High gravity extractive fermentation for enhanced productivity of bioethanol

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Abstract

Bioethanol is a renewable alcohol fuel produced from sugary substrates via fermentation processes. Accumulation of ethanol in the fermentation broth inhibits cell growth and further production of ethanol. Recovering ethanol from a dilute broth is expensive. Ethanol inhibition of fermentation may be reduced by continuously removing it as it is formed.

This work focussed on production of bioethanol from glucose using the anaerobic bacterium *Zymomonas anaerobia*. This bacterium produces ethanol more rapidly than does the conventional yeast fermentation. The aim was to assess the impact of continuous in-situ removal of ethanol on the productivity of batch and continuous high-gravity fermentations. High-gravity fermentations use a medium with a high concentration of sugar to reduce production volume (bioreactor size) and potentially achieve a high productivity of ethanol, if the ethanol concentration in the broth can be kept to below inhibitory levels.

First, the batch fermentation was characterized for glucose tolerance, ethanol tolerance, optimal production temperature and biocompatibility with solvents and adsorbents that could be used for in-situ removal of ethanol. High gravity media containing 50–300 g L^{-1} glucose were used to characterize batch and continuous fermentations with a view to identifying the best fermentation conditions for a detailed study. The optimal fermentation temperature was found to be 35 °C and the maximum tolerable initial glucose (i.e. without causing substrate inhibition) was 150 g L^{-1}.

In continuous high-gravity fermentations, six different dilution rates (D = 0.05–0.30 h^{-1}) were tested, but steady-state operation proved to be impossible at the lowest dilution rate: the fermentation showed a highly consistent oscillatory behaviour that was ascribed to ethanol toxicity. Use of higher dilution rates could overcome oscillations by washing out the ethanol from the bioreactor, but this reduced ethanol productivity as glucose and biomass also washed out. Strategies for removing ethanol in-situ while operating at such a dilution rate as to achieve a high ethanol productivity, were assessed by using liquid-liquid extraction and adsorption on polymer resins as methods for removing ethanol as it was being produced.

In the absence of in-situ removal of ethanol, the lowest operable steady-state dilution rate (i.e. without oscillations) was 0.15 h^{-1}. With in-situ removal of ethanol, the dilution rate for stable steady state operation could be reduced to 0.05 h^{-1}. At a dilution rate of 0.15 h^{-1}, the steady-state ethanol concentration was 42.5 g L^{-1} and the biomass concentration was 1.49 g L^{-1}. In the absence of in-situ ethanol removal, the ethanol concentration, but not ethanol productivity, was highest at a dilution rate of 0.3 h^{-1} although much residual glucose remained.

In in-situ batch extractive fermentations, all extraction solvents tested improved biomass concentration, glucose consumption and ethanol concentration relative to control, but iso-octadecanol was clearly the most effective solvent. For batch in-situ extractive fermentation with iso-octadecanol, the ethanol yield on glucose was 0.485±0.005 g g^{-1}, or comparable to to a yield of 0.468±0.005 g g^{-1} for the control culture, but the ethanol productivity was distinctly higher than for the control culture. Of the various polymer resins tested in batch fermentations for in-situ removal of ethanol by adsorption, Dowex Optipore L-493 appeared to be somewhat better than the control (i.e. no resin).

The best extraction solvent (i.e. iso-octadecanol) and the best adsorption resin (i.e. Dowex Optipore L-493) were separately assessed for ethanol removal in continuous fermentations. Continuous removal of ethanol both by adsorption and solvent extraction allowed a steady-state operation of the continuous fermentations at a dilution rate of 0.05 h^{-1} — the dilution rate at which steady-state operation had proved impossible in control fermentation (i.e. without in-situ removal of ethanol). This confirmed the mechanism used to explain the oscillatory behaviour of the fermentation and showed that in-situ ethanol removal permitted steady-state operation at dilution rates that would not allow such operation in the absence of ethanol removal. At a dilution rate of 0.05 h^{-1}, an extraction solvent flow rate of 300 mL h^{-1} provided the highest total ethanol productivity and ethanol yield on glucose while keeping the solvent use to a minimum.

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Abbreviations

A	-	Cross sectional area of the column (m^2)
C_{Aq}	-	Aqueous phase concentration of ethanol (g L^{-1})
C_e	-	Equilibrium concentration of ethanol in glucose solution (g L^{-1})
D	-	Dilution rate (h^{-1})
D_p	-	Particle size (m)
E	-	Maximum ethanol concentration (g L^{-1})
E_A	-	Steady state ethanol concentration in aqueous phase (g L^{-1})
E_S	-	Steady state ethanol concentration in solvent phase (g L^{-1})
Fs	-	Flow rate of solvent (mL h^{-1})
g	-	Acceleration due to gravity (m s^{-2})
H	-	Height of expanded resin bed (m)
H_o	-	Bed height of original settled resin at superficial flow velocity of zero
K_a	-	Distribution coefficients of solute a in the solvent
K_b	-	Distribution coefficients of solute b in the solvent
K_d	-	Partition coefficient
K_F	-	Freundlich constant
K_L	-	Langmuir constant
K_r	-	Partitioning coefficient of ethanol on resin
K_s	-	Limiting substrate concentration (g L^{-1})
L	-	Specific loading
n	-	Freundlich coefficient
P_E	-	Ethanol productivity (g L^{-1} h^{-1})
P_{EA}	-	Steady state ethanol productivity in the aqueous phase ($gL^{-1}h^{-1}$)
P_{ES}	-	Steady state ethanol productivity in the solvent phase ($gL^{-1}h^{-1}$)
P_{ET}	-	Total ethanol productivity (g L^{-1} h^{-1})
P_x	-	Biomass productivity (g L^{-1} h^{-1})
q_e	-	Equilibrium adsorption capacity of ethanol on the adsorbent (g kg^{-1})
Q_F	-	Medium flow rate (mL h^{-1})
Q_L	-	Flow rate (mL h^{-1})

Q_S	-	Solvent flow rate (mL h^{-1})
q_m	-	Maximum adsorption capacity of ethanol on the adsorbent (g kg^{-1})
q_p	-	Average specific ethanol production rate (g g^{-1} h^{-1})
q_s	-	Average specific glucose uptake rate (g g^{-1} h^{-1})
R	-	Ideal gas constant (J mol^{-1} K^{-1})
R^2	-	Regression coefficient
S	-	Glucose concentration (g L^{-1})
S_o	-	Initial substrate concentration (g L^{-1})
T	-	Absolute temperature (K)
t	-	Time (h)
t_0	-	Initial time (h)
t_f	-	Time at equilibrium (h)
V_L	-	Liquid volume in the reactor (L)
V_s	-	Volume of solvent (L)
X_{max}	-	Biomass concentration (g L^{-1})
X_r	-	Mass of resin per unit volume of aqueous phase (g L^{-1})
W_o	-	Weight of aluminium pan and sample before drying (g)
W_f	-	Weight of aluminium pan and sample after drying (g)
$Y_{p/s}$	-	Ethanol yield on substrate (g g^{-1})
$Y_{x/s}$	-	Biomass yield on substrate (g g^{-1})
μ	-	Specific growth rate (h^{-1})
ε	-	Void volume of expanded bed
σ_L	-	Liquid density (kg m^{-3})
ε_m	-	Minimum bed void volume of a settled bed of spherical particles (m)
σ_p	-	Solid density (kg m^{-3})
$[E]_{aq}$	-	Ethanol concentration in the aqueous phase (g L^{-1})
$[E]_{org}$	-	Ethanol concentration in the organic phase (g L^{-1})
μ_{max}	-	Maximum specific growth rate (h^{-1})
ΔG	-	The adsorption Gibbs free energy (kJ/mol)

ρ	-	Density (kg/m^3)
β	-	Selectivity

CHAPTER 1

INTRODUCTION

1.1 Rationale and importance of the study

Sustainable production of fuel ethanol from renewable carbohydrates is a potential option for replacing certain liquid transport fuels derived from petroleum (Hagerdal *et al.*, 2006; Kim and Dale, 2004; McMillan, 1997; Sun and Cheng, 2002; Szczodrak and Fiedurek, 1996; Vertes *et al.*, 2007). World use of petroleum is expected to reach 97 million barrels per day by 2015. Transportation sector accounts for nearly 68% of the total consumption of petroleum (EIA, 2010b). In view of declining petroleum reserves (EIA, 2010a) and the adverse environmental impact of a continued use of petroleum, sustainable sources of renewable energy are needed. Ethanol produced from renewable carbohydrates by fermentation processes, or bioethanol, is a proven liquid fuel that can be used alone in internal combustion engines to power motor vehicles. Bioethanol can be produced from a variety of agricultural materials including sugars and biopolymers (e.g. starch, cellulose) which can be hydrolysed to fermentable sugars. Bioethanol can potentially replace 32% of the global gasoline consumption if used as an E85 blend in passenger vehicles (EIA, 2010a).

Production of ethanol from sugar and starch materials is expanding rapidly (Figure 1.1). This growth is expected to further accelerate once commercial production of bioethanol from abundant lignocellulosic materials becomes feasible.

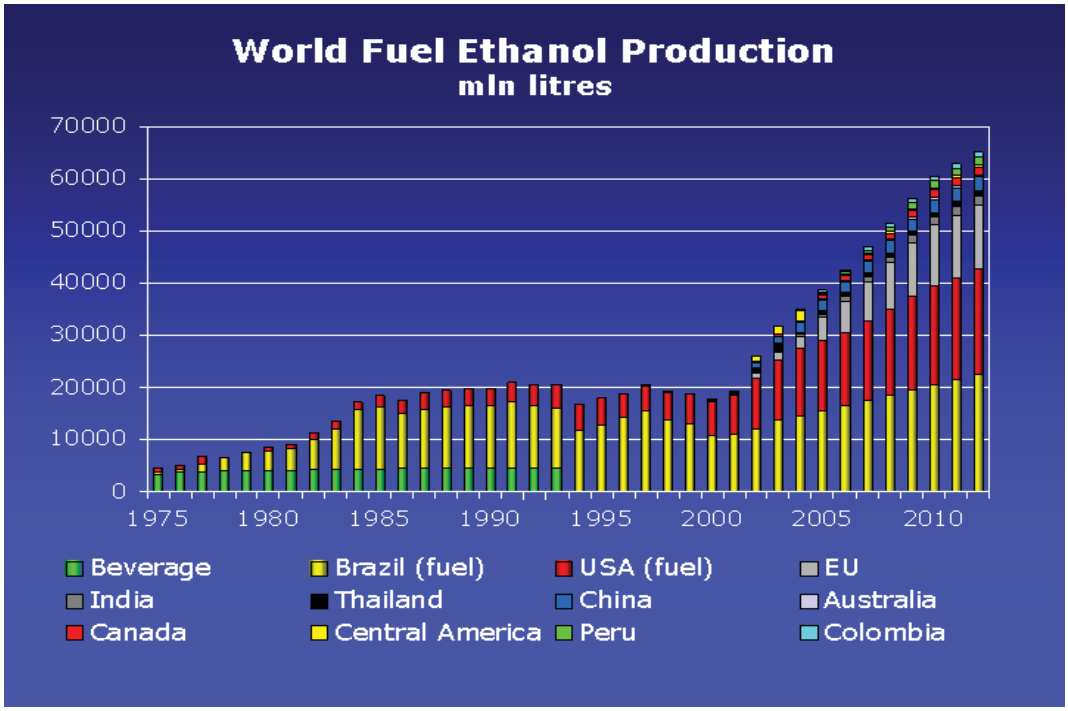


Figure 1.1 World ethanol production (From the website of World Fuel Ethanol, www.distill.com)

Bioethanol is relatively expensive to produce because the sugar fermenting microorganisms cannot withstand a high concentration of ethanol. This limits the maximum attainable concentration of ethanol in the fermentation broth and necessitates ethanol recovery from a dilute fermentation broth. This is expensive. As the concentration of ethanol in a fermentation broth increases its further production is inhibited even though the broth may contain a large amount of residual sugar. This feed-back inhibition of ethanol production can be reduced by removing the ethanol from the fermentation broth as it is being produced.

This research is focused on an inexpensive bacterial fermentation of glucose to ethanol by using anaerobic bacterium *Zymomonas anaerobia* ATCC 29501. A highly concentrated glucose medium (i.e. high gravity fermentation) is used to reduce the expense of thermal sterilization of a large volume of a dilute medium. Furthermore, schemes are evaluated for continuous removal of the ethanol from fermentation broth in situ during the fermentation. Liquid-liquid extraction and solid-liquid extraction (or adsorption) methods are examined for continuous ethanol recovery so as to reduce feed-back inhibition of ethanol production. Toxicity of extraction solvents and adsorption

resins to the fermentation microorganism is examined for selection of a suitable removal system. Both batch and continuous fermentation schemes are examined. The main novelty of the research is in its focus on high-gravity fermentations, selection of solvents and resins that have not been previously considered, and a comparative characterization of batch and continuous operational methods of the fermentation systems of interest.

1.2 Research approach

Fermentation processes are often impeded by an accumulation of the product in the bioreactor. Integration of fermentation and a product recovery step can accelerate the product formation and improve the product yield. Among the different possible approaches for integrating the fermentation and product recovery steps, in situ liquid-liquid extraction and solid-liquid extraction appear to have the greatest potential.

So far not much work appears to have been done on high-gravity fermentation of *Z. anaerobia* specially in continuous cultures. The impact of the in situ ethanol recovery on this fermentation has not be examined before. When a high-gravity medium is used, the microorganism is potentially subjected to a strong substrate inhibition at the beginning of the fermentation and product inhibition towards the end of the fermentation. Both these can adversely affect the performance of such a fermentation system. In view of these considerations, batch fermentations were first used at different initial sugar concentrations to establish the substrate inhibition effects. Separate batch experiments with added ethanol were used to characterize the product tolerance. Various potential extraction solvents and resins were experimentally evaluated for their ability to remove ethanol from fermentation broth and their biocompatibility with the biocatalyst (i.e. the microorganism).

Using a suitable solvent and suitable resin, separate experiments were carried out to evaluate the effects of the in situ product recovery in batch and continuous fermentations. Control fermentations (i.e. without in situ product recovery) were carried out in all cases for comparison.

1.3 Research objectives

The major objectives were as follows:

1. To characterize the effects of substrate (glucose) concentration and product (ethanol) concentration on the bacterium *Zymomonas anaerobia* ATCC 29501 for use in high-gravity fermentations.

2. To characterize batch and continuous high gravity fermentations of glucose to ethanol by *Z. anaerobia*.

3. To identify suitable solvents and adsorption resins for in situ ethanol recovery from *Z. anaerobia* high-gravity fermentations.

4. To characterize the kinetics of batch and continuous high-gravity fermentations implemented with in situ recovery of ethanol.

1.4 Thesis outline

This thesis comprises five chapters: Chapter 1, introduce the rationale for the study, the research approach and the objectives of research. Chapter 2 provides a comprehensive literature review of ethanol fermentation with reference to the microorganism of interest and the theory of the relevant extractive fermentations. Chapter 3 explains the materials and methods used in this study. Chapter 4 presents the experimental results and discusses them. Chapter 5 deals with the conclusions of the study and some recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Bioethanol

Bioethanol is ethanol (ethyl alcohol) produced from agricultural materials through microbial action. Bioethanol has the potential to replace petroleum derived liquid fuels in some applications. Bioethanol can be produced sustainably from renewable resources such as cellulose, sugarcane, molasses, potatoes, corn and barley (Chandel *et al.*, 2007; EIA, 2010a). If bioethanol is used to displace petroleum fuels, it has the potential to reduce net emission of carbon dioxide, the principal greenhouse gas. Production of bioethanol uses the same basic technology as used in making alcoholic beverages. Ethanol is low in toxicity, volatility and photochemical reactivity (Wyman, 1996). It burns to produce carbon dioxide and water (Wyman, 1996).

Bioethanol is already widely used as fuel in Brazil, United States and some European countries. The largest producers are Brazil (37% of global global production), USA (33% of global production) and European Unions with 14% of global production. Today, ethanol production requires about 50 percent less energy than it did in the early 1980s. Technology of producing bioethanol is continually improving. For example, ethanol yield from corn has increased by more than 22% in the USA compared to 1980. Similarly, in the USA, the capital costs of constructing an ethanol from corn facility have decreased from more than $2.00 per gallon of annual production capacity in the 1980s to less than $1.50 per gallon in 2010 (EIA, 2010b). Ethanol contains less energy than gasoline and is still relatively expensive for widespread use as a transport fuel.

2.2 Properties and uses of ethanol

Ethanol (CH_3CH_2OH) is a colorless liquid with a characteristic odor and taste. It boils at 78.4 °C. It is miscible with water in all proportions and is difficult to separate from water. Pure ethanol is also known as absolute ethanol or anhydrous ethanol. Ethanol forms a constant-boiling mixture, or azeotrope, with water. Aqueous azeotrope of ethanol

contains 95% (by vol) ethanol and 5% (by vol) water and boils at 78.15 °C. As the boiling point of this azeotrope is below that of pure ethanol, absolute ethanol cannot be obtained by a simple distillation. However, if benzene is added to 95% ethanol, a ternary azeotrope of benzene, ethanol, and water, with a boiling point of 64.9 °C, can be formed. As the proportion of water to ethanol in this azeotrope is greater than in 95% ethanol, the water can be removed from 95% ethanol by adding benzene and distilling off this azeotrope (Chandel *et al.*, 2007; Leary, 2000). Due to the small amounts of benzene that remain, the absolute ethanol prepared by this process is poisonous.

Bioethanol is used mainly as a motor fuel and fuel additive. In the US, automobiles manufactured since 1998 have been equipped to enable them to run on either gasoline or E85, a mixture of 85% ethanol and 15% gasoline. E85, however, is not yet widely available (EIA, 2010a). Ethanol is produced also by petrochemical methods, but this is not bioethanol. Chemically derived ethanol is commonly known as industrial ethanol and used extensively as a solvent (Shakhashiri, 2006) and a precursor of other chemicals.

2.3 Microorganisms used in making bioethanol

Baker's yeast *Saccharomyces cerevisiae* is most commonly used to make ethanol from sugars such as sucrose, fructose and glucose. Other yeasts are used to a lesser extent. Yeasts have a number of disadvantages. For example, they require expensive highly aerobic conditions to generate the biomass for use in a subsequent microaerobic fermentation; are limited to a relatively low temperature and therefore a slow rate of ethanol production; and have a low tolerance for ethanol (Panesar *et al.*, 2006). Because of these limitations, other microorganisms have been tested for producting ethanol (Asther and Khan, 1983; Asther and Khan, 1985; Baratti and Bu'lock, 1986; Gunasekaran and Raj, 1999; Kamini and Gunasekaran, 1989; Kosaric *et al.*, 1982). Numerous genetically modified microorganisms are being developed for bioethanol production from mixed sugars. Naturally occurring bacteria such as *Zymomonas mobilis* are recognized to be good producers of ethanol. Rogers *et al.* (1979) first reported on the great potential of *Z. mobilis* for ethanol production. Since then, this bacterium has been the focus of much attention. Table 2.1 compares ethanol production by *Z. mobilis* and yeast.

Table 2.1 Comparation between *Z. mobilis* and yeast for ethanol production (Panesar *et al.*, 2006)

Parameters	*Zymomonas mobilis*	Yeast
Conversion of sugar to ethanol (%)	96	96
Maximum ethanol concentration (%)	12	12
ATP yield (per mole glucose)	1	2
Ethanol production rate (g/g.h)[a]	5.67	0.67
pH range	3.5-7.5	2-6.5
Optimum temperature (°C)	25-30	30-38

[a] Batch fermentation with 10% glucose

Z. mobilis has a faster specific rate of fermentation compared to yeast and uses the Entner-Doudoroff (ED) glycolic pathway to convert glucose, sucrose and fructose to ethanol while yeast uses the Embden-Meyerhof pathway (EMP) (Dawes and McGill, 1971). The EMP produces two moles of ATP from one mole of glucose compared to the one mole of ATP produced by the ED pathway (Dawes and McGill, 1971). Thus, *Z. mobilis* must ferment twice the amount of glucose compared to the yeast to acquire the same amount of energy (Baratti and Bu'lock, 1986). Based on Gunasekaran *et al.* (1999) and Panesar *et al.* (2006), the advantages of the *Z. mobilis* over various yeasts can be summarized as follows;

- Higher specific rate of substrate uptake
- Higher ethanol yield
- Lower biomass production
- Higher volumetric sugar uptake rate
- Higher ethanol tolerance
- No requirement of oxygen to maintain cell viability during the fermentation

A disadvantage of *Z. mobilis* is its limited substrate range (glucose, fructose and sucrose) and by-product formation. Attempts are being made to overcome this by genetic engineering of *Z. mobilis* (Yanase *et al*., 2005).

