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Isolation of Butyric Acid-Degrading Bacterium, Serratia marcescen and its Potential for Bioremediation

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Biodegradation of butyric acid was investigated in mineral salt medium (MSM) with a novel bacterial strain B6a. The molecular identification based on 16S rRNA sequence analysis revealed the bacterial strain B6a as Serratia marcescen. The bacterial strain was able to grow in the MSM supplemented with 1,000 mgL^{-1} of butyric acid as a sole source of carbon and energy. At pH 7 and 30 ^oC under continuous shaking of 110 rpm, 1,000 mgL^{-1} of butyric acid was completely degraded within 24 h. The modified Gompertz model was used to describe the bacterial growth. These results suggest that the application of Serratia marcescen could be a promising biodegradation strategy for butyric acid in pit latrines.

1. Introduction

Poor sanitation contributes to pressing health problems that adversely impacts billions of people around the world (Seetharam, 2015). Poor sanitation is associated with bacterial, viral, and parasitic infections, including diarrhoea, trachoma polio, cholera, and schistosomiasis. It is estimated that 33 % of the world's incidences of diarrheal diseases, has higher mortality rate in young children more than HIV/AIDS, tuberculosis and malaria combined, could be reduced by improvement in sanitation alone (Bartram and Cairncross, 2010). Investment in sanitation has higher economic returns which are estimated at USD 5.5 for every dollar invested in comparison to USD 2.0 for every dollar invested in water (Hutton, 2012).

Despite the enormous potential benefits of improved sanitation, at the close of the curtain of the Millennium Development Goals (MDGs) in 2015, more than 2.3 $x10^9$ people still lived without access to any type of improved sanitation facility such as toilets or latrines. About 892 M people still continued to practice open defecation either by preference or necessity (WHO and UNICEF, 2017).

In low income settings, particularly rural areas and informal settlements in the developing countries pit latrine still remains and continue to be the most predominant means of human excreta disposal (Thye et al., 2011). This could be attributed to its low-cost, simplicity of construction, little or no water usage, and ease in operation and maintenance (Government of India, 2016). Globally, about 1.77 × 10^9 people use different forms of pit latrines as sanitation technological option of human excreta disposal (Nakagiri, et al., 2016). However, emission of nuisance odours is one of the inherent performance challenges associated with pit latrines (Obeng, et al., 2016). This is one of the factors that impede the usage and adoption of pit latrines. This encourages people to shun them for health risky open defecation.

Butyric acid is one of the predominant odorous components commonly detected from pit latrine emissions that have been identified to contribute significantly to pit latrine nuisance odours (Chappuis et al., 2016). In its pure state it has an extremely pungent smell of sweet rancid which makes it quite complex to handle (Sheridan, et al., 2003). In recent years, various low cost technologies have emerged in the developing countries in order to attenuate odours emitted from pit latrines. These include; use of naturally scented substances, pouring wood ash, disinfectants, pesticides, oil, laundry and soapy water, detergents, car-battery acids and modified latrine designs such as ventilated improved pit (VIP) latrine, urine-diversion and composting toilets and water seal latrines (Rheinländer et al., 2013). However, these strategies and technologies are associated with their own social, economic and technological shortfalls. Bioremediation, the use of microorganisms or microbial processes to degrade environmental contaminants or to prevent pollution (Sardrood, et al., 2013) may be an attractive alternative to the existing odour control techniques and strategies. The technology is extensively used because

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of its low cost and high efficiency (Farag and Soliman, 2011) and environmental sustainability (Prasciolu et al., 2017). There is no detailed information in literature on the use of microorganisms to deal with pit latrine odours. The present work reports the isolation and identification of butyric acid degrading bacterial strain from pit latrine faecal sludge in Mpumalanga, South Africa. Its growth and butyric acid degradation capability at set environmental conditions are also reported.

2. Materials and methods

2.1. Chemicals

Analytical grade chemicals were used in this study. Butyric acid (≥ 99%) was purchased from Sigma Aldrich Inc., St Louis, MO, USA. Sodium hydroxide (NaOH) and all chemicals and reagents used for preparation of growth medium were purchased from Merck Chemical (Pty) Ltd, Gauteng, South Africa. Deionised water was prepared by Purelab Flex 18.2Ω purification system, ELGA, UK).

