Continuous Succinic acid Production by *Actinobacillus Succinogenes*: Suspended Cell and Biofilm Studies in an Anaerobic Slurry Reactor

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Continuous Succinic acid Production by *Actinobacillus Succinogenes*: Suspended Cell and Biofilm Studies in an Anaerobic Slurry Reactor

by

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Synopsis

Succinic Acid (SA) was continuously produced using glucose and a $Mg_2CO_3(OH)_2$ slurry as feed. Glucose feed concentrations of 20 and 40 g ℓ^{-1} were employed with corresponding $Mg_2CO_3(OH)_2$ slurry concentrations of 60 and 120 g ℓ^{-1}. The reactor pH was passively maintained between 6,4 and 6,8 by the buffer properties of the slurry in conjunction with the pH adjusted glucose feed. The suspended cell (SC) reactor was operated at 37°C with dilution rates varying between 0,04 $h^{-1}$and 0,6 h^{-1}. Groperl® particles were used as inert supports in the biofilm reactor; dilution rates of 0,11 h^{-1} to 1 h^{-1} were investigated.

Two SC fermentations were conducted for the 20 g ℓ^{-1} glucose feed concentration and one for the 40 g ℓ^{-1}. All SC fermentation runs were operated in excess of 12 days, while the biofilm run lasted 6,5 days. Fermentations were terminated only after contamination by lactic acid bacteria was observed.

SC fermentations with the glucose feed concentration of 20 g ℓ^{-1} achieved a maximum SA productivity of 5,2 g $\ell^{-1}h^{-1}$ at 0,6 h^{-1} with a corresponding SA yield of 0,65 g g^{-1}. SC fermentations with the glucose feed concentration of 40 g ℓ^{-1} achieved a maximum SA productivity of 3,76 g $\ell^{-1}h^{-1}$ at 0,4 h^{-1} with a SA yield of 0,82 g g^{-1}. The results were comparable to the other continuous studies with *Actinobacillus succinogenes*, despite the fact that either biofilms or membranes were employed in these studies.

The preliminary biofilm study demonstrated the capability of *A. succinogenes* to produce SA in high productivities and yields. SA productivities and yields for the dilution rates of 0,33 h^{-1} and 1,0 h^{-1}, were 5,72 g $\ell^{-1}h^{-1}$ (0,95 g g^{-1}) and 12 g $\ell^{-1}h^{-1}$ (1,0 g g^{-1}), respectively. The biofilm reactor at 0,33 h^{-1} achieved twice the SA productivity of the SC reactor at 0,3 h^{-1} with a 42 % increase in SA yield.

Keywords: continuous succinic acid production, *Actinobacillus succinogenes*, biofilm

Nomenclature

α_S	Growth associated substrate utilization term	g g^{-1}
α_{SA}	Growth associated succinic acid formation term	g g^{-1}
β_S	Non growth associated/maintenance substrate utilization term	h^{-1}
β_{SA}	Non growth associated succinic acid formation term	h^{-1}
C_{BP}	By-product concentration	g ℓ^{-1}
C_{BPO}	Inlet by-product concentration	g ℓ^{-1}
C_P	Total acid concentration	g ℓ^{-1}
$C_{P,max}$	Maximum total acid concentration	g ℓ^{-1}
C_S	Substrate concentration	g ℓ^{-1}
C_{SO}	Inlet substrate concentration	g ℓ^{-1}
C_{SA}	Succinic acid concentration	g ℓ^{-1}
C_{SAO}	Inlet succinic acid concentration	g ℓ^{-1}
C_X	Dry weight cell concentration	g ℓ^{-1}
C_{XO}	Inlet dry weight cell concentration	g ℓ^{-1}
D	Dilution rate	h^{-1}
$f(C_P)$	Total acid concentration effect	-
$f(C_S)$	Substrate concentration effect	-
K_S	Substrate concentration where $\mu = 0.5\ \mu_{max}$	g ℓ^{-1}
R_{BP}	Volumetric rate of by-product formation	g ℓ^{-1} h^{-1}
R_S	Volumetric rate of substrate consumption	g ℓ^{-1} h^{-1}
$\widehat{R}_S$	Specific rate of substrate utilization (based on dry cell mass)	g g^{-1} h^{-1}
R_{SA}	Volumetric rate of succinic acid production/ productivity	g ℓ^{-1} h^{-1}
$\widehat{R}_{SA}$	Specific rate of succinic production (based on dry cell mass)	g g^{-1} h^{-1}

R_X	Volumetric cell growth rate	g ℓ^{-1} h^{-1}
μ	Specific growth constant	h^{-1}
μ_{max}	Maximum specific growth constant	h^{-1}
$Y_{BP/X}$	Mass of by-products produced per mass of dry cells formed	g g^{-1}
$Y_{S/X}$	Mass of substrate consumed per mass of dry cells formed	g g^{-1}
$Y_{SA/X}$	Mass of succinic acid produced per mass of dry cells formed	g g^{-1}

Acronyms

PCS	Plastic Composite Supports
PP	Polypropylene
SA	Succinic Acid
SC	Suspended Cells
SS	Steady State

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Chapter 1

Introduction

In the new era of sustainability, renewable biomass is considered to be a viable replacement for fossil fuels. With a change in raw material, traditional refineries will be replaced by biorefineries with the aim of producing an equivalent range of fuels and chemicals. On the fuel side bioethanol is a fast growing industry, while on the chemical side development is in its infancy. To stimulate the development of biorefineries the U.S. Department of Energy (2004) identified 12 platform chemicals, which could be produced from biobased feedstocks to replace petrochemical products and manufacture value added products.

1,4 butanedioic acid/succinic acid (SA) is a precursor to numerous high value chemicals (Beauprez *et al.*, 2010); therefore SA was identified as one of the 12 platform chemicals (U.S. Department of Energy, 2004). SA is commercially produced by the partial oxidation of butane, followed by hydrogenation of the intermediate, maleic anhydride. The cost of butane is approximately 850 - 950 US $ ton^{-1} (Argus International LPG, 2011), the overall carbon yield is less than 40 % and the hydrogenation process produces many pollutant by-products that makes the purification of SA costly.

Alternatively SA can be produced from glucose by fermentation. The price of glucose (98% pure from corn syrup) is approximately 220 - 250 US $ ton^{-1} (Tongaat Hulett, 2011), and carbon yields are theoretically higher than 1 (1,31 g SA (g glucose)$^{-1}$) since CO_2 is taken up from the environment. These figures make it clear that the fermentation process offers enormous possibilities, and hence there is intense research on the topic in many leading academic and industrial environments.

Several companies are currently producing biological SA, however, their projected production is capable of only supplying 0,2 % of the projected SA market (Bomgardner *et al.*, 2011, Luo *et al.*, 2010). With the prospect of SA becoming a bulk scale commodity, efficient and low cost production processes will become crucial. Moreover, continuous industrial scale production will be preferred over current batch processes.

Actinobacillus succinogenes, *Mannheimia succiniciproducens,* A*naerobiospirillum succiniciproducens* and recombinant *Escherichia coli* are the most documented bacterial strains used in SA production. These microbes produce SA using the glycosis intermediate phospho*enol*pyruvate in combination with a carbonate source to form oxaloacetate from which SA is formed via the reverse section of the tri-carboxylic acid (TCA) cycle. 2 mol of SA can be produced from 1 mol of glucose (organic carbon) and 2 mol of CO_2 (inorganic carbon) provided that all carbon flux is towards SA production.

Actinobacillus succinogenes is a prominent SA strain because of its ability to produce high SA titers, use a wide range of carbon sources, and tolerate high concentrations of organic acids. An extensive range of batch and fed-batch studies have been performed using *Actinobacillus succinogenes,* while only two continuous studies have been reported in open literature; the first employed cell immobilization on plastic support tubes (Urbance *et al.*, 2004) and the second used membranes to increase suspended cell concentrations (Kim *et al.*, 2009). Both studies used continuous CO_2 sparging as the inorganic carbon source.

In this study a continuous process was developed where the inorganic carbon was supplied in the form of a $Mg_2CO_3(OH)_2$ slurry. The reactor pH was maintained by utilizing the buffer properties of the slurry in conjunction with the pH adjusted glucose feed. The majority of the work was performed in a chemostat setup, where no attempt was made to enhance immobilization of *A. succinogenes* cells. In the final continuous run Groperl® support particles were used to enhance immobilization in order to perform a preliminary test of the biofilm potential of the reactor system.

Chapter 2

Literature

2.1 Succinic acid

2.1.1 Emerging market

1,4 butanedioic acid/succinic acid (SA) is used in the manufacturing of foods, pharmaceuticals, cosmetics and is an intermediate in the production of gamma-butyrolactone, 1,4 butanediol and tetrahydrofuran (Beauprez *et al.*, 2010). SA was classified by the U.S. Department of Energy (2004) as one of the top 12 biomass derived platform chemicals.

SA is commercially produced by the partial oxidation of butane, followed by hydrogenation of the intermediate, maleic anhydride. The cost of butane is approximately 850 - 950 US $ ton^{-1} (Argus International LPG, 2011), the overall carbon yield is less than 40 % and the hydrogenation process produces many pollutant by-products that makes the purification of SA costly.

Alternatively SA can be produced from glucose by fermentation. The price of glucose (98% pure from corn syrup) is approximately 220 - 250 US $ ton^{-1} (Tongaat Hullet, 2011), and carbon yields are theoretically higher than 1 (1,31 g SA (g glucose)$^{-1}$) since CO_2 is taken up from the environment. These figures make it clear that the fermentation process offers enormous possibilities, and hence there is intense research on the topic in many leading academic and industrial environments.

Biological SA production is poised to replace the petrochemical production of maleic anhydride (2,2 million tons yr^{-1}) (U.S. Department of Energy, 2004) and supply the emerging market for SA and its derivatives (30 million tons yr^{-1}) (Luo *et al*, 2010). BioAmber, Reverdia, Myriant Technologies and DSM-Purac are companies that are at the forefront of industrial SA production; their combined production is expected to reach 70,000 tons yr^{-1} by 2013 (Bomgardner *et al.*, 2011). The projected production is capable of only supplying 0,2 % of the total SA market. For SA to become a bulk scale commodity, research into continuous industrial scale production is required to replace current batch processes.

2.1.2 Microorganisms

Numerous microorganisms have been isolated and metabolically engineered for the fermentative production of SA. Gram-negative bacteria; *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens* and recombinant *Escherichia coli* are the prominent high titer producers of SA (Song & Lee, 2006).

Anaerobic fermentations are more commonly used in the production of SA by the microorganisms; however processes involving recombinant *E. coli* have used both aerobic and anaerobic processes. Aerobic fermentations were used to achieve high cell concentrations and then SA production was accomplished anaerobically (Wang *et al.*, 2011, Jiang *et al.*, 2010).

The Gram-positive bacterium, *Enterococcus faecalis* RKY1 has been documented to be an excellent SA producer from fumaric acid. Wee *et al.* (2002) reported a SA productivity and yield of 17,1 g ℓ^{-1} h^{-1} and 0,54 g g^{-1} for their continuous hollow fiber reactor operated at a dilution rate of 0,4 h^{-1}.

Fungi species such as *Aspergillus niger, Aspergillus fumigatus, Byssochlamys nivea, Lentinus degener, Paecilomyces varioti, Penicillium viniferum* and yeast *Saccharomyces cerevisiae* also produce SA, however, not in high titers or yields (Song & Lee, 2006).

2.1.3 Prominent biological production studies

The effectiveness of a biobased SA production process hinges on the efficiency of the microorganism to convert the substrate to SA, without excessive by-product formation and with minimal substrate usage towards cell growth and maintenance of cell functions. 2 mol of SA can be produced from 1 mol of glucose and 2 mol of CO_2 (yield of 1,31 g SA produced per 1 g of glucose consumed) provided that all carbon flux is towards SA production.

The use of vastly different carbon sources, nutrient sources and mineral salts affects the microorganism's effectiveness in producing SA; therefore further discussion is limited to literature where glucose was used as the major organic carbon source.

Table 2.1 gives a summary of the highest reported performances of *A. succinogenes, M. succiniciproducens*, *A. succiniciproducens,* and recombinant *E. coli* in the production of SA using different reactor configurations. Further discussion is focused on *A. succinogenes*; except for direct comparisons of continuous processes.

Table 2.1: Performance of bacterial strains on SA production

Organism	Medium	pH control and Inorganic carbon source	Reactor type	Substrate conversion	SA			Cell conc.	By-products	Reference
			Time (h) /Dilution rate (h^{-1})		Titer	Productivity	Yield			
	($g\ \ell^{-1}$)			(%)	($g\ \ell^{-1}$)	($g\ \ell^{-1}\ h^{-1}$)	($g\ g^{-1}$)	($g\ \ell^{-1}$)	($g\ \ell^{-1}$)	
A. succinogenes CGMCC 1716	Gluc(99,5) YE(10) CSL (5) MS	6,5 (1,5 M Na_2CO_3) CO_2(0,15 vvm)	Batch (NR)	100	**75,1**	NR	0,76	NR	Ace(13,5)	Li *et al.* (2011)
	Gluc(80) YE(10) CSL (5) MS	6,8 (1,5 M Na_2CO_3) CO_2(0,15 vvm)	Batch (34)	81	51,6	**1,5**	0,76	3,5	Ace(10,3)	Xi *et al.* (2011)
ATCC 55618	Gluc(40) YE(6) CSL (10) MS	6,5 (1,5 M Na_2CO_3) CO_2(0,15 vvm)	Repeat Batch PCS Biofilm (38,5)	98	33,9	0,9	**0,87**	NR	NR	Urbance *et al.* (2004)
CGMCC 1593	Gluc(25[a], 10-15[b]) YE(5) CSL (3) MS	6,5 (1,5 M Na_2CO_3) CO_2(0,15 vvm)	Fed-Batch (48)	NR	**60,2**	**1,3**	0,75	7,5	Ace(9,2)	Liu *et al.* (2008)
	Gluc(50[a], 30-35[b]) YE(5) CSL (3) MS		Fed-Batch (48)	NR	52	1,1	**0,82**	6	Ace(4,2)	
ATCC 55618	Gluc(20) YE(6) CSL (10) MS	6,5 (1,5 M Na_2CO_3) CO_2(0,15 vvm)	Continuous PCS Biofilm (0,2 - 1,2 h^{-1})	81	7,3	**8,8**	0,45	NR	NR	Urbance *et al.* (2004)
	Gluc(60) YE(20)	6,8 (acid & base) CO_2(saturated)	Continuous SC Membrane (0,2 - 0,5 h^{-1})	55	**18,6**	3,7	0,56	**16,1**	Ace(7,4) For(5,8)	Kim *et al.* (2009)

Bold: highest reported value for reactor type to date, Gluc: Glucose, YE: Yeast Extra, CSL: Corn Steeped Liquor, MS: Mineral Salts, Ace: Acetic acid/Acetate, For: Formic acid/Formate, PCS: Plastic composite support, SA: Succinic acid/ Succinate, SC: Suspended Cell, NR: Not Reported, Cell conc.: dry cell weight per liter, [a]: initial substrate concentration in batch reactor, [b]: substrate concentration maintained in fed batch reactor

Table 2.1: continued

Organism	Medium (g ℓ^{-1})	pH control and Inorganic carbon source	Reactor type Time (h) /Dilution rate (h^{-1})	Substrate conversion (%)	SA Titer (g ℓ^{-1})	SA Productivity (g ℓ^{-1} h^{-1})	SA Yield (g g^{-1})	Cell conc. (g ℓ^{-1})	By-products (g ℓ^{-1})	Reference
M. succiniciproducens KCTC 10626BP	Gluc(18) YE(5) MS	6,5 (28% NH_4OH) CO_2(0,25 vvm)	Batch (14)	100	**13,6**	0,97	**0,78**	1,6	Ace(0,4) Lac(4,8)	Song *et al.* (2007)
KCTC 0769BP	Gluc(18) YE(5) MS	6,5 (28% NH_4OH) CO_2(0,25 vvm)	Batch (7)	100	8,8	**1,26**	0,49	**3,3**	Ace (3,6) For(4) Lac(1)	Song *et al.* (2008)
	Gluc(20) YE(20)	6,8 (acid & base) CO_2(saturated)	Continuous SC Membrane Recycle (0,1 - 0,3 h^{-1})	100	12,8	**1,28**	0,64	**5**	Ace(4)	Kim *et al.* (2009)
A. succiniciproducens	Gluc (20) CSL(10) MS	6,5 (2 M Na_2CO_3) CO_2(0,25 vvm)	Batch (13,5)	100	**17,8**	**1,3**	**0,89**	**3,5**	Ace (3,7)	Lee *et al.* (2000)
	Glc (20) YE(5) MS	6,5 (1,5 M Na_2CO_3) CO_2(0,15 vvm)	Continuous SC Membrane Filtration (0,032-0,63 h^{-1})	100	14,3	3,3	0,72	**6,5**	Ace (3,5)	Lee *et al.* (2008)
ATCC 29305	Gluc(19) YE(2,5) MS	6,5 (1,5 M Na_2CO_3) CO_2(0,25 vvm)	Continuous SC (0,032-0,63 h^{-1})	65	9,7	5,6	0,87	1,5	Ace (3,1)	Lee *et al.* (2009)
	Gluc(38) YE(5) MS			57	**15,9**	**6,5**	**0,89**	1,1	Ace (4,8)	

