack of the organic contaminant and at the end of the respiratory chain (Fritsche and Hofrichter, 2000; Olajire and Essien 2014) as depicted in Figure 2.2.

The initial intracellular attack and the availability of oxygen are the primary rate-limiting factors (Atlas and Philp, 2005). Generally, aerobic biodegradation is the most rapid and complete degradation process of the majority of the pollutants and is more preferred to anaerobic biodegradation (Eskander and Saleh, 2017). Under anaerobic conditions, the microorganisms use inorganic compounds such as nitrate (NO_3^-), sulphate (SO_4^{2-}), ferric iron (Fe^{3+}), manganese (Mn^{3+}, Mn^{4+}), bicarbonate (HCO_3^-) and carbon dioxide (CO_2) as an alternative terminal acceptor to oxygen (Rayu *et al*., 2012).

2.11 Factors influencing biodegradation of organic pollutants

All biodegradation approaches depend on having right microorganisms, which have physiological and metabolic capabilities, to degrade the pollutant. These microorganisms have to be in the right place coupled with right prevailing environmental conditions that favour the metabolic activities of the microorganisms (Juwarkar *et al*., 2010). A number of environmental and biological factors that been recognised to influence the biodegradation ability or metabolism of the microorganisms. Some of the environmental factors can be controlled or modified to optimize the environment for biodegradation by either suppressing or stimulating growth of the contaminant-degrading microorganisms while others cannot be modified (Juwarkar *et al*., 2010). The environmental and biological factors that could influence biodegradation are summarised as follows:

2.11.1 Temperature

Temperature plays a critical role in determining the physico-chemical of the pollutant, physiology and diversity of the microorganisms (Jain *et al.*, 2011) in a number of ways. Notably, temperature affects the metabolic activities of contaminant-degrading microorganisms (Naseri *et al.*, 2014). Generally, there is a direct relationship between changes in temperature and rate in microbial activities until its maximum level at an optimum temperature. The rate of biochemical reactions in the cell of microorganisms almost gets doubled for every 10 ^{o}C increase in temperature (Thapa *et al*., 2012; Niti *et al.*, 2013). However, this relationship is not linear beyond optimum temperature (Ali, 2010). Further increase in temperature above optimum temperature results into the abrupt drop in the biodegradation activities of the microorganisms. This is due to slower growth and reproductive rate and denaturation or deactivation of the enzymes responsible for degradation (Ali 2010; Naseri *et al*., 2014). There is also a lower temperature limit at which the microorganisms can withstand for their metabolic activities. and below which they become metabolically inactive (Niti *et al.*, 2013, Abatenh *et al.,* 2017*)*. The temperature range at which biodegradation activity can occur and the optimum temperature level at which the biodegradation rate is at its maximum, vary from one microorganism to another. Temperature also influences the solubility of pollutant; solubility of pollutant increases with an increase in temperature, which consequently increases the bioavailability and mass transfer of the contaminant molecules to microbial cells (Aislabie *et al*., 2006; Alegbeleye *et al*., 2017). In contrast, when temperature increases, amount of dissolved oxygen reduces which in turn reduces the metabolic activities of aerobic mesophilic microorganisms. Furthermore, at high temperature some pollutant change into a new compound (s) that accumulate and often appears to be more lethal than the parent compound (s) , as a result they inhibits the biodegradation ability of the microorganisms (Ghosal *et al*., 2016).

2.11.2 pH

pH is also a very important factor in the biodegradation of the pollutant. The pH of the environment can be highly variable and must be taken into account to enhance biodegradation activities (Al-Hawash *et al.*, 2018). Most microorganisms can survive only within a certain pH range because it has its own impact on microbial metabolic activity. Different microorganisms prefer different environments with different pH values. pH affects the occurrence and distribution of microorganisms in the environment. The group of microorganisms that are metabolically active in the environments with pH values below 5 are known as acidophiles, and those, which have optimal growth rate at pH above 9, are known as alkaliphiles. The microorganisms that can tolerate environments with pH within one or two units of the neutral pH of 7 are neutrophiles (Jin and Kirk, 2018).

Generally, yeast and fungi exhibit biodegradation activities in the acidic pH range or neutral pH while heterotrophic bacteria show a strong preference for nearly neutral or alkaline conditions (pH 6-8) (Ali, 2010; Li *et al*., 2017). Environmental pH variations below or above the optimal pH range and sudden changes in the pH of the contaminated matrix significantly inhibit microbial growth and the biodegradation activities by interfering with cell membrane transport, catalytic reaction balance and the capability of microorganisms to accomplish their enzymatic activities (Li *et al*., 2017, Al-Hawash *et al*., 2018). The pH changes in the environment also interferes with gas solubility, macro- and micro-nutrients availability and bioavailability as well as the chemical structure of the organic pollutant (Juwarkar *et al*., 2010).

2.11.3 Concentration and structure of the contaminant

The rate at which contaminant-degrading microorganisms degrade the contaminant depends upon contaminant characteristics including chemical structure and concentration of the pollutant. Contaminant concentration is important as microbial growth and activity is inhibited by the contaminant itself, particularly at elevated concentrations. On one hand, when the concentration is too high, the pollutant may have toxic effects on the indigenous microorganisms. This may lead to a prolonged acclimation times and even to an inhibition of the biodegradation process. Owing to toxicity of the pollutant to the microbial cells, the production of microbial biomass is inhibited at higher contaminant concentrations (Kao *et al*., 2005; Agarry *et al*., 2008; Juwarkar *et al*., 2010). On the other hand, minimum contaminant concentration below which it may prevent induction of the catabolic genes of the degrading bacteria. (Fetzner, 2002; Adams *et al.,* 2015).

The ease with which pollutant can be biodegraded also depends on the contaminant structure. The number, type, cyclicity and position of alkyl substituents as well as the extent of branching

determine the contaminant structure. Organic pollutant with increasing number of alkyl substituents and/or branching are less biodegradable compared to unsubstituted and less branched ones due to their hydrophobicity, poor aqueous solubility and bioavailability (Koshlaf and Ball, 2017). Generally, there is inverse relationship between the molecular weight of the contaminant and the biodegradation rate (Wammer and Peters 2005; Han *et al*. 2008; Maletić *et al*., 2011). However, in a multi-substrate biodegradation of more readily degradable organic pollutant and more recalcitrant and larger molecular weight organic pollutant, the biodegradation of the former is inhibited while that of the latter is enhanced (Guha *et al.,* 1999; Couling *et al*., 2010).

2.11.4 Nutrient availability

Nutrients, such as nitrogen (N), phosphate (P) and potassium (K) and in some cases iron, are vital constituents for an efficient biodegradation of pollutant (Al-Hawash *et al*., 2018). Microorganisms involved in biodegradation activities need the nutrients for their cellular metabolism and growth (Singh *et al*., 2017). However, the environment contaminated with organic pollutant are generally rich in carbon while in deficiency of other inorganic nutrients like N, P and K depending on contaminant composition and biodegradation activities. Thus, sometimes, this limited availability of inorganic nutrients become a limiting factor for biodegradation of the pollutant. Generally, nutrients are supplemented in both *in-situ* and *ex-situ* bioremediation of contaminated environments for stimulating or enhancing contaminant degradation by microbial communities (Carter *et al*., 2006; Juwarkar *et al*., 2010). The addition of supplemental nutrients through the application of urea, phosphate, NPK fertilisers, ammonium and phosphate salts enhanced the biodegradation of pollutant (Zaidi and Imam, 1999;Boopathy, 2000; Børresen and Rike, 2007; El-Bestawy and Albrechtsen, 2007; Ron and Rosenberg, 2014). However, excessive amounts of inorganic nutrients can also inhibit the biodegradation activity of microorganisms.

Inhibition of microbial biodegradation activities because of high concentration levels of nutrients such as NPK have also been reported (Tatarko and Bumpus, 1998; Kaushik and Malik, 2009; Chandra *et al.,* 2013; Koshlaf and Ball, 2017). This is usually because the addition of the supplemental nutrients to the contaminated environment disturbs C: N: P ratio that negatively affect oxygen availability to the microorganisms (Varjani and Upasani, 2017). Moreover, the addition of supplemental nutrients can result in failure of bioremediation. This could be attributed to the variable and complex composition of the contaminated matrix as well as other variables such nitrogen reserves and the presence of nitrogen fixing bacteria (Chandra *et al.*, 2013). It is, therefore, important to evaluate the stoichiometric relationship between nutrients and pollutant so that the optimal amounts and proportion of different

nutrients are added to accomplish an effective bioremediation based on oxygen-limiting conditions (Carter *et al.*, 2006; Chandra *et al*., 2013). The optimal C: N: P ratio is theoretically in the range of 100:10:1 to 100:1:0.5 (Chandra *et al.*, 2013). However, in nutrient limited wastewaters, Biochemical Oxygen Demand (BOD): N: P ratio is the theoretical ratio that is often applied as a standard for nutrient addition (Chandra *et al*., 2013).

2.11.5 Bioavailability

Bioavailability is generally referred to as the tendency of the part of an individual chemical (nutrient, substrate or toxicant) in environment to be physico-chemically accessible and taken up or transformed by microorganisms (Al-Hawash *et al*., 2018). Fundamentally, the biodegradation of organic pollutant is only realizable when the pollutants are in dissolved state. Therefore, low aqueous soluble or sparingly aqueous soluble or absorbed pollutant lead to the necessity of mass transfer from the non-aqueous to the aqueous phase (Vig *et al*., 2003). Bioavailability is partly mediated by the degree of partitioning between the non-aqueous phase and the aqueous phase of the contaminated matrix (Phillips *et al*., 2005).

Pollutants that have low bioavailability are known as hydrophobic pollutant. These pollutants are very often of low aqueous solubility, which make them not accessible to the degrading microorganisms hence limit their availability to the microorganisms (Al-Hawash *et al*., 2018). On the other hand, pollutant with high water solubility pass very rapidly from a non-aqueous phase and often in closer proximity to the degrading microorganisms in the aqueous phase (Phillips *et al*., 2005). The application of surfactants, which is either synthetically or microbically produced, is the alternative that enhances the availability of pollutant for biodegradation (Providenti *et al*. 1993; Boopathy 2000; Santos *et al.* 2011).

Contaminant bioavailability also depends on the degree contact between the contaminant and the non-aqueous phase of the contaminated matrix (Foght *et al.*, 2001). The longer the duration the contaminant is in contact with the non-aqueous phase, the more it becomes bound and resistant to chemical extraction, and may be irreversibly sorbed to the non-aqueous phase. Because of this, the contaminant aqueous concentration and bioavailability to biodegrading microorganisms are reduced, therefore, inhibiting biodegradation (Huesemann *et al.*, 2002; Bamforth and Singleton 2005). This phenomenon is referred to as ageing of the contaminant (Bamforth and Singleton, 2005). There are two stages involved in the biodegradation of pollutant in the contaminated matrix. The biodegradation of pollutant is generally higher in the early stages of the bioremediation process. This is as a function of molecules of the pollutant being physically available to the microbial cells. This is because the molecules of the pollutant are non-sequestered, as a result, are easily bioavailable to

microorganisms. In the absence of this, biodegradation of the contaminant will be governed by the bioavailability of the contaminant to the microorganisms (Okere and Semple, 2012).

However, in the second stage of the bioremediation process, the contaminant bioavailability is limited due to sequestration in the solid phase of the contaminated matrix. In this stage, the molecules become substantially inaccessible to biodegrading microorganisms and even to extracellular degradative enzymes with time. Therefore, the bioavailability of the pollutant will be limited by the amount of the pollutant being released to an accessible site (Alexander 2000; Huesemann *et al*., 2002; Koshlaf and Ball, 2017). The rate of desorption and diffusion from the accessible sites would govern the rate at which the pollutant become available to biological components of the environment. In some cases, when the second stage has been reached biodegradation ceases during the rest of bioremediation process (Koshlaf and Ball, 2017).

2.11.6 Oxygen availability

Oxygen supply can be one of the rate-limiting factor of aerobic bioremediation of organic pollutant. In large-scale aerobic bioremediation of organic pollutant, an aeration system is regarded, therefore, as a critical component in the designing process (Crawford and Crawford, 2005). Aerobic microorganisms do not use molecular oxygen primarily as the terminal electron acceptor for aerobic respiration only, but also as a co-substrate in oxygen-catalysed for the microbial degradative of numerous organic chemicals. The availability of dissolved oxygen plays crucial role in deciding the physiological growth of the aerobic microorganisms as well as influence the biodegradation rate of organic pollutant. Therefore, proper oxygen supply is a prerequisite for aerobic catabolic reactions to take place (Shaler and Klecka, 1986; Miller, *et al*. 2004; Xu *et al*., 2005; Balcke *et al*., 2008).

Generally, the aerobic respiration of bacteria is not affected by the critical oxygen concentration. Critical dissolved oxygen concentration is defined as the concentration value for half-maximal rate of oxygen uptake of the microbial cells observed at saturating levels. Generally, it has been demonstrated that flocculant microbial cultures have higher critical dissolved oxygen concentration, usually in the range of 0.5 mg/L, as compared to dispersed microbial cultures. Moreover, above the critical dissolved oxygen concentration any elevation in oxygen levels has no effect on the microbial respiration rates (Gaudy and Gaudy,1980).

2.12 Biological odour treatment

Current odour pollution approaches can treat a wide variety of odour causing compounds at higher concentrations; however, control of odorous air with low concentrations is quite difficult

or impossible. Biological methods for odour control of waste gaseous emissions are cost effective when low concentrations are involved (Bajpai, 2014). As discussed in section 2.9.1, Biodegradation is the most important process for organic contaminant mass reductions. The biodegradation has been successfully applied for the abatement of odours with high elimination efficiencies in many instances. Few examples are outlined in this section.

Ho *et al*. (2007) studied algae derived odour causing compounds, in particular, 2-methylisoborneol (MIB) and geosmin in drinking water. These compounds are recalcitrant to convectional water treatment. In this study, 200 ng/L of MIB and 50 ng/L of geosmin were observed to be removed completely through biodegradation process. Four bacteria, a *Pseudomonas* spp., an *Alphaproteobacterium* spp., a *Sphigomonas* spp. and an *Acidobacteriaceae* member were identified as the most likely involved in the biodegradation processes of the compounds through the biological sand filtration.

Schhlegelmilch *et al.*(2005) investigated biological waste gas treatment plants using two different bioscrubber/biofiltration combinations and different biofilter materials were tested. The results of the study revealed that the biofilters were mainly resposble for the efficient biodegradation of the odour caising compounds. Wan *et al. (*2010) studied the use of microoorganisms for the biological treatment of an odorous, strong and colourless volatile organic sulfourous compound (VOSC) liquid, ethanethiol. A novel bacterium isolated from the cultivated sludge in a domestic wastewater treatment plant identified as *Lysinibacillus sphaericus* was found to degrade completely 4 mg/L ethanethiol at 30 ^{o}C and pH=7 within 96 h.

Chin *et al*.(2010) investigated the removal of volatile fatty acids such as butyric acid, acetic acid, valeric acid and caproic acid. These volatile fatty acids are components of odours emitted from various industries were examined using a biofiltration system. Bacteria for the study was isolated from rivers and cow farm to degrade volatile fatty acids. Four bacteria, *Acnetobacter calcoacetius* C6, *Burkholdeira cepacia* C4, *Waitersia paucula* B3 and *Wautersia paucula* C7 were identified as they acquired the highest degradation rates and specific growth rates for butyric acid, acetic acid, valeric acid and caproic acid among the thirteen butyric acid degrading bacterial strains. However, *Actinetobacter calcoaceticus* C6 had the highest degradation rates and growth and was able to degrade various volatile fatty acids. Under optimal conditions, the bacterium degraded completely 1371 mg/L of butyric acid, 452 mg/L of propanoic acid and 1399 mg/L of valeric acid found in wastewater by the biofiltration system.

With the recorded success of biological treatment of a seemingly endless of odour causing

compounds in the laboratory and the few reported cases for the treatment of odours in the real environment, it is becoming a popular approach for odour treatment systems. The increased attention seems justified in view of increased awareness of non-green processes and a subsequent global change towards environmentally friendlier processes. For low cost sanitation technologies in developing countries such as pit latrine, development of a biological odour treatment system that could potentially degrade odour-causing compounds and act as a deodourant with the production of other environmental pollutants would be an added benefit.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 General materials and supplies

3.1.1 Analytical reagents and supplies

All chemicals used in this study were of analytical grade, the highest purity available. Ethanol (≥99%), HCl 37% w/w), acetone, methanol, toluene and NaCl were purchased from Merck Chemical (Pty) Ltd, Gauteng, South Africa. Butyric acid (99%), Dimethyltrisulfide (DMTS) (≥98%), indole (≥99%) p-Cresol (99%), Isopropyl disulphide (96%), 2-Ethylbutyric acid (99%), 4-isopropylphenol (98%) and dichlorodimethylsilane (98%) were purchased from Sigma Aldrich Inc., St Louis, MO, USA. Glycerol, granular NaOH, HPLC grade H_2SO_4 (98%) were purchased from Glassworld, South Africa. Ultrahigh purity (UHP) He (99.999%) was purchased from Afrox, Johannesburg, South Africa. Distilled water was prepared by Water Still system (Daihan Labtec. Co. Ltd, Kyonggi-Do, Korea). Ultra-filtered, deionised water (18.2 MΩ) was prepared by a Purelab Flex purification system (ELGA Lab Water Ltd., UK.) Stelised deionised water (18.2 MΩ) was used to to make 6 M NaOH solution.

SPME manual holders, fibres and polytetrafluoroethylene (PTFE) coated magnetic stir bars were obtained from Supelco (Bellefonte, PA, USA). In this study, five commercially available fibres used were: 24 ga and 1 cm fused silica/SS 85 µm Polyacrylate (PA), 100 µm Polydimethylsiloxane (PDMS), 24 ga and 1cm stableflex/SS 85 µm Carboxen–PDMS (CAR–PDMS), 65 µm PDMS–divinylbenzene (PDMS–DVB) and 50/30 µm DVB/CAR/PDMS. Amber glass screw cap 20 mL vials and their caps equipped with PTFE /silicone septa (20 mm) were purchased from Restek Corporation (Bellefonte, PA, USA).

Glassware was thoroughly cleaned with soap and rinsed with distilled water followed by acetone and dried at 50 ^oC in the oven until they are absolutely dried. The glassware was covered with aluminium foil before autoclaving them at temperature of 121 ^oC and pressure of 115 kg/cm^2 for 15 min.

3.1.2 Growth media

3.1.2.1 Nutrient broth

Nutrient broth was composed of 1.0 g meat extract, 2.0 g yeast, 5.0 g peptone and 8.0g NaCl. The nutrient broth was prepared by dissolving 16 g nutrient broth powder in 1 L of 18.2 MΩ deionized water and autoclaved at temperature of 121 ^oC and pressure of 115 kg/cm^2 for 15 min. It was then allowed to cool down to room temperature prior to use.

3.1.2.2 Nutrient agar

Nutrient agar was composed of 1.0 g meat extract, 2.0 g yeast, 5.0g peptone, and 8.0g NaCl and 15.0 g agar. The nutrient agar was prepared by dissolving 31 g in 1 L of 18.2 MΩ deionized water and autoclaved at temperature of 121 ^{o}C and pressure of 115 kg/cm^2 for 15 min. It was then cooled down to a temperature of 40-50 ^{o}C prior to dispensing in pre-sterilized petri dishes for colony development. The prepared nutrient agar could be kept in the refrigerator at 4 ^{o}C for a maximum of two weeks for use.

3.1.2.3 Mineral salt medium

Mineral Salt Medium (MSM) consisted of: 2.722 g KH_2PO_4; 0.535 g NH_4Cl; 0.049 g $MgSO_4$; 4.259 g Na_2HPO_4; 0.114 g Na_2SO_4 per litre of 18.2 MΩ deionised water. The MSM was supplemented with 1 mL of trace element solution per litre of MSM solution. The trace element solution consisted of 0.0128 g $NiCl_2$; .549 g $CaCl_2$; 0.0124 g H_3BO_3 6.9505 g $FeSO_4$; 0.0358 g $CuCl_2$; 0.0136 g $ZnCl_2$; 0.0103 g NaBr; 0.0121 g $NaMoO_2$; 0.0198 g $MnCl_2$; 0.0166 g KI and 0.0238 g $CoCl_2$ per litre of 18.2 MΩ deionised water (Roslev *et al.,* 1998). For degradation and cell growth studies, MSM was supplemented with 1000 mg/L butyric acid. The pH of the medium was adjusted to 7.0, by titration with 6.0 M NaOH, which was prepared with 18.2 MΩ deionised water and sterilised by autoclaving at 121 ^{o}C for 15 min. The pH was determined using Hach HQ40D portable multi-meter (Hach, Colorado, USA).

3.2 Identification and characterisation of volatile compounds

3.2.1 Chemical and supplies

NaCl, deionised water, He, SPME manual holder and 50/30 µm CAR/DVB/PDMS fibres were used in this work. Details about the quality of all reagents used in this work are as provided in section 3.1.1. The fibres were conditioned at 260 ^{o}C for 1 h.

3.2.2 Sampe collection and preparation

Feacal sludge samples were collected from pit latrines in a semi-rural mining area of Kendal in Mpumalanga Province in South Africa (26^{o}5’24’’S 28^{o} 58’17” E). Pit latrines are the common means of human waste disposal, for the residents of the area. Feacal sludge samples in containment structure like pit latrines are normally heterogeneous (Niwagaba *et al*., 2014). To characterise VOCs and odourants from feacal sludge taken from the containment structure. A representative sample was collected by taking multiple samples horizontally at different points and vertically at different depths 0 cm (top surface) middle (15 cm from the top) and bottom (30 cm from the top surface) in 8 pit latrines in winter (May) and in summer (October) in 2015.

The sampled pit latrines were designated as PXW or PXS. Where P, X, W and S denote pit latrine, pit latrine number, winter and summer, respectively. Samples were collected using a sterilised graduated auger-like equipment manufactured purposely for collection of the feacal

sludge. The reading was known by the mark of the top surface that was registered on the equipment. All non-feacal wastes (such as diapers, stones, clothes, metals, plastic bags etc.) were removed.

The samples for each pit latrine were put in a 500 mL PET-polymer, gas tight bottle that contained about 1000 g of feacal sludge and the placed in a cooler box with ice to prevent volatilisation and microbial activities. *In situ* relative humidity (RH) and sample pH were taken using Edison digital temperature, humidity and dew point multimeter (The Power Factory, Wigston, England)

The samples were immediately transported to the laboratory for analysis. Sample preparation for extraction of volatile compounds was done within eight hours from the time of collection. In cases when it was not feasible to analyse the samples within eight hours, the samples were refrigerated at 4 ^{o}C prior to analysis. Prior to HS-SPME protocol, samples were thawed at room temperature and thoroughly vortexed with a mixer to obtain a composite sample.

3.2.3 HS-SPME procedure

A feacal sample of 1 g was placed in a 20 mL amber glass vial with 5 g of NaCl. NaCl used in this study was heated in an oven at 300 ^{o}C for 12 h prior to use to get rid of all impurities. A small magnetic polytetraflu-orothylene (PTFE)-coated stirring bar was also added. Then deionised water was added to the sample while the vial was being gently shaken until the sample volume was 10 mL. The vial was tightly closed with a PTFE-coated silicone septum. The vial was placed in a 100 mL beaker filled with 50 mL of water and then put on a thermostatted block with a stirrer. The SPME fibre was inserted into the headspace for extraction time of 24 h at room temperature while the sample was stirred at a constant rate of 1000 rpm. After extraction, the fibre was removed from the sample vial and inserted into the injection port of the GC for desorption for 5 min. The SPME fibres were preconditioned for 10 min at the desorption temperature of 250 ^{o}C to prevent carry-over effect. Each sample was used for one analysis and it was discarded thereafter. Vials containing deionised water were used as blank samples.

3.2.4 Analytical instrumentation

Analysis of VOCs and odourants was performed by a Gas Chromatograph (GC) system, Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) coupled to a Pegasus 4D Time of Flight Mass Spectrometry (GC-ToFMS) system (LECO, St Joseph MI, USA). Separation of the extracted compounds was performed by equipping the GC- ToFMS system with a fused silica capillary column as specified in Table 3.1.

The injection port equipped with SPME Borosilicate Glass specifically designed narrow straight liner of 0.75 mm ID and deactivated by the manufacturers (Restek Corporation, USA)

to increase linear velocity and introduce analytes onto the GC column in a narrow band, thus leading to the sharper peak of the analytes (Sigma Aldrich, 1997). The SPME fibre was manually injected and the sample extracts were thermally desorbed in the slit/splitless injector. Only compounds with a probability of 95% match to a chemical compound in the National Institute of Standards and Technology (NIST) 08 library were scored. Details of GC and MS operating conditions used for analysis are presented in Table 3.1.

Table 3-1: GC and MS operating condition for analysis of volatile organic compounds and odourants

GC and MS conditions	
Columm	30 m x 0.25 ID x 0.25 µm Stabilwax (Crossbond®Carbowax®polyethylene glycol)(Restek Corp. Bellefonte, PA, USA)
Inlet injector temperature	250 ºC
Initial oven temperature	40 ºC (2 min)
Ramp 1	80 ºC (2 ºC/min)
Ramp 2	140 ºC (20 ºC /min)(2 min)
Desorption time	5 min
Injection mode	Splitless
Mobile phase	Ultrahigh purity (UHP) helium (99.999%)
Mobile phase flow rate	1 mL/min
Solvent delay	3 min 10 s
Mass scan range	35-450 m/z
Ion source temperature	230 ºC
Transfer line temperature	240 ºC
Electron ionisation voltage	-70 eV
Acquisition rate	20 spectra/s
Chromatogram acquisition and data processing software	LECO ChromaTOF™ (LECO, St. Joseph, MI, USA)
Database library	National Institute of Standards and Technology (NIST) 08

3.3 Optimisation of HS-SPME for GC-ToFMS analysis of pit latrine key odourants

3.3.1 Chemical and supplies

Details about the quality of all reagents specification of other supplies used in this work are as provided in section 3.1.1.

3.3.2 Standard and internal standard preparation

Standard stock solution of 10 000 mg/L of butyric acid, DMTS, indole, p-Cresol and internal standards, Isopropyl disulphide, 2-ethylbutyric acid and 4-isopropylphenol in 10 mL solution in 10 mL volumetric flasks separately and protected from light by coating it with aluminium foil and stored at 4 ºC. The standard stock solutions were being stored for not more than 1 week. The working solutions of the reference standards of a concentration of 0.5 mg/L were prepared

daily by measuring 5 µL of the stock solution into a 100 mL volumetric flask followed by dilution with ultra-pure water to the 100 mL mark.

3.3.3 SPME fibre selection

SPME fibre screening was done prior to execution of the optimisation of SPME parameters for determination of the four key odourants of interest in this study. The SPME fibres that were used were those described in section 3.1.1. Prior to their first use the fibres; PDMS and PDMS/DVB were thermally conditioned at 250 ^{o}C for 30 min while CAR/PDMS, DVB/CAR/PDMS and PA were thermally conditioned at 300 ^{o}C, 270 ^{o}C and 280 ^{o}C for 30 min respectively as specified by the manufacturer. This was done with the splitter left open to lessen the quantity of impurities entering the column. This was done in order to stabilise the solid phase (Godayol *et al.,* 2011) as well as to get rid of contaminants.

Immediately prior to use, all glassware, headspace vials and magnetic stirrers used in this specific work were salinized for 1 h in a solution of dichlorodimethylsilane (approximately 10%, v/v, in toulene), thoroughly washed with toluene and methanol and oven-dried at 105 ^{O}C for 1 h to avoid the absorption the compounds to the glass surface.

A 10 mL volume of the reference standard mixture of 0.5 mg/L with an appropriate amount of IS (Isopropyl disulphide, 2-ethylbutyric acid and 4-isopropylphenol) was placed in 20 mL amber glass vials with 5 g of NaCl. The NaCl was added to vary the properties of the boundary phase and to reduce the solubility of hydrophilic compounds in the sample (Silva *et al.,* 2014). The pH of the solution was modified to 2 with HCl. The pH was measured by HQ11d digital pH/ORT meter (Hach, Loveland, Colorado, USA). A small magnetic PTFE- coated magnetic stir bar was also added. The vial was tightly closed with a PTFE-lined silicone septum. The vial was heated with a heater-stirrer plate (Heidolph Instruments GmbH and Co, Germany) accommodated in a 100 mL beaker filled with 50 mL of water to adjust the extraction temperature. Thus, the sample was not directly heated, with temperature controlled with a thermometer suspended in the water. The sample was incubated for 20 min at 40 ^{o}C to facilitate the transfer of the analytes from the mixed standard solution to the headspace, thus speeding up extraction. After this, the SPME fibre was inserted into the headspace for extraction time of 30 min while the sample was stirred at a constant rate of 600 rpm.

After extraction the SPME fibre was retracted into the needle of the SPME manual holder syringe and the needle was removed from the sample vial and inserted into the GC injection port of the GC for desorption, at the constant needle depth of 3.5 cm, and analysis. Moreover, between the analyses the SPME fibres were baked out for 10 min at 290 ^{o}C. Blanks were run by thermal desorption for 5 min in the injection port and GC analysis was subsequently done

to ensure that any analyte thermally desorbed i.e. to eliminate carry over from the erstwhile runs, to such an extent that a clean chromatogram is obtained under normal run conditions.

The selection was based on the simplified criterion function of Zuba *et al.,* (2002) as described by Hamm *et al.,* (2003) (equation 3.1):

$$F_{ij} = \frac{\sum_i H_{ij}}{\frac{1}{k}\sum_{ij} H_{ij}} \quad (3.1)$$

where H_{ij} is the peak height of i analyte with the use of j fibre coating.

$F_{ij}$is the concentration capability factor of the fiber *j*.

k is the number of fibres

3.3.4 Optimisation experimental design

A two-level factorial design was employed to determine the influence of all the experimental variable factors that were studied in order to ascertain a list of the main effects and interaction effects between them that had influence on response values (chromatographic peak areas). This was supported by an analysis of variance (ANOVA) that showed which effects were statistically significant at 95% confidence level.However, Pareto charts generated by Minitab statistical software (Release 17; Minitab Inc., PA, USA) were used to graphically interpret the results..Furthermore, to achieve the real optimal HS-SPME extraction conditions of the influential factors in the simultaneous determination of the four key odourants of interest in this study the response surface models were built (Welke *et al*., 2012). A face-centred cube central composite design (CCD) with centre points was used. The generalized response –surface model to describe the variation in the response values was given by equation 3.2:

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (3.2)$$

where Y is the response value predicted by the model; β_o is a constant; andβ_i, β_{ii} and β_{ij} are the linear, quadratic and interaction coefficients, respectively. In this model, X_1, X_2 and X_3 are the independent variables.

3.3.5 Optimisation of HS-SPME procedure

After optimisation, a 10 mL volume of the reference standard mixture of 0.5 mg/L with its pH adjusted to 2 was placed in 20 mL amber glass vials with 5 g of NaCl. A small magnetic PTFE-coated magnetic stir bar was also added. The vial was tightly closed with a PTFE-lined silicone septum. The vial was placed in a 100 mL beaker filled with 50 mL of water and then put on a thermostatted block with a stirrer. The sample was equilibrated for 20 min at 40 ^{o}C in order to facilitate the transfer of the analytes from the sample solution to the headspace, thus speeding

up extraction. After this, the SPME fibre was inserted into the headspace for extraction time of 30 min while the sample was stirred at a constant rate of 800 rpm. After extraction the SPME fibre was retracted into the needle of the SPME manual holder syringe and the needle was removed from the sample vial and inserted into the injection port of the GC for desorption, at the constant needle depth of 3.5 cm, and analysis. Moreover, to avoid cross-contamination between the analyses the SPME fibres were baked out for 10 min at 290 ^{o}C and blanks were run by thermal desorption for 5 min in the injection port and GC analysis was subsequently done to confirm that all components were thermally desorbed. For each tested condition all analyses were performed in triplicates. To prevent losses deactivation of all glassware and vials was carried out by silanisation with of dichlorodimethylsilane (approximately 10%, v/v, in toulene), thoroughly washed with tuelene and methanol and oven-dried at 105 ^{O}C for 1 hour in order to avoid the absorption the compounds to the glass surface.

3.3.6 GC/TOF-MS analysis conditions

The analysis of volatile compounds was performed by a Gas Chromatograph (GC) system, Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) coupled to a Pegasus 4D Time of Flight Mass Spectrometry (GC-ToFMS) system (LECO, St Joseph MI, USA). Separation of the extracted compounds was performed by equipping the GC- ToFMS system with a 30 m x 0.25 ID fused silica capillary column (Restek Corp. Bellefonte, PA, USA) having a 0.25 µm film thickness of Stabilwax (Crossbond®Carbowax®polyethylene glycol).

The injection port equipped with SPME Borosilicate Glass specifically designed narrow straight liner of 0.75 mm ID and deactivated by the manufacturers (Restek Corporation, USA) to increase linear velocity and introduce analytes onto the GC column in a narrow band, thus leading to the sharper peak of the analytes (Sigma Aldrich., 1997). The SPME fibre was manually injected and the sample extracts were thermally desorbed in the slit/splitless injector that was configured in splitless mode at an inlet injector temperature of 250 ^{o}C for 5 min. Then, SPME fibre was baked at the same temperature for 10 min. Details of GC and MS operating conditions for the analysis of the key pit latrine odourants are presented in Table 3.2.

Table 3-2: GC and MS operating condition for analysis of four key pit latrine odourants

GC and MS conditions	
Columm	30 m x 0.25 ID x 0.25 µm Stabilwax (Crossbond®Carbowax®polyethylene glycol)(Restek Corp. Bellefonte, PA, USA)
Inlet injector temperature	260 ^{o}C
Initial oven temperature	40 ^{o}C (1 min)
Ramp 1	100 ^{o}C (30 ^{o}C/min)
Ramp 2	200 ^{o}C (15 ^{o}C /min)
Ramp 3	240 ^{o}C (20 ^{o}C /min) (3 min)
Desorption time	5 min

Injection mode	Splitless
Carrier gas	Ultrahigh purity (UHP) helium (99.999%)
Mobile phase flow rate	1 mL/min
Solvent delay	3 min
Mass scan range	35-450 m/z
Ion source temperature	230 °C
Transfer line temperature	240 °C
Electron ionisation voltage	-70 eV
Acquisition rate	20 spectra/s
Chromatogram acquisition and data processing software	LECO ChromaTOF™ (LECO, St. Joseph, MI, USA)
Database library	National Institute of Standards and Technology (NIST) 08

The analysis of the target odourous compounds was performed in the selected ion monitoring (SIM) mode. Their mass fragment used for determination and the relative retention time for determination of each compound are indicated in Table 3.3. One quantifier and three qualifiers were monitored, the **ion in bold** is the quantifier while the others are qualifiers

Table 3-3: Standards and internal standards (IS) and their quantification

Compound	**CAS No.**	**Molecular weight**	**Boiling point (ºC)**	**RT (min)**	**Ions (m/z)**
Butyric acid	107-92-6	88.11	162	7:29	41,42,43,**60**,73
DMTS	3658-80-8	126.26	58	5:40	45,47,64,79,**126**
p-Cresol	106-44-5	108.14	202	10:37	77,79,90,**107**,108
Indole	120-72-9	117.15	254	12:48	63,89,90,**117**,118
2-ethylbutyric acid (IS)	1988-09-8	116.16	99	4:26	41,43,66,**108**,150
Isopropyl disulfide (IS)	4253-89-8	150.31	176	6:31	41,43,66,108,**150**
4-isopropylphenol (IS)	99-89-8	136.19	212	6:45	77,91,103,**121**,136

The electron multiplier was set to an auto tune procedure. A solvent delay time of 3 min 10 s was used to avoid overloading the mass spectrometer with ethanol.

3.3.7 Data acquisition and chemometric analysis

The GC-ToF-MS was operated by LECO ChromaToF 4.50 data acquisition and processing software (LECO Corp, St Joseph MI, USA). TICs obtained were processed using automated peak find at S/N threshold of 100 with a library search in normal and forward mode. Components identification was based on comparison of both the retention times and mass spectra with those of the Willey 275 and NIST (USA) 92-Mainlib and Replib Mass spectral

libraries on the full spectra generated from the authentic standards with the similarity percentage of at least 90% under the identical experimental conditions. The two-level factorial experimental design and the CCD for SPME method development and analysis of variance (ANOVA) of the collected data were accomplished by employing Minitab statistical software (Release 17; Minitab Inc., PA, USA).

3.4 Isolation and identification of butyric acid degrading bacterial strains

3.4.1 Feacal sludge sample collection and preparation

Feacal sludge samples were collected as described in section 3.2.2 with some modifications in that the samples were collected once from a depth of 0 (surface) to 10 cm. The samples were immediately transported to the laboratory and preserved at 4 ^{o}C prior to use.

A mass of approximately 100 g of feacal sludge sample was suspended in a pre-sterilised 2 L Schott bottle with 1 L of sterile 18.2 MΩ deionised water prepared by Purelab Flex purification system (ELGA Lab Water Ltd, UK). The mixture was vigorously vortexed for 5 min and the suspended solids were allowed to settle down for 10 min. The supernatant was subsequently filtered through sterilised cotton wool (Dischem, South Africa) in a sterilised funnel for complete removal of the top layer (scum). The cotton wool was replaced after every 100 mL of the supernatant is filtered to avoid cotton wool compacting when wet. The aliquot of the filtrate obtained therefrom was preserved at 4 ^{o}C prior to use for bacterial isolation.

3.4.2 Enrichment, isolation and purification

The enrichment culture technique was used to isolate butyric acid-degrading bacteria (Liu *et al.* 2014). One hundred and fifty millilitres of sterile MSM supplemented with 500 mg/L of butyric acid as the sole carbon source and 1000 µL of supernatant (obtained from a 100 g of feacal sludge that was thoroughly vortexed in 1 L of deionised water) in 250 mL Erlenmeyer volumetric flask were incubated in a rotary incubator at 150 rpm and 30 °C in the dark for 24-48 h. An aliquot of 1000 µL was sub-cultured onto a fresh MSM with 500 mg/L butyric acid every 24 h and incubated under the same conditions. The culture was enriched by a series of four consutive enrichments in the fresh medium were carried out under the same condition to enrich a butyric acid-degrading microbial consortium. Then a series of dilution of the enriched inoculums from the flask were conducted. Ten 10 mL sterile test tubes were obtained and labelled test tube 1 through 10 and then 4.5 mL of MSM was added to each test tube and then 0.5 mL of the inoculums with bacterial suspension into test tube 1. At each step the solution was thoroughly mixed using the vortex before proceeding to the next step. This continued in this fashion until the original bacterial suspension was serially diluted into test tube 10. Then 100 µL of 7 to 10-fold serially diluted bacterial cell suspension was spread on nutrient agar plates to obtain pure colonies subsequently the sterile nutrient agar plates were incubated in

static incubator at 30 °C for 24-48 h in the dark. For purification morphologically distinct colonies obtained based on characteristics of colour and size were streaked at least three times on fresh nutrient agar slants and incubated for 24-48 h in the static incubator at 30 °C in the dark in preparation for 16 rRNA sequence identification

3.4.3 DNA extraction

For 16S rRNA analysis, DNA was extracted from isolates using the boiling method from a 16 h pre-grown cell suspensions of the pure cultures wherein the DNA was extracted by suspending a single bacterial colony from nutrient agar in sterile distilled water and incubating the cell suspension for 10 min at 100 °C in water bath. The polymerase chain reaction (PRC) was used for amplification of 16S rRNA gene for sequencing and phylogenetic evaluation. The solution was centrifuged at 14 000 x g for 30 s and supernatant was eliminated. The pellet was re-suspended in molecular biology-grade water (Eppendorf, Hamburg, Germany) and centrifuged at 14,000 x g for 30 s.

The amplification of the genes coding for 16S rRNA and sequencing was performed by using a set of forward and reverse primers, complementary to highly conserved region of the 16S rRNA. Briefly, each 25 µL PCR mixture was consisting of aliquots of 50-100 ng template DNA, 10 µM/µl of forward primer (27F: 5' GAG TTT GAT CCT GGC TCA G 3') (modified from Edwards *et al.,* 1989) and reverse primer (1492R: 5' GGT TAC CTT GTT ACG ACT 3') (Lane, 1991), 25 mM $MgCl_2$ (Separation Scientific, Johannesburg, South Africa), 2.5 mM of each deoxyribonucleotide triphosphate (dNTP)(Fermentas, Massachusets, USA), 0.1 U/µL of Super-Therm Taq DNA polymerase and reaction buffer (Separation Scientific, Johannesburg, South Africa).

Following polymerase activation, the cycling conditions were as follows: initial denaturation performed at 94 °C for 10 min, thereafter there were 30 cycles of denaturation at the same temperature of 94 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 °C for 1 min, subsequently a final elongation step at 72 °C for 5 min.

3.4.4 Phylogenetic analysis

The sequences were evaluated using ChromasLite v2.01 (Technelysium, Queensland, Australia) and BioEdit v7.05 (Hall, 1999). The 16S rRNA sequences were compared to all the nucleotide sequences included in GenBank® database maintained by National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) searches at http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990; Benson *et al*., 2004). The Multiple Alignment using Fast Fourier Transform (MAFFT) online version (Katoh *et al*.

2002) was used for the alignment of the 16S rRNA sequences of isolates and closest reference strains.

Phylogenetic trees were constructed using the best-fit evolutionary model parameters determined by MEGA version 6 (Tamura *et al*., 2013) and the best of the Nearest-Neighbour Interchange (NNI) and Subtree-Pruning-Regrafting (SPR) search algorithms were applied for tree searching. Branch support was evaluated using bootstrap analyses based on the same model parameters and was estimated using 100 pseudoreplicates.

3.4.5 Degradation and bacterial growth conditions

To investigate the degradation of butyric acid as well as the growth of bacterial strains with butyric acid as a carbon and energy source, 1 mL of bacterial strain pure seed culture (OD_{600} = 2.0) (equivalent biomass, mg/L, for each of the bacterial strain are provided in Table S3.1 in appendix 1 was inoculated into 150 mL each of the MSM supplemented with 1000 mg/L butyric acid in 250 mL Erlenmeyer flask in triplicates. The experiments were aseptically conducted. Similarly, abiotic MSM with the same concentration of butyric acid was used as a control in triplicates. All the reactors were incubated at 30 ^{o}C on a temperature controlled rotary shaker in the dark at 110 rpm for 24 h. The samples were aseptically withdrawn at regular time intervals of 4 h to determine both butyric acid concentration and optical density (OD). Samples for determination of bacterial growth were withdrawn from the reactor before and at 4, 8, 12, 16, 20 and 24 h after starting incubation while for determination of butyric acid concentration, samples were withdrawn at 4, 8, 12, 16, 20 and 24 h after starting the incubation. From this procedure, the degradation efficiencies of the bacterial strains were determined with equation 3.3 (Gutarowska *et al*. 2014):

$$D_e = \left(\frac{A_c - A_s}{A_c}\right) x \ \ 100\% \tag{3.3}$$

where D_e, A_c and A_s are the butyric acid degradation efficiency (%), the initial concentration of butyric acid (mg/L) in the abiotic culture at t_n (0, 4, 8, 12, 16, 20 and 24 h) and the concentration of butyric acid (mg/L) in the biotic culture at t_n (0, 4, 8, 12, 16, 20 and 24 h), respectively.

3.4.6 HPLC analysis

The aliquot (6 mL) of culture medium was withdrawn from the enrichment flasks at 4 h time intervals and was centrifuged at 9,000 rpm for 10 min at room temperature, using a Minispin centrifuge of Eppendorf AG type (Hamburg, German). The supernatant was subsequently filtered through Milipore Millex-GV Hydrophilic PVDF 0.22 µm membrane and dispersed into 2 mL HPLC vial prior to analysis.

Residual butyric acid was determined by a Waters Alliance 2695 Separation Module High Performance Liquid Chromatography (HPLC) system (Waters Corporation, Milford, MA, USA) in triplicates to determine the residual butyric acid concentration. The system was equipped with a low-pressure mixing pump, an inline degasser, an auto-sampler with programmable temperature control (samples held at 5 °C) and a Waters 2998 Photodiode array detector (PAD) equipped with micro UV cell (Waters Corporation, Milford, MA, USA).

An HPLC mobile phase of 0.02 M H_2SO_4) was used. The mobile phase was prepared by diluting 1.1 mL of 18.4 M H_2SO_4 with 18.2 MΩ deionised water to a final volume of 1.0 L. This was filtered through a Nylon 5-micron membrane before injection into the HPLC. Sample injection volume of 10 µL was used for all analyses. The stationery phase was an Aminex HPX-87H87H ion-exclusion organic acid, 300 mm × 7.8 mm, 9 µm particle size column (Bio-Rad Laboratories, Berkeley, CA, USA) ran with an isocratic flow rate of 1 mL/min at a column temperature of 60 ^{o}C. The detection of the peaks was monitored at a wavelength of 210 nm. Retention time for butyric acid was 12.2 min and the total run time was set at 15 min. Chromatographic data were processed by Empower2 Build 2154 software (Waters Corporation, Milford, MA, USA).

Qualitative and quantitative data were obtained by comparing the peak area and peak height to butyric acid standard compound with known concentration. The concentration of butyric acid was deduced from an external calibration curve.

3.4.7 Bacterial growth measurements

The concentration of bacteria in the samples were quantitatively determined by spectrophotometric monitoring in which the optical density at a single wavelength λ = 600 nm (OD_{600}) using a UV Lightwave II spectrophotometer (Labotec, Gauteng, South Africa) was measured. The macro quartz cuvette with optical path length of 10 mm (Thomas Scientific, South Africa) was used to carry the aliquots in the sample chamber of the spectrophotometer. The optical density measurements were taken using an abiotic MSM cuvette as a reference. The dry weight method was applied to estimate biomass in mg/L. The generated calibration equations of each bacterial strain are listed in Table S3.1 in appendix 1.

3.5 Construction of bacterial consortia

3.5.1 Bacterial consortia development

The isolates for the consortium development were selected based on two categories. The first category comprised of three isolates that were able to degrade butyric acid completely within 20 h. The second category comprised of three isolates that were able to degrade butyric acid within 24 h. Accordingly, 19 different bacterial consortia were indigenously constructed using

the selected bacterial isolates by applying a combinational statistical formula which is denoted by equation 3.4 (Katdare and Patil, 2014):

$$\binom{n}{r} = \frac{n!}{r!\,(n-r)!} \tag{3.4}$$

where $\binom{n}{r}$ is the combinatorial symbol, read as "n choose r", n is the total number of bacterial strains and r is number of bacterial strains in each consortium. The bacterial consortia were developed by aseptically mixing in 1/1(v/v) (1 mL of pure bacterial cell suspension with absorbance of 2.0 (OD_{600})) into a 50 mL pre-sterilised centrifuge tube (Greiner Bio-One, Kremsmünster, Austria). The mixture was then vigorously vortexed to ensure homogenous distribution of all bacterial strains.

3.5.2 Butyric acid degradation by pure cultures and bacterial consortia

The experiments were carried out by inoculating 1 mL of bacterial consortium or pure bacterial cultures into 150 mL each of MSM supplemented with 1000 mgL^{-1} of butyric acid as a sole carbon source in a sterile 250 mL Erlenmeyer volumetric flask in triplicates. Abiotic MSM with the same butyric acid concentration was used as the control in triplicates. After sealing with aseptic cotton wool, the flasks were incubated in the dark at 30 ºC in a temperature controlled rotary shaker at an agitation rate of 110 rpm for 24 h. Samples were taken aseptically at regular 4 h time intervals to determine the butyric acid concentration as well as the optical density. The samples for determining the butyric acid degradation were taken at time, t = 4, 8, 12, 16, 20, 24 h while the samples used to determine the bacterial growth i.e. measuring the optical density were taken at time t = 0, 4, 8, 12, 16, 20, 24 h. The suspensions were vortexed and centrifuged for 10 min at 10,000 rpm at 4ºC. The supernatant from each sample was analysed by HPLC as described in section 3.4.6. The degradation ability was expressed as the percentage of butyric acid degraded in relation to the remaining butyric acid in appropriate abiotic control samples based on equation 3.3. in section 3.4.5.

3.5.3 Effect of environmental parameters on bacterial growth and butyric acid degradation

Effects of temperature, pH and inoculum size on butyric acid degradation by and growth of *S.marcescens* and *B.cereus* were investigated. Bacterial strain cell suspension was inoculated in 250 mL Erlenmeyer flasks, in triplicates, containing 150 ml MSM supplemented with 1000 mg/L of butyric acid as a sole source of carbon and kept at varied temperatures, initial pH values, and initial inoculum concentrations and incubated for 16 h by inoculating OD_{600}= 2.0 biomass of each of the bacterial strains separately unless otherwise stated. The effects of temperature on butyric acid degradation and bacterial growth were assessed at

various temperatures of 25, 30, 35, 40 and 45 °C at pH 7 and 110 rpm. The effects of initial pH value on butyric acid degradation and bacterial growth were assessed with MSM initial pH values of 5, 6, 7, 8, 9 and 10 at 30 ^{o}C and 110 rpm. The initial pH values were obtained by titration of concentrated HCl or 6M NaOH. To assess the effect of initial inoculums concentrations on butyric acid degradation and bacterial growth, MSM was inoculated with 1 ml of cell suspension with varied inoculum sizes of 0.5, 1.0, 1.5, 2.0 and 2.5 at 30 ^{o}C, pH7 and 110 rpm. Abiotic controls were also set up for each experiment. After 16 h, butyric acid degradation efficiencies in the respective cultures' flasks were determined based on the equation.3.3.

3.6 Bacterial growth modelling

3.6.1 Inoculum preparation

Vials were moderately thawed in a static incubator (Merck, South Africa) at 35 ^{o}C to prepare the frozen cultures for the assays. A 100 µL of the thawed culture was transferred to 300 ml of MSM supplemented with 500 mgL^{-1} in in a sterile 1000 mL Erlenmeyer volumetric flask (reactor) and in a shaking incubator (LabCon, Texas, USA), at 110 rpm and 30 ^{o}C in an aerobic condition by plugging with sterile cotton wool. Due to the presence of some freeze-damaged cells, this culture was not used for growth experiments. The inocula for use in assays were prepared by transferring 100 µL of each culture to 300 mL of MSM supplemented with 500 mg/L and incubating aerobically as per procedure mentioned above for 16 to 20 h prior to the time of the experiment conception. At the time of the experiment conception, the cell suspension of each culture was harvested by centrifugation at 9000 rpm, 4 ^{o}C for 10 min using Sorvall Lynx 6000 centrifuge (Thermo Scientific, Germany) and the supernatant was discarded. The cell pellet was rinsed thrice with sterile physiological saline solution (0.085% (w/v) NaCl solution) and centrifuged as per procedure mentioned above. The optical density at 600 nm (as explained below) for pure cultures was adjusted to 2.0 by resuspension in sterile MSM prior to inoculation.

3.6.2 Bacterial growth assays

A volume of 1 mL of prepared inoculum suspension of pure cultures of *B.cereus* and *S.marcescens* was sceptically added to 150 mL of MSM supplemented with 1000 mg/L in in a sterile 250 mL Erlenmeyer volumetric flask. All reactors were incubated in a temperature-controlled shaking incubator in the dark at 110 rpm and observed for at least 24 h . Constant temperatures were set at 25, 30, 35, 40 and 45 ^{o}C to study the effect of temperature. Samples for the determination of optical density were aseptically withdrawn from the reactors at a regular interval of 4 h with the initial withdrawal performed immediately after inoculation This was doneuntil the stationary phase was reached. All assays were performed in triplicates for

each temperature. For each replicate assay performed in triplicate, a mean of the triplicates of each sampling time point was used to determine estimates of the bacterial growth. The concentration of bacteria in the samples were quantitatively determined as described in section 3.4.7.

3.6.3 Primary and sondary modelling

The growth of *B. cereus* and *S.marcescens* measured from absorbance that was recorded in 4 min intervals in all the bioreactors for aerobic batch cultures was plotted against incubation time. The modified logistic (Zwietering *et al.,* 1990), modified Gompertz (Gibson *et al*.,1987) and Richards (Richards, 1959) models (for simplicity, hereinafter referred to as logistic model Gompertz model, Richards model, respectively) expressed according to the equations (3.5) (3.6) and (3.7), respectively.

$$y = \frac{a}{[1 + exp(b - cx)]} \tag{3.5}$$

$$y = a * exp[-exp(b - cx)] \tag{3.6}$$

$$y = a\{1 + v * exp[k * (\Tau - x)]\}^{(-1/v)} \tag{3.7}$$

where a , b , c , v , k and $\Tau$ are mathematical parameters

The above sigmoidal functions have been applied to empirically describe the growth of microbial cultures, however, the models were derived so that the mathematical parameters, a , b , c , v , k and $\Tau$ in equations (3.5), (3.6) and (3.7) have microbiological relevance. The re-parameterised modified logistic (equation 3.5), modified Gompertz (equation 3.6) and Richards (equation 3.7) models are given by equations (3.8), (3.9) and (3.10), respectively (Zwietering *et al.*, 1990):

$$y = \frac{A}{\left\{1 + exp\left[\frac{4\mu_{max}}{A}(\lambda - t) + 2\right]\right\}} \tag{3.8}$$

$$y = A \ \ exp\left(-exp\left(\frac{\mu_{max} e}{A}(\lambda - t) + 1\right)\right) \tag{3.9}$$

$$y = A\left\{1 + v.exp(1 + v).exp\left[\frac{\mu_{max}}{A}(1 + v)\left(1 + \frac{1}{v}\right).(\lambda - t)\right]\right\}^{(-1/v)} \tag{3.10}$$

where y is the bacterial concentration at time t , A is the asymptotic value as t decreases indefinitely, λ [h] is the lag phase duration, μ_{max} [h^{-1}] is the maximum specific growth rate, v

is the shape parameter and *e* is the Euler's constant, which is the base of the natural logarithm, equal to 2.718.

Growth kinetic parameters of biological relevance A, μ_{max} and λ were computed from the logistic model mathematical parameters as follows:

$$A = a \tag{3.11}$$

$$\mu_{max} = \frac{bc}{4} \tag{3.12}$$

$$\lambda = \frac{(b-2)}{c} \tag{3.13}$$

The Ratkowsky (Square root) model (Ratkowsky *et al.,* 1982) and the inverse Ratkowsky model (Zwietering *et al*., 1991; Dobrić and Bååth, 2018) defined by equations (3.14) and (3.15), respectively, were used to describe the maximum specific growth rate, μ_{max} and lag phase duration, λ , respectively, as a function of incubation temperature.

$$\sqrt{\mu_{max}} = b\ (T - T_{min}) \tag{3.14}$$

(3.15)

$$\sqrt{\frac{1}{\lambda}} = b\ (T - T_{min})$$

μ_{max} [h^{-1}] is the maximum specific growth rate, λ [h] is the lag phase duration, *T* [^oC] is the temperature, T_{min} [^oC] is a conceptual minimum temperature for microbial growth, is the intercept between the model and the temperature and *b* [$h^{0.5}\ ^oC^{-1}$] is an empirical parameter (slope of the regression line).

The growth data of *B.cereus* and *S.marcessen* obtained at isothermal conditions were fitted non-linearly to determine mathematical growth kinetic parameters using ORIGIN 9.0 software (OriginLab Corporation, Northampton, MA, USA) that uses the Levenberg-Marquardt algorithm. This algorithm uses least square estimation wherein the sums of square of residuals between the predicted and experimental values are minimized. Microsoft Excel software

(Microsoft Corporation., Redmond, WA, USA) was used to fit the square root of μ_{max} and inverse of λ versus incubation temperature and calculating their goodness of fit parameters, Pearson's coefficient of determination (R^2) and root mean square error (RSME).

3.6.4 Model performance evaluation

The performance of the primary models was statistically evaluated by considering the R^2 and RMSE using equations (3.16) and (3.17), respectively:

$$R^2 = 1 - \left[\sum_{i=1}^{N}(y - \hat{y})^2 / \sum_{i=1}^{N}(y - \bar{y})^2\right] \tag{3.16}$$

where N is the number of observations, y is the observed values at the i^{th} temperature, $\hat{y}$ is the predicted values at the i^{th} temperature and $\bar{y}$ is the mean of the predicted values.

$$RMSE = \left[\sum_{i=1}^{N}(\hat{y} - y)^2 / N\right]^{1/2} \tag{3.17}$$

where N is the number of observations, y is the observed values at the i^{th} temperature and $\hat{y}$ is the predicted values at the i^{th} temperature.

R^2 is often used as an overall measure of the prediction attained. The higher the value ($0< R^2 <1$), the better is the prediction by the model. The lower the value of RMSE, the better the adequacy of the model to describe the experimental data (Ross, 1996). In addition, three other complementary criteria based on the information theory (Shanon,1948), viz. Akaike Information Criterion (AIC) (Akaike, 1973) and its sond sond-order variant commonly known as corrected Akaike Information Criterion (AIC_c) (Cavanugh, 1997) and Bayesian Information Criterion (BIC) (Schwarz,1978) were also considered to evaluate the models based on generalizability i.e. the best predicting model using the equations (3.18), (3.19) and (3.20), respectively.

$$AIC = nIn[(\sigma^2)] + 2k \tag{3.18}$$

$$AIC_c = AIC + \frac{2k(k+1)}{n-k-1} \tag{3.19}$$

$$BIC = \ nIn[(\sigma^2)] + kIn(n) \tag{3.20}$$

in which

$$\sigma^2 = \frac{RSS}{n} = \sum_{i=1}^{n} (\hat{y} - y)^2 / n \tag{3.21}$$

where σ^2 is the estimate of error variance from fitting the full model, n is the number of observations; k is the number of parameters in the model and RSS is the residual sum of squares in the model. In the application of these criteria to several models, the best model is the one with the lowest AIC, AIC_c or BIC values.

When the candidate models are known, the best model is chosen by comparing their respective Akaike weights. Generally, the best model is the one with the highest Akaike weights. For M candidate models, the Akaike weight of the i^{th} model to estimate the model plausibility and is computed as equation (3.22) (Serment-Moreno *et al.,* 2015):

$$W_i = exp\left[-(1/2)\Delta_i\right] \Big/ \sum_{k=1}^{M} exp\left[(-1/2)\Delta_k\right] \tag{3.22}$$

where for all of the candidate models the difference between AICc values was given by $\Delta_i, = \mathrm{AICc}_i - \mathrm{AICcmin}, i = 1, 2...$ *M* and AICc_{min} represents the minimum AICc value in all M candidate models.