2.2. Culture media

Nutrient broth (NB) was composed of 1.0 g meat extract, 2.0 g yeast, 5.0 g peptone and 8.0 g sodium chloride in 1 L of distilled water. Nutrient agar (NA) was composed of 1.0 g meat extract, 2.0 g yeast, 5.0 g peptone, 8.0 g sodium chloride and 15.0 g agar in 1 L of distilled water.
Mineral salt medium (MSM) contained the following: 0.535 g NH_4Cl; 4.259 g Na_2HPO_4; 2.722 g KH_2PO_4; 0.114 g Na_2SO_4; 0.0493 g $MgSO_4$ and 1 mL of trace element solution also in 1 L of MSM solution. The trace element solution contained the following: 5.549 g $CaCl_2$; 6.950 g $FeSO_4$; 0.0136 g $ZnCl_2$; 0.0341 g $CuCl_2$; 0.0103 g NaBr; 0.0121 g $NaMoO_2$; 0.0198 g $MnCl_2$; 0.0166 g KI; 0.0124 g H_3BO_3; 0.0238 g $CoCl_2$ and 0.0128 g $NiCl_2$ in 1 L distilled water (Roslev et al., 1998). The media were then autoclaved at 121 ^oC at 2 atm for 15 min. The pH was adjusted to 7.0 by titration with NaOH before use.

2.3. Isolation and identification of butyric acid degrading bacteria

Faecal sludge samples in this work were collected from the pit latrines in the semi-rural area of Kendal in Mpumalanga, South Africa. MSM was used as the enrichment medium amended with butyric acid (500 mgL^{-1}) as a sole carbon source to isolate butyric acid degrading bacteria. 100 g of faecal sludge sample was mixed with 1L of sterile distilled water and filtered. 1 mL of supernatant obtained from this was aseptically inoculated into each sterile cotton plugged 250 mL Erlenmeyer flask with 150 mL of the 500 mg/L butyric acid supplemented MSM. The cotton plugged Erlenmeyer flasks were incubated at 30 ^oC for 24 h at 110 rpm in the dark.
The single colonies were streaked onto nutrient agar plates, incubated at 30 ^oC for 24 h. The process was done successively until near pure culture of each identified colony was realised. All the pure isolates were aseptically cultivated in nutrients broth at 30 ^oC and harvested at their mid-exponential phase. The bacterial cells were harvested at the speed of 6,000 rpm at 4 ^oC for 10 min using Sorvall Lynx 600 centrifuge (ThermoFisher Scientific, Osterode am Harz, German). The cells were preserved with 30% glycerol at -80 ^oC until use. The butyric acid degrading isolates were identified by 16S rRNA genotype fingerprinting. Partially sequenced amplified 16 rRNA fragment was compared with other gene sequences in Gen Bank using a basic BLAST of the National Center for Biotechnology Information (NCBI) gene library. This was aligned with gene sequence of the isolates (Tamura et al., 2013).

2.4. Bacterial growth and butyric degradation studies

For bacterial growth and degradation studies the 1 mL of 2.0 (OD_{600}) suspension of cells that were harvested at exponential phase was aseptically transferred to 250 mL Erlenmeyer volumetric flask with 150 mL of the 1,000 mgL^{-1} butyric acid amended MSM. The pH of the MSM was adjusted to 7.0. The flasks were plugged with sterile cotton wool and incubated in the dark at 30 °C, agitation rate of 110 rpm for incubation period of 24 h. All the experiments were performed in triplicates. Simultaneously abiotic (control without inoculum) experiments were also carried out in triplicates. Aliquots were withdrawn at regular intervals of 4 h for determination of optical density and residual butyric acid concentrations.
Analysis of the bacterial growth curve was done using the modified Gompertz model to determine the bacterial growth kinetic parameters. The model is described according to the Eq(1) (Zwietering et al., 1990):

$$Y_t = A + \mathrm{C}.\exp[-\exp(b(m-t)] \quad (1)$$

in which $Y_t = In(\frac{N}{N_o})$, N is the population density as a function of time and t is time, N_o is the initial population density at time=0, A is the upper asymptote, m is the time at which the absolute growth rate is maximal (time at inflection), C is the asymptotic amount of growth as t approaches infinity and b is the rate constant of growth measured at the m time. In addition, the maximum specific growth rate,μ_m in Eq(2) was derived as follows:

$$\mu_m = \frac{b.C}{e} \quad (2)$$

in which asymptote value, a, in Eq(3) is reached for time is approaching infinity, and the lag time , λ, Eq (3) was derived as follows:

$$\lambda = m - \frac{1}{b} \quad (3)$$

The curve fitting was performed using Origin 2018 data analysis and graphing software (Originlab Corporation, Northampton, MA, USA) with 95% confidence intervals for all the parameters. The model was fitted to the observed experimental data by nonlinear regression with a Levenberg Marquardt algorithm.