2.4 The genus *Zymomonas*

Zymomonas sp. are facultative anaerobic Gram-negative prokaryotes that have been isolated from alcoholic beverages in tropical countries. *Zymomonas* bacteria have been isolated from plant saps of agave and sugarcane. They have been found in palm wine, cocoa beans and ripening honey (Sahm *et al.*, 2006; Sprenger, 2005; Swings and Ley, 1977). In Europe "cider sickness" has been associated with bacteria (Barker and Hillier 1912). Millis (1956) showed that the cause of cider sickness was *Zymomonas* sp. In the past about 20 different names have been used for the bacterium now known as *Zymomonas mobilis*.

The genus *Zymomonas* consists of two species (Kluyver *et al.,* 2001; Shimwell, 1937). These are *Zymomonas mobilis* and *Zymomonas anaerobia*. Table 2.2 shows the original basis for differentiation between *Z. mobilis* and *Z. anaerobia*. The main distinction between them is the ability of *Z. mobilis* to ferment sucrose whereas *Z. anaerobia* can ferment only glucose and fructose (Dadds *et al.*, 1973; Gunasekaran and Raj, 1999; Lee *et al.*, 1981b). In addition, the vitamin requirements of the two species are slightly different; *Z. anaerobia* requires pantothenate whereas *Z. mobilis* requires *p*-aminobenzoic acid, folic acid, biotin and cyanocobalamin.

Table 2.2 Difference between the two species of the genus *Zymomonas* (Carr and Passmore, 1971)

Characteristics	*Z. mobilis*	*Z. anaerobia*
Size (μm)	1.4–2.0 × 4.0–5.0	1.0–1.5 × 2.0–3.0
Yield conversion (1 mol glucose)	1.6 moles of ethanol, 1.8 moles of CO_2, traces of lactate	1.8 moles of ethanol, 1.9 moles of CO_2, some acetaldehyde
Ability to use sucrose	Ferments sucrose and produces levan	Does not ferment sucrose
Requirement for lipic acid and biotin	Not required	Essential

Growth in the presence of ammonium nitrogen only	Good growth	Poor growth

The following characteristics have been noted for *Z. mobilis* (Rogers *et al.*, 1982; Sahm *et al.*, 2006):

- Cells are plump and rod shaped with rounded ends (2-6 µm long and 1-1.4 µm in diameter)
- Cells occur singly and in pairs
- Cells are Gram-negative
- No spores, capsules, intracellular lipids or glycogen are produced
- Anaerobic, but can tolerate some oxygen
- Either motile or non-motile
- Contains pentacyclic triterpenoids (hopanoid), vaccenic acid and sphingolipid in cell membranes

2.5 Carbohydrate metabolism

2.5.1 Glucose and fructose

Zymomonas mobilis can use only glucose, fructose and sucrose as substrates. The catabolism of glucose to ethanol in *Z. mobilis* follows an anaerobic Entner-Doudoroff pathway as shown in Figure 2.1 (Dawes and McGill, 1971). A characteristics of this pathway is an energy yield of 1 mol ATP per mole of glucose fermented (Dawes and McGill, 1971). This is half the energy yield of the Embden-Meyerhof pathway in yeasts. As a consequence, *Z. mobilis* produces less biomass than yeast and more carbon is funnelled to fermentation products (Dien *et al.*, 2003).

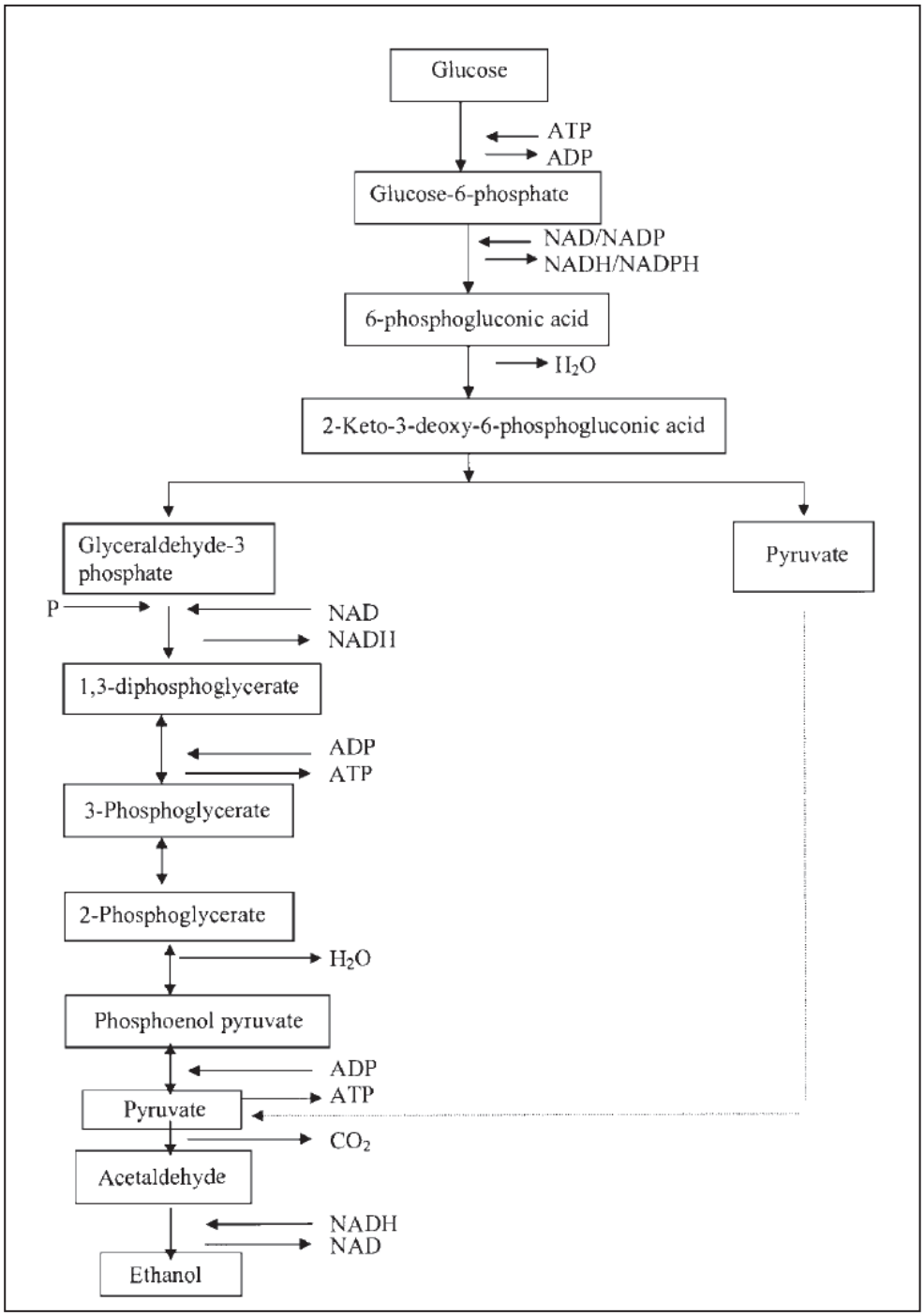


Figure 2.1 Entner-Doudoroff pathway in *Zymomonas mobilis* (Panesar *et al.*, 2006)

Only 2-2.6% of glucose and fructose consumed by *Zymomonas* is converted to biomass and the rest is almost quantitatively converted to ethanol and carbon dioxide (Dawes and

McGill, 1971). This genus has been reported to produce up to 1.58-1.93 mol of ethanol per mol of glucose consumed in anaerobic conditions.

In aerobic conditions, the yield of ethanol is reduced to 1.6 mol of ethanol per mol of glucose. However, the exact yield depends on the strain and the culture conditions (DeMoss and Raps, 1962). Ethanol yields as high as 0.49 grams ethanol produced per gram of glucose consumed (or 1.92 mol ethanol per mol glucose) have been reported (Baratti and Bu'lock, 1986; Kosaric *et al.*, 1982; Lee and Rogers, 1983; Lee *et al.*, 1980). In ethanol production by *Z. anaerobia* NCIB 8777 the yield was found to be much lower because of formation of glycerol and dihydroxylacetone (Dawes and McGill, 1971).

Z. mobilis is able to grow in media with a high glucose concentration of 100-250 g L^{-1} (Rogers *et al.*, 1979). Some strain are able to tolerate a glucose concentration of up to 400 g/L (Rogers *et al.*, 1982). It is therefore clear that any *Zymomonas* strain intended for ethanol production needs to be evaluated on an individual basis.

2.5.2 Sucrose

Z. mobilis is able to use sucrose as a carbon source. This ability distinguishes it from *Z. anaerobia*. The ethanol yield from glucose and fructose tends to be high, almost 94% of theoretical, but the yield on sucrose may be as low as 70% of theoretical (Lyness and Doelle, 1983). The maximum ethanol yield on glucose is 92 – 96% of the theoretical yield (Rogers *et al*., 1982). Theoretically, a maximum of 2 moles of ethanol can be produced from a mole of glucose. This is equivalent to 92.15 g of ethanol being produced from 180.16 g of glucose. Thus, the maximum theoretical yield of ethanol on glucose is 0.51 g g^{-1}.

In *Z. mobilis* fermentation of sucrose ethanol yield is reduced due to formation of fructose oligomers (levan) and sorbitol (Bai *et al.*, 2007; Dawes and McGill, 1971; Gunasekaran and Raj, 1999). The first step of sucrose metabolism is its hydrolysis to glucose and fructose. The latter is used to produce levan. Both growing and non-growing cells form levan (Dawes *et al.*, 1966). In a batch fermentation with an initial sucrose concentration of 250 g L^{-1}, nearly 20 g L^{-1} of levan was produced. Other by-products of sucrose fermentation are sorbitol and gluconate (Kluyver and Niel, 2001).

2.5.3 Other carbohydrates

Z. mobilis appears to be able to grow on raffinose (Rogers *et al*., 1982) but not on maltose, rhamnose, xylose, lactose, mannitol, dulcitol, galactose, saccharose, ribose, arabinose and trehalose (Belaich and Senez, 1965; Carr, 1974). No other monosaccharides, disaccharides, polysaccharides or fatty acids are metabolized by *Zymomonas* sp.

2.6 Growth and conservation conditions

2.6.1 Temperature

The optimum temperature for growth of the *Zymomonas mobilis* strains is between 25 and 30 °C. Nearly 74% of the strains are able to grow at 38 °C but only 5% grow at 40 °C (Swings and Ley, 1977). Growth is slow at 15 °C and absent at 4 °C (Millis, 1956).

2.6.2 pH

The optimum pH range for growth of most *Zymomonas* strains is 3.5 - 7.5 (Swings and Ley, 1977). A relative acid tolerance is a typical feature of *Zymomonas* sp. (Swings and De Ley, 1977).

Table 2.3 summarizes the ranges of temperature and pH previously studied in fermentations of *Zymomonas* species.

Table 2.3 Ranges of temperature and pH studied in *Zymomonas* fermentations

Microorganism	Strain	Temperature (° C)	pH	Agitation (RPM)	Fermentation time (h)	Reference
Zymomonas mobilis	ATCC 10988	30, 32.7, 35	6.0 controlled by adding NH_4OH	100	48	(Fiescho and Humphrey, 1983)
Zymomonas mobilis	ATCC 29191	30, 33, 36	5.0-5.5 controlled by adding 2M KOH	200	12	(Stevnsborg *et al.*, 1986)
Zymomonas mobilis	ATCC 10988	27, 30, 32, 34.5	5.0 controlled by adding NH_4OH	150	15	(Laudrin and Goma, 1982)
Zymomonas mobilis	ATCC 10988	21, 25, 30, 35, 40, 45, 50	4.8, 4.4, 3.8, 3.0, 5.3, 6.1, 7.0	Not reported	8-20	(Bajpai and Margaritis, 1986)
Zymomonas mobilis	ATCC 10988	30, 34, 37, 40	5.0, 6.0, 7.0, 7.5, 8.0	Not reported	16	(King and Hossain, 1982)
Zymomonas mobilis	ATCC 29191	30, 37, 40, 50	5.0 controlled by adding 1N NaOH	100	70	(Huang and Chen, 1988)
Zymomonas mobilis	ATCC 29191	30	3.5, 4.5, 5.0, 5.5, 6.0,6.5,7.0	200	Not reported	(Lawford *et al.*, 1988)
Zymomonas anaerobia	ATCC 29501	28, 32, 34, 35, 36, 38	4.5, 5.1, 5.6, 6.0, 6.5	140	23	(Kosaric *et al.*, 1982)

2.7 Inhibitory factors

Z. mobilis is able to grow aerobically, but the ethanol yield in aerobic growth is only about one-third of the value achievable in anaerobic conditions (Sprenger, 2005). Therefore, oxygen may be considered to have an inhibitory effect especially at a high sugar concentration. Under aerobic conditions some of the ethanol produced is oxidized to acetate (Belaich and Senez, 1965). In aerobic conditions, the specific growth rate *Z. mobilis* is reduced because of production of acetaldehyde and acetic acid (Bringer *et al.*, 1984; Tanaka *et al.*, 1990). Carbon dioxide also inhibits biomass production, increases the residual glucose concentration (Burrill *et al.*, 1983; Nipkow *et al.*, 1985) and suppresses ethanol production.

Ethanol is the strongest inhibitor of its own production in *Z. mobilis* fermentation (Burrill *et al.*, 1983) and this limits both the rate of production and the maximum attainable concentration of ethanol. Accumulation of ethanol to above a certain concentration progressively decreases the rate of further sugar conversion to ethanol as well as the rate of cell growth (Jöbses and Roels, 1986b). A build up of ethanol affects the composition of phospholipids in the cell membranes and decreases the ratio of lipids to proteins. An increased concentration of ethanol increases the permeability of the plasma membrane in *Z. mobilis* (Carey and Ingram, 1983; Ingram and Buttke, 1984; Osman and Ingram, 1985).

2.8 Continuous steady-state fermentations

Several studies of continuous "steady-state" fermentation of *Z. mobilis* have reported occurrence of "oscillations" in concentrations of substrate, biomass and ethanol during fermentations conducted at a substrate concentration of greater than 130 g/L (Ghommidh *et al.*, 1989; Jöbses *et al.*, 1986a; Lee *et al.*, 1979; McLellan *et al.*, 1999). Oscillation did not occur if the concentration of glucose in the feed was kept at 100 g/L (Lee *et al.*, 1979). Similar oscillations have been reported also in certain other high-gravity fermentations (Bai *et al*., 2004; Bai *et al*., 2007; Thomas *et al*., 1994).

These oscillations have been linked to the inhibitory effect of ethanol (Jöbses *et al.*, 1986a). As the ethanol concentration in the bioreactor increases, inhibition suppresses growth and the biomass concentration is reduced by washout and consequently the glucose concentration increases (Lee *et al.*, 1979). Once the ethanol concentration returns

to below an inhibitory level, biomass production increases, glucose concentration decreases and the cycle repeats at a steady dilution rate.

Studies suggest that in *Z. mobilis* fermentation a high rate of increase in ethanol concentration has an intense inhibitory effect (Daugulis *et al.*, 1995), but the ethanol concentration exposure history of *Z. mobilis* cells has no effect on their ability to produce ethanol. Thus, the cells are inhibited by a high ethanol concentration, but retain their ability to produce ethanol at a high rate once the ethanol concentration is reduced to below inhibitory levels.

2.9 In situ product recovery

Production of ethanol in a conventional batch or continuous fermentation is limited by the toxicity of the product to the producing microorganism. As a consequence, fermentation broths typically attain only a low concentration (e.g. 5-15% by volume) of ethanol. Therefore, it is necessary to start with a relatively dilute solution of glucose (e.g. not more than 10 g L^{-1}), so as to achieve complete conversion within a reasonable time (Freeman *et al.*, 1993; Stark and Von Stockar, 2003). Ethanol recovery from a dilute broth is expensive.

Traditional distillation processes are frequently used to concentrate ethanol from fermentation broths, but these processes are highly energy intensive. A low ethanol concentration in the broth greatly increases the cost of ethanol recovery by distillation (Phillips and Humphrey, 1982). A low maximum ethanol concentration in the broth means that a large volume of the broth must be produced and processed for a specified production capacity of ethanol. This increases both the cost of the equipment and cost of the processing (Daugulis, 1988). The problem of product inhibition can be reduced by continuously removing the product as it forms (Daugulis *et al.*, 1991; Freeman *et al.*, 1993; Roffler *et al.*, 1984). Operations of fermentation and product recovery can be integrated in a single step to provide in-situ product removal. Most of the research on in situ recovery of ethanol in fermentation processes has focused on yeast (*S. cerevisiae*) fermentations. Relatively little work has been done on *Zymomonas* fermentations.

The technologies for in-situ product recovery include: evaporative recovery in a vacuum fermentation; use of pervaporation; extractive fermentation using water immiscible solvents and adsorption of the product on resins. These methods can be used

to eliminate product inhibition and minimize the energy requirement for product recovery (Phillips and Humphrey, 1982; Roffler *et al.*, 1984; Woodley *et al.*, 2008). The technologies of in-situ product recovery in ethanol fermentation are briefly summarized next.

2.9.1 Vacuum and flash fermentations

Relatively volatile products such as ethanol and butanol can be removed in situ from an aqueous broth by carrying out the fermentation under reduced pressure (vacuum fermentation or vacuumferm). A reduced pressure lowers the boiling point of the aqueous broth so that ethanol is essentially boiled off at the fermentation temperature. An alternative strategy is to carry out the fermentation at normal atmospheric pressure, but continuously recycle the broth from the fermentor to an external "flash" chamber held under vacuum. In this "flashferm" operation, the ethanol is volatalized from the fermentation broth in the flash chamber. In both vacuumferm and flashferm systems, the fermentation broth always remains at the normal fermentation temperature of 30 – 37 °C for the yeast fermentation (Ramalingham and Finn, 1977); Phillips and Humphrey, 1982). The fermentor headspace (vacuumferm) or the flash chamber (flashferm) are kept under a vacuum of 35 to 75 mmHg (Phillips and Humphrey, 1982). Both these processes have been shown to reduce inhibition of the fermentation by ethanol and enhance its productivity in highly concentrated solutions of sugars. Both vacuum and flash fermentations require expensive equipment (vacuum pump, compressors, external loops, etc.).

Flash fermentation has been applied for ethanol recovery in fermentations of *Z. mobilis* (Roger *et al.*, 1981; Lee *et al.*, 1981) and the yeast *Candida acidothermophilum* (Ghose *et al.*, 1984). The *Z. mobilis* fermentor operated at atmospheric pressure and an external recycle loop with a vacuum vessel (50 mm Hg) was used for ethanol removal (Lee *et al.*, 1981). An ethanol productivity of 85 g/l.h was attained in continuous recycle experiments with 200 g/L of initial glucose concentration.

Both vacuumferm and flashferm processes are energy intensive because of the need to operate a vacuum compressor. Accumulation of non-volatile toxic substances in the broth during long term operation limits the productivity of both vacuumferm and flashferm operations. Both of these processes appear to be uneconomic (Daugulis, 1994; Roffler *et al.*, 1984) compared to conventional fermentation – distillation operation.

2.9.2 Gas stripping

Gas stripping is a simple method for recovering a volatile product from the fermentation broth. Products such as ethanol can be continuously recovered from the culture broth by sparging it with CO_2 or other suitable gas. The ethanol rich gas emerging from the fermentor is then passed through a condenser to condense the ethanol vapour. In some cases, the broth from the fermentor may be recycled through a separate stripper.