Bold: highest reported value for reactor type to date, Gluc: Glucose, YE: Yeast Extra, CSL: Corn Steeped Liquor, MS: Mineral Salts, Ace: Acetic acid/Acetate, For: Formic acid/Formate, Lac: Lactic acid/Lactate, SA: Succinic acid/ Succinate, SC: Suspended Cell, NR: Not Reported, Cell conc.: dry cell weight per liter

Table 2.1: continued

Organism	Medium (g ℓ^{-1})	pH control and Inorganic carbon source	Reactor type / Time (h) /Dilution rate (h^{-1})	Substrate conversion (%)	SA Titer (g ℓ^{-1})	SA Productivity (g ℓ^{-1} h^{-1})	SA Yield (g g^{-1})	Cell conc. (g ℓ^{-1})	By-products (g ℓ^{-1})	Reference
E. coli DC1515	Gluc (40)[a]	O_2(4 vvm), 7(10M NaOH & 10% H_2SO_4)	Aerobic Batch Cell Growth (11)	NR	**116,2**	1,6	**1,13**	**19,2**	Ace(11,4) Lac(1,8) Eth(3,4)	Wang *et al.* (2011)
	Gluc (10)[b]	6,7($MgCO_3$) CO_2(2 vvm)	Anaerobic Fed-Batch (75)							
AFP111	Gluc (13) [a]	O_2(4 vvm), 7(10M NaOH & 10% H_2SO_4)	Aerobic Batch Cell Growth (30)	NR	101,2	**1,9**	1,07	12	NR	Jiang *et al.* (2010)
	Gluc (20) [b]	6,8($Mg_2CO_3(OH)_2$) CO_2(3,5 vvm)	Anaerobic Fed-Batch (53)							

Bold: highest reported value for reactor type to date, Gluc: Glucose, Ace: Acetic acid/Acetate, Lac: Lactic acid/Lactate, Eth: Ethanol, SA: Succinic acid/ Succinate, NR: Not Reported, Cell conc.: dry cell weight per liter, [a]: initial substrate concentration in batch reactor, [b]: substrate concentration maintained in fed batch reactor

2.2 *Actinobacillus succinogenes*

2.2.1 Microorganism description

Actinobacillus succinogenes is a Gram-negative, rod shaped or occasionally filamentous, facultative anaerobic, capnophilic and non-motile bacterium. Three different culture collections of *A. succinogenes* have been reported; *A. succinogenes* (ATCC 55618), *A. succinogenes* (CGMCC 1593) and *A. succinogenes* (CGMCC 1716). *A. succinogenes* (CGMCC1593) posses the same catabolic pathways as *A. succinogenes* (ATCC 55618), however, they differ in their physiological and biochemical characteristics (Liu *et al.*, 2008). The differences between *A. succinogenes* (CGMCC 1716) and *A. succinogenes* (ATCC 55618) have not been described.

A. succinogenes (ATCC 55618) is a chemoheterotroph that grows on common heterotrophic media at an optimum temperature and pH of 37 ^{o}C and 6,8, respectively. The microorganism is a natural SA producer capable of producing 130 g ℓ^{-1} of SA (Guettler *et al.*, 1996). *A. succinogenes* is also a moderate osmophile capable of tolerating high glucose concentrations of upto 160 g ℓ^{-1} and can ferment a broad range of carbon sources such as glucose, fructose, mannitol, arabitol, sorbitol, sucrose, xylose and arabinose under anaerobic conditions (Van der Werf *et al.*, 1997).

2.2.2 Metabolic pathways

Figure 2.1 summarises the metabolic pathways and enzymes that *A. succinogenes* uses in fermentations (McKinlay *et al.*, 2005; Van der Werf *et al.,* 1997). Phospho*enol*pyruvate (PEP) is the branch point between the TCA pathways and the fermentative pathways leading to lactate and other fermentation products. The enzyme phospo*enol*pyruvate carboxykinase (PEPCK) is responsible for oxaloacetate formation; the crucial step in the formation of succinate.

Podkovyrov & Zeikus (1993) purified and characterized PEPCK from *A. succiniciproducens*; PEPCK displayed Michaelis-Menten kinetics for the substrate PEP and the cosubstrates HCO_3^- and ADP. *A. succinogenes* can use CO_2, HCO_3^- and CO_3^{2-} as inorganic carbon sources; CO_2 can permeate the cell membrane and be used directly, however HCO_3^- and CO_3^{2-} need ATP for transportation into the cell (Xi *et al.*, 2010).

Van der Werf *et al.* (1997) studied the effect of inorganic carbon concentrations on the product distribution for glucose fermentations by *A. succinogenes* (ATCC 55618); inorganic carbon was supplied as poorly soluble $MgCO_3$. At low inorganic carbon levels (0,1 mol C/mol glucose) the flux of PEP was observed to favor the C_3 pathway forming lactate and ethanol. Under high inorganic carbon levels (1 mol C/mol glucose) the C_4 pathway was favored; the succinate concentration was 4 times greater than at low inorganic carbon levels.

The importance of a strictly anaerobic fermentation strategy in SA production was further illustrated by Li *et al.* (2010). The authors observed that initial aerobic cultivation and subsequent anaerobic fermentation with *A. succinogenes* (CGMCC1593) produced high lactic acid titers, while strictly anaerobic cultivation and fermentation was observed to produce high SA titers.

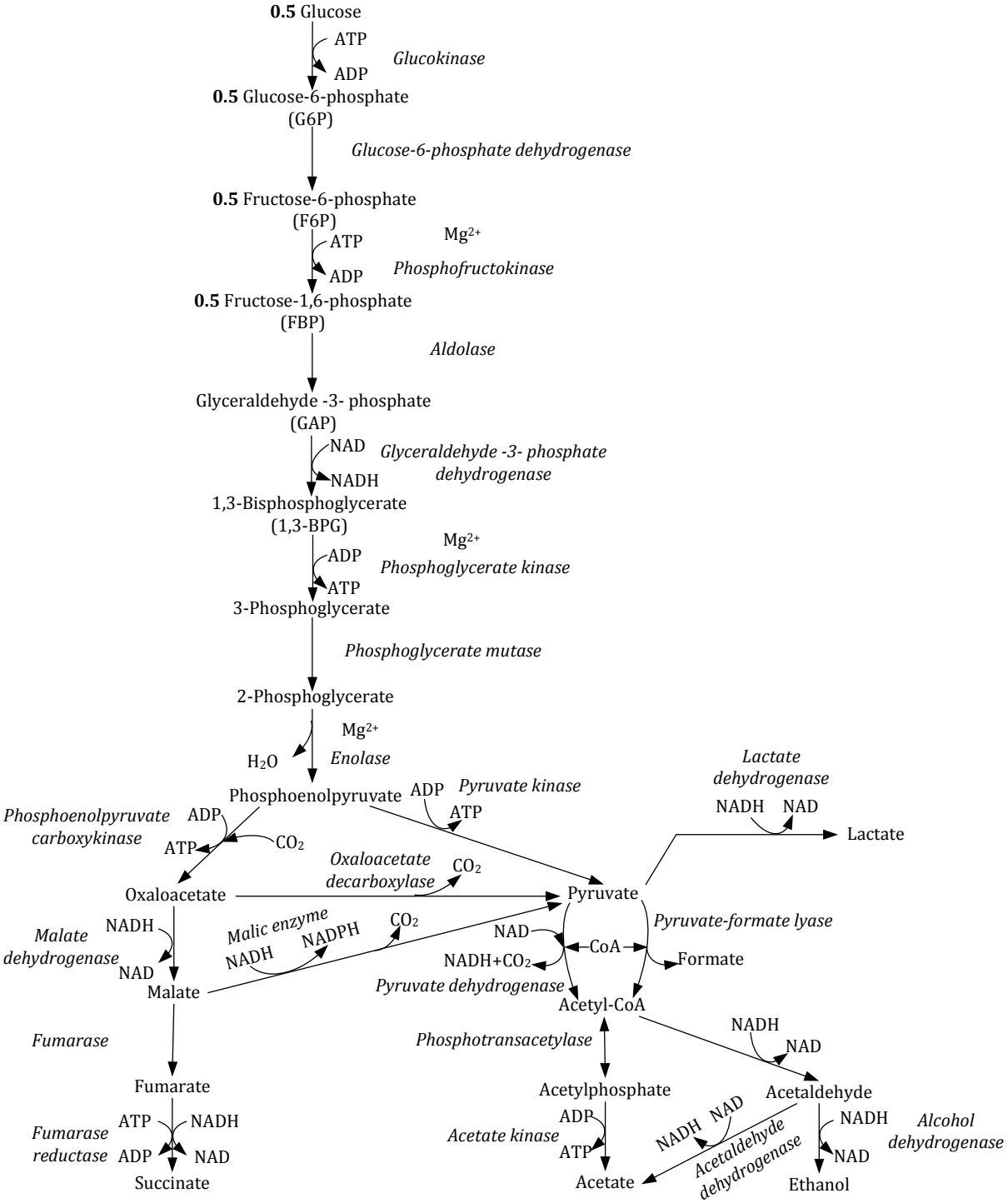

Figure 2.1: *Actinobacillus succinogenes* metabolic pathways

2.2.3 Nutritional requirements

Table 2.2 represents the complex media proposed by Guettler *et al*. (1996) for use in *A. succinogenes* fermentations. The use of complex media increases the cost of the fermentation process; therefore, it is necessary to reduce media complexity without compromising SA productivity and *A. succinogenes* cell growth. In order to alter the fermentation media, the effects of various media components must be understood.

Table 2.2: Complex SA medium

Medium component	Compound	Concentration
Media component	Heavy corn steep liquor	10 g ℓ^{-1}
	Bacto yeast extract	6 g ℓ^{-1}
	Sodium acetate	1,36 g ℓ^{-1}
	Hemin	0,5 g ℓ^{-1}
	Standard salts solution	10 mℓ ℓ^{-1}
Standard salts solution	NaCℓ	1 g ℓ^{-1}
	K_2HPO_4	3 g ℓ^{-1}
	$MgCl_2 \cdot 6H_2O$	0,2 g ℓ^{-1}
	$CaCl_2 \cdot H_2O$	0,2 g ℓ^{-1}
Sugars	Dextrose	20 g ℓ^{-1}
	Xylose	1 g ℓ^{-1}
Phosphates	$NaH_2PO_4 \cdot H_2O$	1,6 g ℓ^{-1}
	Na_2HPO_4	0,31 g ℓ^{-1}
Vitamins	B_{12}	0,001 g ℓ^{-1}
	Biotin	0,02 g ℓ^{-1}
	Folic acid	0,02 g ℓ^{-1}
	Thiamine	0,05 g ℓ^{-1}
	Riboflavin	0,05 g ℓ^{-1}
	Niacin	0,05 g ℓ^{-1}
	Pantothenate	0,05 g ℓ^{-1}
	p-aminobenzate	0,05 g ℓ^{-1}
	Lipoic acid	0,05 g ℓ^{-1}
	B_6	0,1 g ℓ^{-1}
Fatty acids	1,4-naptho quinine	0,01 g ℓ^{-1}
	Methyl butyrate	0,2 mℓ ℓ^{-1}
	Valerate	0,2 mℓ ℓ^{-1}
	Isobutyrate	0,2 mℓ ℓ^{-1}
	Isovalaerate	0,2 mℓ ℓ^{-1}

2.2.3.1 Effect of medium components

Effect of yeast extract & corn steeped liquor

A. succinogenes (ATCC 55618) cell growth increases with increased yeast extract concentration; however, SA yields are poor. SA yield can be improved by the use of corn steeped liquor. The use of optimal concentrations of yeast extract and corn steeped liquor in *A. succinogenes* (ATCC 55618) fermentations have been shown to promote cell growth and improve SA yields (Guettler *et al.*, 1996).

Effect of cations

Liu *et al.* (2008) studied the effects of sodium, calcium and magnesium ions on cell growth and SA production by *A. succinogenes* (CGMCC1593); the cation concentrations varied from 0 – 0,3 mol ℓ^{-1}.

Na^+ is involved in the formation of trans-membrane pH gradient, cell motility and intracellular pH regulation. Liu *et al.* (2008) observed that Na^+ concentrations of 0,05 mol ℓ^{-1} promoted high cell and SA concentrations, whilst in Na^+ deprived mediums the cell and SA concentrations were reduced. Na^+ concentrations greater than 0,1 mol ℓ^{-1} were observed to decrease cell and SA concentrations.

Ca^{2+} concentrations greater than 0,05 mol ℓ^{-1} inhibited cell growth, whilst Mg^{2+} concentrations affected neither cell growth nor SA production (Liu *et al.*,2008).

Effects of anions

Liu *et al.* (2008) reported that $C\ell^-$, SO_4^{2-} and PO_4^{3-} ions slightly affected cell growth and SA production, however, the extent of these effects were not quantified.

2.2.3.2 Complex media alteration

Urbance *et al.* (2003) studied the effect of medium components on SA titers and yields in batch fermentations by *A. succinogenes* (ATCC 55618). Table 2.2 represents the complex media that they used; $MgCO_3$ (80 g ℓ^{-1}) was used for pH buffering and as an inorganic carbon source.

Their results are presented in Table 2.3; they demonstrated that a highly complex medium (Table 2.2) is not necessary to achieve high SA titers and yields. Therefore, the authors recommended the use of the medium given in Table 2.4.

Table 2.3: SA medium evaluation

Test Treatment	SA	
	Titer ($g\ \ell^{-1}$)	**yield ($g\ g^{-1}$)**
SA medium (Table 2.2)	14,4	0,74
No fatty acids	12,5	0,63
No vitamins	15,1	0,77
1mℓ ℓ^{-1} Cane molasses, no vitamins	18,1	0,91
No xylose	19,3	0,96
No phosphates	11,1	0,57
No standard salts	15,1	0,76
No hemin	15,3	0,77
Dried bovine heme, no hemin	10,5	0,53
Dried bovine RBC, no hemin	11,9	0,6
Ardamine Z YE, no Bacto YE	13,8	0,69
Ardamine Z YE, no Bacto YE, no fatty acids, no vitamins	17,4	0,87
Ardamine Z YE,no Bacto YE, no fatty acids, no vitamins, no xylose, no hemin	14	0,70

Table 2.4: Revised SA medium

Compound	Concentration ($g\ \ell^{-1}$)
Glucose	20
Ardamine Z yeast extract	6
Heavy corn steeped liquor	10
Sodium acetate	1,36
$NaC\ell$	1
K_2HPO_4	3
$MgC\ell_2$	0,02
$CaC\ell_2$	0,02
Na_2HPO_4	0,31
NaH_2PO_4	1,6

2.3 Succinic acid production by *Actinobacillus succinogenes*

2.3.1 Batch studies

Table 2.5 is a summary of the extensive batch studies conducted with *A. succinogenes* using glucose as the major organic carbon source; different inorganic carbon sources and methods of pH control are reported.