A new weight as a reference indicator as proposed by Shi and Ge (2010), which is referred to as α in this study, was used. This weight integrates the aforementioned indicators to choose the best model and was estimated using equation (3.23):

$$\alpha_i = \beta_i \Big/ \sum_{k=1}^{M} \beta_k \tag{3.23}$$

where α_i is the weighed mean of standardized indicators, i = 1, 2,….,*M* and β_i was computed based on equation (3.24). In this study, R^2_{adj}, BIC and AIC_C were chosen to compute β_i.

$$\beta_i = \frac{1}{3}\left[\frac{\left|R^2_{adj,i} - R^2_{adj,min}\right|}{R^2_{adj,max} - R^2_{adj,min}} + \frac{|BIC_i - BIC_{max}|}{BIC_{max} - BIC_{min}} + \frac{|AICc_i - AICc_{max}|}{AICc_{max} - AICc_{min}}\right] \tag{3.24}$$

where y_{max} represents the maximum y value in all M candidate models, y_{min} represents the minimum y value in all *M* candidate models y_i represents the y value of the i^{th} candidate model and i =1, 2,…., *M*.

3.6.5 Statistical analysis

The statistical significant differences of the μ_{max} and λ of *B.cereus* and *S.marcessens* were checked by using one-way analysis of variance (ANOVA) and Tukey's post hoc analysis at a $\alpha \leq 0.05$. Growth parameters for both bacterial strains were also compared to each other. Statistical analyses were carried out in SPSS 25.0 for Windows (SPSS Inc., Chicago, USA).

CHAPTER FOUR

4 IDENTIFICATION AND CHARACTERISATION OF ODOURANTS AND OTHER VOLATILE ORGANIC COMPOUNDS FROM PIT LATRINE FEACAL SLUDGE

4.1 Background

Resource-constrained communities in developing countries rely on on-site sanitation facilities such as pit latrines for low cost, hygienic and safe disposal of human wastes. Approximately, 1.77 billion people depend on such facilities on a daily basis (Graham and Polizzotto, 2013). This number of pit latrine users is expected to rise as countries work towards achieving SDG 6.2 (Ravenscroft *et al.*, 2017). Thisincrease will not only result in more pit latrines but also an exacerbation of environmental challenges associated with pit latrine particularly odour emissions. A study conducted by Bakare *et al*. (2012) revealed that anaerobic digestion is the principal pathway for waste components of pit latrine. Malodours associated with anaerobic processes are one of the deterrents to consistent use or adoption of pit latrines. Filthy smelling forces current users and would be adopters and users to prefer open defecation, a practice that promotesthe spread of oral-feacal related diseases. (Mercer *et al*., 2019). This makes elimination or at least reduction of compounds that cause malodours acritically important endeavour as it helps to improve the user-friendliness of pit latrines and enhances sanitation adaptation for all the people to ensure the the goal of universal sanitation is realised.

Generally, most odourous compounds are organic in nature and are volatised from the solid or liquid material where they are generated (Miner, 1977). The anaerobic decomposition of biodegradable organic matter contained in waste by bacteria result in generation of malodourous products that can be either intermediate or end products (Filipy *et al*., 2006). Although VOCs represent less than 1 % of total gaseous emissions, some have been identified as responsible for odours (Moreno *et al.*, 2014). VOCs are among the most hazardous air pollutants and long term exposure to some such as benzene, toluene, ethylbenzene and xylene (BTEX) are toxic and carcinogenic (Moreno *et al.*, 2014; Schreiner *et al.*, 2017). Moreover, VOCs play a role in photochemical smog formation hence they indirectly contribute to global warming (Schiavon *et al.*, 2017).

Current odour abatement technologies applied throughout the developing countries are relatively ineffective. One of the reasons for their ineffectiveness is that malodourous compounds are eliminated or neutralised after they have already been partitioned into the gas

phase. It is, therefore, more desirable to control odour at the source by preventing gas-phase partitioning and potentially avert malodourous emissions from the pit latrines (Mercer *et al.*, 2019) and characterisation of VOCs emitted by pit latrines is a crucial step for the odour control process. Few studies have identified and quantified volatile gasses emitted from pit latrines (Lin *et al.*, 2013; Chappuis *et al.*, 2015; Obeng *et al.* 2016; Chappuis *et al*., 2018; Obeng *et al.*, 2019). However, for most of these studies, the attention was focussed specific selected malodourants and primarily concerned with malodourants in the off-gases emitted from pit latrines. In addition, although management of odourants in the liquid phase is an attractive proposition, those studies on liquid phase VOC odourant characterisation of pit latrine feacal sludge have been insufficient. Consequently, our scientific knowledge about the actual composition of liquid phase feacal sludge odourants and volatile organic compounds is far from sufficient to provide a scientific basis for mitigating malodourous emissions from pit latrines.

A broad overview identification of the VOCs hitherto detected in pit latrine sludge would provide insights into potential odour-active compounds that contribute to pit latrine malodours. In addition, the knowledge of the composition of VOCs emitted from pit latrine feacal sludge can also be valuable to assess the environmental impacts of the volatiles inside pit latrines as well as ambient air of their vicinities. The purpose of this work is, therefore, to identify malodourants and volatile organic compounds emanating from pit latrine feacal sludge without their deep quantification. The results obtained from this work could be fundamental to focus for future research to develop alternative and effective odour abatement technologies. to diminish the more critic volatile organic compounds that are responsible for pit latrine malodourous emissions. The results will also form part of an integrated solution to offer the pit latrine users nuisance-free breathable air latrines and an important step in the elimination of open defecation and help to attenuate pit latrine odour emission related public health risks.

4.2 Results and discussion

Materials and methods used to achieve the objectives of this chapter are found in section 3.2.

4.2.1 Composition of the VOC emissions

VOCs are any organic chemical compounds which contain at least one carbon and hydrogen atoms in their molecular structures and that have with a high vapour pressure of greater than 2mm of mercury (0.27 kPa) at 25 ^{o}C, are present in the atmosphere as gases emitted from certain solids or liquids (Dewulf *et al.*, 2002; Mirzaei *et al*., 2016). In this study showed that pit latrines feacal sludge emit a plethora of chemically divergent of VOCs and inorganic compounds throughout the eight pit latrines as shown in Figure 4.1. Even though a wide

spectrum of VOCs were detected, only the VOCs that had their structures identified with 95% or greater match with NIST library were listed in this study.

It is worth noting that some of the compounds that were detected in feacal sludge samples were also present and detected in the blank samples. In view of this, it was deemed to omit all the compounds detected in the blanks from the peak list. A variety of compounds, 358 VOCs and odourants in total, were identified in the feacal sludge samples. The variety of compounds detected in the present study are greater than what is reported by Lin et al. (2013) who conducted a comparable study but with a sample size of 16 pit latrines. The highernumber of VOCs detected in the present study can be attributed to the salting-out effect that is facilitated by the addition of inorganic salt such as NaCl in the samples. Generally, it is known that the addition of salt enhances the transfer of compounds from the aqueous to air phase during SPME extraction (Liu *et al.*, 2018). This allowed more VOCs to partition into the vial headspace consequently improving their extraction yield. Moreover, the SPME extraction temperature employed in the study by Lin *et al*. (2013) was higher than the temperatures used in this study. Although high temperature enhances the partitioning of the analytes from the liquid sample to the headspace, the same can result in the distortion of the VOC composition through thermal artefact formations and loss of highly volatile compounds.

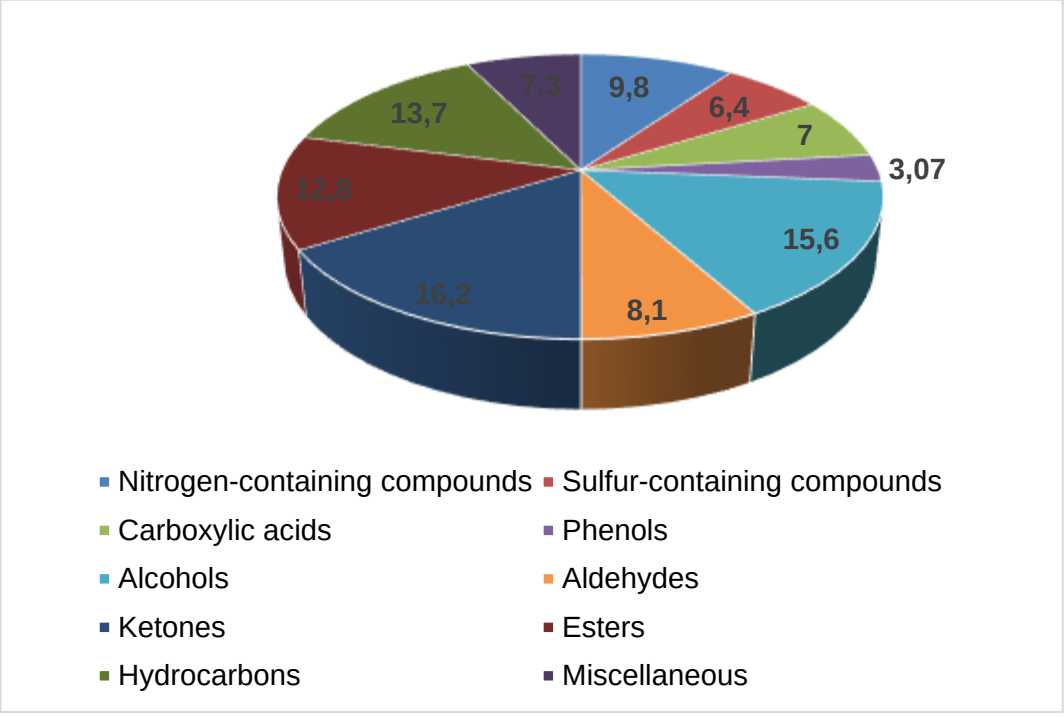


Figure 4-1: VOC chemical functional group distribution for all compounds identified qualitatively

It is often difficult to analyse VOC species because they were quite numerous and to alleviate this difficulty, the compounds detectedwere allocated into one of the nine chemical functional groups of the compounds based on their major moieties. The groups were nitrogen-containing compounds, sulfur-containing compounds, carboxylic acids, phenols, aldehydes, ketones, alcohols, hydrocarbons, esters and miscellaneous as presented in Table S4.1. As shown in Figure 4.1, ketones were found to be the most abundant species among the VOCs detected as listed in Table S4.1 followed by alcohols, hydrocarbons, esters, nitrogen-containing compounds, aldehydes, carboxylic acids, sulphuric-containing compounds and phenols in that order. The large number of ketones detected in this study can be because of the decarboxylationof the analogous oxo-acids by bacteria in the human gut (de Lacy Costello *et al.*, 2014). On the other hand, the large number of alcohols may be attributed in part to reduction of the analogous fatty acid, bacterial degradation of amino acids and carbohydrates (Garner *et al*., 2007) as well as the high levels of alcoholic beverages comsumption in South Africa (WHO, 2019). South Africa is one of the highest alcohol consuming countries in Africa with alcohol per capital consumption (APC) of 9.3 (WHO, 2019). To some certain extent results from the t present study are inconsistent with Lin *et al* (2013) found when the carried out a comparable study on faecal sludge collected from pit latrines in Uganda, South Africa, Kenya and India. Some of the compounds detected in this study have also been reported by other researchers to be present in headspace of human feacal samples, (Garner *et al*., 2007; Reade *et al.*, 2014) and human urine, (Smith *et al.*, 2008; Bouatra *et al.*, 2013; Wagenstaller and Buettner, 2013; Mochalski and Unterkofler, 2016). The results of this study suggest that liquid phase of pit latrine feacal sludge contains a very complex mixture of VOCs and that a vast collection of VOCs upon their production dissolve in water that is found in pit latrines.

As shown in Table S4.1 in appendix 2, it was observed that the type and number of volatiles detected were affected by season (winter and summer). Nearly all pit latrines exhibited a similar trend with a greater number of compounds w detected in summer than in winter. The number of volatiles detected in summer ranged between 56 (P4S) and 66 (P8S) and average of the volatiles was found to be 48. On the other hand, the number of the volatiles detected in winter ranged between 80 (P1W) and 33 (P7W) and average number of 46. The results show that in summer, P4 had the largest number of volatiles detected while P8 has the smallest number of the detected volatiles. In winter, P4 had the largest number of volatiles and P7 had the smallest number of volatiles. Despite differences in the specific categories, it was observed that, for instance, butyric acid and indole had the highest frequency of detection in summer while p-Cresol had the same frequency of detection during summer and winter. The observed trend is probablydue to changes in environmental conditions in the pit latrine.

4.2.2 Physicochemical parameters of the detected VOCs

The compounds that emitted from all the pit latrines in this study were mostly organic in nature with very few compounds such as sulphur dioxide and nitrous oxide being inorganic. Therefore, the major focus of interest was to consider the physicochemical properties of the detected VOCs. Physico-chemical properties are important parameters in the evaluation of the fate, distribution and behaviour of the VOCs, as polluting chemical compounds, in the different environmental compartments (Bobadilla *et al*., 2003). The determination of the VOCs's physical and chemical properties are also considered vital for the development of appropriate VOC control strategies through either minimising their emissions or maximising their removal (Yue and Li, 2013).

In this study, the physical and chemical properties that were taken into consideration include the number of carbon atoms in the molecular structures, boiling point (bp.), vapour pressure (v.p.), water solubility, octanol–water partitioning coefficient and Henry's law constant (HLC). This approach is divergent from the approaches used in previous studies (Lin *et al.*, 2013; Chappuis *et al.*, 2015; Obeng *et al.* 2016; Chappuis *et al*., 2018; Obeng *et al.*, 2019) that did not consider any of these properties. To the best of the researcher's knowledge, this is the first attempt to characterise pit latrine VOCs based on their physical and chemical characteristics. The physicochemical characteristics of the VOCs such as water solubility, octanol–water partition coefficient, HLC and vapour pressure influence the ability of the compounds to either become airborne or adsorb/ absorb to the organic material of the emission source and subsequently such properties greatly influencethe overall composition of VOCs (Lehtinen and Veijanen, 2011). Additionally, in relation to biological treatment of volatile odourous compounds, water solubility, octanol–water partition coefficient, HLCs and vapour pressure, together with hydrophobicity, are important as they determine if the VOCs can be degradeded by microorganisms (Lewis *et al.*, 1994).

The physicochemical properties such as vapour pressure and water solubility were compared at the same temperature of 25 ^{o}C to enable objective comparison. This is important since these two parameters are temperature dependent. The higher the temperature, the higher and the water solubility and vapour pressure and vice versa. Furthermore, the units of measurement for each physicochemical data were the same for comparison. The physico-chemical properties data of all the detected VOCs such as molecular weight, vapour pressure, aqueous solubility, HLC, and octanol-water partition coefficient (K_{ow}) was not experimentally determined or estimated through calculations rather were obtained from the literature (Mackay

et al., 1992; Cai *et al.*, 2006; Sander, 2015) and public database such as PubChem (PubChem, undated).

4.2.2.1 Number of carbons

Pit latrine emissions contain a variety of VOCs, which range from very volatile organic compounds (VVOCs) to semi-volatile organic compounds (SVOCs) that range from C1 to C24. In this study, the VOCs with C1-C5, C6-C17 and C18-C24 were classified as VVOCs, VOCs and SVOCs, respectively (Salthammer, 2016). This classification scheme is usually relieson the number of carbons in the alkyl chain. Generally, as the number of carbons (Cs) increases in the alkyl group, the volatility of the compound decreases. Figure 4.2 shows the distribution of the VOCs based on the number of carbons. Overall, 29.0% of compounds were classified as VVOCs, 68% as VOCs and 3.0 % asSVOCs and thr average carbon number of 8.

As shown in Figure 4.3, for all the chemical functional groups except sulfur-containing compounds (SCCs), most of the compounds can be classified as VOCs. For SCCs most of the compounds (86%) were classified under VVOCs whilst all the phenols were classified as VOCs. These results are reflective of the fact that, notwithstanding the chemical functional group the majority of the VOCs have less than 18 carbons in their chemical structures.

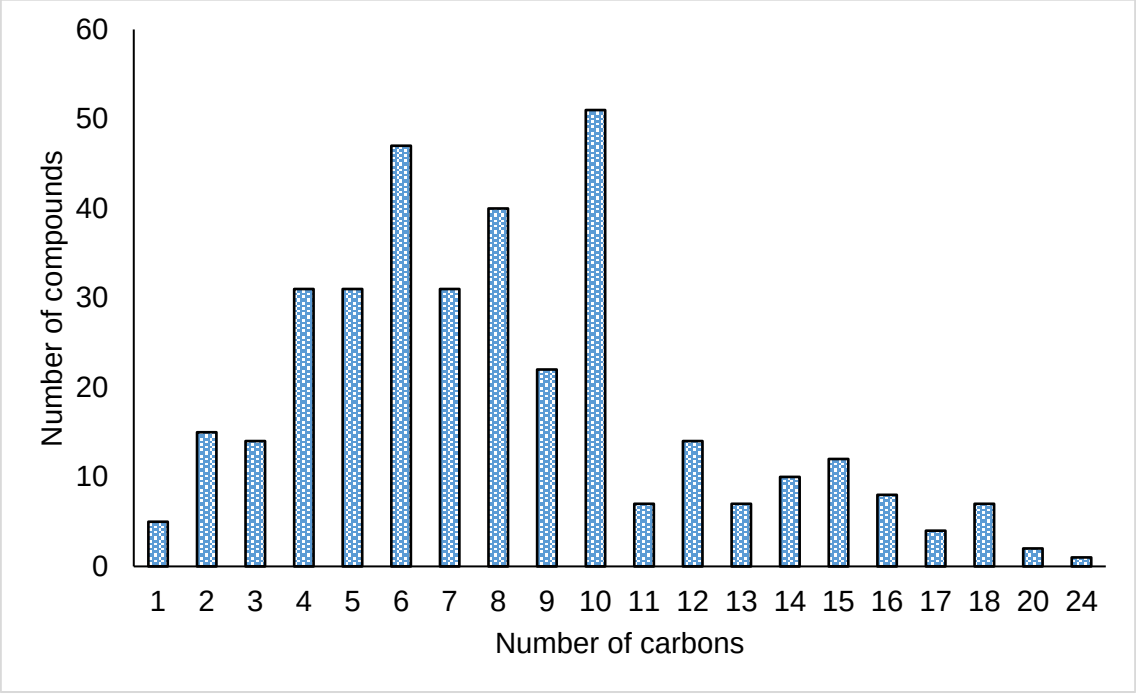


Figure 4-2: Distribution of VOCs by number of carbons

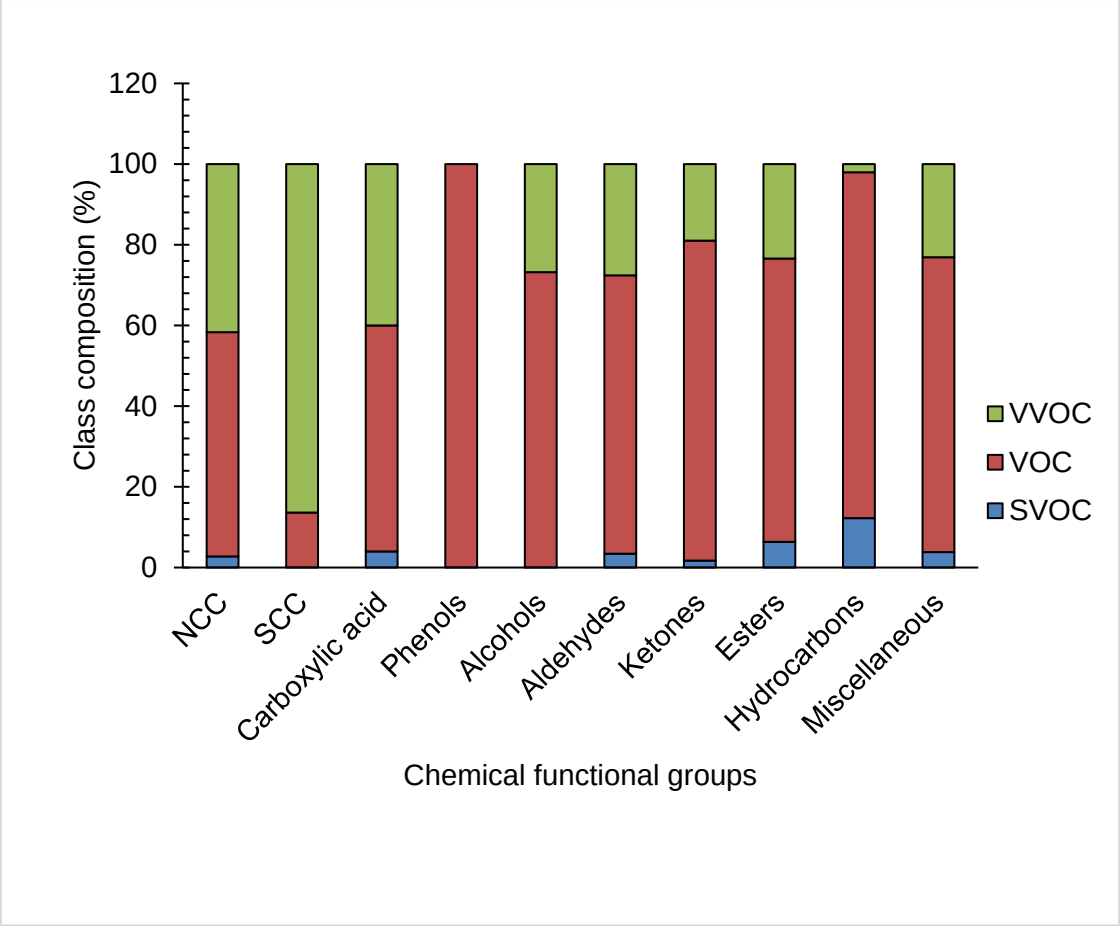


Figure 4-3: Distribution of VOCs by number of carbons according to chemical functional

4.2.2.2 Boiling points

Based on the World Health Organisation (WHO) classification, the VOCs were also characterised according totheir respective boiling points (bps) (Król *et al*., 2010). The bp of the compounds ranged from −89 to 454.13 ºC and the mean bp. was 180.17 ºC. As shown in Figure 4.4, 13.6 % of the compounds were classified as VVOCs with bp. ranging from -89 to 100 ºC.. The compounds with bp. between > 100 and 250 ºC made up 66.9% of the compounds that were classiofied as VOCs. Finally, compounds with greater that 250 ºC were classified as SVOCs.y. It is interesting to recall that all extractions were conducted at 25 ºC and the results indicate that pit latrine feacal sludge even at ambient temperatures can emit a wide range of VOCs. It also has to be noted that the fecacal sludge were kept at 25 ◦C during sampling and the analysis has showed that 0.8% of compounds had bp. <25 ◦C. It is high likely that such compounds, those with bp <25 ºC, were underreported. This is because those compounds with bp below the ambient temperature are so volatile that they are hardly extracted on the fibres and they exist almost entirely as gases in the air rather than in the fecal sludge or on the fibre surfaces. As shown in Figure 4.5 using bp to classify the compounds, bpSCCs could be classified as VVOCs and VOCs. Carboxylic acids and phenols could be

classified as VOCs and SVOCs. The remaining compounds were present in other chemical functional groups were in all classes. However, it was noted that most of the compounds in all chemical functional groups were classified as VOCs.

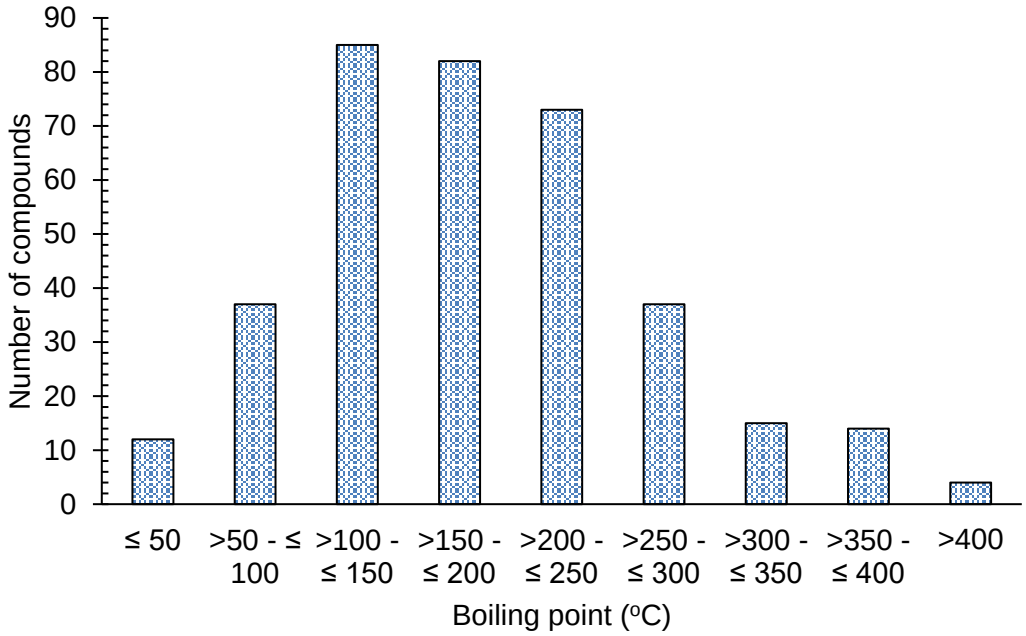


Figure 4-4: Distribution of VOCs by boiling point

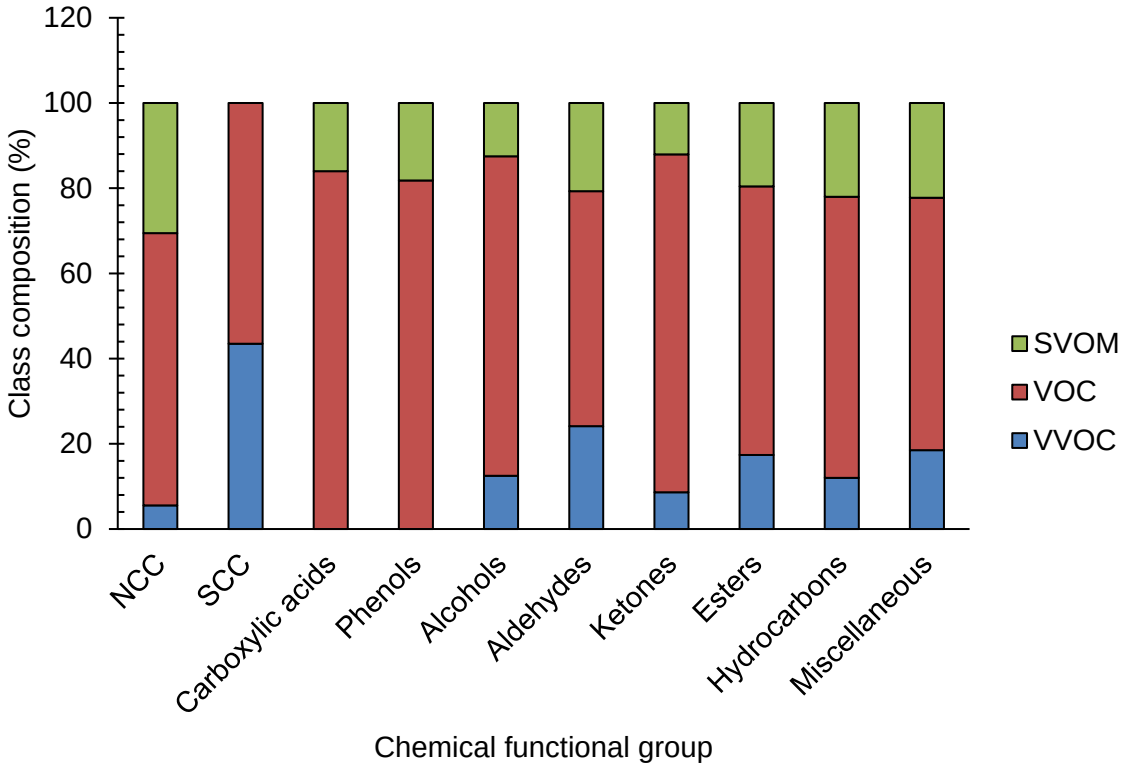


Figure 4-5: Distribution of VOCs by boiling points according to chemical functional groups

4.2.2.3 Water solubility

The aqueous solubility of a chemical compound is an important molecular property that plays an important role in environmental applications and their knowledge is, therefore, required. Regarding the VOCs, their emission is a function of their water solubility at 25 ^{o}C. Generally, hydrophobic compounds are likely to be more volatile than hydrophilic compounds (Peng *et al.,* 2003). Consequently, hydrophilic compounds are found in higher concentrations in the aqueous phase while hydrophobic compounds are found in higher concentrations in the air phase. It can be reasoned that the solubility of t is an importantproperty of the compounds since it determines where respective compounds are more likely to be found, in the aquous phase or gaseous phase. However, mostVOCs are largely hydrophobic and sparingly soluble in water; their solubility may be enhanced by the presence of water-miscible co-solvents such as methanol and butanol or the presence of suspended organic solids aqueous solution (Li *et al*., 1992; Lin and Chou, 2006).

In this study as shown in Figure 4.6, water solubility of 122 VOCs could not be found in literature. This is probably because of difficulties associated with determining the solubility of VOCs using conventional methods at technical, analytical and scientific level, since determining water solubility of VOCs is time consuming and complicated (Zhang *et al*., 2016; Birch *et al.,* 2019). The aqueous solubility figures available in literature as shown in Figure 4.6 indicate that solubility of VOCs varies widely from insoluble to 2.25x10^{6} mg/L. Again from the

same literature, 20% of the compounds detected in this study were water insoluble, 23.3%. as defined by Ney (1995) had low water solubility (less than 10 mg/L) whilst 22.5% and 34.2 % of the compounds have moderate (10-1000 mg/L) and high (more than 1000 mg/L) water solubility, respectively. The higher proportion of the compounds with high water solubility is not a new phenomenon. For instance, Cai *et al.* (2006) found that majority (64%) of swine barn particulate matter were highly soluble in water.

Fugure 4.7 shows the distribution of water solubility according to chemical functional groups. The results indicated that more than 50% of nitrogen containing compounds and carboxylic acids have high water solubility whilst sulfur-containing compounds, phenols and alcohols had more moderate water solubility while aldehydes, ketones, hydrocarbons and miscellaneous have low water solubility. Organic compounds with low and medium water solubility without segregation are considered as VOCs (Rathbun, 2000).

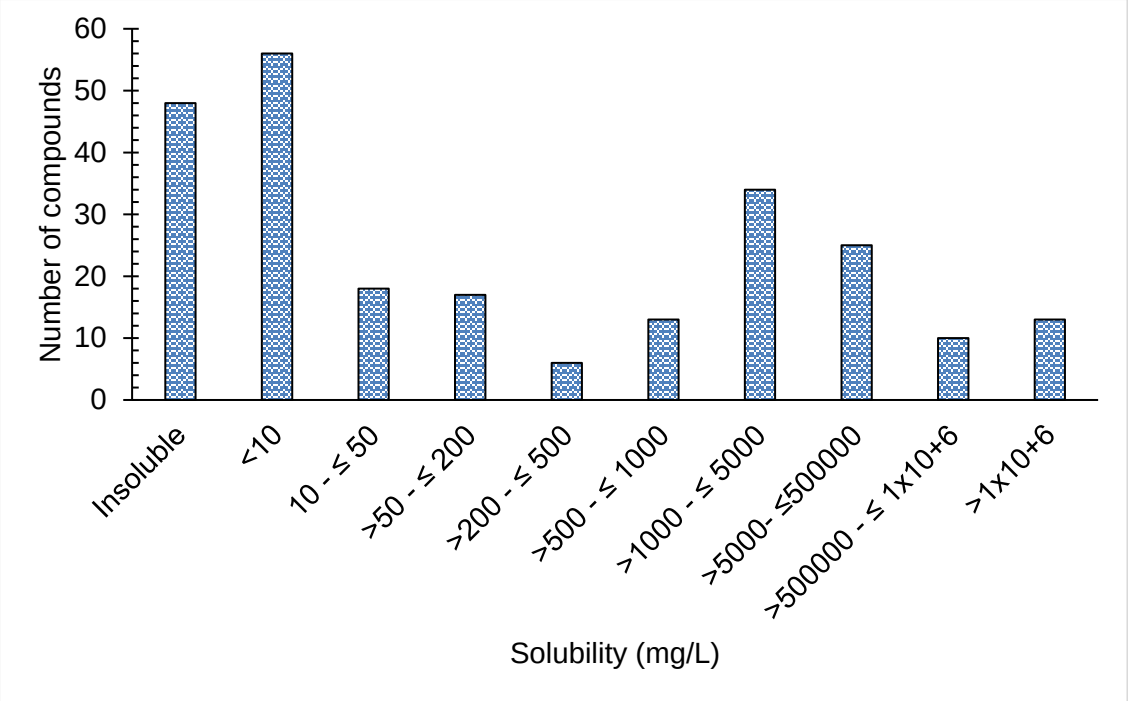


Figure 4-6: Distribution of VOCs by solubility in water

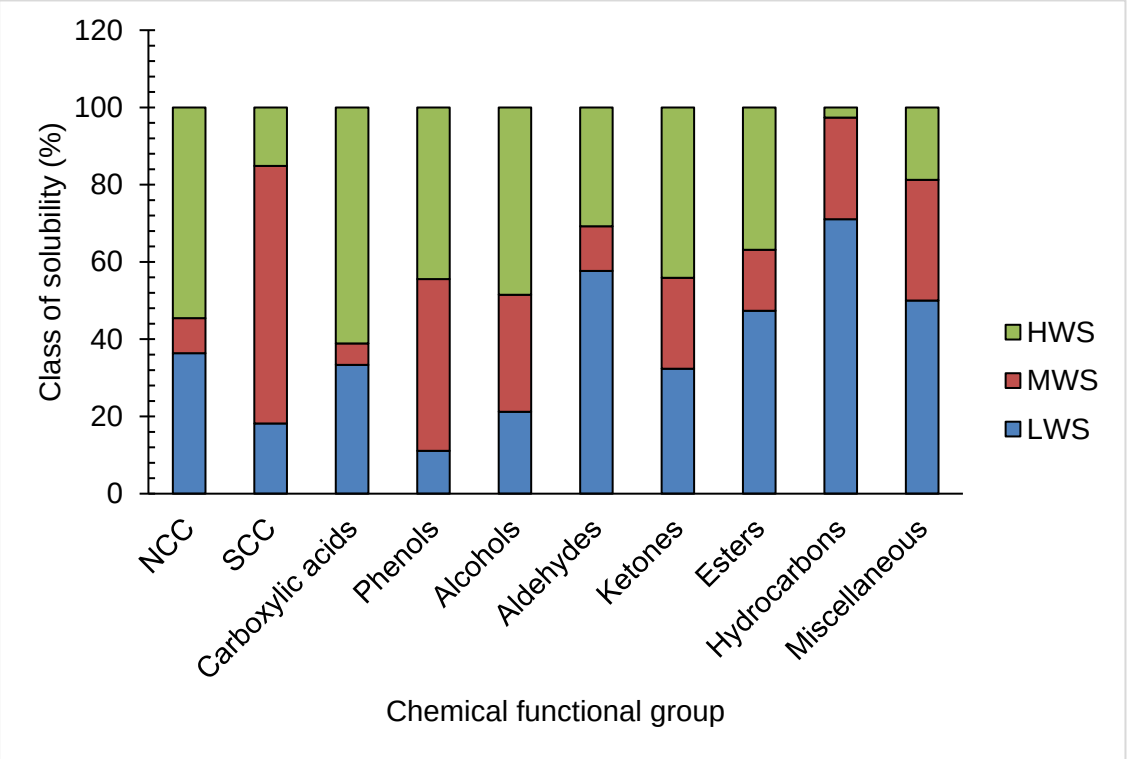


Figure 4-7: Distribution of VOCs by water solubility according to chemical functional group

4.2.2.4 Vapour pressures

In addition to water solubility of the compounds, vapour pressure is another crucial parameter that can determine their tendency to partition into the atmosphere. Vapour pressure is a measure of the ability of a compound to volatilise into the air from the aqueous phase or solid phase at a given temperature of a vapour in thermodynamic equilibrium in a closed system (Mackay *et al*., 2006). It is, therefore, reasoned that the knowledge of the water vapour pressures of VOCs is indispensable if one wants to know or predict the environmental fate of the compounds. As shown in Table S4.1, similar to what was observed for water solubility, the vapour pressure at 25 ^{o}C of 105 VOCs identified in this study could not be found in literature. As shown in Figure 4.8, for those compounds whose vapour pressures are available in literature, their range varies widely from 4 x 10^{-7} mmHg to 4.29 x 10^{4} mmHg. It is observed that 53 % of the compounds have their vapour pressure of more than 1 mmHg. This implies that these compounds exist exclusively in the gaseous phase at room temperature, therefore, virtually impossible to control their release into the air (Spicer *et al*., 2002). Due to high vapour pressure under normal conditions of these compounds, they can play a significant role in the quality of air emitted from pit latrines. According to the definition used by Spicer *et al* (2002), 3.1% of the compounds were categorised as VVOCs, 67.1% as VOCs and 29 % as SVOCs.

Using the same definition, the results of vapour pressure analysis for the chemical functional groups summarized in Figure 4.9 indicate that larger proportion of carboxylic acids can be classified asSVOCs, the larger proportion of SCCs, ketones, esters, hydrocarbons, NCCs and aldehydes as VOCs. VVOCs were only identified in SCCs, aldehydes and miscellaneous compounds. The results suggest that majority of the carboxylic acids can exist in both particle and vapour state.

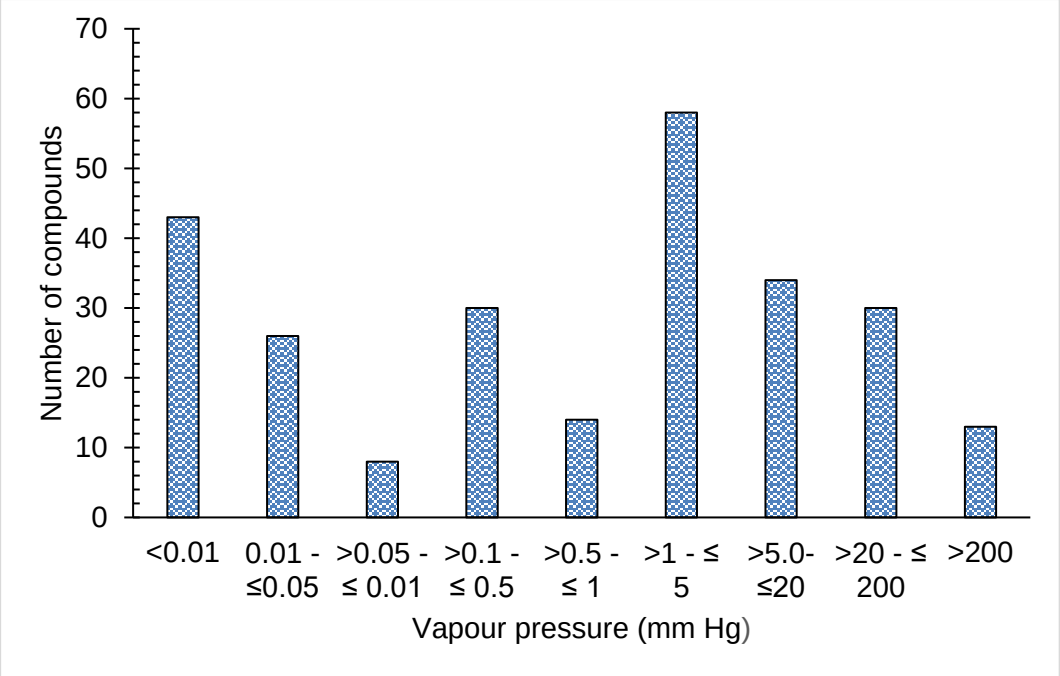


Figure 4-8: Distribution of VOCs by vapour pressure

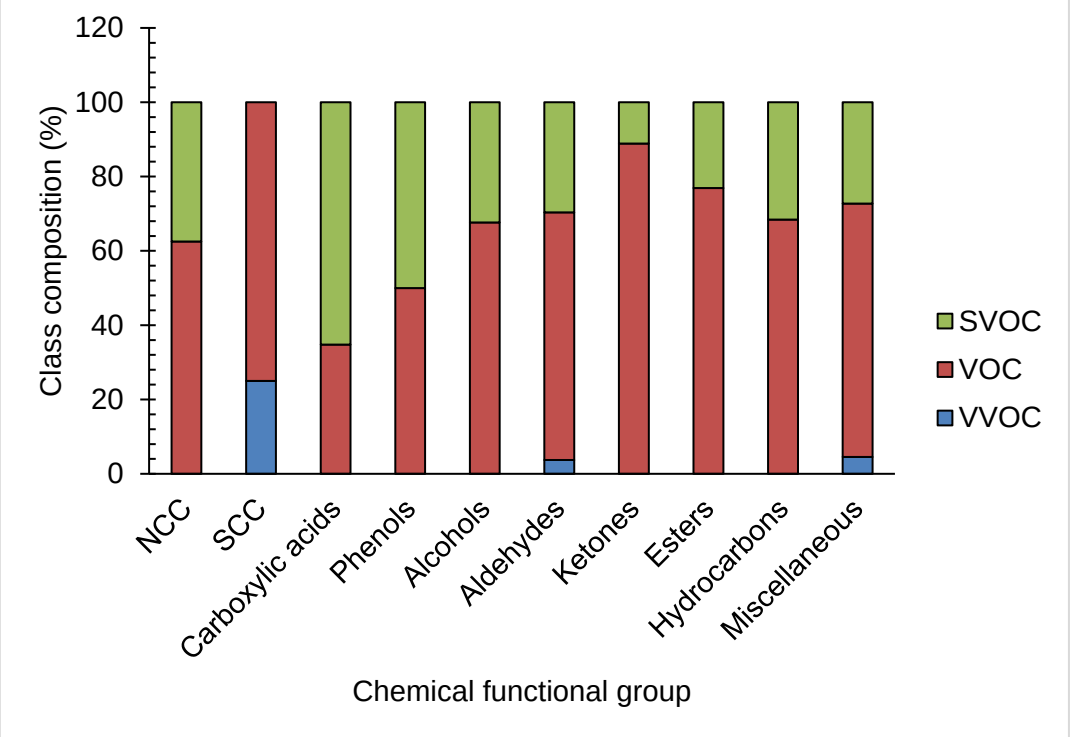


Figure 4-9: Distribution of VOCs by vapour pressure according to chemical functional group

4.2.2.5 Henry's law constants

The distribution of organic compounds between the gaseous and aqueous phases constitute an important route in the dispersion of polluting agents and is often expressed by Henry's law constant (HLC) (Dumont *et al*., 2010). HLC for compounds in water represents a crucial parameter as it helps one to predict the fate and distribution of compounds in the environment. This is consequently important for the development of successful solutions to environmental pollution (Bobadilla *et al*., 2003). HLC of a compound can be calculated using its vapour pressure and water solubility or determined through experiments (Hansen *et al.,* 1993).

Results from the analyses as shown in Table S4.1 indicates that HLCs for 211 compounds could not be found in literature. This is huge huge gap in data could be because it is costly to experimentally determine the HLCs of the compounds and consequentially, fewer HLCs are reported in literature (Brennan *et al.,* 1998; Altschu *et al*., 1999). As shown in Figure 4.10, in this study, using the definition ofEPA (2012), 8.2 % of the compounds are classified as very volatile from water (with HLC of more than 10^{-1} atm-m^3/mole) and 12.9% of the compounds are classified as volatile from water (with HLC of between 10^{-1} and 10^{-3} atm-m^3/mole). About 40.8% of the compounds are classified as moderately volatile from water (with HLC of between 10^{-3} and 10^{-5} atm-m^3/mole) while the remaining compounds were slightly volatile from water (with HLC of more than 10^{-5} atm-m^3/mole). In addition, as shown in Figure 4.11, all the phenols were slightly volatile from water. In addition, NCCs and alcohols were largely slightly volatile from water while sulphur-containing compounds and miscellaneous compounds were volatile from water.

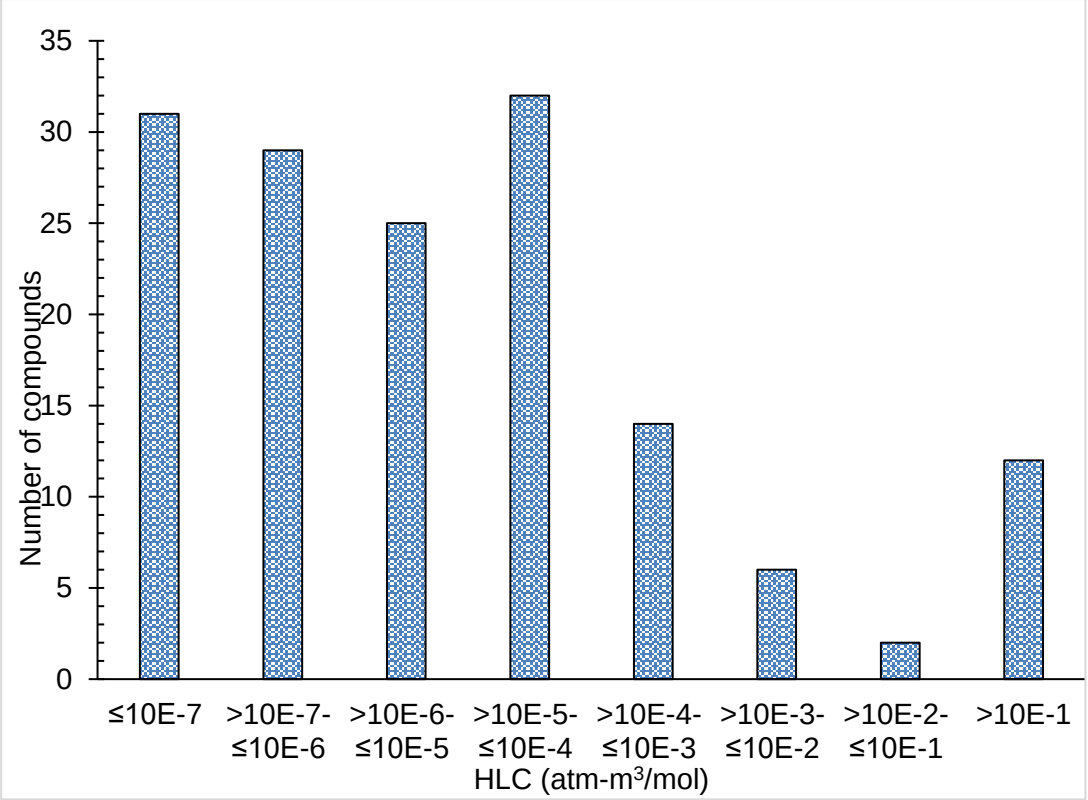


Figure 4-10: Distribution of VOCs by Henry's law constants

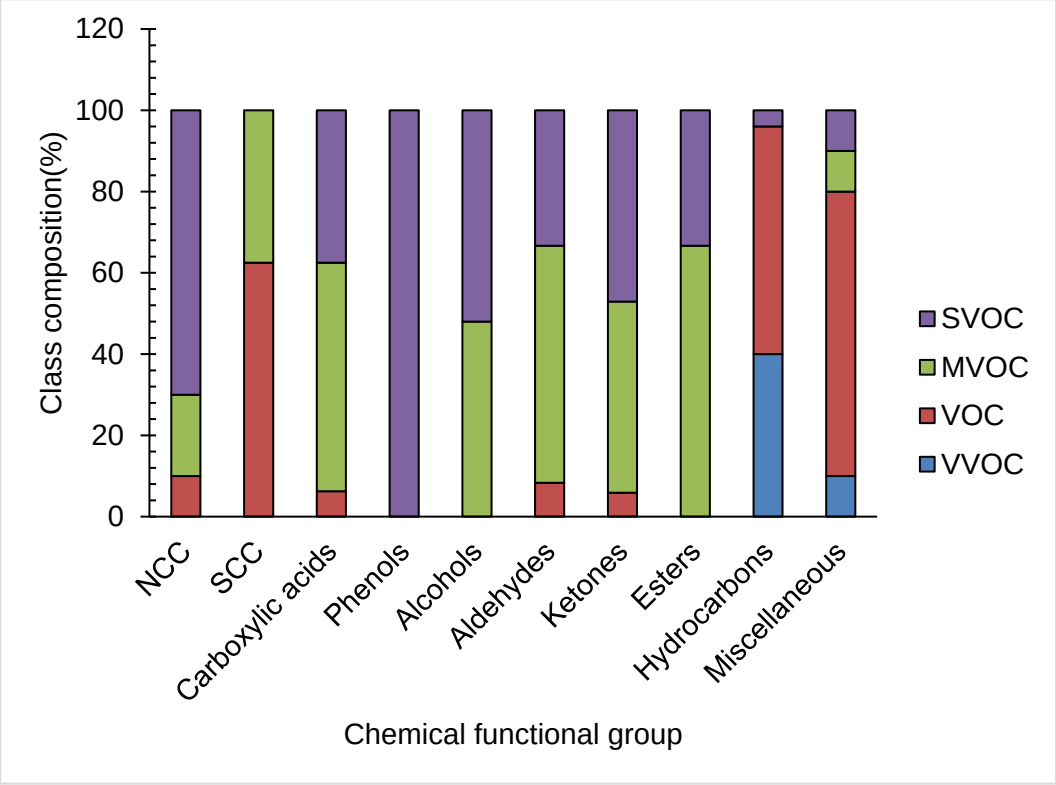


Figure 4-11: Distribution of VOCs by Henry's law constant according to chemical functional group

Due to their high HLCs, these compounds tend to partition predominantly into the atmosphere and the aqueous phase mass-transport resistance (Earl *et al.,* 2003) usually governs their evaporation rate from water. About 5.3% of the compounds were moderately volatile from water (with HLC of between 10^{-3} and 10^{-5} atm-m^3/mole). The compounds with intermediate HLCs can be taken from the source of generation by the routes of both aqueous and gaseous phases. About 8.3% of the compounds were classified as slightly volatile from water (with HLC of between 10^{-5} and 10^{-7} atm-m^3/mole). For these compounds, partitioning is predominantly into the aqueous phase and the air phase-mass transport resistance (Earl *et al.*, 2003) usually governs the rate at which they evaporate.

4.2.2.6 Octanol-water partition coefficients

Hydrophobicity is used to indicate the physical property of a compound that governs its partitioning behaviour between octanol (which represents the lipids or fats in biological organisms) and water. In addition to water solubility discussed in section 4.2.2.3, the octanol-water partition coefficient (K_{ow}) is also used to confidently determine hydrophobicity of compounds. However, a compound's K_{ow} and it water solubility are inversely related. Thus, high K_{ow} is a characteristic of a compound, which preferentially partitions into non-aqueous phase rather than aqueous phase. The octanol-water partition coefficient is defined as in equation 4.1 (Mazzobre *et al*., 2005):

$$K_{ow} = \left(\frac{C_{oct}}{C_{wat}}\right) \tag{4.1}$$

where C_{oct} is the molar concentration of the organic compound in the octanol phase [mol/L] and C_{wat} is the molar concentration of the organic compound in the water phase [mol/L] when a two phase (octanol/water) system is at equilibrium.

Knowledge of K_{ow} is useful for predicting the tendency of the compounds to migrate from water to the atmosphere and partitioning into biological organism's cell membranes. In other words, like water solubility and vapour pressure discussed in sections 4.2.2.3 and 4.2.2.4 respectively, K_{ow} is concerned with the equilibrium distribution of the compounds between phases. In terms of treatment of VOC, K_{ow} of a compound influences its biological properties such biodegradation and bioaccumulation potential as well as its exposure and toxicity in the aqueous environment (Jianlong *et al*., 2004). It should be noted that the K_{ow} values of the compound span widely with many orders, hence the decadic logarithm of K_{ow} values are often used. In this study, the logK_{ow} values of certain 103 compounds were not found in the literature. The logK_{ow} values of the identified compounds varied widely from -1.35 (hydrophilic) to 8.69 (extremely hydrophobic). This wide variation could be primarily attributed to differences in

behaviour of the compounds in the water phase and not in octanol (Mackay and Shiu, 1990). Because of the $logK_{ow}$ values of organic compounds being mostly related to water solubility, the compounds identified in this study were further characterised based on their affinity for water as defined by EPA (2012). In this study, the K_{ow} values of 104 compounds were not found in the literature. Overall, as shown in Figure 4.12, about 23 % of the compounds are highly soluble in water (with $logK_{ow}$ values of less than 1.0), thus, they are hydrophilic. About 57 % of the compounds were moderately soluble in water and about 20% are not very soluble in water (with $logK_{ow}$ values of more than 4.0) (hydrophobic). About 0.3% of the compounds are not readily bioavailable. This suggests that the compounds would not be available for microbial uptake as microbes such as bacteria use only the compounds in the aqueous phase.

The distribution of octanol-water partition coefficient according to chemical functional groups is given in Figure 4.13. The results based on octanol-water partition coefficients show that in all the classes of the compounds except SCCsand NCCs, the majority of the compounds can be categorised as VOCs. Up to 90% phenols were VOCs whilst 73 % SCCs are in the class of. VVOCs.

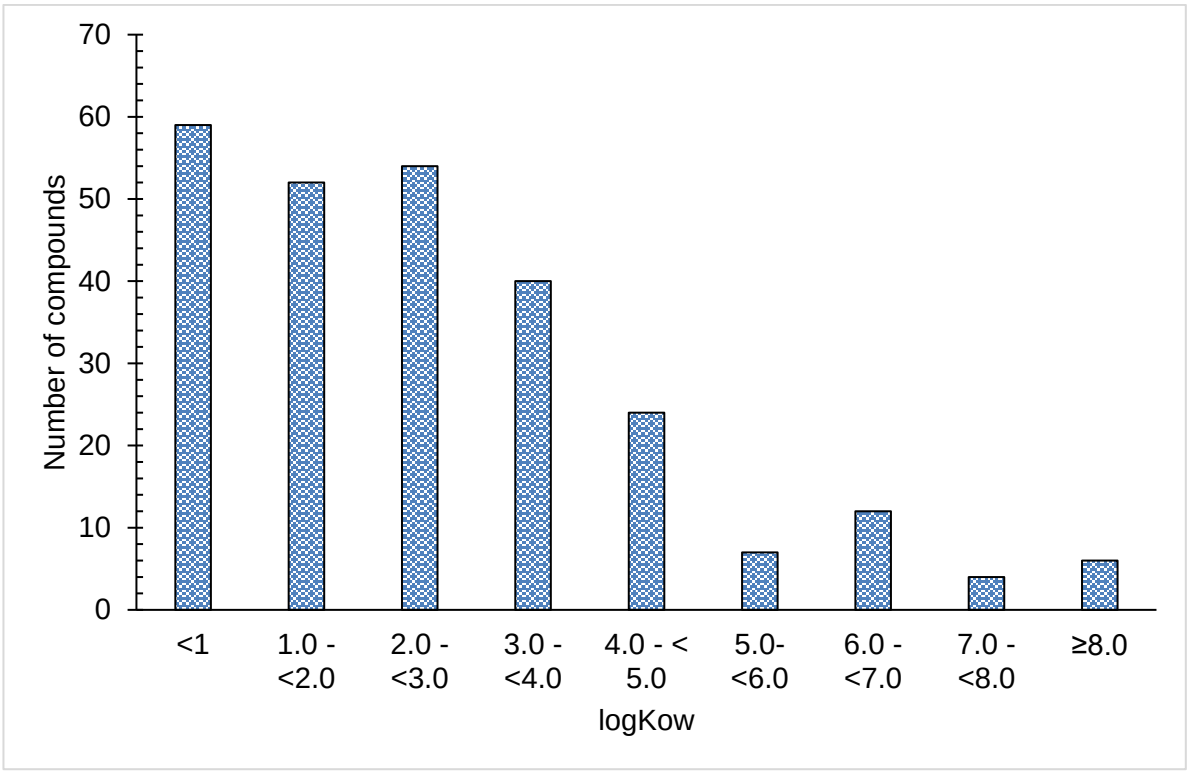


Figure 4-12: Distribution of VOCs by octanol-water partition coefficient

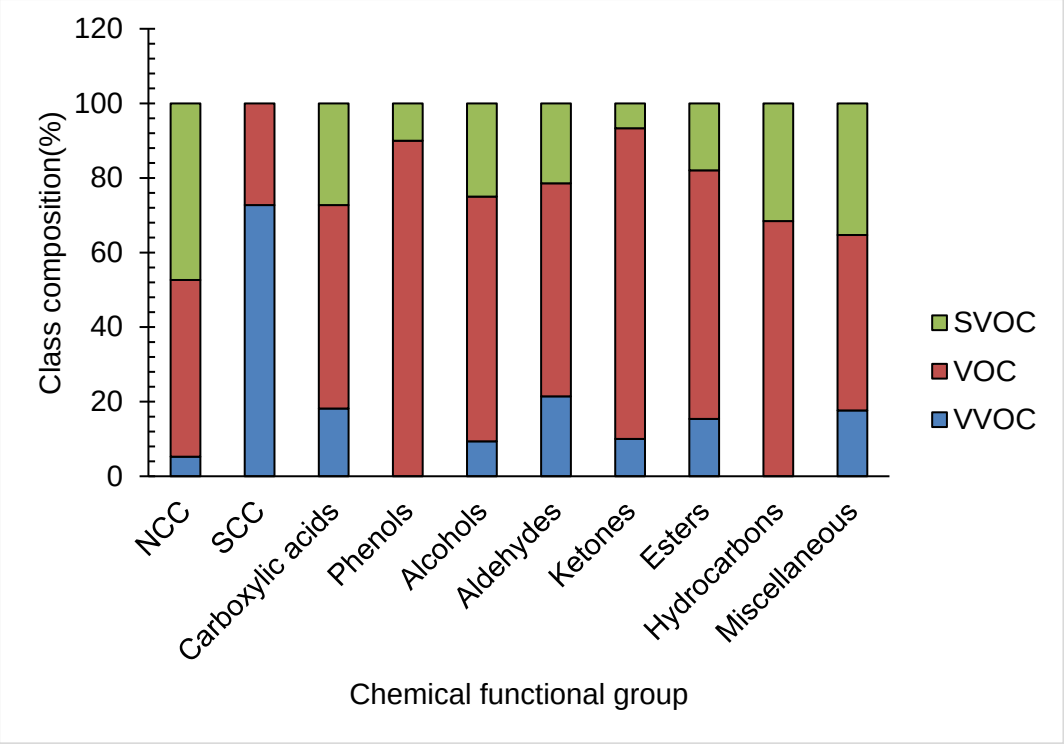


Figure 4-13: Distribution of VOCs by octanol-water partition coefficients according to chemical functional group

4.2.3 Temporal and spatial variation of the compounds

In the present study, the compounds that had a frequency of at least 50% were considered as most frequently occurring compounds. As shown in Table 4.1, twenty-one compounds were identified. All the compounds but 3-methyl-thiophene have previously been identified as VOCs from pit latrines (Lin *et al*., 2013). All other compounds except alpha-pinene, toluene, methyl thioacetate and ethylformate have also been identified as compounds frequently emitted from pit latrines (Lin, *et al*., 2013). Toluene, alpha-pinene, DMTS and limonene are some of the most odourous compounds in wastewater treatment plants (Lehtinen and Veijnen, 2011). All the compounds detected fall somewhere in betweenthe VVOC to VOC spectrum based on their physico-chemical characteristics. However, despite the fact that their distinctive odour character as individual compounds as shown in Table 4.2, their olfactory impact and contribution to the nuisance odours from pit latrines is not known and may be complex. This is because as shown in section 4.2.1 the odour emission is determined bya mixture of VOCs. Additionally, other compounds may be present in very low concentrations to be detected by the method used in this study but may still contribute towards the overall odour perception. As seen in Table S4.1 in appendix 2 and Table 4.1 the pit latrine emissions generally contain a mixture of VOCs with variable concentrations and compositions and it is impossible to predict the olfactory impact of the odours emitted frompit latrines. The effect of either an independent

or an additive or an antagonistic or a synergetic can be observed (Baker, 1963; Naddeo *et al*., 2013). According to Fisher *et al*., (2017), the presence of other VOCs, even at low concentration and below their odour threshold limit, is still crucial since VOCs can affect the overall odour character of an emission stream. Moreover, it was difficult to elucidate the potential of the volatiles to be odourous at the source of emission or determine their sensorial relevance despite their frequency of detection. This is because the OAVs could not be computed in the absence of the actual quantification of concentration of the volatiles as henceforth discussed.