CO_2 stripping of the broth for removing ethanol from a stirred tank fermentor was reported by Walsh *et al.* (1983). In this case, ethanol was recovered from the carrier gas stream by adsorbing the volatile components onto activated carbon. In another study Dale *et al.* (1985) recovered the ethanol by condensation of the recycle gas stream. CO_2 stripping can be combined with a gas-lift fermentor to strip ethanol as well as mix the broth.

Taylor *et al.* (1995) operated a continuously fed yeast fermentor with ethanol removal but stripping for 150 days. This process was further scaled up to a 30 L pilot fermentor (Taylor *et al.* 1998). Ethanol was stripped in an external packed column using CO_2 as the stripping gas. A simplified scheme of this process is shown in Figure 2.2. Similar systems have been used for removal of acetone and butanol from batch, fed batch and continuous fermentations of *C. beijerinckii* BA101 (Ezeji *et al.*, 2003; Ezeji *et al.*, 2004; Groot *et al.*, 1992). No studies have been reported for *Z. mobilis* fermentation.

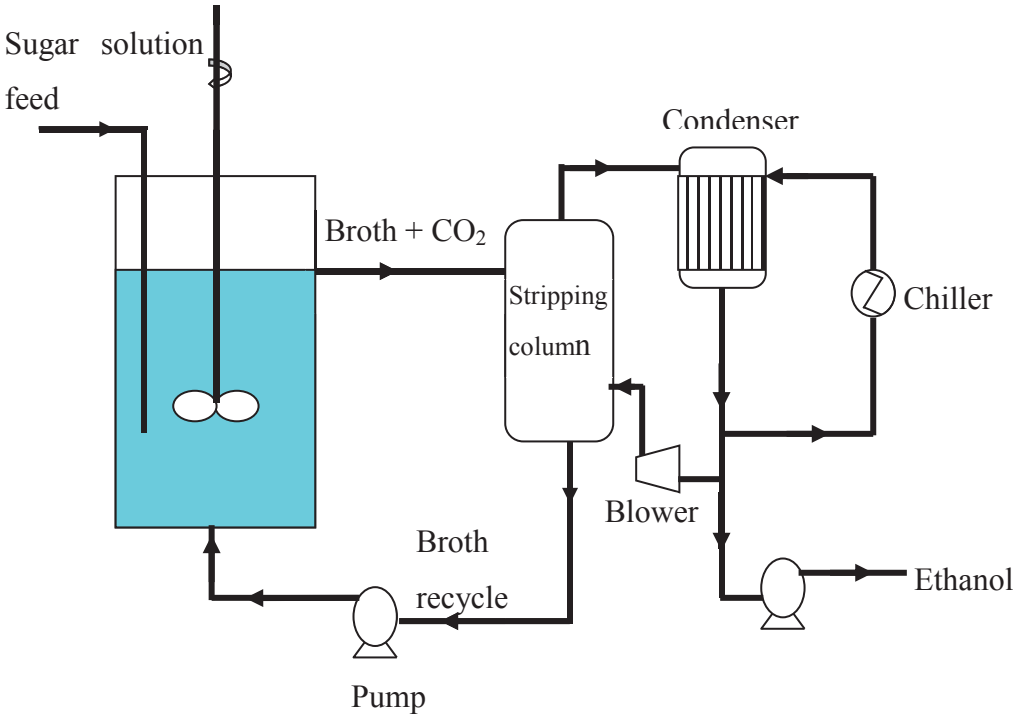


Figure 2.2 Fermentation coupled with gas stripping (after Cardona and Sánchez, 2007)

Liu and Hsien-Wen (1990) demonstrated the use of gas stripping to enhance the steady-state cell concentration for a given dilution rate. The substrate consumption was increased by increasing the stripping factor for a given dilution rate and ethanol concentration in the broth was reduced for a fixed dilution rate. The productivity of ethanol was shown to increase with an increase in the stripping factor for a given dilution rate.

Gas stripping has its associated drawbacks. For example, evaporation of water during stripping increases the energy required in the condensation step. Complete condensation of alcohol from the gas is associated with a complete condensation of water (Vane, 2008). Gas stripping does not appear to be used commercially for ethanol or butanol separation from fermentation broths.

2.9.3 Pervaporation

Pervaporation is a combination of evaporation and a membrane separation process. In pervaporation a liquid stream containing two or more components is contacted with one side of a membrane which selectively allows a volatile component to permeate to the other side. The other side of the membrane is heated, or purged with gas, to continuously

vaporize and remove the volatile component (Ezeji *et al.*, 2004; Vane, 2005). Schemes used in ethanol recovery from whole fermentation broth and cell free broth are shown in Figure 2.3.

Although pervaporation for removal of ethanol (Groot *et al.*, 1992; Ikegami *et al.*, 2007; Ikegami *et al.*, 2009; Kaseno and Kokugan, 1998; Mori and Inaba, 1990; Nomura *et al.*, 2002; O'Brien and Craig Jr, 1996; O'Brien *et al.*, 2000; O'Brien *et al.*, 2004) and butanol (Favre *et al.*, 1996; Gallego-Lizon *et al.*, 2002; Groot *et al.*, 1992; Groot *et al.*, 1984; Izak *et al.*, 2008; Larrayoz and Puigjaner, 1987; Qureshi and Blaschek, 1999) from fermentation broths has been extensively studied, it has not become commercially established because of the cost of the membranes and the need to periodically replace them (Cardona and Sánchez, 2007; Daugulis, 1988).

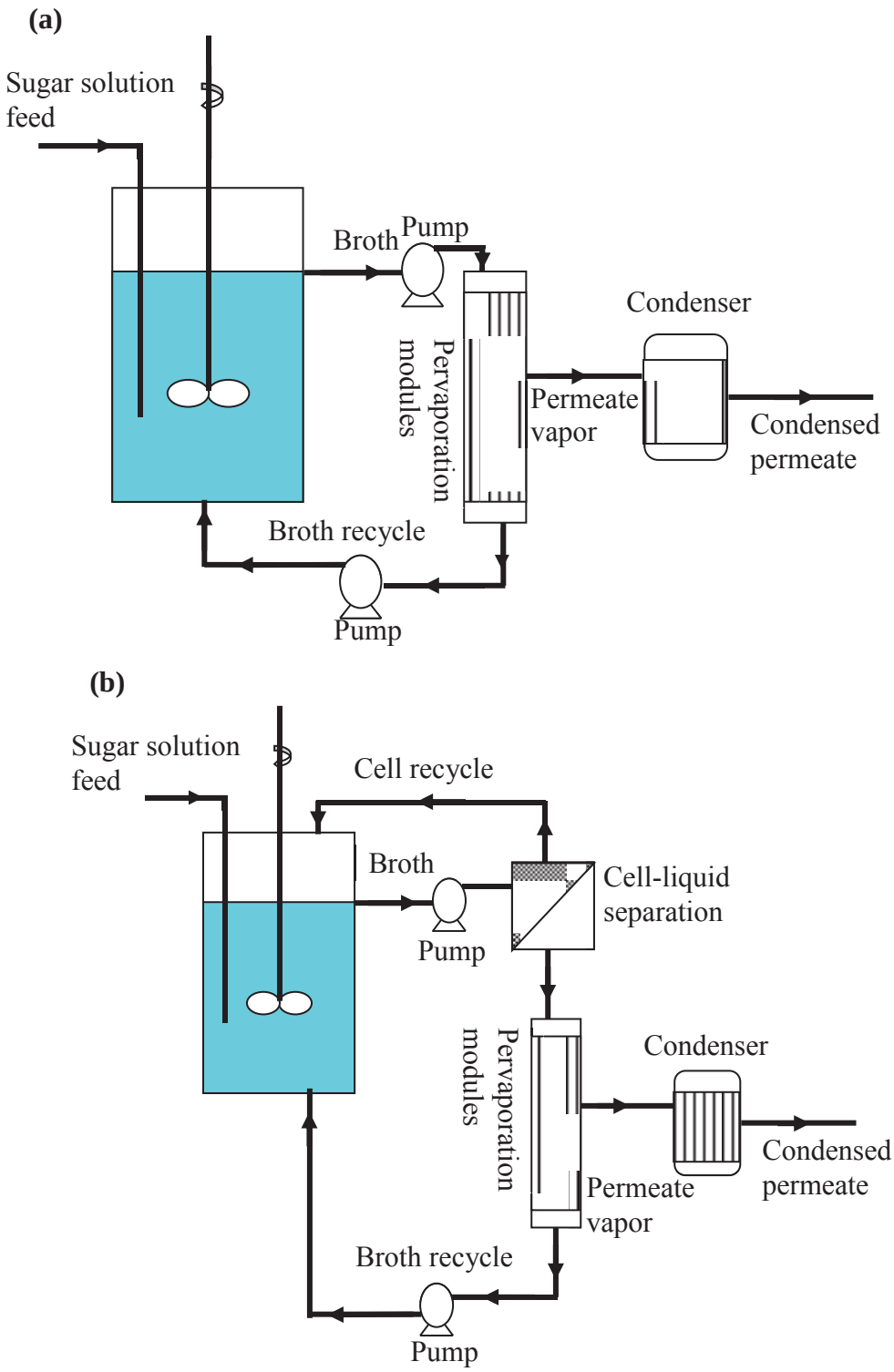


Figure 2.3 Process configuration for an ethanol fermentation integrated with pervaporation: a. ethanol recovery directly from fermentation broth by pervaporation; b. ethanol recovery by pervaporation from a cell-free broth (after Groot *et al.*, 1992).

Pervaporation coupled to fermentation has been shown to improve fermentation performance (Groot *et al.*, 1992; Kaseno *et al.*, 1998; Noruma *et al.*, 2002). Attempts to improve pervaporation membranes by making then more selective and less prone to fouling are continuing (Lewandowska and Kujawski, 2007; Ikegami *et al.*, 2002; Ikegami *et al.,* 2003).

2.9.4 Liquid-liquid extraction

Liquid - liquid extraction separates components based on differences in their solubilities in two immiscible solvents. The fermentation broth is contacted with a water immiscible organic solvent and the component of interest (ethanol in this case) is partly removed in the organic solvent. Distillation is then used to recover the component of interest from the relatively volatile organic solvent. This can reduce the cost of ethanol recovery as organic solvents boil at relatively low temperature compared to water and generally have a lower enthalpy of vaporization than water (Boudreau and Hill, 2006; Geankoplis, 2003). Solvent extraction is widely used for product recovery in many fermentation processes (Boudreau and Hill, 2006; Jassal *et al.*, 1994; Job *et al.*, 1989; Koullas *et al.*, 1999; Matsumura and Markl, 1984; Offeman *et al.*, 2005; Offeman *et al.*, 2008; Solimo *et al.*, 1989). A solvent for use in extraction of ethanol from a fermentation broth should ideally have (Bruce *et al.*, 1991a; Cho Koullas *et al.*, 1999; Shuler, 1986):

- a high separation factor for ethanol
- a high capacity for ethanol to minimize solvent use
- a low solvent solubility in the aqueous phase to minimize solvent loss
- a sufficient density difference between the solvent phase and the aqueous phase to permit a rapid separation of phases
- a good chemical stability
- a low boiling point (vapour pressure)
- a low toxicity toward the fermentating microorganisms
- an efficient method for ethanol recovery from the solvent
- a low cost

In its simplest form, liquid-liquid extraction involves agitation of two phases together followed by gravity separation in a separating funnel. The solute transfers from the

aqueous phase to the other solvent. The solvent containing the solute is processed further to recover the solute.

To evaluate the feasibility of separating ethanol by liquid-liquid extraction (solvent extraction), distribution coefficient or partition coefficient of ethanol needs to be known (Zhang *et al.*, 1991). In addition, the selectivity of the solvent for ethanol may be important (Zhang *et al.*, 1991) so that the other components (e.g. sugars) are not removed from the fermentation broth by the solvent. These parameters are explained next.

Partition coefficient or distribution coefficient is the ratio of the concentrations of the solute in the organic solvent and the aqueous phase. Partition coefficient (K_d) can be determined from the following equilibrium equation:

$$K_d = \frac{[E]_{org}}{[E]_{aq}} \qquad Equation\ 2.1$$

In the above, $[E]_{org}$ is the equilibrium concentration of the solute in the upper (organic) phase and $[E]_{aq}$ is the equilibrium concentration of solute in the lower aqueous phase (Doran, 1995). If $K_d > 1$, the solute favours the upper phase; if $K_d < 1$, the solute is concentrated in the lower phase. Partitioning is influenced by the size, electric charge and hydrophobicity of the solute molecules as well as the properties of the extraction solvent. A solvent with a large K_d is more effective in extracting and concentrating the target solute.

Solvent selectivity (or relative separation) is considered an important factor in selecting a solvent for extraction. Selectivity is the ability of the solvent to preferentially extract the desired component from the broth as compared to the other components present. The relative selectivity β is calculated as follows (Coulson *et al.*, 2002):

$$\beta = \frac{K_a}{K_b} \qquad Equation\ 2.2$$

Where K_a and K_b are distribution coefficients of solute a and b in the solvent. Compound b is often water in extractive fermentation. Ideally, as little water should be extracted into the solvent as possible.

Extractive fermentations using organic solvents have been extensively investigated (Bruce *et al.*, 1991b; Jassal *et al.*, 1994; Koullas *et al.*, 1999; Matsumoto *et*

al., 2004). Toxicity of extraction solvents (extractant) to the fermentation microorganisms always has been a key issue. Toxicity considerations generally eliminate many low molecular weight water immiscible organic solvents from the list of potentially useful extraction solvents (Yabannavar and Wang, 1991).

Table 2.4 lists some of the solvents that have been used as extractants. Oleyl alcohol has been the solvent of choice in most extractive fermentations. This is because oleyl alcohol is non-toxic, has a very high relative selectivity for ethanol but has a low partition coefficient (less than 0.4). A low partition coefficient means that a lot of solvent must be used to efficiently extract ethanol out of the fermentation media and this of course results in a low concentration of ethanol in the solvent. According to Kirbaslar *et al.* (2001), dodecanol has a high boiling point and is a possible separating agent for use with dilute aqueous ethanol solutions. According to Offeman and Stephenson (2006), lauryl alcohol (1-dodecanol) has a high toxicity to microorganisms and a relatively high solubility in the aqueous phase. Goma and Minier (1981) successfully used a plug flow reactor and dodecanol as extractant to increase the ethanol productivity in fermentation of glucose by yeast. 2-Ethyl-hexanol is also a good extactant because it has a good phase separation capability, a distribution coefficient of more than 0.4, a high boiling point and is relatively inexpensive (Offeman *et al.*, 2005). Matsumura and Markl (1984) investigated the effects of several solvents on various microorganisms and found polypropylene glycol P-1200 to be biocompatible with all the tested microorganisms.

Table 2.4 Solvents used as extractants in fermentations

Solvent	Substrates and products	Microorganism	Reference
Oleyl alcohol	Glucose to acetone and butanol	*Clostridium acetobutylicum* (ATCC 824)	(Roffler *et al.*, 1988)
	Glucose to lactic acid	*Lactobacillus delbrieckii*	(Yabannavar and Wang, 1991)
	Glucose to ethanol	*S. cerevisae*	(Daugulis *et al.*, 1994)
	Glucose to	*Clostridium*	(Roffler *et al.*,

	butanol	*acetobutylicum* (ATCC 824)	1987)
	Glucose to ethanol	*Zymomonas mobilis* (ATCC 29191)	(Bruce and Daugulis, 1992)
Alamine 336 in oleyl alcohol	Glucose to butyric acid	*Clostridium tyrobutyricum* (ATCC 25755)	(Daugulis *et al.*, 1995)
Dodecanol	Glucose to ethanol	*S. cerevisae*	(Wu and Yang, 2002)
Oleic acid	Glucose to ethanol	*S. cerevisae*	(Munro *et al.*, 1984)
2-Etyhl-hexanol	Not reported	Not reported	(Goma and Minier, 1981)
Polypropylene glycol P-1200	Glucose to ethanol	*Schizocassharomyces pombe* IFO0344	(Hill *et al.*, 1994)
		S. cerevisiae IFO0309 *Candida brassicae* IFO 1664	(Egan *et al.*, 1988)
		S. uvarum ATCC 26602	(Offeman *et al.*, 2006)
		Zymomonas mobilis IFO 13766	(Matsumura and Markl, 1984)

Recently, vegetables oils and their derivatives have been investigated for use as extractants in fermentations. Table 2.5 lists some of these non toxic solvents. Based on Offeman *et al.* (2006), castor oil has a high distribution coefficient for ethanol compared to the other vegetables oils studied and may be non toxic. Rahman *et al.* (1995) also used vegetable oils such as soybean oil as extraction solvents.

Solvent extraction studies have generally disregarded the solvent biocompatibility issue or have addressed it from the perspective of a yeast fermentation. Unfortunately, the

solvent biocompatibility depends on the microorganism used in the fermentation process (Roffler *et al.*, 1991) and microorganism specific studies are needed. As barely any work has been done on in situ recovery of ethanol in *Z. mobilis* fermentations, potential extraction solvents which have been reported to be nontoxic to other microorganisms were used for biocompatibility tests with *Z. mobilis* in this work.

Table 2.5 Extraction solvents, sources and characteristics (Offeman *et al.*, 2006)

Extractant	Predominant fatty acid species	Grade	Source[a]	Characterization[b]
Coconut oil	Lauric [C12:0]	Refined, 76°F	Alnor Oil	44-52% lauric, <10% unsat, FFA <0.05%, SV 250-264, IV 6-12
Olive oil	Oleic [C18:1]	Edible,	Welch, Holme & Clark	72.6% oleic, FFA 0.12 wt%, IV 83.1, SV 194.3
Castor oil	Ricinoleic [12-hydroxy C18:1]	Neutralized	Alnor Oil	87.1% ricinoleic, FFA 0.33%, IV 85.1, SV 178.7, HV 161.6
Safflower oil	Linoleic [C18:2]	Edible, high	Alnor Oil	75% linoleic, FFA 0.04%
Methyl laurate	Lauric	linoleic acid	Aldrich	99.9%
Methyl oleate	Oleic		Aldrich	99.8%
Ethyl oleate	Oleic		Aldrich	99.6%
Butyl oleate	Oleic		Spectrum	65%
Methyl ricinoleate	Ricinoleic		Spectrum	99.5%
Methyl linoleate	Linoleic		Sigma	99.5%
Lauryl alcohol	Lauric	ACS reagent	Aldrich	98.52%
Oleyl alcohol	Oleic	Jarcol 95B	Jarchem	93% oleyl, SV 0.15,HV 207, IV 91.3
Ricinoleyl alcohol	Ricinoleic		MP Biomedicals	89.1% ricinoleyl

[a]Alnor Oil, Valley stream, NY; Welch, Holme & Clark, Newark, NJ; Sigma-Aldrich, St Louis, MO; Spectrum Chemicals and Laboratory Products, Gardena, CA; Jarchem, Newark, NJ; MP Biomedicals, Aurora, OH.
[b]Characterization definitions: FFA = free fatty acids, expressed as oleic acid; IV = iodine value; SV saponification value; HV hydroxyl value.

2.10 Adsorption

Adsorption is the use of solid sorbents for in-situ product recovery. Solids that are used to adsorb gases or dissolved substances are known as adsorbents and the adsorbed molecules are collectively referred to as adsorbate. Adsorption is a surface phenomenon in which the absorbate attaches to the absorbent via electrostatic interactions, van der Waals forces,

reactive binding, etc., depending on the nature of the absorbent and the adsorbate (Doran, 1995).