Table 2.5: Batch SA production by *Actinobacillus succinogenes*

A. succinogenes	Medium	pH control and Inorganic carbon source	Fermentation time	Substrate conversion	SA			Cell Conc.	By-products	Reference
					Titer	Productivity	Yield			
	(g ℓ^{-1})		(h)	(%)	(g ℓ^{-1})	(g ℓ^{-1} h^{-1})	(g g^{-1})	(g ℓ^{-1})	(g ℓ^{-1})	
ATCC 55618	Gluc(40) YE(6) CSL (10) MS	6,5 (1,5 M Na_2CO_3) CO_2(0,15 vvm)	38,5	98	33,9	0,88	**0,87**	NR	NR	Urbance *et al.* (2004)
	Gluc(85) YE(15) MS Supplements	6.8 (10M Na_2CO_3) CO_2(0,5 vvm)	34	71	47,6	1,4	0,8	4,8	Ace(8,9) For(3,4) Pyr(4,8)	Lin *et al.* (2008)
CGMCC 1593	Gluc(25) YE(15) MS Supplements	6.8 ($MgCO_3$) CO_2(0,1 vvm)	14	100	19	1,4	0,76	7,4	Ace(3,1)	Liu *et al.* (2008)
	Gluc(50) YE(15) MS Supplements		32	98,4	40,5	1,3	0,82	6,4	Ace(4,5)	
CGMCC 1716	Gluc(85) YE(10) CSL (5) MS	6,8 (3 M Na_2CO_3) CO_2(3 vvm)	70	79	48	0,69	0,69	**7,4**	Ace(12,5) For(8,8)	Fang *et al.* (2011)
	Gluc(99,5) YE(10) CSL (5) MS	6,8 ($MgCO_3$)	NR	99,6	**75,1**	NR	0,76	NR	Ace(13,5)	Li *et al.* (2011)
	Gluc(80) YE(10) CSL (5) MS	6,8 (1,5 M Na_2CO_3) pCO_2(0,1 MPa)	34	81,3	51,6	**1,52**	0,76	3,5	Ace(10,3)	Xi *et al.* (2011)

Bold: highest reported value for reactor type to date, Gluc: Glucose, YE: Yeast Extra, CSL: Corn Steeped Liquor, MS: Mineral Salts, Ace: Acetic acid/Acetate, For: Formic acid/Formate, Pyr: Pyruvate, SA: Succinic acid/Succinate, NR: Not Reported, Cell conc.: dry cell weight per liter

2.3.2 Repeat batch / Fed batch studies

Repeat batch studies

Repeat batch fermentations are performed at the end of a batch fermentation where the fermentation medium is decanted but a small volume of the broth is left within the reactor to serve as an inoculum. The bath reactor is then refilled with fresh fermentation medium and the batch fermentation is restarted; this process is repeated several times.

During decanting, the suspended cells are washed out; therefore it is crucial to maintain a high cell concentration in the reactor to ensure rapid fermentations. Cell immobilizations by entrapment and attachment have been studied as a means of cell retainment. Cell entrapment in calcium alginate or κ-carrageenan beads is costly and has disadvantages such as bead swelling, bead disintegration and increased mass transfer limitations.

A preferable method of cell retainment is attachment immobilization; where cells attach onto solid surfaces to form "biofilms". Biofilms are naturally occurring forms of cell immobilization; which form when microbial communities embedded in an extracellular polymer matrix (EPM) attach on an inert support (Qureshi *et al.*, 2005). Plastic composite support (PCS) tubes (Table 2.6) were developed by Urbance *et al.* (2003) to promote *A. succinogenes* (ATCC 55618) biofilm formation.

Table 2.6: Plastic composite supports tubes composition

Component	Composition (wt %)
polypropylene	50
dried ground soybean hulls	35
dried bovine albumin	5
soybean flour	2,5
ardamine Z yeast extract	2,5
dried bovine red blood cells	2,5
bacto peptone	2,5

Urbance *et al.* (2004) studied repeat batch fermentation of glucose by *A. succinogenes* (ATCC 55618) suspended cells (SC) and PCS biofilms. SC repeat batch fermentations were performed for glucose concentrations: 20, 40, 80 and 160 g ℓ^{-1}. The experiments were conducted in 250 mℓ flasks. The SC flask fermentations were inoculated with 10 mℓ fresh inoculums. All the SC flask fermentations were conducted for 72 h and were repeated 4 times.

PCS biofilm repeat batch fermentations were performed for glucose concentrations: 20-160 g ℓ^{-1} in 20 g ℓ^{-1} increments. The PCS fermentations were performed in a bioreactor which was operated at 38°C with the pH maintained at 6,8 with 10 N NaOH. CO_2 was continuously sparged at ≥0,25 vvm and the reactor contents were stirred at 150 rpm. No fresh inoculum was introduced in the PCS biofilm fermentations, the biofilm served as the 'inoculum'. The PCS biofilm repeat batch fermentations were conducted twice.

Table 2.7 presents the four prominent results the authors acquired; they showed that PCS biofilms did improve SA production. The authors determined that the optimal glucose concentration for PCS biofilm fermentations was 40 g ℓ^{-1} based on the high SA yield.

Table 2.7: Repeat batch fermentation results

Initial glucose	**Fermentation time**	**Substrate Conversion**	**SA**		
			Titer	**Productivity**	**Yield**
(g ℓ^{-1})	**(h)**	**(%)**	**(g ℓ^{-1})**	**(g ℓ^{-1} h^{-1})**	**(g g^{-1})**
SC fermentations					
20	72	100	9,8	0,1	0,52
40	72	80	12,3	0,2	0,42
PCS fermentations					
40	38,5	98	33,9	0,9	0,87
60	48,2	96	35,1	0,7	0,68

Fed batch studies

In fed-batch fermentations, nutrients are continuously or semi-continuously fed while the fermentation broth is retained such that the broth volume increases with substrate feeding. At the end of the fed batch period the whole or a portion of the broth is harvested and sent for downstream processing. Control of the nutrient feed rate offers control on overflow metabolism which diverts carbon flow to undesired by-products. Furthermore, control of the substrate concentration prevents biomass and desired product substrate inhibition. Higher product titers and yields can therefore be obtained (Villadsen *et al.*, 2011: 411-419).

Liu *et al.* (2008) using *A. succinogenes* (CGMCC1593) performed fed-batch fermentations at 37 °C. $MgCO_3$ (65 g ℓ^{-1}) was used as a pH buffer and inorganic carbon source. The authors also used CO_2 as an alternative inorganic carbon source; CO_2 was sparged at 0,10 vvm.

Two fed-batch fermentations were conducted; one was maintained at a glucose concentration range of 10-15 g ℓ^{-1} with an initial glucose concentration of 25 g ℓ^{-1}. The other was maintained at a glucose concentration range of 30-35 g ℓ^{-1} with an initial glucose concentration of 50 g ℓ^{-1}. A concentrated glucose solution of 600 g ℓ^{-1} was used to maintain the specified glucose concentration ranges. The fermentations were conducted for 48 h. The authors avoided the withdrawal of the fermentation broth except when sampling.

The results obtained by the authors are given in Table 2.8. The SA productivities of the fed batch reactors maintained at 10-15 g ℓ^{-1} and 30-35 g ℓ^{-1} were 48,7 % and 28,5 % higher compared to their respective batch fermentations.

Table 2.8: Fed batch fermentation results

		10 - 15	30 - 35
SA	**Titer (g ℓ^{-1})**	60,2	52,1
	Yield (g g^{-1})	0,75	0,82
	Productivity (g ℓ^{-1} h^{-1})	1,3	1,1

2.3.3 Continuous flow studies

In continuous SC reactors the interplay between the bacteria growth rate, SC concentration, retention time and cell washout rate leads to a steady state where process variables do not change with time (Lewandowski, Beyenal & Stookey, 2004).

In continuous flow biofilm reactors steady state requires that the biofilm micro-scale processes (biofilm thickness, density, viable cell concentration and amount of extracellular polymer substances produced) and the macro-scale processes reach a stage where they do not change with time. However, biofilm structures and processes are ever changing through processes of attachment, growth, detachment and lysis (Lewandowski *et al.*, 2004).

Urbance *et al.* (2004) used the RSA medium (Table 2.4) to study the fermentations of glucose by *A. succinogenes* (ATCC 55618) SC and biofilms. The reactor was operated at 38°C with the pH maintained at 6,8 with 10 N NaOH. CO_2 was continuously sparged at ≥0,25 vvm and the reactor contents were stirred at 125 rpm and 150 rpm. Dilution rates varying from 0,2 to 1,2 h^{-1} in 0,2 h^{-1} increments were studied. The biofilm reactor used eight plastic composite support (PCS) tubes (Table 6) or six polypropylene (PP) tubes, which were stacked perpendicularly and attached to the agitator shaft.

Samples were collected from the reactor after five working volume exchanges, after which the dilution rate was increased. This procedure indicates that the authors assumed that the

reactor reached steady state after five working volumes. For the suspended cell studies the assumption might have been accurate; however, according to Lewandowski *et al.* (2004) this assumption might not have been accurate for the biofilm studies.

Biofilm and SC experiments from Urbance *et al.* (2004) that attained the highest SA productivities are presented in Figure 2.2. The list below aids in the interpretation of Figure 2.2 and Figure 2.3.

- Diagonal line: represents the maximum glucose consumption rate
- Data point: represents the reported volumetric SA production rate (productivity)
- Top of vertical bar: represents the reported volumetric glucose consumption rate
- Yield: SA productivity divided by the corresponding glucose consumption rate
- Titer: SA productivity divided by the corresponding dilution rate

The Urbance *et al.* (2004) data presented in Figure 2.2 are scattered; the SC studies achieved higher SA productivities and yields compared to the PCS and PP biofilm studies for dilution rates 0,6 h^{-1}. The PP biofilm reactor achieved nearly similar productivities compared to the PCS biofilm reactor for dilution rates 0,2 - 0,8 h^{-1} and outperformed PCS biofilm reactor at 1 h^{-1}.

Prominent continuous SA production studies have also been reported by Lee *et al.* (2009) and Kim *et al.* (2009). Lee *et al.* (2009) conducted SC continuous fermentation studies with *A. succiniciproducens* (ATCC 29305). Dilution rates of 0,032-0,63 h^{-1} were investigated with glucose feed concentrations of 19 and 38 g ℓ^{-1}; some of their results are presented in Figure 2.2 and Figure 2.3, respectively. Lee *et al.* (2009) achieved high SA productivities and yields with cell concentrations reaching a maximum of 1,6 g ℓ^{-1}.

Kim *et al.* (2009) studied continuous SA production by *A. succinogenes* (ATCC 55618) and *M. succiniproducens* (KCTC 0769BP); an external cell recycle system was used to increase SC concentrations. Experiments with *M. succiniproducens* and *A. succinogenes* used glucose feed concentrations of 20 and 60 g ℓ^{-1}, respectively. Dilution rates of 0,2–0,5 h^{-1} and 0,1-0,3 h^{-1} were investigated. Results from the experiments with 20 and 60 g ℓ^{-1} glucose concentrations are presented in Figure 2.2 and Figure 2.3, respectively.

Kim *et al.* (2009) managed to achieve high cell concentrations of upto 16,4 g ℓ^{-1} in the *A. succinogenes* experiments, however their SA productivities and yields were not superior compared to Lee *et al.* (2009) who achieved much lower cell concentrations.

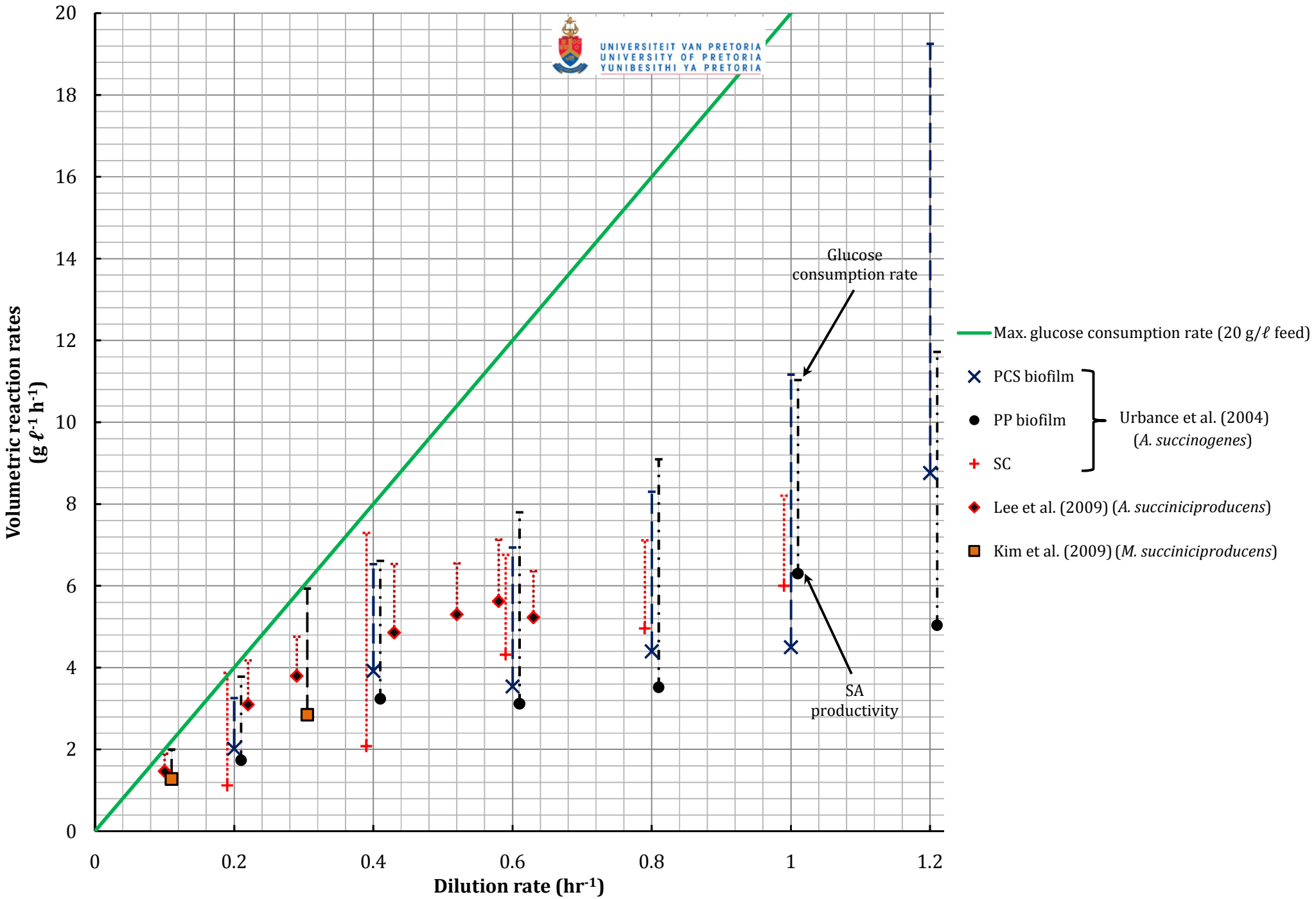

Figure 2.2: Continuous SC and biofilm studies; 20 g ℓ^{-1} glucose feed concentration

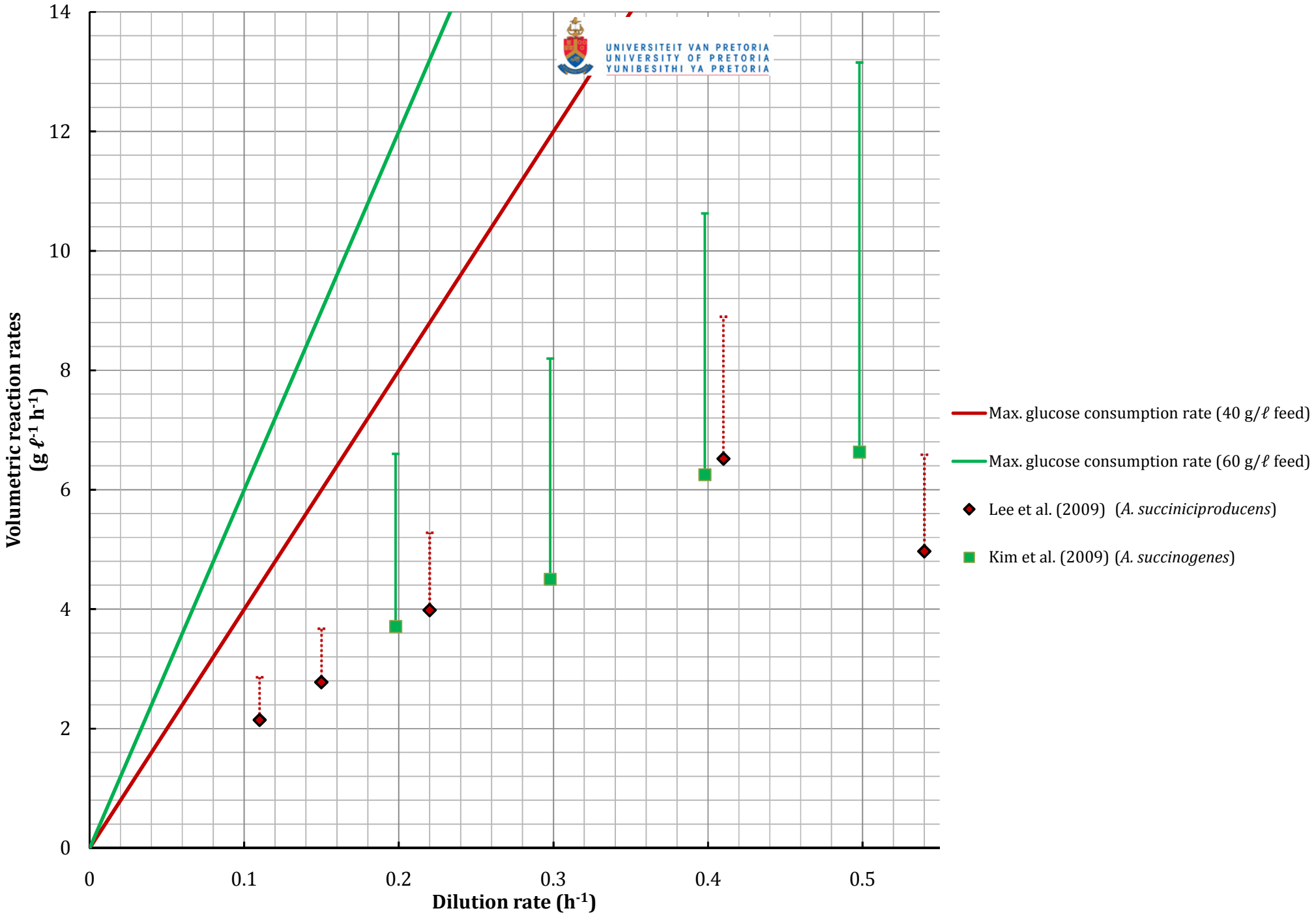

Figure 2.3: Continuous SC studies; 40 and 60 g ℓ^{-1} glucose feed concentrations

Chapter 3

Experimental

3.1 Microorganism

Actinobacillus succinogenes (ATCC 55618) acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ) was used in all the studies. After 24 h of growth in tryptone soy broth (TSB) at 37°C, aseptic culture samples were stored at −75°C in 2 mℓ microbank™ vials which contained treated beads and a special cryopreservative solution.