2.5. Analytical procedure

Butyric acid degradation was determined by Waters Alliance 2695 Separation Module HPLC system (Waters Corporation, Milford, MA, USA) equipped with an Aminex HPX-87H87H ion-exclusion organic acid, 300 mm × 7.8 mm, 9 µm particle size column (Bio-Rad Laboratories, Berkeley, CA, USA) with an HPLC mobile phase of 0.02 M sulphuric acid (H2SO4). Retention time for butyric acid was 12.2 min and the total run time was set at 15 min. at a flow rate of 1 mL/min and at 60 °C. The detection of the peaks was achieved at the wavelength of 210 nm using Waters 2998 Photodiode Array detector (PAD) equipped with micro UV cell (Waters Corporation, Milford, MA, USA). Bacterial growth in the medium was spectrophotometrically monitored by measuring the optical density (OD) at single wavelength λ = 600nm (OD_{600}) using a UV Lightwave II spectrophotometer (Labotec, Gauteng, South Africa).

3. Results and discussion

3.1. Isolation and identification of butyric acid degrading bacteria

After separation and purification, nine bacterial strains that capable of utilising butyric acid as the sole source of carbon and energy isolated from pit latrine faecal sludge. All the bacterial strains were capable of utilising butyric acid as a sole source of carbon and energy. All these bacterial strains were further investigated to ascertain the butyric acid degradation efficiency as pure cultures. Among the strains, the bacterial strain designated as B6a was grown in sterile 150 mL of MSM in cotton plugged 250 mL Erlenmeyer volumetric flask supplemented with 1,000 mgL^{-1} of butyric acid. The isolated strain was identified based on 16S rRNA sequence analysis. On this basis, the strain B6a was identified as Serratia marcescen as illustrated in the constructed phylogenetic tree in Figure 1 and shared the highest sequence homology of 100 % that evidently matched with Serratia marcescen DSM 30121 (Gen- Bank Accession No.: AJ2334431). 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.

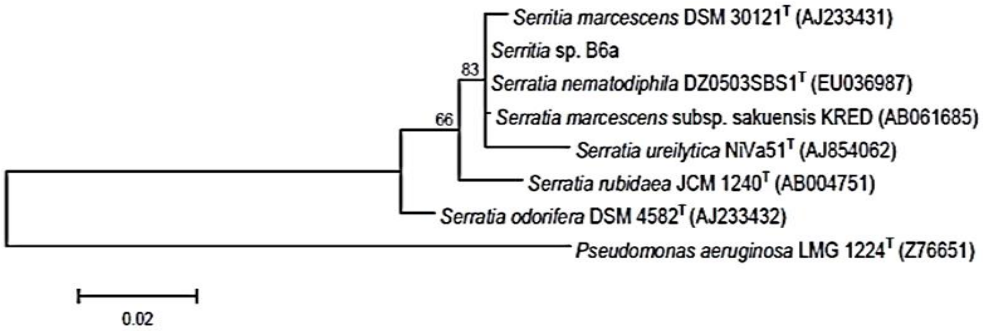

Figure 1: Phylogenetic tree based on 16S rRNA gene sequences showing genetic relationship of strain B6a with taxonomically related strains. GeneBank access number of each strain is given in parentheses