Adsorption can be either of a physical nature (i.e. no formation of covalent bonds between the adsorbent and adsorbate) or may involve chemical interaction (e.g. the binding between an antibody and antigene). In physical adsorption, weak forces such as van der Waals forces are dominant; however, in ion exchange adsorption strong ionic interactions occur (Shuler and Kargi, 2002). There is no chemical specificity in physical adsorption. The type of adsorbent used depends on the application. For example, activated carbon is a widely use nonspecific adsorbent for organics in wastewater treatment. Similarly, ion exchange adsorbents are widely used in recovery of charged molecules such as amino acids, proteins, certain antibiotics and some vitamins. Adsorption capacity (i.e. the amount of material adsorbed per unit mass of adsorbent) varies depending on the adsorbent, the adsorbate, the physicochemical conditions, and the surface properties of the adsorbent and adsorbate (Shuler and Kargi, 2002). Porous adsorbents (e.g. activated carbon, polymer resins) offer extremely large surface areas for adsorption compared to nonporous adsorbents. The criteria used for selection of sorbents for in situ product removal from a fermentation broth are the following (Bui *et al.*, 1985; Carton *et al.*, 1998; Groot and Luyben, 1986; Lencki *et al.*, 1983; Maddox, 1982; Milestone and Bibby, 1981; Oumi *et al.*, 2002):

1. Selectivity; the sorbents must selectivity adsorb the product with minimal adsorption of water, glucose, etc.
2. Desorption characteristics; the desorption of the product must be easy at a low temperatute
3. Sterilizability; the sorbents must be sterilizable and heat stable
4. Toxicity; the sorbents should be nontoxic
5. Cost; the sorbents must be inexpensive and reuseable multiple times

Among the sorbents used for adsorption of alcohols are activated carbon (Bui *et al.*, 1985; Lee and Wang, 1982; Qureshi and Blaschek, 1999; Wang *et al.*, 1981), silicalites (Bui *et al.*, 1985; Carton *et al.*, 1998; Groot and Luyben, 1986; Lencki *et al.*, 1983; Maddox, 1982; Milestone and Bibby, 1981; Oumi *et al.*, 2002) and polymeric resins (Groot and Luyben, 1986; Nielsen *et al.*, 2010; Nielsen *et al.*, 1988; Pitt Jr *et al.*, 1983; Qureshi and Blaschek, 1999; Qureshi *et al.*, 2005). Bui and Verykios *et al.* (1985) used silicalite, activated carbon and zeolite for ethanol adsorption. They reported that

silicalite was the most suitable adsorbent for the in-situ removal of ethanol from fermentation broths because of its selectivity compared to activated carbon and zeolites.

Polymer resin materials have attracted attention in recent years for in situ product recovery (Gao and Daugulis, 2010; Nielsen *et al.*, 2010; Nielsen and Prather, 2009; Qureshi *et al.*, 2005). Polymer resins have the advantages of good stability (physical, chemical and biological), improved biocompatibility, complete immiscibility with the fermentation broth and potential for reuse (Huck and Bonn, 2000; Nielsen and Prather, 2009; Rehmann *et al.*, 2007). Among polymer resins, poly (styrene-co-divinylbenzene) resins have shown the greatest adsorption potential for n-alcohols due to their highly nonpolar nature (Nielsen and Prather, 2009).

In biotechnology processes, adsorption can be implemented in different ways. Possible approaches are shown in Figure 2.4. Resins can be added directly to the bioreactor or placed in a separate vessel through which the broth is circulated (Figure 2.4) (Holst and Mattiasson, 1991; Roffler *et al.*, 1984).

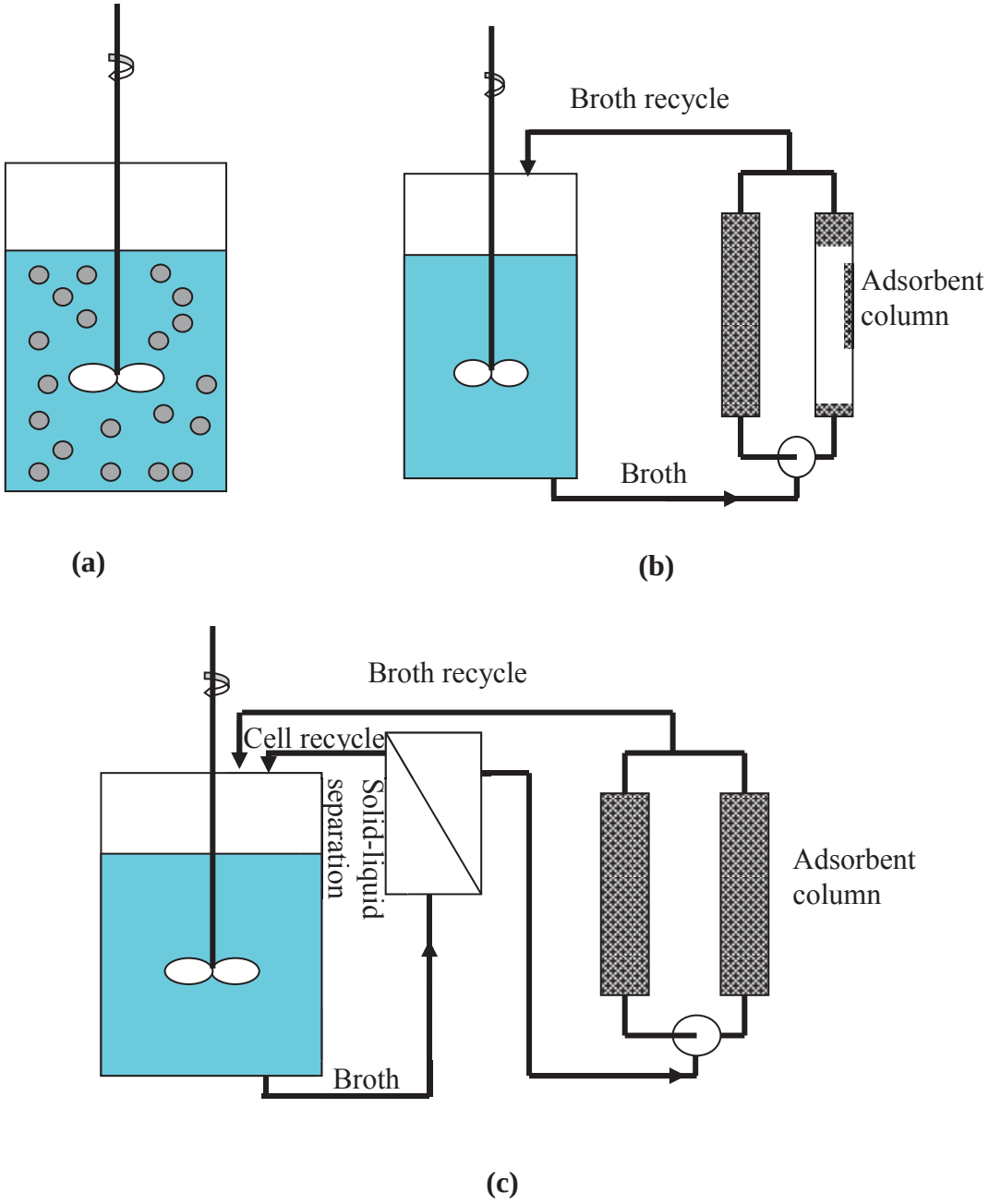


Figure 2.4 Possible methods of using solid sorbents in an extractive fermentation: a) adsorption inside the fermertor; b) adsorption from the whole broth but outside the fermentor; c) adsorption from cell-free broth outside the fermentor (after Holst and Mattiasson, 1991).

Having the resin suspended in the bioreactor poses difficulties with separation of the resin from cells and this method cannot be used in a long term continuous fermentation.The second approach involving recycling the broth from the bioreactor through external adsorption columns is more flexible and allows the columns to be

changed easily and regenerated. The third approach of recycling the cell-free broth through an adsorption column avoids the general fouling of the sorbent bed by the cells, but requires the use of a cell–liquid separation step.

When an external column of resin is used, it may be operated as a fixed bed or fluidized bed. Fixed bed columns can plug and foul easily as the resin bed acts as a filter that may trap the cell. To overcome this problem, a fluidized bed is preferred. In a fluidized bed, the resin particles are separated from each other and suspended in an upflow of the broth (Gailliot *et al.*, 1990; Scott, 1993). The adsorption capacity of sorbents for ethanol varies with the conditions in the medium, the concentration of ethanol and the temperature. Adsorption values of around 100 mg of ethanol per g sorbent have been reported (Milestone and Bibby, 1981; Pitt Jr *et al.*, 1983).

Ion exchange resins have been used for in situ recovery of fermentation products that can be made to exist in an ionic form. Examples of such products are lactic acid, amino acids and salicylic acid. Ethanol does not belong to this category of products and, therefore, cannot be recovered by ion exchange resins.

CHAPTER 3

MATERIALS AND METHODS

This research focused on the use of the facultative anaerobic gram-negative bacterium *Zymomonas anaerobia* ATCC 29501 for producing ethanol by high gravity fermentation. Preliminary work in shake flasks was used to establish an upper limit on tolerance of the bacterium to the concentration of the C-source (glucose). At the highest acceptable glucose concentration, experiments were done to characterize the bacterial susceptibility to ethanol inhibition so as to establish the need and requirement for in situ removal of ethanol in future extractive fermentations. Batch fermentations were used to identify the optimal fermentation temperature. Finally, extractive batch and continuous fermentations were conducted using selected solvents and resins as the extractants. For liquid-liquid extraction, several solvents were experimentally evaluated for ability to extract ethanol and biocompatibility with *Z. anaerobia.* Similarly, adsorption resins were experimentally tested for continuous in situ recovery of ethanol. The relevant materials and methods are explained in this chapter.

3.1 Microorganism, maintenance and preparation

The bacterium *Z. anaerobia* ATCC 29501 (DSM 473) was obtained from the American Type Culture Collection, USA (www.atcc.org). Pure stock cultures were maintained on agar slants at 4 °C. The maintenance agar medium was made using deionized water and had the following composition (g L^{-1}): glucose 20; yeast extract 10; KH_2PO_4 1; $(NH_4)_2SO_4$ 1; $MgSO_4$ 0.5; and agar 15. The medium was sterilized by autoclaving (121 °C, 15-min). Freshly inoculated agar plates were anaerobically incubated at 30 °C for 5 –7 days in an anaerobic jar (Gas Pak EZ Anaerobe Container System, Maryland, USA; www.bd.com) and then stored at 4 °C. The microorganism was subcultured every 2 weeks. This stock culture was used for inoculum preparation throughout this study.

Inocula were asceptically prepared by transferring a streak of colonies from a petri plate to a 25 mL flask containing 10 mL of sterile glucose-based medium (see Section

3.2). The flask was incubated at 30 °C for 24 hours. After incubation, 5 mL of the culture broth was poured into a 250 mL flask containing 100 mL of sterile medium and allowed to incubate at 30 °C for 8–24 hours (depending on the initial glucose concentration) with gentle agitation at 100 rpm. The cell concentration of the inoculum was standardized to an absorbance of approximately 1.0 at 650 nm (Ultraspec 2000, model 80-2106-00 spectrophotometer; Pharmacia Biotech Inc, Piscataway, NJ, USA). All fermentations were inoculated using the above inoculum at a level of 10% by volume.

3.2 Culture medium

The media used in fermentations and the preparation of inoculum contained the specified concentration of glucose (50–300 g L^{-1}) and the following in distilled water 10 g L^{-1} yeast extract; 1 g L^{-1} KH_2PO_4; 1 g L^{-1} $(NH_4)_2SO_4$ and 0.5 g L^{-1} $MgSO_4.7H_2O$ (Kosaric *et al.*, 1982). The initial pH of the media was adjusted to 5.5 by adding 2N H_2SO_4 (Spangler and Emert, 1985). The media were then sterilized by autoclaving at 121 °C for 15 minutes.

3.3 Solvents and resins

Several solvents were experimentally evaluated for possible use in extractive fermentation and biocompatibility with *Z. anaerobia.* These solvents and their characteristics are show in Table 3.1. Prior to use, the solvents were purged with nitrogen gas (New Zealand Industrial Gases Ltd, Palmerston North, New Zealand) for 15-min in order to remove any dissolved oxygen. Thorough removal of oxygen was important because the fermentations were anaerobic and oxygen is highly soluble in water–immiscible organic extraction solvents. The adsorption resins used in various experiments are shown in Table 3.2. The resins were dried at 37 °C in an incubator (Contherm Digital Series Five Incubator, Lower Hutt, New Zealand, Cat. No: 245M) for 72 h prior to use to remove excess moisture. After drying, the resins were cooled to room temperature in a desicator and weighed (1 g of each resin). The resins were sterilized with the liquid media by autoclaving for 15 min at 121 °C.

Table 3.1 Solvents and their characteristics

Solvent	Source	Composition	Boiling point, ºC	Specific gravity, (20 ºC /20 ºC)
Castor oil	Sigma-Aldrich www.sigmaaldrich.com	89.5% Ricinoleic Acid 4.2% Linoleic Acid 3.0% Oleic Acid 1.0% Stearic Acid 1.0% Palmitic Acid 0.7% Dihydroxystearic Acid 0.3% Linolenic Acid 0.3% Eicosanic Acid	313	0.957 - 0.961

Solvent	Source	Composition	Boiling point, ºC	Specific gravity, (20 ºC /20ºC)
Soy oil	New World www.newworld.co.nz	54.5% Linoleic Acid 22.4% Oleic Acid 10.5% Palmitic Acid 8.3% Linolenic Acid 4.3% Stearic Acid	150	0.92
1-Dodecanol	Sigma-Aldrich www.sigmaaldrich.com	98% 1-Dodecanol	259	0.833
2-Ethly hexanol	Sigma–Aldrich www.sigmaaldrich.com	95% 2-Ethyl haxanol	179	0.83
Oleyl alcohol (Adol 85)	Fluka www.sigmaaldrich.com	60% Oleyl alcohol	330–360	0.85

Solvent	Source	Composition	Boiling point, ºC	Specific gravity, (20 ºC /20ºC)
2-Octyl-1-dodecanol	Sigma–Aldrich www.sigmaaldrich.com	97% Octyl-1-dodecanol	234–238	0.838
Isostearyl alcohol	Nissan Chemical www.nissanchem.co.jp	99% Iso-octadecanol	330 –260	0.847
Polypropylene glycol P-1200	Fluka www.sigmaaldrich.com		185 –189	1.00

Table 3.2 Resins and their characteristics

Trade name	Chemical name	Source	Dry density (g/mL)	Particle size (mesh)	Surface area (m^2/g)	Average pore diameter, (Angstroms)	Pore volume (mL/g)	Cost (NZD/kg)
Dowex Optipore L-493	Poly(styrene-*co*-divinylbenzene)	Dow www.dow.com	0.68	4.6	1100	46	1.16	414
Amberlite XAD-16	Poly(styrene-*co*-divinylbenzene)	Rohm and Haas www.rohmhaas. com	1.08	20-60	900	100	1.82	317
Poly(styrene-*co*-divinylbenzene)	Poly(styrene-*co*-divinylbenzene)	Sigma–Aldrich www.sigmaaldrich.com	0.29	80-100	300-400	0.0085		(164/25g)
Poly(4-vinylpyridine)	Poly(4-vinylpyridine)	Sigma –Aldrich www.sigmaaldrich.com	1.15	100-200	650			937

3.4 Cleaning of glassware

Prior to use, all glassware was washed in hot Pyroneg detergent (Diversey-Wallace Ltd, Papatoetoe, New Zealand), rinsed in tap water, and then in distilled water, and dried in an oven (Contherm, Lower Hutt, New Zealand, Cat. No: 245M) at 105 °C.

3.5 Equipment sterilization

All bottles, measuring cylinders and tubings were sterilized by autoclaving at 121 °C for 15 min. Pipette tips were sterilized at 121 °C for 15 min. The 3-L bioreactor unit (including pH and dissolved oxygen (DO) probes and membrane air filters attached) containing the medium was autoclaved at 121 °C for 15 min. The solid–liquid extraction column and tubing were sterilized separately at 121 °C for 15 min. The bioreactor and the solid-liquid extraction column were connected asceptically after they had cooled to room temperature. Aseptic inoculation was then carried out.

3.6 Glucose tolerance studies

Glucose tolerance of *Z. anaerobia* ATCC 29501 was examined in fermentations carried out in 2-L shake flasks (1-L working volume). In different experiments the initial glucose concentration in the medium ranged from 50 to 300 g L^{-1}. The other components in the medium were as noted in Section 3.2. The flasks containing the media were sterilized by autoclaving at 121 °C for 15 minutes. The cooled flasks (30 °C) were inocoluted with the starter culture (Section 3.1). The starter culture had been grown in medium with the same initial glucose concentration as in the medium being inoculated. The mouth of each flask was sealed with cotton wool and aluminium foil. The inoculated flasks were incubated in an Infors Multitron shaker (Infors AG CH-4103 Bottmingan; www.infors-ht.com) at 100 rpm agitation and 30 °C. Samples were taken every 2 hours during the lag phase and every 1 hour during exponential growth phase. The duration of fermentation depended on the initial glucose concentration (Kosaric *et al.*, 1982; Roger *et al.*, 1980). The initial glucose concentration that gave the highest maximum ethanol productivity and allowed a maximum consumption of the glucose was considered to be optimal and was used for

future batch and continuous in situ extractive fermentations. All experiments were repeated at least three times in order to establish reproducibility. The experiments were highly reproducible.

3.7 Ethanol tolerance studies

Inhibition of *Z. anaerobia* by ethanol was experimentally examined to establish tolerance limits and the requirements for in situ ethanol removal from the fermentation broth. The culture medium used was as previously described (Section 3.2) but with a glucose concentration of 150 g L^{-1} that had been shown to be the highest tolerated (see Section 3.6). Fourteen shake flasks (2-L) with the culture medium (1-L) were sterilized by autoclaving (121 °C, 15-min) and cooled to 30 °C. 100 mL of inoculum was added to each shake flask. Pure ethanol (0, 20, 40, 60, 80, 100, 120 g L^{-1}) was asceptically added to the flasks either at the start of the fermentation (time = 0 h) or 10 h after fermentation began. The inoculum had been standardized as described in Section 3.1. The culture was incubated at 30 °C for 24 hours (Huang and Chen, 1988). Samples were taken every 2 hours and ethanol concentration, biomass concentration and residual sugar were measured.

3.8 Effect of an anaerobic atmosphere

All shake flask fermentations were conducted in stoppered flasks, but some of the early fermentations (Section 3.6, Section 3.7) had an atmosphere of air above the liquid. To see if this may have affected the results, two sets of identical fermentation were performed in shake flasks such that one set of flasks was stoppered but had an air atmosphere above the liquid (control flasks) and the second set of flasks had the headspace purged with nitrogen (Figure 3.1). The anaerobic flasks were purged with pure nitrogen before and after inoculation and during the fermentation. The nitrogen flow rate was controlled at 0.3 LPM. All flasks had an identical sterile medium (1 L, Section 3.2) with 150 g L^{-1} glucose. Flasks were inoculated with 100 mL of the earlier mentioned standardized inoculum and incubated at 30 °C (100 rpm agitation) as specified earlier. Every 2 h samples were withdrawn and ethanol concentration, biomass concentration and residual sugar concentration were measured. The experiments were repeated for reproducibility checks.