Table 4.1 also shows the peak area counts of the detected VOCs and other compounds. For economic reason, no standard were available to obtain the actual concentrations, therefore, peak areas were used. However, the peak area counts still enabled the researcher to carry out a valid comparative analysis of the VOCs between pit latrines and within the same pit latrine over time. This subsequently allowed the identification of the general trends in the behaviour of pit latrine emission. It is evident that the VOCs emitted from pit latrine feacal sludge varied across the eight pit latrines as well as within the same pit latrine across time. However, it was difficult to compare concentrations between different compounds using the peak area counts. This is because the area of a peak is proportional to the concentration of a specific compound. The variations in the concentration and types of the compounds are probably because of intrinsic differences in environmental conditions in the latrine pits prior tosampling. This is because the method for extraction of the volatiles was standardised and controlled and the samples were exposed to same incubation conditions i.e. temperature, extraction time, ionic effect etc.

Meteorological factors such as temperature and relative humidity could affect VOC emissions, therefore, at the time of sampling the ambient temperatures in the area were measured as 18.5 ±1 ^{o}C and 30±1 ^{o}C in winter and summer, respectively. The ambient relative humidity was also measured as 51 % and 32 % in winter and summer, respectively. Although the meteorological conditions were the same outside the pit latrines, it was observed that temperatures and relative humidity in different pit latrines varied markably as shown in Table 4.1. The temperature in the latrine pits varied between 14 ^{o}C and 18 ^{o}C and from 23 ^{o}C to 29 ^{o}C in winter and summer, respectively. The recorded relative humidity also varied significantly across pit latrines and ranged between 28% and 48% in winter and between 13% and 30% in summer. It is also worth noting that the temperatures and relative humidity in the pits were consistently lower than what was recorded in the vicinity. The results of this study are in consistent with what was found by Nakagiri *et al* (2017) who reported that temperatures and relative humidity are consistently higher around the pit latrine structures. The *in situ* pH of pit latrine feacal was between 5.8 and 8.8 during winter while during summer was between 6.4

and 8.3. Previous studies have reported pH ranges of between 5.2 and 8.2 (Irish *et al*., 2013), 5.0 and 11.8 (Nakagiri *et al*., 2017), 7.31 and 9.01(Wood, 2013), and between 5.3 and 7.5 (Rose *et al*., 2015).

The aforementioned results imply that the environmental conditions in latrine pits vary significantly. The environmental conditions inside the latrine pits may influence the physical and bio-chemical processes inside latrine pits, which in turn, affect the VOC emissions. It can therefore be postulated that changes in temperature and humidity modify the processes in pit latrines though this needs to be verified. However, previous study showed that variations in humidity and temperature cause variations in the intensity of perception of the odours of pure substances (Bocca and Battiston, 1964). Sun *et al.* (2010) monitored seasonal variations of odour and gas concentrations, emissions, and they reported that seasonal odour, gas concentrations and emissions were significantly affected by sampling month and ambient temperature. Hence, it is postulated that the differences in VOC species and concentrations amongst different pit latrines or betweentwo seasons might due to differences in environmental conditions, composition of the wastes in terms of type and volume as well as the stage of digestion of the feacal sludge at the time of sample collection.

Besides, there is a large heterogeneity in contents within and between different pit latrines due to such factors as diet, surrounding soil type and, users' practices and management activities (Torondel, 2010). The individual variation in contents across pit latrines and seasons coupled with the fact that uncontrolled environments possibly contribute to the complex heterogeneity of volatile compounds emitted from pit latrines. In addition to pH and temperature discussed above, moisture, oxygen, characteristics of the surrounding soil, pit latrine dimensions, complexity of macromolecules therein, presence of inhibitory substances, microflora and macroflora composition, microbial biomass, redox status, present in pit latrines are also affect the digestion of pit latrine contents hence the type and quantity of VOCs (Torondel, 2010). Further work is clearly required to explore how differentenvironmental factors affect the types and concentration of VOCs emitted from pit latrines.

It may be seen in Table 4.1 that 15 of the most frequent occurring compounds identified in the present study are generally characterised by unpleasant odours even at relatively low odour threshold values and can also result in adverse human health effects dependent on concentrations. The possible sources of the compounds are mostly due to anaerobic and aerobic microbial activity, particularly microbial degradation of organic matter, either in the pit latrine or in the gut.

Table 4-1: Occurence of volatiles in pit latrine feacal sludge and their peak area counts

Compounds	Pit Latrines (Peak Area)																Frequency
	P1W	P1S	P2W	P2S	P3W	P3S	P4W	P4S	P5W	P5S	P6W	P6S	P7W	P7S	P8W	P8S	
1-propanol	1.6E+07	5.6E+06	ND	1.8E+05	9.5E+08	1.2E+06	ND	1.7E+06	1.5E+05	ND	1.1E+06	ND	2.2E+08	1.7E+08	ND	ND	10
2-butanone	1.3E+05	ND	2.3E+06	ND	9.8E+04	ND	4.3E+05	1.5E+06	ND	6.9E+07	6.3E+06	ND	3.8E+09	ND	ND	ND	8
2-methylbutyric acid	ND	2.6E+07	2.1E+06	2.7E+08	ND	ND	5.6E+06	7.3E+05	ND	1.9E+06	1.4E+06	5.0E+06	4.5E+06	4.4E+08	2.7E+07	ND	10
3-methyl-thiophene	ND	3.9E+05	ND	2.9E+06	3.4E+06	ND	1.3E+05	ND	3.0E+06	4.5E+05	1.3E+07	5.6E+06	1.8E+07	ND	5.6E+06	3.4E+08	11
alpha-pinene	3.5E+06	ND	2.4E+06	ND	1.9E+05	5.3E+06	9.4E+05	3.3E+07	ND	3.6E+06	4.7E+07	ND	ND	5.4E+07	3.4E+07	ND	10
Butyric acid	7.8E+08	1.0E+10	ND	1.7E+07	9.5E+08	2.8E+06	3.5E+06	7.1E+04	8.0E+06	1.7E+06	ND	2.1E+06	4.9E+07	7.2E+07	4.9E+09	8.0E+07	14
Dimethyl disulphid	ND	ND	1.5E+07	ND	1.8E+05	ND	1.7E+09	1.9E+08	4.6E+05	ND	1.2E+06	ND	ND	ND	3.4E+07	2.3E+07	8
Dimethyl trisulfide	4.3E+06	1.5E+07	1.0E+06	4.3E+06	ND	8.5E+06	1.0E+10	7.7E+08	2.0E+06	1.4E+08	ND	ND	2.6E+07	ND	ND	4.6E+07	11
Ethyl acetate	6.3E+05	ND	5.1E+04	ND	2.6E+05	ND	2.3E+06	ND	2.1E+06	ND	ND	6.9E+06	ND	5.4E+08	ND	5.6E+08	8
Ethyl formate	ND	1.2E+05	ND	ND	3.5E+05	2.6E+06	8.1E+05	6.5E+04	ND	1.5E+06	ND	4.3E+06	2.7E+08	ND	2.6E+08	ND	9

Indole	2.3E+07	1.1E+09	ND	1.9E+09	1.3E+08	4.0E+09	2.4E+09	9.1E+07	1.7E+07	1.4E+08	4.9E+09	1.7E+07	ND	9.4E+09	3.4E+09	1.4E+09	14
Isobutyric acid	4.8E+06	3.4E+06	ND	ND	ND	4.7E+06	1.9E+07	ND	3.5E+05	ND	6.8E+06	1.8E+06	8.9E+07	ND	4.8E+06	3.4E+07	10
Limonene	7.8E+05	6.3E+06	ND	9.4E+08	4.7E+08	1.4E+07	3.6E+06	4.1E+04	2.5E+06	1.7E+07	ND	8.7E+08	1.1E+09	ND	ND	5.6E+06	12
Methyl thioacetate	ND	2.8E+06	ND	1.7E+07	3.8E+06	5.7E+06	7.8E+06	ND	2.6E+06	ND	4.8E+07	5.6E+06	ND	4.7E+07	4.9E+07	3.7E+07	11
p-Cresol	1.4E+08	1.1E+09	1.9E+06	8.4E+07	6.2E+08	4.2E+05	1.2E+09	4.9E+04	ND	2.9E+08	4.1E+06	3.9E+08	6.3E+07	2.3E+08	9.4E+08	ND	14
Phenol	2.8E+06	1.6E+06	3.2E+05	5.2E+07	5.1E+08	1.1E+07	ND	2.6E+04	ND	3.4E+07	ND	2.3E+07	ND	ND	ND	ND	8
Propionic acid	ND	1.1E+05	2.7E+05	1.4E+05	ND	ND	ND	ND	3.8E+07	2.8E+06	ND	2.9E+06	1.8E+07	ND	8.4E+07	8.5E+07	9
Skatole	2.5E+05	ND	2.9E+08	1.8E+07	ND	8.9E+06	5.3E+04	8.1E+04	ND	5.1E+07	ND	4.7E+07	ND	ND	ND	ND	8
Toulene	1.4E+07	ND	2.3E+07	1.4E+07	ND	4.2E+06	3.0E+07	1.7E+06	1.6E+07	1.1E+09	ND	ND	ND	ND	ND	ND	8
Temperature	16	29	17	23	18	19	15	21	15	22	16	22	14	26	14	28	
Relative Humidity	32	23	47	20	46	30	35	26	28	19	38	23	43	13	34	16	
pH	7.2	8.3	7.5	6.8	8.8	7.1	8.3	6.9	6.7	7.9	5.8	8.1	7.6	6.4	8.8	7.4	

Table 4-2: Most frequently occurring compounds and their odour character, ODT, possible source and human health effects

Compound	Retention time (min)	Odour character	ODT (mg/L)	Possible sources	Adverse human health effects	References
Indole	28:48	Feacal, nauseating	0.00030	Microbial degradation of L-tryptophan	Causes glomerular sclerosis, haemolysis, improper oviduct functioning and chronic arthritis	Gostelow *et al.*.(2001); Sato *et al*. (2001); Nagata and Takeuchi, (2003); Arora *et al*., (2015); Ma *et al.*, (2018)
Butyric acid	07:23	Rancid, sweet	0.00019	Microbial anaerobic acidogenesis of biodegradable organic materials	Causes mild skin irritation	Gostelow *et al*. (2001); Sato *et al*. (2001); Nagata and Takeuchi 2003; Lehtinen (2012); Jung *et al.* (2016)
p-Cresol	07:00	Phenolic	0.000054	Microbial degradation of tyrosine	Possible human carcinogen and has adverse effects on the central nervous system, cardiovascular system, lungs, kidney and liver	Nagata and Takeuchi (2003); Singh *et al.* (2008); Lehtinen (2012)
Limonene	15:04	Pleasant lemon-like	0.038	Citrus fruits and flavouring agents in food stuff	Causes skin irritation	Misra *et al.*(1996); Filipsson *et al*., (1998); Hensen and Eggert, (2003); Nagata and Takeuchi (2003); Lehtinen (2012)
Methyl thioacetate	09:08	sulfurous, cheesy	21	Microbial degradation of L-methionine	Causes eyes, respiratory system, and skin irritation	Bonnarme *et al.*(2001); Ranau and Steinhart (2005); Du *et al.* (2011)

3-methylthiophene	13:09	Sulphurous, onion, roasted,	360	Foods such as cooked onion, coffee	Causes skin and eye irritation	Shimoda and Shibamoto (1990); USGA (1999); Ranau and Steinhart (2005)
2-methylbutyric acid	06:23	Fruity, sourish[a]	-	Microbial derived from L-leucine and L-isoleucine	-	Nimitkeatkai *et al*.,(2005)
Isobutyric acid	13:34	Rancid, butter, cheese	0.0015	Microbial anaerobic acidogenesis of biodegradable organic materials	Causes nose, throat and lungs irritation causing coughing, wheezing and/or shortness of breath.	USGA (1999); Nagata and Takeuchi (2003); Sánchez-Palomo, *et al.* (2010)
1-propanal	12:13	Pungent, unpleasant			Sensory irritation	USGA (1999)
Alpha-pinene	05:23	Pine	0.018	From vegetable food stuff	Skin, eyes, mucous membranes irritation and lung symptoms	Nagata and Takeuchi, (2003); Lehtinen (2012); Lasekan and Lazeez (2014)
Propionic acid	12:57	Sour, pungent	0.0057	Microbial anaerobic acidogenesis of biodegradable organic materials	Causes eyes, nose, throat and skin irritation	USGA (1999); Nagata and Takeuchi (2003); Ranau and Steinhart, (2005); Lehtinen (2012)
Ethyl formate	17:16	Fruity odour	2.7	Not found	Causes eyes, nose, throat and skin irritation	USCG (1999); Nagata and Takeuchi (2003)
Dimethyldisulfide	04:45	Decayed cabbage	0.0020	Microbial degradation of methionine and carbon disulphide from cysteine and cysteine and also produced by actinomycetes	Causes pulmonary irritation, liver damage and has haematological effects dependent on concentration and exposure time	Gostelow *et al*. (2001); Nagata and Takeuchi (2003); Alberta Environment (2004); Lehtinen (2012)
Skatole	14:23	Feacal, nauseating	0.0000056	Microbial degradation of	Bioactivation of skatole to reactive intermediates	Gostelow *et al.* (2001); Sato *et al.* (2001); Chen *et al*.(2006)

				tryptophan through indole-3-acetic acid	initiate apoptosis in human lung cell lines	
2-butanone	12:46	Sweet, pleasant, pungent	0.006	From manufactured products such as paints, coatings, resins, and printing materials	Causes moderate eye, nose, throat and skin irritation	USCG (1999); Yuwono *et al*. (2002); Chen *et al*.(2015)
Ethyl acetate	08:09	Fragrant	0.87	Microbial anaerobic acidogenesis of biodegradable organic materials	Causes headache, irritation of respiratory passages and eyes, dizziness and nausea, weakness, loss of consciousness	USCG (1999); Nagata and Takeuchi (2003); Ranau and Steinhart, (2005)
Toluene	04:53	Sweet, pungent	0.33	Microbial anaerobic degradation of phenylalanine	Causes eye, nose, throat and skin irritation	Nagata and Takeuchi, (2003); Chen *et al*., (2006); Lehtinen (2012)

Torondel *et al*. (2016) concluded that microbial diversity and structure variation between pit latrines and over time and are influenced by pH, temperature and moisture content. With respect to odour generation, changes in environmental conditions that affect microbial diversity and activity will likely influence the production of the odourous compounds Furthermore, Torondel (2010) indicated that microbial decomposition process in pit latrines are influenced by temperature, moisture content, characteristics of the surrounding soil, pit latrine dimensions, complexity of macromolecules, oxygen, inhibitory substances, microflora and microflora. Because of the differences in microbial diversity between pit latrines and variation in microbial composition and structure over time within the same pit latrine and variations in other environmental variables it is very likely that pit latrines would emit VOCs of distinct types and concentrations. The emission of microbially-produced VOCs is biologically dynamic and strongly affected by the microbial species and environmental growth conditions and phase (Contarino *et al*., 2019). The VOC production by a variety of microorganisms through a highly connected catabolytic pathways in a terrestrial ecosystem has been observed in a number of studies (Leff and Fierer, 2008; Sun *et al.,* 2010; Batty *et al*., 2016; Zhao *et al*., 2016; Huang et al., 2019). On the other hand, other studies have shown that some microbial species can readily use a wide array of VOCs as a source of carbon and energy as either a single substrate or a mixture (Lu *et al*., 2010; Bushnaf *et al*., 2011; Priya and Philip, 2013; Datta *et al.,* 2014; Bak *et al*., 2017; Dabslaw and Engesser, 2018; Mokhtari *et al*., 2019). This may represent a significant sink for VOCs (Bushnaf *et al*., 2011). Additionally, the microbial production or utilisation of VOCs is also dependent on the existence of the carbon substrates and environmental growth conditions. Further work is clearly warranted to evaluate the effects of the environmental variables and microbiota associated with pit latrine feacal sludge on the production of VOCs.

4.3 Summary

Pit latrines are associated with emissions of volatile organic compounds (VOCs) and inorganic gases. The emission of volatile organic compounds (VOCs) from the pit latrines have been implicated as the main cause of malodourous air quality inside the pit latrines and their immediate precincts. The objective of this study was to identify and characterise liquid phase volatile organic compounds (VOCs) from pit latrine feacal sludge. VOCs were analyzed in winter (May) and in summer (October) in 2015 from eight pit latrines, in order to further understand emissions of VOCs from the pit latrines. Headspace Solid Phase Microextraction (HS-SPME) using Carboxen-divinylbenzene polydimethylsiloxane (CAR/DVB/PDMS) fibres was used to extract the VOCs from pit latrine feacal sludge after equilibration with vial headspace. Gas Chromatography- Time of Flight Mass Spectrometry (GC-ToF-MS) system was applied for analysis. Up to 358 volatiles, including ketones, alcohols, hydrocarbons, esters, nitrogen-containing compounds, aldehydes, carboxylic acids, sulphuric-containing compounds and phenols, detected from pit latrine faecal sludge. Nineteen odourants, indole, butyric acid, p-Cresol, alpha-pinene, skatole, dimethyldisulfide, dimethyltrisulfide, phenol, methyl thioacetate, propionic acid, 2-butanone, isobutyric acid, ethyl acetate, ethyl formate, limonene, toluene, 1-propanol, 2-methylbutyric acid, and 3-methylthiophene, were considered as the most common VOCs emitted from pit latrines. This study provides an insight of the nature of volatile compounds responsible for odorous emissions from pit latrines. The study has demonstrated that there is a wide range of compounds with quite varied chemical functionalities and the concentrations and profile of these VOCs is vary spatially and temporally. These variations are a result of, among other factors, variations in environmental conditions, diet of latrine users, soil type the latrine is built on, and management regimes. Overall, the spatial and temporal diversity of VOCs identified in this study reveals the necessity of further field studies to better understand the VOCs profile of pit latrine emissions. Due to the spatial and temporal variability in types and concentrations of components of odour emissions, no universal odour abatement technique is recommended for pit latrine deodorisation. The results of this study provide vital information upon which further research towards developing pit latrine off-odour deodorisation techniques and strategies can be premised. Finally, it will be

imperative to investigate the source of the volatile compounds identified within the present study. One might speculate about their direct origin from endogenous sources, but also from foods as free odorous compounds contained therein, or about their potential occurrence as anaerobic or aerobic digestion products from more complex pit latrine contents.

CHAPTER FIVE

5 MULTIVARIATE OPTIMISATION OF HEADSPACE SOLID PHASE MICROEXTRACTION (HS-SPME) OF FOUR KEY PIT LATRINE ODOURANTS

5.1 Background

Pit latrine odour nuisance is a result of chemically complex emission of predominantly volatile organic compounds (Lin et *al*. 2013) and other inorganic compounds such as hydrogen sulphide, ammonia nitrogen, (Mara,1984; Obeng *et al*., 2016), sulphur dioxide and nitrous oxide (in the current study). These compounds are from various chemical classes such as organo-sulphurs, nitrogenous compounds, aromatics, phenols, alcohols, aldehydes, ketones, esters and hydrocarbons (Lin *et al*., 2013; Mercer *et al.,* 2019). The human olfactory impact of each odourous compound is dependent on its chemico-physical properties, concentration and its human odour detection threshold (ODT), which varies considerably from one compound to another (Binieka and Caroli, 2011; Arcari *et al*., 2017).

Most often, the discrimination and identification of odourous-active compounds in a complex mixture of volatile compounds is more difficult task for humans. This requires caution because of (i) sensory limitations as a result of competition for receptor sites and cells at the periphery when there are more than four odourants in a mixture and (ii) Instrumental limitations in the identification of the odourants, which may co-elute with many volatile compounds and are often present in trace concentrations (Jinks and Laing, 1999; Théron *et al*, 2010). Consequentially, the establishment of the relationship between VOCS and olfactory characteristics becomes the most complex aspect of odour study. For pit latrines, however, a recent study (Chappuis *et al.,* 2016) identified butyric acid, DMTS, indole and p-Cresol as the four odourants that contribute significantly to pit latrine malodourous emissions. These compounds were also

found to be one of the most the most frequently occurring VOCs in the samples of pit latrine feacal sludge analysed in this study. However, the human odour detection thresholds of these compounds are in trace and ultra-trace concentrations to be identified and quantified below the limits of conventional techniques of detection. Consequently, studies on the analysis of VOCs in pit latrines are still a challenge due to their low concentrations. In view of this, an accurate and reliable analytical method that pre-concentrates the analytes prior to chromatographic analysis is crucial.

Solid Phase Micro-extraction (SPME) is a popular solvent-free sample pre-concentration technique that is a simple, sensitive, reliable, inexpensive, time efficient and easy-to-automate for the analysis of volatile compounds (Souza-Silva, 2013). The SPME sample pre-concentration technique offers certain advantages over other well established and widely used traditional sample preparation techniques for extraction of organic analytes from aqueous matrices such as solid phase extraction (SPE) (Andrade-Eirora *et al*., 2016), purge and trap (Abeel *et al.*,1994), and liquid-liquid extraction (LLE) (Pena-Pereira *et al*., 2009). This is in terms of procedure, accuracy, sensitivity, repeatability, simplicity, speed, cost and greenness (Piri-Mughadam *et al*., 2016). However, the different possible configurations of SPME and sample preparation steps directly affects the results of the analysis. The SPME method optimisation has been accomplished by a traditional univariate procedure, in which one experimental factor is studied separately at a time while other factors are held constant. This can lead to erroneous conclusions about the importance of certain factors on the extraction process because interactions between factors are not being considered. A multivariate optimisation procedure that allows simultaneous variation of all experimental factors has become popular. This enables the investigation of main effects as well as interaction effects influencing the SPME process (Polo *et al*., 2005).

Hence, the objective of this work was to develop a simple and reliable method based on HS-SPME-GC-ToFMS for the simultaneous determination of four odourants (butyric acid, DMTS, p-Cresol and indole) that are predominantly responsible for malodourous smell perception in

pit latrines. The chemical formulas and the main physical– chemical properties of the target odourants are listed in Table S4.1. Although HS-SPME has been previously applied for the analysis of VOCs emanating from pit latrines (Lin *et al*., 2013; Chappuis *et al*., 2015) to the best of our knowledge, comprehensive investigation of all parameters that may affect to the simultaneous analysis of the four key odourants by HS-SPME- GC/ToF-MS using multivariate approach has not been carried out. Development of a sensitive and repeatable technique for detecting these compounds in pit latrine feacal sludge is crucial for odourous air control. In the current study, a comprehensive investigation was carried out to optimize the HS-SPME as a fast and simple extraction method with a target limit of detection of below the human ODT for all the target compounds.

The goal of this study was to develop a reliable HS-SPME method that would be then applied for simultaneous analysis of microbial deodourisation of the four key odourants using bacterial strains that had been isolated and identified in our laboratory.

5.2 Results and discussion

Materials and methods used to achieve the objectives of this chapter are found in section 3.3.

5.2.1 Selection of SPME fibre

The sensitivity and selectivity of SPME extraction technique depends primarily on the value of the distribution constant for analytes partitioned between the sample matrix and fibre coating material dependent upon its type of stationary phase and also on its polarity and thickness (Risticevic *et al.,* 2010). Therefore, fibre coating type is one of the key factors in the extraction efficiency of analytes. For this reason, in this study the extraction efficiency of the five commercially available SPME fibre coatings described in section 3.1.1 were evaluated for their extraction efficiency of the targeted odourous compounds in order to select the fibre coating for this application. Each fibre was exposed to the headspace under same condition of 20 min of equilibrium time, 40^{o}C of extraction temperature, 30 min. of extraction time, ionic strength at NaCl saturation point (500g/L), sample volume of 10 mL in 20 mL amber glass vial, constant stirring rate of 800 rpm and pH of 2. NaCl was heated to the temperature of 300 ^{o}C for 24 h

prior to use to eliminate possible risk of organic contaminations of the sample (He *et al*., 2000; Spietelun *et al.,* 2013).

The differences in the GC peak height obtained exposed the behaviour of each type of fibre coating used for each fibre in respect to their extraction capacity of the compounds under investigation. The selection was fundamentally based on the following simplified criterion function of Zuba *et al*., (2002) as described in equation 5.1 by Hamman *et al*. (2003):

$$F_{ij} = \frac{\sum_i H_{ij}}{\frac{1}{k}\sum_{ij} H_{ij}} \quad (5.1)$$

where H_{ij} is the peak height of i analyte with the use of j fibre coating.

$F_{ij}$is the concentration capability factor of the fibre *j.*

k is the number of fibres

The F_{ij} values for the five fibres obtained from equation 5.1 using the four compounds of interest in this study are presented in Table 5.1. As a result, it was demonstrated that DVB/CAR/PDMS and CAR/PDMS fibre coatings showed much higher extraction efficiency for the compounds under investigation than the other (PDMS/DVB, PDMS and PA) fibre coatings.

Table 5-1: Performance values of the criterion function F_{ij} calculated for five different fibre coatings for butyric acid, DMTS, indole and p-Cresol

Fibre coating	F_{ij} for butyric acid	F_{ij} for DMTS	F_{ij} for indole	F_{ij} for p-Cresol	F_{ij} for all
DVB/CAR/PDMS	1.95	2.21	1.73	1.69	1.90
CAR/PDMS	2.44	1.93	1.69	1.83	1.97
DVB/PDMS	0.47	0.78	1.44	1.43	1.03
PDMS	0.07	0.08	0.12	0.04	0.08
PA	0.07	0.00	0.01	0.01	0.02

However, on one hand DVB/CAR/PDMS had higher extraction efficiency for DMTS and indole than CAR/PDMS. On the other hand, CAR/PDMS had much higher extraction efficiency for butyric acid and p-Cresol compared to all the evaluated fibre coatings. This could be because

the DVB/CAR/PDMS fibre comprises of a layer of DVB suspended in PDMS over a layer of CAR also suspended in PDMS. Consequently, the higher molecular weight compounds (DMTS and indole were retained in the pores of the outer DVB layer and the lower molecular weight compounds (butyric acid and p-Cresol) travelled through this layer and were retained by the micropores in the inner coating of CAR (Torrens *et al*., 2004; Welke *et al*., 2012). Besides, high sensitivity of these fibre coatings also showed much better repeatability. Furthermore, CAR/PDMS has been reported that it does not favour production of oxidation products at high temperatures (Pia Gianelli, *et al.,* 2002). On the basis of the overall performance as shown in Table 5.1, the CAR-PDMS was selected for the subsequent optimisation experiments.

5.2.2 Preliminary univariate experiments

HS-SPME preconcentration efficiency of analytes out of either the samples or the headspace of the samples onto a fused silica fibre coated with a polymeric phase is dependent upon such different experimental parameters as; fibre coating type and thickness, extraction temperature, extraction time, equilibrium time, sample volume, ionic strength, sample pH, desorption time, etc. (Risticevic *et al.,* 2008). Several preliminary univariate experiments were conducted on the mixed standard solution of 500 mg/L to investigate the potential effect of each factor on the response variable (mean chromatographic peak area of each compound of interest in this study as well as to determine the boundary levels for each factor.

The preliminary results showed that experimental factors as stirring rate, sample volume, equilibrium time had insignificant influence on the response variables. For instance, an evaluation of different stirring rates is reported in Figure 5.1a, where insignificant among 600 rpm or 800 rpm or 1000 rpm can be seen. This fact could be explained by the equilibrium of the analytes was reached faster hence rendering agitation ineffective. However, such experimental factors as extraction time, extraction temperature, sample pH and ionic strength had significant influence on the response variables. For instance, an evaluation of different extraction times as reported in Figure 5.2b, where significant difference among 10min or 20 min or 30 min can be easily observed.

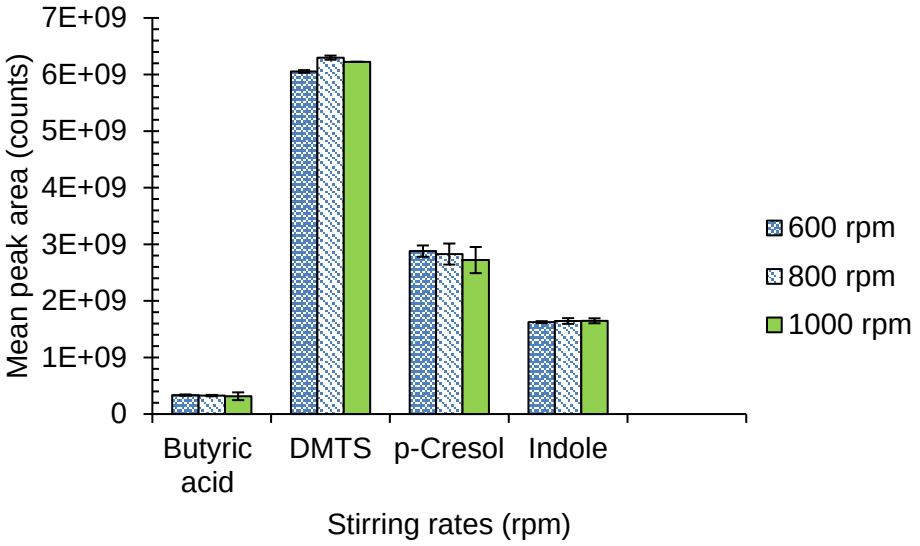


Figure 5-1a: Comparison of compounds extraction in duplicates at different stirring rates

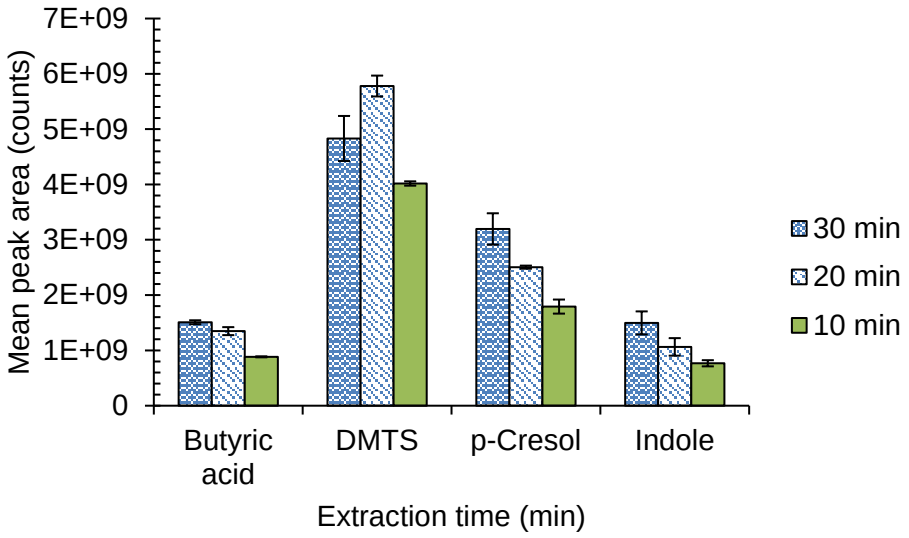


Figure 5-1b: Comparison of compounds extraction in duplicates at different extraction times

5.2.3 SPME parameters optimisation

Based on the preliminary tests that were performed prior to optimisation, four parameters that were chosen as potentially influencing the SPME efficiency were; extraction temperature, extraction time, sample pH and ionic strength (NaCl concentration). The factors and their levels (low (−), centre (0) and high (+)) for each factor are designated in Table 5.2. The low and high levels designated for each factor were also chosen based on the experience gathered in preliminary experiments. In particular, the pH range was narrow to enhance the extraction of

butyric acid since lowering of pH would make it to be present in the solution in its neutral form which has a greater affinity for the fibre (Ábalos *et al*., 2000).

Table 5-2: Factor levels in the 2^4 factorial experimental designs for HS-SPME optimisation

Factor(units)	Low(-)	Centre(0)	High(+)
Extraction temperature (°C)	40	55	70
Extraction time (min)	10	20	30
Sample pH	1	1.5	2
NaCl concentration (mg/mL)	0	250	500

The sample volume of 10 mL, equilibrium time of 10 min and constant stirring rate of 800 rpm, were kept constant for all the experiments. The effects of these factors from a low level to a high level value were investigated on response values (chromatographic peak area of DMTS, butyric acid, p-Cresol and indole).

5.2.4 Screening by 2^4 full factorial design

The initial screening design was done to identify the factors that had main effects and interaction effects as well as to estimate the degree of the effects on the responses of each of the targeted compounds (DMTS, butyric acid, p-Cresol and indole) in the appropriate experimental realm as indicated in Tables 5.2 and 5.3.

Table 5-3: Coded experimental matrix of the 2^4 factorial design

Standard order	Run order	Extraction temperature(°C)	Extraction time(min)	Sample pH	Ionic strength(mg/ml)
25	1	-1	-1	-1	1
36	2	0	0	0	0
14	3	1	-1	1	1
16	4	1	1	1	1
18	5	1	-1	-1	-1
33	6	0	0	0	0
1	7	-1	-1	-1	-1
30	8	1	-1	1	1
23	9	-1	1	1	-1
34	10	0	0	0	0
7	11	-1	1	1	-1
21	12	-1	-1	1	-1
13	13	-1	-1	1	1
19	14	-1	1	-1	-1

26	15	1	-1	-1	1
32	16	1	1	1	1
20	17	1	1	-1	-1
27	18	-1	1	-1	1
22	19	1	-1	1	-1
17	20	-1	-1	-1	-1
3	21	-1	1	-1	-1
35	22	0	0	0	0
8	23	1	1	1	-1
6	24	1	-1	1	-1
5	25	-1	-1	1	-1
11	26	-1	1	-1	1
9	27	-1	-1	-1	1
12	28	1	1	-1	1
2	29	1	-1	-1	-1
10	30	1	-1	-1	1
4	31	1	1	-1	-1
31	32	-1	1	1	1
15	33	-1	1	1	1
29	34	-1	-1	1	1
28	35	1	1	-1	1
24	36	1	1	1	-1

A 2^4 full factorial design was therefore used to generate an experimental design matrix as shown in Table 5.3 to optimize HS-SPME process. A total of 36 experiments (2^4 =16) were performed in duplicates. Additionally, four centre points, one for each factor were also conducted in order to quantify experimental error (Sousa *et al*., 2006). The intervals for the four factors as listed in Table 5.2 were selected based on the preliminary experimental results. Furthermore, all the analyses were undertaken in randomized manner in order to reduce the effect of extraneous or 'nuisance' variables in the actual responses (Mejìas *et al*., 2002; Ma *et al*., 2013; Di Carro *et al.,* 2015) whereas centre points were spread throughout the experimental matrix.

The matrix design permitted to interpret the results using analysis of variance (ANOVA) at the 95% confidence level and graphic tools usually known as Pareto charts were generated to detect which main factors as well as interactions among the factors are statistically significant.

The results of the experiments for the four odourants are presented in Table 5.4. The results of the ANOVA in terms of the p-values and F-ratios are presented in Table 5S1 in appendix 3. All factors that had p-values of equal to or less than 0.05 are statistically significant. However, the results for both main factors and interactions among factors obtained from the 2^4 factorial design are further graphically visualized using Pareto charts of the effects as shown in Figures 5.2a, 5.2b, 5.2c and 5.2d for butyric acid, DMTS, p-Cresol and indole, respectively. In Pareto charts, the length of each bar is correlated to the absolute value of its associated standardized main effects and interaction effects. The standardized effect is calculated by dividing the estimated effect of each factor or interaction by its standard error. Vertical dotted line in the chart is the reference line. In this study, the reference line was calculated from the variance of each effect, 15 degrees of freedom and at α=0.05. This implies that the effect of the factor or interaction beyond this line were statistically significant at 95% confidence level and vice versa. The shaded bar in the Pareto charts indicates the effect of the main factor or interactions have a positive influence while the unshaded bar indicates a negative influence. Furthermore, the length of the bar from the line determines the magnitude of the importance of the factors or interactions in the HS-SPME process.

As can be seen, the Pareto chart of the effects for DMTS in Figure 5.2b indicated that apart from the three-way interactions of extraction temperature, sample pH and ionic strength (ACD) having dismal statistically significance, none of the factors and two-way, four-way interactions and other three-way interactions were statistically significant on the response values (chromatographic peak areas).

Table 5-4: Experimental results for the 2^4 factorial design

Std order	Run order	Chromatographic peak areas (asu)			
		Butyric acid	DMTS	Indole	p-cresol
25	1	88 694 110	2 390 020 595	364 134 857	646 010 643
36	2	89 716 490	9 685 646	565 167 093	655 475 739
14	3	154 099 310	1 088 509 236	1 285 470 823	1 401 747 470
16	4	304 447 420	1 437 771 470	1 645 409 706	1 908 257 961
18	5	52 169 827	1 399 764 157	339 521 867	377 894 150
33	6	129 270 084	8 994 604	582 099 236	798 261 762
1	7	12 724 402	1 040 278 327	46 521 500	40 213 872
30	8	233 977 155	2 278 761 074	1 376 234 934	1 639 741 922
23	9	11 440 376	748 154 204	56 220 772	45 981 041
34	10	70 409 315	5 993 760	617 281 366	722 192 892
7	11	15 841 849	1 170 632 217	78 912 883	61 254 313
21	12	12 744 945	966 999 192	33 941 905	31 165 797
13	13	60 837 551	1 322 582 143	507 054 305	554 883 590
19	14	15 775 438	1 091 458 886	57 413 763	55 004 674
26	15	137 467 754	2 610 968 146	1 196 252 353	1 546 984 615
32	16	315 234 593	2 272 705 489	1 702 801 454	1 953 831 682
20	17	64 470 581	1 523 625 913	478 432 868	541 025 143
27	18	66 818 120	2 217 016 052	644 377 562	853 953 044
22	19	41 493 929	1 452 371 443	293 319 271	303 718 982
17	20	6 842 206	576 450 960	37 013 107	29 834 991
3	21	6 142 073	533 815 160	60 173 589	39 211 266
35	22	130 835 599	9 293 224	665 274 427	844 579 652
8	23	28 226 538	1 246 017 741	417 869 639	361 025 793
6	24	23 221 975	1 044 426 001	226 541 986	220 162 321
5	25	12 057 321	985 289 982	37 829 957	33 032 407
11	26	85 512 453	2 497 020 099	690 660 767	928 565 195
9	27	54 500 932	2 114 957 553	282 293 998	507 320 937
12	28	256 046 479	96 725 763	1 610 660 166	1 923 681 797
2	29	48 062 154	1 395 187 795	253 785 858	299 600 099
10	30	198 337 165	187 872 108	1 435 705 345	1 620 318 085
4	31	77 382 275	1 709 328 240	585 050 581	612 836 115
31	32	98 392 751	1 326 683 435	740 061 781	812 569 236
15	33	50 544 998	857 325 185	562 698 814	626 358 352
29	34	28 612 019	557 226 643	463 570 372	443 631 686
28	35	243 165 307	100 700 699	1 555 890 987	1 849 069 157
24	36	41 747 979	1 494 403 734	360 864 914	375 320 208

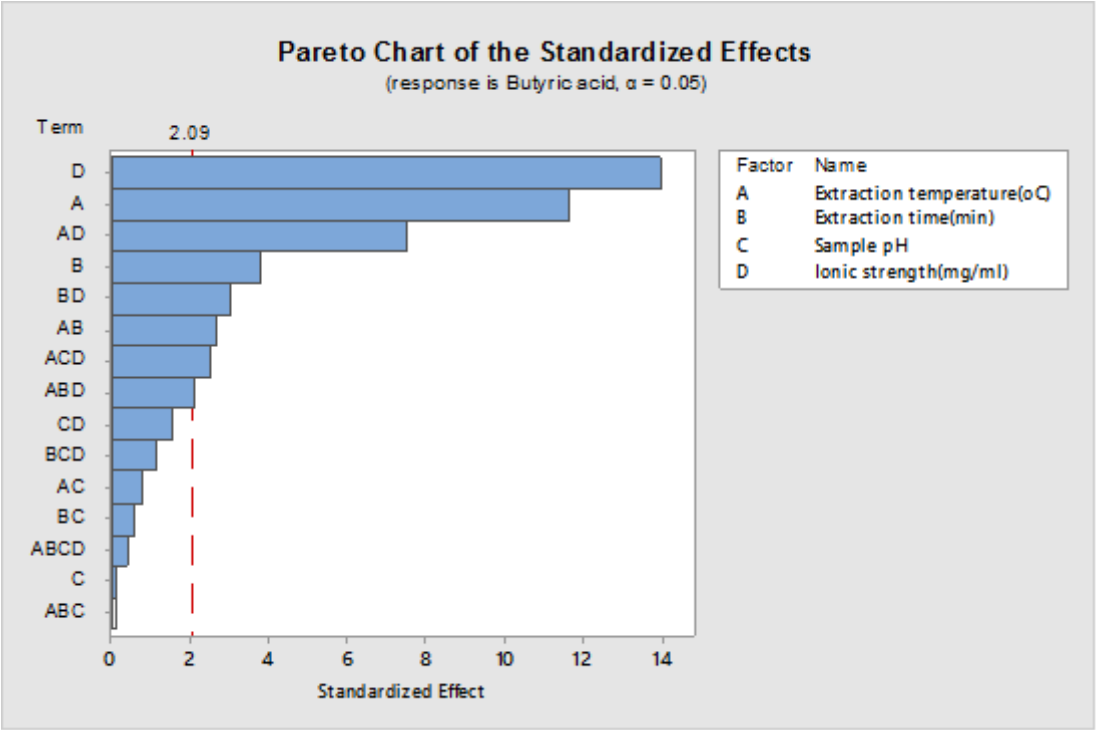


Figure 5-2a: Pareto chart of the standardized effects of extraction temperature, extraction time, sample pH and ionic strength on the chromatographic peak area of butyric acid

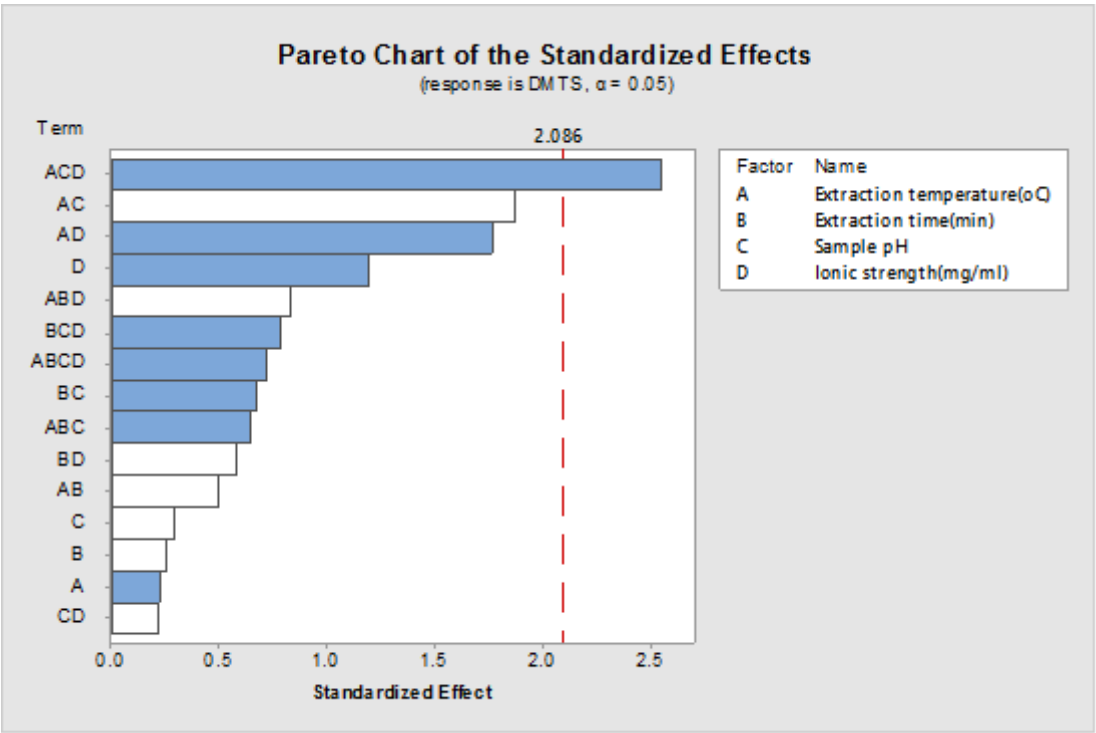


Figure 5-2b: Pareto chart of the standardized effects of extraction temperature, extraction time, sample pH and ionic strength on the chromatographic peak area of DTMS

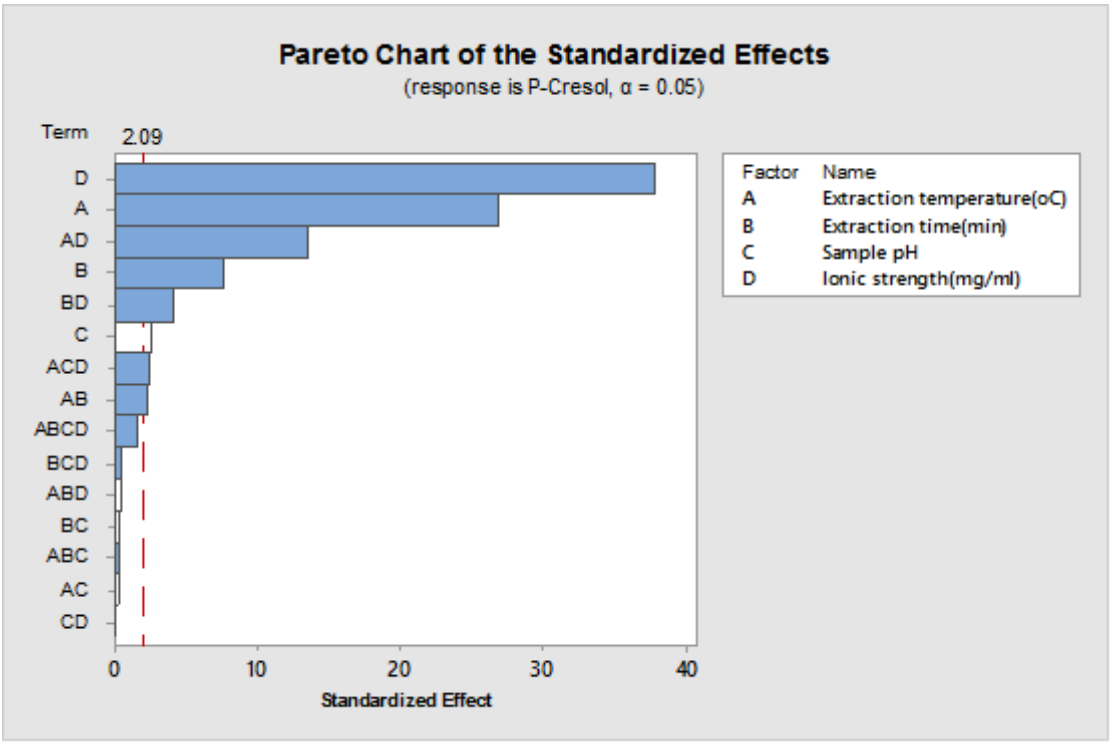


Figure 5-2c: Pareto chart of the standardized effects of extraction temperature, extraction time, sample pH and ionic strength on the chromatographic peak area of p-Cresol

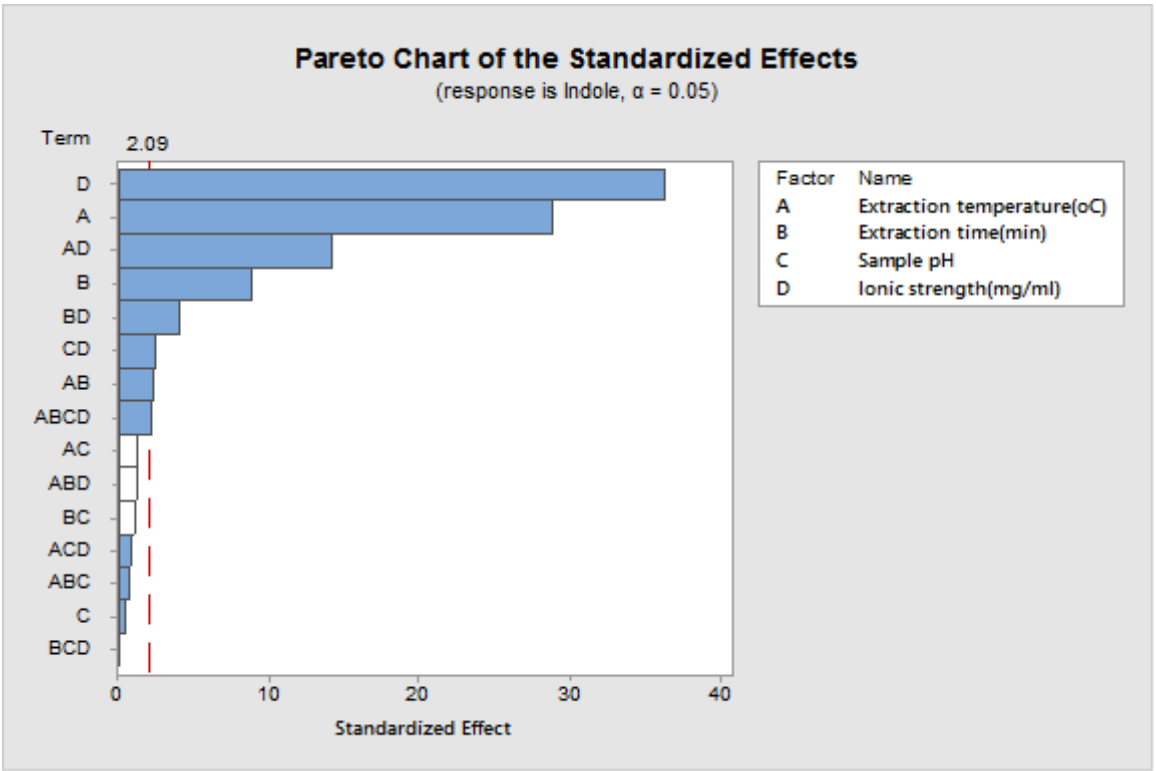


Figure 5-2d: Pareto chart of the standardized effects of extraction temperature, extraction time, sample pH and ionic strength on the chromatographic peak area of indole

This entails that the optimal conditions for HS-SPME extraction process of DMTS is certain only if the factors such as extraction temperature, sample pH and ionic strength are simultaneously optimised as one entity. This is because the factors are not independent from each other. This behaviour could be attributed to its volatility, because DMTS is very volatile as well as hydrophobic hence its migration from the sample matrix to the headspace is not governed the experimental factors. All Pareto charts of the effects for butyric acid, p-Cresol and indole (Figures 5.2a, 5.2c and 5.2d, respectively) but for DMTS (Figure 5.2b), indicate that ionic strength (NaCl concentration) was the most important factor, indicating statistical significance with a positive influence for butyric acid, p-Cresol and indole.

With reference to extraction temperature, it was shown that it was the second most important factor for HS-SPME process, it was statistically positive significant for butyric acid, p-Cresol and indole. Extraction time also presented statistical positive all the three compounds (butyric acid, p-Cresol and indole), whereas sample pH was statistically significant with a negative influence for only p-Cresol. This means that the higher the sample pH as a factor involves a significant decrease of chromatographic peak areas of p-Cresol while for the other compounds had no significant effect on their chromatographic peak areas. Subsequently, this insinuates that the sample pH should be set to a low value only for deionization of butyric acid to enhance its affinity for the SPME fibre as already indicated in section 5.2.3.

With reference to two way interactions, that between extraction temperature and ionic strength (AD), extraction time and ionic strength (BD) and extraction temperature and extraction time (AB), in that descending order of magnitude of importance, were statistically significant with a positive influence for butyric acid, p-Cresol and indole, whereas interaction between sample pH and ionic strength (CD) was statistically significant with a positive influence only for indole. In regard to three-way interactions, that among extraction temperature, sample pH and ionic strength (ACD) was statistically significant with a positive influence for DMST as indicated above, butyric acid and p-Cresol, whereas that among extraction temperature, extraction time and ionic strength (ABD) was statistically positive for only butyric acid. Other less important

interactions were that of four-way among extraction temperature, extraction time, sample pH and ionic strength (ABCD). This was statistically significant with a positive effect for only indole. The higher values involve a significant increase of chromatographic peak areas of the compounds if the factors and interactions that were found to be statistically positive significant.

5.2.5 Response surface methodology optimisation

In order to estimate the linear effects, the interactions between pairs of variable factors and the quadratic effects, a 2^3 factorial design (after taking out the sample pH as one of the factors) was extended to CCD with the total number of design points used were according to the experimental equation 5.2 (Ebrahimzadeh *et al*., 2011):

$$N = 2^f + 2f + C \tag{5.2}$$

where N is number of experiments, f is the number of factors and C is the number of central points

The axial distance, α, was selected as 1.682. This was for establishing the rotatability condition i.e. identical information in all directions produced by design and a rotation of the design about the origin. This does not change the variance contours (Naccarato *et al*., 2014).

As shown in Table 5.4, a complete design matrix of 20 (2^3+ (2 × 3) + 6) randomized experiments was generated. Thus, it had 3 factors (extraction temperature, extraction time and ionic strength) with 6 center points in cube, which gave five degrees of freedom, in order to reduce the risk of missing non-linear relationship in the middle of the intervals as well as for determination of confidence intervals (Lundstedt *et al*., 1998). It also had eight cube points and augmented with six axial points to estimate curvature. The ranges of the three considered factors were as follows: 40–70 ^{o}C for extraction temperature, 10–30 min for extraction time, 100-550 mg/mL of NaCl for ionic strength. The factors and their levels axial (-α), low (−), centre (0), high (+) and axial (+α) for each factor and the experimental results are presented in Table 5.4. All the analyses were conducted with all other variable factors kept the same as in 24 full

factorial design screening as indicated in section 5.2.3, however, the sample pH was reduced to 1.5.

Due to the fact that the higher-order terms such as three-factor interactions and four-factor interaction terms were presumed to be negligible in relation to main effects and were used to get an estimate of random variation on response variables, two –factor interaction terms in the modeling design to get a model that could be used to ascertain the factor settings that optimized the responses, they were not considered in the CCD. However, all linear terms of both factors that form the two- way interaction were included in the model. The generalized response surface model to describe the relationship between the observed chromatographic peak areas (Y) and the experimental factors takes the form of a polynomial equation 5.2 (Roosta *et al.,* 2015):

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (5.2)$$

where β_o is a constant; and β_i, β_{ii} *and* β_{ij} are the linear, quadratic and interaction coefficients, respectively. In this model, X_i and X_j are the independent variables.

A response surface methodology (RSM) is the common strategy used to summarize the results of CCD experiments (Demirci, 2014). In this study, the response surfaces are presented in three- dimensional (3-D) representation with two selected factors in Figures 5.3a through 5.3h were used to in order evaluate the effects of independent variables (extraction time, extraction temperature and ionic strength) on the simultaneous HS-SPME extraction of the response variables (the four odourants). To investigate the two-way interaction effects of two selected independent variables on the peak areas of the four odourants, the 3-D response surface plots were generated by varying the two selected factors within their experimental range while the third factor was held constant at its central point value.

Figure 5.3a shows the response surface plot for butyric acid peak area versus extraction temperature and extraction time. As can be seen the highest peak area obtained was corresponding to an extraction temperature of about 58 ^{o}C and an extraction time of about 23 min. Both variables were positive significant ($p<0.05$) in the model, an indication that the butyric acid peak area was clearly influenced by both variables. Similar behaviour was exhibited by indole as shown in Figure 5.3b. Figure 5.3c shows the response surface plot for butyric acid peak area versus extraction temperature and NaCl concentration. As can be seen, the optimum peak area was achieved at an extraction temperature of about 76 ^{o}C and NaCl concentration of about 450 mg/mL. Similar responses were also exhibited by indole and p-Cresol as shown in Figures 5.3d and 5.3e, respectively. In all these cases, NaCl concentration exerted more positive influence than extraction temperature. Figures 5.3f, 5.3g and 5.3h show the response surface plots for butyric acid, p-Cresol and indole peak areas, respectively, versa extraction time and NaCl concentration. Butyric acid, p-Cresol and indole exhibited optimal peak area at extraction times of 26 min, 35 min and 33 min, respectively, and NaCl concentrations of 420 mg/mL, 500 mg/mL and 470 mg/mL, respectively. In all these cases, NaCl concentration also exerted positive influence more that extraction time.