3.2 Fermentation medium

Table 3.1 describes the composition of the fermentation medium used in all fermentation studies. For experiments conducted with 40 g ℓ^{-1} of glucose, only the glucose concentration was doubled, whilst the other component concentrations were maintained as described in Table 3.1. The Organic and Mineral Salt (MS) mediums were autoclaved separately at 121°C for 2 hrs and mixed aseptically.

Table 3.1: Organic-Mineral salt (MS) composition in fermentation studies

	Compound	Concentration (g ℓ^{-1})
Organic	Glucose	20
	Yeast extract	6
	Corn steep liquor	10
Mineral salt (MS)	Na_2HPO_4	0.31
	NaH_2PO_4	1,4
	NaAc	1,36
	NaCℓ	1
	K_2HPO_4	1,5
	$MgC\ell_2.6H_2O$	0,2
	$CaC\ell_2.2H_2O$	0,2

2 mol of SA can be produced from 1 mol of glucose and 2 mol of inorganic carbon provided that all the carbon flux is used for SA production. The $Mg_2(CO_3)(OH)_2$ slurry concentration was calculated for the inorganic carbon to be in 25 % excess to the required amount at full glucose conversion with a SA yield of 1 g (g glucose)$^{-1}$. Table 3.2 demonstrates how the slurry concentrations were calculated; the 20 g ℓ^{-1} of glucose experiment is presented as an example.

Table 3.2: Calculation of the $Mg_2CO_3(OH)_2$ concentration

C_{SO}	20 g ℓ^{-1}
SA Yield	1 g SA (g glucose)$^{-1}$
C_{SA}	20 g ℓ^{-1}
Molar SA Conc.	0,17 mol ℓ^{-1}
Molar inorganic Carbon Conc.	0,17 mol ℓ^{-1}
25 % Excess Molar inorganic Carbon Conc.	0,212 mol ℓ^{-1}
Molar Mg_2CO_3 $(OH)_2$ Conc.	0,424 mol ℓ^{-1}
Mass Mg_2CO_3 $(OH)_2$ Conc.	60 g ℓ^{-1}

The excess carbonate was supplied to buffer the medium pH at 6,8 during acid production. For the 20 and 40 g ℓ^{-1} glucose experiments the slurry concentrations were calculated to be 60 and 120 g ℓ^{-1}, respectively. The slurry was sterilized at 121°C for 2 hrs. The $Mg_2(CO_3)(OH)_2$ slurry pH was 11; therefore prior to autoclaving the Organic-MS medium pH was adjusted to 2 using 32 % (w/w) HCℓ to achieve a pH of 7,8 after mixing with the slurry (no reaction).

3.3 Batch bioreactor

Batch fermentations were conducted in 250 mℓ Duran flasks. The flasks were prepared by adding 120 mℓ of the $Mg_2(CO_3)(OH)_2$ slurry and sterilizing at 121°C for 2 h. 60 mℓ of the organic and mineral salt mediums were sterilized separately and mixed aseptically. The organic and mineral salt mediums were then mixed with the $Mg_2(CO_3)(OH)_2$ slurry to achieve the concentrations described in Table 3.1. Initial glucose concentrations of 20 and 40 g ℓ^{-1} were investigated with their corresponding $Mg_2(CO_3)(OH)_2$ slurry concentrations.

The flasks were inoculated with 10 mℓ of a 24 h *A. succinogenes* culture grown in TSB broth. The flasks were placed in an incubator at a temperature and stirrer rate of 37 °C and 80 rpm. Each glucose concentration had a replication of two flasks. Samples were taken from each flask and analyzed for glucose, fermentation products and SC concentrations; average concentrations were reported.

3.4 Continuous bioreactor

Figure 3.1 represents the bioreactor setup used for all the continuous fermentation experiments. The reactor was constructed from glass (40 mm outer diameter, 3,2 mm thick and 115 mm long) with the top and bottom sections made from aluminum. The top and bottom aluminum distributors had 1 mm diameter holes drilled on the intersecting lines of concentric circles (4 mm apart) and diameter lines (6 lines, 15° apart).

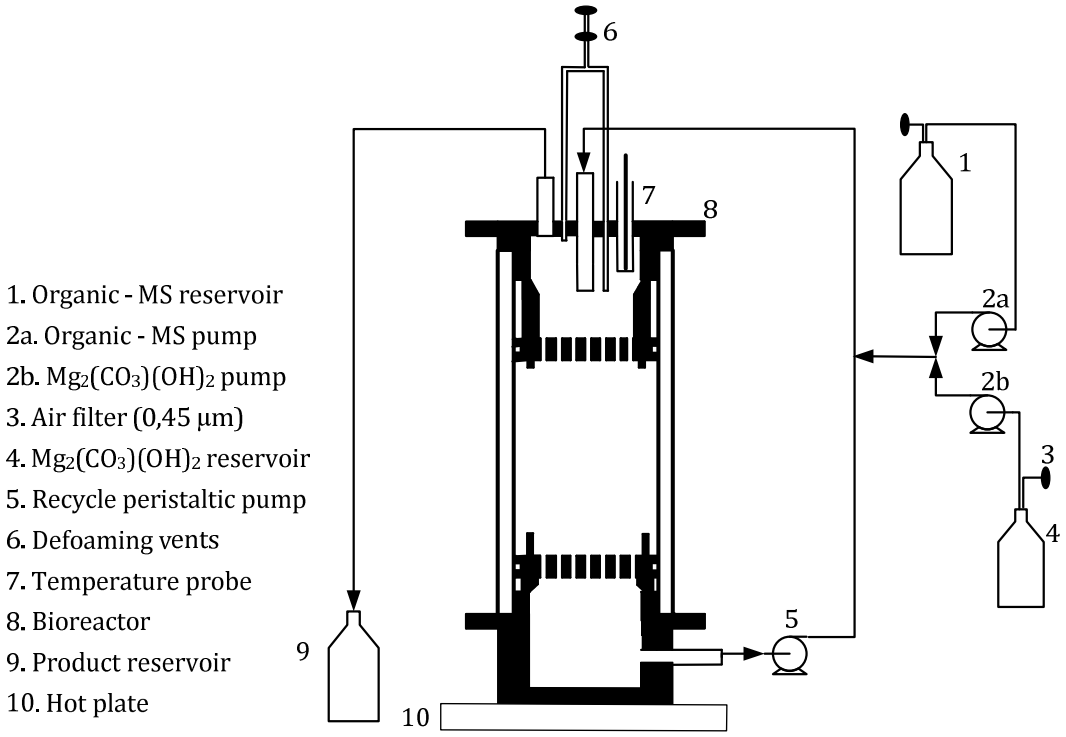

Figure 3.1: Bioreactor setup

3.4.1 Experimental conditions

The working reactor volume was determined by filling the reactor setup with water then disassembling and measuring the volume of water contained therein. For SC studies the distributors were removed. The SC reactor had a working volume of 160 mℓ with 90 % of the volume contained in the glass section.

The Organic-MS, $Mg_2(CO_3)(OH)_2$ and recycle pumps were Watson Marlow peristaltic pumps models 120U, 520s, and 323s, respectively. Table 3.3 describes the tube sizes, flowrates and dilution rates used in the SC studies. The dilution rate was defined as the total flowrate into the reactor divided by the empty reactor volume. The recycle flowrate was maintained at 12,500 mℓ h^{-1} for all fermentation runs. The linear flow rate within the glass section at the prescribed recycle rate was 3 mm s^{-1}.

Table 3.3: Pump flowrates and dilution rate calculation

Pump	Internal tube diameter (mm)	Min-Max inlet flowrates (mℓ h^{-1})	Dilution rate (h^{-1})
Organic-MS (2a)	1,6	3,3 – 48,4	0,04 – 0,6
$Mg_2(CO_3)(OH)_2$ (2b)	1,6	3,1 – 46,8	

The flowrates of the Organic-MS and $Mg_2(CO_3)(OH)_2$ streams were essentially equal for all the dilution rates investigated; therefore the concentration of the components in the Organic-MS reservoirs were doubled in order for the feed concentrations to be equal to those described in Table 3.1. Feed glucose concentrations of 20 and 40 g ℓ^{-1} were investigated with their corresponding $Mg_2(CO_3)(OH)_2$ reservoir concentrations of 60 and 120 g ℓ^{-1},respectively.

The Organic-MS and $Mg_2(CO_3)(OH)_2$ reservoirs were equipped with a medium delivery line, medium transfer line and 0,45 µm air filter. The medium delivery line was used as the feed line into the reactor and the medium transfer line was used to refill the reservoir as medium levels decreased. The reservoir contents were stirred at 80 rpm to maintain homogenous suspensions.

Temperature in the reactor was controlled at 37°C by use of a Heidolph EKT Hej-con thermostat and a Heidolph MR 3001 K hot plate. A stainless steel sheath was constructed to house the temperature probe to prevent contamination of the medium.

The 1,5 m vents depicted in Figure 3.1 were used for defoaming the reactor medium. The outlet of the defoaming line was equipped with two 0,45 µm air filters to prevent contamination of the reactor contents.

3.4.2 Operational procedure for continuous bioreactor

Figure 3.2 shows the protocol used in the continuous bioreactor. Continuous fermentations were conducted for dilution rates of 0,04 - 0,6 h^{-1}. Time spans where minimal concentration variation occurred were considered to represent steady state behavior; the dilution rate was increased only after a steady state was reached.

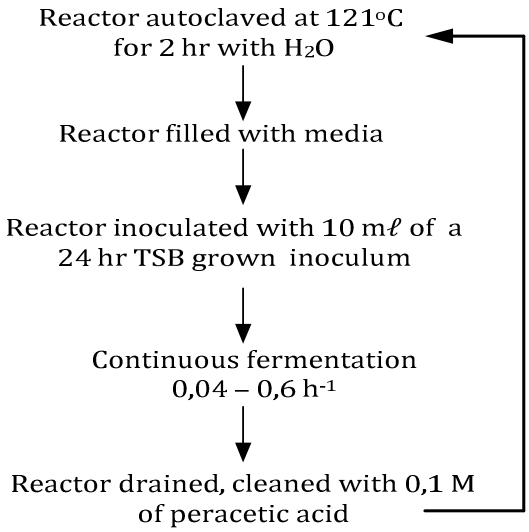

Figure 3.2: Operation protocol for continuous SA fermentations

3.4.3 Biofilm run

Perlite is a naturally occurring siliceous rock; when heated to temperatures above 1000 ^{o}C the rock expands to 20 times its original volume. Groperl®, part of the Genulite™ grade is a perlite used extensively in horticulture because of its high absorption of water.

Biofilm studies were conducted using Groperl® particles as inert supports where biofilm attachment could occur. 2,5 grams of Groperl® particles with equivalent diameters of 3 – 4 mm and a dry bulk density of 100 kg m^{-3} were used.

Preliminary flow tests showed that downward fluidization of the particles required a recycle flowrate of 37,500 mℓ h^{-1}. The high recycle flowrate would have caused high shear rates and attrition in the reactor which would have lead to low biofilm formation on the support particles, therefore the biofilm reactor was operated as an upflow packed bed; which required a significantly lower recycle rate of 12,500 mℓ h^{-1}. For the biofilm reactor the distributors and Groperl® particles were placed in the glass section of the reactor. The biofilm reactor had an empty volume of 115 mℓ, dilution rates of 0,11 h^{-1} to 1,0 h^{-1} were investigated.

3.5 Analytical methods

3.5.1 Fermentation analysis

4 mℓ samples of the reactor effluent stream were collected aseptically every 12 hrs. 1 mℓ of the sample was centrifuged at 13,4×10^{3} rpm using the Eppendorf centrifuge MiniSpin® for 90 seconds to separate the cells and $MgCO_3$ as pellets at the bottom of the centrifuge tubes. The supernatant was withdrawn from the centrifuge tubes and filtered using 0,2 µm cellulose acetate filters and analysed by high performance liquid chromatography (HPLC).

The Agilent® 1260 Infinity HPLC was used in conjunction with an Agilent® 1260 refractive index detector. Glucose and the fermentation products were separated in a single injection by a Bio-Rad Aminex HPX-87H column (300×7,8 mm). Sulfuric acid aqueous solution (0,3 mℓ ℓ^{-1}) was used as the mobile phase at a flow rate of 0,6 mℓ min^{-1} with an injection volume of 20 µℓ. All separations were conducted at a column temperature of 60°C.

3.5.2 Suspended cell concentration

Three empty centrifuge tubes were weighed on an Ohaus Adventurer AR2140 electronic balance with an accuracy of 10^{-4} grams. 1,0 mℓ of the reactor sample was poured into each of the three centrifuge tubes. The 1,0 mℓ samples were mixed with 0,5 mℓ of 32% (w/w) HCℓ to react with the $MgCO_3$ in the samples.

After complete $MgCO_3$ dissolution, the samples were centrifuged and the supernatant removed. The centrifuge tubes were then dried in an oven at 90^{o}C for 24 hrs; the drying

period was proven to be long enough to ensure no further weight changes. The tubes were reweighed and the mass recorded. The tube mass was subtracted from the resulting mass to ascertain the mass of cells in the 1,0 mℓ sample. The average cell concentration was reported from the three tubes. All cell concentrations reported are dry cell weight concentrations. In the continuous studies SC concentrations were measured only when steady state was achieved.

Chapter 4

Results and Discussion

4.1 Batch fermentations

The results of the batch fermentations for the initial glucose concentrations of 20 and 40 g ℓ^{-1} are shown in Figure 1(a) and Figure 1(b), respectively. The SA yield was defined as the mass of SA formed per mass of glucose reacted. The SA productivity was calculated as the SA produced per fermentation time elapsed.

Figure 1(a) shows that the lag time observed in the 20 g ℓ^{-1} fermentations lasted for at least 5 hrs, thereafter fermentation commenced and full glucose conversion was achieved after 25 hrs. The final SA concentration and yield were 16,3 g ℓ^{-1} and 0,82 g g^{-1}, respectively. Acetic acid was the only by-product; the final concentration was 3,5 g ℓ^{-1}. The cell concentration was observed to increase to a maximum of 3,5 g ℓ^{-1} after 27 hrs of fermentation. Over the course of the fermentation run the medium pH dropped from an initial value of 7,8 to 6,8 at the end of the fermentation run.

Figure 1(b) shows that the lag time observed in the 40 g ℓ^{-1} fermentations lasted for at least 12 hrs. The final SA concentration and yield were 28,8 g ℓ^{-1} and 0,72 g g^{-1}, respectively. The final acetic acid concentration was 2,9 g ℓ^{-1}; lower than observed in Figure 1(a). The medium pH was observed to decrease from an initial 7,8 to 6,4 at the end of the fermentation run. The cell concentration increased to a maximum of 7 g ℓ^{-1} after 46 hrs of fermentation.