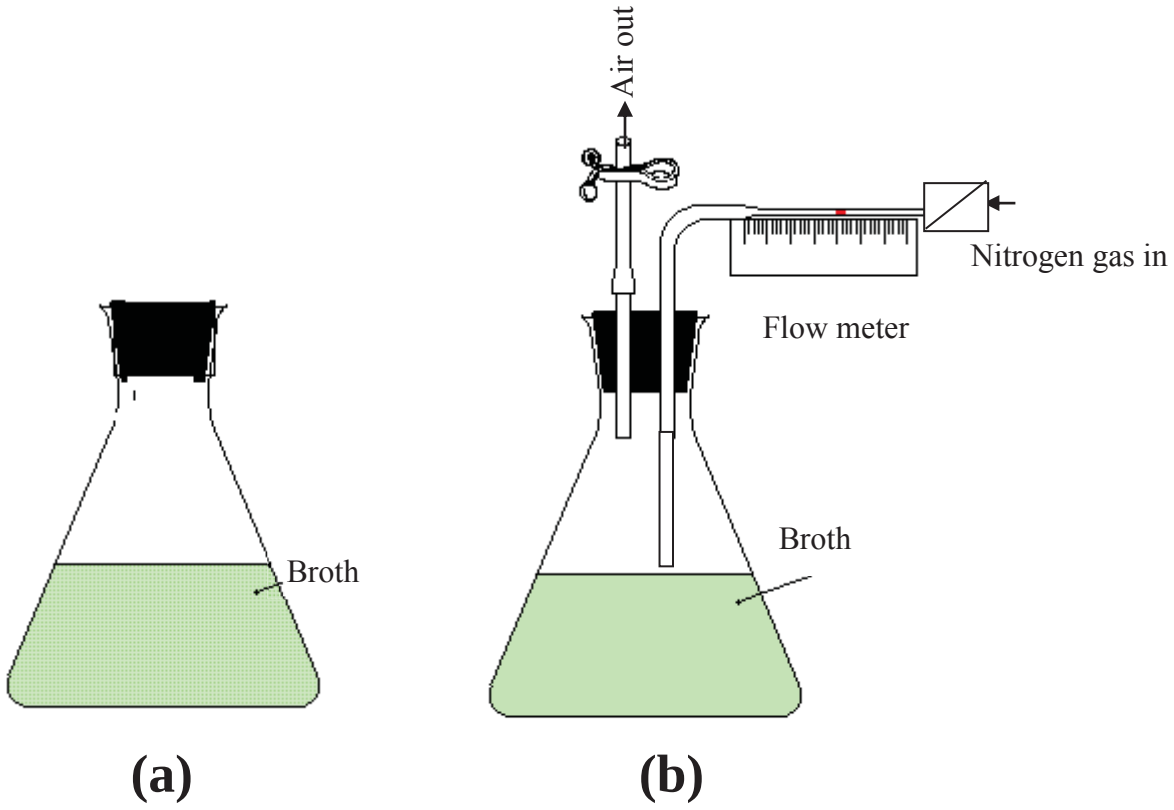


Figure 3.1 An anaerobic fermentation (a) with an air atmosphere and (b) with purging with nitrogen

3.9 Effect of temperature

The optimal temperature for the fermentation was established by temperature controlled (30 °C to 40 °C) batch fermentations conducted in a 3-L stirred bioreactor (BIOFLO 110 New Brunswick Scientific, East Brunswick, NJ, USA; www.nbsc.com). The internal diameter of the electrically jacketed glass bioreactor vessel was 0.13 m. The vessel was fully baffled with 4 vertical baffles spaced equidistance around the periphery. The baffle width was 17 mm. A central shaft supported two 6-bladed Rushton disc turbine agitators. The agitators were identical with a diameter of 52 mm and were spaced 0.1 m apart on the shaft. The lower agitator was located 35 mm above the bottom of the vessel. The working volume was kept at 1.0 L. The sterile bioreactor was inoculated with 100 mL (10% by vol) of the earlier specified inoculum (Section 3.1). The initial glucose concentration in the medium was 150 g L^{-1}. The fermentation

temperature was controlled within ±0.2 °C of the set point temperature. The agitation speed was maintained at 100 rpm. All fermentations were run as an aseptic anaerobic cultures by flushing the headspace of the fermentor with nitrogen at a constant flowrate of 0.3 LPM. Each batch fermentation was run for 17 hours. Samples were taken periodically. All experiments were repeated at least three times.

3.10 Batch bioreactor fermentations

A 3-L stirred bioreactor was used throughout this study (Section 3.9). All fermentations were run as aseptic anaerobic batch cultures. The gas inlet and exhaust ports on the bioreactor were installed with sterile hydrophobic membrane filters (0.2 μm; either Sartorious, Gottingen, Germany, or Millipore, Bedford, MA, USA). The assembled bioreactor filled with the earlier specified liquid medium (150 g L^{-1} glucose concentration; Section 3.2) was autoclaved (121 °C, 20-min) with the pH and the dissolved oxygen electrodes installed. The pH electrode (Ingold gel-filled electrode, model no. 465-35-SC-P-K9/270/9848; Mettler-Toledo, www.mt.com) had been calibrated using pH 7.0 and pH 4.0 buffers prior to autoclaving. The pH was monitored, but not controlled. The initial pH was always 5.5. The dissolved oxygen (DO) electrode had been installed to check for an absence of oxygen in the bioreactor. The DO electrode had been calibrated at 35 °C in the sterilized medium bubbled with the nitrogen gas until the dissolved oxygen level had declined to approximately 0% (Chisti, 1989; Chisti, 2010). Then, the air was bubbled in until the concentration of dissolved oxygen had attained saturation at 100%. Fermentation was run as aseptic anaerobic culture by flushing the headspace of the fermentor with nitrogen at a constant flowrate of 0.3 LPM until the dissolved oxygen reading declined to 0%. The nitrogen purge had stopped before inoculation commenced. The dissolved oxygen was always monitored but not controlled. The working volume was always 1 L. The standardized inocolum volume was 100 mL. The agitation speed was a constant 100 rpm and the temperature was controlled at 35 °C as had been found optimal (Section 3.9). Samples were taken periodically and stored at 4 °C for further analysis. The storage period did not exceed 3 days.

3.11 Continuous bioreactor fermentations

Constant volume continuous stirred tank anaerobic fermentations were carried out at various dilution rates to characterize glucose consumption, biomass production and ethanol production. All continuous fermentations used the aforementioned 3-L stirred bioreactor (Section 3.9).

Fermentations were conducted asceptically at 35 °C at a constant volume V_L of approximately 1 L. The headspace of the fermenter was flushed with nitrogen at a constant flow rate of 0.3 LPM. The agitation speed was always 300 rpm. Continuous fermentations commenced in the batch mode, as outlined in Section 3.10, prior to being switched to continuous feeding of substrate at 10 h. The sterile feed medium was introduced at a controlled flow rate (Q_L) by means of a peristaltic pump. The dilution rate D was Q_L/V_L (h^{-1}). The pH was continually measured but not controlled. The feed medium with initial glucose concentration of 150 g L^{-1} was used. The volume in the fermentor was controlled by operating the overflow effluent peristaltic pump twice as fast as the feed pump. Continuous fermentation began at the lowest dilution rate value of 0.05 h^{-1}. Dilution rates of up to 0.3 h^{-1} were tested. At least 5 samples were collected at each dilution rate following the attainment of steady state conditions. Steady state was assumed to exist only after a minimum of three residence times had elapsed and the samples taken on successive days had similar levels of substrate and product concentrations. At each steady state, samples were taken periodically and analyzed to determine the concentrations of glucose, cell mass and ethanol.

The above mentioned continuous fermentations were initially carried out at feed glucose concentration of 150 g L^{-1} with various dilution rates. Another set of continuous fermentations was carried out in at feed glucose concentration ranging from 80 g L^{-1} to 150 g L^{-1} at the fixed dilution rates of 0.05 h^{-1} and 0.01 h^{-1} to identify the minimum glucose concentration that led to oscillation around a ‘steady state’. Identification of the oscillation regime was necessary to design future batch and continuous in situ extractive fermentations to prevent oscillations at the feed sugar concentration that would otherwise cause oscillations.

3.12 Pump calibration

The medium pump and the harvest pump used in continuous fermentations were calibrated by measuring of water/solvent (i.e. iso oxtadecanol) flow (100 mL measuring cylinder) for various lengths of time (stop watch) at various settings (0 –100 %) of the pumps. The rotational speed (0 –100% of full speed) of the pump and the flow rate were linearly correlated (Figure 3.2). Therefore, the relationships between dilution rates and pump settings were linear (Figure 3.2).

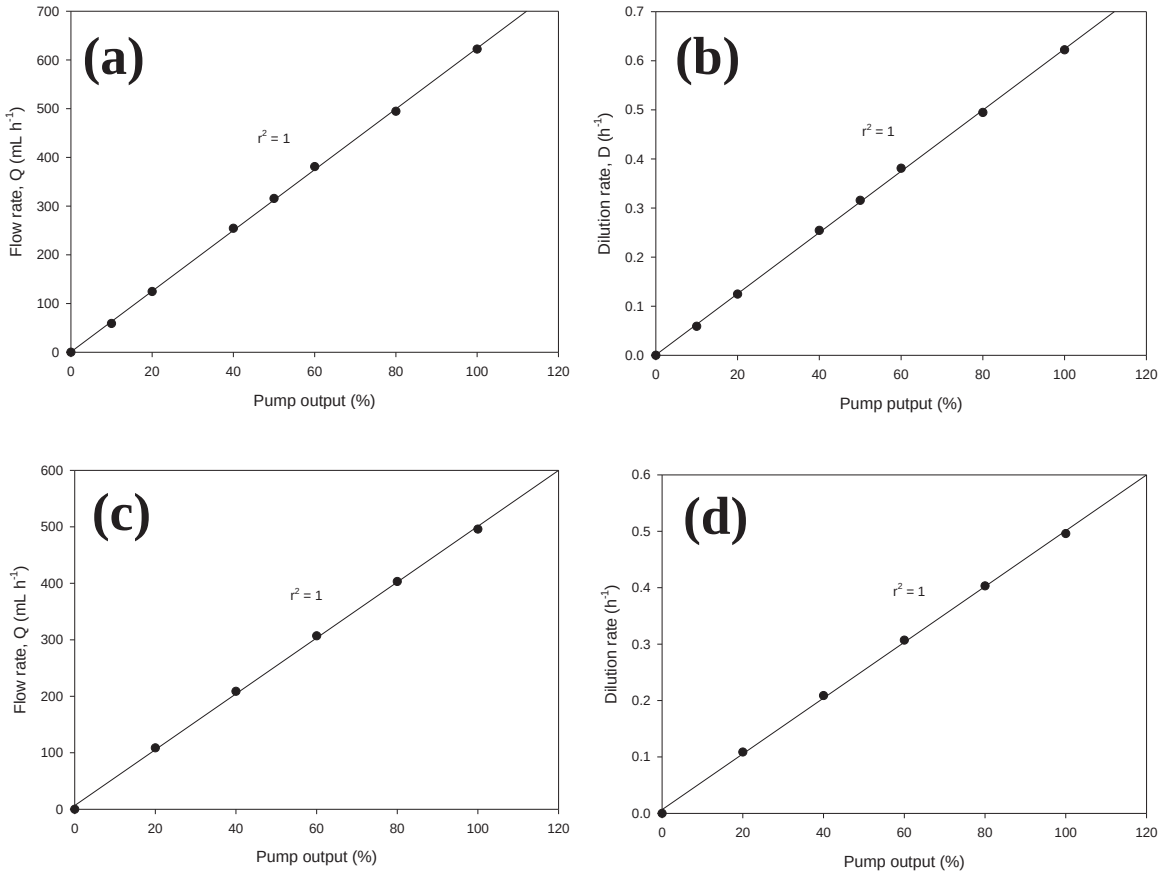


Figure 3.2 News Brunswick BioFlo 210 pump calibration graphs: (a) feed flow rate (mL h^{-1}); (b) dilution rate of feed (h^{-1}); (c) solvent flow rate (mL h^{-1}); (d) dilution rate of solvent (h^{-1}).

3.13 Distribution coefficients of ethanol

Distribution coefficients, or partition coefficients, of ethanol in various solvents were determined over an initial ethanol concentration in the range of 0–100 g L^{-1} in the aqueous medium. Thus, 5 mL of a standard aqueous solution of ethanol was placed in a 20 mL test tube containing 5 mL of the water-immiscible organic solvent (extractant solvent). Triplicate tests were conducted for each extractant. The tubes were gently twirled for 10 min using a magnetic stirrer. Then, they were incubated at 35 °C for approximately 24 h. The tubes were then centrifuged at 2000 × g for 10 min to ensure a complete separation of phases. The ethanol concentration in each phase was then determined by gas chromatography (model GC 6000 Vega Series 2; Carlo Erba Instruments, Milan, Italy). The distribution coefficient (K_d) was calculated as the ratio of the concentration of ethanol in the organic phase to that in the aqueous phase, thus:

$$K_d = \frac{[E]_{org}}{[E]_{aq}} \qquad \text{Equation (3.1)}$$

Where $[E]_{org}$ is ethanol concentration in the organic phase and $[E]_{aq}$ is the ethanol concentration in the aqueous phase (Daugulis and Kollerup, 1986).

3.14 Biocompatibility experiments

A solvent used for product removal in an in situ extractive fermentation inevitably comes into contact with the microorganism responsible for the fermentation. It is therefore essential to ensure that the solvent used is not only a good extractant but is also biocompatible with the microorganism being used. Therefore, the various solvents (Table 3.1) were evaluated for biocompatibility with *Z. anaerobia* using the shake flask biocompatibility test described by Kollerup and Daugulis (1986) and Bruce *et al.* (1992). Thus, 125 mL shake flasks containing 50 mL of the sterilized glucose medium (150 g L^{-1} glucose, Section 3.2) were inoculated with a 5 mL of standardized inoculum of *Z. anaerobia*. The flasks were incubated in a shaker (35 ºC, 100 rpm) for 12 hours. At this point the cells were vigorously growing. Solvent (10 mL) was now added to each flask except the control flasks. After a further 30 hours of incubation, the

fermentation broth in the flasks was sampled. The samples were analyzed for ethanol concentration, residual glucose concentration, biomass concentration and cell viability (Section 3.18.6).

3.15 Extractive fermentations

3.15.1 In-situ batch extractive fermentations

Extractive batch fermentations were carried out asceptically in the 3-L stirred bioreactor (Section 3.9) with a working volume 2-L (1:1 ratio of solvent and fermentation broth, by volume). Figure 3.3 shows a schematic diagram of the extractive fermentation system used. Fermentation medium (Section 3.2) with an initial glucose concentration of 150 g L^{-1} was metered into the bioreactor and maintained under a nitrogen atmosphere (nitrogen flow rate of 0.3 LPM purged the headspace of the fermentor). The bioreactor was inoculated by pumping in 100 mL of a vigorously growing inoculum. The temperature and the agitation rate were maintained at 35 ºC and 150 rpm, respectively. Initial pH was 5.5.

Solvents that had been found to be nontoxic to *Z. anaerobia* were used for in-situ batch extractive fermentations. The extraction commenced after inoculation. Solvent was pumped into the bioreactor near the bottom and rose through the aqueous phase to form a layer at the top. Both the solvent phase and the aqueous phase were agitated by the impellers located in the respective phases. At regular intervals, samples were withdrawn from the two phases separately and measured for ethanol concentration, biomass concentration and residual sugar concentration.

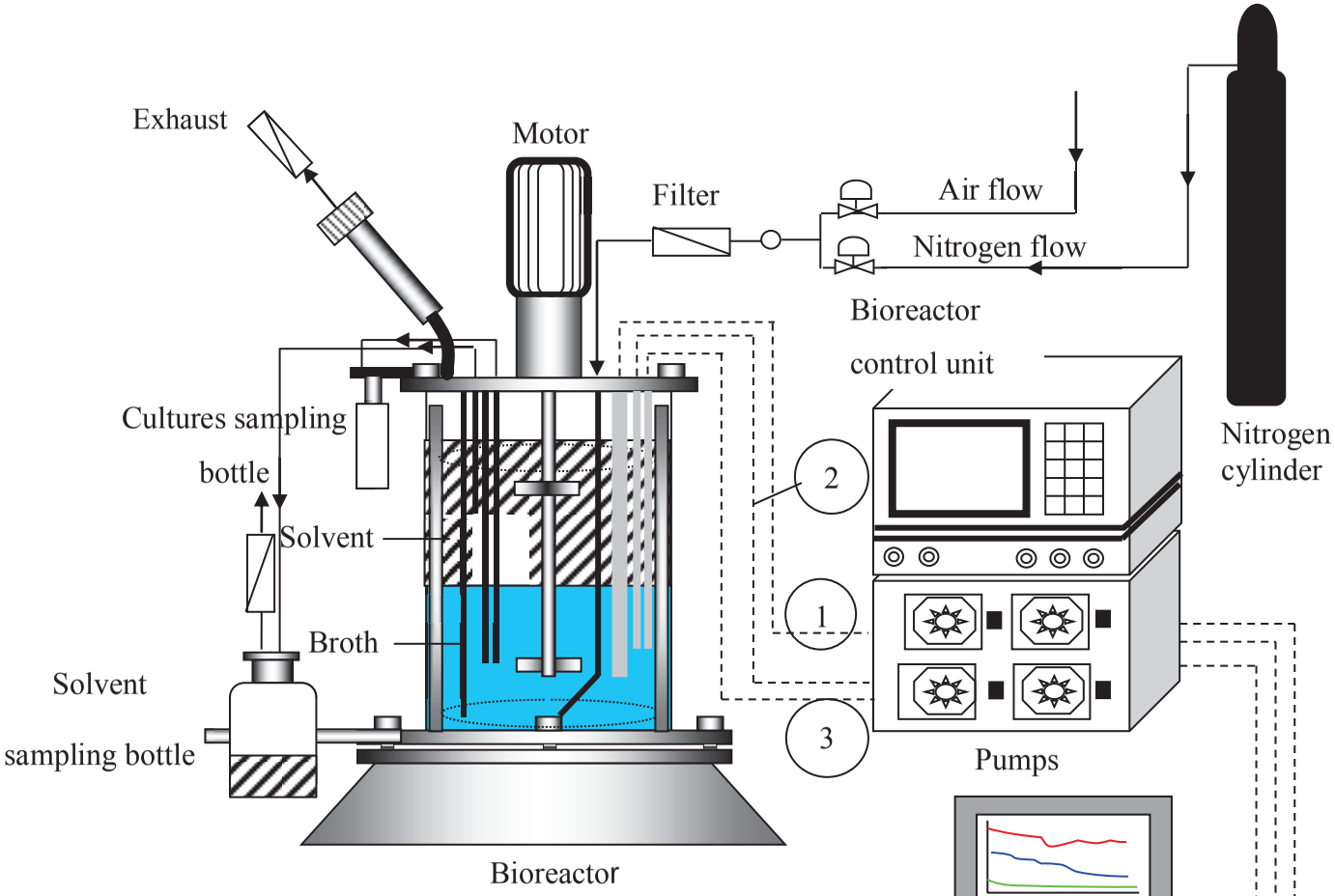


Figure 3.3 Batch solvent extractive fermentation set up (adapted from Sulaiman *et.al*, 2011)

3.15.2 Continuous extractive fermentations

Figure 3.4 shows the experimental set up used in in-situ continuous extractive fermentations. A 3-L stirred bioreactor (Section 3.9) with working volume of 2-L (1:1 ratio of solvent and fermentation broth by volume) was used. The two 6-bladed Rushton disc turbine impellers were operated at a constant speed of 150 rpm such that one of the impeller mixed the fermentation broth and the other mixed the solvent. No pH control was used but the initial pH was adjusted to 5.5. The bioreactor was equipped with the pumps and other accessories for extractive fermentation involving an aqueous phase (the bottom phase) and an organic solvent phase (upper phase).

The fermentation began as a nonextracted batch at an initial sugar concentration of 150 g L^{-1} using the medium described in Section 3.2. After 10 hours of batch fermentation, the solvent was added to the required volume and continuous flow of the solvent and the feed began at preset flow rates. The solvent was introduced at the bottom of the bioreactor underneath the lower impeller and dispersed through the aqueous phase and formed an upper layer allowing for the withdrawal of only the solvent by overflow pumping. The feed flow began at the same time as the solvent flow. The aqueous broth was harvested from a pipe that extended to about halfway depth of the aqueous broth in the bioreactor. The solvent removal pump was run a little faster than the solvent feed pump to maintain a constant level in the bioreactor. The medium feed and harvest flow pumps had exactly matched flow rates to maintain a constant volume of the aqueous broth in the bioreactor. The solvent containing dissolved ethanol was continuously removed from the top of the solvent layer. Samples of the fermentation broth were taken at regular interval and analyzed for biomass concentration, ethanol concentration and glucose concentration. Similarly, the solvent phase was sampled and measured for the concentration of ethanol.

The effluent solvent from the bioreactor was collected, regenerated and reused for extractive fermentations. In solvent regeneration, an effluent solvent batch (~ 1.5 L) was first placed in a 2-L separating funnel and separated from any residual aqueous broth. The upper solvent phase removed from the separating funnel was placed in a rotary evaporator (Heidolph Instruments, Schwabach, Germany; www.heidolph-instruments.com) and heated at 80 ºC to distill off the ethanol. The latter was condensed and collected separately. Once all the ethanol had been distilled off, the residual solvent was (~500 mL) cooled to room temperature and

centrifuged (18000 × g, 20 min) to remove any suspended solids and water droplets. The recovered upper phase contained the regenerated solvent that was reused in the fermentations.