Table 5-5: Factors and value levels used in the central composite design

Run order	Extraction temperature (ºC)	Extraction time (min)	Ionic strength (mg/mL)	Chromatographic peak area (asu)			
				Butyric acid	DMTS	p-Cresol	Indole
1	55	36.82	300	538 404 501	95 603 754	2 012 830 798	2 059 770 765
2	55	3.18	300	219 306 798	48 855 915	416 018 876	429 442 707
3	40	10	150	65 526 033	9 282 967	111 140 918	115 447 463
4	55	20	300	731 485 119	96 581 805	1 328 128 801	1 267 540 676
5	70	10	450	396 452 041	32 776 201	1 211 126 914	964 534 870
6	55	20	300	760 023 324	94 510 078	1 378 570 403	1 241 211 469
7	55	20	552.3	383 553 953	944 846 879	895 847 035	1 293 486 814
8	40	10	450	179 759 463	17 939 813	426 509 547	330 860 971
9	55	20	300	739 213 580	98 270 752	1 237 656 944	1 207 316 226
10	55	20	300	696 852 491	102 770 403	1 330 083 118	1 145 491 049
11	70	30	450	783 526 989	137 057 333	2 219 961 998	2 033 880 446
12	55	20	300	686 083 433	119 471 550	1 297 919 481	1 177 621 176
13	55	20	47.7	574 432 988	24 453 885	1 461 283 685	1 222 434 946
14	70	30	150	419 024 598	41 826 115	1 384 528 999	1 268 140 840
15	70	10	150	276 580 417	33 318 298	709 913 668	584 397 815
16	55	20	300	695 680 878	99 071 069	1 272 878 150	1 147 406 401
17	80.23	20	300	494 052 419	119 320 198	1 873 494 406	1 715 148 773
18	29.77	20	300	157 220 796	67 729 762	308 544 511	260 076 196
19	40	30	450	309 573 526	29 150 448	970 954 226	805 000 513
20	40	30	150	192 537 943	26 144 181	367 332 049	331 099 584

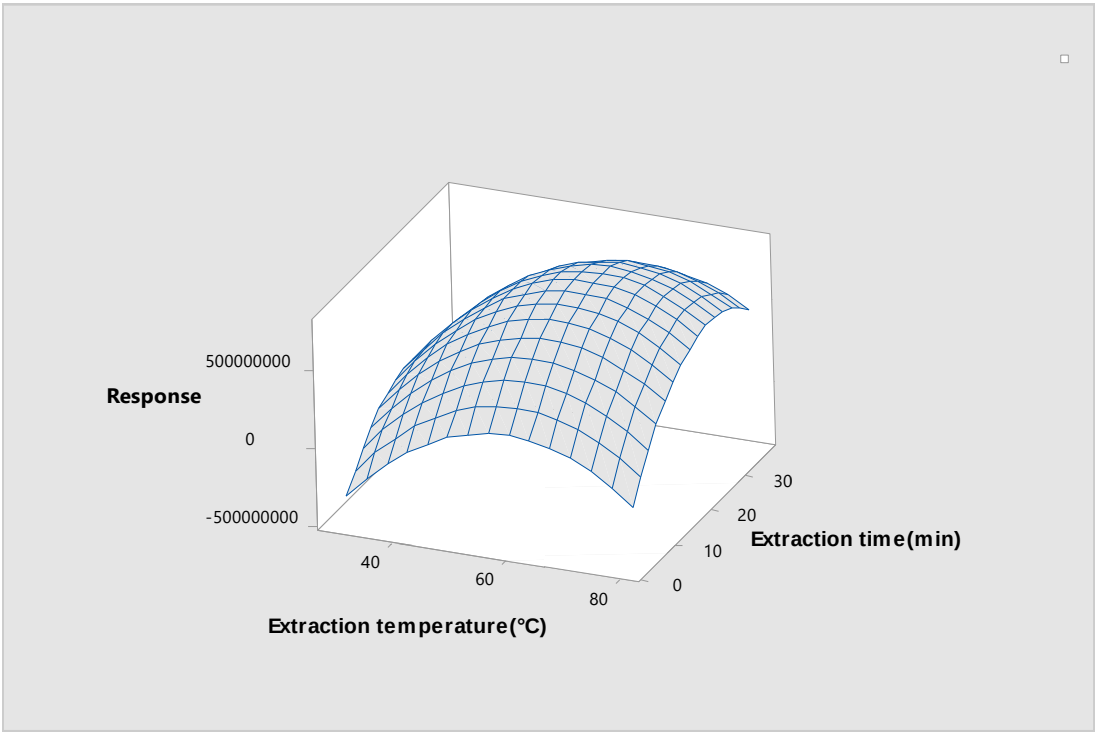


Figure 5-3a: 3D response surface plot of butyric acid peak area versus extraction temperature and extraction time

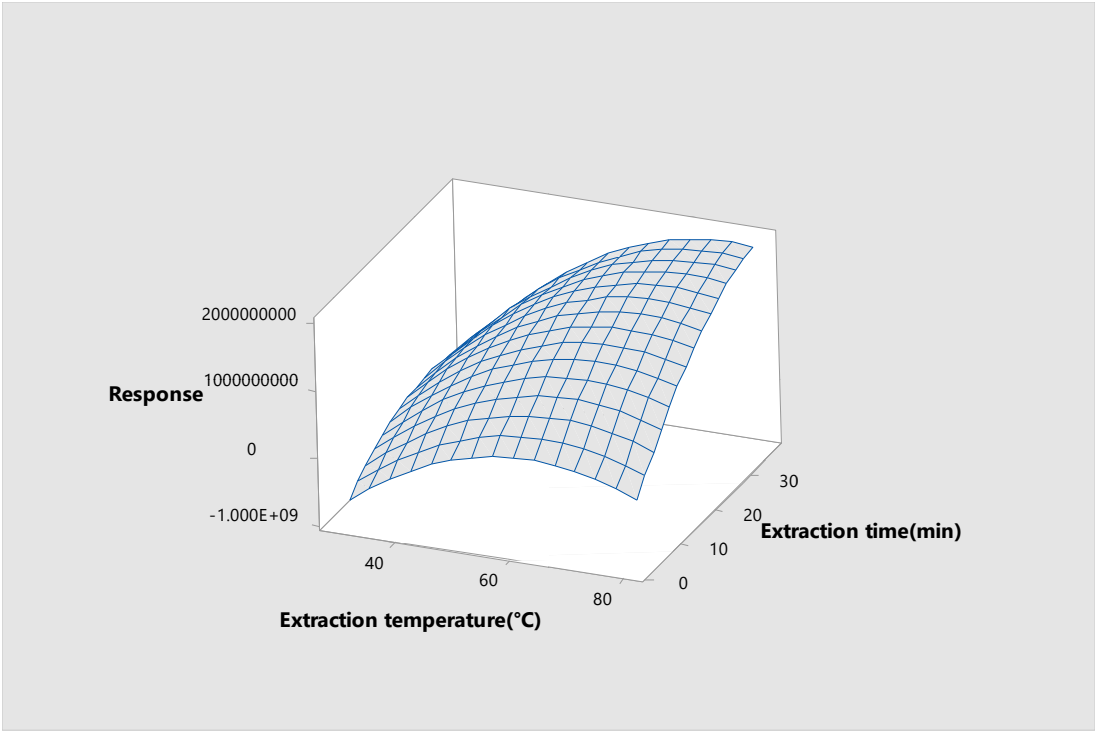


Figure 5-3b: 3D-response surface plot indole peak area versus extraction temperature and extraction time

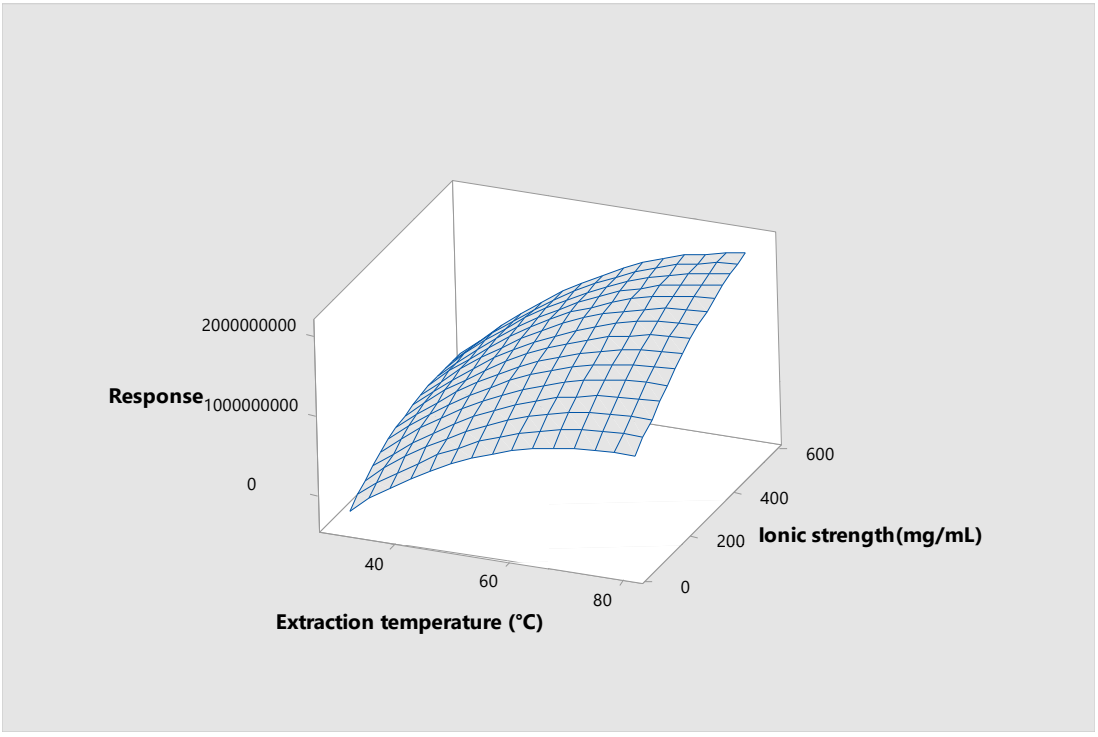


Figure 5-3c: 3D-response surface plot of butyric acid peak area versus extraction temperature and ionic strength

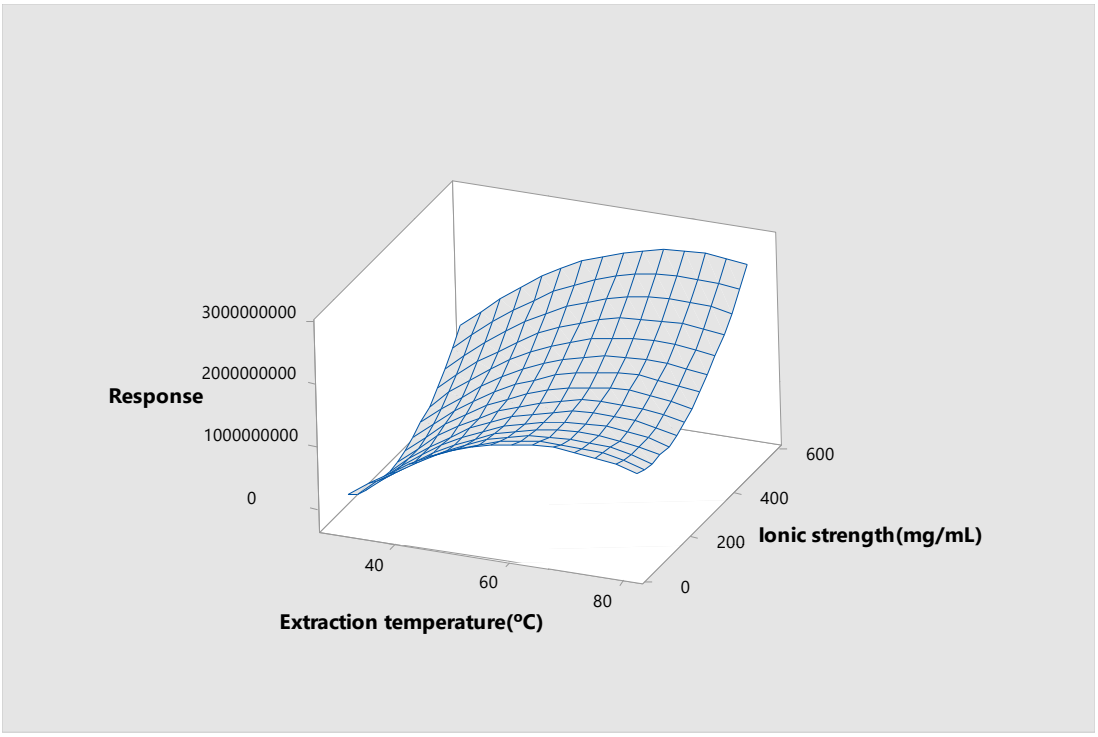


Figure 5-3d: 3D-response surface plot of indole peak area versus extraction temperature and ionic strength

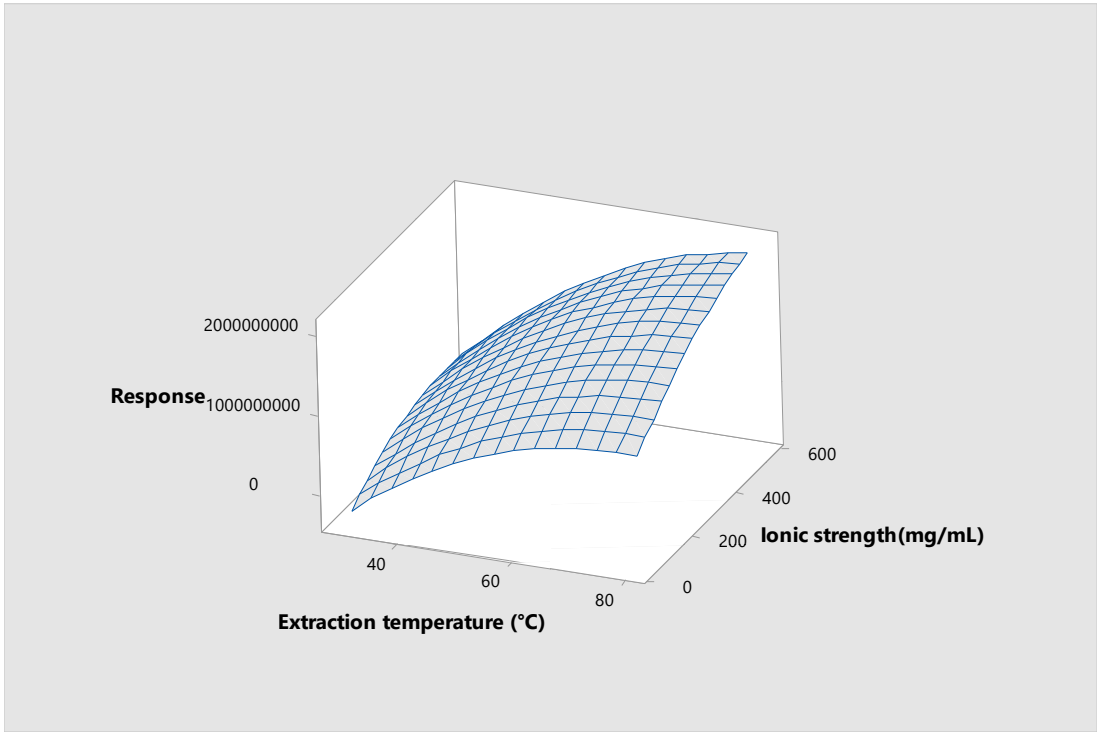


Figure 5-3e: 3D-response surface plot of p-Cresol peak area versus extraction temperature and ionic strength

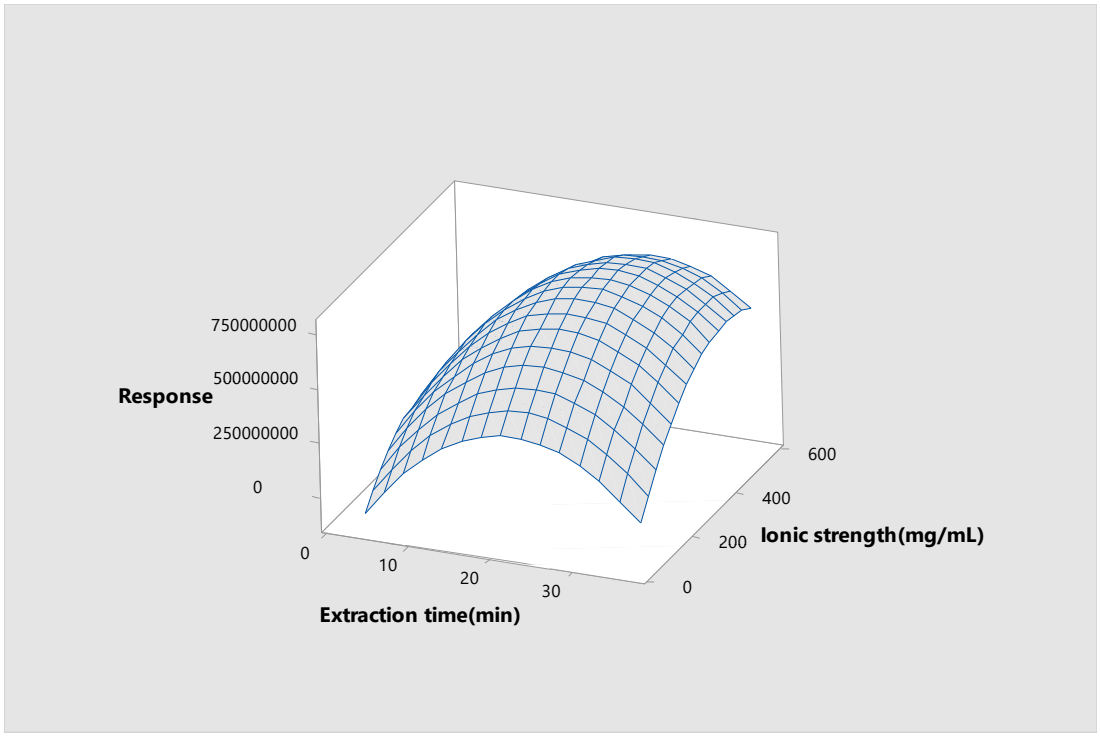


Figure5-3f: 3D-response surface plot of butyric acid peak area versus extraction time and ionic strength

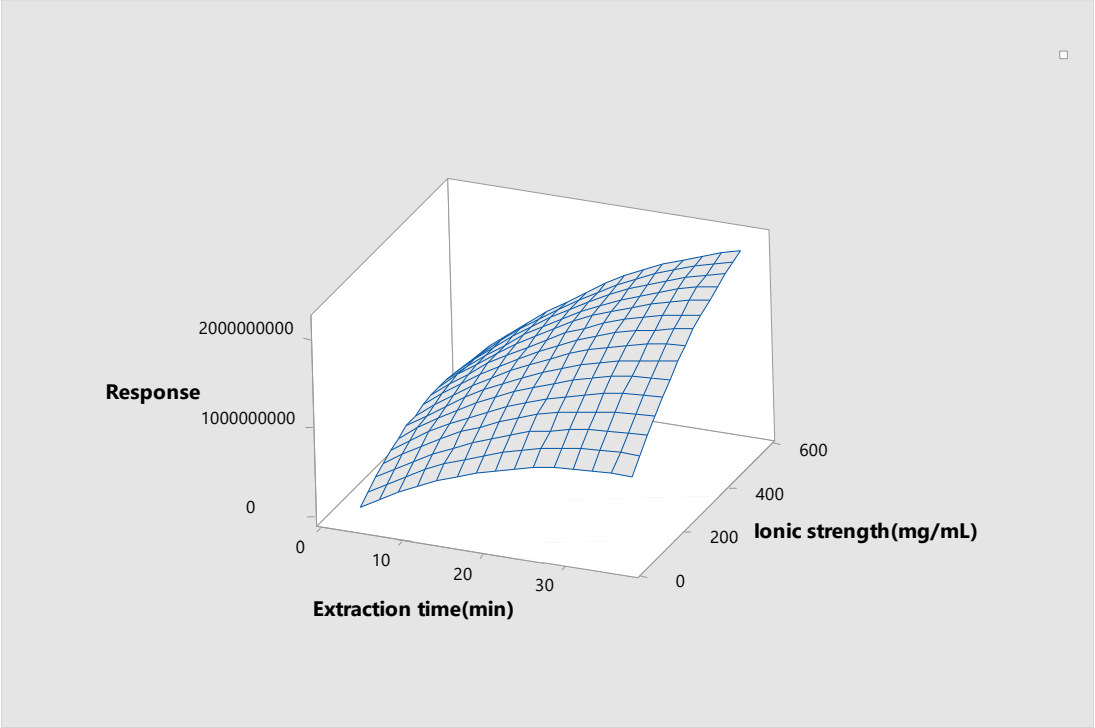


Figure 5-3g: 3D-response surface plot of p-Cresol peak area versus extraction time and ionic strength

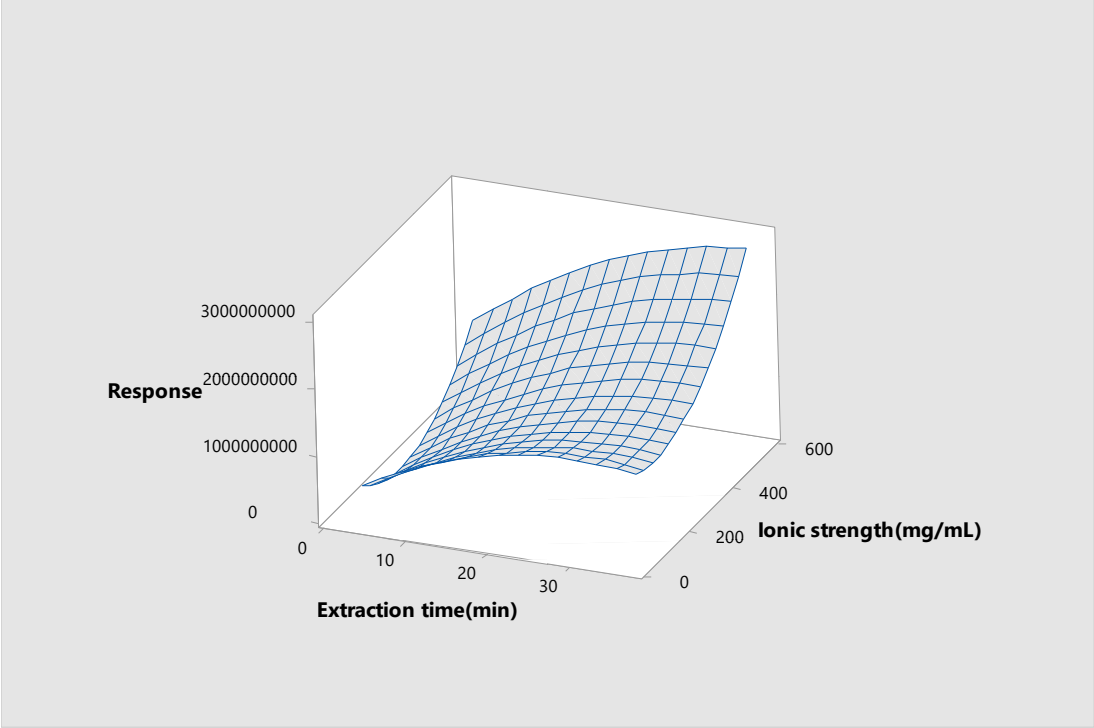


Figure 5-3h: 3D-response surface plot of indole peak area versus extraction time and ionic strength

5.2.6 Multiple responses optimisation

It can be easily realised from the response surface plots generated that the optimal extraction conditions for single responses were localized in different regions. It was, therefore, necessary to find the common experimental region that would simultaneously satisfy all the four responses. Hence Harrington (1965) and Derringer (1994) approach based on desirability function, commonly known as Derringer desirability function, was used to identify the optimal HS-SPME extraction conditions that would provide 'most desirable' combination of four responses- chromatographic peak areas for the odourants under investigation. In this approach an individual response ($\hat{y}_i$) is transformed into a dimensionless desirability function denoted by d_i ($\hat{y}_i$), a measure of how close the fitted value with the optimal settings of the factors is to the desired value. In this study, the optimal HS-SPME extraction conditions would be considered as successfully achieved if the yields of all the four odourants had reached their maximum peak area values, which was the desired objective function, therefore, the composite desirability function of the type the larger is the best is expressed as equation 5.3 (Carro and Lorenzo, 2001):

$$d_i\big((\hat{y}_i)(x)\big) = \begin{cases} 0 & \text{if } y_i(x) < L_i \\ \left(\dfrac{y_i(x) - L_i}{U_i - L_i}\right)^w & \text{if } L_i \le y_i(x) \le U_i \\ 1 & \text{if } y_i(x) > U_i \end{cases} \tag{5.3}$$

where d_i is the individual desirability of the i^{th} response, y_i is i^{th} response value, L_i and U_i are the lower and upper specification limits respectively and w is the weight coefficient.

Then, as proposed by Harrington (1965), the composite desirability function (D) is determined by taking into account the geometric mean of all individual desirability values which is expressed as equation 5.4:

$$D = \{(d_1 Y_1) * d_2(Y_2) * \ldots\ldots\ldots\ldots * d_k(Y_k)\}^{1/k} \tag{5.4}$$

where D is the composite desirability, d_i (i=1, 2, ….k) k is the number of responses.

Initially, the numerical optimisation module using Mintab17 software was utilized to set the goals for all the response variables and the experimental factors. The desired responses of the chromatographic peak area of butyric acid, DMTS, p-Cresol and indole were maximum, hence, the goal was set to maximize and all the responses during the optimisation process were given equal weight and importance of 1, which were not discriminating between output responses. Then, the composite desirability function (D) was generated. The optimal HS-SPME process conditions as shown in Figure 5.4a led to individual desirability scores for butyric acid, DMTS, indole and p-Cresol as 0.967, 0.273, 0.937 and 1.000, respectively, and the composite desirability score (D) of 0.7100. These were achieved at the following values: extraction temperature of 65.45 ^{o}C; extraction time of 27.31 min and NaCl concentration of 552.30 mg/mL. Under these optimized conditions, the chromatographic peak areas of butyric acid, DMTS, indole and p-Cresol, were estimated by RSM models to be 7.596E+09, 2.644E+08, 3.097E+09 and 2.233E+09 arbitrary surface units (asu), respectively. However, for practical purposes, by taking into consideration the operating parameters of most equipment, as shown in Figure 5.4 b, the optimal HS-SPME process conditions were modified as follows: extraction temperature of 65 ^{o}C, extraction time of 28 min and NaCl concentration of 550 mg/mL, which achieved a composite desirability score of 0.7053. Under the modified optimisation criteria, the extraction yields of the analytes expressed as the chromatographic peak areas were 7.593E+09 asu for butyric acid, 2.579E+08 asu for DMTS, 3.097E+09 asu for indole and 2.249E+09 asu for p-Cresol.

As a result of some technical challenges, estimation of method quality parameters with respect to limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy and repeatability for the suitability and performance of HS-SPME-GC-ToFMS for simultaneous quantitative determination of the four odourants was not conducted. Hence, further research is warranted to validate the method. However, the optimized conditions adopted in the HS-SPME by use of the chemometric approach were applied for simultaneous extraction of all the four odourants in milli-Q water containing 0.01µg/L of each odourant. The results of the extracts

are depicted in the typical chromatogram shown in Figure 5.5a and their corresponding mass spectra are shown in Figure 5.5b. The concentrations are generally 10 to 100 folds below the ODT of the odoants reported in the literature (PubChem, undated; Le Cloirec *et al*., 1994; Boon and Vincent, 2003). This signifies that the adopted HS-SPME method has potential to detect the mixture of these key pit latrine odourants at concentrations at which the offensive odour is no longer detectable to the human olfactory system. Hence, the method could be a valuable tool for application in pit latrine odour control.

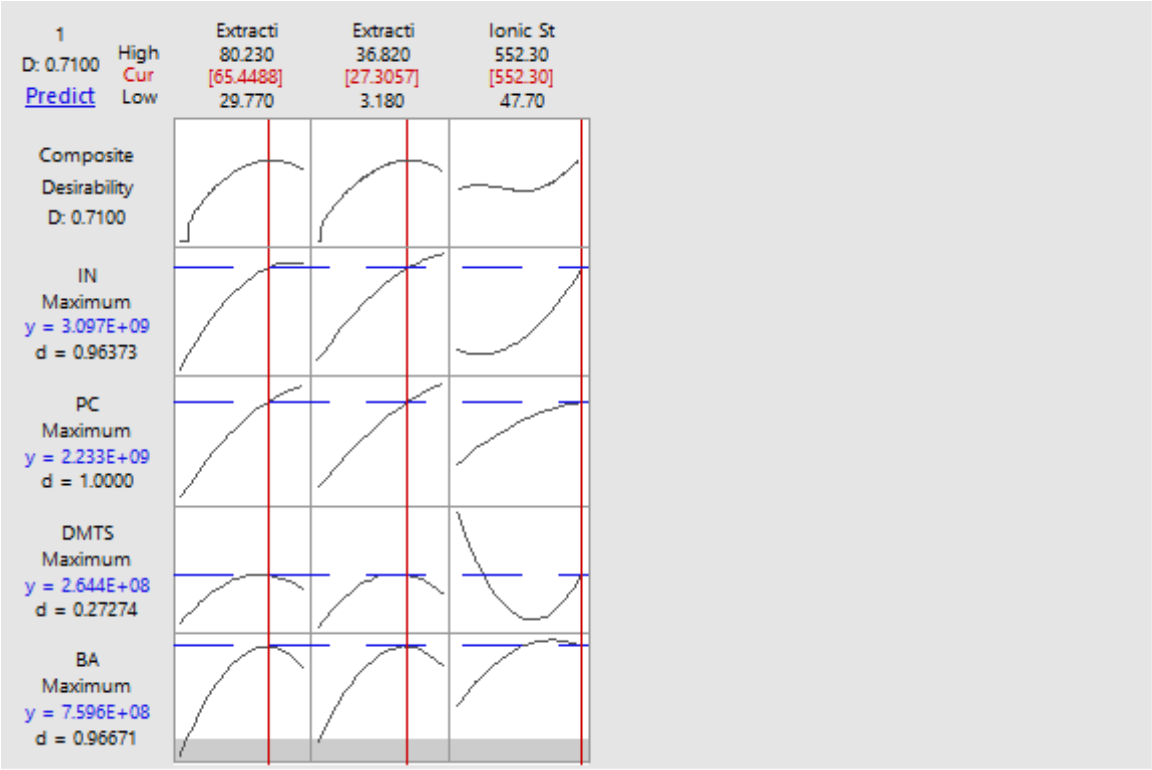
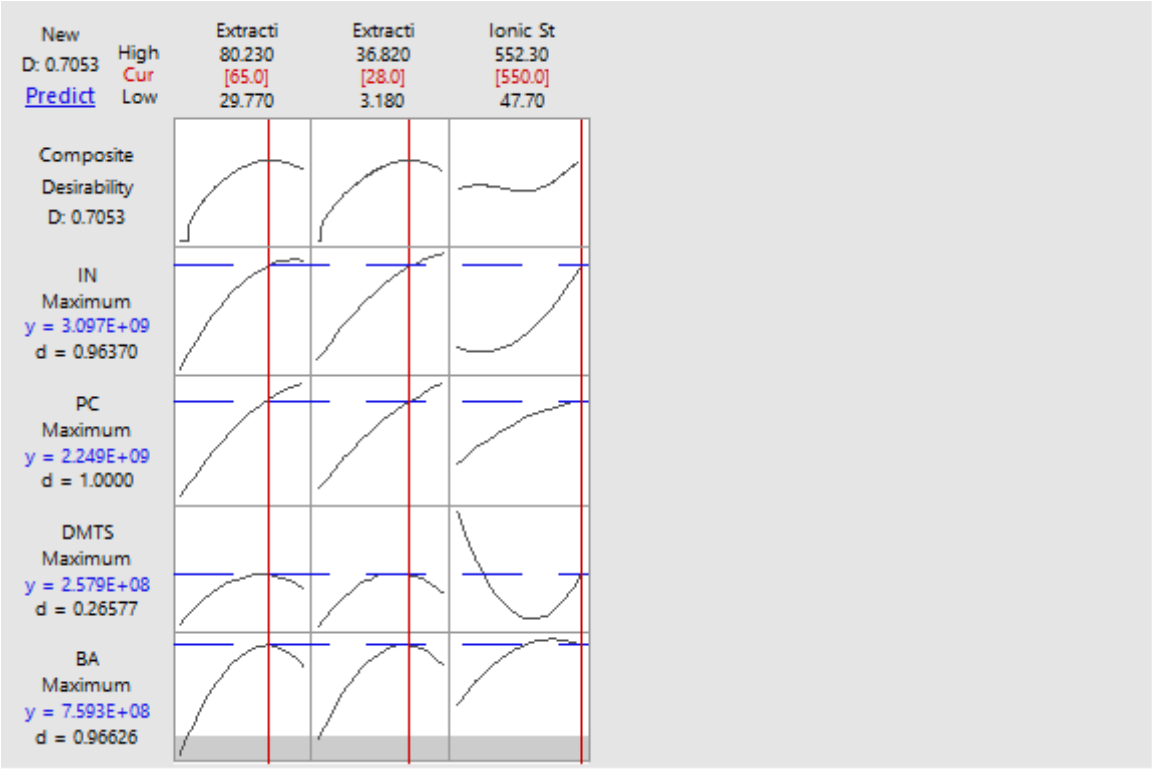


Figure 5-4a: Graphical representation of the composite desirability function, D, for butyric acid, DMTS, indole and p-Cresol



Figur5-4b: Graphical representations of the composite desirability function, D, for butyric acid, DMTS, indole and p-Cresol after modification of HS-SPME process variables

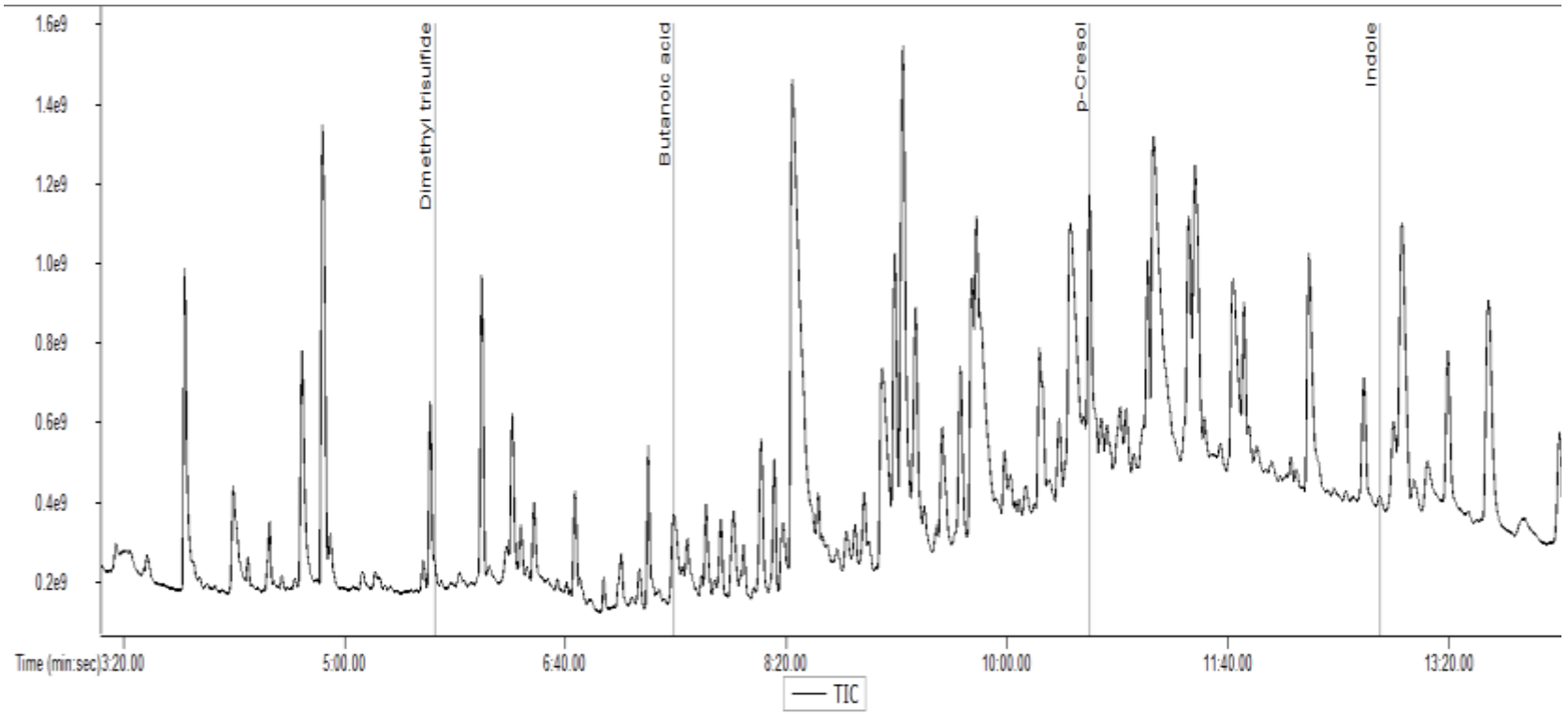


Figure 5-5a: HS-SPME-GC-ToFMS chromatogram of Milli-Q water containing 0.01µg/L of each odourant

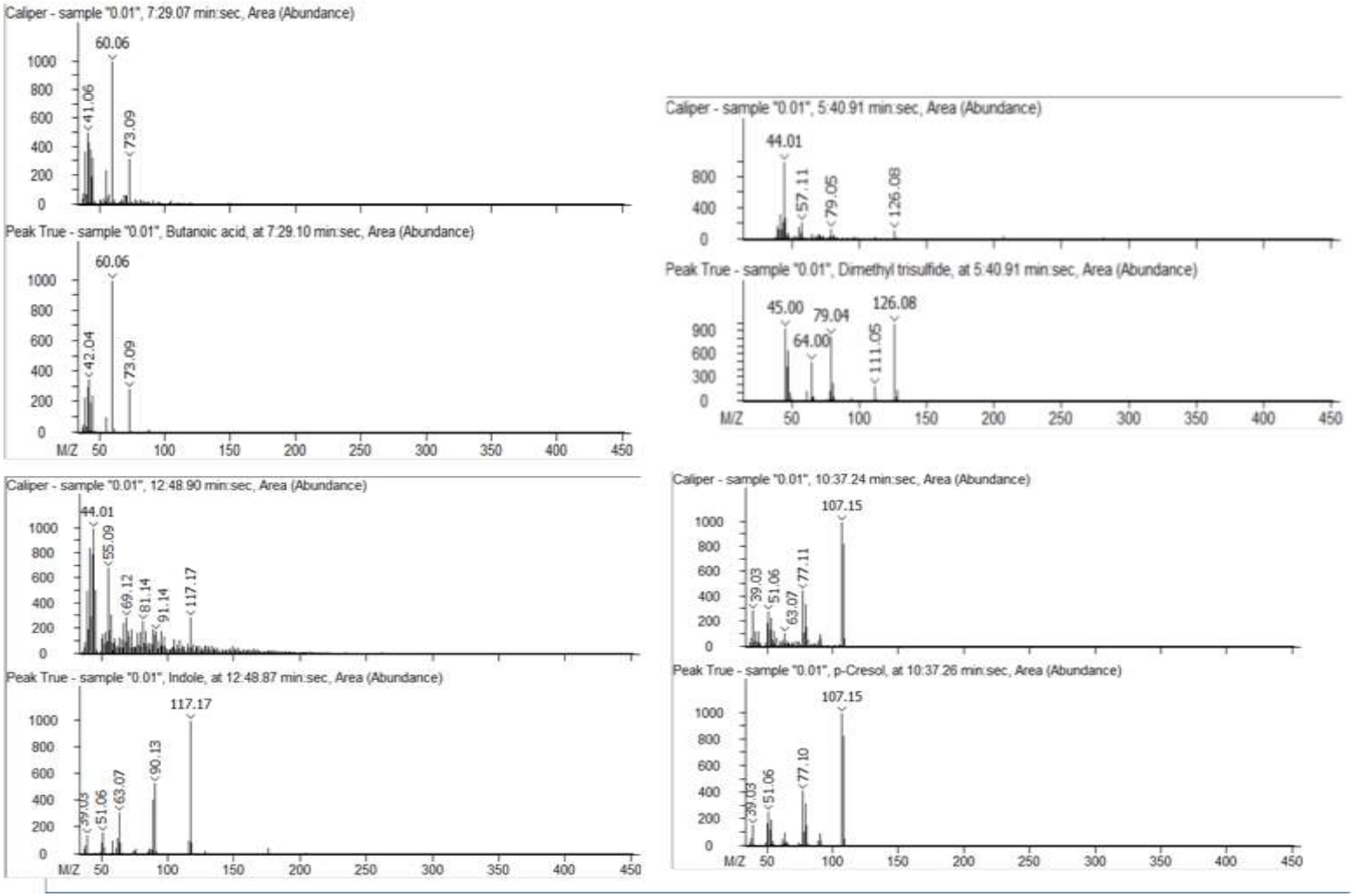


Figure 5-5b: Mass spectra of butyric acid, DMTS, indole and p-Cresol

5.3 Summary

This chapter focused on multivariate optimisation of HS-SPME extraction efficiency for rapid and reliable simultaneous analysis of four key pit latrine odourants (butyric acid, DMTS, indole and p-Cresol) using GC-ToF-MS. Five commercially available SPME fibre coatings were tested. Parameters that influence the extraction efficiency of HS-SPME such as sample volume, sample pH, extraction time, extraction temperature, equilibrium time, ionic strength and stirring rate were investigated using a univariate optimisation approach and a 2^4 factorial screening design.

Subsequently, the parameters that had a significant positive effect on the analytical response (chromatographic peak area) of the target analytes such as extraction temperature, extraction time and ionic strength (NaCl concentration) were further considered for simultaneous optimisation with CCD based on RSM and Derringer's desirability function. The desirability function indicated that the optimal conditions for optimisation of HS-SPME extraction for analysis of key pit latrine odourants were obtained with sample volume 10ml in 20 mL vial, stirring rate of 800 rpm, equilibrium time of 10 min, extraction time of 28 min, extraction temperature of 65 ^{o}C, ionic strength of sodium chloride of 550 mg/mL.

However, due to some technical challenges, the reliability and performance of the proposed HS-SPME procedure was not evaluated under the optimum process conditions derived with respect to quantitative parameters such as LOD, LOQ, linearity, precision, accuracy and repeatability. The proposed method was, however, successfully applied to experimentally detect all the four odourants in Milli-Q water containing 0.01 µg/L of each of them. Notwithstanding technical challenges, the proposed HS-SPME procedure for the detection, identification and quantification of the four key pit latrine odourants is an opportunity worth further research.

CHAPTER SIX

6 ISOLATION, MOLECULAR IDENTIFICATION AND BIODEGRADATIVE CAPACITY OF BUTYRIC ACID DEGRADING BACTERIA FROM PIT LATRINE FEACAL SLUDGE

6.1 Background

Emissions of offensive odours from pit latrine remain a critical deterrent to investment, uptake and consistent use of pit latrines in resource-constrained households in developing countries (Obeng *et al.,* 2016). Several studies among others (Grimason *et al*.,2000; Lundblad and Hellström 2005; Diallo *et al*., 2007; Le *et al*., 2012; Tsinda *et al*., 2013; Obeng *et al.,* 2015) have also implicated offensive odours as one of the impediments to effective sanitation promotion for low income communities in developing countries.

In the present study as well as a previous study conducted by Chappuis *et al.*(2016) butyric acid ($C_4H_8O_2$) is one of the volatile compounds that have been identified in pit latrine emissions that predominate and significantly contribute to unpleasant smell. Butyric acid is a four-carbon SCFA, which is one of the intermediate products of anaerobic digestion, in which complex soluble organic materials are reduced to a methane (CH_4) and carbon dioxide (CO_2) mixture as the main final products (Siegert and Banks 2005). This process comprises of a continuum of metabolic reactions (hydrolysis, acidogenesis and methanogenesis) because of a complex intimate relationship between the acid-forming species and the methane-producing species of bacteria (Lee *et al.,* 2015). Butyric acid, in its pure state as an individual compound, exhibits an idiosyncratic smell of sweet rancid (Sheridan *et al.,* 2003; Otten *et al.,* 2004), which makes it offensive to handle. It is one of the volatile compounds that have a very low olfactory threshold (Sheridan *et al.,* 2003), unpleasant smell that causes nuisance to population in the pit latrine vicinity and contribute significant to the air pollution inside the pit latrine. Therefore, deodourization of butyric acid, as one of the pit latrine odourants, oriented at the point of emission is considered preferable for enhanced and sustainable sanitation in low income settings (Nakagiri *et al.,* 2016).

A range of technologies has been in existence that have traditionally been used to treat odourous air emitted from pit latrines in low-income households in developing countries. These include; use of naturally fragrance occurring substances, addition of wood ash, antiseptics, insecticides, lubricants, laundry and soapy water, motor-battery acids, detergents and

modified latrine designs such as; ventilated improved pit (VIP) latrine, urine-diverting dry and ecological sanitation toilets and pour flush latrines (Rheinländer *et al*., 2013). Nevertheless, these technologies to a greater degree have not provided the desired results, as they are associated with their own social, economic, institutional and technological challenges.

Biological treatment of environmental pollutants, including odourants, either *in situ* or *ex situ*, has gained popularity in the past few decades. This is due to certain competitive advantages offered over the conventional physical and chemical treatment methods. Biological treatment is relatively efficient and cost-effective technology for environmental pollution attenuation, and uses microorganisms to reduce, oxidize or eliminate pollutants (Sheridan *et al*., 2003). According to Muñoz *et al*., (2015) biological technologies for odour removal are based on the capability of microorganisms to convert a wide variety of odourous compounds such as butyric acid into harmless and odourless compounds. When supplied with oxygen, bacteria in pit latrine feacal sludge could utilise odourous compounds as source of carbon and energy and oxidise them to odourless by- products. In this case, simply Burgess *et al.* (2001) can express the butyric acid transformation process:

$$C_4H_8O_2 + O_2 \rightarrow^{\text{via bacteria}} \text{more bacterial cells} + CO_2 + H_2O$$

Bacteria capable of degrading malodourous compounds may be an attractive surrogate pit latrine odour control to use in low-income settings in developing countries. However, detailed information on bacteria that degrade odour-causing compounds, including butyric acid in pit latrines is scarce and very little is known about their degradative capacity.

It is against this backdrop that this chapter focuses on enrichment, isolation and phylogenetical identification of indigenous bacterial strains from pit latrine feacal sludge from South Africa that have capabilities to utilise butyric acid as a sole source of carbon and energy and further determine their butyric acid degradation efficiencies. To the best of our knowledge and after a thorough search in the literature, the use of aerobic bacteria isolated from pit latrine feacal sludge for degradation of butyric acid has not been reported in literature yet.

6.2 Results and discussion

Materials and methods used to achieve the objectives of this chapter are found in section 3.4.

6.2.1 Isolation and molecular identification of the bacterial strains

In this study, indigenous aerobic bacterial strains capable of utilising butyric acid as a sole carbon and energy source were successfully isolated from pit latrine feacal sludge. There were

a total of twenty-four morphologically distinct bacterial colonies that were isolated. The isolates were further screened for their butyric acid degrading ability using MSM supplemented with 1000 mg/L butyric acid. Of the 24 bacterial isolates tested, 9 bacterial isolates demonstrated pronounced growth in butyric acid supplemented MSM as pure cultures after enrichment and purification. The bacterial isolates were designated as Ba, B1a, B1b, B6a, B5a, B7a, C4c, CrNb and CrNc for identification purposes. To identify selected isolates, the RNA sequence analyses of the PCR products from the 16S rRNA gene of the isolates were obtained, submitted and compared with other genes in GenBank using a basic BLAST of the National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequencing was used because it is present in virtually all bacteria and its role has not temporarily changed (Garcha *et al*. 2016). Further, the identification is more objective as optimal growth and microbial viability are not the prerequisites (Reller *et al*., 2007). Comparative phylogenetic dendrograms generated based on 16S rRNA gene sequences of the isolates with closely related species revealed that the bacterial isolates Ba, B1a, B1b, B6a, B5a, B7a, C4c, CrNb and CrNc clearly marched with *Alcaligenes* sp. strain SY1 (AS), *A.animicus* (AA), *P.aeruginosa* (PA), *S.marcescens* (SM), *A.xylosoxidans* (AX), *B.cereus* (BC), *L.fusiformis* (LF), *B.methylotrophicus* (BM) and *B.subtilis* (BS), respectively. Their phylogenetic dendrograms showing the closest NCBI (BLASTn) relatives based on the 16rRNA gene sequence were constructed by the neighbour-joining method as shown in Figure 6.1.

The highest sequence homology (% identity) of each bacterial strain and their closely related strains are also presented in Table 6.1. As described by Stackebrandt and Goebel (1994) the 16SrRNA sequence with higher than 97% identity with known sequences as it is the case in this study are considered homologous with the known bacterial species. The identification of the high percentage of *Bacillus* genus related strains is probably because *Bacillus* strains are not difficult to cultivate in the medium used in this study, or environmental conditions in the pit latrines in Kendal, South Africa are favourable for their survival and growth (Zhang *et al.*, 2010). To the best of our knowledge and after thorough search in the literature, this is the first time all these bacterial strains but members of genus *Pseudomonas* have been reported to utilise butyric acid as the sole carbon and energy source (Sheridan *et al.,* 2003) and *Bacillus* sp in a mixture of other VFAs (Yun and Ohta 1997). Since they are indigenous organisms, they are more likely to survive and to be active than exogenous bacterial strains when introduced into pit latrine environments in South Africa or similar environments. The introduced exogenous bacterial strains are more likely to be subjected to intense competition, predation, or parasitism after their release into the target environment (Han *et al*., 2015), in this case the pit latrine

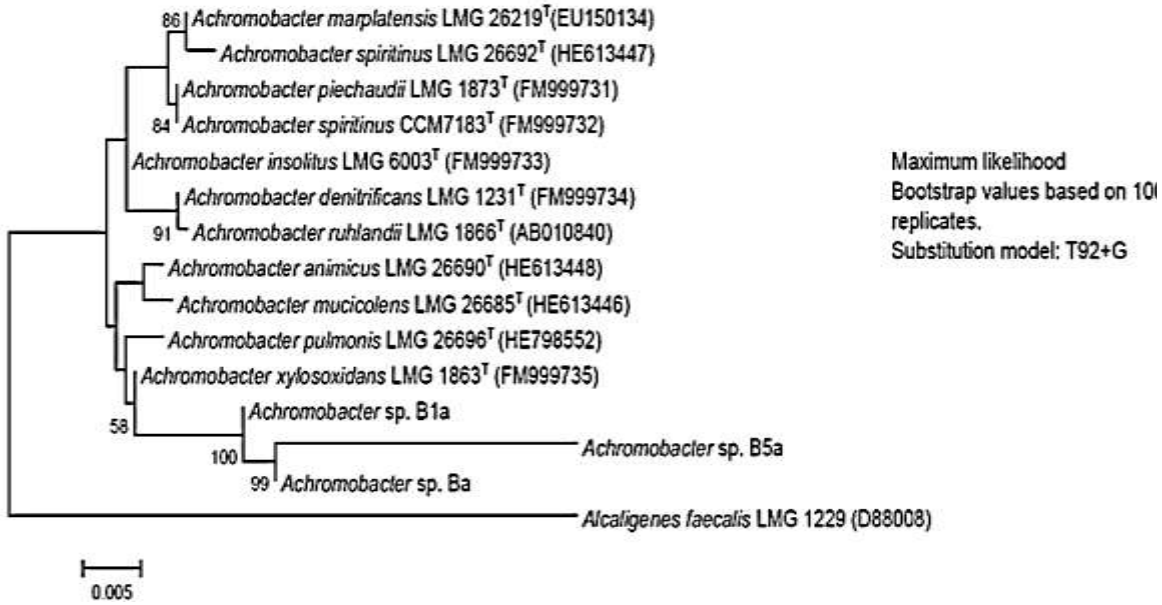


Figure 6-1a: Phylogenetic tree for *Alcaligenes* sp. SY1, *A.animicus* and *A.xylosoxidans* and related strains based on 16s rRNA gene sequences

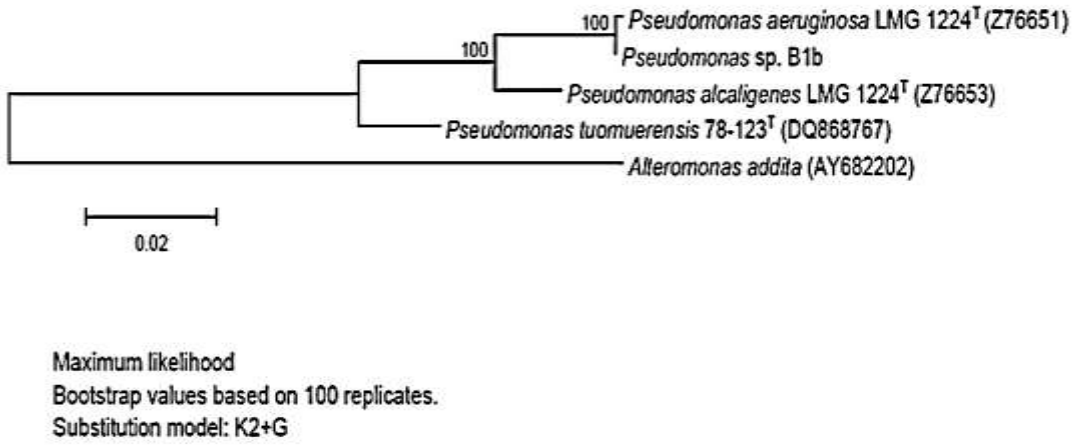


Figure 6-1b: Phylogenetic tree for *P.aeruginosa* and related strains based on 16s rRNA gene sequences

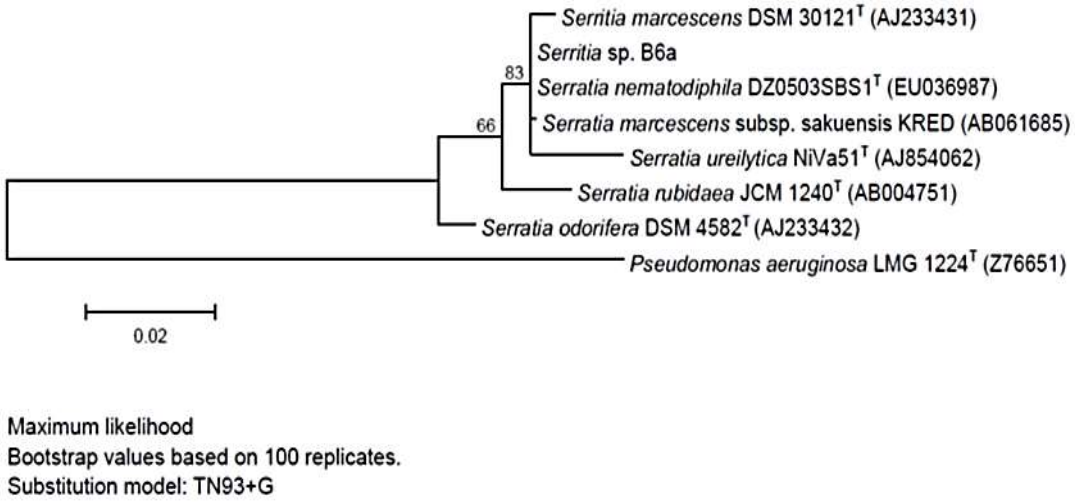


Figure 6-1c: Phylogenetic tree for *S.marcescens* and related strains based on 16s rRNA gene sequences

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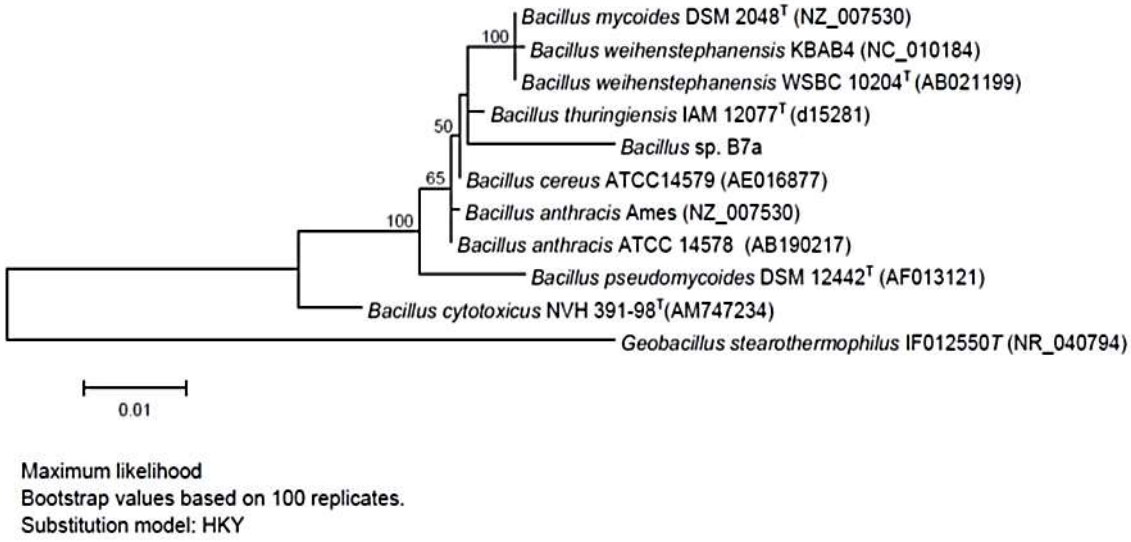

Figure 6-1d: Phylogenetic tree for *B.cereus* and related strains based on 16s rRNA gene sequences

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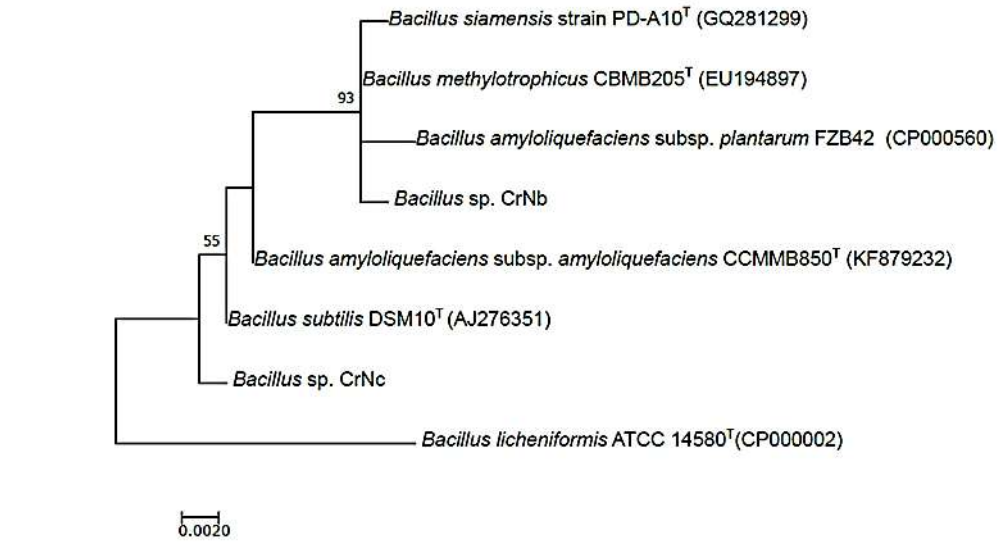
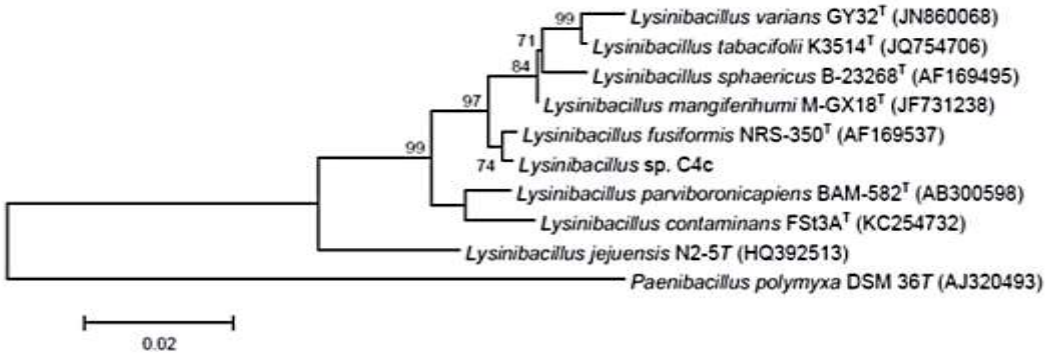


Maximum likelihood
Bootstrap values based on 100 replicates.
Substitution model: HKY

Figure 6-1e: Phylogenetic tree for *B.methylotrophicus* and *Bacills subtilis* and related strains based on 16s rRNA gene sequences



Maximum likelihood
Bootstrap values based on 100 replicates.
Substitution model: K2+G

Figure 6-1f: Phylogenetic tree for *L.fusiformis* and related strains based on 16s rRNA gene sequences

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Table 6-1: Closest relatives of the 16S rRNA gene sequences of bacterial isolates

No	Isolate designation	Closest hit	Accession No.	Homology (%)
1	Ba	*Alcaligenes* sp. strain SY1		99
2	B1a	*A.animicus* LMG26690^T	HE613448	99
3	B1b	*P.aeruginosa* LMG 1224^T	Z76651	100
4	B5a	*A.xylosoxidans* LMG 26686^T	FM999735	93
5	B6a	*S.marcescens* DMS 30121^T	AJ233431	100
6	B7a	*B.cereus* ATCC14579	AE016877	100
7	C4c	*L.fusiformis* NRS-350^T	AF169537	100
8	CrNb	*B.methylotrophicus* CBMB205^T	EU194897	100
9	CrNc	*B.subtilis* DSM10^T	AJ276351	100

Some bacterial strains of these genera are known to be involved in degradation of a number of different odourants that have also been identified in this study as components of pit latrine emissions. For instance, Klenheinz *et al.,* (1999) applied *A.xylosoxidans* to a biofiltration unit for use in degradation of α-pinene as a source of carbon and energy in which 90% removal of α-pinene was achieved. Liang *et al*. (2015) found that *B.cereus* could effectively remove dimethyl disulphide within 96 h under optimal conditions with temperarature of 30 ^oC, pH 7 and 200 rpm. Alesia (2014) observed that *L.fusiformis* was deemed as the most efficient sulphur-oxidising bacterial strain that led to reduction of odour during acid mine water treatment. Gutarowska *et al.,* (2014) showed the feasibility of using *B.subtilis* as one of the active microorganisms for poultry manure deodourisation.