3.2. Bacterial growth and butyric acid degradation

Figure 2 shows the degradation of butyric acid by Serratia marcescen in MSM supplemented with 1,000 mg/L of butyric acid. The results showed the significant differences in degradation of butyric acid between MSM inoculated with the bacterial strain (biotic) and the one without bacterial inoculation (abiotic). There was no degradation of butyric acid in the first 4 h after incubation. However, there was a sharp increase in degradation from 4 to 20 h after incubation. Then there was decrease in degradation between 20h and 24 h. There was no degradation that was observed in un-inoculated control. This showed that the bacterial strain B6a was proficient

for biodegradation of butyric acid. The complete degradation of butyric acid in this study is important. This is primarily due to the fact that even at low concentrations butyric acid has high odour nuisance values and can be perceived by people at long distance from the point of emission that is attributed to its very low odour detection threshold (Sheridan, et al., 2003). Butyric acid is one of the short chain fatty acids (SCFAs) that have infinite solubility in water (Hudges, 1934). The high degradation of butyric acid could be attributed to its hydrophilicity and bioavailability (Kristansen et al., 2011).

The relationship between bacterial growth and butyric acid degradation were investigated. The growth of and butyric acid degradation by Serratia marcescen was conducted at 30 ^{o}C, pH 7 and agitation rate of 110 rpm for incubation period of 24 h. The bacterial growth was expressed by the absorbance value (OD_{600}) while the butyric degradation was expressed as degradation rate (%).

As observed in Figure 2 the bacterial strain grew well in the MSM supplemented with 1,000 mgL^{-1} of butyric acid, in which butyric acid was used as sole source of carbon and energy for growth under incubation conditions. It is evident that growth of marcescen was basically consistent with the degradation rate of butyric acid. This clearly indicates that the degradation of butyric acid was principally dependent on the growth of Serratia marcescen. The growth for the bacterial strain showed from 0 to 5 h, the curve was shallow and there was negligible or no growth in bacterial cells.

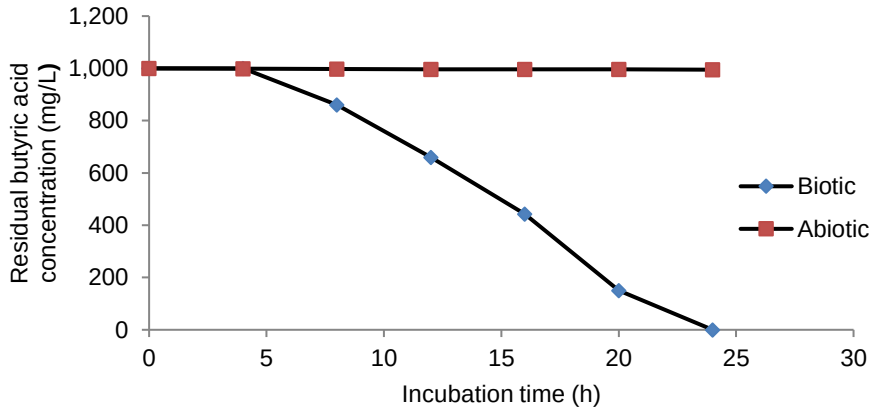

Figure 2: Residual butyric acid concentrations during the incubation period

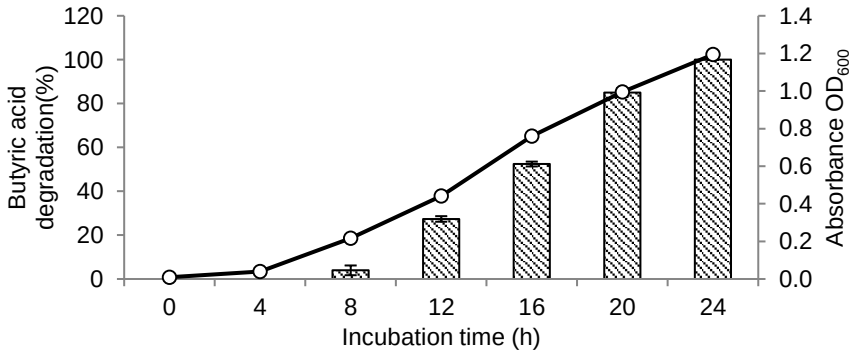

Figure 3: Growth for butyric acid degrading bacterium, Serratia marcescen and degradation of butyric acid. Butyric acid degradation rate; bar graphs: bacterial growth; line graph.

It is presumed that this period allows the adaptation required for bacterial cells to begin to get acclimated to new environmental conditions. This phenomenon is generally found during batch culture, in which a typical bacterial growth curve goes through physiological processes which are manifested in five distinct phases of growth: lag phase, the delay before the start of exponential growth; exponential phase, where cell division takes place at a constant rate; stationary phase, when conditions become adverse for growth and bacterial cell division ceases , death phase, when cells lose viability; and, finally, long-term stationary phase, which can prolong for years (Rolfe, et al., 2012).