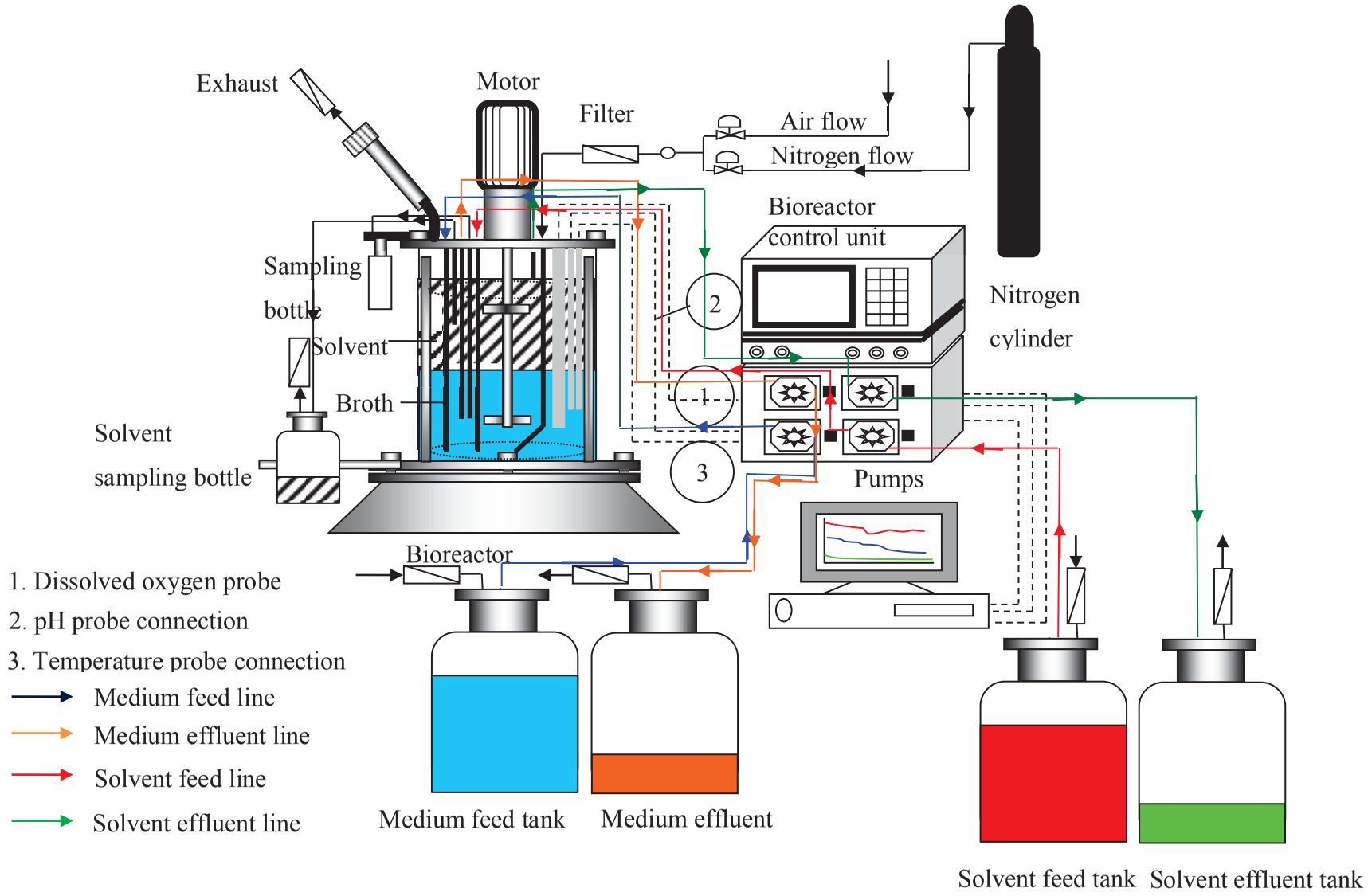


Figure 3.4 Flow scheme of the in situ solvent extractive continuous fermentation up (modified from Sulaiman *et al.*, 2011).

3.16 Adsorption resins

Commercially available adsorption resins (Table 3.2) were experimentally evaluated to assess their ability to take up ethanol from the fermentation broth. Dowex Optipore L-493, Amberlite XAD 16, poly (styrene-co-divinylbenzene) and poly (4–vinylpyridine) were used and had been purchased from Sigma Aldrich (www.sigmaaldrich.com). To allow for a comparison of performance, all calculations were based on the dry weight of the resins. Prior to use, all resins were dried in desiccator at 37 ºC for 72 h to remove any moisture. No further pretreatment was used on the resins as received from the supplier. Resins were sterilized in specified liquid media by autoclaving for 15 min at 121 ºC.

3.16.1 Adsorption equilibria (specific loadings and partition coefficients)

Experiments were performed in sterile 25 mL shake flasks containing 10 mL of an aqueous solution of absolute ethanol with an initial concentration ranging from 10–50 g L^{-1}. An accurately known amount of the solid sorbent was then added to the flasks. The liquid to solid ratio was 10:1 by weight, in all experiments. The resin suspension in the stoppered flasks was equilibrated for 24 h at 35 ºC with agitation at 250 rpm. The ethanol concentration in the medium was measured before the resin was added and after equilibrium had been attained. The specific loading (L) and the partitioning coefficient (K_r) were calculated by the following relationships (Nielsen and Prather, 2009):

$$L = \frac{C_{Aq}\big|_{tf} - C_{Aq}\big|_{t0}}{X_r} \qquad \text{Equation (3.2)}$$

$$K_r = \frac{L}{C_{Aq}\big|_{tf}} \qquad \text{Equation (3.3)}$$

where C_{Aq} is the aqueous phase concentration of ethanol, X_r is the mass of resin per unit volume of the aqueous phase, t_o is the start time, t_f is the time after equilibrium had been attained.

3.16.2 Glucose adsorption isotherm

The equilibrium adsorption of glucose on the resins was also measured. For this, aqueous solutions of glucose with an initial concentration in range of 0 to 200 g L^{-1} were used. Solutions were equilibrated with the specified mass of the resins for 24 h at 35 ºC and 250 rpm agitation in stoppered flasks. The liquid to solid ratio was 10:1 by weight for all experiments.

3.17 Batch solid-sorbent extractive fermentations (shake flasks)

The various adsorption resins were first screened for satisfactory performance in shake flask fermentations. In-situ batch extractive fermentations were carried out in 100 mL shake flasks with a working volume of 50 mL. The mass ratio of the broth to the resins was always 10:1. The control fermentations were conducted without the resin. All resins used had been dried at 37 ºC for 72 h. Fresh medium with an initial glucose concentration of 150 g L^{-1} was dispensed into shake flasks, adsorption resin was added (except for control). They were then sterilized at 121 ºC for 15-min. 5 mL of a vigorously growing *Z. anaerobia* inoculum was added, and the headspace of the flasks was flushed with sterile nitrogen. The flasks were stoppered and incubated at 35 °C, 100 rpm. The initial pH was always 5.5. Asceptic samples (resin free) were taken periodically for measuring the concentrations of the residual glucose, the biomass and ethanol.

3.18 Continuous solid-sorbent extractive fermentations

These were carried out in the aforementioned (Section 3.9) 3-L stirred bioreactor attached to an external fluidized bed adsorption column containing the resins (Figure 3.5). The working volume of the bioreactor was 1-L. The fluidized bed adsorption column had the dimensions shown in Figure 3.6. The broth from the bioreactor was recirculated continuously through the adsorption

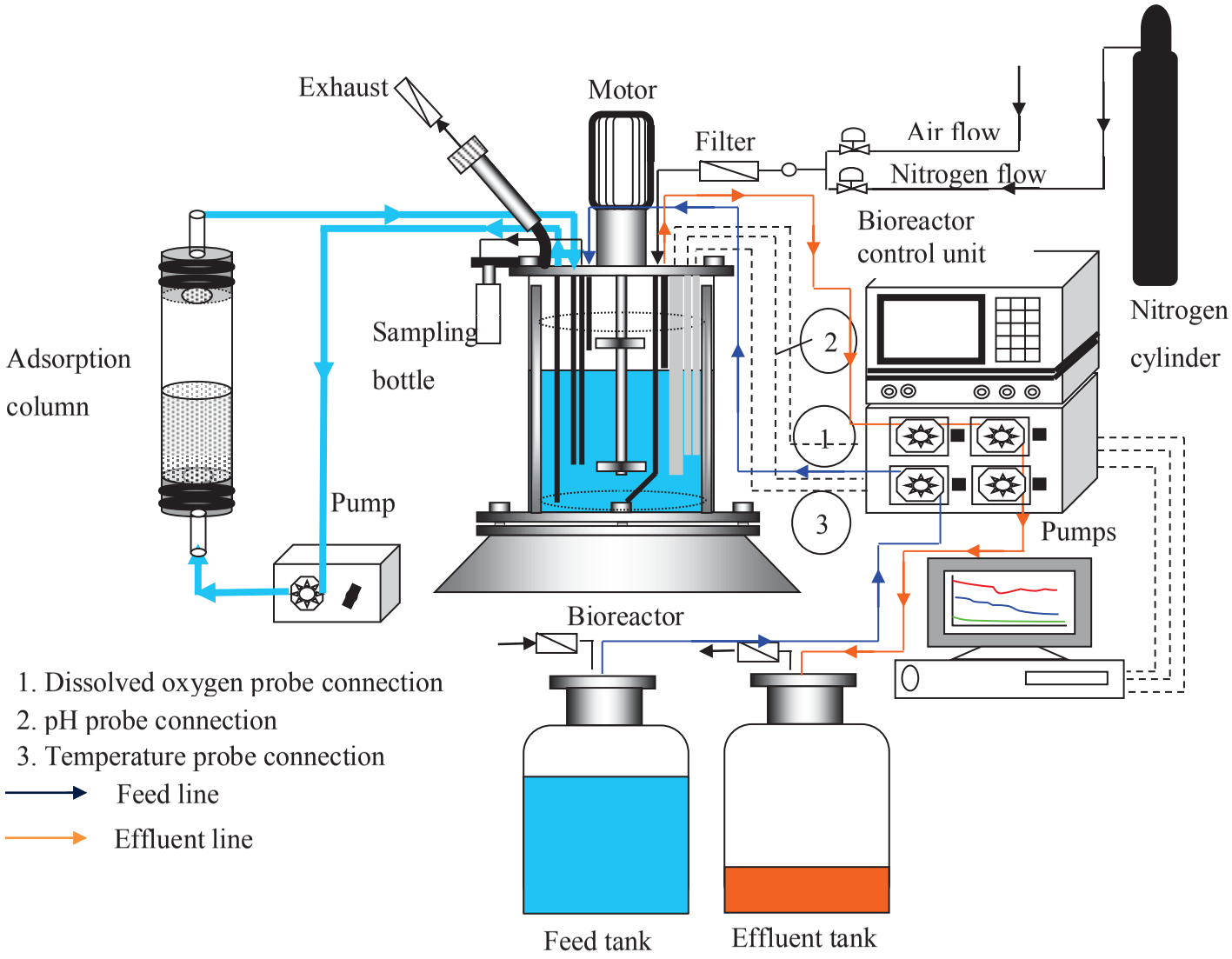


Figure 3.5 Continuous adsorptive fermentation set up (modified from Sulaiman *et al.*, 2011).

column using a peristaltic pump (Masterflex pump controller; model no. 7554-60, supplied by Cole Parmer Instrument Co., Chicago, Illinios 60648). The recirculation flow was fixed at 0.22 L min^{-1}. The recirculation commenced after 10 h of initiation of the fermentation. A specified resin that had been found to be nontoxic to *Z. anaerobia* and was effective in extracting ethanol was used in the adsorption column. 100 g of resin in a fluidized bed adsorption system was used to prevent clogging of the adsorption column with microbial solids. In a fluidized bed, the spacing among the resin particles allows free flow of the fermentation broth (Gailliot *et al.*, 1990).

The adsorption column was made of borosilicate glass and was jacketed for temperature control. The resin was retained in the column by means of wire mesh screen (mesh size 10 = 2.0 mm). The column and the resin were autoclaved (121 °C, 20 min) together, cooled to room temperature, and connected to the bioreactor aseptically using sterile silicone tubing. The fermentation began as a batch, without the broth being recirculated through the adsorption column.

The fermentation was initiated by inoculating the bioreactor with 100 mL of the standardized (Section 3.1) inoculum. The fermenter temperature was controlled at 35.0±0.2 °C and the agitation speed was 150 rpm. The headspace was continuously flushed with sterile nitrogen at a flow rate of 0.3 LPM. After 10 h of a batch fermentation, sterile fresh medium was fed continuously to the top of the bioreactor using sterile silicone tubing and peristaltic pump at a set flow rate. The pH was monitored, but not controlled. The volume in the bioreactor was kept constant by overflow pumping of the harvest broth. The overflow pump ran twice as fast as the feed pump. Dilution rate of 0.05 h^{-1} was used. Samples were taken periodically and analyzed for ethanol, residual glucose, and dry cell weight. Adsorbent particles in the fluidized column were changed every day with an equal amount of fresh sterile adsorbent particles. Used particles were washed and regenerated by heating at 80 °C in rotary evaporator (Heidolph Instruments, Schwabach, Germany; www.heidolph-instruments.com) for 12 h. The regenerated particles were cooled to room temperature before being dried at 37 °C for 72 h and autoclaved (121 ºC, 15 min) for reuse.

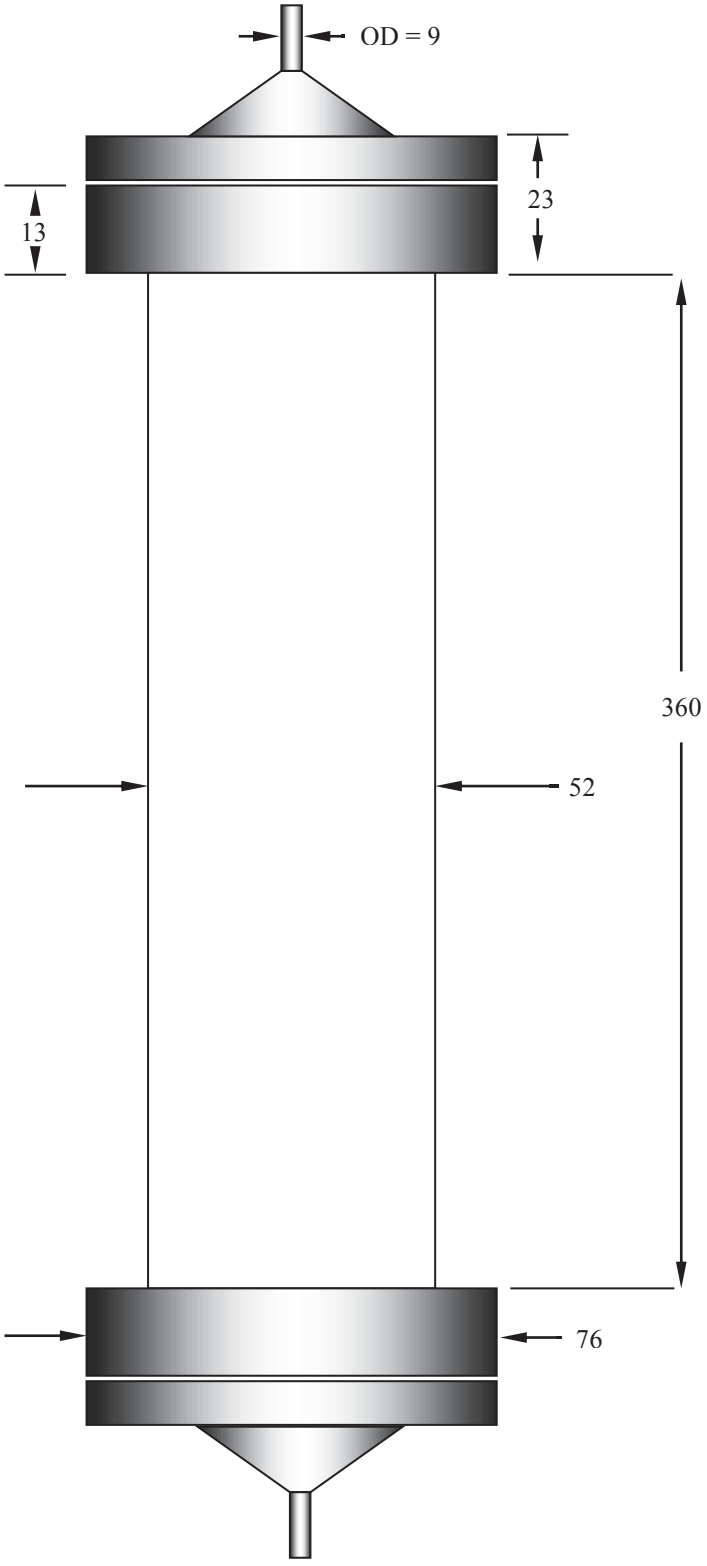


Figure 3.6 The fluidized bed glass column. Dimensions in mm.

3.19 Analytical methods

3.19.1 pH measurements

Routine pH measurements were made using an Orion pH meter (Boston, USA; Model 420A, S/N: 006880; www.thermoscientific.com) which was calibrated with pH 4.0 and 7.0 buffers prior to use.

3.19.2 Preparation of samples

A fermentation broth samples (25 mL) was centrifuged (18000 × g, 15 min) immediately after sampling (Asther and Khan, 1983) using a high speed refrigerated centrifuge (Hitachi CR-22GII refrigerated centrifuge, Hitachi Koki Co., Ltd., Tokyo, Japan) operated at 4°C. The supernatant was decanted and stored at 4°C for ethanol and glucose analyses. These analyses were done within 3 days of sampling.

3.19.3 Glucose concentration

Glucose concentration was estimated by DNS method (Miller, 1959) using glucose as a standard. In the DNS method, 3,5−dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicyclic acid by a reducing sugar such as glucose and the amount of amino-nitrosalicylic acid produced is proportional to the amount of the sugar present. The DNS solution was prepared by dissolving 10 g of 3,5−dinitrosalicylic acid, 2 g phenol, and 0.5 g sodium sulfite in 1 liter of distilled water (Wang, 2007). 3 mL of DNS solution was placed in a test-tube and mixed with 3 mL of the sample. The mixture was heated in a water bath at 90 °C for 15 minutes. The colour obtained was stabilized by adding 1 mL of a 40% solution of Rochelle salt to the mixture. The test tubes were then cooled under running tap water and brought to ambient temperature. The characteristic colour obtained was measured at 575 nm (Ultraspec 2000, model 80-2106-00 spectrophotometer; Pharmacia Biotech Inc., Piscataway, NJ, USA) against a blank. The blank had been prepared exactly as above, except that the sample was replaced by 3 mL of distilled water. The

concentration of glucose was determined by using a calibration curve obtained for a range of precisely known glucose concentrations.

3.19.3.1 Standard calibration curve for glucose

A standard glucose solution was prepared by dissolved 1 g of D(+) anhydrous glucose in 1 L of distilled water. Various volumes of the standard glucose solution were mixed with distilled water to prepare dilutions of known glucose concentration. The total volume of each diluted sample was 3.0 mL.

Glucose in the diluted standard solutions was determined by using the DNS colorimetric method (Section 3.18.3). A graph of absorbance versus the known glucose concentration was plotted (Figure 3.7). Subsequently, the glucose concentration of an unknown sample was estimated from the mean measured absorbance, using the equation of the standard curve:

$$\text{Concentration (g L}^{-1}) = \frac{\text{Absorbance}}{0.0048} \qquad \text{Equation (3.4)}$$

Care was taken to ensure that the sample had an absorbance reading of 0.7 or lower. If the reading was higher than 0.7, the sample was diluted with distilled water and reanalyzed by the DNS method.