The isolation or identification of indigenous bacterial strains present in pit latrine feacal sludge and with capabilities to degrade other odourants found therein is very crucial to the application of these bacterial strains in deodourisation process. However, there is still need for further research on how the presence of numerous odourants as source of carbon and energy for the degrading bacteria would affect the deodourisation process.

6.2.2 Butyric acid degradation by pure bacterial cultures

The ability of the bacterial strains to utilise butyric acid as a sole source of carbon and energy at 30 ^oC and pH 7 was investigated. The choice of pH 7 was because heterotrophic bacteria show a strong preference for nearly neutral or alkaline conditions (pH 6-8) (Ali, 2010; Li *et al.*, 2017) while 30 ^oC was to reduce the amount of butyric acid lost through vaporisation. As shown in Figure 6.2, the initial 1000 mg/L of butyric acid can be biodegraded effectively by the indigenous pure bacterial strains as it can be observed that it was completely degraded within 20-24 h. However, the degradation rates varied from one bacterial strain to another. The bacterial strains, *A.xylosoxidans, B.subtilis, L.fusiformis, B.cereus, P.aeruginosa* and *B.methylotrophicus* completely degraded 1000 mg/L butyric acid within 20 h while *A.animicus, S.marcescens and Alcaligenes* sp. strain SY1 completely degraded butyric acid within 24 h.

The reason for the differences in degradation efficiencies is unclear. Therefore, probably further studies to elucidate the genes involved in butyric acid degradation in these bacterial strains, along with studies to determine the butyric acid degrading pathway should be undertaken. It is anticipated that this will explain the disparities in degradative capacities of the bacterial strains.

Previous studies (Bourque *et al.,* 1987; Yun and Ohta, 1997; Chin *et al.,* 2010) have found that many bacterial strains can degrade butyric acid. For instance, Bourque *et al.,* (1987) isolated *Acinetobacter calcoaceticus, Alcaligenes feacalis* and *Arthrobacter flavescens* from swine waste. The bacterial strains were able to aerobically degrade butyric acid completely in the presence of other volatile fatty acids (VFAs) such as acetic acid, propionic acid, isobutyric acid and valeric acid and phenol and *p*-cresol after incubation at 29 ^{o}C and 200 rpm within 3 to 5 days. Yun and Ohta (1997) isolated bacterial strains identified as *Bacillus* sp, *Rhodococcus* sp and *Staphylococcus* sp from seed culture which was used for the treatment of animal faeces which exhibited growth on butyric acid in the presence of other VFAs after incubation of 37 ^{o}C and medium pH of 8.0 for 2 days. Conversely, in these previous studies, butyric acid was not the sole source of carbon. Only Chin *et al*. (2010) isolated bacterial strains identified as *Acinetobacter calcoaceticus, Wautersia paucula, Burkholdeira cepacia* which have the ability to degrade 1000 mg/L butyric acid as a sole source of carbon and energy. The complete degradation of butyric acid was achieved within 18 h for *Acinetobacter calcoaceticus* while the other strains it was achieved within 30-55 h at 30 ^{o}C and pH 7.0. The present study and the aforementioned previous studies clearly show that a greater diversity of bacterial genera have the capacity to degrade butyric acid but they are yet to be identified.

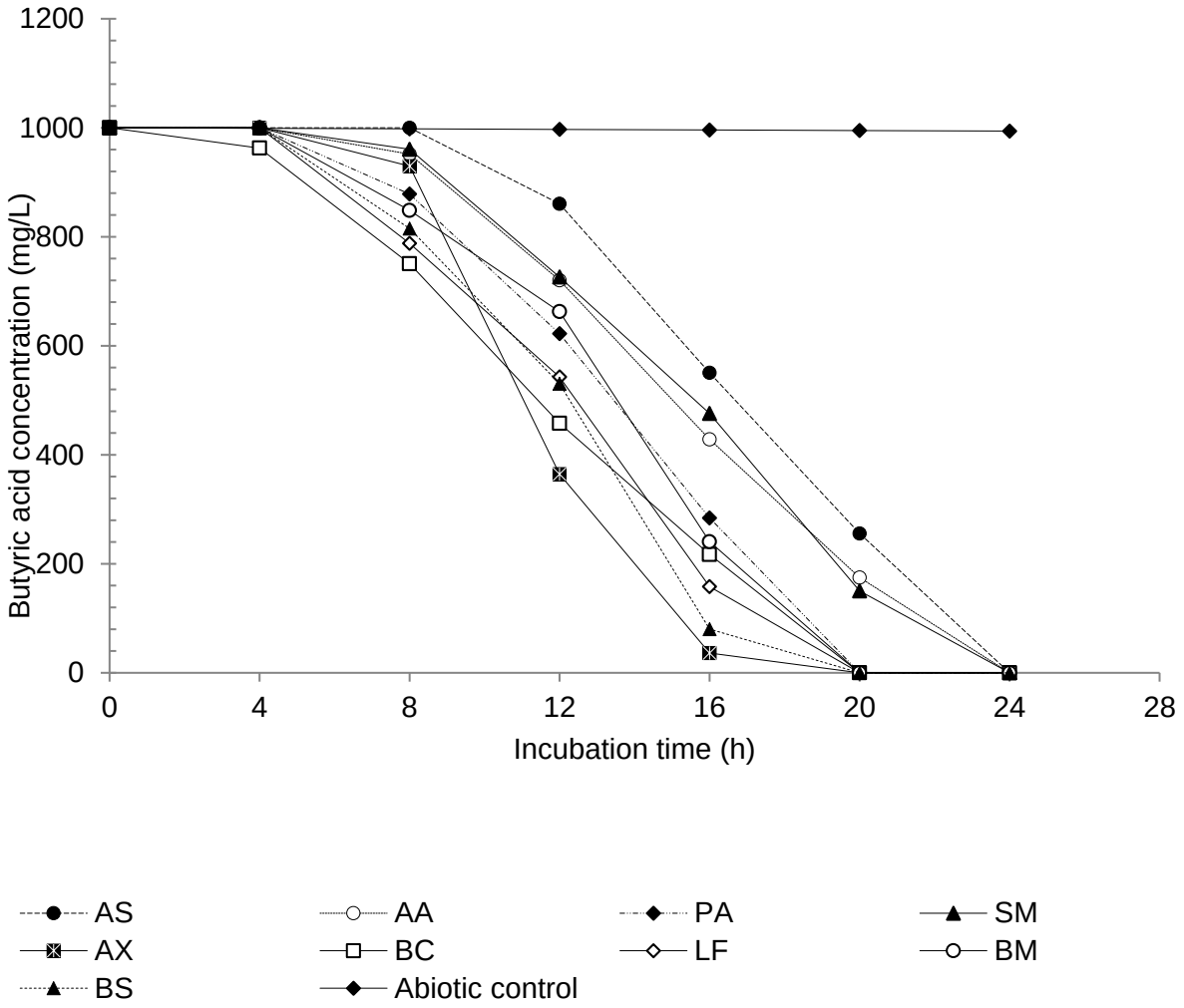

Figure 6-2: Butyric acid degradation kinetics by different bacterial isolates

The complete degradation of butyric acid in this work is important. This is primarily because even at low concentrations, butyric acid is one of the VFAs that has high odour nuisance index. Its odour can even create problems at a receptor of odour nuisance at distances far away from the points of emission. This is attributed to its very low odour detection threshold (Sheridan *et al*., 2003). Butyric acid is one of the SVFAs which infinitely dissolves in aqueous solution (Hughes,1934). Hence, the high degradation of butyric acid could be attributed to its high rates of dissolution and solubility in water which determines its bioavailability (Kristiansen *et al.,* 2011).

As shown in Figure 6.2, in the control experiments, the concentration of butyric acid remained almost stable from 1000 mg/L to 996.99 mg/L during the incubation for 24 h. The loss of butyric that resulted from abiotic process was insignificant. This could be attributed to either surface

volatilisation losses or photo-degradation due to exposure to light during sample withdrawals that was inevitable.

The butyric acid degradation and growth potential of the bacterial strains were investigated in detail. Although it was not known that these are their optimal growth conditions, all the strains showed remarkable ability to grow well at pH 7.0, 30 ^{o}C and agitation rate of 110 rpm and butyric acid concentrations 1000 mg/L provided as a sole source of carbon and energy with initial seed culture of 2.0. The increase in cell density of each bacterial strain as expressed by its absorbance value measured at 600 nm was positively correlated to degradation efficiency of butyric acid as illustrated in Figure 6.2. The Pearson correlation coefficients were in the range of 0.990 (*A.animicus*) to 0.999 (*L.fusiformis*) at $p < 0.01$. Bacterial cell density was increased with incubation time in all the bacterial strains, reaching the maximal density at different times that ranged from 0.990±0.01 to 1.25±0.004 within 20 to 24 h dependent on the bacterial strain as can be seen in Figure 6.3.

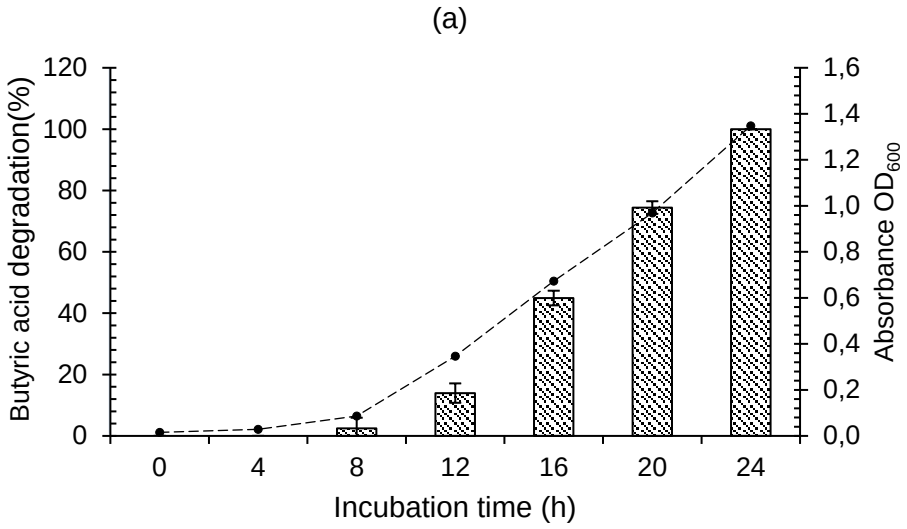

Figure 6-3a: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *Alcaligenes sp*. strain SY1

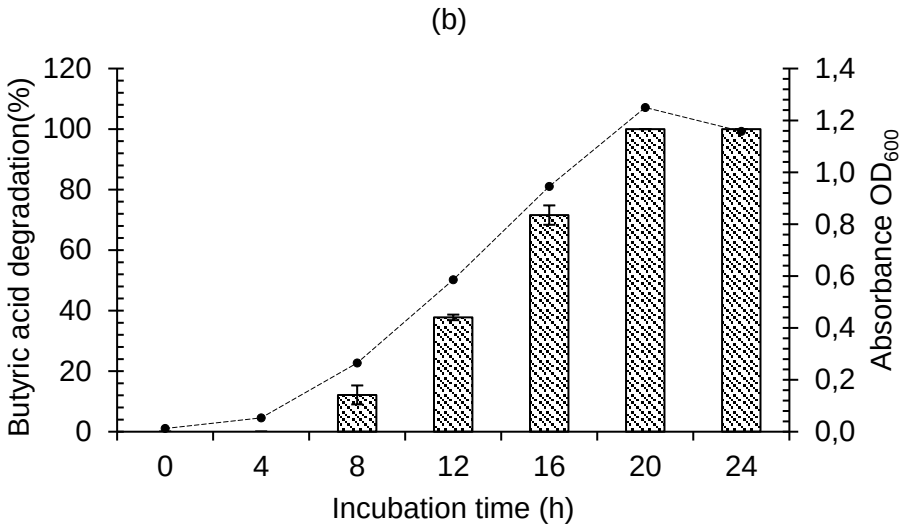

Figure 6-3b: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *P.aeruginosa*

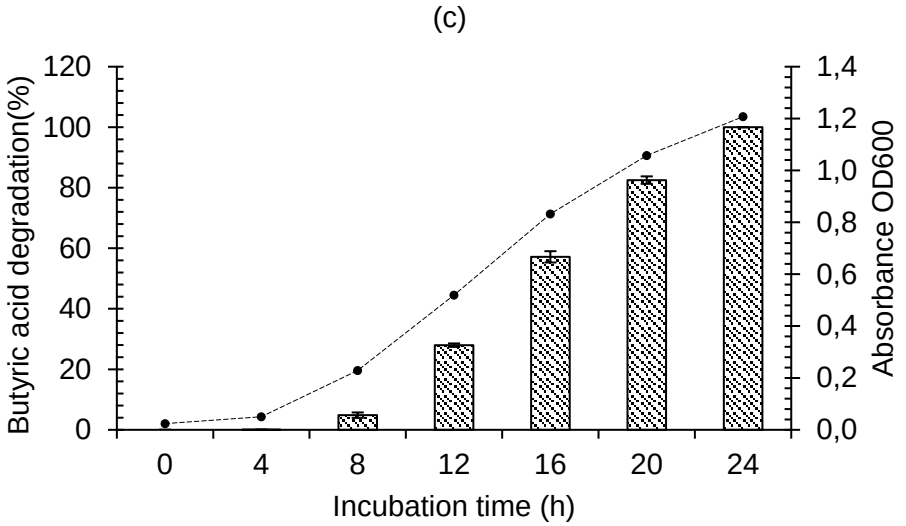

Figure 6-3c: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *A.animicus*

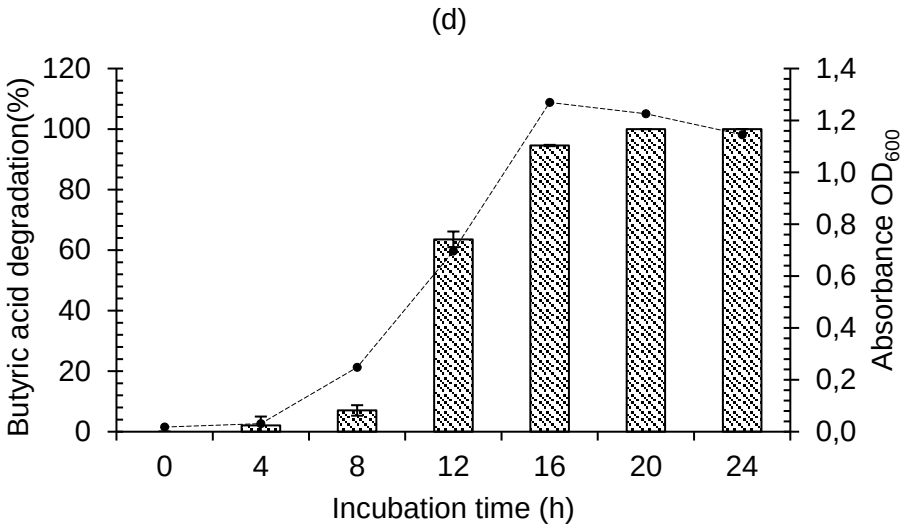

Figure 6-3d: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *A.xylosoxidans*

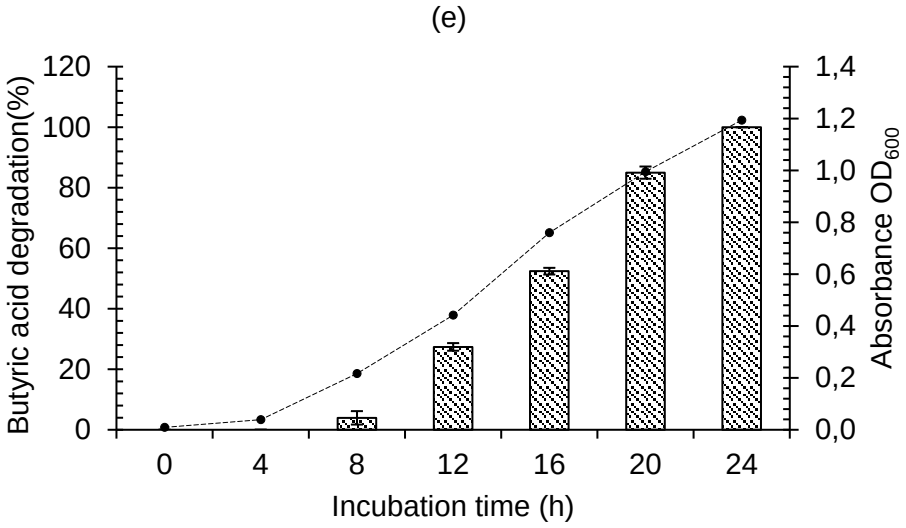

Figure 6-3e: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *S.marcescens*

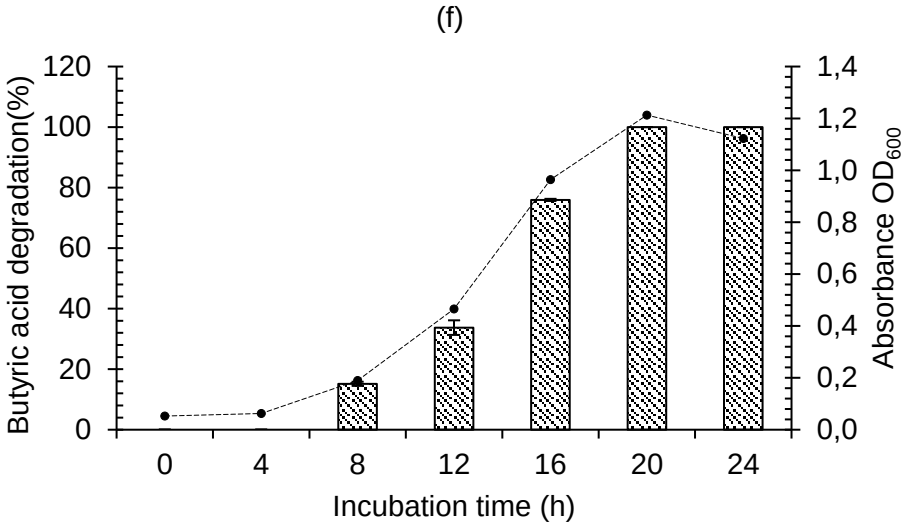

Figure 6-3f: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *B.methylotrophicus*

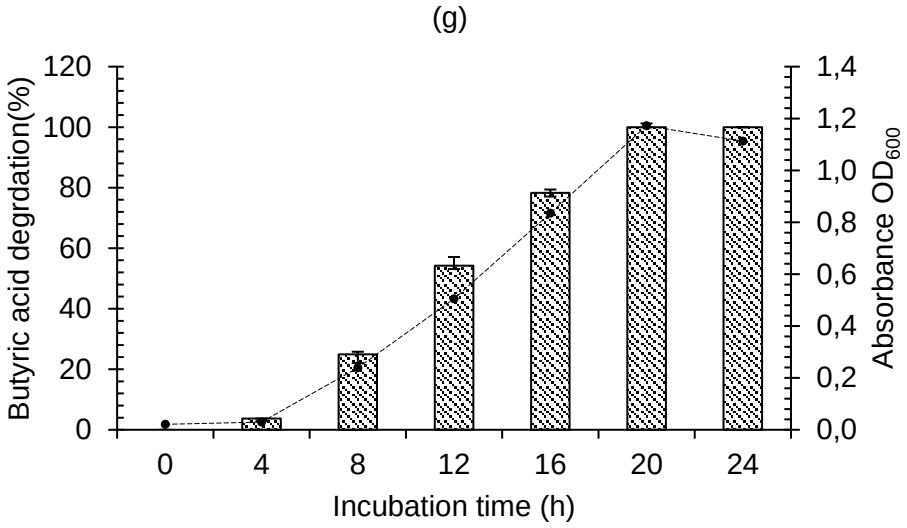

Figure 6-3g: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *B.cereus*:

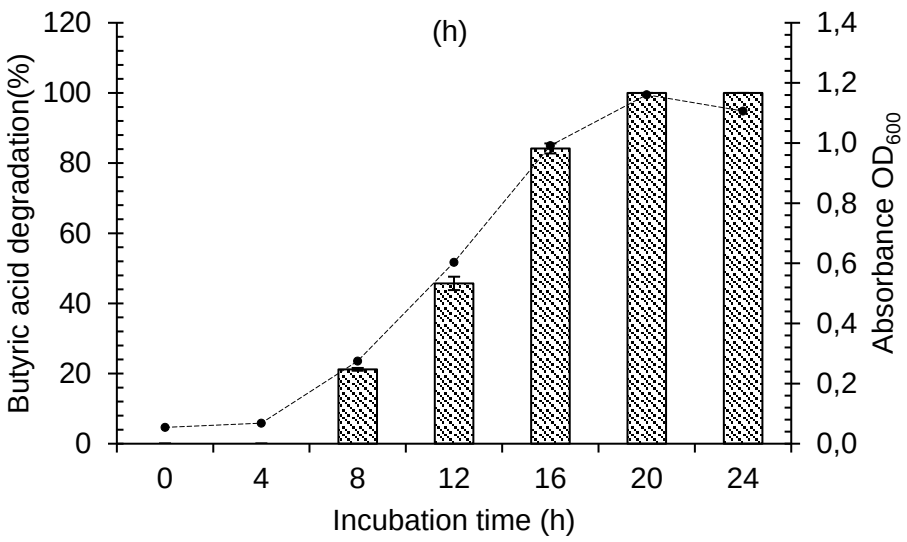

Figure 6-3h: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *L.fusiformis*

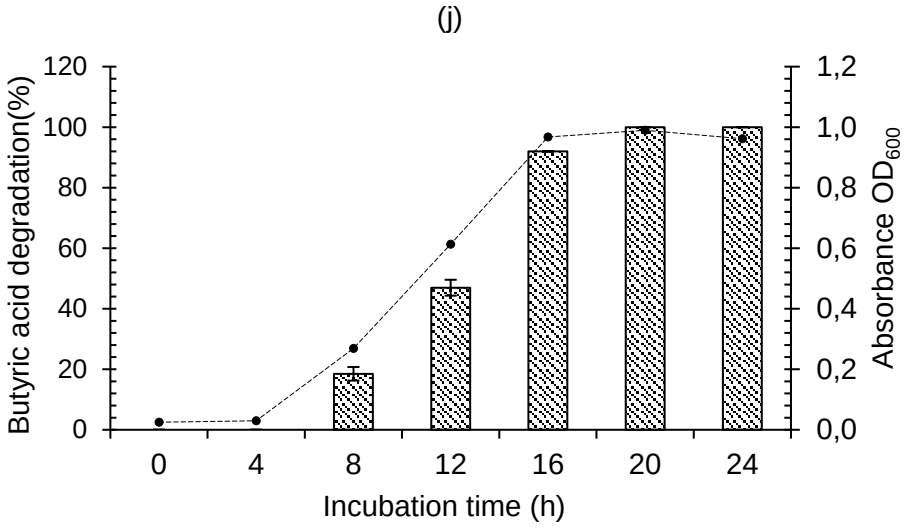

Figure 6-3i: Butyric acid degradation and bacterial growth under pH 7, 30 ^{O}C and 110 rpm against incubation time of *B.subtilis*

Butyric acid was degraded by all the bacteria strains as shown in Figure 6.2 and Figure 6.3. However, during the lag phase particularly 4 h after incubation, no bacterial strains but *A.xylosoxidans* and *B.cereus* manifested the degradation of butyric acid as determined by the HPLC. It is assumed that this lag phase allows the bacteria to adapt to the new environmental conditions required for bacterial cells to begin cell division (Baranyi *et al.*, 1993). Although there were variations in degradation efficiencies of butyric acid between bacterial strains during the duration of the lag phase, the high degradation efficiencies were observed in the

exponential phase of growth for all the bacterial strains. Thus, 95 to 100% of the butyric acid degradation occurred in this phase. Generally, there was a very high increase in butyric acid degradation efficiencies of the bacterial strains near the mid-exponential growth phase and decreased as the cultures aged towards the early stationary phase. This is consistent with Kotler *et al*. (1993) previous observations that bacterial cells in their exponential growth phase rapidly consume the available nutrients in most nutritionally defined media and then ceases to grow exponentially.

6.3 Summary

Butyric acid is one of the volatile organic compounds that significantly contribute to malodour emission from pit latrines. The purpose of this work was to isolate and identify bacterial strains that have the capability to degrade butyric acid and determine their butyric acid degradation efficiencies. Pure cultures of bacterial strains capable of degrading butyric acid were isolated from pit latrine feacal sludge using an enrichment technique and were identified based on 16S rRNA analysis. The bacterial strains were cultured in mineral salt medium (MSM) supplemented with 1000 mg/L butyric acid, as a sole carbon and energy source, at 30 ^{o}C, pH 7 and 110 rpm under aerobic growth conditions. Bacterial strains were phylogenetically identified as *Alcaligenes* sp. strain SY1, *A.animicus, P.aeruginosa, S.marcescens, A.xylosoxidans, B.cereus, L.fusiformis, B.methylotrophicus* and *B.subtilis*. The bacterial strains in pure cultures degraded butyric acid of 1000 mg/L within 20-24 h. This work highlights the potential for use of these bacterial strains in microbial degradation of butyric acid for deodourisation of pit latrine feacal sludge.

CHAPTER SEVEN

7 CONSTRUCTION OF AEROBIC BACTERIAL CONSORTIA FOR *IN VITRO* BIODEGRADATION OF BUTYRIC ACID

7.1 Background

The importance of reducing malodours in pro-poor sanitation technologies such as pit latrine has not received much attention, despite their widespread use with respect to developing countries. Most of the literature related to odour reduction in wastewater focusses on high-tech sanitation technologies. Bioremediation is the use of microorganisms to transform or mineralise hazardous organic materials to harmless or less hazardous compounds (Muller *et al*., 1991). In this process microorganisms obtain energy from oxidation of primary substrate i.e. carbon, which is converted to innocuous end products such as CO_2, H_2O, inorganic salts, some VOCs and microbial biomass by assimilating part of the carbon into new cell material (Nicolai and Janni, 2001).

Microorganisms are the main agents that play a significant role in degrading pollutants in the environment (Das and Chandran, 2011). Microorganisms capable of degrading malodourous compounds may be an attractive alternative to the existing odour control techniques and strategies currently used in low-income settings. Previous studies (Bourque *et al*., 1997; Yun and Ohta, 1997; Chin *et al*., 2010) have found that many bacterial strains can degrade butyric acid. However, despite increased attention internationally in bioremediation, there is limited information on the biodegradation of butyric acid in the environment, particularly in pit latrine feacal sludge. The importance of reducing malodours in pro-poor sanitation technologies suach as pit latrines has not received much attention, inspite of their widespread use with respect to developing countries. Most of the literature related to odour control in wastewater focus on high-tech sanitation technologies.

This study revealed that *A. xylosoxidans*, *B. subtilis*, *L. fusiformis, B.cereus*, *P.aeruginosa*, *B. methylotrophicus*, *S.marcescens*, *A.animicus* and *Alcaligenes* sp. strain SY1 strains

isolated from pit latrine feacal sludge samples could grow using butyric acid as a sole source of carbon and energy (Njalam'mano and Chirwa, 2019). However, bacteria in natural environments do not live in seclusion but they dynamically interact with many other bacterial strains in complex multispecies communities (Mukherjee and Bardoloi, 2011). Successful biodegradation of butyric acid may require the concerted efforts of the multispecies community working as bacterial consortia. However, the degradation capacity of a constructed bacterial consortium is not necessarily a simple direct summation of the degradation capacities of the individual constituent butyric acid degrading bacterial strains. The application of bacterial consortia has gained more attention in the recent years in the removal of other environmental pollutants other than butyric acid including organophosphate insecticide parathion (Gilbert et al., 2003), acid blue 113 (Nachiyar *et al*., 2012) and petroleum hydrocarbons (Xia *et al.,* 2019) among many others. This is because of competitive advantage that bacterial consortia have in that they can metabolise a wider range of environmental pollutants as compared to individual bacterial strains (Hamzah *et al*., 2013). In view of this background, this chapter focuses on the elucidation of the cooperative biodegradation activity of mixed bacterial population, which have been artificially constructed, in the biodegradation of butyric acid. For this to be achieved, the following objectives were derived;

(1) To evaluate and compare the butyric acid degradation efficiencies of the bacterial consortia formulated using different combinations and their respective individual bacterial strains, and

(2) To evaluate the effect of initial inoculation concentration, temperature and pH on the growth and butyric acid degradation efficiencies of the constituent bacterial strains of the most efficient bacterial consortium.

7.2 Results and discussion

Materials and methods used to achieve the objectives of this chapter are found in section 3.5.

7.2.1 Selection of the bacterial strains

Effective biodegradation of butyric acid entails the presence of an acclimatized microbial

population capable of degrading butyric acid. Bacterial isolates were consequently obtained from composite feacal sludge from the pit latrines that had butyric acid as one of the emitted compounds responsible for malodours. The detailed representative total ion chromatogram (TIC) of the feacal sample is shown in Figure 7.1.

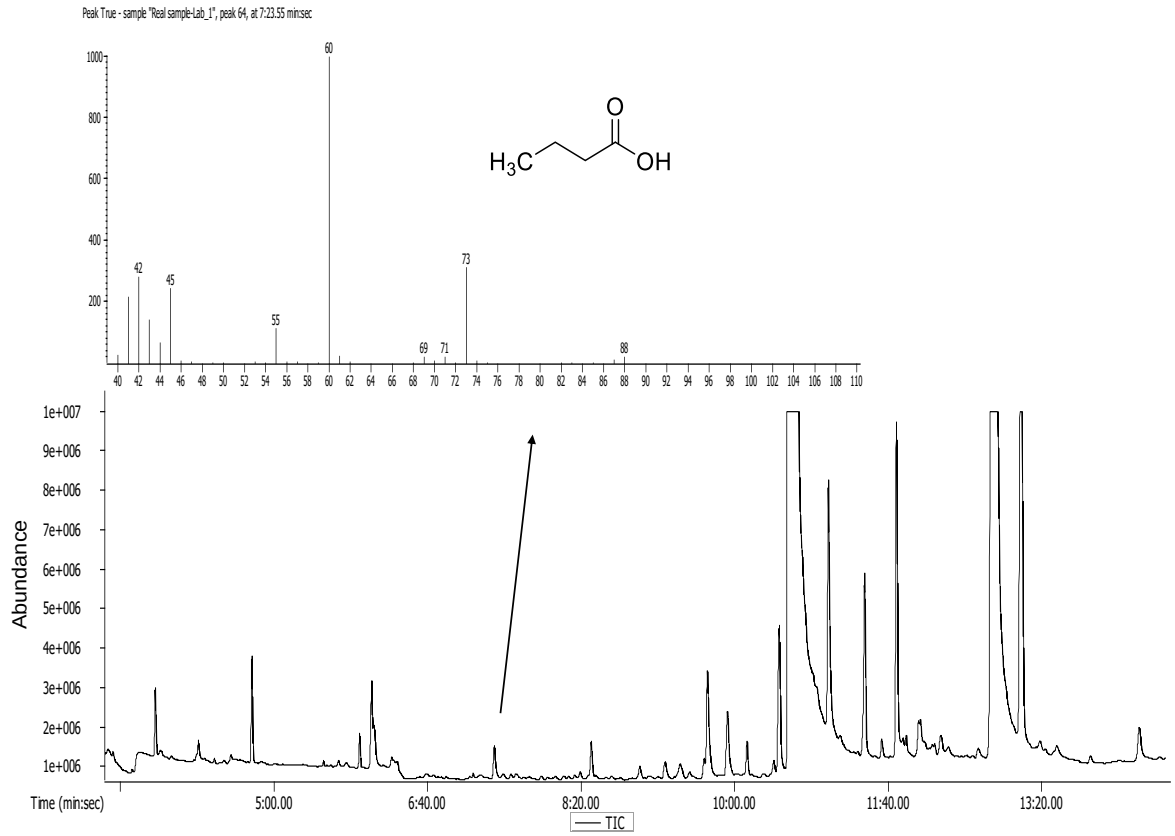

Figure 7-1: Detailed total ion chromatogram of feacal sludge sample

It is assumed that bacterial strains have better potential for the degradation of butyric acid in pit latrines when they are adapted to condition ofsimilar environments. In our previous work, nine bacterial strains were found to possess butyric acid degrading capabilities (Njalam'mano and Chirwa, 2019). However, in this work only six bacterial strains were selected. The butyric acid-degrading bacterial strains were phylogenetically identified based on the 16S rRNA gene sequencing are shown in Table 7.1.

The bacterial strains were selected because they are commonly associated with pit latrine feacal sludge in addition to their butyric acid degradation effieciencies. According to previous studies found in the literature,for instance, Rao *et al*. (2015), in their study on the estimation of nitrous oxide (N_2O) release from pit toilets in Mulbagal town, Karnataka, India revealed that

the microbial denitrification reaction was facilitated by *Pseudomonas* sp., *Serratia* sp., *B.cereus*, *B. subtilis* and *Achrobacter* sp. Similarly, Déportes *et al.* (1998) and Carrington (2001) have indicated that *Bacillus* sp, *Pseudomonas* sp, and *Serratia* sp were bacteria of epidemiological concern that are associated with feacal sludge. Thus, isolation of the indigenous bacterial strains that are acclimated to the local environmental conditions of pit latrine feacal sludge are vital for the microbial proficiency of butyric acid removal in the pit latrine or analogous environments (Wu *et al*., 2013).

Table 7-1: Bacterial strains selected for construction of bacterial consortia

No	Isolate designation	Closest hit	Accession No.	Homology (%)
1	Ba	*Alcaligenes* sp. strain SY1		99
2	B1a	*A.animicus* LMG26690^T	HE613448	99
3	B1b	*P.aeruginosa* LMG 1224^T	Z76651	100
4	B5a	*A.xylosoxidans* LMG 26686^T	FM999735	93
5	B6a	*S.marcescens* DMS 30121^T	AJ233431	100
6	B7a	*B.cereus* ATCC14579	AE016877	100

7.2.2 Butyric acid degradation by pure bacterial strains

To take the role as butyric acid attenuation agents, the bacterial strains ought to have the capacity to grow in an environment that contains a high concentration of butyric acid. Butyric acid degradation by individual pure bacterial strains was assessed at an initial butyric acid concentration of 1000 mg/L in a defined MSM. The choice of 1000 mg/L butyric acid concentration in the present study was based on butyric acid concentration used for experiments in Njalam'mano and Chirwa (2019). According to Lin *et al*. (2013), 90% of pit latrines surveyed in Durban, Nairobi, Kampala and Pune and the model toilet had butyric acid concentration between 46.2 and 1042 mg/L. The comparison of butyric acid degradation efficiencies by the individual bacterial strain cultures and their corresponding bacterial growth curves are shown in Figure 7. 2a and and their corresponding bacterial growth curves are shown in Figure 7.2b.

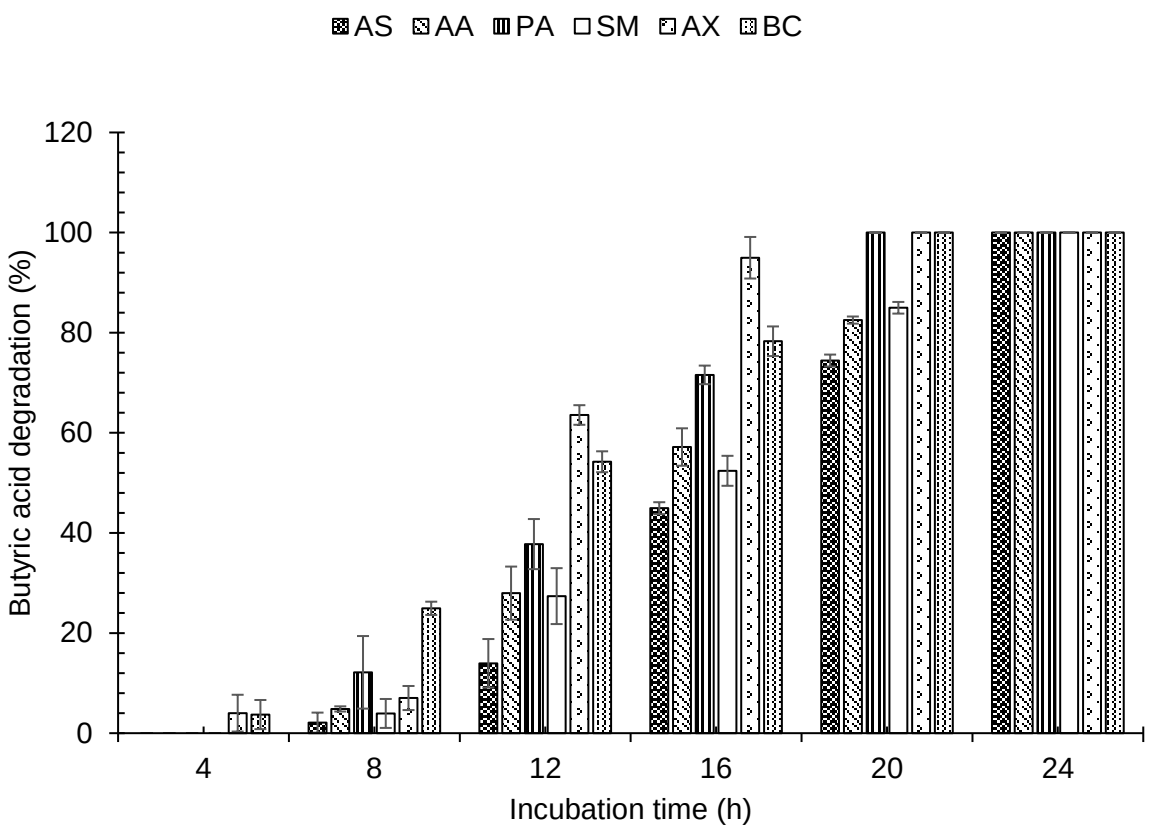

Figure 7-2a: Butyric acid degradation efficiencies by different bacterial strains

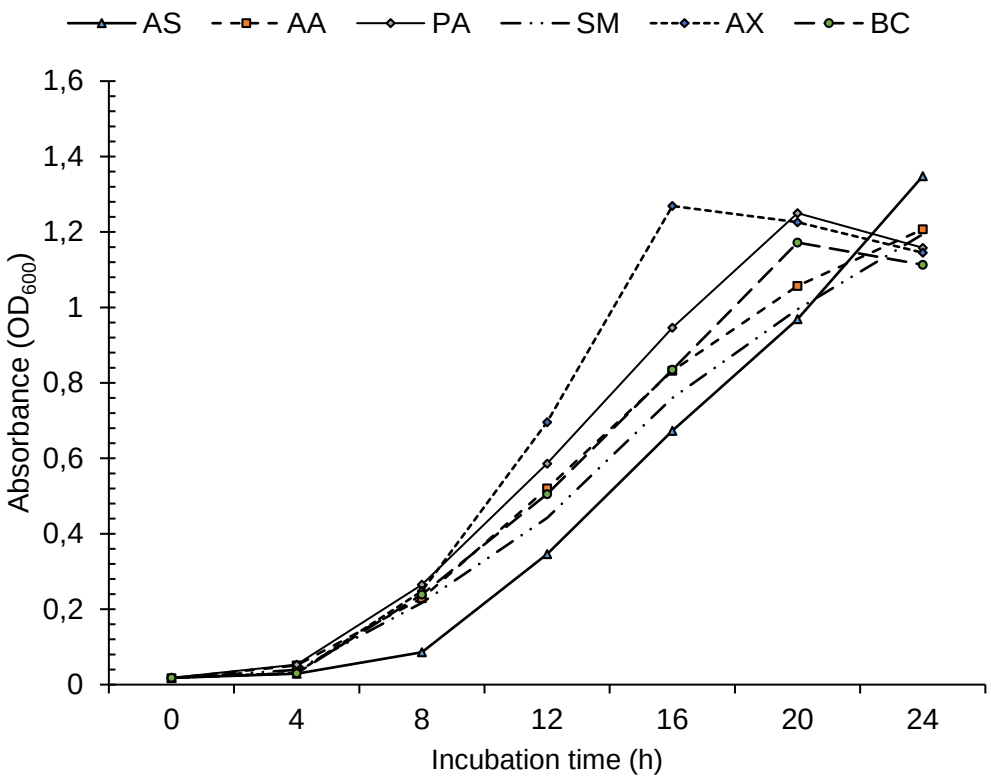

Figure 7-2b: Growth kinetics of different bacterial strains

The results show that butyric acid biodegradation occurred in each of the bacterial strains as measured by HPLC analysis and comparison to abiotic controls (samples without bacterial inoculum). The butyric acid degradation efficiency results reflect the relationship between bacterial growth and butyric acid degradation. In all the experiments, the removal of butyric acid was accompanied by a concomitant increase in bacterial growth even though the length of the lag phase varied between the bacterial strains. After 4 h the results showed that *A. xylosoxidans* degraded 4.0% of butyric acid and *B.cereus* degraded 3.7% of butyric acid when compared with the abiotic control. At the end of 8 h, *B.cereus, P.aeruginosa, A.xylosoxidans, A.animicus, S.marcescens* and *Alcaligenes* sp. strain SY1 degraded 24.9%, 12.16%, 7.03%, 4.84%, 3.94% and 2.10%, respectively, when compared with the abiotic control. The results suggest that the inoculum degradation efficiencies of butyric acid past 8 h incubation time were in the descending order as follows: *A.xylosoxidans* > *B.cereus* >*P.aeruginosa* >*S. marcescens* >*A.animicus* >*Alcaligenes* sp. strain SY1 compared to the abiotic control. However, it is also evident from Figure 7.1 that each bacterial strain showed variations in

butyric acid degradation efficiencies at different times. It was observed that the rates of degradation were high in the exponential phase of bacterial growth. Figure 7.2 (a) clearly shows that all three bacterial strains; *P.aeruginosa*, *A.xylosoxidans* and *B.cereus* could perform complete degradation of 1000 mg/L butyric acid within 20 h of incubation. Whilst bacterial strains; *Alcaligenes* sp. strain SY1, *A.animicus*, and *S.marcescens*, could degrade 1000 mg/L of butyric acid completely within 24 h of incubation. No butyric acid degradation was observed in the abiotic controls. Studies regarding bacteria degrading butyric acid have been reported in literature (Bourque *et al*., 1987; Yun and Ohta, 1997; Chin *et al*., 2010). Nevertheless, with the exception of the study by Chin *et al*. (2010), all these studies were performed with butyric acid as a sole source of carbon. To the best of our knowledge and after a thorough literature search none of the bacterial strains used in this work have been specifically reported as butyric acid degrading bacteria.

7.2.3 Degradation of butyric acid by bacterial consortia

Characteristically, the application of individual bacterial strains for biodegradation does not represent the real situation of environmental microorganisms during biodegradation of butyric acid in pit latrine feacal sludge. This is because in real environmental settings biodegradation relies on cooperative metabolic activities of mixed microbial populations. There are no studies in the literature regarding the construction of bacterial consortia from bacterial strains isolated directly from pit latrine feacal sludge for biodegradation of butyric acid. Nineteen different bacterial consortia that were constructed involving the selected bacterial strains in five different combinations are presented in Table 7.2.

The successful bacterial consortium was established based on the compatibility of the individual component bacterial strains of the consortium. Hence, there was an absence of any antagonism among constituent bacterial strains to concomitantly accomplish all the metabolic processes for enhanced degradation (Sarkar *et al*., 2013). From the 19 constructed bacterial consortia, the best performing bacterial consortia were selected based on comparatively higher butyric degradation efficiency in relation to the mean degradation efficiencies of the individual component bacterial strains studied after 16 h of incubation under the same

environmental conditions. The 16-h incubation time was chosen for comparison because some of the bacterial consortia had already achieved 100% butyric acid degradation within 16 h incubation.

Table 7-2: Computation of bacterial consortia using the six selected butyric acid-degrading bacteria

Bacterial strains group	Computed bacterial consortia using combinations	Consortium designation
Top two best bacterial strains in category 1 and best bacterial strains in category 2 [AX, SM, BC]	[AX, SM] [AX, BC] [SM, BC]	C1 C2 C3
Top two best bacterial strains in category 1 and top two best bacterial strains in category 2 [AX, SM, BC, AA]	[AX, SM, BC] [AX, SM, AA] [AX, BC, AA] [SM, BC, AA]	C4 C5 C6 C7
All three bacterial strains in category 1 and top two best bacterial strains in category 2 [AX, SM, BC, AA, PA]	[AX, SM, BC, AA] [AX, SM, BC, PA] [AX, SM, AA, PA] [AX, BC, AA, PA] [SM, BC, AA, PA]	C8 C9 C10 C11 C12
All three bacterial strains in category 1 and all three bacterial strains in category 2 [AX, SM, BC, AA, PA, AS]	[AX, SM, BC, AA, PA] [AX, SM, BC, AA, AS] [AX, SM, BC, PA, AS] [AS, SM, BC, PA, AA] [AX, BC, AA, PA, AS] [SM, AX, AA, PA, AS]	C13 C14 C15 C16 C17 C18
All six bacterial strains	[AX, SM, BC, AA, PA, AS]	C19

Butyric acid degradation as examined after 16 h of incubation as measured by HPLC analysis is shown in Figure 7.3. The butyric acid degrading bacteria in all the samples achieved the degradation efficiencies in the range of 55.6 to 100% after 16 h of incubation as monitored by the HPLC. The concentration of butyric acid in all the treatments varied throughout the incubation period monitored as can be seen in Figure 7.2 a. Samples inoculated with bacterial consortia, C1, C2 and C3 achieved 100% butyric acid degradation within 16 h incubation. However, the bacterial consortium, C3, (combination of *S.marcescens* and *B.cereus*) had the highest butyric acid degradation efficiencies at all sampling times (4h, 8h, and 12h). This wascompared to the other bacterial consortia that degraded butyric acid completely within 16 h.. Moreover, the butyric acid degradation efficiency of consortium, C3 was higher than the individual butyric acid degradation efficiencies of *S.marcescens* and *B.cereus* of 52.4% and 78.3%, respectively and the difference from the mean degradation efficiencies of the two individual constituent bacterial strains was much higher compared to that of C1 and C2.

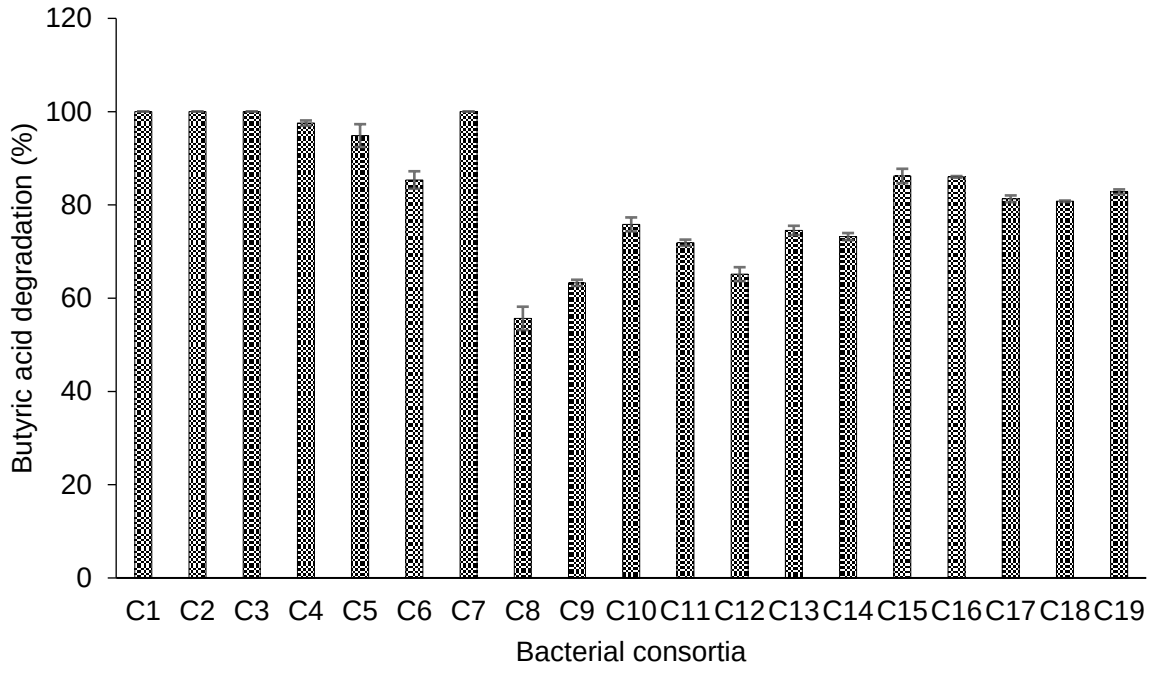

Figure 7-3: Butyric acid degradation by different constructed bacterial consortia after 16 h of incubation at pH 7, 30 °C and 110 rpm

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, The phylogenetic trees of *S.marcescens* and *B.cereus* showing the closest NCBI (BLASTn) relatives based on the 16S rRNA gene sequence are shown in Figure 7.4. Substantial degradation efficiencies of butyric acid of 86.0%, 99.8% and 97.6% were also achieved in the samples inoculated with bacterial consortia, C4, C7 and C16, respectively.The butyric acid degradation efficiencies of C7 and C16 are the same.This was also higher compared to the butyric acid degradation efficiencies of the individual component bacterial strains. It was apparent that the biodegradation of butyric acid by these consortia were more effective as they outperformed the individual component bacterial strains of the consortia. The results suggest that bacterial synergism may be indispensable for butyric acid degradation in the pit latrine feacal sludge where the bacterial strains were isolated. It is widely recognised in the field of microbiology that coordinated bacterial consortia have the potential to be more productive, robust and effective to environmental fluctuations than individual pure bacterial cultures (Brenner *et al*., 2008). This is undoubtedly because of the concerted activities of the individual component bacterial strains of the consortium. The interspecific interactions within the constructed bacterial consortia that coxswained to improve degradation ability were not determined.

Table 7-3: Degradation efficiencies of individual bacterial strains and their bacterial consortia

Degradation efficiencies of individual bacterial strains (%)						Mean efficiency (%)	Consortium	Degradation efficiencies of consortia (%)
AX	SM	BC	AA	PA	AS			
94.97	52.41	-	-	-	-	73.69	C1	100
94.97	-	78.29	-	-	-	86.63	C2	100
-	52.41	78.29	-	-	-	65.35	C3	100
94.97	52.41	78.29	-	-	-	75.22	C4	97.56
94.97	52.41	-	57.16	-	-	68.18	C5	94.86
94.97	-	78.29	57.16	-	-	76.81	C6	85.33
-	52.41	78.29	57.16	-	-	62.62	C7	99.89
94.97	52.41	78.29	57.16	-	-	70.71	C8	55.66
94.97	52.41	78.29	-	71.57	-	74.31	C9	63.28
94.97	52.41	-	57.16	71.57	-	69.03	C10	75.82
94.97	-	78.29	57.16	71.57	-	76.24	C11	71.89
-	52.41	78.29	57.16	71.57	-	64.85	C12	65.15
94.97	52.41	78.29	57.16	71.57	-	70.88	C13	74.53
94.97	52.41	78.29	57.16	-	44.94	65.55	C14	73.21
94.97	52.41	78.29	-	71.57	44.94	68.44	C15	86.21
-	52.41	78.29	57.16	71.57	44.94	60.87	C16	86.01
94.97	-	78.29	57.16	71.57	44.94	69.39	C17	81.31
94.97	52.41	-	57.16	71.57	44.94	64.21	C18	80.79
94.97	52.41	78.29	57.16	71.57	44.94	67.39	C19	82.82

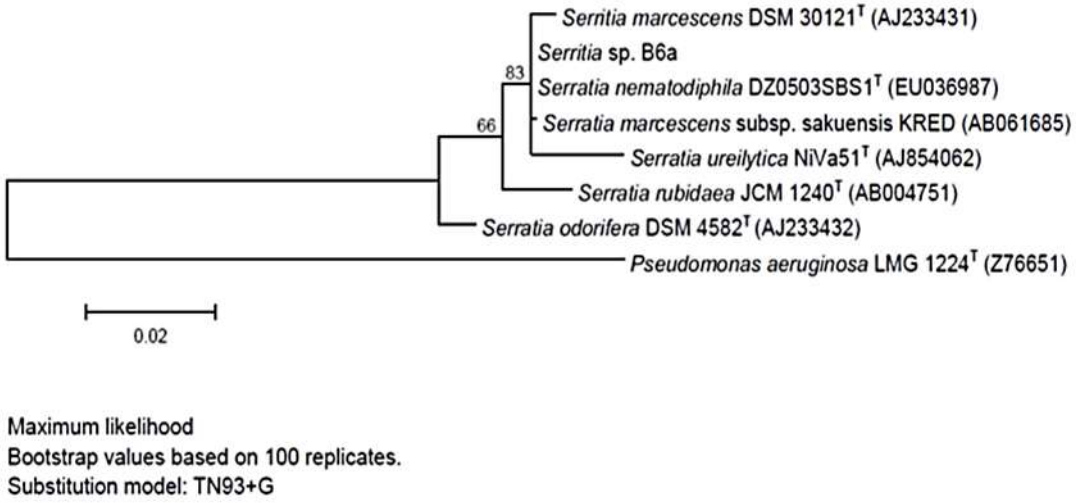

Figure 7-4a: Phylogenetic tree for *S.marcescens* and related strains based on 16s rRNA gene sequences. Bootstrap values were based on 100 replicates

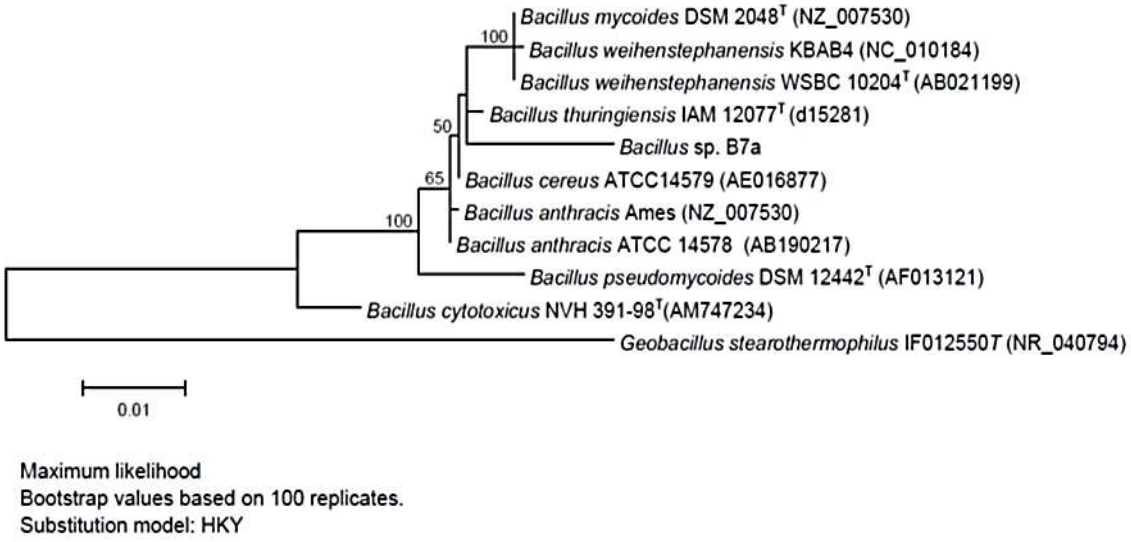

Figure 7-4b: Phylogenetic tree for *B.cereus* and related strains based on 16s rRNA gene sequences. Bootstrap values were based on 100 replicates

However, Deng and Wang (2016) have postulated numerous mechanisms that promote synergetic interactions between constituent members of the degradative mixed communities in nature, but in the present study, two possible mechanisms may be offered at this point based on previous reports *inter alia*:

(i) metabolic and physiological inadequacies of one bacterial strain in the consortium are compensated for by the presence of other bacterial strains in the consortium with the appropriate complementary physiology, which are able to provide the appropriate metabolic benefit to all bacterial strains involved (Dejonghe *et al.,* 2003).