The results presented in Figure 1(a) and Figure 1(b) demonstrated the effectiveness of the $Mg_2(CO_3)(OH)_2$ slurry to act as a pH buffer and inorganic carbon source for SA fermentations. The duration of the 20 and 40 g ℓ^{-1} glucose fermentations were 34,5 hrs and 75,5 hrs, respectively. Despite the long fermentation periods, the fermentation strategy did not affect SA titers, yields and cell concentration. The following could have been responsible for the long fermentation periods:

- high concentration of chloride ions in the Organic-MS medium
- growth of the inoculum in an generic tryptone soy broth (TSB) medium
- lack of quantification of the active cell concentration within the inoculums

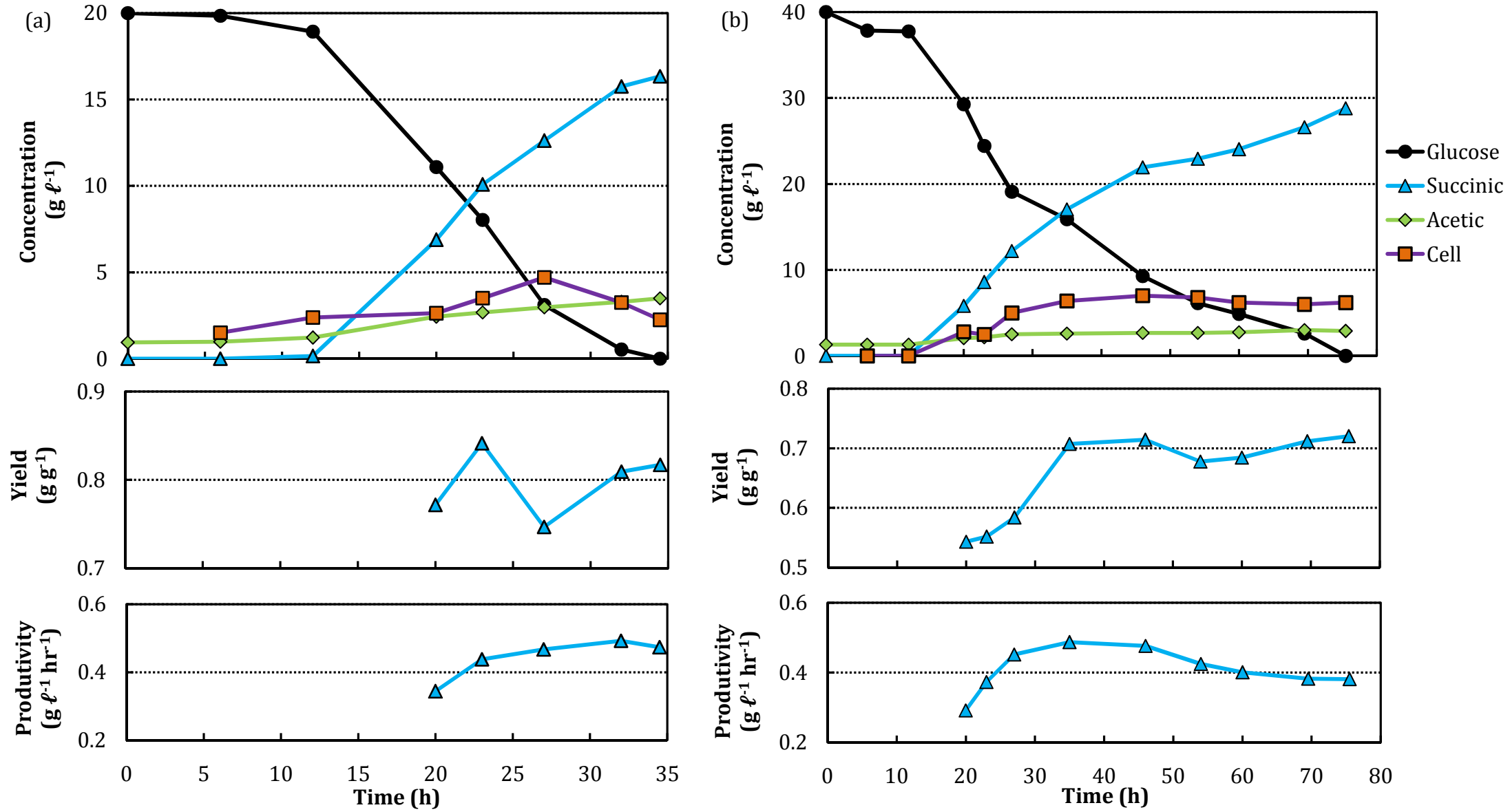

Figure 4.1: Batch fermentation results for initial glucose concentrations of (a) 20 g ℓ^{-1} and (b) 40 g ℓ^{-1}

4.2 Continuous fermentations

4.2.1 Suspended cell

Two suspended cell (SC) fermentations were conducted for the 20 g ℓ^{-1} glucose feed concentration and one for the 40 g ℓ^{-1}. Fermentation was terminated only when contamination occurred or when glucose conversion was below 30 %. The time dependant data of all the continuous SC runs are given in Figure 4.2, Figure 4.3 and Figure 4.4.

Figure 4.2 shows the SC fermentation data accumulated over 12 days of continuous operation for the glucose feed concentration of 20 g ℓ^{-1}. After 14 hrs of continuous fermentation at a dilution rate of 0,04 h^{-1} the concentration of glucose and SA were 0 and 14,8 g ℓ^{-1}, respectively. The pH of the outlet stream was 6,8 which as discussed by Guettler *et al.* (1996) was conducive for SA fermentation. The reactor operated at full glucose conversion for 10 hrs, however at event (a) the glucose concentration was observed to increase accompanied with the washout of fermentation products. After 26 hrs, the glucose and SA concentrations reached 7,9 and 9 g ℓ^{-1}, respectively. The cause of this phenomenon was unknown, however it was clear that complete cell death had occurred and as a result fermentation had terminated.

Event (b) indicated in Figure 4.2 was the point where the reactor was re-inoculated and the dilution rate increased to 0,08 h^{-1}; it was thought that by preventing full glucose conversion cell death would not occur. After event (b), the reactor resumed normal operation, after 57 hrs the glucose and SA concentrations were 0,4 and 14,2 g ℓ^{-1}, respectively. The pH of the reactor's outlet stream after re-inoculation decreased from 6,8 to 6,4, the pH drop was due to the increase in by-product concentrations as shown by Figure 4.2.

When the dilution rate was increased from 0,08 h^{-1} to 0,12 h^{-1} the $Mg_2(CO_3)(OH)_2$ slurry concentration in the reservoir was increased from 60 to 80 g ℓ^{-1}, for the remainder of the run reported in Fig 4.2. This was done to increase the reactor pH from 6,4 to 6,6. After event (b) no cell death events were observed.

SA was the main product during the whole fermentation run; concentration and yields varied within a range of 14,8 – 10,4 g ℓ^{-1} and 0,62 - 0,84 g g^{-1}, respectively. The lactic acid concentration was observed to increase with increased dilution rate to a maximum of 5,7 g ℓ^{-1} at 0,28 h^{-1}. The concentrations of acetic and formic acids were observed to decrease with increased dilution rate. The dry cell concentrations ranged from 0,55 - 3,3 g ℓ^{-1}, however, the minimum cell concentration was measured after cell death had occurred.

The experiment was terminated after 12 days because the process became contaminated with lactic acid producing bacteria. The contamination was identified by the complete conversion of glucose to lactic acid.

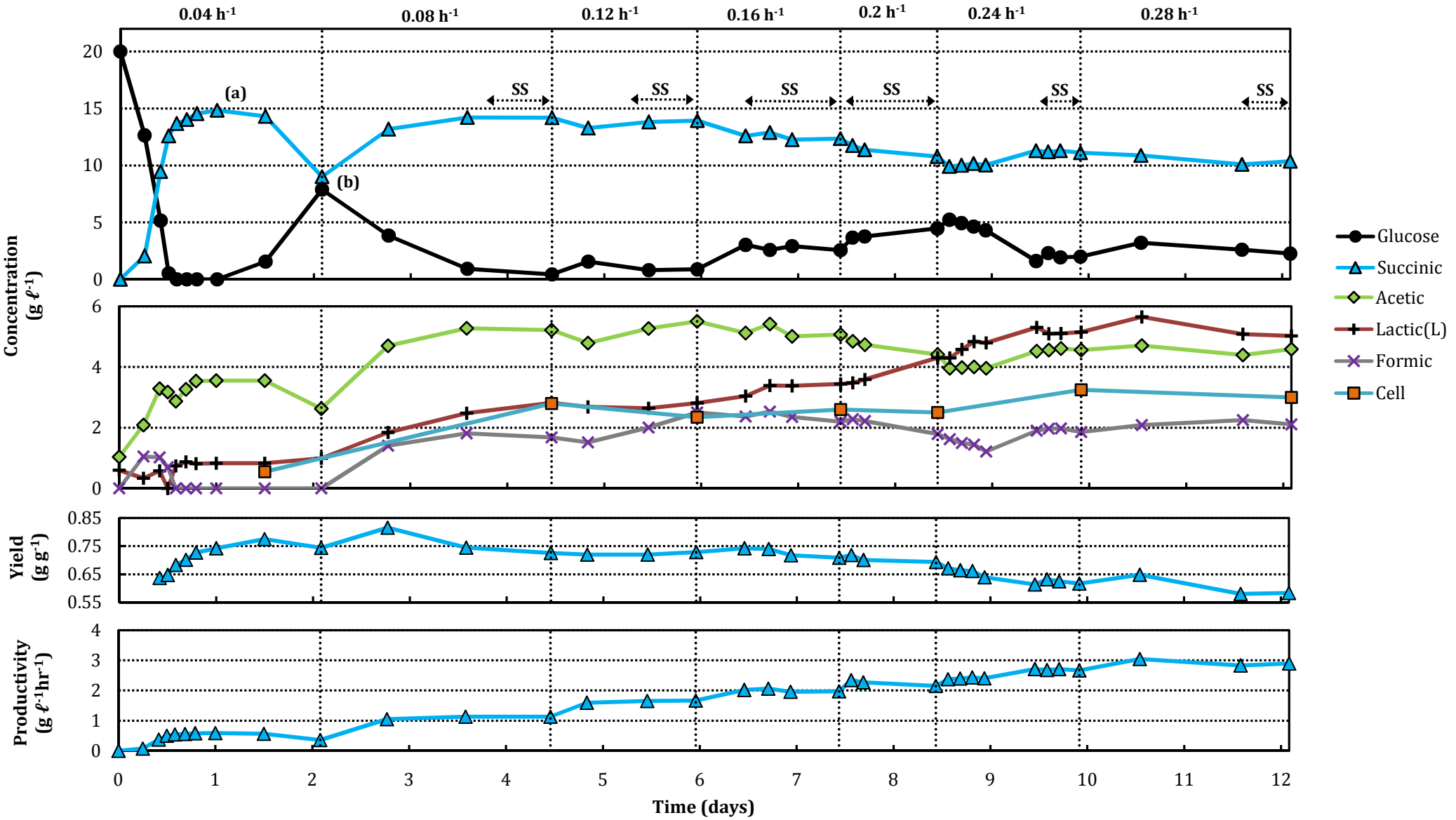

Figure 4.2: Effect of dilution rate on continuous SA production (20 g ℓ^{-1} feed glucose); steady states indicated as SS

After the experiment reported in Figure 4.2 was terminated the effect of increased glucose feed concentration on SA production was studied. Figure 4.3 shows the results of the continuous fermentations conducted with an initial feed glucose concentration of 20 g ℓ^{-1} followed by an increase to 40 g ℓ^{-1}. The discontinuity in Figure 4.3 at 6,5 days indicates the glucose feed change.

The experimental run conducted with a glucose feed concentration of 20 g ℓ^{-1} displayed the cyclic behavior previously observed in Figure 4.2. The reactor was started at a dilution rate of 0,04 h^{-1}, once glucose was observed to reached full conversion the dilution rate was increased to 0,08 h^{-1}. Event (a) in Figure 4.3 shows the point where the dilution rate was increased from 0,04 h^{-1} to 0,08 h^{-1}; this was done to avoid the cell death that was observed in Figure 4.2.

The increase in dilution rate to 0,08 h^{-1} did not lead to stable operation as observed after event (a) in Figure 4.2. In fact, the reactor completely washed out by the time event (b) in Figure 4.3 was reached. At event (b) the reactor was re-inoculated and full glucose conversion was achieved after 13,5 hrs of operation, after 5,5 hrs of operation at full glucose conversion the same trend of glucose re-accumulation accompanied with product washout was observed. After 12,5 hrs of glucose re-accumulation the flow into the reactor was stopped and batch operation was conducted for 6,5 hrs. However, there were no changes in concentrations at the end of the batch operation; indicating that there were no cells alive in the reactor to perform fermentation.

The reactor was re-inoculated at event (c) and the dilution rate increased to 0,12 h^{-1}. After event (c) the concentration of by-products; acetic, lactic, and formic acids were observed to increase above those concentrations obtained at 0,04 h^{-1} and 0,08 h^{-1}. The pH of the medium decreased to 6,5 due to the increased by-product concentrations. After 2,5 days from event (c) steady state operation was observed.

After the glucose feed change to 40 g ℓ^{-1}; the performance of the reactor did not improve significantly; the SA concentration increased from 13,8 to 15 g ℓ^{-1} while the concentrations of the by-products increased more significantly. The increase in glucose concentration increased the cell concentration by 10% to maximum of 3,6 g ℓ^{-1} at 0,12 h^{-1}.

There was no notable increase in the reactor performance when the glucose feed concentration was doubled from 20 to 40 g ℓ^{-1}, this could have been caused by nutrient limitations because only the glucose concentration was double whilst the other components of the fermentation medium were maintained at the same concentrations.

The experiment operated aseptically for 14 days and was terminated because the glucose conversion was 28 %; lower than the 30 % glucose conversion criteria required for the termination of aseptic experiments.

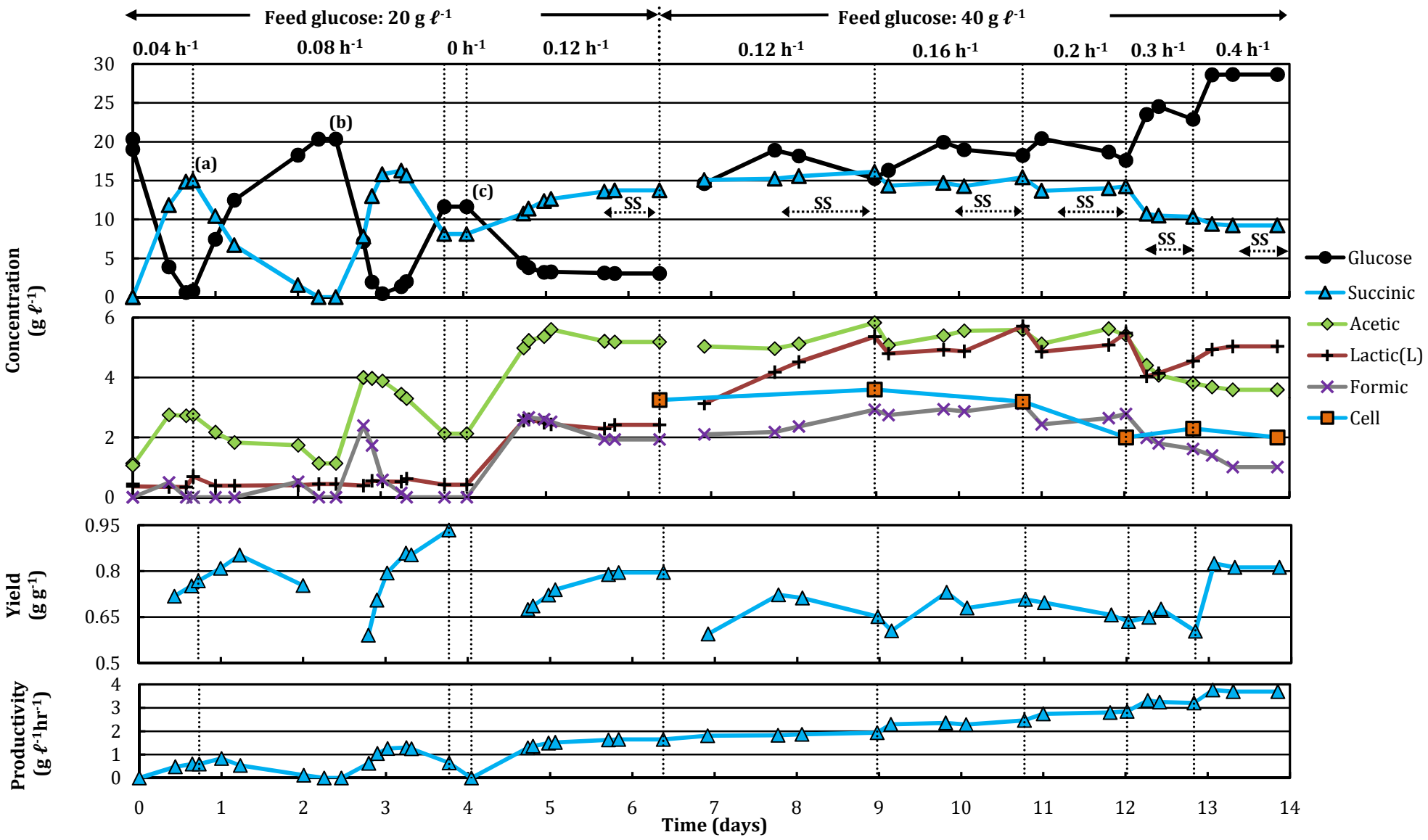

Figure 4.3: Effects of 20 and 40 g ℓ^{-1} glucose feed concentrations and dilution rate on SA production; steady states indicated as SS

The data acquired from Figure 4.2 were for low dilution rates (0,12 h^{-1} - 0,28 h^{-1}), it was therefore deemed necessary to study the effect of dilution rates greater than 0,3 h^{-1} for comparison with data acquired from Figure 4.3.