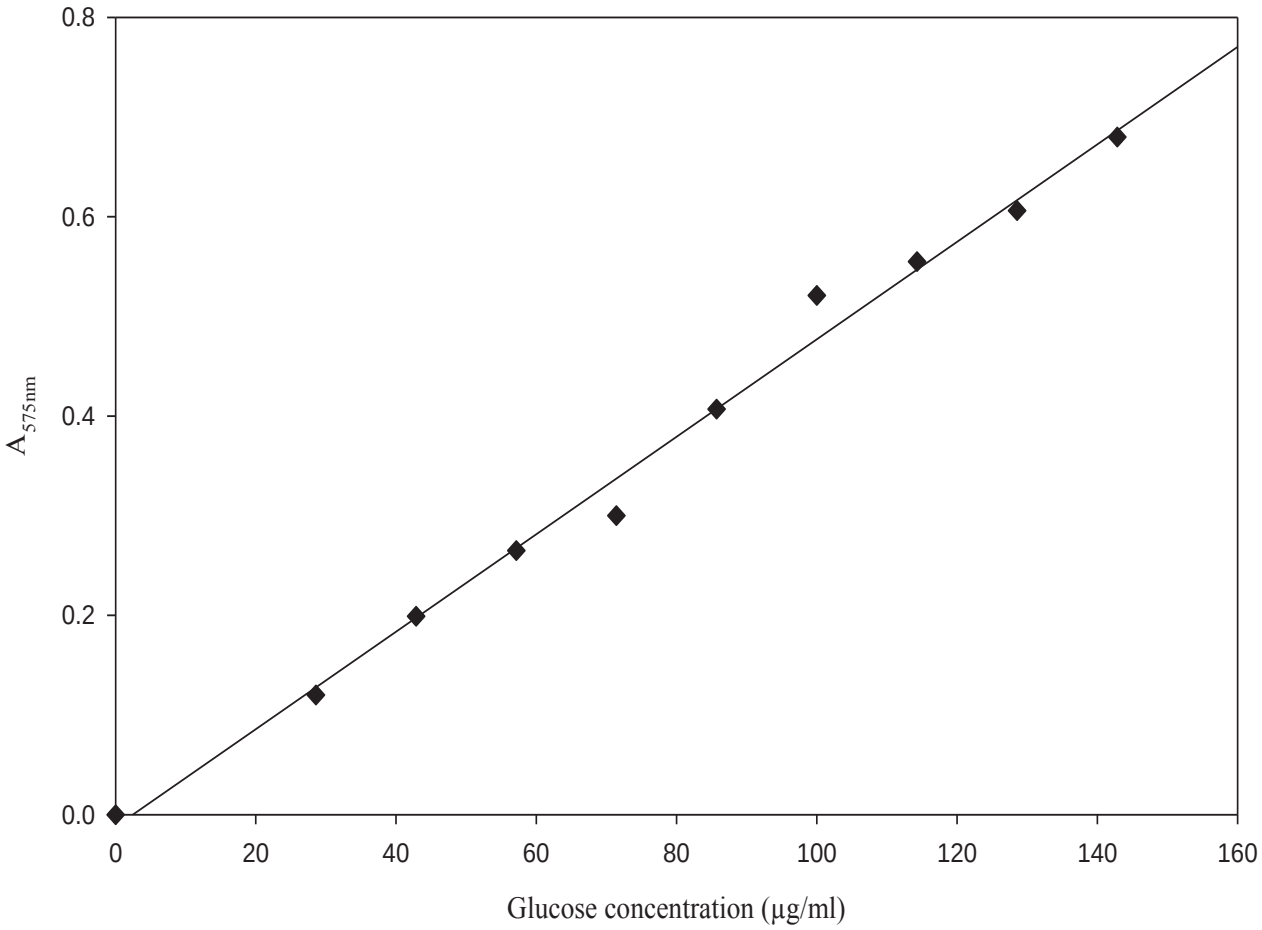


Figure 3.7 The standard curve of glucose

3.19.4 Biomass concentration

The biomass concentration was determined by centrifuging (18000 × g, 15 min) a 25 mL sample of the broth. The supernatant was then decanted off. The pellet was re-suspended in 25 mL of distilled water and then centrifuged as above. The supernatant fluid was again decanted off. The pellet was resuspended again using 25 mL of distilled water. The washed cell suspension was placed in an aluminium dish, weighed, dried overnight (24 hr) at 110 °C in an incubator oven (Contherm Digital Series Five Incubator, Lower Hutt, New Zealand, Cat. No: 245M), and weighed again after cooling to room temperature in a desicator. The dry cell weight (DCW) was calculated as follows:

$$\mathrm{DCW} = \frac{[\mathrm{W_O}] - [\mathrm{W_f}]}{\mathrm{V}} \times \left(\frac{1000\ \mathrm{mL}}{1\ \mathrm{L}}\right) \qquad \text{Equation (3.5)}$$

Where W_o is the weight of aluminium pan and sample before drying (g), W_f is weight of aluminium pan and sample after drying (g) and V is volume (mL) of sample. A calibration curve was prepared for adsorbance (650nm; Ultraspec 2000, model 80-2106-00 spectrophotometer; Pharmacia Biotech Inc., Piscataway, NJ, USA) of the diluted (1:5 dilution) culture broth versus dry cell weight (undiluted) (Figure 3.8) and was linear up to an absorbance of about 1.0. Linear regression of the line in Figure 3.8 was used to establish the following relationship between DCW and spectrophotometric adsorbance:

$$\text{Dry biomass concentration } (\mathrm{g\ L^{-1}}) = \frac{\mathrm{A_{650}}}{5.04\ \times 10^{-1}} \qquad \text{Equation (3.6)}$$

The regression coefficient for the above equation was 0.999.

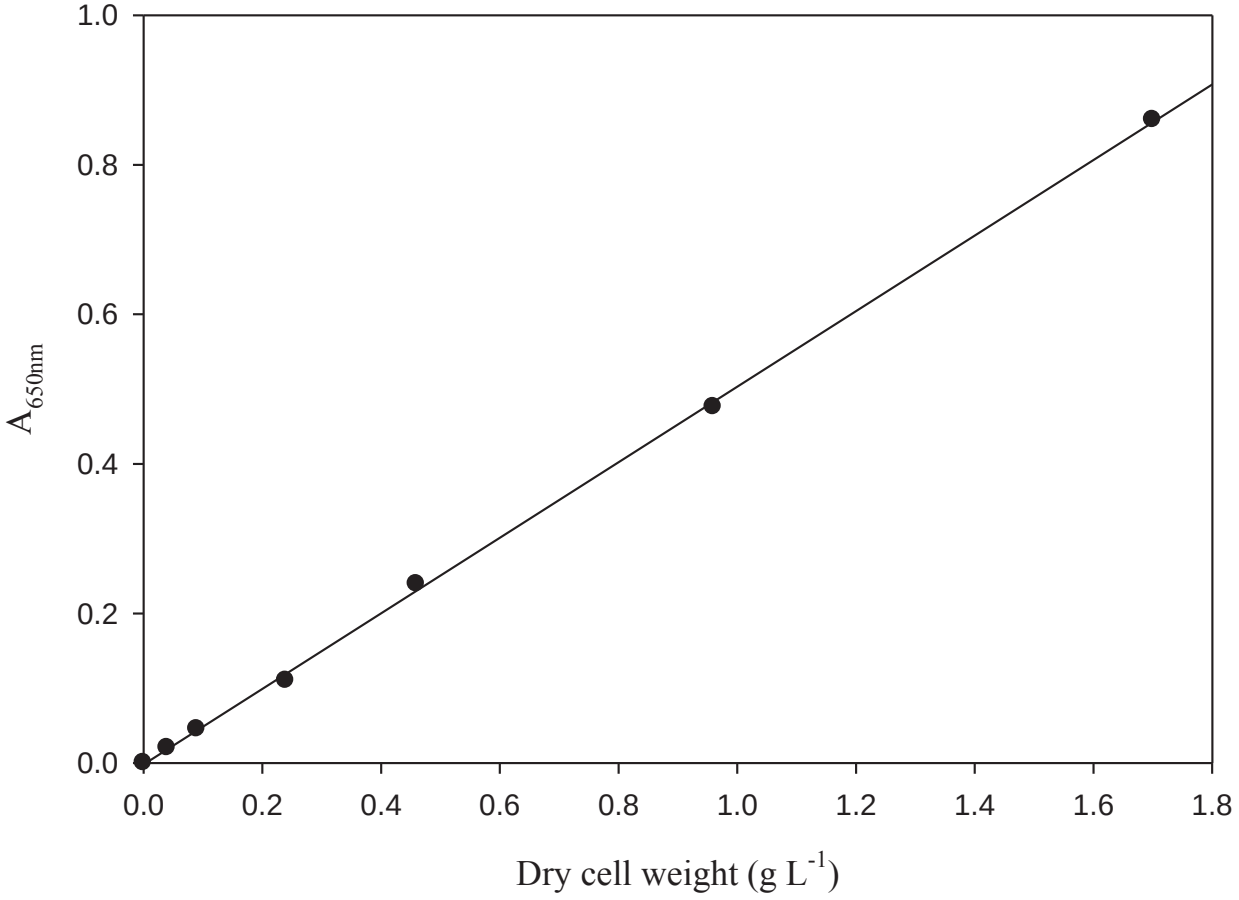


Figure 3.8 Calibration curve for A_{650nm} versus dry cell weight.

3.19.5 Ethanol concentration

Ethanol concentration in the broth supernatant and other samples was analyzed using gas chromatography (model GC 6000 Vega Series 2; Carlo Erba Instruments, Milan, Italy) fitted with a flame ionization detector and chromato-integrator (model D-2500; Hitachi, Tokyo, Japan). The column oven operated isothermally at 180 °C. The flame ionization detector and injection ports were kept at 200 °C. The nitrogen was used as carrier gas at flow rate of 40 mL min^{-1} and combustion gases were a mixture hydrogen and air. The standard calibration curve for ethanol had been made just before sample was injected into GC. Standard ethanol solutions were prepared in the concentration range of 20 to 100 g L^{-1} by diluting absolute ethanol with

deionized water. Before the injection, the sample had been prefiltered through a 0.45 μm membrane filter. 2 μL of the sample volume was injected into the GC. The concentration of the ethanol in the sample was calculated by comparing the relative area under ethanol peak with the standard curve data from solutions of known ethanol concentrations. Duplicate samples were used in all measurements.

3.19.6 Cell viability

Cell viability was measured by plate counts. Appropriate serial dilutions of the broth were prepared in the culture medium and known volumes were plated on maintenance agar plates. The agar medium had been prepared as described in Section 3.1. The plates were incubated at 35°C for 48 h. The number of colonies produced was counted using a Colony Counter (Suntex Instruments. Co Ltd, Taipei, Taiwan, Model 560; www.suntex.com.tw). The number of colony forming units (CFUs) per milliliter (mL) of samples were calculated as follows:

$$\text{CFUs per mL} = \text{number of colonies} \times \text{dilution factor}$$

3.19.7 Fermentation kinetics

The equations used in calculating the various fermentation kinetic parameters (Doran, 1995) were as follows:

Specific growth rate, μ:

$$\mu = \frac{1}{(t_2 - t_1)} \ln\left(\frac{X_2}{X_1}\right) \qquad \text{Equation (3.7)}$$

where X_1 is the biomass concentration at time t_1 and X_2 is the biomass concentration at time t_2 during exponential growth (t_1 and t_2 depended on an initial concentration of glucose).
Average specific glucose consumption rate, q_s:

$$q_s = -\frac{\Delta S}{\Delta X_t} \qquad \text{Equation (3.8)}$$

where ΔS is the glucose consumed by time t and ΔX is the increase in biomass concentration by time t.

Maximum biomass yield on substrate, $Y_{X/S}$:

$$Y_{X/S} = -\frac{\Delta X}{\Delta S} \qquad \text{Equation (3.9)}$$

where $Y_{X/S}$ was calculated at the maximum biomass concentration X_{max}.

Maximum biomass productivity, P_x:

$$P_x = \frac{X_{max} - X_0}{t} \qquad \text{Equation (3.10)}$$

where P_x was calculated at time t corresponding to the maximum biomass concentration X_{max} in the broth. X_o was the initial biomass concentration for the fermentation.

Final ethanol yield on substrate, $Y_{P/S}$

$$Y_{P/S} = -\frac{\Delta P}{\Delta S} \qquad \text{Equation (3.11)}$$

where ΔP was the increase in ethanol concentration during the fermentation.

Final ethanol productivity, P_E :

$$P_E = \frac{E_f - E_o}{t_f} \qquad \text{Equation (3.12)}$$

where E_0 was the initial concentration of ethanol, E_f was the final concentration of ethanol and t_f was the length of the fermentation.

Average specific ethanol production rate, q_p:

$$q_p = \frac{\Delta E}{\Delta X_{max} t} \qquad \text{Equation (3.13)}$$

where q_p was calculated at time t corresponding to the maximum biomass concentration. ΔE was the increase in ethanol concentration by time t.

The kinetics parameters of the continuous extractive fermentations were calculated as follows (Doran, 1995):

Steady state biomass yield on substrate, $Y_{X/S}$:

$$Y_{X/S} = -\frac{X}{\Delta S} \qquad \text{Equation (3.14)}$$

where $Y_{X/S}$ was calculated at the steady state condition of average biomass concentration X and $\Delta S = S_0 - S$ where S_0 was the substrate concentration in the feed and S was the steady state substrate concentration in the bioreactor.

Steady state biomass productivity, P_x:

$$P_X = \frac{X Q_F}{V_F} \qquad \text{Equation (3.15)}$$

where P_x is calculated at steady state biomass concentration X in the fermentation. Q_F was medium flowrate (mL/h) and V_F was medium volume (mL) in the bioreactor.

Steady state ethanol yield on substrate, $Y_{P/S}$

$$Y_{P/S} = \frac{(E_A Q_F) + (E_S Q_S)}{(\Delta S Q_F)} \qquad \text{Equation (3.16)}$$

where E_A and E_S are the steady state ethanol concentrations in the aqueous phase and the solvent respectively. Q_F and Q_S are the medium flowrate and the solvent flowrate (mL/h) respectively. $\Delta S = S_f - S$, where S_f is the substrate concentration in the feed and S is the steady state substrate concentration in the aqueous phase of the bioreactor.

Steady state ethanol productivity in the aqueous phase, P_{EA}:

$$P_{EA} = \frac{E_A Q_F}{V_F} \qquad \text{Equation (3.17)}$$

where E_A is the steady state ethanol concentration in the aqueous phase, Q_F is the medium flowrate (mL/h) and V_F is the volume of medium in the bioreactor.

Steady state ethanol productivity in the solvent, P_{ES}:

$$P_{ES} = \frac{E_S Q_S}{V_S} \qquad \text{Equation (3.18)}$$

where E_S is the steady state ethanol concentration in the solvent, Q_S is the solvent flowrate (mL/h) and V_S is the volume of solvent in the bioreactor.

Total ethanol productivity, P_{ET}:

$$P_{ET} = \frac{E_A Q_F}{V_F} + \frac{E_S Q_S}{V_s} \qquad \text{Equation (3.19)}$$

where E_A and E_S are the steady state ethanol concentrations in the aqueous phase and the solvent respectively. Q_F and Q_S are the steady state medium flowrate and the solvent flowrate (mL/h), respectively. V_F and V_S are the volumes of the medium and the solvent in the bioreactor, respectively.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Fermentation characterization

This chapter deals with a characterization of batch and continuous fermentations of *Z. anaerobia* with a view to identifying the best fermentation conditions for a detailed study. In-situ extractive fermentations (liquid-liquid and solid-liquid extraction) are characterized. Toxicity of extraction solvents and resins is assessed for identifying those that are biocompatible with *Z. anaerobia* and are able to extract the ethanol satisfactorily. The oscillation behavior of continuous steady state fermentations is characterized in detail to identify conditions that cause it.

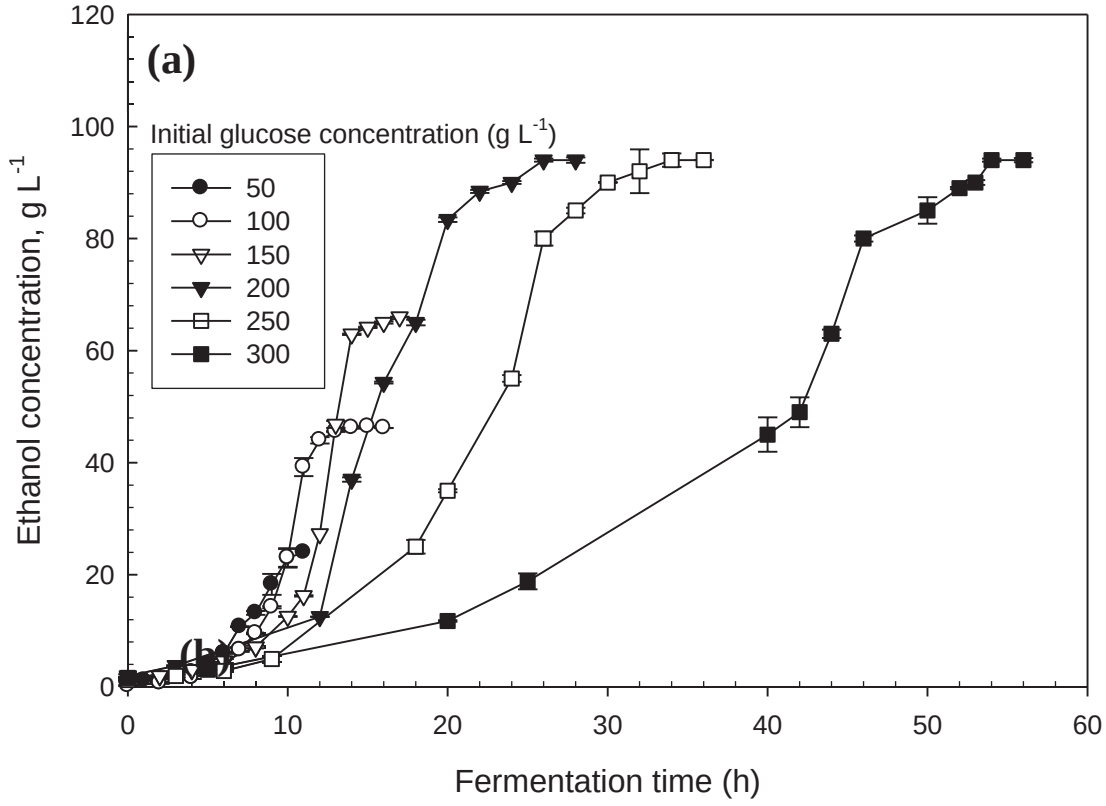
4.1.1 Effect of high glucose concentrations on fermentation

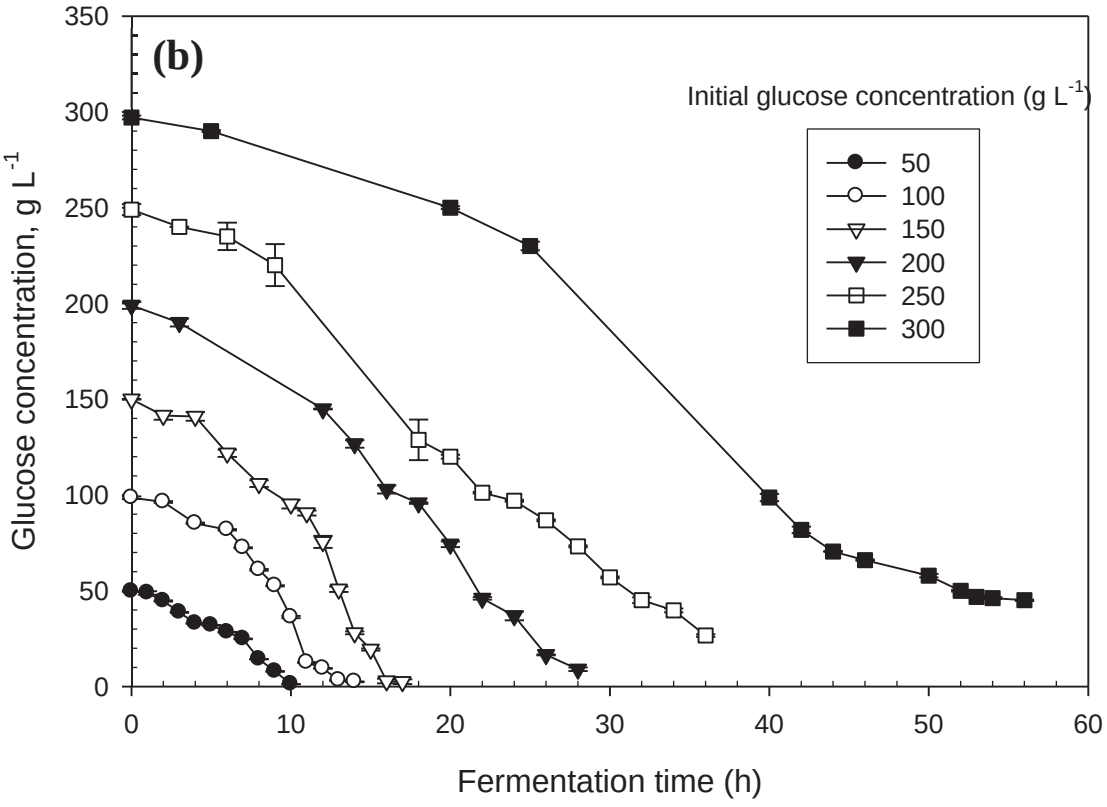
Zymomonas anaerobia was grown in the glucose medium (Section 3.2) in batch culture in shake flasks (Section 3.2) at 30 °C. The initial glucose concentration in the medium varied in different flasks from 50 g L^{-1} to 300 g L^{-1}. The effects of initial glucose concentration on production of ethanol, concentration of glucose and cell growth are shown in Figure 4.1a, Figure 4.1b and 4.1c, respectively. Based on the figures, the maximum attainable ethanol concentration in the broth increased from 24 ± 1.7 g L^{-1} to 94 ± 0.4 g L^{-1} with an increase of initial glucose concentration from 50 g L^{-1} to 200 g L^{-1}. A further increase in the initial glucose concentration up to 300 g L^{-1} did not increase the final concentration of ethanol (Figure 4.1a). Rate of ethanol production during exponential growth generally increased with an increase in initial concentration of glucose from 50 to 150 g L^{-1}. Increase in initial glucose concentration to ≥ 150 g L^{-1} actually decreased the rate of ethanol production (Figure 4.1a) and the rate of glucose utilization (Figure 4.1b).