(ii) associated metabolism, wherein one bacterial strain in the consortium take up the intermediates of the metabolic pathway released during the degradation of butyric acid. The intermediates may be toxic and may hinder the metabolic activities, thus appears to protect the other constituents of the bacterial consortium from toxicity that would otherwise accrue from the accumulation of the metabolites (Ghazali *et al*., 2004).

Several studies of biodegradation potency of bacterial consortia exhibiting similar results have been reported earlier. Thus, for instance, a defined consortium of indigenous *Pseudomonas* sp and actinobacteria offered a synergetic activity for effective PAHs removal capabilities when compared to their pure cultures (Isaac *et al.,* 2015). A mixed bacterial consortium described by Sathishkumar *et al.* (2008) in which *Bacillus* sp. IOS17, *Corynobacterium* sp. BPS2-6, *Pseudomonas* sp. HPS2-5 and *Pseudomonas* sp. BPS1-8 incubated together showed superior growth and degradation of crude oil to individual bacterial strains. Saratale *et al*. (2010) reported evidently higher degradation and decolorization efficiency for a mixture of reactive dyes by a bacterial consortium of *Proteus vulgaris* and *Micrococcus glutamicus* compared to the use of individual bacterial strains. Similarly, Tizntzun-Camacho *et al*. (2012) found low hexadecane degradation efficiencies by pure cultures of *Xanthomonas* sp., *Acinobacter bouvetti* and *Defluvibacter lusatiensis*, which noticeably enhanced (79±3%) when such bacterial strains were grown together. The concerted metabolic potential of the mixed cultures to degrade butyric acid has also been reported by, Kristiansen *et al.* (2011), wherein uncharacterised bacterial strains only identified as members of phyla; *Microbacterium, Gordonia, Acetobacteria, Rhodococus, Propionibacteria, Janibacter, Alpha-, Beta-,* and *Gamma proteobacteria* were used. The results showed that up to 70% reduction of organic acids, including butyric acid, in the presence of other odourous compounds in a full-scale biological air filter treating air from a pig facility. Similarly, Sheridan *et al.* (2003) used a mixed

aerobic microbial culture consisting of two fungi and five bacterial strains of phylum, *Gamma*-Proteobacteria identified as members of genera; *Moraxella, Enterobacter* and *Pseudomonas*, which were isolated from under a diesel storage tank, for degradation of butyric acid from waste exhaust air.

Other bacterial consortia; C5, C6, C8, C9, C10, C11, C12, C13, C14, C15, C17, C18 and C19 exhibited lower degradation efficiencies compared to the degradation efficiencies of at least one of the individual component bacterial strains that made up each of them as shown in SI Table 1. The possible explanation could be that bacterial strains were engaged in competition for a pool of limited available resources such as space, dissolved oxygen and nutrients. This is very common for constituent bacterial strains with analogous nutritional requirements, within the consortium (Hibbing *et al.,* 2010; Foster and Bell, 2012). Studies that have demonstrated that the combined efforts of consortia may not always have a synergetic effect for all the substrates are also found in the literature. Kumar and Phillip (2006) reported that degradation of endosulfan was performed potently better in monocultures of three bacterial strains, *Staphylococcus* sp, *Bacillus circulans*-I, and *Bacillus circulans*-II than by them in a consortium. Guo et al. (2005) observed similar results, which demonstrated that the isolate of *Paracoccus sp* was more efficient in the degradation of pyrene than that of the mixed cultures.

7.2.4 Environmental factors affecting the growth of and butyric acid biodegradation by bacterial consortium, C3

Effective biodegradation can only be achieved when environmental conditions are favourable for microorganisms' metabolic activities (Matsumura *et al.,* 1989). In the present study, the factors such as; temperature, pH and inoculum size were considered for each of the bacterial strains of bacterial consortium, C3, to elucidate how they can affect the accomplishment of the butyric acid biodegradation process.

7.2.4.1 Effect of incubation temperature

In microbiology, it is well established that biological processes such as aerobic metabolism and growth are known to exhibit environmental temperature dependence (Schulte, 2015).

Temperature influences the rates of enzymatically catalysed reactions and the diffusion rate of the substrate to the cell (Torondel, 2010). Both *S.marcescens* and *B.cereus* degraded significant quantities of butyric acid at incubation temperatures (25, 30, 35 and 40 °C) at 16 h of incubation as shown in Figure 7.5. The results show that the growth and butyric acid degradation of both *S. marcescens* and *B.cereus* were optimal at an incubation temperature of 30 °C. It was observed that there was a slight gradual decrease in the degradation efficiencies of butyric acid when the incubation temperature decreased from 30 to 25 °C.

The degradation efficiencies of *S.marcescens* and *B.cereus* decreased from 72.41 to 70.42 % and 78.29 to 66.37%, respectively, at 16 h of incubation. This could be due to reduced catalytic capacity at lower temperatures (Schulte, 2015). However, the bacterial growth and butyric acid degradation efficiencies decreased when the incubation temperature increased by the same 5 °C with comparatively higher decreased degradation efficiencies at 16 h of incubation. This suggests the bacterial strains were much less sensitive to low temperatures than high temperatures. There was a drastic decrease in bacterial growth and butyric acid degradation efficiencies at 16 h of incubation with an increase of incubation temperature from 40 to 45 °C. This could be attributed to denaturation of proteins at high temperatures. This is because with a further rise in temperature, the components with heat sensitivity such as enzymes, which are secreted outside the cell into the surrounding medium to perform metabolic processes, are irreversibly denatured and growth rates drop quickly and cause inhibition and then mortality (Kaleli and Islam, 1997).

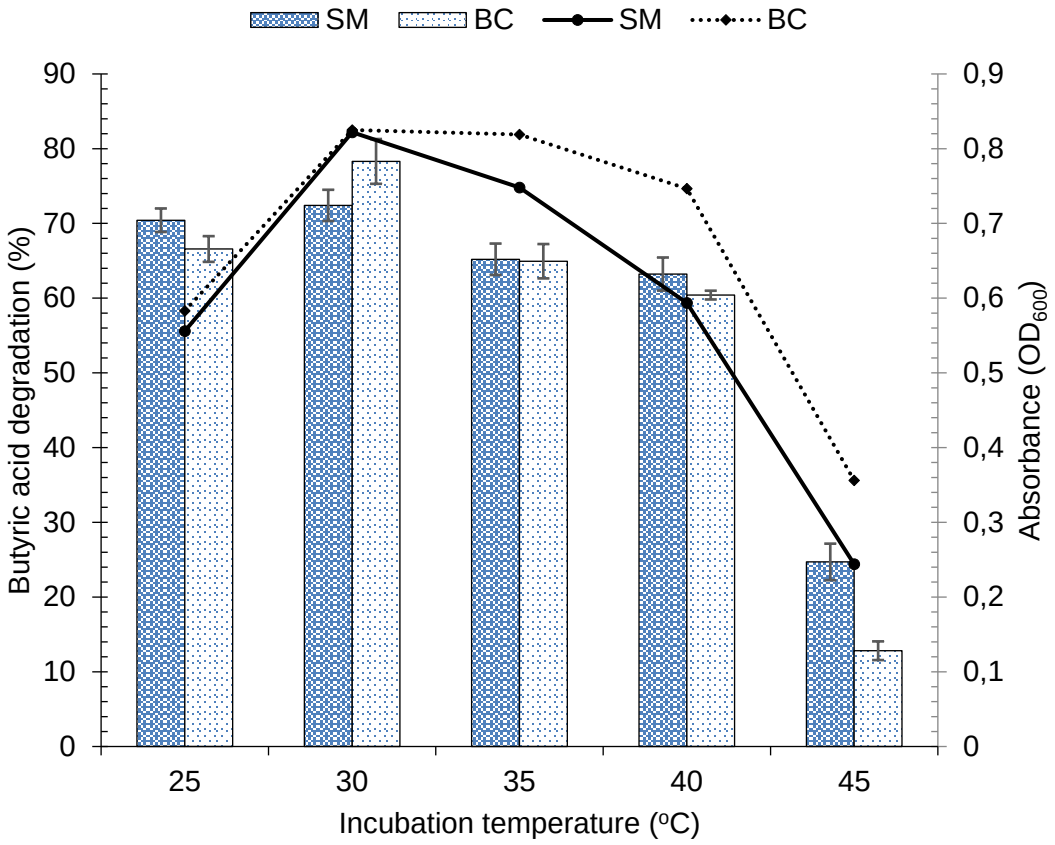

Figure 7-5: Bacterial growth and degradation of butyric acid after 16 h of inoculation by *S. marcescens* and *B.cereus* at different incubation temperatures: Butyric acid degradation; bar graph: bacterial growth; line graph

Furthermore, with increasing temperature, the solubility of oxygen is decreased in aqueous phase, and as a result, the metabolic activity of aerobic microbes is reduced (Ghosal *et al*., 2016). This is supported by the previous report that temperatures higher than the organism's optimum temperature range causes cell death, which is fast, while lower temperatures still result in cell death rate, which is slower (Sumitha, 2014). The complete degradation of butyric acid was observed in the inoculated flasks incubated at 30 °C at 16 h of incubation. These results are in accordance with the work of Chin *et al.* (2010) who reported that a temperature of 30 °C was an optimal incubation temperature for degradation of the butyric acid by *Acinetobacter calcoaceticus* at pH of 7 under aerobic conditions. Literature available regarding temperature values inside pit latrines is limited. However, a study by Sherpa *et al*.

(2009) found that the temperature of feacal sludge sampled from urine-diverting dehydrating toilets with ash as a primary additive in Kathmandu Valley, Nepal was in a range of 19.5-32.8°C. Similarly, Nabateesa *et al*. (2017) investigated the temperature of feacal sludge inside pit latrines in Kampala, Uganda. The temperature was found to be in a range of 22.3-30.7°C with an overall mean of 25.4°C with higher temperatures in the top layer and decreasing with depth. According to Nwaneri *et al*. (2008), aerobic processes inside pit latrines occur in the top layers of feacal sludge portions of pit contents. Therefore, the temperature and the aerobic nature of feacal sludge in the top layer of pit contents can favourably support the metabolic activities of the bacterial strains since it provides the mesophilic temperature range at which both *S.marcescens* and *B.cereus* optimally grow and degrade butyric acid.

7.2.4.2 Effect of initial pH of the medium

pH is another important parameter for microbes and different species prefer different pH values. Environmental pH has a strong effect on their cell metabolism and growth. Figure 7.6 indicates the effect of medium pH on the bacterial growth and butyric acid degradation rates over an initial medium pH of 5 to 10

The pH range was carefully chosen to mimic the range of pH values found in pit latrine feacal sludge in the range of environmental settings according to previous studies (Rose et al., 2015; Zuma et al., 2015; Nabateesa et al., 2017). However, the pH values of feacal sludge are more complex since they are influenced by numerous factors (Zuma et al., 2015).

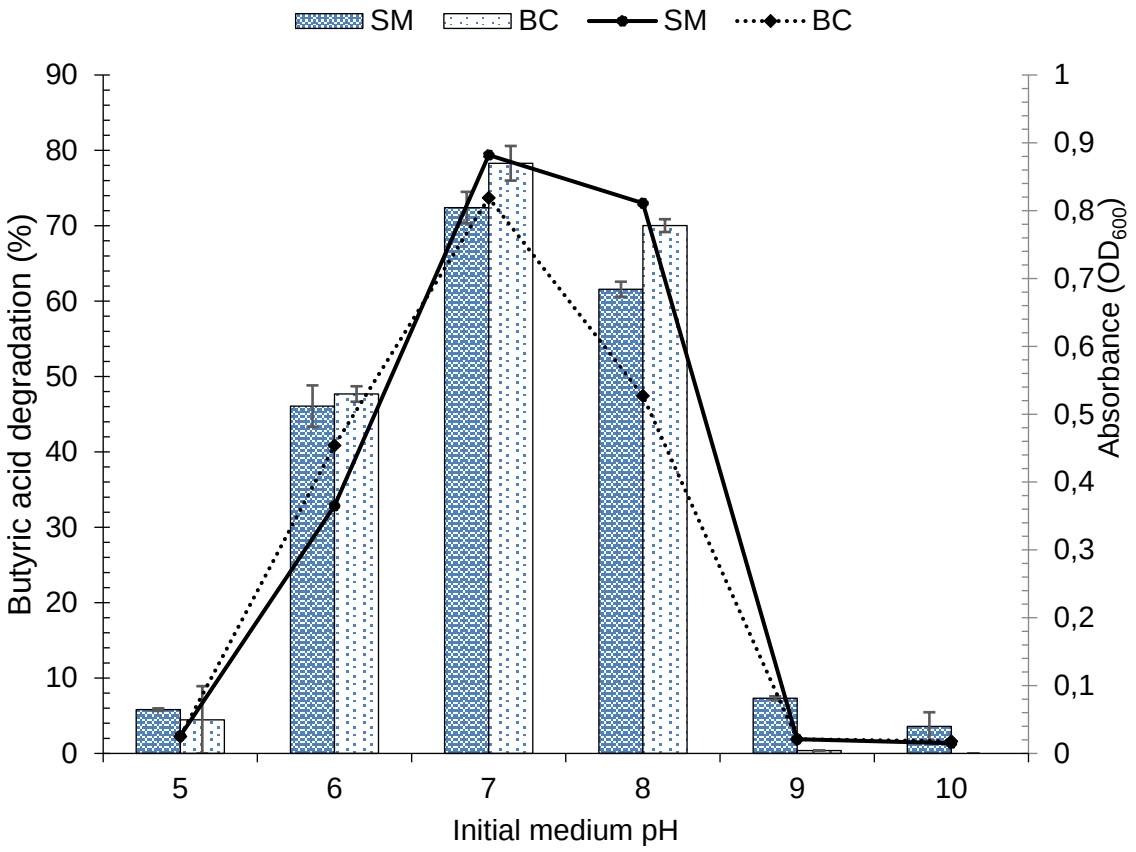

Figure 7-6: Bacterial growth and degradation of butyric acid after 16 h of inoculation by *S.marcescens* and *B.cereus* at different initial medium pH: Butyric acid degradation; bar graph: bacterial growth; line graph

In samples inoculated with *S.marcescens* or *B.cereus*, substantial bacterial growth and butyric acid degradation were observed at an initial pH of 6 to 8 at 16 h of incubation. An increase in the initial pH from 6 to 8 significantly increased the bacterial growth of both bacterial strains and butyric acid degradation. In the range of initial pH investigated the highest bacterial growth and butyric acid degradation for both *B.cereus* and *S.marcescens* were achieved at an initial medium pH of 7 at 16 h of incubation. This suggests that the butyric acid-degrading enzymes have their optimal enzymatic activity in neutral surroundings implying that the bacterial strains are neutrophiles. Coincidentally, the optimal degradation of butyric acid as the sole carbon source inoculated with *Acinetobacter calcoaceticus, Burkholdeira cepacia* and *Wautersia paucula* was accomplished at pH 7.0 (Chin *et al*., 2010).

The pH values outside the range of 6 to 8, the bacterial strains exhibited a characteristic sensitivity to pH that inhibits bacterial growth and butyric acid degradation. It is, however, noteworthy to mention that the bacterial strains might have mechanisms to modify the pH of the medium. It was noted that the pH of the culture medium with initial pH values in acidic condition increased with incubation time and shifted towards the optimal pH. On the other hand, the pH of the culture medium with initial pH values of extreme alkaline condition decreased with incubation time and shifted towards the optimal pH (data not shown). The increasing and lowering of pH of the culture medium could be due to the production of organic acids and ammonia, respectively, as metabolic products (Chin *et al*., 2010; Ratze and Gore, 2018). However, further research is required to understand the mechanisms that the bacterial strains employ to modify the extremes of medium initial pH.

7.2.4.3 Effect of initial inoculum size

To ascertain the effect of initial inoculation size on the degradation of butyric acid and bacterial growth the initial inoculum sizes were varied from 0.5 to 2.5. Only 1 mL in a volume of cell suspension prepared with these optical densities was used. This means that different inoculum sizes affected the initial population of bacteria in the medium. As shown in Figure 7.7, an increase in initial inoculum sizes of *B.cereus* from 0.5 to 2.0 after 16 h of incubation the bacterial growth increased marginally, and the butyric acid degradation efficiencies varied but not significantly.

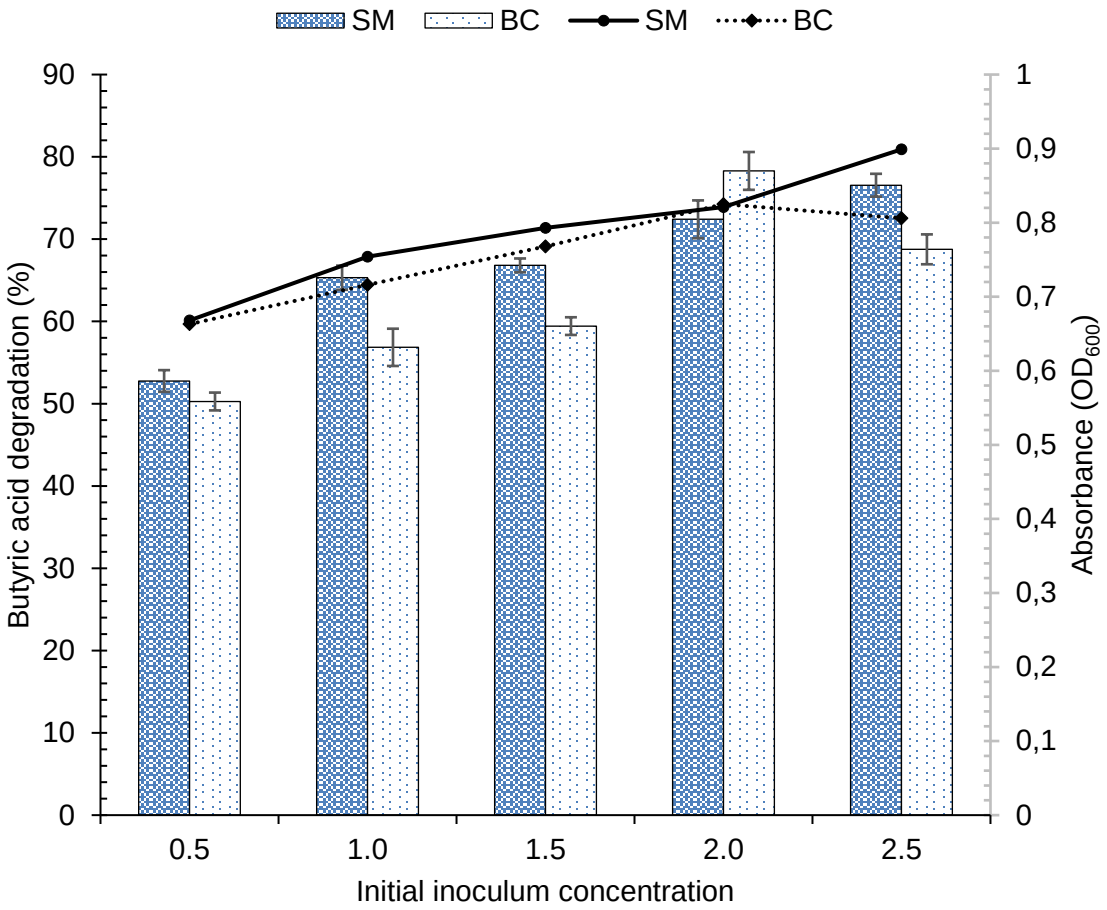

Figure 7-7: Bacterial growth and degradation of butyric acid after 16 h of inoculation by *S.marcescens* and *B.cereus* with different initial inoculum sizes: Butyric acid degradation; bar graph: bacterial growth; line graph

The optimal degradation efficiency was reached at 2.0. However, with an increase in inoculation concentration above 2.0 there was a decrease in butyric acid degradation as well as bacterial growth. The decrease in degradation efficiency with further increase in initial inoculum size is not a new phenomenon. Increasing inoculum size of *Bacillus thuringiensis* did not result into enhanced dimethyl phthalate (DMP) degradation (Surhio *et al*., 2014). This is because bacterial population rise also intensifies the bacterial competition for such resources as substrates, oxygen, space etc and, therefore, restricts bacterial growth when these resources are depleted in the medium (Jensen *et al*., 2014).

The results show that an increase in inoculum size from 0.5 to 1.5 did not reduce the lag phase

to help the batch system to attain the exponential growth phase rapidly to attain significant butyric acid degradation. This could be because the initial densities of the bacteria were not sufficiently large to ensure quick proliferation and biomass synthesis in the cultivation (Zaidi *et al*., 1996) While for *S.marcescens*, the optimum degradation was attained with inoculum size of 2.5. For economic and comparison purposes the inoculum size was not increased. The increase in initial inoculum size of *S.marcescens* resulted in increased butyric acid degradation and bacterial growth. Bildan and Monomania (2002) reported that the aerobic degradation of dichlorodiphenyltrichloroethane (DDT) by *S.marcescens* DT-1P increased with an increase in inoculum size in liquid culture. The differences between the two bacterial strains could be attributed to the fact that dissimilar bacterial strains have different population sizes that can do rapid and complete butyric acid degradation.

7.3 Summary

This chapter focused on the development of efficient bacterial consortia to biodegrade butyric acid, one of the odour-causing compounds that contribute significantly to pit latrine malodours. A laboratory study was undertaken to determine the biodegradation of butyric acid by pure bacterial strains. Six bacterial strains isolated from pit latrine feacal sludge *A.xylosoxidans*, *B.cereus*, *P.aeruginosa*, *S.marcescens*, *A.animicus* and *Alcaligenes* sp. strain SY1 were selected for the study based on their efficiency of butyric acid utilization. Nineteen bacterial consortia of different combinations were prepared using the above bacterial strains for degradation studies. The individual bacterial strains and bacterial consortia were compared by culturing in mineral salt medium (MSM) supplemented with 1000 mg/L butyric acid, as a sole carbon and energy source, at pH 7, 30 °C and 110 rpm under aerobic conditions. Some bacterial consortia showed better degradation than the individual bacterial strains. The study has shown that the effectiveness of the constructed bacterial consortia to enhance butyric acid degradation is not simply a result of the adding together of the individual component bacterial strains' degradation capacities of the consortium. A co-culture of *S. marcescens* and *B.cereus* was selected as the most efficient consortium compared to individual constituent bacterial strains and other bacterial consortia in which 1000 mg/L butyric acid in liquid culture

was completely degraded within 16 h of incubation. This may be the first instance in which 1000 mg/L of butyric acid degradation has been achieved in a short incubation time of 16 h. Temperature of 30 °C and pH 7 were found to be optimum for maximum degradation for both *S.marcescens* and *B.cereus*. The inoculation sizes of 2.0 and 2.5 were optimal for maximum degradation for *B.cereus* and *S. marcescens*, respectively. Although *in vitro* studies may not accurately reflect butyric acid biodegradation occurring *in situ,* this study suggests that the biodegradation process studied here has potential application for attenuation of butyric acid related malodours emanating from pit latrine. These results might lead to the development of better deodorization technologies. This work is of international value, as it will contribute to knowledge and progress on the bioremediation of odours emitted from pit latrine feacal sludge, hence leading to improved sanitation uptake in developing countries.

CHAPTER EIGHT

8 MODELLING GROWTH KINETICS OF MESOPHILIC AEROBIC BUTYRIC ACID DEODOURANT BACTERIA UNDER DIFFERENT SUB-OPTIMAL ISOTHERMAL CONDITIONS

8.1 Background

The intrinsic environmental variables such as pH, temperature and water activity have capacity to stimulate or retard bacterial growth. In microbiology, it is well known that temperature is a cardinal abiotic factor that influences microbial growth and biological reactions (Ratkowsky *et al*., 1982; Torondel, *et al*., 2010). Coincidentally, temperature is a factor that can greatly vary within pit latrines (Torondel *et al*., 2016; Nabateesa *et al*., 2017). Temperature is difficult to manipulate and in the pit latrine, butyric acid degradation will be limited by seasonal changes in temperature. In addition, this has an effect of the butyric acid biodegradation efficiency of the identified bacterial strains. Hence, optimisation of the likelihood and extent of butyric acid biodegradation in pit latrines requires a major advance in understanding of the bacterial growth dynamics consequences of environmental temperature. The resultant knowledge of growth kinetics of *B.cereus and S.marcescens* in the liquid medium supplemented with butyric acid would lead to better understanding of their survivability and multiplication under various natural pit latrine thermal conditions.

The most common means of bacterial growth assessment can be carrying out experiments either *in vivo* or *in vitro*, of which it is laborious and costly. Predictive modelling is a field of research in microbiology has received considerable interest as a tool for potential growth prediction of particular microorganisms under a variety of environmental conditions based upon the premise that the microbial reactions to environmental variables are reproducible

(Pla *et al*., 2015). Besides, this is the method that can be used to obtain reliable first estimates of microbial growth, survival and various critical temperatures, which is is rapid and inexpensive (Fernandez *et al*., 1997). In predictive microbiology, several mathematical models, which vary greatly in theory and complexity, have been developed to estimate the growth of microorganisms in different media and under different conditions. However, currently, to the best of our knowledge, comprehensive studies on the growth kinetics modelling of *B.cereus and S.marcescens* during butyric acid biodegradation under different isothermal conditions have not been reported and this information is necessary for their potential growth assessment.

The objective of this study was to explore the effect of temperature on the growth of two bacterial species of interest in butyric acid deodourisation: *B.cereus and S.marcescens*. For this purpose, the growth curves of *B.cereus and S.marcescens* individually were obtained using the modified logistic, Gompertz and Richards models. The performance of the models was compared to select the best-fit model using statistical tools and information theory criteria (ITC). The selected best-fit model was applied to estimate the parameters of microbiological relevance such as asymptotic value, lag-phase duration and maximum specific growth rate, which can be used to predict bacterial population dynamics with respect to temperature and butyric acid as a sole source of carbon.

8.2 Results and discussion

Materials and methods used to achieve the objectives of this chapter are found in section 3.6. The growth kinetics of *B.cereus* and *Serattia marcescens* grown as pure cultures in liquid medium with 1000 mg/L of butyric acid as a sole source of carbon were assessed. The study of bacterialgrowth kinetics as butyric acid biodeodourants through predictive modelling is not found in the published literature.

8.2.1 Primary model and curve fitting

In the current study, three non-linear equations of logistic, Gompertz and Richards were fitted to the experimental data obtained under four and five different isothermal conditions

for *B. cereus* and *S. marcessens,* respectively, to describe their growth**.** These models are numerically easier to handle as opposed to mechanistic models (Thakur 1991), for instance, the Monod and Michaelis-Menten based models that are preferred for systems to be scaled-up consistently These equations have been long recognised not as mere equations but as models that describe bacterial growth**.** The two bacterial species were selected for this study because their co-culture had collectively achieved the best butyric acid degradation efficiency as compared to all other bacterial consortia (Njalam'mano *et al.*, 2020). The growth data for both bacterial species were obtained until the stationary phase was reached. However, due to some technical challenges encountered with the orbital incubator such as failure to maintain incubation temperatures below 25 ^{o}C the effect of incubation temperature throughout the entire biokenetic range could not be evaluated. Hence, the incubation temperatures of 25, 30, 35, 40 and 45 ^{o}C were considered for this study. Additionally, turbidimetric technique (i.e. OD) was used to monitor the microbial growth, therefore, it could have been difficult to measure the OD due to water condensation and cells aggregation under 20 ^{o}C as observed by Fermanian *et al.* (1994). According to experimental observations, *B. cereus* could not grow at the incubation temperature of 45 ^{o}C. Instead, a rhythmic bacterial growth was observed. This rhythmic growth suggests that the bacterial species was only able to survive without significant growth at this incubation temperature. On this premise as recommended by Buchanan and Phillips (1990), in order to achieve a better fit of the models with the experimental data, the experimental data obtained at 45 °C for *B. cereus* were excluded for consideration in this study. However, the isothermal conditions between 25 and 40 ^{o}C permitted the apparent growth of both *B. cereus* and *S. marcessens.* Graphically, as can be seen in Figures 8.1 (a) to (i) and Appendix 4, all the three models described most of the experimental data sufficiently to each individual growth curve for both *B. cereus* and *S. marcessens* under isothermal conditions used in this study.

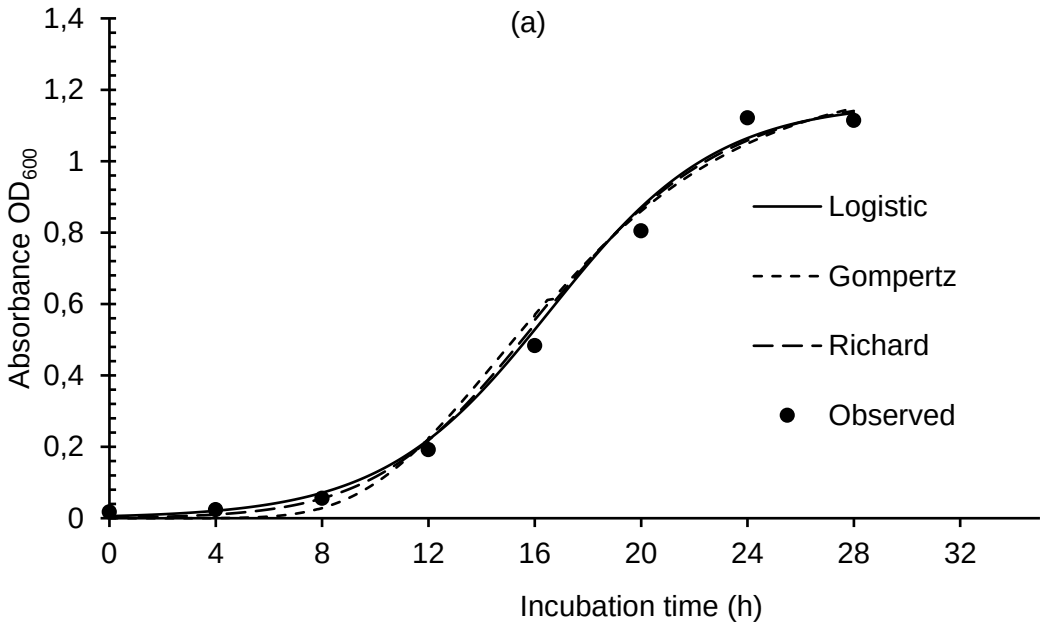

Figure 8-1a: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *B.cereus* growing at 25 °C

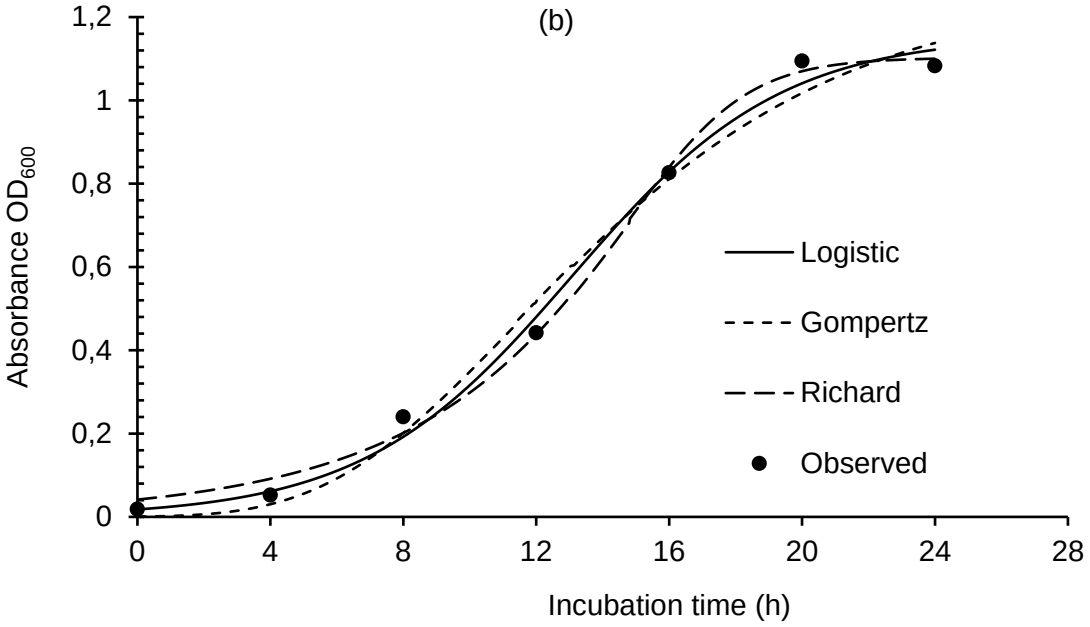

Figure 8-1b: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of B.cereus growing at 30 °C

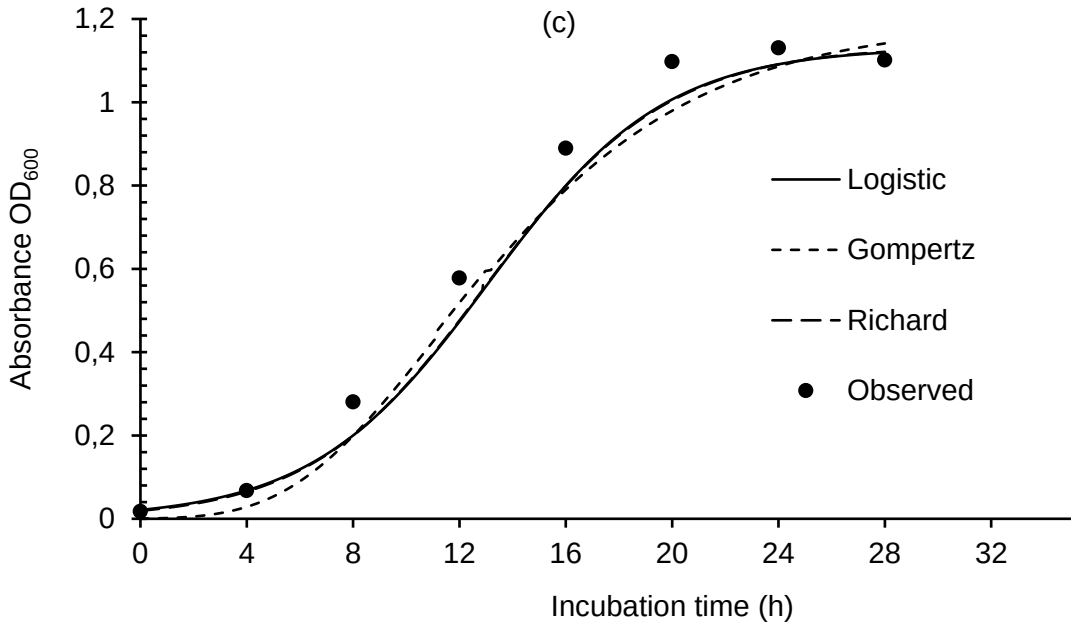

Figure 8-1c: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *B.cereus* growing at 35 ºC

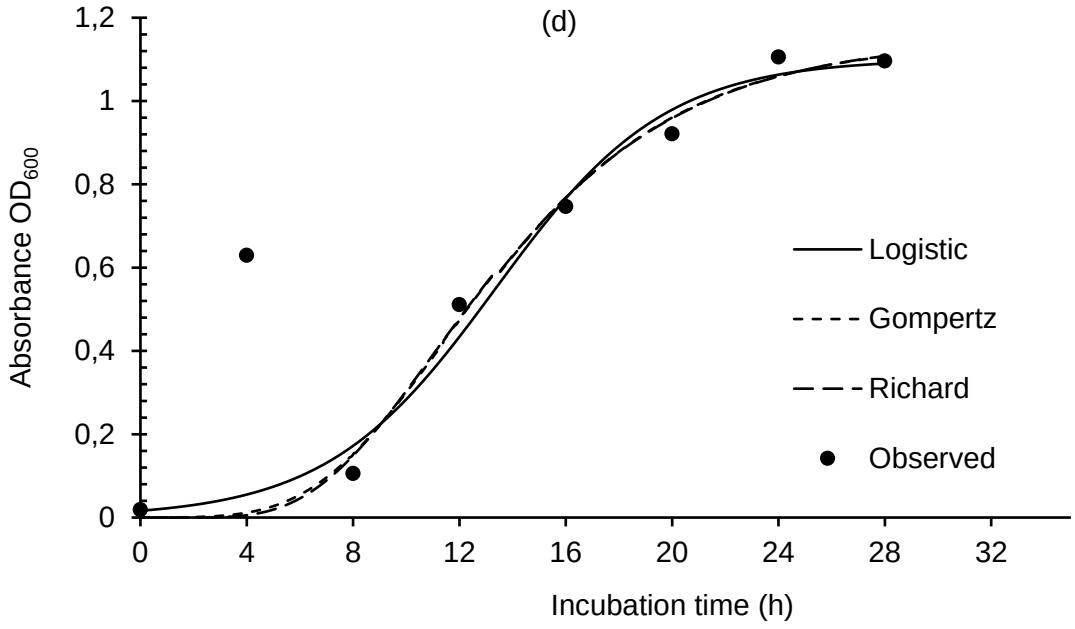

Figure 8-1d: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *B.cereus* growing at 40 ºC

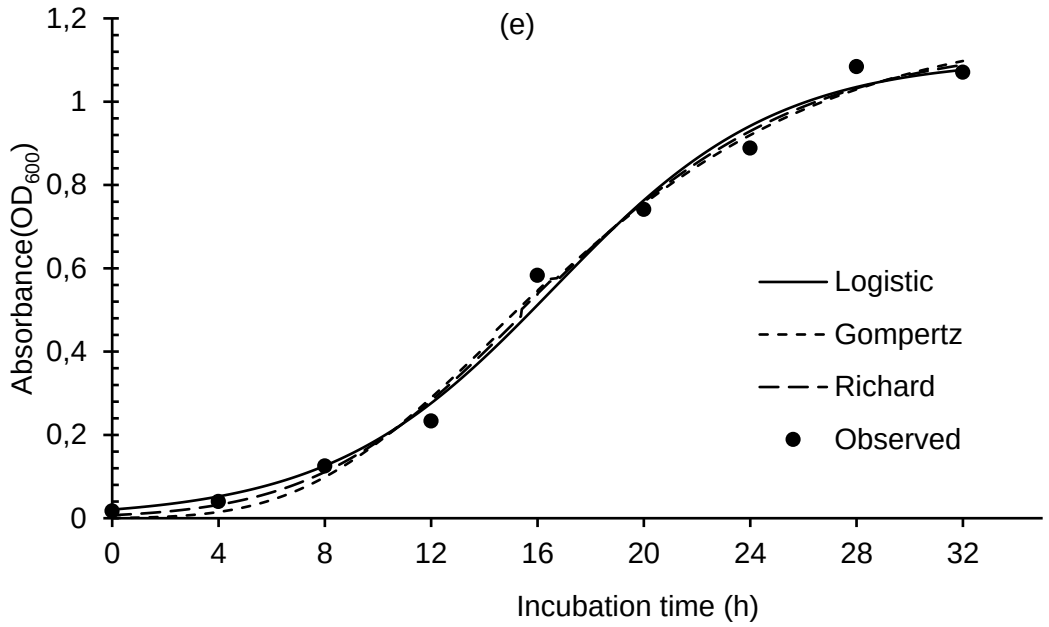

Figure 8-1e: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *Serattia marcescens* growing at 25 °C

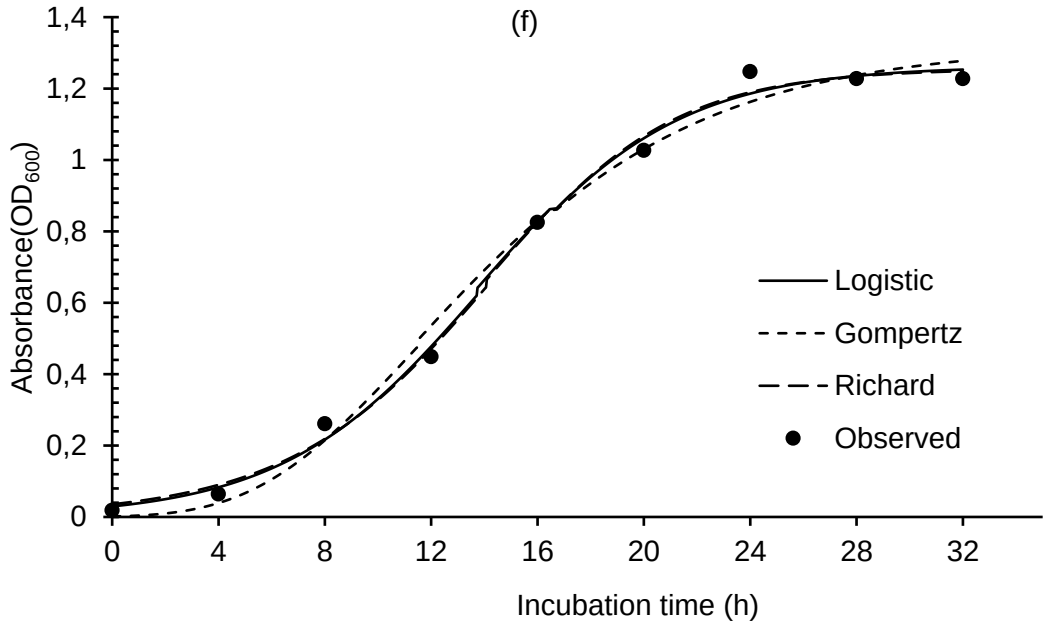

Figure 8-1f: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *S.marcescens* growing at 30 °C

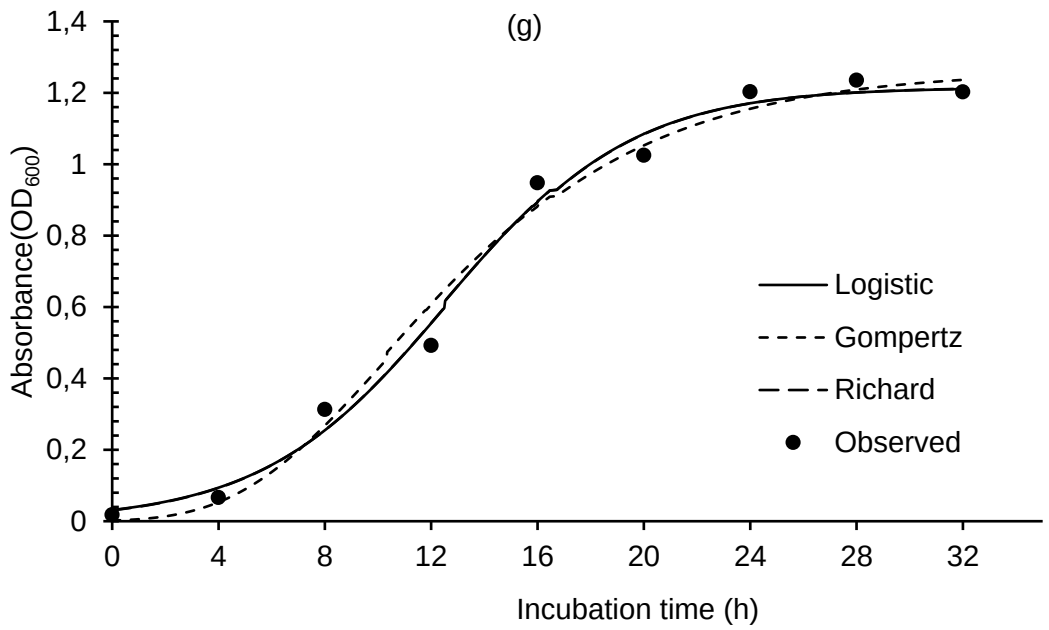

Figure 8-1g: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *S.marcescens* growing at 35 °C

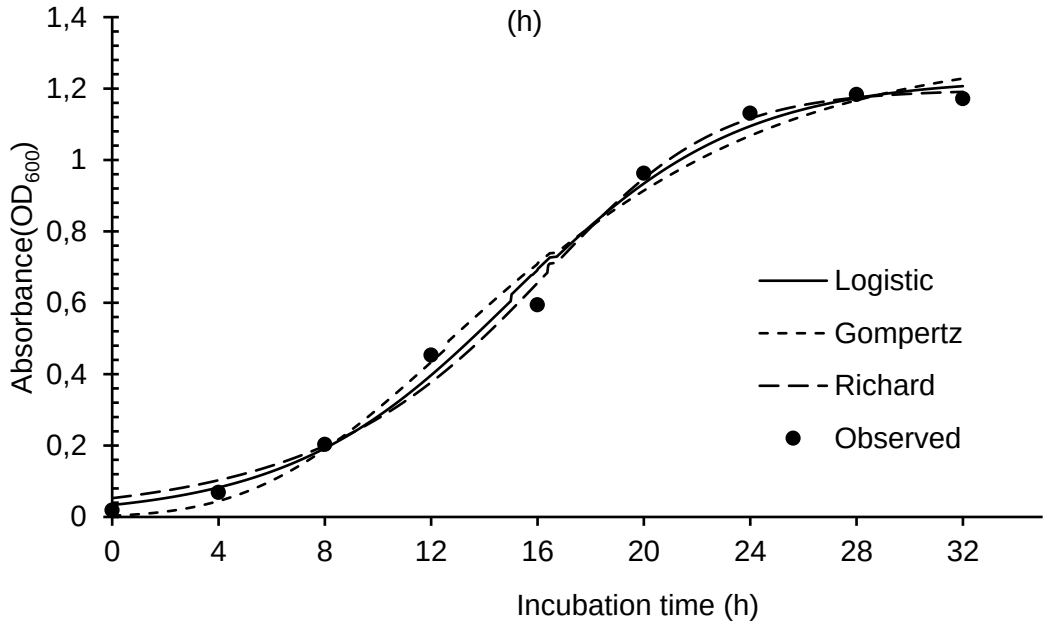

Figure 8-1h: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *S.marcescens* growing at 40 °C

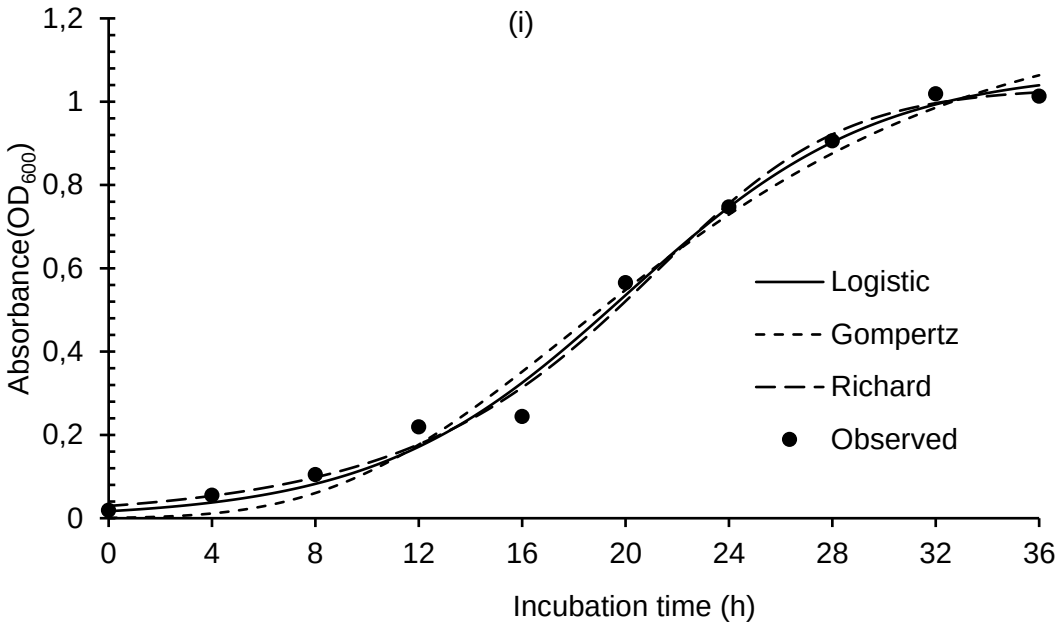

Figure 8-1i: Fitting of the logistic, Gompertz and Richards models to bacterial concentration of *S.marcescens* growing at 45 °C

Based on a modified logistic model Gompertz model, Richards model expressed according to the equations (3.5) (3.6) and (3.7), respectively, mathematical parameters, *a, b, c* and *v* for bacterial growth were predicted as shown in Table 8.1. and appendix 4.

Table 8-1: Mathematical parameter estimates for the different growth models in *S.marcescens* and *B.cereus* at different isothermal conditions (standard errors are in parentheses)

Model	Strain	Parameter	Temperature (°C)				
			25°C	30°C	35°C	40°C	45°C
Logistic	*B.cereus*	*a*	1.162 (0.026)	1.138 (0.055)	1.101 (0.020)	1.081 (0.047)	-
		b	5.684 (0.008)	4.602 (0.003)	5.457 (0.014)	4.489 (0.034)	-
		c	0.344 (0.027)	0.365 (0.060)	0.521 (0.055)	0.339 (0.057)	-
Gompertz		*a*	1.127 (0.089)	1.216 (0.127)	1.112 (0.023)	1.137 (0.087)	-

		b	3.117 (0.017)	2.298 (0.052)	3.450 (0.010)	2.345 (0.038)	-
		c	0.210 (0.034)	0.209 (0.059)	0.371 (0.042)	0.204 (0.050)	-
Richards		*a*	1.045 (0.029)	1.090 (0.026)	1.105 (0.025)	1.056 (0.049)	-
		b	7.375 (0.069)	16.038 (1.037)	4.764 (0.165)	6.852 (0.475)	-
		c	0.431 (0.109)	1.087 (0.837)	0.467 (0.165)	0.487 (0.299)	-
		v	2.612 (0.700)	6.285 (4.793)	1.705 (0.868)	2.962 (1.950)	-
Logistic	*S.marcescens*	*a*	1.104 (0.047)	1.263 (0.029)	1.216 (0.036)	1.229 (0.044)	1.078 (0.046)
		b	3.954 (0.020)	3.730 (0.009)	3.630 (0.018)	3.576 (0.016)	4.141 (0.017)
		c	0.141 (0.002)	0.166 (0.023)	0.178 (0.028)	0.139 (0.024)	0.110 (0.022)
Gompertz		*a*	1.195 (0.076)	1.322 (0.058)	1.263 (0.056)	1.315 (0.088)	1.220 (0.129)
		b	2.040 (0.015)	1.921 (0.012)	1.866 (0.016)	1.777 (0.019)	1.981 (0.031)
		c	0.141 (0.021)	0.166 (0.023)	0.178 (0.028)	0.139 (0.024)	0.110 (0.023)
Richards		*a*	1.147 (0.095)	1.255 (0.037)	1.216 (0.047)	1.196 (0.047)	1.034 (0.129)
		b	2.769 (0.129)	4.182 (0.091)	3.577 (0.164)	5.704 (0.202)	6.476 (0.163)
		c	0.179 (0.075)	0.295 (0.084)	0.285 (0.109)	0.346 (0.144)	0.304 (0.121)
		v	1.393 (0.702)	2.235 (0.733)	1.972 (0.922)	3.058 (1.335)	3.018 (1.209)

Also statistically, both *B. cereus* and *S. marcessen* experimental data had strong fitting for all candidate models. As shown in Tables 8.2 and 8.3, *B. cereus* had R^2 and R^2_{adj} values of greater than 0.987 and 0.981, respectively and RMSE of less than 0.048. Similarly, *S. marcescens* had R^2 and R^2_{adj} values of greater than 0.986 and 0.982, respectively and RMSE of less than 0.046. The calculated RMSEs for *B. cereus* and *S. marcessens* were less than 0.048 and 0.046, respectively. Table 8.2: Statistical indices for *B.cereus* obtained under different isothermal conditions.

Table 8-2: Statistical indices for *B.cereus* obtained under different isothermal conditions

Temperature [°C]	Primary Model	R^2	R^2_{adj}	AIC	AIC_C	BIC	w_i	α_i
25	Logistic	0.998	0.997	-56.67	-50.67	-56.43	0.961	0.750
	Gompertz	0.994	0.991	-48.64	-42.64	-48.40	0.017	0.000
	Richards	0.998	0.997	-56.44	-43.11	-56.12	0.022	0.250
30	Logistic	0.991	0.987	-38.50	-30.50	-38.66	0.872	0.658
	Gompertz	0.981	0.972	-33.53	-25.53	-33.69	0.072	0.026
	Richards	0.997	0.995	-45.09	-25.09	-45.30	0.056	0.316
35	Logistic	0.996	0.994	-50.90	-44.90	-50.66	0.566	0.532
	Gompertz	0.996	0.994	-50.34	-44.34	-50.10	0.428	0.468
	Richards	0.996	0.993	-49.14	-35.80	-48.82	0.006	0.000
40	Logistic	0.988	0.983	-43.06	-37.06	-42.83	0.855	0.595
	Gompertz	0.981	0.973	-39.33	-33.33	-39.10	0.132	0.111
	Richards	0.989	0.981	-41.97	-28.63	-41.65	0.013	0.294

Table 8-3: Statistical indices for *S.marcessens* obtained under different isothermal conditions

Temperature [°C]	Primary Model	R^2	R^2_{adj}	AIC	AIC_C	BIC	w_i	α_i
25	Logistic	0.992	0.989	-52.96	-48.16	-52.37	0.428	0.461
	Gompertz	0.992	0.989	-53.46	-48.66	-52.87	0.550	0.360
	Richards	0.993	0.988	-52.23	-42.23	-51.44	0.022	0.179
30	Logistic	0.996	0.994	-56.00	-51.20	-55.41	0.924	0.664
	Gompertz	0.992	0.989	-50.10	-45.30	-49.51	0.048	0.035
	Richards	0.996	0.993	-54.21	-44.21	-53.42	0.028	0.301
35	Logistic	0.991	0.988	-50.33	-45.53	-49.74	0.746	0.726
	Gompertz	0.989	0.985	-48.01	-43.21	-47.24	0.234	0.164
	Richards	0.991	0.986	-48.33	-38.33	-47.54	0.020	0.110
40	Logistic	0.991	0.988	-52.08	-47.28	-51.49	0.840	0.615
	Gompertz	0.990	0.986	-48.24	-43.44	-47.65	0.124	0.080
	Richards	0.993	0.989	-50.97	-40.97	-50.18	0.036	0.305
45	Logistic	0.992	0.990	-61.38	-57.38	-60.47	0.846	0.593
	Gompertz	0.985	0.980	-54.89	-50.89	-53.98	0.033	0.000
	Richards	0.994	0.990	-61.49	-53.49	-60.28	0.121	0.407

The goodness-of-fit of the three primary models were analysed by comparing the corresponding statistical indices for each model. Tables 8.2 and 8.3 shows the mean of other statistical indices, AIC, BIC and AIC_c values, obtained under isothermal conditions at different incubation temperatures for *B.cereus* and *S. marcessens*, respectively.

One-way ANOVA of R^2, R^2_{adj}, AIC, BIC and AIC_c values of the three primary models indicated that their means were not statistically different with p-values of greater than 0.05 at each isothermal condition. This is common because the models are fundamentally identical. The three- parameter logistic and Gompertz models are an exceptional case of the Richards model that has a temperamental inflection point described by the shape parameter, v. The four-parameter Richards models is equivalent to logistic or Gompertz model if the shape parameter is 2.0 or 1.0, respectively (Nahashon et al., 2006). Moreover, Tjørve and Tjørve (2010) indicated that Richards model is the generalisation of logistic model and Gompertz model in addition to von Bartelanffy model (von Bertalanffy, 1938) and negative exponential, and further demonstrated how these models are nested in the Richards model. Generally, as shown in Table 8.1, the estimated shape parameters of Richards model were close to 1.0 or 2.0 except in two cases. This suggests that all the candidate models were equally appropriate for fitting the growth curves and with similar accuracy. These results are in concurrence with the results obtained by other researchers (Zwietering et al., 1990; Annaradurai et al., 2000; Çelekli and Yavuzatmaca, 2009; Tornuk et al., 2014).