Figure 4.4 shows the fermentation data accumulated over 14 days of continuous aseptic operation; the fermentation run was terminated after the medium became contaminated with lactic acid producing bacteria.

The cell death events observed at 0,04 h^{-1} and 0,08 h^{-1} in Figure 4.2 and Figure 4.3 lead to the alteration of the initial dilution rate to 0,12 h^{-1} in order to achieve steady operation as observed in Figure 4.3. However, after 1 day of continuous fermentation event (a) was observed; showing that cell death had occurred despite there being an ample supply of glucose. Ten hours after event (a) the outlet glucose concentration was equal to the feed concentration, therefore at event (b) the reactor was re-inoculated.

However, event (c) occurred 1,8 days after re-inoculation, the causes of the cell death were still unknown because the cells had ample supply of glucose. The reactor was re-inoculated again at event (d) and left to run. Samples after event (d) were not taken because it was assumed that cell death and washout would reoccur. After 4,4 days (event (e)) the concentrations of glucose and the acids were similar to those observed at event (c), however, cell death and product washout never occurred. The reactor was operated stably at a glucose concentration of 0,5 g ℓ^{-1} for 11 hrs with no incidents of cell death and product washout.

SA was the main product during the whole fermentation run, concentrations and yields varied within a range of 13,8 – 8,7 g ℓ^{-1} and 0,54 - 0,82 g g^{-1}. The lactic acid concentration was observed to increase with increased dilution rate to a maximum of 5,8 g ℓ^{-1} at 0,4 h^{-1}. The acetic and formic acid concentrations were observed to decrease with increased dilution rate. The dry cell concentrations ranged from 2-2,6 g ℓ^{-1}.

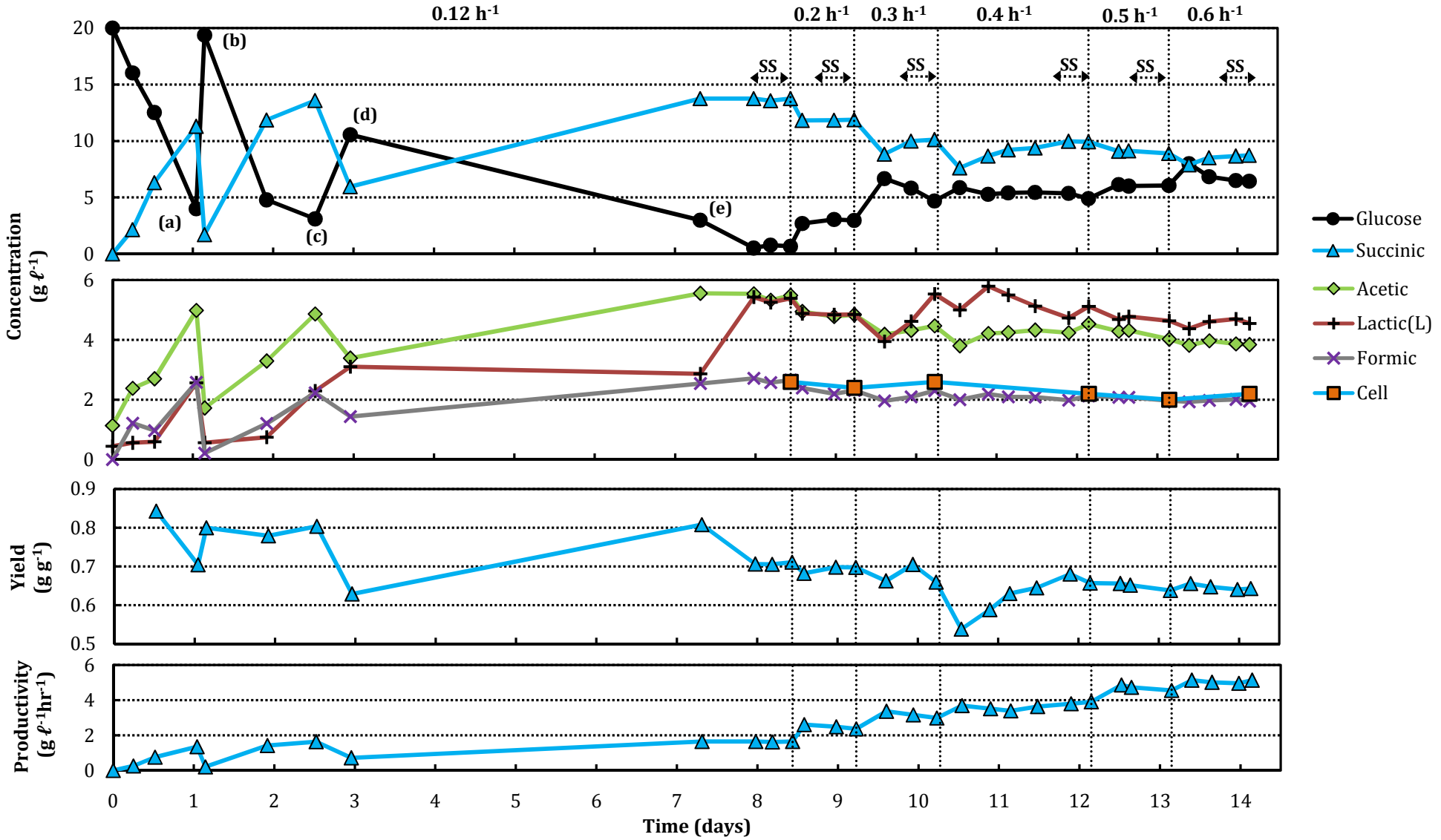

Figure 4.4: Effect of high dilution rates on continuous SA production (20 g ℓ^{-1} feed glucose); steady states indicated as SS

4.2.2 Biofilm reactor run

The biofilm study was conducted over a 6,5 day period, for the glucose feed concentration of 20 g ℓ^{-1}. The results of the fermentation run are reported in Figure 4.5. Dilution rates of 0,11 to 1 h^{-1} were investigated; the experiment served as a preliminary test for the performance enhancement that biofilms may have on SA production.

Events (a) and (b) in Figure 4.5 illustrate the times where the reactor was re-inoculated to counteract possible cell death events that might have occurred prior to re-inoculation. After 4 days of unsteady operation the dilution rate was increase to 0,33 h^{-1}, after which the reactor's performance ceased to be erratic. Initially, the acid concentrations decreased and glucose concentration increased, this was attributed to the 3 orders of magnitude increase in dilution rate. After 7 hrs of operation the SA concentration was observed to increase and glucose concentration decreased.

Over the course of 2 days the SA concentration increased from 7,3 to 17,1 g ℓ^{-1} whilst maintaining yields of 0,95 g g^{-1}; SA productivity reached 5,7 g ℓ^{-1} h^{-1}. The biofilm reactor at 0,33 h^{-1} achieved twice the steady state SA productivity of the SC reactor at 0,3 h^{-1} (Figure 4.4) with a 42 % increase in SA yield.

The biofilm reactor had not reached a steady state, even after 15 residence times, implying that the biofilm colonization of the supports was increasing over the 2 days. Establishing steady state operation was not the primary goal of the biofilm run; hence SC concentrations were not measured. On the 6th day the dilution rate was increased from 0,33 h^{-1} to 1 h^{-1}. The SA yield increased to 1 g g^{-1} whilst the SA concentration decreased to 12 g ℓ^{-1}, the corresponding SA productivity was 12 g ℓ^{-1} h^{-1}.

The packed bed biofilm reactor was clogged after 12 hrs of operation at 1 h^{-1}; clogging was caused by the buildup of solids from the $Mg_2(CO_3)(OH)_2$ slurry and cells. Clogging lead to pressure buildup which caused the bottom of the reactor to pop off, spilling the reactor's contents. After this incident the biofilm experiment was terminated.

The SA productivities and yields achieved in the biofilm reactor run were significantly higher than those reported in Figure 4.2, Figure 4.3 and Figure 4.4. The effectiveness of the Groperl® particles was linked to the particle's rough surfaces and crevices providing conducive zones for biofilm attachment.

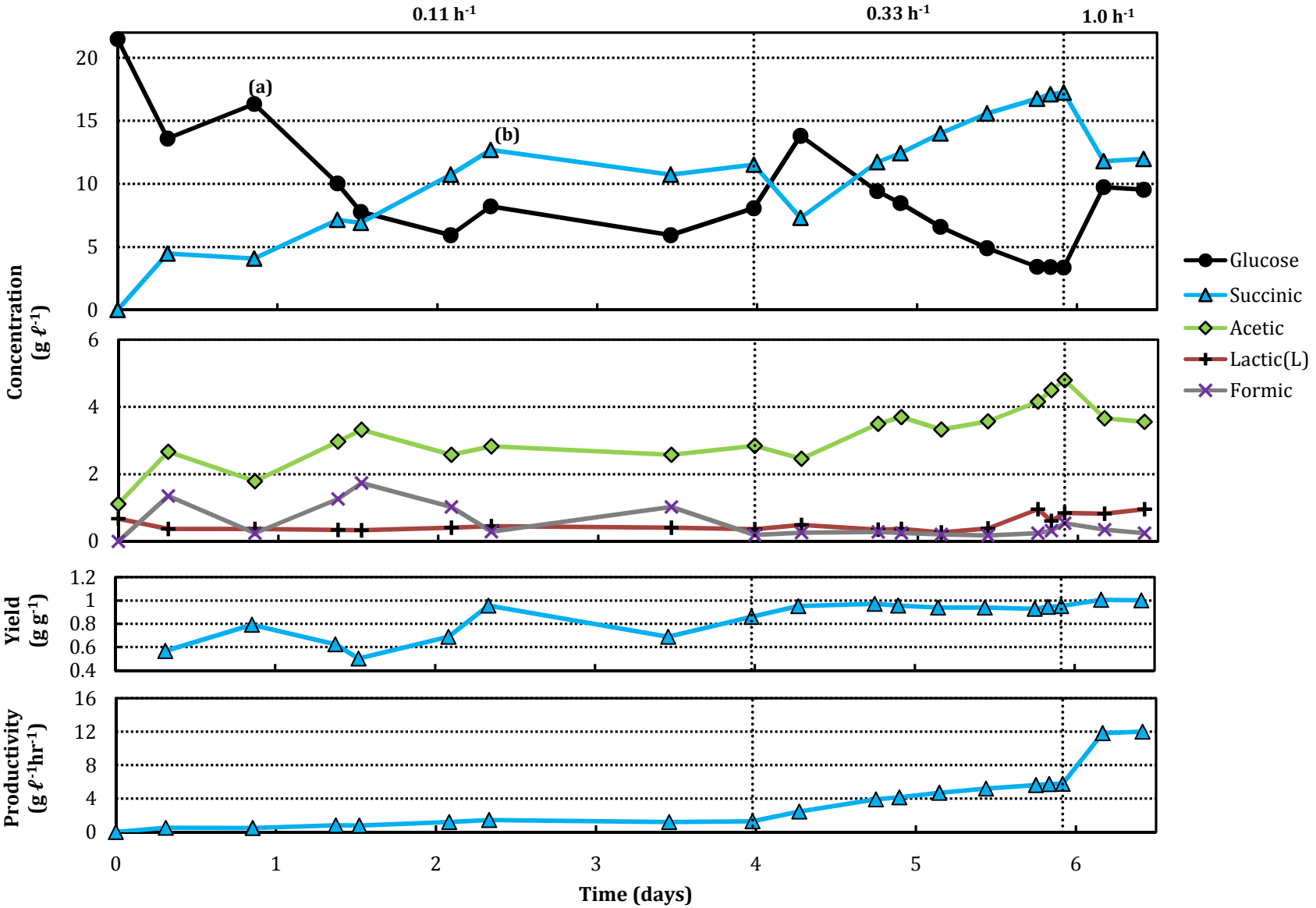

Figure 4.5: Effect of dilution rate on continuous SA production in a biofilm reactor (20 g ℓ^{-1} feed glucose)

4.3 Steady state analysis

4.3.1 Chemostat steady state data

Steady state is a condition that requires that all measured state variables remain constant independent of time. Time spans where minimal concentration variation occurred were considered to represent steady state behavior and are indicated by SS on Figure 4.2, Figure 4.3 and Figure 4.4.

Steady state data for the 20 g ℓ^{-1} glucose feed experiments were obtained from Figure 4.2 and Figure 4.4. In Figure 4.2 steady state operation was achieved for dilution rates of 0,08 – 0,28 h^{-1}. In Figure 4.4, steady state behaviour was observed from 0,12 – 0,6 h^{-1}. Therefore, steady state data for the 20 g ℓ^{-1} glucose feed experiments were considered for dilution rates 0,12 – 0,6 h^{-1}.

Steady state data obtained for dilution rates 0,12 h^{-1} and 0,2 h^{-1} from Figure 4.2 and Figure 4.4 were reported in Figure 4.6(a) to demonstrate the repeatability of the steady state concentrations. Figure 4.6(a) and Figure 4.6(b) represent the steady state data from the experiments conducted with a glucose feed concentration of 20 and 40 g ℓ^{-1}, respectively.

Figure 4.6(a) and Figure 4.6(b) show that concentrations of SA and by-products decreased with increased dilution rate. Figure 4.6(a) shows that cell concentrations increased with dilution rate upto 0,2 h^{-1} after which the cell concentrations decreased and maintained concentrations of 2 g ℓ^{-1} for dilution rates of 0,31-0,6 h^{-1}. Figure 4.6(b) also shows that constant cell concentrations were maintained for dilution rates of 0,2 – 0,4 h^{-1}.

The increase in glucose feed concentration from 20 to 40 g ℓ^{-1}, did not significantly increase the steady state SA and cell concentrations. The minimal improvement in performance could be indicative of a nutrient limitation because only the glucose concentration in the feed medium was doubled whilst all other concentrations were maintained at constant levels.