In this study, a modified Gompertz model (Eq (1)) was adopted to estimate mathematical parameters, *a, b* and *c* for bacterial growth prediction. The model described the growth kinetics of Serratia marcescen from the lag phase to the stationary phase (Baty et al., 2004). The parameters of biological meaning such as lag time (λ), maximum specific growth rate (μ_m) and asymptotic growth level (A) as presented in Table 1 were also computed by fitting the model to the experimental data based on Eq(2) to Eq(4) as derived by Zwietering et al., (1990) as described in section 2.4. The model was chosen because unlike the classical Gompertz model, it has a term of time delay introduced which enables it to fit a sigmoidal pattern of growth, which is similar pattern most bacteria follow as noted in most published articles (Mytilinaios, et al., 2012). The model was re-parameterised in such a way that those parameters such as μ_m, λ and A, that are microbiologically significant, can be more conveniently determined (Zwietering et al., 1990). It is considered as the best sigmoidal model to describe bacterial growth data (Baty et al., 2004). The lag time, maximum specific growth rate and asymptotic growth level computed are presented in Table 1. The values of the parameters might be overestimated as the model is known for overestimation of lag time and maximum specific growth rate as one of the major drawback to its use (Baty et al., 2004). It is worth mentioning that the growth curves for *the* bacterial strain did not reach the stationary phase hence their asymptotic growth levels provided by the model could not be estimated with certainty.

Table 1: Growth parameters and their 95% confidence limits and coefficient of determination (R^2), and RMSE of the fit obtained with modified Gompertz model for the average OD growth curves of Serratia marcescens at pH 7.0, 30 ^oC, 110 rpm in MSM supplemented with 1,000 mgL^{-1} butyric acid inoculated with 1 mL of 2.0 (OD_{600}) cells

Bacterial strain	λ[h]	$\mu_m[h^{-1}]$ (OD_{600})	a (OD_{600})	m[h]	b[h^{-1}]	R^2	RMSE
S. marcescens	5.75	0.07	1.55 (±0.07)	13.54 (±0.46)	0.23 (±0.05)	0.999	0.0003

The model provided the expected values for the growth parameters and fitted the data properly, as evidenced by the statistics analysed. As can be seen in Table 1, according to goodness-of-fit criterion, the coefficient of determination (R^2), to evaluate fitting of the modified Gompertz model, was found to be high (0.999). The high R^2 values obtained in this study imply that the modified Gompertz model adequately describes the bacterial strains' growth curves of the observed experimental data. Root mean square error (RMSE) value in Table 1 to check the model's performance revealed that it gave a consistently better goodness-of-fit to the observed experimental data for Serratia marcescen. The RMSE value obtained was 0.0003. This value is smaller than some that have been reported in the literature. The smaller RMSE value reflects the appropriateness of the model in this study. This signifies that the estimated growth parameters for the bacterial strain obtained on the basis of OD measurements in this study can be more conveniently assessed.

4. Conclusions

Butyric acid-degrading bacterium identified as Serratia mascescens was isolated from pit latrine faecal sludge. The isolated strain is a novel, efficient butyric acid degrader, which may be useful in the development of an environmentally friendly microbial degradation process. Degradation rate of butyric acid was found to be corresponding with bacterial growth. The bacterial strain was able to completely degrade 1000 mgL^{-1} butyric acid within 24 h at pH 7.0, 30 ^oC and 110 rpm. The growth pattern of the bacterial strain was well described by the modified Gompertz model in which parameters of microbiological relevance, maximum specific growth rate (μ_m), lag time (λ) and asymptotic value (a) were accurately estimated from OD measurements. Further investigation should be carried out to ascertain the effect of various environmental parameters such as temperature, pH, and butyric acid concentration on butyric acid degradation by Serratia mascescen *so* as to obtain the optimal degradation of the bacterial strain. The present study has provided the basis for further studies to understand butyric acid degradation in an effort to attenuate the emission of malaodours from pit latrines hence enhancing the usage and adoption in the developing countries with an aim of meeting Sustainable Development Goal (SDG) target on sanitation.

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