8.2.2 Growth model selection

Even though the statistical analysis showed that the statistical indicators were not statistically different, further analysis was done in which Akaike weight, w_i, and z-weight, z_i, were applied to choose the best significant model that have the ability to fit the actual growth pattern of *B.cereus* and *S.marcessens* between available predicted sigmoidal curve-fitting candidate models. The use of these two weight values have shown to be valuable tools for the determination of the performance of other predictive models (Shi and Ge, 2010; Arbab and Mcneill, 2011; Arbab *et al*., 2016; Pachú *et al*., 2018). Both

approaches prescribe that the model that maximises the given criterion value is to be selected. Contrary to the aforementioned studies, in this study R^2 and RMSE were not applied because these statistical indices are not corrected for the number of parameters in the candidate models (Angilletta, 2006). In this study, because Richards model can define more numbers of parameters to growth is always advantageous in modelling, hence had highest R^2 and lowest RMSEs in all cases. Moreover, AICc was used in lieu of AIC. This is because the former is preferred to later when only small sample sizes thus relatively small *n* with respect to *k* (*n/k*<40) are available (Sugiura, 1978; Burnham and Anderson, 2002) as is the case in this study. Hence, R^2_{adj}, BIC and AICc were the only statistical indices that were used to calculate the z-weight values for comparison of the models of different number of parameters.

The calculated z-weight values and w for the candidate models in this study are presented in also presented in Tables 8.2 and 8.3 for *B. cereus* and *S. marcessens,* respectively, under each incubation temperature. The modified logistic model was the best for *B.cereus* when the bacterial strain was incubated at temperatures of 25, 30 and 35 ^oC according to the normalised weight based on AICc, Akaike weights and the integrated weight, α_i of each model. A weight evidence of 0.541 and 0.457, 0.912 and 0.414, 0.909 and 0.474 and 0.913 and 0.510, respectively supported this model. However, under the incubation temperature of 40 ^oC, the modified Gompertz model was the best. The model was supported by a weight of 0.913 and 0.510, respectively. Similarly, for *S. marcessens, the* modified logistic model was the best under three incubation temperatures of 30, 40 and 45 ^oC. This was supported by a weight of 0.896 and 0.601, 0.598 and 0.535 and 0.873 and 0.507, respectively. However, under two incubation temperatures of 25 and 35 ^oC, the modified Gompertz model was the best supported by a weight of 0.550 and 0.436 and 0.543 and 0.474, respectively. The results clearly indicate that generally, there is no universal best model to characterise the growth pattern of these two butyric acid deodourant bacterial strains. Besides, the selection of the best model may vary dependent

upon the type of the organism and the environmental conditions i.e. incubation temperature.

It should be noted that the model selection approach revealed that the z-weight and the integrated weight for each model under each incubation temperature yielded the same results but *B. cereus* under the incubation temperature of 30 ^{o}C. Under the incubation temperature of 30 ^{o}C there were difference in the behaviour of the model selection approach in the sense that the Akaike weight favoured the modified logistic model while the integrated weight favoured the Richards model. According to Burnham and Anderson (1998) and Burnham (2002), the AICc, which already includes a greater penalty for model complexity, tends to favour models with less number of predictor variables as is evident in this study. Moreover, in accordance with the perspective of parsimony embodied in Ockham's razor (Ratskowsky, 1993), Richards model is less desirable and, therefore, the modified logistic model is a parsimonious approximating model. Generally, Richards's model had considerably least support among the set of candidate models. It had a weight evidence of between 0.5 and 1.1% and between 11.8 and 49.3% for logistic model according to Akaike's weights. Gompertz model has essentially no support. Succinctly, in three out of four data sets and in four out of five data sets for *B. cereus* and *S. marcessens,* respectively, the modified logistic model performed well. It is, therefore, intuitively clear that the Richards and modified Gompertz models cannot be justified to describe growth pattern to close reality hence the modified logistic model was selected. This model has been successfully used to model growth of bacteria (Kreyeschmidt *et al.,* 2010; Amodu *et al.,* 2016).

8.2.3 Derivation of growth parameters

In this study the estimates of parameters of microbiological relevance, asymptotic value, a, maximum specific growth rate, μ_{max}, and lag time, λ, as presented in Tables 8.4 and 8.5 were computed using the mathematical parameters in Tables 8.1 for both *B. cereus* and *S. marcessens,* obtained by fitting the experimental data using the modified logistic model expressed as equation 3.8.

Table 8-4: Derived values of biological parameters, A, μ_{max} and λ for *B.cereus* computed from the best-selected model under each incubation temperature

Temperature [°C]	Biological parameters		
	A	μ_{max} [h^{-1}]	λ[h]
25	1.162	0.100	10.71
30	1.138	0.104	7.13
35	1.101	0.143	6.64
40	1.081	0.092	7.34

Table 8-5: Derived values of biological parameters, A, μ_{max} and λ for *S.marcessens* computed based on the best-selected model under each incubation temperature

Temperature [°C]	Biological parameters		
	A	μ_{max} [h^{-1}]	λ[h]
25	1.104	0.039	13.86
30	1.263	0.052	10.42
35	1.216	0.054	9.16
40	1.229	0.043	11.34
45	1.078	0.030	19.46

The parameters were computed according to the aforementioned equations 7, 8 and 10. The use of a single model is highly recommended for comparison purposes. From Tables 8.4 and 8.5, it can be seen that incubation temperature had an insignificant noticeable effect on asymptote (A) (maximum population density) in comparison to μ_{max} and λ. However, the bacterial strains had different durations to reach maximum population densities as shown in Figures 8.1 (a-i). Even, Krist *et al* (1998) noted, for maximum population densities of microbial cultures of substrate-limited batch cultures for some microorganisms, that this was a commonly observed phenomenon.

Generally, with increasing incubation temperature, μ_{max} drastically increased for both bacterial species. Values of maximum specific growth rates ranged from 0.093 at 25 °C to 0.079 at 40 °C for *B. cereus* and 0.085 at 30 °C to 0.057 at 45 °C for *S. marcessens*. The value at 25 °C was lower than at 30 °C for *S. marcessen*. It is worth noting that *B. cereus*

grew faster than *S. marcessens* at all incubation temperatures. Asymptotic values for both bacterial strains followed the similar trend. Similarly, Kandhai *et al*. (2006) observed that the specific growth rates of *Entrobacter sakazakii* were found markedly increasing with increase in temperature. It was also shown that the lag time decreased with increasing temperature from 25 ^{o}C to 35 ^{o}C and followed by the lag time increase with increasing temperature. The lag time was decreasing with increase in incubation temperatures. However, at 40 ^{o}C and above, the lag time started increasing with increase in incubation temperatures. This could be due to heat lability of the bacterial enzymes that have their catalytic efficiency reduced that is induced by thermal inhibition of enzyme secretion, exoenzyme formation and enzyme activity as temperature increases (Feller *et al.,* 1994). Moreover, the lag time of *B. cereus* was longer than that of *S. marcescens*.

8.2.4 Secondary modelling

The maximum specific growth rates and lag phase durations of both *B. cereus* and *S. marcessens s* as a function of temperature were fitted using the square root Ratkowsky and inverse Ratkowsky equations, respectively, based on the predictive data from the modified logistic model as shown in Table 8.4. There are other secondary models such as Arrhenius model (Zwietering *et al.,* 1991), polymonial model (IFR, 2004), Cardinal model (Rosso *et al*., 1993) and others that could have been used to evaluate the effect of incubation temperature on the maximum specific growth rates and lag phase durations. The choice of the model was owing to the fact that the microbial interpretation of its parameters is easy, providing more insight into the behaviour of the bacterial species (Baranyi *et al*., 2017). Figures 8.2a and 8.2b, and 8.3a and 8.3b depict the effect of incubation temperature on the maximum growth rate and lag phase durations computed for *B. cereus* and *S. marcessens*, respectively. Table 8.6 shows the secondary models generated for the dependency of maximum specific growth rates and lag times and the statistical indices for the validation of the secondary models for the growth data of both *B. cereus* and *S. marscessens*.

Table 8-6: Established secondary models depicting the dependency of maximum specific growth rate and lag time on incubation temperature of *B. cereus* and *S.marcescens*

Bacterial species	Secondary model	R^2	RMSE
B.cereus	$\sqrt{\mu_{max}} = 0.125 \ \ (T - 3.26)$	0.980	0.003
	$\sqrt{\frac{1}{\lambda}} = \ \ 0.015 \ \ (T - 5.09)$	0.997	0.007
S.marcessens	$\sqrt{\mu_{max}} = 0.0092 \ \ (T - 6.96)$	0.966	0.007
	$\sqrt{\frac{1}{\lambda}} = \ \ 0.139 \ \ (T - 6.45)$	0.960	0.011

The square root of maximum growth rate, μ_{max}, at these incubation temperatures for both *B. cereus* and *S. marcescens* showed high linearity with high correlation when plotted with a Ratkowsky model (Ratkowsky *et al*., 1982) as shown in Figures 8.2a and 8.2b.

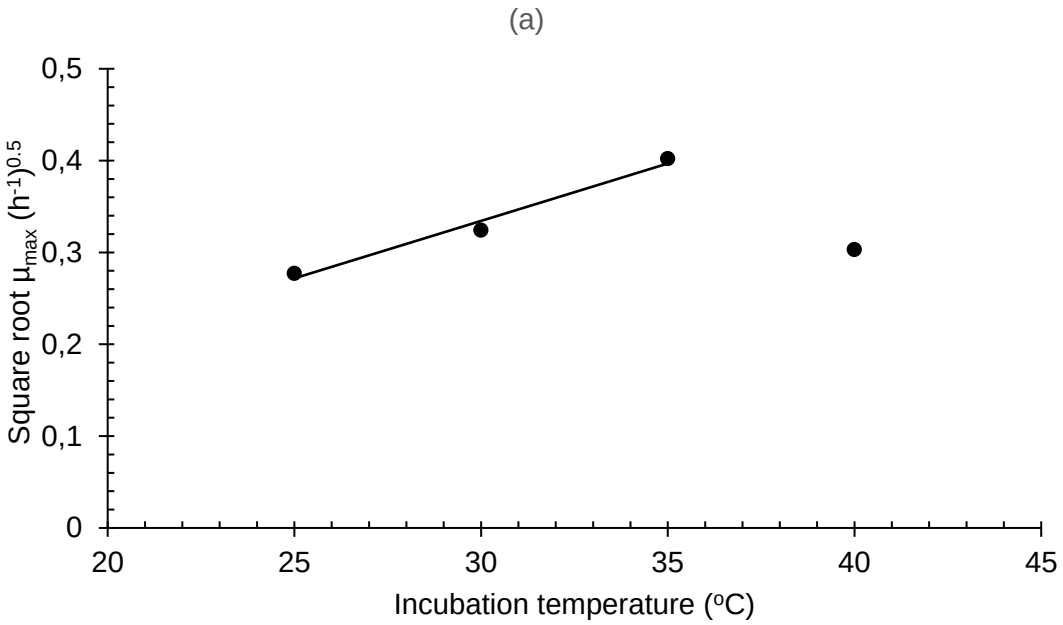

Figure 8-2a: Influence of incubation temperature on the maximum specific growth rate of *B.cereus*

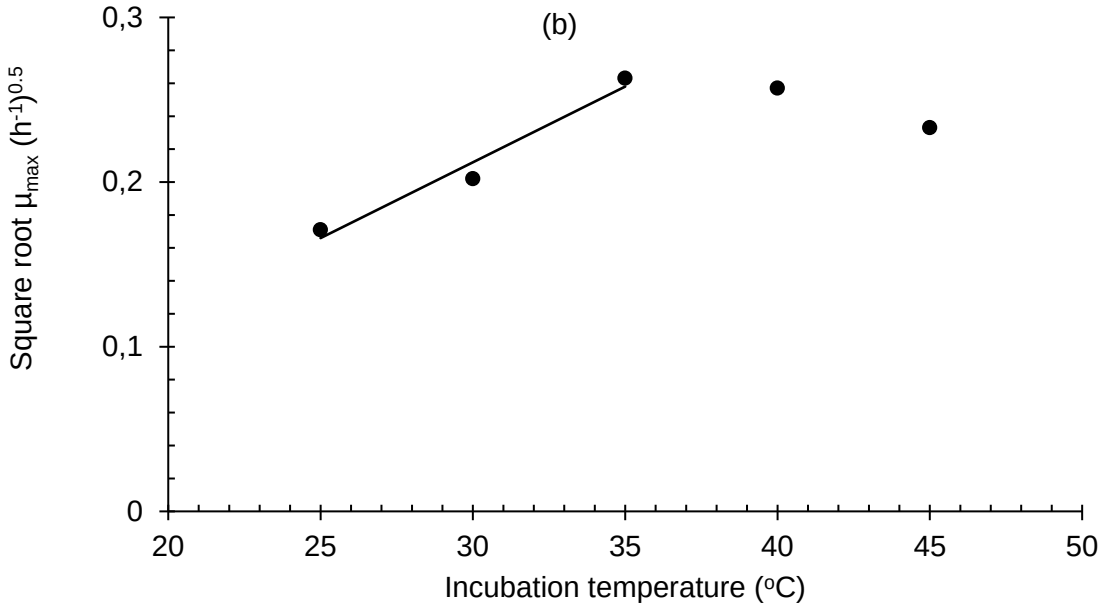

Figure 8-2b: Influence of incubation temperature on the maximum specific growth rate of *S.marcescens*

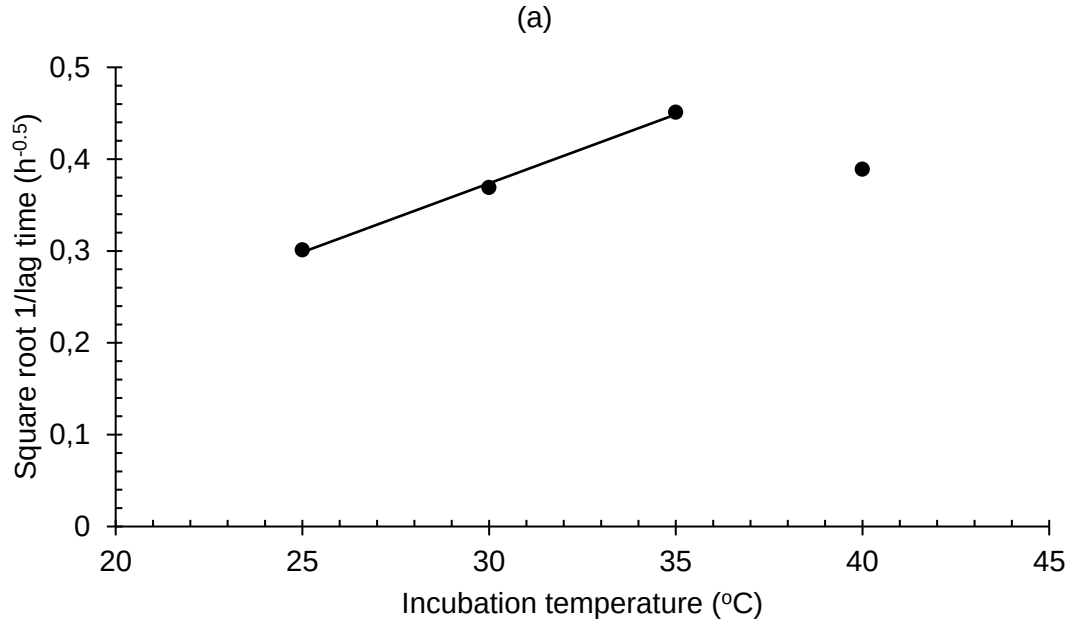

Figure 8-3a: Influence of incubation temperature on the lag phase duration of *B.cereus*

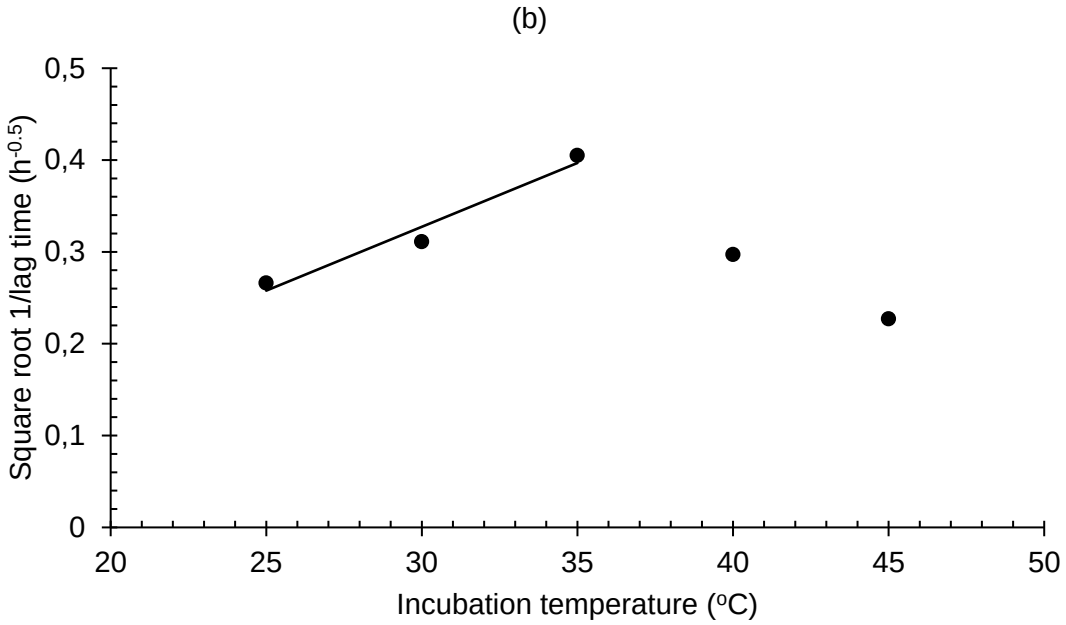

Figure 8-3b: Influence of incubation temperature on the lag phase duration of *S.marcescens*

The linear relationship of the results in form of $\sqrt{\mu_{max}}$ versus incubation temperature and $\sqrt{\frac{1}{\lambda}}$ versus incubation temperature with high R^2 values (≥0.960) and low RMSE values (<0.011) were obtained for incubation temperature of 25 °C in this study up to 35 °C or thereabout at which the optimal maximum specific growth rate was attained for both *B.cereus* and *S.marcessens*. However, as incubation temperature passes 35 °C, an exponential decrease in the maximum specific growth rates and a decrease of lag phase duration occurred for both the bacterial species.

As earlier indicated this could be attributed to inactivation or denaturation of proteins, instability or no synthesis of RNA or inhibition. As such only data points up to 35 °C for which the decrease in the maximum specific growth rate and increase in the lag phase durations had not yet occurred were taken into consideration. Thus, the last data point at 40 °C for *B. cereus* and two last data points, 40 and 45 °C for *S. marcessens* were eliminated because of deviation from the straight line. This is also in consideration that the

secondary models used are valid exclusively for the sub-optimal temperature, which is the temperature range from minimum temperature (T_{min}) to optimal temperature (T_{opt}), for microbial growth (Gospavic *et al*., 2008). These optimal growth temperature values obtained in this study are close to optimal growth temperatures reported by Choma *et al.* (2000) of 31 ^{o}C for *B. cereus* cultivated in courgette broth, Banerjee and Ghoshal (2010) of 37 ^{o}C for *B. cereus* in the degradation of phenol. Bidlan and Manonman (2002) occurred at 37 ^{o}C in the aerobic degradation of dichlorophenyltrichloroethane (DDT) by *S. marcescens* and Bubelovà *et al.* (2015) observed μ_{max} at 37 ^{o}C in *in vitro* production of putrescine and cadaverine production through the growth of *S. marcescens.* The small discrepancies in the optimal temperature could be attributed to the different cultural conditions as well as the interstrain differences.

The maximum specific growth rates of *B.cereus* were more sensitive to variations in incubation temperature in comparison to lag phase duration. In other words, the variation in incubation temperature significantly influenced the maximum specific growth rates greater than the lag phase durations. In contrast the lag phase durations were more sensitive to incubation temperature than the maximum specific growth rate for *S. marcescens*. Essentially, this difference in growth behaviour is not unprecedented because *B. cereus* and *S. marcescens* are dissimilar bacterial strains. There are published studies in literature in which differences in growth behaviour among bacterial strains of the same species have been reported. For instance, Sooltan *et al.* (1987) and Wong and Cheng (1988) reported that notwithstanding that *B.cereus* isolates of the same species and cultured under the same conditions exhibited significant differences in their growth kinetics behaviour.

Based on the regression line obtained the minimum temperature, T_{min}, for *B. cereus* and *S. marcescens* in Table 8.6 were estimated to be 3.26 and 6.96 ^{o}C, respectively. Similarly, when the inverse Ratkowsky model estimated the T_{min} for *B. cereus* and *S. marcessens* were 5.09 and 6.45 ^{o}C, respectively. The similarity of the T_{min} calculated for maximum

growth rate and lag time using Ratkowsky model and Inverse Ratkowsky model, respectively, in this study suggests that temperatures below 35 ^{o}C have the same effect for each of the bacterial strains. It is noteworthy that the square root of maximum specific growth rates and inverse of lag phase duration lead to determination of similar minimum temperature. It is noticeable that the estimated theoretical minimum temperature, T_{min} in this study fall within theoretical minimum growth temperature range of mesophilic bacteria, of which *B. cereus* and *S. marcescens* are, as listed by Ratkowsky et al. (1982). Previous studies (Irish *et al.* 2013; Nabatessa *et al*., 2017; Nakagiri *et al*., 2017) revealed that the measured *in situ* temperature of the samples of pit latrine feacal sludge in their work varied between 21 and 33 ^{o}C. Therefore, taking into consideration the estimated T_{min} and T_{opt} for both *B. cereus* and *S. marcescens* in this study, the temperature range of pit latrine feacal sludge falls within, implies that the bacterial strains can favourably thrive.

8.3 Summary

The odourous emissions from pit latrines have become a priority issue as far as adoption and consistent use of pit latrines as sanitation systems in the developing countries is concerned. Bioremediation of the odourous compounds has attracted significant importance and is being constantly carried out using novel microbial species. Butyric acid biodegradation is a deodourisation process and to gain further insights into the relationship between the efficient butyric acid biodeodourisation efficiencies and a bacterial consortium of *B.cereus* and *Serattia marcescens* mathematical modelling of the growth dynamics were used in this study at five different isothermal conditions of 25, 30, 35, 40, and 45 °C. A set of three sigmoidal models; modified logistic, Gompertz, and Richards were used and compared to select the best model that describe well the observed bacterial growth data. Performance of these models were evaluated based on various statistical criteria such as coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}),), Bayesian Information Criterion (BIC), Akaike Information Criterion (AIC) and corrected Akaike Information Criterion (AICc). Moreover, the maximum growth rates and lag times derived from the best-selected model were fitted to Ratkowsky model and inverse Ratkowsky

model to determine the influence of incubation temperature on maximum growth rates and lag times, respectively. The modified logistic model was the best model in modelling the growth curve of *B.cereus* and *S.marcescens* based on Information Theory Criterion. The parameters constants determined from primary and secondary models used in this study will be a substantial help for the future development of further tertiary models that are useful for bacterial growth prediction under non-isothermal conditions. This is the first study of predictive modelling of the influence of isothermal conditions on the growth parameters of microbiological significance of *B.cereus* and *S.marcessens* during biodegradation of butyric acid. The ability to predict bacterial growth dynamics with respect to butyric acid as a sole carbon source and temperature is beneficial for butyric acid deodourisation optimisation in pit latrines. This study contributes to a better understanding and control of the biodegradation processes and aids to elucidate how and to what degree the feacal sludge temperature will affect with the growth behaviour of the bacterial strains.

CHAPTER NINE

9 CONCLUSIONS AND RECOMMENDATIONS

9.1 Summary and conclusions

Pit latrines are basic form of on-site dry sanitation systems that are more commonly used and sometimes the only available improved option of safe human waste containment in low-income peri-urban and rural areas throughout developing countries. However, there is a growing concern that volatiles emanating from pit latrines, which are characterised by offensive odour perception, is one of the barriers to their utilization by the expected users. Thus, users are compelled to prefer open defecation. Open defection triggers a wide array of detrimental consequences for the environment and human health. Limiting of the sources and mitigating the human health and environmental impacts of malodours, therefore, epitomizes an important public health undertaking. Because of this development, it is indispensable to develop novel and appropiate solutions for this new challenge, but these solutions should be those that are ecofriendly and economically viable in the rural and informal settlements of low-income countries.

In recent years, interest in microbial remediation of environmental pollutants, such as odour causing compounds, has been steadily becoming popular because of its efficiency, cost-effectiveness and environmentally friendliness. In this context, the application of microbial odour control techniques has potential to cater for the needs and holds optimism for pit latrine deodourization in developing countries.

For low cost sanitation technologies such as pit latrines, the development of a biological odour treatment that could potentially degrade odour causing compounds and act as a deodorants with the production of other environmental pollutants would be an added benefit The primary focus of the current study was, therefore, to investigate the potential microbial control of pit latrine odourant emissions. This result of this study has shown that there are bacterial strains that have potential to degrade odourous compounds such as

butyric acid. This finding represents an excellent approach for microorganisms to tolerate and biologically deodorize pit latrines. There is a potential to develop practical applications of engineering processes for bioremediation of odours in liquids phase if this can be adopted. With biological odour treatment methods, reactor engineering is anticipated to be simple and cheaper.

The following is a summary of the conclusions derived from the study:

1. A wide variety of organic volatiles were identified by HS-SPME-GC-ToF-MS in samples of pit latrine feacal sludge. Up to 358 volatiles including ketones, alcohols, hydrocarbons, esters, nitrogen-containing compounds, aldehydes, carboxylic acids, sulfur-containing compounds and phenols were released from pit latrine feacal sludge.
2. The ketones were the most common VOC species in the samples of pit latrine feacal sludge. This was followed by alcohols, hydrocarbons, esters, nitrogen-containing compounds, aldehydes, carboxylic acids, sulphuric-containing compounds and phenols in this order.
3. Nineteen compounds; indole, butyric acid, p-Cresol, alpha-pinene, skatole, dimethyldisulfide, dimethyltrisulfide, phenol, methyl thioacetate, propionic acid, 2-butanone, isobutyric acid, ethyl acetate, ethyl formate, limonene, toluene, 1-propanol, 2-methylbutyric acid, and 3-methylthiophene were considered as the most frequently occurring organic odour stimuli.
4. The study has revealed that pit latine organic volatiles have spatial and temporal variabilities both qualitatively and quantitatively which could be due to inherent variations of feacal sludge characteristics, physico-chemical properties of the compounds and the prevailing environmental conditions in the pit latrines.
5. The application of chemometric tools such as the experimental design, response surface methodology (RSM) and Derringer's desirability function have shown to be a powerful tool to achieve a rapid, simple, sensitive and cost effective simultaneous determination of four key pit latrine odourants; butyric acid, dimethyltrisulfide, indole and p-Cresol.
6. The optimal conditions for optimisation of a proposed HS-SPME extraction procedure for simultaneous determination of the four key pit latrine odourants were obtained with CAR/DVB/PDMS fibre, sample volume 10 mL in 20 mL vial, stirring rate of 800 rpm, equilibrium time of 10 min, extraction time of 28 min, extraction temperature of 65 ^{o}C, pH=2 and NaCl concentration of 550 mg/mL.

7. The proposed HS-SPME extraction procedure has been successfuly applied to experimentally detect 0.01 µg/L of the mixture of butyric acid, dimethyltrisulfide, indole and p-Cresol contained in Milli Q-water. This concentration is 10 to 100 folds lower than the compounds' human odour detection thresholds found in literature.
8. The study was successful in isolating and identifying nine (9) bacterial strains, which are novel in butyric acid degaradation studies. The bacterial isolates were found to have 93 to 100% identities and these include; *Alcaligenes* sp. strain SY1, *A.animicus, P.aeruginosa, S.marcescens, A.xylosoxidans, B.cereus, L.fusiformis, B.methylotrophicus* and *B.subtilis*.
9. All the bacterial strains tested were indegeneous strains obtained from feacal sludge of pit latrines showed potential to remove butyric acid completely with varied efficiencies. *A.xylosoxidans, B.subtilis, L.fusiformis, B.cereus, P.aeruginosa* and *B.methylotrophicus* had potential to remove 1000 mg/L compeletely within 20 h while *A.animicus, Serattia marcescens* and *Alcaligenes* sp. strain SY1 could achieve the same within 24 h, at an incubation temperature of 30 ^{o}C, agitation rate of 110 rpm and pH 7.
10. The degradation efficiencies of butyric acid of the bacterial strains were in the order of *A.xylosoxidans* > *B.subtilis*> *L.fusiformis*> *B.cereus* > *P.aeruginosa*> *B.methylotrophicus*> *S. marcescens* >*A.animicus* >*Alcaligenes* sp. strain SY1.
11. During the development and utilisation of butyric acid-degrading bacteria, in order to fully describe the degradation capability and to develop procedures for their use in bioremediation, such environmental factors as temperature, pH, aeration and inoculation concentrations of the bacteria involved should be taken into considerations
12. This study has revealed the effectiveness of some of the bacterial consortia to enhance butyric acid degradation in comparison to their constituent bacterial strains. Based on the current study, the co-culture of *S.marcescens* and *B.cereus* is found to be the effctivebutyric acid degrading consortium. The co-culture has the capacity to degrade 1000 mg/L of butyric acid at neutral pH and temperature of 30 ^{o}C within 16 h. This may be the first instance in which 1000 mg/L of butyric acid degradation has been achieved in a short incubation time of 16 h.
13. Both *B.cereus* and *S.marcescens* have shown potential to survive and perform well within the pit latrine environment, with respect to pH of bweeten 5 and 8 and temperature of between 25 and 40 ^{o}C, which flunctuates over time.
14. Three primary sigmodal models, modified logistic, Gompertz and Richards successfully fitted the growth data of *B.cereus* and *Serattia marcescens* separately in the liquid medium supplemented with butyric acid as a sole source of carbon under isothermal conditions between 25 and 40 ^{o}C. The modified logistic model

performed better than the modified Gompertz and Richards models to describe the effect of incubation temperature on microbial growth of *B.cereus and S.marcessens*.

15. Modelling of μ_{max} and ʎ of *B.cereus* and *S.marcessens* were accurately performed using a linear relationship between the square root and inverse square root of the parameters, respectively, and temperature within the suboptimal temperatures range as described by Ratkowsky *et al*.(1982). Hence, the equations to describe this relationship have been developed which may be valid to predict μ_{max} and ʎ at sub-optimal temperatures. These models, if validated for their predictive ability, can be applied to develop tertiary models to predict growth curves of *B.cereus* and *Serattia marcescens* under fluctuating temperature conditions in the pit latrines.

9.2 Recommendations

Based on the findings of this study the following are recommendations outlined for further research:

1. The spatial and temporal diversity of VOCs identified in this study reveals the necessity for further field studies to have a better understanding of the VOCs profile of pit latrine emissions. It will also be imperative to investigate the source of the volatile compounds as one might speculate about their direct origin from endogenous sources, but also from foods as free odourous compounds contained therein, or about their potential occurrence as anaerobic or aerobic digestion products from more complex contents of pit latrine.
2. The variations in the characteristics in the pit latrines could drive the changes in the VOC emissions, hence, further work is indispensable to evaluate which of the pit environmental variables have an effect on the types and concentration of the fecal sludge air streams.
3. The proposed HS-SPME extraction procedure is not validated with respect to linearity, precision, repeatability, LOD and LOQ, therefore, further investigation to evaluate the the procedure's applicability in real environment is warranted.
4. The fate of the butyric acid is not known, therefore, further studies to elucidate the degradation pathway of novel butyric acid degrading bacterial strains and the potential optimisation of the biodegradation process should be undertaken.
5. There are many abiotic factors i.e. pH, water activity etc that affect microbial growth, hence, further studies to develop an understanding of how interaction of these factors affect the growth behaviour of the butyric acid degrading bacteria are warranted.

6. It would be necessary to study interactions between the different odour-causing compounds to understand some of the inhibition effects or induction and cometabolism mechanisms. These studies will provide a better insight of the limits of performance of the microbial deodourisation and hence its field-scale implementation
7. The detailed molecular mechanisms of butyric acid degradation by a co-culture of *B.cereus* and *S.marcescens* and their contribution individually in the degradation process are not known, therefore, there is a need to conduct further investigation to envision the role of each plays in the consortium in terms of degradation.
8. The growth of *B.cereus* and *S.marcescens* were modelled individually hence the growth as the consortium as a function of temperature needs to be explored.

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APPENDICES

Appendix 1

Table S3.1: Bacterial strains and calibration curves used to quantify biomass in mg/L from OD

Bacterial strain	Equivalents of 2.0 OD_{600} in mg/L	Calibration equation	R^2
Alcaligenes sp. strain SY1	549.8	$y = 274.9x$	0.994
A.animicus	569.5	$y = 284.8x$	0.993
P.aeruginosa	509.4	$y = 254.7x$	0.996
A.xylosoxidans	575.3	$y = 287.6x$	0.999
S.marcescens	574.6	$y = 287.3x$	0.998
B.cereus	592.2	$y = 296.1x$	0.999
L.fusiformis	540.3	$y = 270.2x$	0.999

Appendix 2

Table S4.1: Compounds detected in the analysed pit latrine feacal sludge and their physico-chemical parameters

Compound name	Chemical formula	No.of C-atoms	Molecular weight (g/mol)	Boiling point (ºC)	Water solubility (mg/L)	Vapour pressure (mmHg)	Octanol-water	Henry's law constant	P1W	P1S	P2W	P2S	P3W	P3S	P4W	P4S	P5W	P5S	P6W	P6S	P7W	P7S	P8W	P8S	Frequency
Nitrogen-containing compounds																									
Ammonium carbamate	$CH_6N_2O_2$	1	78.07	58.8	-	-	-	-						■											1
2-methyltetrazole	$C_2H_4N_4$	2	84.082	171.3	-	-	-	-								■									1
Methylformamide	C_2H_5NO	2	59.068	199	2.25E+06	0.0182	-1.26	6.58E-09												■					1
Acetamide	C_2H_5NO	2	59.068	221	-	-	1,32	1.90E-08															■		1
o-allylhydroxylamine	C_3H_7NO	3	73.095	123.2	-	21.44	1.21	-										■							1
Cyclobutylamine	C_4H_9N	4	71.123	81.5	45	8.36	0.75	-			■										■				2
Butanenitrile	C_4H_7N	4	69.107	117.0	0.498	0.01	2.6	-	■						■										2
2-butenenitrile	C_4H_5N	4	67.091	121.0	-	-	2.56	-								■								■	2
1H-pyrrole	C_4H_5N	4	67.091	131.0	0.37	31.95	-	1.10E-03											■						1
1-methylpyrrole	C_5H_7N	5	81.118	115.0	-	-	-	-					■												1
Pyridine	C_5H_5N	5	79.102	115.3	-	-	2.54	5.48E-09								■									1
2-methylbutyronitrile	C_5H_9N	5	83.134	125.0	-	-	1.4	2.19E-10												■					1
4-pentenenitrile	C_5H_7N	5	81.118	140.0	-	-	-	-														■			1
3-methyl-1H-pyrrole	C_5H_7N	5	81.118	143.0	1.0E+06	6.05	1.2	-	■								■								2
2,5-dimethyl-pyrazine	$C_6H_8N_2$	6	108.144	155.0	-	-	0.63	-	■															■	2
1- methylpiperidine	$C_6H_{13}N$	6	99.177	107.0	-	-	-	-							■										1
2-methylpyridine	C_6H_7N	6	93.129	129.0	-	-	0.63	9.97E-06											■						1
3-methylpyridine	C_6H_7N	6	93.129	144.0	-	-	-	2.35E-05				■												■	2
2,5-dimethyl pyrazine	$C_6H_8N_2$	6	108.144	155.0	-	-	-	-						■											1
3-pyridinecarbonitrile	$C_6H_4N_2$	6	104.112	201.0	-	-	-	-						■											1
2,4-dichloro-benzenamine	$C_6H_5Cl_2N$	6	162.013	245.0	-	-	-	-			■		■												2
Niacinamide	$C_6H_6N_2O$	6	122.127	334	-	20.8	0.65	2.90E-12						■											1
2-amino-Benzonitrile	$C_7H_6N_2$	7	118.139	268.0	5.0E+05	4.2E-04	-0.37	-											■						1
1H-Benzimidazole	$C_7H_6N_2$	7	118.139	360.0	4.5E+04	8.35	0.75	-	■	■															2
Indole	C_8H_7N	8	117.151	253.0	33.00	19.5	0.53	1.39E-06	■	■		■	■	■	■	■	■	■	■	■		■	■	■	14

4,8-diacetyl-di(1,2,5-oxadiazolo)[3,4-b:3,4-e]pyrazine	$C_8H_6N_6O_4$	8	250.174	-	-	-	2.91	-															1
Isoquinoline	C_9H_7N	9	129.162	242.0	-	3.16E-07	4.75	1.90E-03															2
6-methyl-1H-indole	C_9H_9N	9	131.178	260.3	-	-	-	-															1
3-methyl-1H-indole	C_9H_9N	9	131.178	265.0	-	-	-	-															2
7-methyl-1H-indole	C_9H_9N	9	131.178	266.0	3560	0.01	-	-															5
Skatole	C_9H_9N	9	131.178	266.0	-	-	-	-															8
4-methyl-1H-indole	C_9H_9N	9	131.178	267.0	1	0.253	-0.97	-															2
Bis-(ethoxycarbonyl)methoxymethyloxyiminomethane	$C_9H_{15}NO_6$	9	233.22	271.0	-	-	-	-															2
Binapacryl (Endosan)	$C_{15}H_{18}N_2O_6$	15	322.317	436.7	0.498	0.0055	2.6	-															7
Dinocap	$C_{18}H_{24}N_2O_6$	18	364.39	138.0	-	-	-	4.70E-09															1
Sulfur-containing compounds																							
Sulfur dioxide	SO_2	0	64.058	10.0	1.90E+03	154.2	1.81	7.60E-03															1
Methyl mercaptan	CH_4S	1	48.103	6.0	-	-	-	2.60E-03															2
Carbon disulfide	CS_2	1	76.131	46.0	0.4	22.15	2.34	1.62E-03															1
Dimethyl sulfide	C_2H_6S	2	62.13	38.0	-	-	-	1.76E-03															2
Dimethyl disulfide	$C_2H_6S_2$	2	94.19	109.0	3	28.7	1.77	1.70E-03															8
Dimethyl trisulfide	$C_2H_6S_3$	2	126.25	183.0	15.4	1.51	0.78	-															11
Dimethyl sulfoxide	C_2H_6OS	2	78.129	189.0	-	-	-	-															1
Dimethyl tetrasulfide	$C_2H_6S_4$	2	158.31	243.1	8.5	3.0E+03	-	-															3
1-propanethiol	C_3H_8S	3	76.157	68.0	4.3	0.014	2.01	5.81E-03															2
Allyl mercaptan	C_3H_6S	3	74.14	68.0	-	524.45	-	8.22E-03															1
methyl thioacetate	C_3H_6OS	3	90.14	99	2160	359	1.94	-															11
2,4-dithiapentane	$C_3H_8S_2$	3	108.217	147.0	-	-	-	-															2
Ethyl methanesulfinate	$C_3H_8O_2S$	3	108.155	153.0	-	-	-	-															2
2-(methylthio)ethanol	C_3H_8OS	3	92.156	169.0	0.292	0.206	2.87	-															2
Allyl methyl trisulfide	$C_4H_8S_3$	4	152.288	30.0	-	1.028	-	-															2
Methyl propyl sulfide)	$C_4H_{10}S$	4	90.184	95.5	-	0.05	-	-															2
Allyl isothiocyanate	C_4H_5NS	4	99.151	151.9	Insoluble	1.06	1.94	2.41E-03															4
Propyl methyl trisulfide	$C_4H_{10}S_3$	4	154.304	212.7	-	50.82	-	-															1

1,1-bis(methylthio)propane	$C_5H_{12}S_2$	5	136.271	204.0	-	-	-	-															1
3-methyl-thiophene	C_5H_6S	5	98.163	114.0	2.2E+04	502	0.84	-															11
2-methyl-5-(methylthio)-furan	C_6H_8OS	6	128.189	66.0	2.000	-	2.15	-															1
2-ethyl-thiophene	C_6H_8S	6	112.19	134.0	1.0E+06	0.60	-1.35	-															1
Benzothiazole	C_7H_5NS	7	135.186	223.9	-	-	-	-															1
Carboxylic acids																							
Formic acid	CH_2O_2	1	46.025	100.8	0.0014	0.03	2.64	1.12E-07															1
Acetic acid	$C_2H_4O_2$	2	60.052	118.1	2240	8.2E-05	2.26	1.19E-05															1
Propionic acid	$C_3H_6O_2$	3	74.079	141.2	45	0.49	1.18	3.40E-13															9
(acetyloxy)-acetic acid	$C_4H_6O_4$	4	118.088	142.0	1.0E+06	15.7	-0.17	-															1
Isobutyric acid	$C_4H_8O_2$	4	88.106	155.0	-	0.006	-	1.03E-06															10
Butyric acid	$C_4H_8O_2$	4	88.106	163.5	-	-	-	3.40E-11															14
Succinic acid	$C_4H_6O_4$	4	118.088	235.0	167000	1.81	0.94	3.66E-13															6
Isovaleric acid	$C_5H_{10}O_2$	5	102.133	176.5	1.0E+06	15.7	-0.17	9.87E-06															3
2-methylbutyric acid	$C_5H_{10}O_2$	5	102.133	177.0	3400	7.0E-04	1.87	-															11
Pentanoic acid	$C_5H_{10}O_2$	5	102.133	186.0	60000	1.65	0.79	8.97E-07															6
4-methyl-pentanoic acid	$C_6H_{12}O_2$	6	116.16	201.0	4.6	0.039	1.96	-															1
Hexanoic acid	$C_6H_{12}O_2$	6	116.16	205.0	4.81	1.6E-05	4.6	2.82E-06															1
2-hydroxy-benzoic acid	$C_7H_6O_3$	7	138.122	211.0	1.0E+06	42.59	-0.54	-															4
Heptanoic acid	$C_7H_{14}O_2$	7	130.187	223.0	2.82E+03	1.07E-2	2.42	1.03E-06															2
Cyclohexanecarboxylic acid	$C_7H_{12}O_2$	7	128.17	233.0	1.03E+04	0.0435	1.92	8.97E-05															1
Benzoic acid	$C_7H_6O_2$	7	122.123	249.2	0.597	7.22E-07	8.23	3.40E-08															1
2-ethylhexanoic acid	$C_8H_{16}O_2$	8	144.214	228.0	0.04	3.8E-07	7.17	-															1
Octanoic acid	$C_8H_{16}O_2$	8	144.214	237.0	-	0.44		6.58E-05															1
2-methyl-octanoic acid	$C_9H_{18}O_2$	9	158.241	246.5	Insoluble	1.65E-03	3.42	-															1
Nonanoic acid	$C_9H_{18}O_2$	9	158.241	254.0	24.0E+03	1.96E-01	1.39	2.60E-06															2
Trans-2-decenoic acid	$C_{10}H_{18}O_2$	10	170.252	278.6	1.0E+06	3.53	3.53	-															5
Dodecanoic acid	$C_{12}H_{24}O_2$	12	200.32	298.9	83200	1.91E-07	-0.59	2.19E-06															2
Tetradecanoic acid	$C_{14}H_{28}O_2$	14	228.376	250.5	-	3.71E-03	3.05	6.17															1
Hexadecanoic acid	$C_{16}H_{32}O_2$	16	256.43	351.5	1.07	1.40E-06	6.11	-															1
Octadecanoic acid	$C_{18}H_{36}O_2$	18	284.484	361.0	-	-	-	-															1
Phenols																							

Phenol	C_6H_6O	6	94.113	182.0	35	4.77E-03	5.19	2.67E-07	■	■	■	■	■	■		■		■		■					9
p-Cresol	C_7H_8O	7	108.14	202.0	-	-	-	9.87E-07	■	■	■	■	■	■	■	■		■	■	■	■	■	■		14
3-methyl-phenol	C_7H_8O	7	108.14	203.0	-	-	-	-										■							1
m-cresol	C_7H_8O	7	108.14	203.0	4900	0.0372	2.58	1.25E-06						■											1
Guaiacol	$C_7H_8O_2$	7	124.139	205.0	1.25	-	3.49	1.60E-06		■						■									2
4-ethylphenol	$C_8H_{10}O$	8	122.167	218.0	18.7	0.103	1.32	1.20E-06										■							1
3-(1-methylethyl)-phenol	$C_9H_{12}0$	9	136.194	228.0	22700	0.11	1.96	-	■																1
Thymol	$C_{10}H_{14}O$	10	150.221	232.9	21500	0.11	1.94	3.30E-06	■		■		■			■		■				■	■		7
Carvacrol	$C_{10}H_{14}O$	10	150.221	237.7	82800	0.35	1.46	4.11E-06	■	■			■	■		■		■		■					7
2,4-bis(1,1-dimethylethyl)-phenol	$C_{14}H_{22}O$	14	206.329	265.0	98	0.016	3.3	-							■										1
4,4'-(1-methylethylidene)bisphenol	$C_{15}H_{16}O_2$	15	228.291	400.8	120	4.0E-08	3.32	-	■					■											2
Alcohols																									
Methanol	CH_4O	1	32.042	64.7	-	0.0105	-0.83	6.60E-11											■						1
Dimethyl-silanediol	$C_2H_8O_2Si$	2	92.169	122.2	0.30	1.1E-04	6.03	-															■		1
2-propanol	C_3H_8O	3	60.096	82.6	63200	7.0	0.88	4.11E-05	■			■		■		■	■					■			6
1-propanol	C_3H_8O	3	60.096	97.0	4.1E-02	6E-06	6.83	7.05E-06	■	■		■	■	■		■	■		■		■	■			10
Glycerin	$C_3H_8O_3$	3	92.094	290.0	1.0E+03	0.243	-0.92	4.297E-08							■										1
[S-(R*,R*)]-2,3-butanediol	$C_4H_{10}O_2$	4	77.4	90.1	1.67	0.2163	2.62	-	■	■						■									3
2-butanol	$C_4H_{10}O$	4	74.123	100.0	5.9	0.928	2.03	1.35E-07	■		■														2
Isobutanol	$C_4H_{10}O$	4	74.122	108.0	Insoluble	0.531	2.11	9.87E-06					■												1
1-butanol	$C_4H_{10}O$	4	74.123	117.7	22.000	2.2	1.51	8.22E-06										■							1
1,4-butanediol	$C_4H_{10}O_2$	4	90.122	230.0	8.5E+04	10.4	0.76	1.30E-09					■												1
2-pentanol	$C_5H_{12}O$	5	88.15	119.3	1.0E+06	21.0	0.25	1.41E-05	■	■								■		■	■				5
2-methyl-1-butanol	$C_5H_{12}O$	5	88.15	128.7	-	-	-	2.30E-05												■					1
3-methyl-3-buten-1-ol	$C_5H_{10}O$	5	86.132	130.0	-	-	-	-										■							1
Isoamyl alcohol	$C_5H_{12}O$	5	88.148	132.0	181	18.3	0.61	1.41E-05	■							■									2
1-pentanol	C5H12O	5	88.15	138.0	-	-	-	1.30E-05						■											1
4-methyl-3-penten-1-ol	$C_6H_{12}O$	6	100.159	156.0	30	3.13	-	-	■											■					2
1-hexanol	$C_6H_{14}O$	6	102.177	157.0	-	-	-	1.71E-05						■											1
4-methyl-1-pentanol	$C_6H_{14}O$	6	102.175	160.0	-	-	-	-				■													1
Cyclohexanol	$C_6H_{12}O$	6	100.159	161.0	4095	1.028	2.24	7.60E-06									■								1
2-(2-ethoxyethoxy)-ethanol	$C_6H_{14}O_3$	6	134.175	202.0	0.07	-	-	-						■											1

Carbitol	$C_6H_{14}O$	6	134.174	202.0	-	-	-	-									■						1
2,2'-oxybis-1-propanol	$C_6H_{14}O_3$	6	134.175	234.2	1590	0.159	2.97	-						■									1
3-heptanol	$C_7H_{16}O$	7	183.204	156.7	44.6	1	1.19	8.97E-06		■													1
4-heptanol	$C_7H_{16}O$	7	116.20	157	1.0E+7	45.4	0.05	7.59E-06									■						1
1-heptanol	$C_7H_{16}O$	7	116.204	175.8	1379	0.512	2.721	2.60E-05												■			1
1-octen-3-ol	$C_8H_{16}O$	8	128.215	85.0	540	7.94E-2	3	7.59E-05	■				■										2
3-octanol	$C_8H_{18}O$	8	130.23	174.0	3.51E+04	10.245	1.098	9.87E-06										■					1
2-ethyl-1-hexanol	$C_8H_{18}O$	8	130.231	184.0	-	-	-	2.60E-05										■					1
1-Octanol	$C_8H_{18}O$	8	130.231	188.0	4700	0.99	2.22	4.70E-05														■	1
Benzeneethanol	$C_8H_{10}O$	8	122.25	221.0	-	-	-	2.56E-07						■									1
Phenethanol	$C_8H_{10}O$	8	222.164	221.0	-	-	-	2.56E-07										■					1
Methyl salicylate	$C_8H_8O_3$	8	152.147	222.0	5.30E+06	2.37	1.16	-											■				1
1-Nonanol	$C_9H_{20}O$	9	144.258	214.0	-	-	-	-					■										1
Exo-fenchol	$C_{10}H_{18}O$	10	154.25	201.0	-	-	-	-									■						1
Linalool	$C_{10}H_{18}O$	10	154.249	82.5	738	5.02E-02	3.24	4.94E-05				■											1
Menthol	$C_{10}H_{20}O$	10	156.265	105.0	140	0.0227	3.77	1.50E-05									■						1
Beta-fenchol	$C_{10}H_{18}O$	10	152.249	202.9	175.4	-	3.7	-						■									1
1,7,7-trimethyl-(1S-endo)-Bicyclo[2,2,1]heptan-2-ol	$C_{10}H_{18}O$	10	154.00	210.0	-	-	-	-												■			1
Alpha-terpineol	$C_{10}H_{18}O$	10	154.25	213.0	7100	0.04	2.98	2.23E-06		■													1
Iso-borneol	$C_{10}H_{18}O$	10	154.25	214.0	Insoluble	0.069	2.55	-							■								1
2-pinen-4-ol	$C_{10}H_{16}O$	10	152.237	214.9	1.00E+06	0.126	-0,54	-									■						1
Levomenthol	$C_{10}H_{20}O$	10	156.265	216.0	-	-	-	-												■			1
Isomenthol	$C_{10}H_{20}O$	10	156.265	219.0	-	0.657	1.23	-					■			■							2
Citronellol	$C_{10}H_{20}O$	10	156.265	222.0	-	-	-	-			■						■						2
dihydrocarveol	$C_{10}H_{18}O$	10	154.249	225.0	2.67E+04	2.37	1.16	-														■	1
2-decen-1-ol	$C_{10}H_{20}O$	10	156.269	230.3	-	-	-	-	■														1
9-decen-1-ol	$C_{10}H_{20}O$	10	156.265	235.0	490	0.08	3.4	-									■						1
4-(1,1-dimethylethyl)-cyclohexanol	$C_{10}H_{20}O$	10	156.262	214.2	-	-	-	-												■			1
2-undecanol	$C_{11}H_{24}O$	11	172.312	228.0	-	-	-	-	■							■		■					3
Trans-2-dodecen-1-ol	$C_{12}H_{24}O$	12	184.318	283.3	1000	127	-0.77	-														■	1
(E)-2-Tridecen-1-ol	$C_{13}H_{28}O$	13	198.345	283.0	420	7.67E-03	3.4	-			■			■	■	■		■		■		■	7

1-tetradecanol	$C_{14}H_{30}O$	14	214.393	289.0	-	0.0343	2.5	-															1
T-cadinol	$C_{15}H_{26}O$	15	222.366	140.0	880	0.136	2.73	-															2
epicubenol	$C_{15}H_{26}O$	15	222.366	302.0	-	-	-	-															3
1-hexadecanol	$C_{16}H_{34}O$	16	242.447	344.0	-	-	-	-															1
trans-9-hexadecen-1-ol	$C_{16}H_{32}O$	16	210.424	388.7	-	-	-	-															1
Aldehydes																							
Acetaldehyde	C_2H_4O	2	44.053	20.8	612.7	0.552	2.809	2.41E-08															1
Propanal	C_3H_6O	3	58.079	49.0	Insoluble	0.001	4.866	`															1
Butanal	C_4H_8O	4	72.106	76.0	Insoluble	1.823	2.3	-															1
2-methylbutanal	$C_5H_{10}O$	5	86.132	94.0	1.12E+04	49.317	1.267	4.30E-4															1
3-methlybutanal	$C_5H_{10}O$	5	86.132	103.0	Slightly soluble	50	1.23	-															1
Pentanal	$C_5H_{10}O$	5	86.132	103.0	Insoluble	4.884	0.51	-															1
3-Furaldehyde	C_5H_4O	5	96.08	144.0	Insoluble	0.00002	5.037	-															1
Furfural	C_5H_4O	5	96.084	162.0	6950	1.27	1.48	3.80E-06															2
Hexanal	$C_6H_{12}O$	6	100.159	131.0	Insoluble	0.644	0.67	3.10E-03															1
5-methyl-2-Furancarboxaldehyde	C_6H_6O	6	110.11	187.0	7.41E+04	2.21	0.41	2.6															1
(Z)-2-Heptenal,	$C_7H_{12}O$	7	112.17	85.6	Insoluble	0.067	3.701	-															1
Heptanal	$C_7H_{14}O$	7	114.186	154.0	1.0E+06	902	-0.34	2.99E-03															1
Benzaldehyde	C_7H_6O	7	106.122	178.0	Insoluble	0.02	4.989	-															1
(E)-2-Octenal	$C_8H_{14}O$	8	126.2	86.0	Insoluble	1.80	-	-															1
2-ethyl-hexanal	$C_8H_{16}O$	8	128.21	163.0	-	0.39	1.78	8.22E-03															2
Octanal	$C_8H_{16}O$	8	128.212	171.0	7.1E+04	111	0.88	4.70E-03															1
Benzeneacetaldehyde	C_8H_8O	8	120.148	196.0	1.25	3.52	2.29	-															1
Piperonal	C_8H_6O	8	150.131	264.0	5.6E+02	-	3.5	5.48E-07															1
Vanillin	C_8H_8O	8	152.147	285.0	3.06E+05	317	0.59	2.10E-09															1
(E)-2-Nonenal,	$C_9H_{16}O$	9	140.22	88.0	Insoluble	0.256	3.319	-															1
Nonanal	$C_9H_{18}O$	9	142.239	216.9	Insoluble	0.001	7.027	7.34E-04															1
Decanal	$C_{10}H_{20}O$	10	156.265	209.0	-	11.3	1.78	2.30E-03															1
(Z)-2-decenal	$C_{10}H_{18}O$	10	154.249	227.6	Insoluble	0.005	4.216	-															6
Dodecanal	$C_{12}H_{24}O$	12	184.318	270.7	11700	-	1.31	-															1
Hexadecanal	$C_{13}H_{32}O$	13	240.43	297.0	Insoluble	0.006	6.008	-															1

Tetradecanal	$C_{14}H_{28}O$	14	212.372	260.0	96	3.7E-01	3.27	-															1
Lilial	$C_{14}H_{20}O$	14	204.308	275.0	-	1.0E-02	1.05	-															1
2-(phenylmethylene)-octanal	$C_{15}H_{20}O$	15	216.32	176.0	1.56E-03	0.103	3.76	-															1
7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	$C_{18}H_{26}O$	18	258.405	361.9	1.10E+04	1.18E-04	1.21	-															1
Ketones																							
2-butanone	C_4H_8O	4	72.106	80.0	-	-	-	1.22E-06															8
2,3-butanedione	$C_4H_6O_2$	4	86.089	88.0	-	-	1.43	1.35E-05															1
3-hydroxy-2-butanone	$C_4H_8O_2$	4	88.105	148.0	20	4.1E-02	4.09	-															2
1,4-Dioxane-2,6-dione	$C_4H_4O_4$	4	116.072	240.0	-	-	0.4	-															1
3-methyl-2-butanone	$C_5H_{10}O$	5	86.132	95.0	-	-	-	-															1
3-methyl-3-buten-2-one	C_5H_8O	5	84.116	98.0	-	-	-	-															1
2-pentanone	$C_5H_{10}O$	5	86.132	102.2	-	-	-	8.36E-05															5
2,3-pentanedione	$C_5H_8O_2$	5	100.12	112.0	2.0E+05	56.8	-1.34	-															1
(E)-3-Penten-2-one,	C_5H_8O	5	84.116	121.0	76.8	0.27	3.73	-															2
Cyclopentanone	C_5H_8O	5	84.116	131.0	15.5	18.11	1.331	1.20E-05															2
Acetylacetone	C_5H_8O	5	100.116	141.0	-	1.35	2.37	5.81E-06															1
Acetone	C_3H_6O	6	58.079	56.0	367	0.115	3.07	1.23E-07															3
2-methyl-3-pentanone	$C_6H_{10}O$	6	100.159	1160	Slightly soluble	0.5	-0.321	1.52E-03															1
4-methyl-2-pentanone	$C_6H_{12}O$	6	100.159	117.0	Insoluble	-	-0.85	4.30E-09															1
4-hexanone	$C_6H_{12}O$	6	100.159	118.0	-	-	4.42	-															1
2-methyl-1-penten-3-one	$C_6H_{10}O$	6	98.143	118.5	Insoluble	2.029	2.66	-															3
3-methyl-2-pentanone,	$C_6H_{12}O$	6	100.159	119.0	2.23E+05	90.6	0.29	-															7
3-hexanone	$C_6H_{12}O$	6	100.159	123.0	4.28E+03	3.85	1.98	-															3
2-hexanone	$C_6H_{12}O$	6	100.159	127.0	-	11.6	1.38	-															1
2,3-hexanedione	$C_6H_{10}O$	6	114.142	128.0	-	-	-	-															1
2-methylcyclopentanone	$C_6H_{12}O$	6	98.143	139.0	0.371	0.62	3.14	-															3
4-methyl-3-hexanone	$C_7H_{14}O$	7	114.186	138.7	-	-	-	-															1
4-heptanone	$C_7H_{14}O$	7	114.186	143.0	4.3E+04	35.4	0.91	-															3
5-methyl-2-hexanone,	$C_7H_{14}O$	7	114.186	144.0	Insoluble	0.01	5.053	-															1
3-heptanone	$C_7H_{14}O$	7	114.186	149.0	4.28E+03	3.85	1.98	8.97E-05															2
2-heptanone	$C_7H_{14}O$	7	114.186	150.0	-	2.7E+0	-0.36	1.67E-03															1