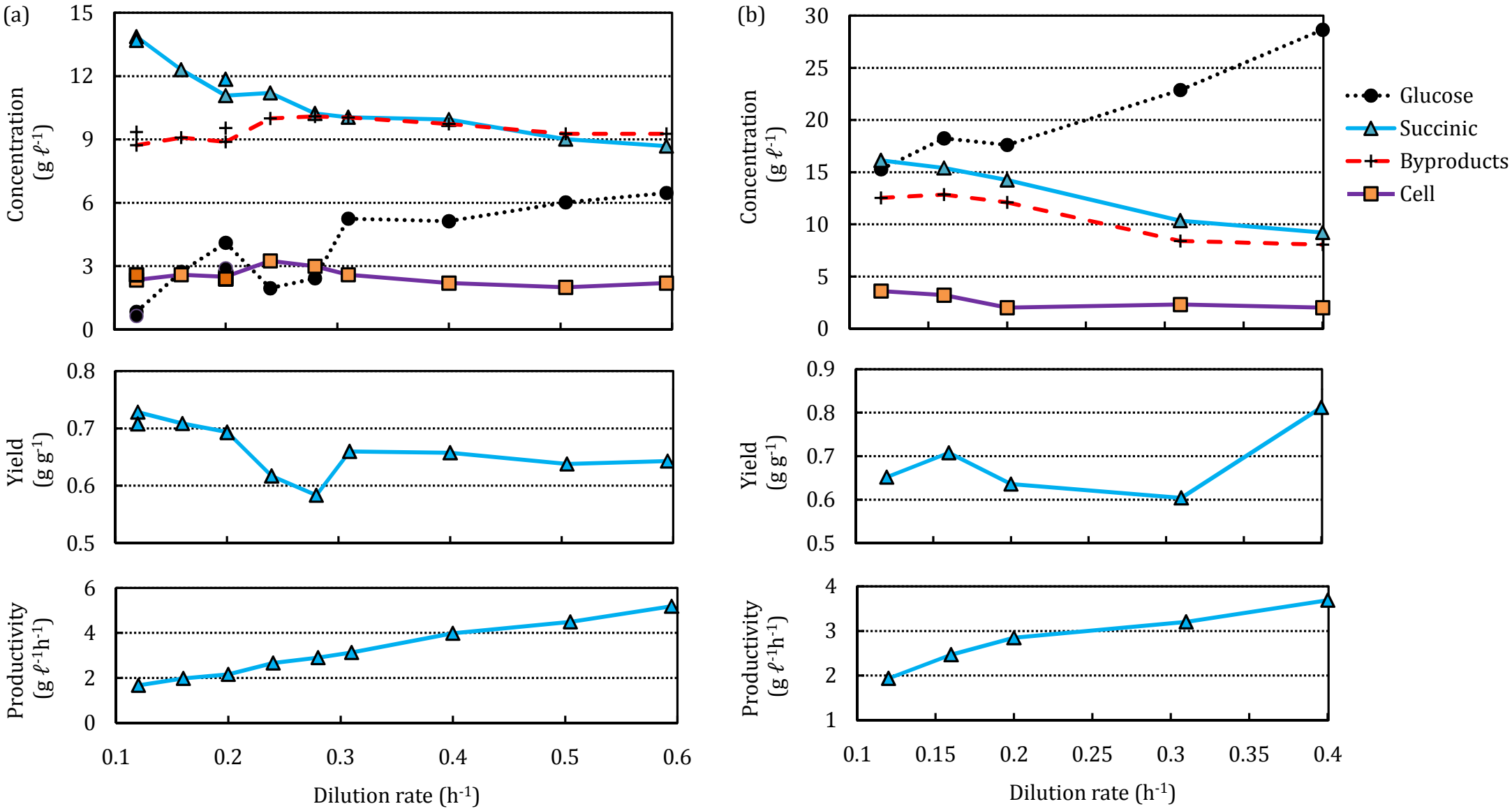

Figure 4.6: Steady state performance of SC fermentations for glucose feed concentrations of (a) 20 g ℓ^{-1} and (b) 40 g ℓ^{-1}

4.3.2 Steady state parameters

Specific succinic acid reaction rate ($\hat{R}_{SA}$) and substrate reaction rate ($\hat{R}_S$)

Product formations and substrate utilization in fermentors are sometimes directly linked to the cell growth rate; in which case the production and consumption are said to be growth related. In some fermentations non-growing cells are also responsible for product formations and substrate consumption for maintenance. Equations 1 and 2 are generic representations of the contributions of the growth and non-growth terms in SA production and substrate utilization.

$$R_{SA}=\alpha_{SA}R_X+\beta_{SA}C_X \quad (1)$$

$$R_S=\alpha_S R_X+\beta_S C_X \quad (2)$$

Equations 3 and 4 are obtained by the division of Equations 1 and 2 by the dry cell concentration (C_X) and the substitution of μ with D (steady state operation). The α and β parameters can therefore be obtained from the experimental data by plotting $\hat{R}_{SA}$ and $\hat{R}_S$ (with a dry cell basis) against the dilution rate.

$$\hat{R}_{SA}=\alpha_{SA}D+\beta_{SA} \quad (3)$$

$$\hat{R}_S=\alpha_S D+\beta_S \quad (4)$$

From Figure 4.7 (a) and (b) it is evident that β_{SA} and β_S are negligible and α_{SA} and α_S are not very sensitive to the glucose feed concentration. For β_{SA} and β_S values of zero; the α_{SA} and α_S values of 4,17 g g^{-1} and 6,35 g g^{-1} represent the best fitted yield parameters of $Y_{SA/X}$ and $Y_{S/X}$, respectively. These values were used to model the steady state data for the 20 g ℓ^{-1} glucose feed reactor in Appendix A.

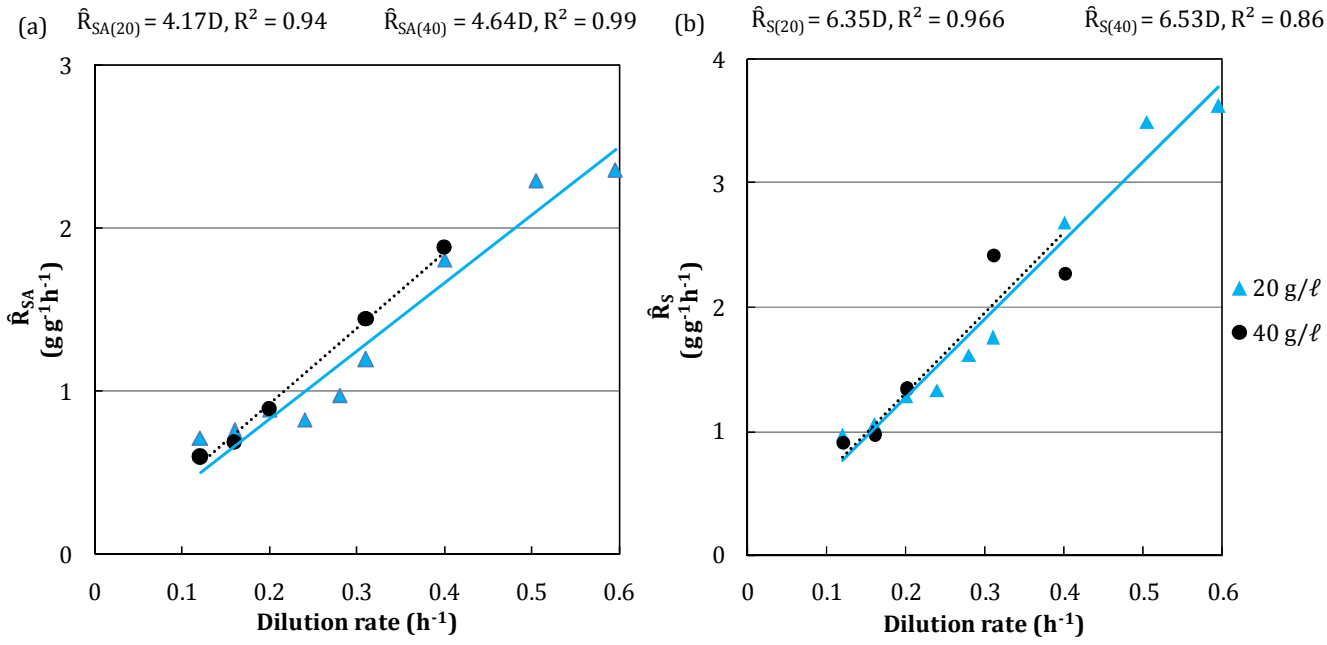

Figure 4.7: Steady state (a) $\hat{R}_{SA}$ and (b) $\hat{R}_S$ for 20 and 40 g ℓ^{-1} glucose concentrations

Specific growth constant (μ)

The specific growth constant (Equation 5) is a function of glucose and product concentrations. The specific growth constant is related to the substrate concentration by the Monod equation given in Equation 6.

$$\mu=\mu_{max} f(C_S)\, f(C_P) \tag{5}$$

$$f(C_S)=\frac{C_S}{K_S + C_S} \tag{6}$$

The experimental observation that increased glucose concentrations did not alter the SA production rates leads to the conclusion that the specific growth constant (μ) is not significantly affected by glucose concentrations; this implies that the growth constant was zero order with respect to the glucose concentration and that there was no substrate inhibition.

In order to mathematically model the total acid inhibition effect the $f(C_S)$ was assumed to be 1 (no substrate effects) and μ was substituted with D (steady state operation) in Equation 5. The relationship between μ and C_P could therefore be determined from experimental data (20 g ℓ^{-1} glucose feed) by plotting the total acid concentration against the dilution rate.

Figure 4.8 shows that the specific growth constant increased significantly as the total acid concentration in the reactor decreased. The total acid inhibition model fitted to the experimental data is given in Equation 7, fitted values of μ_{max} and $C_{p,max}$ were 2,0 $h^{-1}$and 24 g ℓ^{-1}, respectively. The Monod and product inhibition expressions (values excluded) were used to model the steady state data for the 20 g ℓ^{-1} glucose feed reactor in Appendix A.

$$\mu= \mu_{max}\,(1-\frac{C_P}{C_{P,max}}) \tag{7}$$

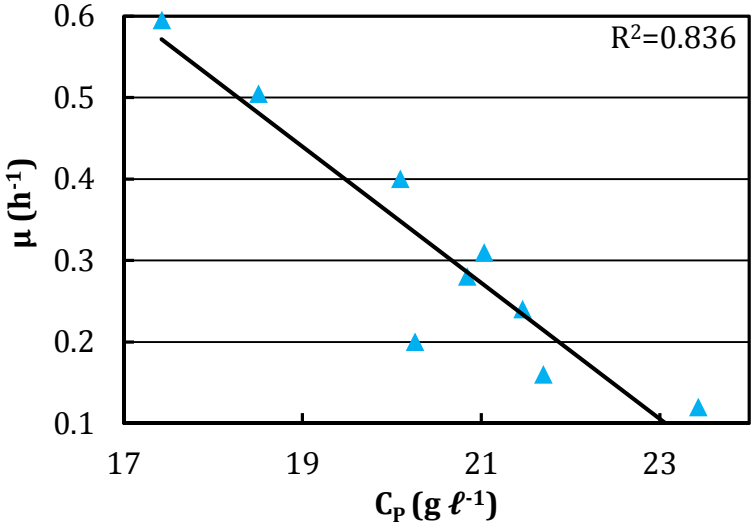

Figure 4.8: Effect of product inhibition on the specific growth constant

4.4 Overall process performance and comparison

Urbance *et al.* (2004) used the same fermentation medium and microorganism (*A. succinogenes*; ATCC 55618) as used in this work, therefore a comparison of performances is warranted.

Figure 4.9 is a repeat of Figure 2.2 with the inclusion of the steady state data presented in this report. Figure 4.9 shows that for dilution rates of 0,2 h^{-1} and 0,4 h^{-1} the Urbance *et al.* (2004) SA productivities and yields were comparable to those achieved in this report with the SC studies. For the dilution rate of 0,6 h^{-1} the SA productivity and yield in this report was higher than those reported by Urbance *et al.* (2004).

These observations call into question whether the PCS tubes offer any superior performances that its inventors have promoted. The composition of the PCS tubes was selected by Urbance *et al.* (2003) to offer alternative nutrient sources for the biofilms formed on the tube surfaces to improve SA yields. However, because the yields from Urbance *et al.* (2004) data shown no significant superiority to those obtained in this report, the claim that PCS tubes improve SA yields is questionable.

The SC data from this report were not superior in either SA productivity or yield to those attained by Lee *et al.* (2009) with *A. succiniproducens*. The Kim *et al.* (2009) results with *M. succiniproducens* presented in Figure 4.9 had similar SA productivities compared with this work despite the high cell concentrations of 8-12 g ℓ^{-1} obtained by Kim *et al.* (2009). The higher SA yields obtained in this work could be attributed to the use of corn steeped liquor which was absent in the Kim *et al.* (2009) studies.

The data presented for the biofilm studies in Figure 4.9 were not obtained at steady state, but were reported to illustrate the superior SA productivities and yields that can be obtained when using *A. succinogenes* biofilms. The SA productivity and yield of 12 g $\ell^{-1}h^{-1}$ and 1 g g^{-1} achieved at 1 h^{-1} were the highest reported in literature for *A. succinogenes*. Although limited, these preliminary results pave the way for further research on *A. succinogenes* biofilms.

The Lee *et al.* (2009) data for 38 g ℓ^{-1} glucose feed concentration presented in Figure 4.10 had higher SA productivities and yields compared to this work. The *A. succinogenes* results presented by Kim *et al.* (2009) also had higher SA productivities; however their yields are lower compared to those achieved in this work.

The Kim *et al.* (2009) membrane process was susceptible to rapid contamination with lactic acid forming bacteria; however, the slurry process developed in this work was more robust as it operated aseptically for upto 14 days before contamination was observed.

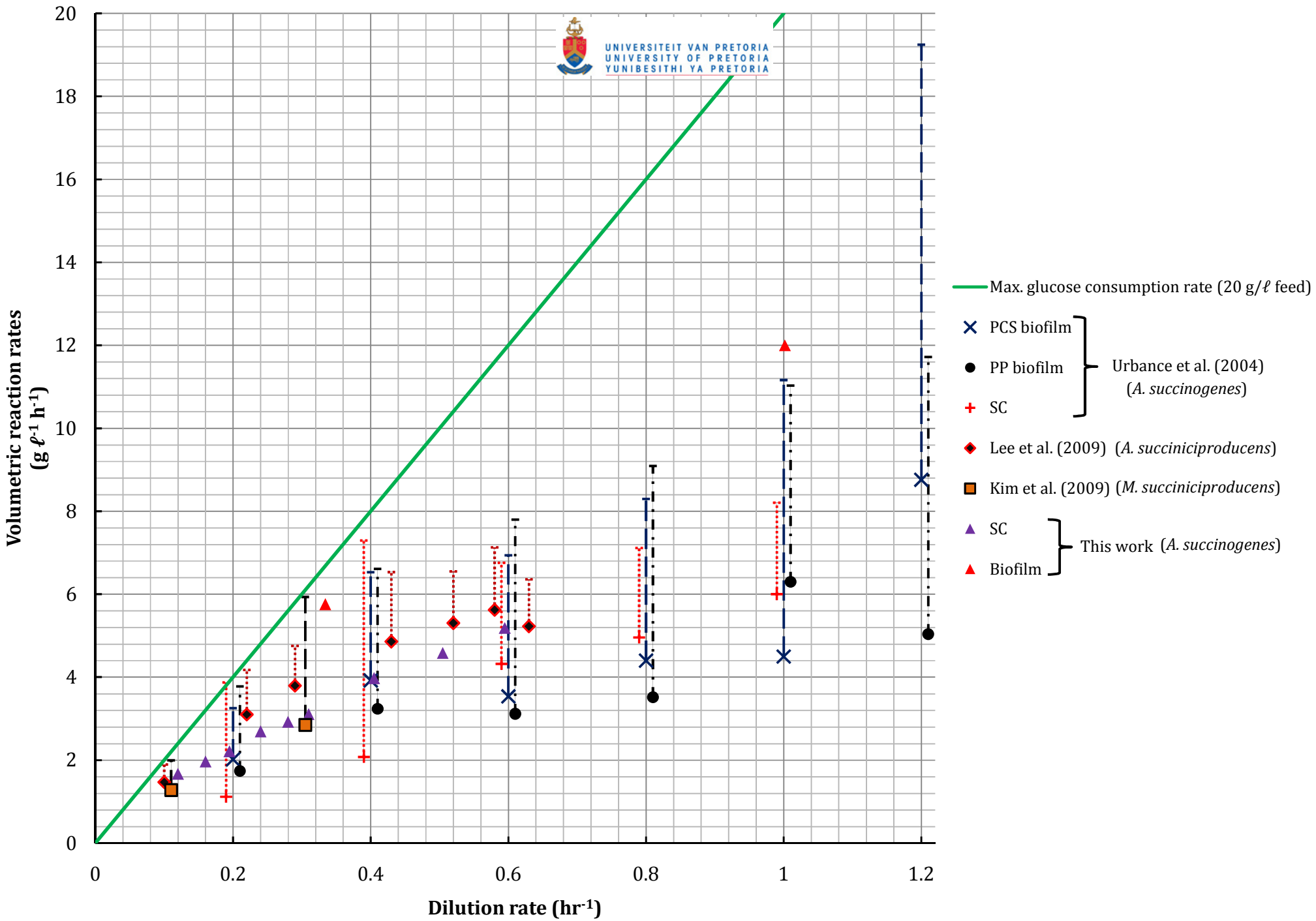

Figure 4.9: Comparison of continuous SA processes; 20 g ℓ^{-1} glucose feed concentration