Trans-3,4-dimethylcyclopentanone	$C_7H_{12}O$	7	112.17	154.8	0.21	11.61	1.331	-															2
1-(2-aminophenyl)-ethanone	C_8H_9NO	8	135.163	255.0	-	-	-	-															1
5-methyl-2-heptanone	$C_8H_{16}O$	8	128.212	159.9	42	-	-	-															1
3-methyl-4-heptanone	$C_8H_{16}O$	8	128.21	159.9	Insoluble	2.444	2.35	-															1
6-methyl-3-heptanone,	$C_8H_{16}O$	8	128.212	164.0	-	2.0	2.22	-															1
3-octanone	$C_8H_{16}O$	8	128.212	167.0	3.19E+03	6.12	2.04	-															1
6-methyl-2-Heptanone	$C_8H_{16}O$	8	128.212	167.0	-	-	-	-															1
6-methyl-5-hepten-2-one	$C_8H_{14}O$	8	126.196	172.0	3.19E+03	6.12	204	-															3
1-methoxy-4-methyl-benzene,	$C_8H_{10}O$	8	122.164	175.0	1900	19.9	1.31	-															1
Acetophenone	C_8H_8O	8	120.148	202.0	5400	5.77	1.88	8.97E-06															2
2-octanone	$C_8H_{16}O$	8	128.212	173.0	-	-	-	-															1
2-nonanone	$C_9H_{18}O$	9	142.239	195.0	-	-	-	3.67E-04															1
Benzyl methyl ketone	$C_9H_{10}O$	9	134.175	214.0	-	-	-	-															1
Cyclohexanone	$C_{10}H_{10}O$	10	98.143	154.0	Very slight	52.5	0.84	1.20E-05															1
Fenchone	$C_{10}H_{16}O$	10	152.233	192.0	-	-	-	-															1
Camphor	$C_{10}H_{16}O$	10	152.23	209.0	-	-	-	-															1
2-decanone	$C_{10}H_{20}O$	10	156.269	212.0	1.37E+03	2.75	2.15	4.70E-03															1
Trans-dyhydrcarvone	$C_{10}H_{16}O$	10	152.237	220.0	-	-	-	-															1
(-)-carvone	$C_{10}H_{14}O$	10	150.218	230.0	-	-	-	-															1
Piperitone	$C_{10}H_{16}O$	10	152.233	233.0	6.13	0.397	1.58	-															2
2-undecanone	$C_{11}H_{22}O$	11	170.292	231.0	1.0E+06	231	-0.24	6.17E-03															4
1-(2,3,4-trimethylphenyl)-ethanone	$C_{11}H_{14}O$	11	162.232	241.9	137	1.93E-03	3.18	-															1
5-heptyldihydro-2(3H)-furanone	$C_{11}H_{20}O$	11	184.275	331.4	73.14	0.82	3.52	-															1
1,1'-(1,3-phenylene)bis-ethanone	$C_{12}H_{18}N_8$	12	274.332	150.0	14.7	13.94	1.487	|-															3
6,10-dimethyl-, (E)-5,9-undecadien-2-one	$C_{13}H_{22}O$	13	194.31	254.0	2270	0.16	1.44	-															1
4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butanone-	$C_{13}H_{22}O$	13	194.131	263.5	1.6E+03	0.65	2.38	-															1
2-tridecanone	$C_{13}H_{26}O$	13	198.345	296.7	25000	4.33	0.81	1.47E-03															1
Benzophenone	$C_{13}H_{10}O$	13	182.218	305.0	9175	11.4	0.38	5.81E-07															1
13-methyl-oxacyclotetradecane-2,11-dione	$C_{14}H_{24}O_3$	14	240.343	191.4	-	-	-	-															3

2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	$C_{14}H_{20}O_2$	14	220.307	309.3	Insoluble	14.217	0.52	-									■						1
1,4-dioxacycloheptadecane-5,17-dione	$C_{15}H_{26}O$	15	270.365	388.3	-	-	-	-										■					1
7-acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	$C_{18}H_{26}O$	18	258.398	361.9	Insoluble	0.057	2.85	-	■														1
Esters																							
ethyl formate	$C_2H_4O_2$	2	60.05	31.5	2.16E-02	2.36E-05	6.5	2.41E-04											■				1
Thiocyanic acid, methyl ester	C_2H_3NS	2	73.117	131.1	Insoluble	6.866	1.806	4.49E-05												■			1
ethyl formate	$C_3H_6O_2$	3	74.078	54.3	356.7	2.906	2.76	2.90E-04		■			■	■	■	■		■		■	■		9
Acetic acid, methyl ester	$C_3H_6O_2$	3	74.078	58.0	-	-	-	6.17E-06							■								1
Ethyl acetate	$C_4H_8O_2$	4	88.11	77.1	-	216.2	0.18	1.34E-04	■		■		■		■		■			■		■	8
Methylpropionate	$C_4H_8O_2$	4	88.11	79.8	-	-	-	1.74E-04						■									1
Ethyl butyrate	$C_6H_{12}O_2$	4	116.16	120.0	Insoluble	10.34	2.823	-								■							1
Allyl Isothiocyanate	C_4H_5NS	4	99.15	148.2	290	0.68	3.333	2.41E-03										■			■		2
Ethylpropionate	$C_5H_{10}O_2$	5	102.132	98.9	Insoluble	0.002	4.405	-	■														1
Methyl butyrate	$C_5H_{10}O_2$	5	102.13	102.0	25	2.24E-04	3.97	2.67E-04					■										1
Propyl acetate	$C_5H_{10}O_2$	5	102.131	102.0	-	-	-	2.18E-04									■						1
Propyl propionate	$C_6H_{12}O_2$	6	116.16	122.0	Insoluble	0.23	3.842	-					■					■					2
Methyl pentanoate	$C_6H_{12}O_2$	6	116.16	126.0	Insoluble	4.42	2.314	-											■				1
n-butyl acetate	$C_6H_{12}O_2$	6	116.16	126.0	11.2	2.01E-05	4.5	-	■		■							■		■		■	5
Ethyl- (E)-crotonate	$C_6H_{10}O_2$	6	114.14	142.0	80000	93.2	0.73	-								■							1
Propanoyl propanoate	$C_6H_{10}O_3$	6	136.097	167.0	2.44E-02	9.35E-05	7.17	-											■				1
Ethyl caproate	$C_8H_{16}O_2$	6	144.21	168.0	1330	3.72	-	-										■					1
Propyl butyrate	$C_7H_{14}O_2$	7	130.187	143.0	4900	12.8	1.85	6.17E-04									■						1
Ethyl valerate	$C_7H_{14}O_2$	7	130.19	145.0	629	1.665	2.823	-							■								1
Butyl propionate	$C_7H_{14}O_2$	7	130.2	146.0	70.1	0.224	3.842	-									■						1
Pentyl acetate	$C_7H_{14}O_2$	7	130.187	149.0	Insoluble	4.269	2.225	3.88E-04									■						1
Methyl caproate	$C_7H_{14}O_2$	7	130.187	149.8	2210	4.80	2.314	3.67E-04				■											1
Ethyl tiglate	$C_7H_{12}O_2$	7	128.171	154.0	88250	200	0.23	-		■								■	■		■		4
Butanoic acid, 2-methylpropyl ester	$C_8H_{16}O_2$	8	144.211	158.0	19200	35.8	1.21	-						■									1
Butyl butyrate	$C_8H_{16}O_2$	8	144.21	165.0	Insoluble	6.04E-05	7.38	-									■						1
Propyl valerate	$C_8H_{16}O_2$	8	144.214	167.0	1.5E+05	32.3	1.29	-			■											■	2
Methyl heptanoate	$C_8H_{16}O_2$	8	144.214	171.0	2.3E+05	585.7	0.03	-												■			1

n-amyl butyrate	$C_9H_{18}O_2$	9	158.241	186.0	Insoluble	1.430	2.823	-															2
Propyl caproate	$C_9H_{18}O_2$	9	158.241	186.0	5.06	-	1.96	-															5
Ethyl heptanoate	$C_9H_{18}O_2$	9	158.238	188.3	62.37	84.04	0.82	4.93E-04															1
Butanoic acid, 1-methylbutyl ester	$C_9H_{18}O_2$	9	158.24	208.0	8.33E+03	11.5	1.78	-															1
Butyl caproate	$C_{10}H_{20}O_2$	10	172.27	61.0	Insoluble	0.048	3.507	-															1
Ethyl caprylate	$C_{10}H_{20}O_2$	10	172.268	207.5	Insoluble	0.569	3.32	-															3
2-Propenoic acid, 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluorooctyl ester	$C_{11}H_5F_{15}O_2$	11	225.084	454.1	Insolube.	1.6	2.823	-															1
4-terpinenyl acetate	$C_{12}H_{20}O_2$	12	196.286	64.9	Insoluble	0.039	3.96	-															1
alpha-terpinyl acetate	$C_{12}H_{20}O_3$	12	196.29	220.0	-	-	-	-															1
n-butyric acid 2-ethylhexyl ester	$C_{12}H_{24}O_2$	12	200.322	231.9	1.73E+03	3.5	2.3	-															1
Benzeneacetic acid, 2-phenylethyl ester	$C_{12}H_{16}O_2$	12	240.297	359.9	18900	35.9	1.24	-															1
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	$C_{12}H_{24}O_3$	12	216.317	365.2	Insoluble	5.96	2.314	-															1
Benzoic acid, 2-hydroxy-, 2-methylbutyl ester	$C_{12}H_{16}O_3$	12	208.25	384.0	-	-	-	-															1
Benzyl benzoate	$C_{14}H_{12}O_2$	14	212.24	323.6	-	-	-	5.48E-06															3
Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	$C_{14}H_{28}O_3$	14	244.37	-	3.22E+04	12.1	0.502	-															1
1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O$	16	278.344	327.0	6.2	4.76E-05	4.11	-															1
Dibutyl phthalate	$C_{16}H_{22}O_4$	16	278.34	340.0	1.02E+05	1.36	0.4	1.061E-06															6
Isopropyl myristate	$C_{17}H_{34}O_2$	17	270.45	167.0	2000	3.70	2.15	-															1
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	17	270.45	332.1	-	-	-	-															3
1,2-benzenedicarboxylic acid, butyl 2-ethylhexyl ester	$C_{20}H_{30}O$	20	334.45	391.2	5.3	13.94	1.804	-															1
Hydrocarbons																							
Pentane	C_5H_{12}	5	72.15	36.1	Insoluble	0.36	4	2.14548															3
3-methylpentane	C_6H_{14}	6	86.178	63.0	-	-	-	1.70159															1
Benzene	C_6H_6	6	80.1	78.1	-	-	3.47	1.86E-10															2
Toluene	C_7H_8	7	92.14	110.6	1.12E-03	5.40	5.15	2.41E-03															8
Octane	C_8H_{18}	8	114.23	125.0	-	-	-	3.18E-03															1

m-xylene	C_8H_{10}	8	106.17	135.9	-	-	-	7.05E-03					■											1
Ethylbenzene	C_8H_{10}	8	106.17	136.0	Insoluble	1.501	4.732	1.70E-03	■								■							2
p-xylene	C_8H_{10}	8	106.16	138.4	1.384	2.8	-	5.19E-03										■						1
Dimethylbenzene	C_8H_{10}	8	106.16	140.0	-	-	-	1.06E-03			■			■						■				3
o-xylene	C_8H_{10}	8	106.16	141.2	17.9	190	3.6	9.87E-05							■						■			2
1-nonene	C_9H_{18}	9	126.243	147.0	3.90	0.0022	6.31	8.22E-03							■									1
alpha-pinene	$C_{10}H_{16}$	10	136.23	156.9	Insoluble	3.1E-02	6.392	-	■		■		■	■	■	■	■	■			■	■		10
3,7-dimethyl-, (Z)-2-octene	$C_{10}H_{20}$	10	140.27	158.7	2.49	4.75	4.83	-	■				■								■	■		4
3,7-dimethyl-2-octene	$C_{10}H_{20}$	10	140.27	158.7	1.79E+03	94.8	2.13	-			■													1
p-cymene	$C_{10}H_{14}$	10	134.21	161.9	0.05011	0.0130	6.3	7.59E-03					■				■							2
Sabinene	$C_{10}H_{16}$	10	136.23	163.0	2.452	1.59	4.386	6.17E-03						■										1
Beta-pinene	$C_{10}H_{16}$	10	136.238	165.0	4.89	2.93	4.16	-									■							1
Delta-3-carene	$C_{10}H_{16}$	10	136.23	170.0	7.48	8.93E-03	4.01	-												■				1
1-decene	$C_{10}H_{20}$	10	140.27	170.6	-	-	-	-					■										■	2
Beta-phelandrene	$C_{10}H_{16}$	10	136.238	171.0	Insoluble	3.72	4.38	-								■								1
1,2,3,4-tetramethyl-benzene	$C_{10}H_{14}$	10	134.22	173.9	-	-	-	-								■								1
Decane	$C_{10}H_{22}$	10	142.29	174.1	Insoluble	0.198	4.57	2.30E-05				■												1
1-p-menthene	$C_{10}H_{18}$	10	138.25	174.5	5.20E-02	1.43	5.01	-	■						■									2
D-limonene	$C_{10}H_{16}$	10	136.24	174.9	3.7E-03	0.135	6.1	-									■							1
Limonene	$C_{10}H_{16}$	10	136.24	176.0	1.9E-03	4.62E-06	4.57	1.67E-03	■	■		■	■	■	■	■	■	■		■	■			■	12
o-cymene	$C_{10}H_{14}$	10	134.22	178.0	170	9.6	3.15	1.10E-03	■															1
1-methyl-4-(1-methylethenyl)-benzene	$C_{10}H_{12}$	10	132.20	186.0	Insoluble	1.0E-02	6.352	-			■		■								■			3
1,2,3,5-tetramethyl-benzene,	$C_{10}H_{14}$	10	134.22	187.9	2.3E-03	2.28E-04	8.69	-						■		■								2
Naphthalene	$C_{10}H_8$	10	128.17	218.0	7.57	1.55	4.57	8.97E-05	■	■		■		■										4
Undecane	$C_{11}H_{24}$	11	156.31	172.9	-	-	-	18.2764						■						■				2
2-methoxy-naphthalene	$C_{11}H_{10}O$	11	158.2	272.0	23.4	1.50	4.1	-		■				■		■				■			■	5
Acenaphthene	$C_{12}H_{10}$	12	154.2	934	-	-	-	1.37E-03							■									1
Dodecane	$C_{12}H_{26}$	12	170.34	216.2	2.1E-05	1.49E-03	8.2	7.59E-03										■						1
Biphenyl	$C_{12}H_{10}$	12	154.212	2550	23.3	1.5	4.38	4.70E-30	■	■													■	3
Tridecane	$C_{13}H_{28}$	13	184.37	234.0	31	0.085	3.3	12.50E-02													■			1
1,3-bis(1-1-dimethylethyl)-benzene	$C_{14}H_{22}$	14	190.324	106.6	0.115	1.67	5.7	-		■								■						2
Beta-caryophyllene	$C_{15}H_{24}$	15	204.36	130.0	160	8.29	3.2	-				■							■					2

alpha-longifolene	$C_{15}H_{24}$	15	204.357	254.0	0.66	14.1	5.18	-			■								■			■		3
Caryophyllene	$C_{15}H_{24}$	15	204.36	262.0	1.62E+02	8.84	3.15	-	■	■				■		■						■		5
Gamma-cadinene	$C_{15}H_{24}$	15	204.36	271.0	1.78E+02	6.61	3.12	-	■	■							■					■		3
Delta-cadinene	$C_{15}H_{24}$	15	204.36	279.0	38	514	3.39	-			■													1
Hexadecane	$C_{16}H_{34}$	16	226.41	286.8	0.135	4.5E-06	4.88	3.80E-03								■								1
Pyrene	$C_{16}H_{10}$	16	202.25	378.2	526	28.4	2.73	1.32E-05	■		■			■			■	■		■				6
Heptadecane	$C_{17}H_{36}$	17	240.475	302.0	Insoluble	2.633	3.94	4.49E-02										■						1
(1-propyloctyl)-benzene	$C_{17}H_{28}$	17	232.4	341.5	0.0047	0.0375	6.73	-	■			■												2
(1-pentylheptyl)-benzene	$C_{18}H_{30}$	18	246.43	364.3	-	-	-	-														■		1
3-methyl-heptadecane	$C_{18}H_{38}$	18	254.502	313.4	6.44E-08	4.07E-06	-	-							■									1
Eicosane	$C_{20}H_{42}$	20	282.56	34.1	-	-	-	1.97E-03						■										1
Tetracosane	$C_{24}H_{50}$	24	338.65	391.0	0.0044	0.412	5.74	-			■				■									2
Miscellaneous																								
Nitrous oxide	N_2O	0	162.013	245.1	-	400	-	0.04E-04											■					1
Ethylene oxide	C_2H_4O	2	110.56	113.0	Insoluble	1.2	2.7	1.70E-03									■							1
Isothiocyanato-methane	C_2H_3NS	2	147.01	177.9	3.1	2.48E-03	4.12	-			■													1
Tetrahydrofuran	C_4H_8O	4	70.091	54.6	156	1.36	3.43	7.05E-05	■			■											■	3
2,3-dyhydrofuran	C_4H_6O	4	122.167	170.0	-	-	-	-			■				■		■							3
Gamma-butyrolactone	$C_4H_6O_2$	4	154.253	176.0	0.0112	2.01E-05	4.5	-					■				■							2
Nickel tetracarbonyl	C_4NiO_4	4	131.175	215.4	-	3.53	-	-												■				1
1,3-dichloro-benzene	$C_6H_4Cl_2$	6	147.01	173.0	125	2.15	3.53	-	■			■			■		■							4
2,4-dichloro-benzenamine	$C_6H_5Cl_2N$	6	96.13	92.0	3500	1.90	2.74	-		■					■									2
1,2-dichloro-benzene	$C_6H_4Cl_2$	6	327.57	180.0	-	1.31	-0.3	-								■								1
Hexamethylcyclotrisiloxane	$C_6H_{18}O_3Si_3$	6	168.19	285.0	0.567	4.29E+04	0.36	1.76E-03				■												1
2,5-dimethyl-furan	C_6H_8O	6	146.14	298.1	0.033	1.05	6.74	-									■							1
1-nitrohexane	$C_6H_{13}NO_2$	6	222.37	324.8	-	-	-	2.19E-03							■									1
Hexyl-hydroperoxide	$C_6H_{14}O_2$	6	278.34	340.0	1000.0	1.62E+02	0.46	-				■												1
2-propyl-furan	$C_7H_{10}O$	7	44.05	115.0	Insoluble	249.98	-0.034	-	■															1
Octamethyl-cyclotetrasiloxane	$C_8H_{24}O_4Si_4$	8	118.176	182.7	1000.0	4.5E-01	-0.64	-				■					■						■	3
1-methoxy-4-methylbenzene,	$C_8H_{10}O$	8	86.08	204.0	-	3.53	-	-						■							■			1
Coumarin	$C_9H_6O_2$	9	73.11	117.0	Insoluble	53	2.24	9.87E-08				■									■			2
2-propanol, 1-chloro-, phosphate	$C_9H_{18}Cl_3O_4P$	9	222.46	133.9	1.6E+10	9.23E-03	2.59	-											■					1

Trans-anethole	$C_{10}H_{12}O$	10	44.013	89.0	-	-	-	-						■										1
Methyltris(trimethylsiloxy)silane	$C_{10}H_{30}O_3Si_4$	10	170.733	43.0	-	-	-	-			■								■					2
1,8-cineole	$C_{10}H_{18}O$	10	310.687	235.0	7.6E+03	3.54	0.94	1.67E-03		■														1
Tetraglyme	$C_{10}H_{22}O_5$	10	432.6	440.3	1.11E-01	0.07	-	-						■				■						2
Dibenzofuran	$C_{12}H_8O$	12	296.616	17.0	-	9.8E-04	1.39	1.41E-05														■		1
Ambrox	$C_{15}H_{26}O$	15	222.281	275.0	-	-	-	4.93E-03								■								1
Dibutyl phthalate	$C_{16}H_{22}O_4$	16	72.11	66.0	527.1	2.0	2.66	1.06E-06					■											1
o-(4-methoxybenzoyl)-o'-(2,2,3,3,4,4,4-heptafluorobutyryl)-1,2-benzenediol	$C_{18}H_{11}F_7O_5$	18	148.205	235.6	-	0.04	-	-						■										1

Appendix 3

Table 5S1: Results of ANOVA indicating the statistical significance of main factors and their interactions

Compound	Statistic	Main factors				Interactions					
		A	B	C	D	AB	AC	AD	BC	BD	CD
Butyric acid	F-ratio	105.340	11.040	0.010	151.040	5.640	0.520	43.720	0.280	7.050	1.870
	p-value	0.000	0.003	0.904	0.000	0.026	0.477	0.000	0.600	0.014	0.183
DMTS	F-ratio	0.040	0.060	0.080	1.230	0.210	3.040	2.700	0.390	0.290	0.040
	p-value	0.835	0.810	0.785	0.277	0.649	0.093	0.113	0.537	0.597	0.840
Indole	F-ratio	762.490	70.360	0.170	1205.340	5.170	1.480	187.300	1.120	14.920	5.470
	p-value	0.000	0.000	0.683	0.000	0.032	0.236	0.000	0.301	0.001	0.000
p-Cresol	F-ratio	605.160	50.770	5.800	1197.150	4.590	0.070	156.800	0.230	14.430	0.028
	p-value	0.000	0.000	0.024	0.000	0.042	0.801	0.000	0.636	0.001	0.950

Appendix 4

Analysis results generated using Minitab statistical software

Nonlinear Curve Fit (Slogistic1) (2019/03/20 20:22:07)

Parameters

		Value	Standard Error	t-Value	Prob>|t|	Dependency
B	a	1,16545	0,0563	20,70177	4,86933E-6	0,69859
	xc	16,5884	0,57914	28,64323	9,71715E-7	0,59035
	k	0,31792	0,04548	6,99039	9,22562E-4	0,46865
C	a	1,15681	0,05451	21,22303	2,91421E-5	0,71938
	xc	13,0826	0,55124	23,73319	1,86898E-5	0,61729
	k	0,31729	0,04259	7,45033	0,00173	0,48897
D	a	1,13155	0,02524	44,83497	1,0421E-7	0,60158
	xc	13,08813	0,31878	41,05673	1,61669E-7	0,47158
	k	0,30218	0,02378	12,70839	5,36368E-5	0,38152
E	a	1,10098	0,04892	22,50346	3,22042E-6	0,58931
	xc	13,36615	0,62095	21,52527	4,01393E-6	0,45815
	k	0,31411	0,05017	6,26034	0,00153	0,37088

Reduced Chi-sqr = 0.00331343343355
COD(R^2) = 0.98919334045465
Iterations Performed = 7
Total Iterations in Session = 7
All datasets were fitted successfully.
Standard Error was scaled with square root of reduced Chi-Sqr.
Some input data points are missing.

Statistics

	B	C	D	E
Number of Points	8	7	8	8
Degrees of Freedom	5	4	5	5
Reduced Chi-Sqr	0,00247	0,00206	8,65367E-4	0,00331
Residual Sum of Squares	0,01236	0,00822	0,00433	0,01657
R-Square (COD)	0,9923	0,99364	0,99716	0,98919
Adj. R-Square	0,98922	0,99046	0,99603	0,98487
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)

Fit Status Code :
100 : Fit converged. Chi-Sqr tolerance value of 1E-9 was reached.

Summary

	a		xc		k		Statistics	
	Value	Standard Error	Value	Standard Error	Value	Standard Error	Reduced Chi-Sqr	Adj. R-Square
B	1,16545	0,0563	16,5884	0,57914	0,31792	0,04548	0,00247	0,98922
C	1,15681	0,05451	13,0826	0,55124	0,31729	0,04259	0,00206	0,99046
D	1,13155	0,02524	13,08813	0,31878	0,30218	0,02378	8,65367E-4	0,99603
E	1,10098	0,04892	13,36615	0,62095	0,31411	0,05017	0,00331	0,98487

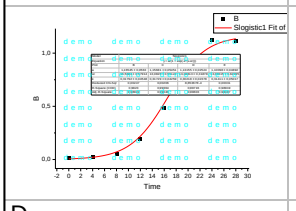
ANOVA

		DF	Sum of Squares	Mean Square	F Value	Prob>F
B	Regression	3	3,51393	1,17131	473,95239	1,47897E-6
	Residual	5	0,01236	0,00247		
	Uncorrected Total	8	3,52629			
	Corrected Total	7	1,6045			
C	Regression	3	3,2995	1,09983	535,02583	1,15773E-5
	Residual	4	0,00822	0,00206		
	Uncorrected Total	7	3,30772			
	Corrected Total	6	1,29343			
D	Regression	3	4,36611	1,45537	1681,7948	6,28044E-8
	Residual	5	0,00433	8,65367E-4		
	Uncorrected Total	8	4,37044			
	Corrected Total	7	1,52515			
E	Regression	3	4,08563	1,36188	411,0172	2,10854E-6
	Residual	5	0,01657	0,00331		
	Uncorrected Total	8	4,1022			
	Corrected Total	7	1,53305			

At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.

Fitted Curves

B



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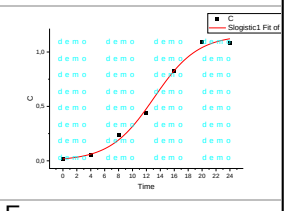

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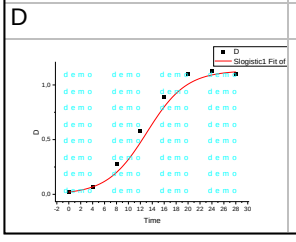

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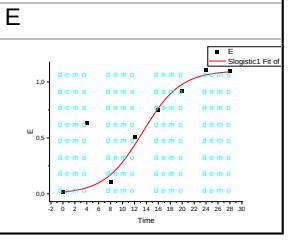
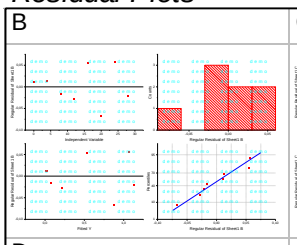


Residual Plots

B



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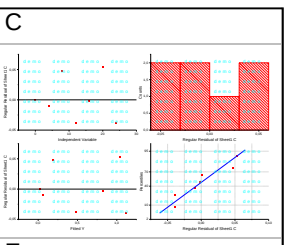

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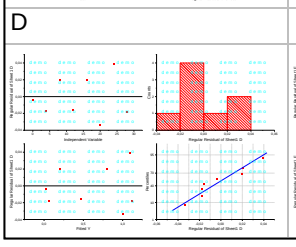

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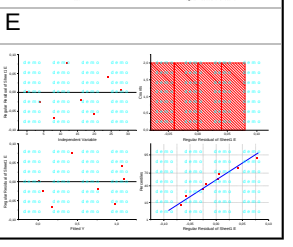


Nonlinear Curve Fit (SGompertz) (2019/03/20 20:22:37)

Parameters

		Value	Standard Error	t-Value	Prob>|t|	Dependency
B	a	1,23808	0,08852	13,98642	3,35949E-5	0,85617
	xc	14,88123	0,59914	24,83761	1,97352E-6	0,6177
	k	0,19728	0,0363	5,4341	0,00286	0,76193
C	a	1,26853	0,13933	9,10453	8,07191E-4	0,8968
	xc	11,44931	0,93216	12,28253	2,52376E-4	0,73215
	k	0,17679	0,04339	4,07428	0,01517	0,80628
D	a	1,19476	0,05103	23,41525	2,6446E-6	0,8014
	xc	11,17854	0,43757	25,54668	1,71604E-6	0,49445
	k	0,1833	0,02318	7,90687	5,20704E-4	0,70497
E	a	1,1451	0,04877	23,4776	2,60994E-6	0,76028
	xc	11,53264	0,42854	26,91117	1,32506E-6	0,42315
	k	0,20513	0,0282	7,2732	7,68254E-4	0,66424

Reduced Chi-sqr = 0.00178021195077
COD(R^2) = 0.99419389438286
Iterations Performed = 6
Total Iterations in Session = 6
All datasets were fitted successfully.
Standard Error was scaled with square root of reduced Chi-Sqr.
Some input data points are missing.

Statistics

	B	C	D	E
Number of Points	8	7	8	8
Degrees of Freedom	5	4	5	5
Reduced Chi-Sqr	0,00258	0,0041	0,00158	0,00178
Residual Sum of Squares	0,01292	0,01639	0,0079	0,0089
R-Square (COD)	0,99195	0,98733	0,99482	0,99419
Adj. R-Square	0,98873	0,98099	0,99275	0,99187
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)

Fit Status Code :
100 : Fit converged. Chi-Sqr tolerance value of 1E-9 was reached.

Summary

	a		xc		k		Statistics	
	Value	Standard Error	Value	Standard Error	Value	Standard Error	Reduced Chi-Sqr	Adj. R-Square
B	1,23808	0,08852	14,88123	0,59914	0,19728	0,0363	0,00258	0,98873
C	1,26853	0,13933	11,44931	0,93216	0,17679	0,04339	0,0041	0,98099
D	1,19476	0,05103	11,17854	0,43757	0,1833	0,02318	0,00158	0,99275
E	1,1451	0,04877	11,53264	0,42854	0,20513	0,0282	0,00178	0,99187

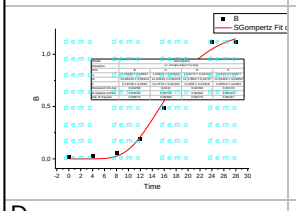
ANOVA

		DF	Sum of Squares	Mean Square	F Value	Prob>F
B	Regression	3	3,51337	1,17112	453,36454	1,65189E-6
	Residual	5	0,01292	0,00258		
	Uncorrected Total	8	3,52629			
	Corrected Total	7	1,6045			
C	Regression	3	3,29133	1,09711	267,73639	4,59653E-5
	Residual	4	0,01639	0,0041		
	Uncorrected Total	7	3,30772			
	Corrected Total	6	1,29343			
D	Regression	3	4,36253	1,45418	920,22186	2,82928E-7
	Residual	5	0,0079	0,00158		
	Uncorrected Total	8	4,37044			
	Corrected Total	7	1,52515			
E	Regression	3	4,0933	1,36443	766,44442	4,46432E-7
	Residual	5	0,0089	0,00178		
	Uncorrected Total	8	4,1022			
	Corrected Total	7	1,53305			

At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.

Fitted Curves

B



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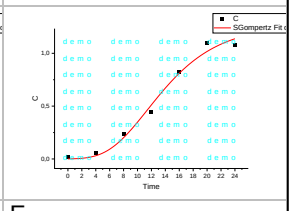

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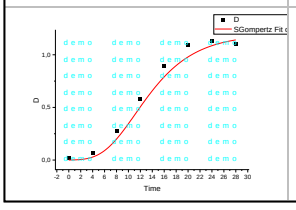

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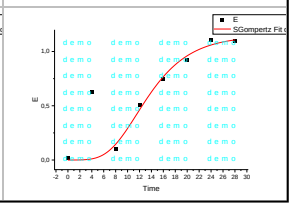
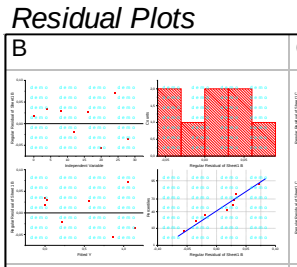
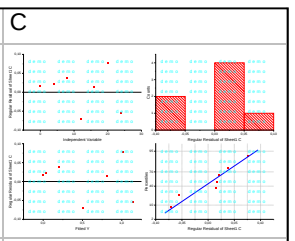


Residual Plots

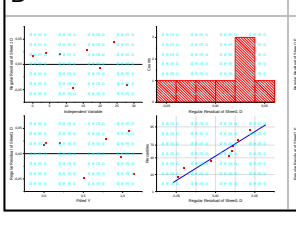
B



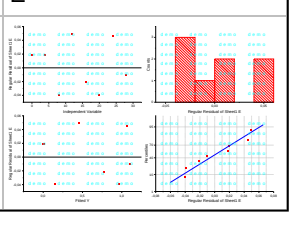
C



D



E



Nonlinear Curve Fit (SRichards2) (2019/03/20 20:23:11)

Parameters

		Value	Standard Error	t-Value	Prob>|t|	Dependency
B	a	1,18936	0,10491	11,33708	3,45103E-4	0,89243
	xc	15,96235	1,51167	10,55944	4,55048E-4	0,92922
	d	1,55454	0,9195	1,69064	0,16617	0,96743
	k	0,26306	0,13295	1,97864	0,11899	0,95418
C	a	1,10253	0,04025	27,39281	1,06778E-4	0,64532
	xc	14,7736	1,12661	13,11337	9,57877E-4	0,9354
	d	4,34892	2,49597	1,74238	0,1798	0,9872
	k	0,66142	0,38787	1,70524	0,1867	0,97768
D	a	1,13534	0,03738	30,37613	6,99665E-6	0,7729
	xc	12,95627	0,8974	14,4376	1,33785E-4	0,91713
	d	1,90369	0,58521	3,25298	0,03129	0,96065
	k	0,29027	0,07592	3,82329	0,01873	0,93183
E	a	1,15065	0,07941	14,48932	1,31914E-4	0,89215
	xc	11,26066	1,85905	6,05722	0,00375	0,96456
	d	0,88978	0,65615	1,35606	0,24658	0,97021
	k	0,19584	0,08191	2,39099	0,07509	0,95943

Reduced Chi-sqr = 0.00210233141973
COD(R^2) = 0.99451464944502
Iterations Performed = 42
Total Iterations in Session = 42
All datasets were fitted successfully.
Standard Error was scaled with square root of reduced Chi-Sqr.
Some input data points are missing.

Statistics

	B	C	D	E
Number of Points	8	7	8	8
Degrees of Freedom	4	3	4	4
Reduced Chi-Sqr	0,00294	0,00156	0,00107	0,0021
Residual Sum of Squares	0,01177	0,00468	0,0043	0,00841
R-Square (COD)	0,99267	0,99638	0,99718	0,99451
Adj. R-Square	0,98717	0,99276	0,99507	0,9904
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)

Fit Status Code :
100 : Fit converged. Chi-Sqr tolerance value of 1E-9 was reached.

Summary

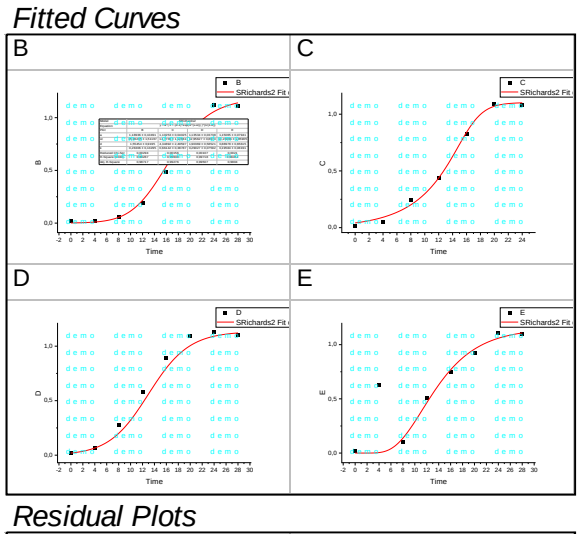
	a		xc		d		k		Statistics	
	Value	Standard Error	Value	Standard Error	Value	Standard Error	Value	Standard Error	Reduced Chi-Sqr	Adj. R-Square
B	1,18936	0,10491	15,96235	1,51167	1,55454	0,9195	0,26306	0,13295	0,00294	0,98717
C	1,10253	0,04025	14,7736	1,12661	4,34892	2,49597	0,66142	0,38787	0,00156	0,99276
D	1,13534	0,03738	12,95627	0,8974	1,90369	0,58521	0,29027	0,07592	0,00107	0,99507
E	1,15065	0,07941	11,26066	1,85905	0,88978	0,65615	0,19584	0,08191	0,0021	0,9904

ANOVA

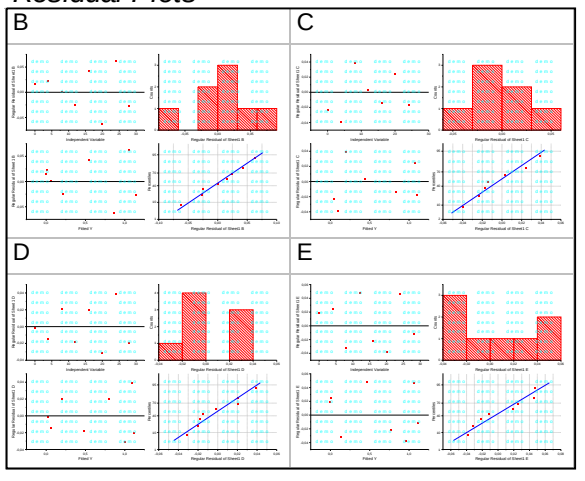
		DF	Sum of Squares	Mean Square	F Value	Prob>F
B	Regression	4	3,51452	0,87863	298,70887	3,33238E-5
	Residual	4	0,01177	0,00294		
	Uncorrected Total	8	3,52629			
	Corrected Total	7	1,6045			
C	Regression	4	3,30304	0,82576	528,90537	1,33098E-4
	Residual	3	0,00468	0,00156		
	Uncorrected Total	7	3,30772			
	Corrected Total	6	1,29343			
D	Regression	4	4,36614	1,09153	1015,77396	2,89993E-6
	Residual	4	0,0043	0,00107		
	Uncorrected Total	8	4,37044			
	Corrected Total	7	1,52515			
E	Regression	4	4,09379	1,02345	486,81576	1,25897E-5
	Residual	4	0,00841	0,0021		
	Uncorrected Total	8	4,1022			
	Corrected Total	7	1,53305			

At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.
At the 0.05 level, the fitting function is significantly better than the function y=0.

Fitted Curves



Residual Plots



Nonlinear Curve Fit (Slogistic1) (10/06/2019 18:51:55)

Parameters

		Value	Standard Error	t-Value	Prob>|t|	Dependency
B	a	1,10391	0,04729	23,34531	4,05156E-7	0,71223
	xc	16,60781	0,67265	24,69	2,90409E-7	0,6079
	k	0,23808	0,02944	8,08615	1,91715E-4	0,48111
C	a	1,26253	0,02932	43,06378	1,04942E-8	0,56846
	xc	13,84203	0,38859	35,62095	3,26357E-8	0,4343
	k	0,26947	0,02336	11,53657	2,55004E-5	0,35367
D	a	1,21509	0,03585	33,89143	4,39364E-8	0,50206
	xc	12,60668	0,50549	24,93933	2,73558E-7	0,36575
	k	0,28797	0,03532	8,15254	1,83192E-4	0,30049
E	a	1,229	0,04439	27,6881	1,46776E-7	0,67463
	xc	15,12476	0,59972	25,21983	2,55945E-7	0,55815
	k	0,23642	0,02663	8,87955	1,13572E-4	0,44658
F	a	1,07819	0,04647	23,20234	7,00803E-8	0,76466
	xc	20,06278	0,72811	27,55452	2,12826E-8	0,68011
	k	0,2064	0,02289	9,01599	4,21673E-5	0,53216

Reduced Chi-sqr = 0.00169234392661
COD(R^2) = 0.99205758265503
Iterations Performed = 7
Total Iterations in Session = 7
All datasets were fitted successfully.
Standard Error was scaled with square root of reduced Chi-Sqr.
Some input data points are missing.

Statistics

	B	C	D	E	F
Number of Points	9	9	9	9	10
Degrees of Freedom	6	6	6	6	7
Reduced Chi-Sqr	0,00214	0,00153	0,00287	0,00236	0,00169
Residual Sum of Squares	0,01286	0,00917	0,01722	0,01417	0,01185
R-Square (COD)	0,9916	0,99564	0,99132	0,99239	0,99206
Adj. R-Square	0,9888	0,99419	0,98843	0,98985	0,98979
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)

Fit Status Code :
100 : Fit converged. Chi-Sqr tolerance value of 1E-9 was reached.

Summary

	a		xc		k		Statistics	
	Value	Standard Error	Value	Standard Error	Value	Standard Error	Reduced Chi-Sqr	Adj. R-Square
B	1,10391	0,04729	16,60781	0,67265	0,23808	0,02944	0,00214	0,9888
C	1,26253	0,02932	13,84203	0,38859	0,26947	0,02336	0,00153	0,99419
D	1,21509	0,03585	12,60668	0,50549	0,28797	0,03532	0,00287	0,98843
E	1,229	0,04439	15,12476	0,59972	0,23642	0,02663	0,00236	0,98985
F	1,07819	0,04647	20,06278	0,72811	0,2064	0,02289	0,00169	0,98979

ANOVA

		DF	Sum of Squares	Mean Square	F Value	Prob>F
B	Regression	3	4,05773	1,35258	631,2867	6,88212E-8
	Residual	6	0,01286	0,00214		
	Uncorrected Total	9	4,07059			
	Corrected Total	8	1,52975			
C	Regression	3	6,5662	2,18873	1432,44589	5,92595E-9
	Residual	6	0,00917	0,00153		
	Uncorrected Total	9	6,57537			
	Corrected Total	8	2,10261			
D	Regression	3	6,6197	2,20657	768,92056	3,81581E-8
	Residual	6	0,01722	0,00287		
	Uncorrected Total	9	6,63692			
	Corrected Total	8	1,98379			
E	Regression	3	5,56529	1,8551	785,40607	3,58119E-8
	Residual	6	0,01417	0,00236		
	Uncorrected Total	9	5,57946			
	Corrected Total	8	1,86194			
F	Regression	3	3,87123	1,29041	762,49796	3,64708E-9
	Residual	7	0,01185	0,00169		
	Uncorrected Total	10	3,88307			
	Corrected Total	9	1,49154			

At the 0.05 level, the fitting function is significantly better than the function y=0.
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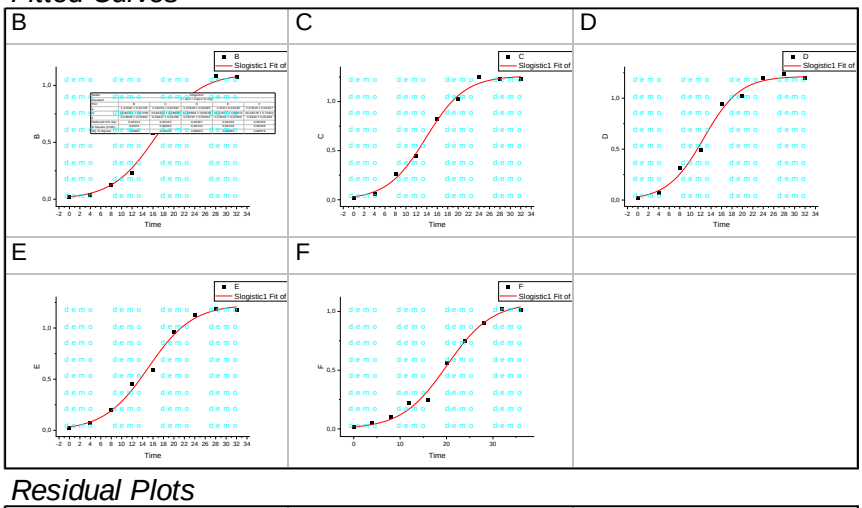
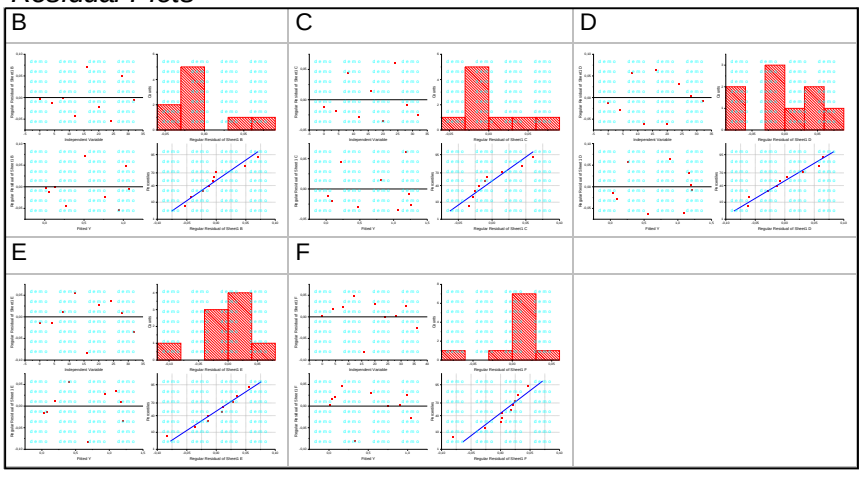
Fitted Curves



Residual Plots



Nonlinear Curve Fit (SGompertz) (10/06/2019 18:53:54)

Parameters

		Value	Standard Error	t-Value	Prob>|t|	Dependency
B	a	1,19476	0,07629	15,65995	4,29534E-6	0,87758
	xc	14,47924	0,7106	20,37605	9,08288E-7	0,67849
	k	0,14089	0,02146	6,56517	5,98413E-4	0,78376
C	a	1,32194	0,05834	22,66017	4,83592E-7	0,76939
	xc	11,60478	0,53828	21,55883	6,50022E-7	0,43732
	k	0,16557	0,02317	7,14561	3,78644E-4	0,67325
D	a	1,26302	0,05551	22,75243	4,72061E-7	0,70935
	xc	10,45727	0,56805	18,40892	1,65623E-6	0,35269
	k	0,17842	0,02792	6,3906	6,91055E-4	0,61683
E	a	1,31468	0,08816	14,91282	5,72249E-6	0,85511
	xc	12,7405	0,79513	16,02311	3,75387E-6	0,61802
	k	0,13945	0,02396	5,81909	0,00113	0,75946
F	a	1,21987	0,12912	9,44765	3,10755E-5	0,92507
	xc	17,97505	1,34312	13,38309	3,04832E-6	0,82108
	k	0,1102	0,02272	4,85037	0,00186	0,83796

Reduced Chi-sqr = 0.00324057809201
COD(R^2) = 0.9847914934777
Iterations Performed = 11
Total Iterations in Session = 11
All datasets were fitted successfully.
Standard Error was scaled with square root of reduced Chi-Sqr.
Some input data points are missing.

Statistics

	B	C	D	E	F
Number of Points	9	9	9	9	10
Degrees of Freedom	6	6	6	6	7
Reduced Chi-Sqr	0,00203	0,00295	0,00371	0,00362	0,00324
Residual Sum of Squares	0,01216	0,01767	0,02228	0,02172	0,02268
R-Square (COD)	0,99205	0,9916	0,98877	0,98833	0,98479
Adj. R-Square	0,9894	0,98879	0,98502	0,98444	0,98045
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)

Fit Status Code :
100 : Fit converged. Chi-Sqr tolerance value of 1E-9 was reached.

Summary

	a		xc		k		Statistics	
	Value	Standard Error	Value	Standard Error	Value	Standard Error	Reduced Chi-Sqr	Adj. R-Square
B	1,19476	0,07629	14,47924	0,7106	0,14089	0,02146	0,00203	0,9894
C	1,32194	0,05834	11,60478	0,53828	0,16557	0,02317	0,00295	0,98879
D	1,26302	0,05551	10,45727	0,56805	0,17842	0,02792	0,00371	0,98502
E	1,31468	0,08816	12,7405	0,79513	0,13945	0,02396	0,00362	0,98444
F	1,21987	0,12912	17,97505	1,34312	0,1102	0,02272	0,00324	0,98045

ANOVA

		DF	Sum of Squares	Mean Square	F Value	Prob>F
B	Regression	3	4,05843	1,35281	667,71911	5,81933E-8
	Residual	6	0,01216	0,00203		
	Uncorrected Total	9	4,07059			
	Corrected Total	8	1,52975			
C	Regression	3	6,5577	2,1859	742,22633	4,24116E-8
	Residual	6	0,01767	0,00295		
	Uncorrected Total	9	6,57537			
	Corrected Total	8	2,10261			
D	Regression	3	6,61463	2,20488	593,69792	8,26823E-8
	Residual	6	0,02228	0,00371		
	Uncorrected Total	9	6,63692			
	Corrected Total	8	1,98379			
E	Regression	3	5,55774	1,85258	511,64661	1,28947E-7
	Residual	6	0,02172	0,00362		
	Uncorrected Total	9	5,57946			
	Corrected Total	8	1,86194			
F	Regression	3	3,86039	1,2868	397,08848	3,53949E-8
	Residual	7	0,02268	0,00324		
	Uncorrected Total	10	3,88307			
	Corrected Total	9	1,49154			

At the 0.05 level, the fitting function is significantly better than the function y=0.
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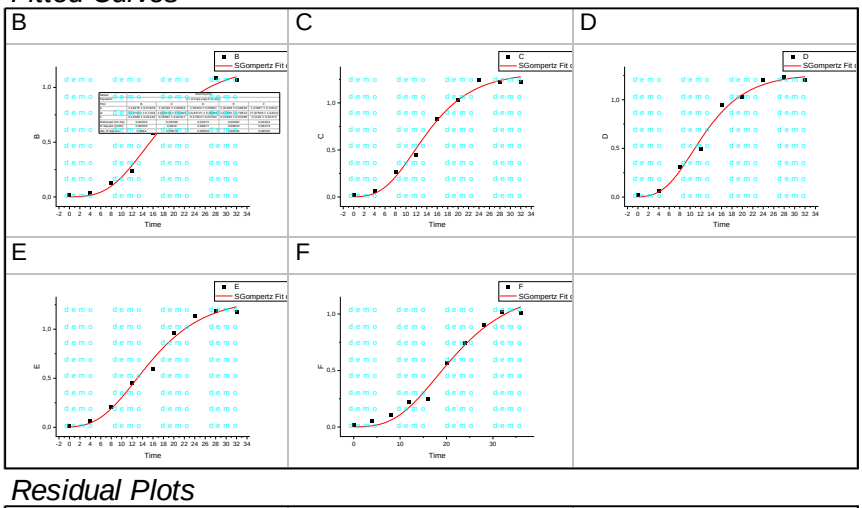
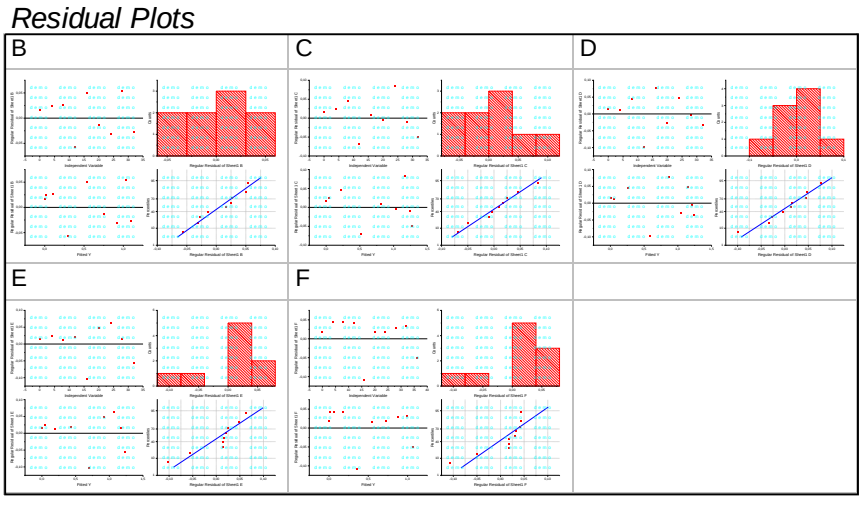
Fitted Curves



Residual Plots



Nonlinear Curve Fit (SRichards2) (10/06/2019 18:55:18)

Parameters

		Value	Standard Error	t-Value	Prob>|t|	Dependency
B	a	1,14654	0,09473	12,10268	6,80208E-5	0,91936
	xc	15,49856	1,70436	9,0935	2,6913E-4	0,93712
	d	1,39252	0,70181	1,98419	0,10402	0,97027
	k	0,17867	0,07543	2,36862	0,06406	0,96149
C	a	1,25519	0,03735	33,60839	4,38485E-7	0,69185
	xc	14,18846	1,07298	13,22337	4,4192E-5	0,91323
	d	2,23464	0,73304	3,04847	0,02847	0,96176
	k	0,29477	0,08443	3,49118	0,01745	0,92818
D	a	1,21596	0,04726	25,72671	1,65721E-6	0,65575
	xc	12,56482	1,50901	8,32652	4,08422E-4	0,91456
	d	1,97193	0,92152	2,13986	0,08533	0,95535
	k	0,28469	0,10884	2,6156	0,04735	0,91407
E	a	1,19633	0,04707	25,4174	1,75984E-6	0,70195
	xc	16,50141	1,3954	11,82556	7,61176E-5	0,91328
	d	3,05836	1,33536	2,29029	0,07062	0,97373
	k	0,34569	0,14493	2,38514	0,06276	0,95147
F	a	1,03408	0,04659	22,19612	5,46814E-7	0,7964
	xc	21,28767	1,3424	15,8579	3,98971E-6	0,91215
	d	3,01779	1,2094	2,49528	0,04683	0,97722
	k	0,30423	0,12117	2,51076	0,04586	0,9607

Reduced Chi-sqr = 0.00160074793654
COD(R^2) = 0.9935606765895
Iterations Performed = 9
Total Iterations in Session = 9
All datasets were fitted successfully.
Standard Error was scaled with square root of reduced Chi-Sqr.
Some input data points are missing.

Statistics

	B	C	D	E	F
Number of Points	9	9	9	9	10
Degrees of Freedom	5	5	5	5	6
Reduced Chi-Sqr	0,00223	0,00179	0,00344	0,00257	0,0016
Residual Sum of Squares	0,01117	0,00896	0,01722	0,01284	0,0096
R-Square (COD)	0,99269	0,99574	0,99132	0,9931	0,99356
Adj. R-Square	0,98831	0,99318	0,98612	0,98897	0,99034
Fit Status	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)	Succeeded(100)

Fit Status Code :
100 : Fit converged. Chi-Sqr tolerance value of 1E-9 was reached.

Summary

	a		xc		d		k		Statistics	
	Value	Standard Error	Value	Standard Error	Value	Standard Error	Value	Standard Error	Reduced Chi-Sqr	Adj. R-Square
B	1,14654	0,09473	15,49856	1,70436	1,39252	0,70181	0,17867	0,07543	0,00223	0,98831
C	1,25519	0,03735	14,18846	1,07298	2,23464	0,73304	0,29477	0,08443	0,00179	0,99318
D	1,21596	0,04726	12,56482	1,50901	1,97193	0,92152	0,28469	0,10884	0,00344	0,98612
E	1,19633	0,04707	16,50141	1,3954	3,05836	1,33536	0,34569	0,14493	0,00257	0,98897
F	1,03408	0,04659	21,28767	1,3424	3,01779	1,2094	0,30423	0,12117	0,0016	0,99034

ANOVA

		DF	Sum of Squares	Mean Square	F Value	Prob>F
B	Regression	4	4,05942	1,01485	454,07877	1,37936E-6
	Residual	5	0,01117	0,00223		
	Uncorrected Total	9	4,07059			
	Corrected Total	8	1,52975			
C	Regression	4	6,56641	1,6416	916,36459	2,3948E-7
	Residual	5	0,00896	0,00179		
	Uncorrected Total	9	6,57537			
	Corrected Total	8	2,10261			
D	Regression	4	6,6197	1,65493	480,65558	1,19711E-6
	Residual	5	0,01722	0,00344		
	Uncorrected Total	9	6,63692			
	Corrected Total	8	1,98379			
E	Regression	4	5,56662	1,39166	541,88694	8,87882E-7
	Residual	5	0,01284	0,00257		
	Uncorrected Total	9	5,57946			
	Corrected Total	8	1,86194			
F	Regression	4	3,87347	0,96837	604,94663	6,04157E-8
	Residual	6	0,0096	0,0016		
	Uncorrected Total	10	3,88307			
	Corrected Total	9	1,49154			

At the 0.05 level, the fitting function is significantly better than the function y=0.
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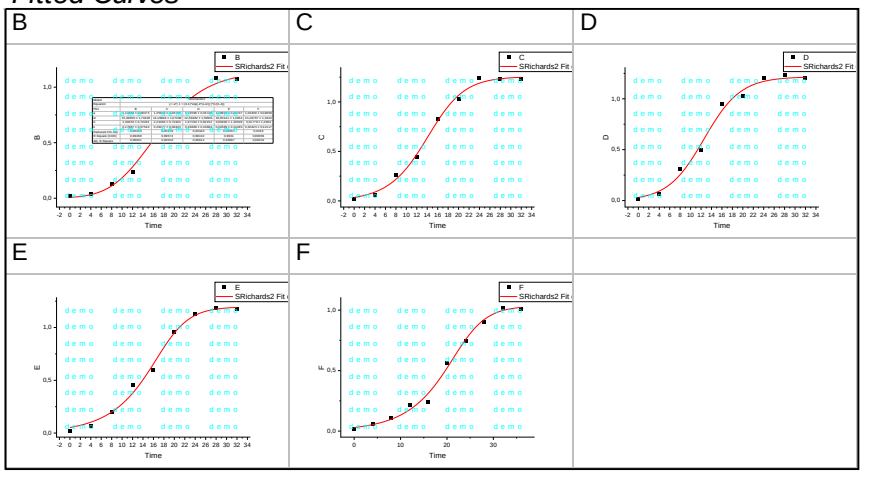
At the 0.05 level, the fitting function is significantly better than the function y=0.

Fitted Curves



Residual Plots