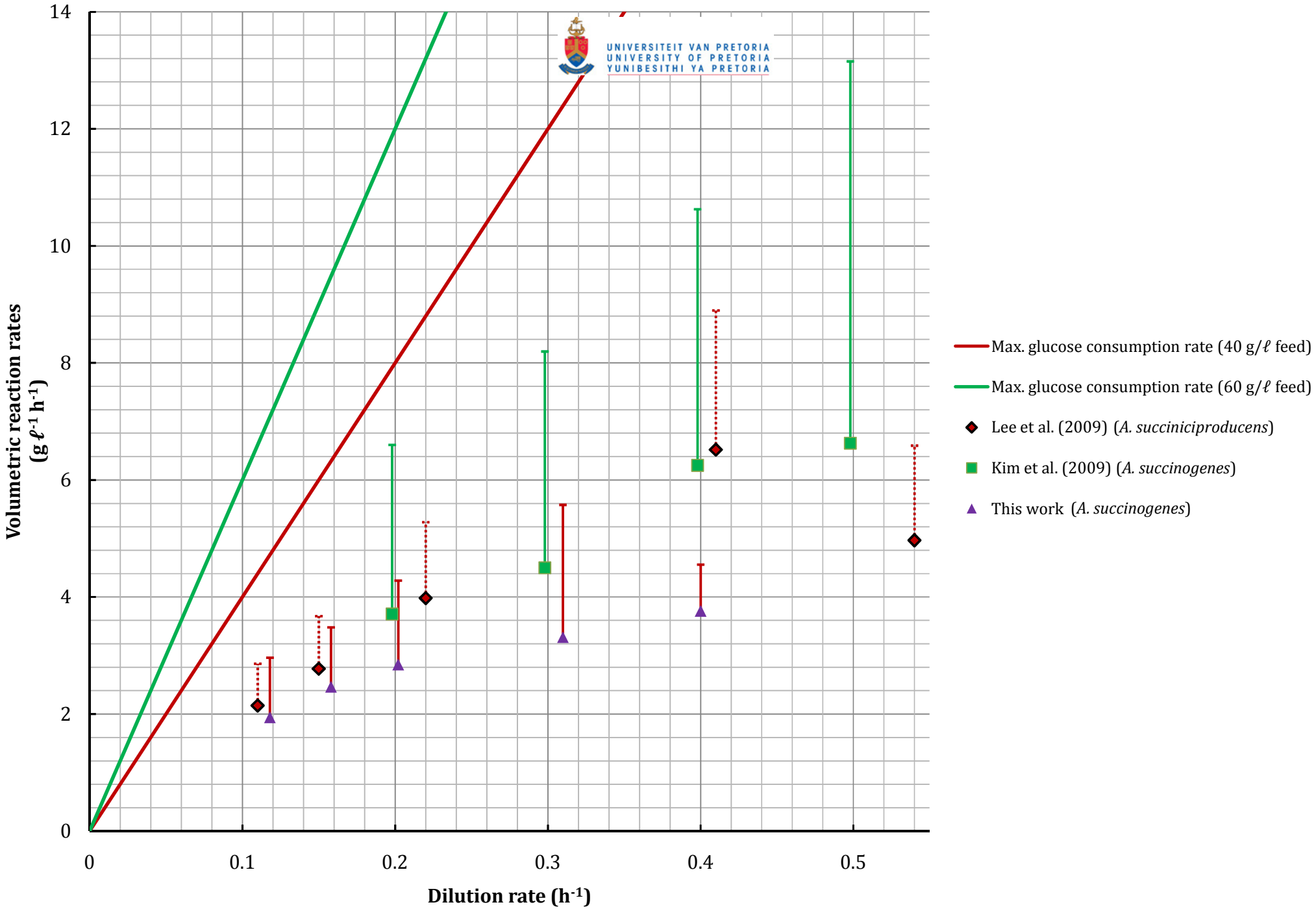

Figure 4.10: Comparison of continuous SA processes; 40 and 60 g ℓ^{-1} glucose feed concentrations

Chapter 5

Conclusions

Batch studies with the initial glucose concentrations of 20 and 40 g ℓ^{-1} achieved succinic acid (SA) concentrations and yields of 16,3 g ℓ^{-1} (0,82 g g^{-1}) and 28,8 g ℓ^{-1} (0,72 g g^{-1}), respectively. The SA productivities for the batch fermentations were 0,47 g $\ell^{-1}h^{-1}$ and 0,38 g $\ell^{-1}h^{-1}$, respectively. The $Mg_2CO_3(OH)_2$ slurry was observed to be an effective inorganic carbon source and was capable of buffering the medium pH in the range of 6,4 - 6,8.

Suspended cell (SC) continuous flow experiments were conducted aseptically for upto 14 days before contamination by lactic acid producing bacteria. The $Mg_2CO_3(OH)_2$ slurry allowed the medium pH levels to be buffered at 6,4 – 6,6 without active control. Fermentations with the glucose feed concentration of 20 g ℓ^{-1} achieved a maximum SA productivity of 5,2 g $\ell^{-1}h^{-1}$ at 0,6 h^{-1} with a corresponding SA yield of 0,65 g g^{-1}. Fermentations with the glucose feed concentration of 40 g ℓ^{-1} achieved a maximum SA productivity of 3,76 g $\ell^{-1}h^{-1}$ at 0,4 h^{-1} with a corresponding SA yield of 0,82 g g^{-1}. The SC results achieved in this work are comparable and even better than other continuous studies which used *A. succinogenes*, despite the fact that either biofilms or membranes were employed in these studies.

SA production in the SC reactor was observed to be growth associated. It was observed that the increase in substrate feed concentration from 20 to 40 g ℓ^{-1} had a negligible effect on the SA production rate. The total acid concentration in the fermentation broth had a severe inhibitory effect on the growth of *A. succinogenes* cells; a linear dependency was observed between the specific growth constant and the total acid concentration.

The preliminary biofilm study demonstrated the capability of *A. succinogenes* to produce SA in high productivities and yields. The SA productivities and yields for the dilution rates of 0,33 h^{-1} and 1,0 h^{-1}, were 5,72 g $\ell^{-1}h^{-1}$ (0,95 g g^{-1}) and 12 g $\ell^{-1}h^{-1}$ (1,0 g g^{-1}), respectively. The biofilm reactor at 0,33 h^{-1} achieved twice the SA productivity of the 20 g ℓ^{-1} glucose feed SC reactor at 0,3 h^{-1} with a 42 % increase in SA yield. Although limited, the biofilm results are very promising and further work on the development of a stable and non-clogging reaction system is recommended.

The anaerobic slurry reactor fermentations with SC were superior to those reported by Urbance *et al.* (2004). The biofilm run achieved the highest reported SA productivities and yields in non-membrane processes; without the use of the PCS tubes recommended by Urbance *et al.* (2003) and Urbance *et al.* (2004).

Chapter 6

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Chapter 7

Appendix

Appendix A1: Reactor modeling

The steady state performances of the 20 and 40 g ℓ^{-1} glucose experiments did not differ significantly; therefore modeling of the steady state data was limited to the 20 g ℓ^{-1} glucose steady state data.

Equations 8-16 were used to model the experimental outlet concentrations C_S, C_X, C_{SA} and $C_{BP,}$ respectively; the rate expressions were given by Equations 9-12. The inlet concentrations C_{XO}, C_{SAO} and C_{BPO} were zero for all the experiments conducted.

The constant $Y_{BP/X}$ in Equations 12 was determined as 4,2 g g^{-1} from the experimental data following the procedure described in Figure 4.7. The parameters values for $Y_{BP/X,}$ $Y_{S/X}$ and $Y_{SA/X}$ are the best fit values for the dilution rates employed. The steady state data showed that the yields were not constant with dilution rates; for example $Y_{SA/X}$ ranged from 5,9 to 3,45 g g^{-1} over the dilution rates 0,12 h^{-1} to 0,6 h^{-1}. Using these average parameters ($Y_{BP/X,}$ $Y_{S/X}$ and $Y_{SA/X}$) was a limitation in the modeling of the steady state experimental data.

$$\mu=\mu_{max}\frac{C_S}{K_S+C_S}\left(1-\frac{C_P}{C_{P,max}}\right) \quad (8)$$

$$R_X=\mu C_X \quad (9)$$

$$R_S=Y_{S/X}R_X \quad (10)$$

$$R_{SA}=Y_{SA/X}R_X \quad (11)$$

$$R_{BP}=Y_{BP/X}R_X \quad (12)$$

$$-R_S+D(C_S-C_{SO})=0 \quad (13)$$

$$R_X+D(C_X-C_{XO})=0 \quad (14)$$

$$R_{SA}+D(C_{SA}-C_{SAO})=0 \quad (15)$$

$$R_{BP}+D(C_{BP}-C_{BPO})=0 \quad (16)$$

Equation 8 defines the substrate and product expressions used to model the specific growth constant. The constants μ_{max}, K_S and $C_{P,max}$ were unknown, therefore these parameters were optimised to fit the experimental data. The Matlab® R2007 function fsolve was used to solve Equations 13-16 for C_S, C_X, C_{SA} and C_{BP} at each experimental dilution rate, whilst the optimisation solver lsqnonlin was used to minimise the sum of the errors by fitting μ_{max}, K_S and $C_{P,max}$ to the experimental data.

The matlab code used for solving and optimising the unknown parameters are given in detail in Appendix A2; also included are the initial guesses and range of values for μ_{max}, K_S and $C_{P,max}$ used in the optimisation.

Figure 4.11 shows the attempt at fitting the described steady state model to the 20 g ℓ^{-1} glucose experimental data. The fitted model did not accurately predict SA and by-product concentrations; however the glucose and cell concentrations were fitted by the model. The optimised μ_{max}, K_S and $C_{P,max}$ values were 1,56 h^{-1}, 0,3 g ℓ^{-1} and 25,6 g ℓ^{-1}, respectively.

The μ_{max} fitted to the data is too high to be a realistic maximum growth rate, typical growth rates in batch reactors range from 0,5-0,8 h^{-1}. The Monod constant K_S was a low value of 0,3 g ℓ^{-1}; therefore for C_S greater than K_S, the specific growth constant (μ) would be independent of the substrate and only influenced by the concentration of products; which was observed from the experiments conducted with 20 and 40 g ℓ^{-1} glucose feed concentrations. The fitted parameters presented in this model are specific to the data obtained from the experiments; extrapolation or application to other data would not be advisable.

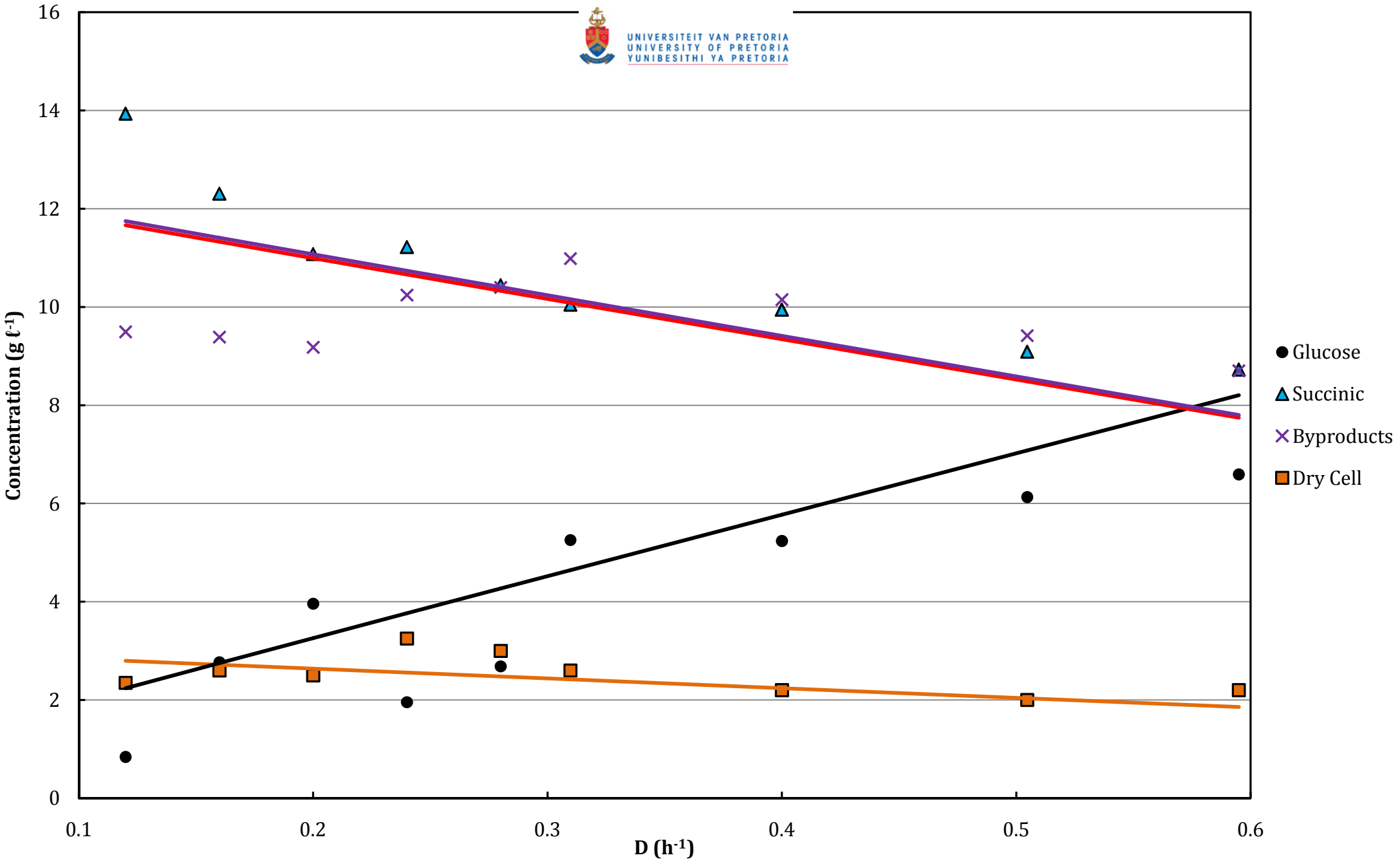

Figure 4.11: Steady state data (20 g ℓ^{-1} feed glucose) fitted by the steady state reactor model

Appendix A2: Matlab® programming code

Optimization routine

```
function f =obj(n)
M=xlsread('Experimental data(20).xlsx');
D=M(:,1);
C_X=M(:,2);
C_S=M(:,3);
C_SA=M(:,4);
C_BP=M(:,5);
```

Inlet conditions

```
C_XO =0;
C_SO=20;
C_SAO =0;
C_BPO=0;
```

Determined parameters

```
Y_S/X =6.8;
Y_SA/X =4.17;
Y_BP/X=4.16;
```

Optimized parameters

```
μ_max =n(1);
K_S =n(2);
C_P,max =n(3);

for i=1:length(D)
   x0=[1; 1; 1; 1];
   x=Solve(C_XO,C_SO,C_SAO,C_BPO,D(i),μ_max,K_S,α_S,α_SA,α_BP,C_P,max,x0);
   C(1,i)=x(1);
   C(2,i)=x(2);
   C(3,i)=x(3);
   C(4,i)=x(4);
end
```

Equation solver

```
function y= Solve(C_XO,C_SO,C_SAO,C_BPO,D(i),μ_max,K_S,α_S,α_SA,α_BP,C_P,max,x0)
options = optimset('Display', 'off');
y = fsolve(@poly, x0, options);
    function y=poly(x)
        y=[(( μ_max *x(2)*x(1))/(K_S +x(2)))*((1-((x(3)+x(4))/C_P,max)))+D*(C_XO -x(1));
        (-Y_S/X *(( μ_max *x(2)*x(1))/(K_S +x(2))))*((1-((x(3)+x(4))/C_P,max)))+D*(C_SO -x(2));
        (Y_SA/X )*(( μ_max *x(2)*x(1))/(K_S +x(2)))*((1-((x(3)+x(4))/C_P,max)))+D*(C_SAO -x(3));
        (Y_BP/X)*(( μ_max *x(2)*x(1))/(K_S +x(2)))*((1-((x(3)+x(4))/C_P,max)))+D*(C_BPO -x(4))];
   end
end
```

Objective function

f=(C(1,:) - transpose(C_X)).^2+(C(2,:) - transpose(C_S)).^2+(C(3,:) - transpose(C_{SA})).^2 + (C(4,:)-transpose(C_{BP})).^2

Optimization solver: lsqnonlin – non linear least squares
start point; n=[1.8 0.5 24]
lower bounds; n=[0.5 0 0.1]
upper bounds; n=[2.3 15 50]