Once the initial glucose concentration exceeded 150 g L^{-1}, glucose was not completely consumed by 30 h (Figure 4.1b). An increase in initial glucose concentration to ≥ 150 g L^{-1} actually slowed biomass growth (Figure 4.1c). In this fermentation, too much glucose clearly limits metabolic activity (Kesava *et al.,* 1995). The inhibitory effect of glucose is related principally to its osmotic effect (Ozmihci and Kargi, 2007). As glucose concentration increases, the water activity in the medium decreases. At high substrate concentrations, the decreased water activity and the onset of plasmolysis combine to decrease the rate of fermentation and ethanol production (Jones and Greenfield, 1986).

The kinetic parameters of the batch fermentation (Figure 4.1a–c) are shown in Table 4.1. The kinetic parameters were calculated according to Doran (1995) and Shuler and Kargi (2006). These results (Figure 4.1a–c, Table 4.1) are consistent with the data reported by Rogers (1979) on *Zymomonas mobilis* ZM4 and Kosaric *et al.* (1982) on *Zymomonas anaerobia* ATCC 29501 as used in this work. Based on Table 4.1, the maximum value of ethanol yield on substrate, the highest average biomass specific ethanol production rate, all occured at an initial glucose concentration of 150 g L^{-1}. At this initial glucose concentration, an ethanol concentration of 66 ± 0.2 g L^{-1} was obtained by 16 h of fermentation (Figure 4.1a). Based on this experimental data (Figures 4.1a–c, Table 4.1), an initial glucose concentration of 150 g L^{-1} was clearly the optimal value for a batch fermentation for maximum ethanol productivity, yield and final concentration. Initial glucose concentration values of ≥ 150 g L^{-1} adversely affected the growth rate and ethanol production (Figure 4.1).

The error bars in Figure 4.1 demonstrate an excellent reproducibility of the fermentations. The error bars are based on three parallel fermentations at any given set of condition. Figure 4.2 based on data shown in Table 4.1, clearly shows that an increasing concentration of glucose in the entire range studied actually reduced the specific growth rate of the bacterium, but the peak productivity of ethanol occurred at a glucose concentration of around 150 g L^{-1} and not under conditions of maximum growth rate. In view of the results, the optimum initial concentration of glucose of 150 g L^{-1} was selected for use in all future experiments.


(a)
Initial glucose concentration (g L-1)
50
100
150
200
250
300
Ethanol concentration, g L-1
Fermentation time (h)
0
20
40
60
80
100
120
0
10
20
30
40
50
60


(b)
Initial glucose concentration (g L-1)
50
100
150
200
250
300
Glucose concentration, g L-1
Fermentation time (h)
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60

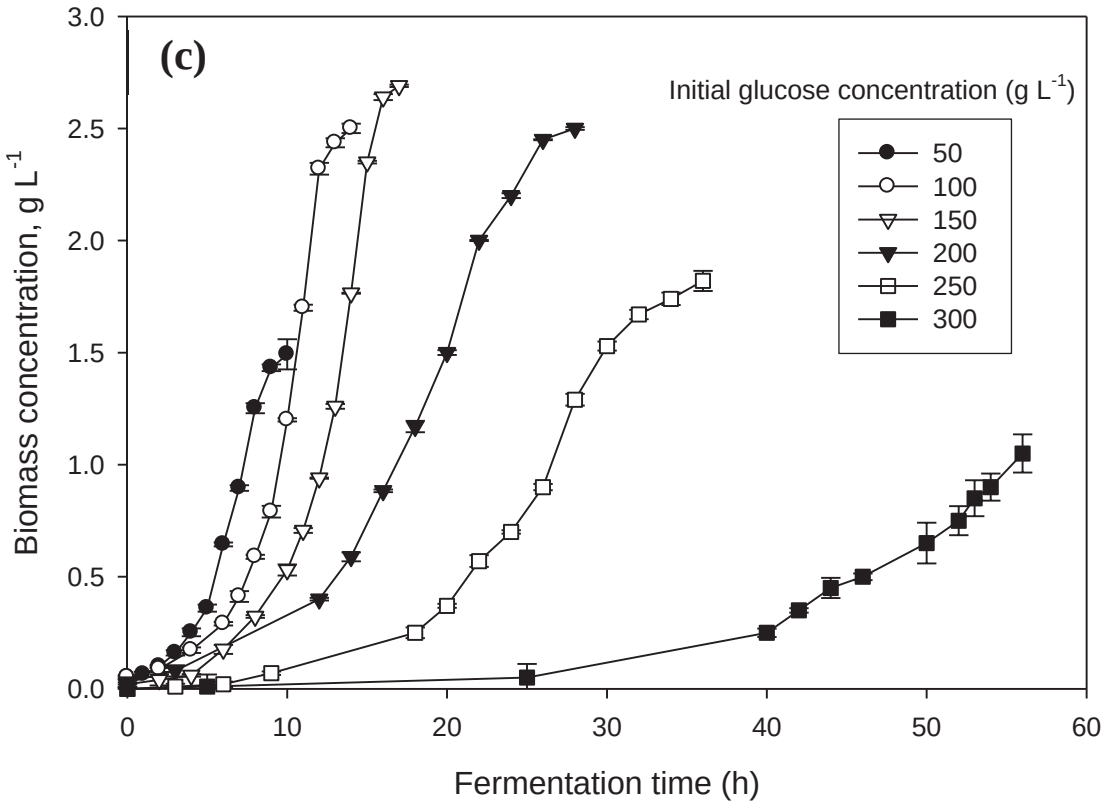


Figure 4.1 The effects of initial glucose concentrations on (a) ethanol production; (b) glucose utilization and (c) biomass growth. Each curve in the above graphs is based on three replicate fermentations. Each measurement in each fermentation was made in triplicate. Therefore, nine measurements were used to calculate the averages and standard deviations.

Table 4.1 Kinetic parameters of *Z. anaerobia* in batch culture

Parameter	Initial glucose concentration (g L^{-1})					
	50	100	150	200	250	300
Maximum biomass productivity, P_x (g L^{-1} h^{-1})	0.149 ± 0.007	0.142 ± 0.001	0.131 ± 0.001	0.089 ± 0.000	0.050 ± 0.001	0.018 ± 0.002
Maximum biomass yield on glucose, $Y_{x/s}$ (g g^{-1})	0.031 ± 0.000	0.021 ± 0.000	0.015 ± 0.001	0.013 ± 0.000	0.008 ± 0.000	0.004 ± 0.000
Maximum ethanol productivity, P_E (g L^{-1} h^{-1})	2.400 ± 0.010	3.580 ± 0.048	4.067 ± 0.008	3.860 ± 0.012	3.270 ± 0.048	1.740 ± 0.012
Maximum ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.492 ± 0.004	0.486 ± 0.005	0.463 ± 0.005	0.459 ± 0.008	0.450 ± 0.007	0.342 ± 0.002
Maximum specific growth rate, μ (h^{-1})	0.401 ± 0.018	0.333 ± 0.005	0.281 ± 0.010	0.227 ± 0.015	0.183 ± 0.010	0.132 ± 0.016
Average specific glucose uptake rate, q_s (g g^{-1} h^{-1})	3.260 ± 0.148	3.480 ± 0.046	3.870 ± 0.038	3.200 ± 0.031	2.270 ± 0.096	1.330 ± 0.35
Average biomass specific ethanol production rate, q_p (g g^{-1} h^{-1})	1.540 ± 0.135	1.580 ± 0.032	1.630 ± 0.010	1.590 ± 0.008	1.430 ± 0.052	1.340 ± 0.134

All data are based on three replicate fermentations.

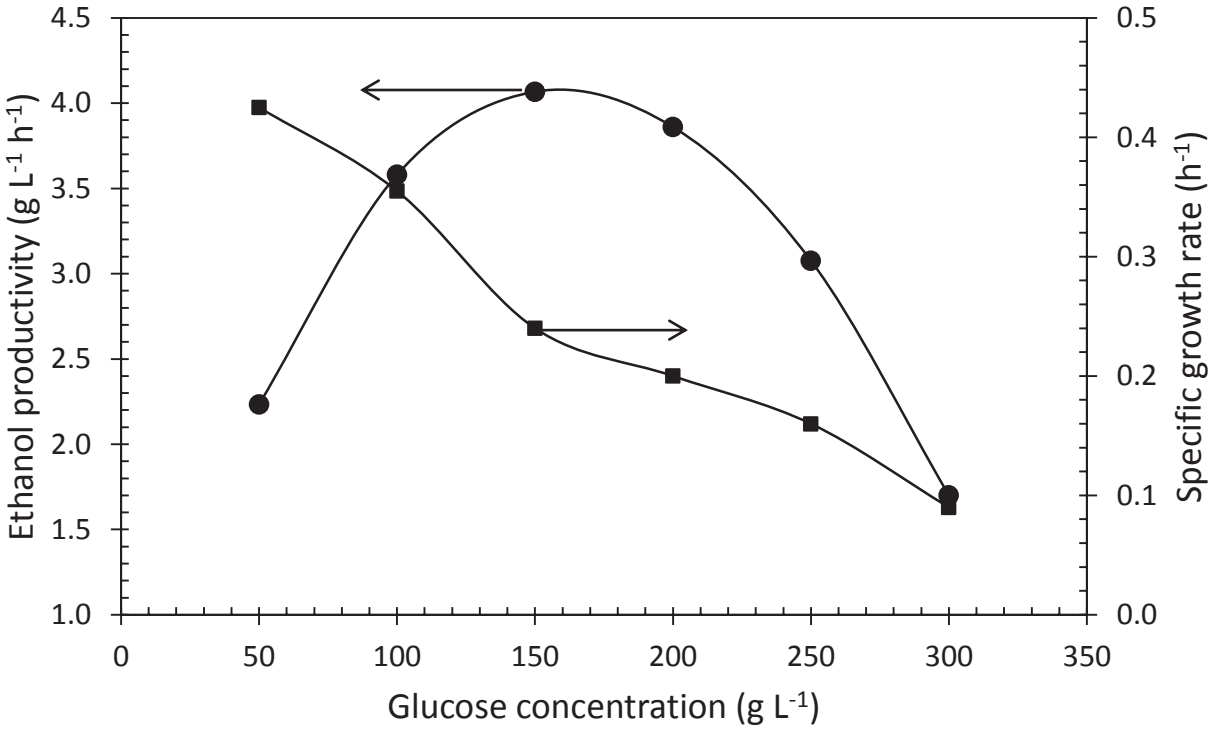


Figure 4.2 Effect of initial glucose concentration on ethanol productivity and specific growth rate in batch fermentations. Data from Table 4.1.

4.1.2 Inhibition by ethanol

To establish ethanol tolerance of *Z. anaerobia*, the batch fermentation was characterized with ethanol added at various levels. This is discussed in this section. Ethanol produced by a microorganism during fermentation has been reported to be much more toxic to it than ethanol added to the culture (Brown *et al.,* 1981; Dasari *et al.,* 1990; Luong, 1984; Roels and Jobses, 1985). This is because cellular metabolic machinery is exposed to ethanol produced within the cell and then excreted. In contrast, externally added ethanol appears not to be transported into the cell to a major extent, as the net flow of ethanol is from the inside to the outside. The toxic effect

of ethanol on microorganisms is associated with its damaging effect on cellular membranes. A high concentration of ethanol alters membrane properties and retards transport through membranes (Ingram, 1975; Ingram and Osman, 1985; Ingram, 1986; Hobley and Pamment, 1994).

Separate experiments were conducted with ethanol added at the start of the fermentation (i.e at 0 h) and near mid way point (10 h) of the fermentation. The results are shown in Figure 4.3 and Figure 4.4. All fermentations used an initial glucose concentration of 150 g L^{-1}. The culture temperature was always at 30 °C. From the data in Figure 4.3, control culture (no added ethanol) attained a final ethanol concentration of nearly 60 g L^{-1} (6% vol/vol), but all fermentations supplemented with ethanol at the beginning of the fermentation failed to produce much ethanol. For example, the fermentation with an ethanol supplementation at 2% (vol/vol) (20 g L^{-1} initial ethanol concentration) attained a final ethanol concentration of only about 35 g L^{-1} (Figure 4.3). Ethanol supplementation reduced both the glucose consumption rate and the biomass growth rate (Figure 4.4). Clearly, an ethanol concentration of 2% vol/vol was strongly inhibitory if supplemented at the start of the fermentation. Ethanol supplementation reduced the specific growth rate of biomass in a concentration dependent manner as shown in Figure 4.5. Ethanol inhibited growth likely because of its inhibitory effect on glycolytic enzymes such as alcohol dehydrogenase and pyruvate decarboxylase as is known to occur in the yeast *Saccharomyces cerevisiae* (Huang and Chen, 1987; Ingram, 1975; Ingram and Buttke, 1984; Novak, 1981; Hobley and Pamment, 1994). The effects of added ethanol on fermentation kinetics are summarized quantitatively in Table 4.2 based on the data discussed above (Figure 4.3–4.5).

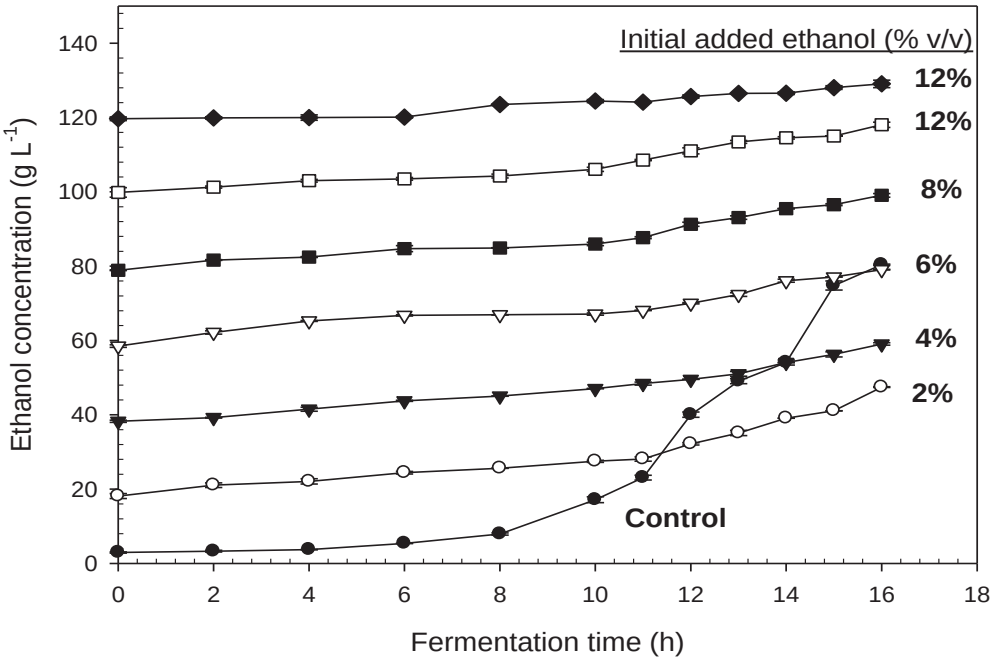


Figure 4.3 Effect of ethanol on its production. No ethanol was added to control cultures. Each curve is based on three replicate fermentations.

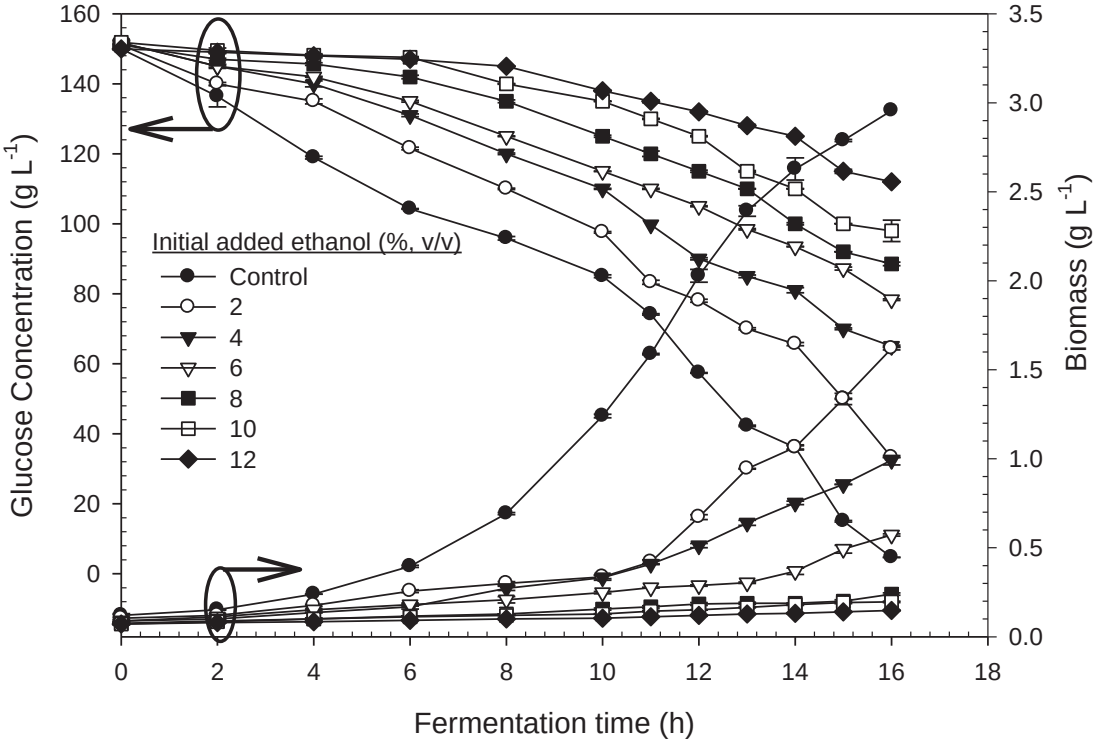


Figure 4.4 Effect of ethanol on glucose consumption and biomass production. No ethanol was added to control cultures. Each curve is based on three replicate fermentations.

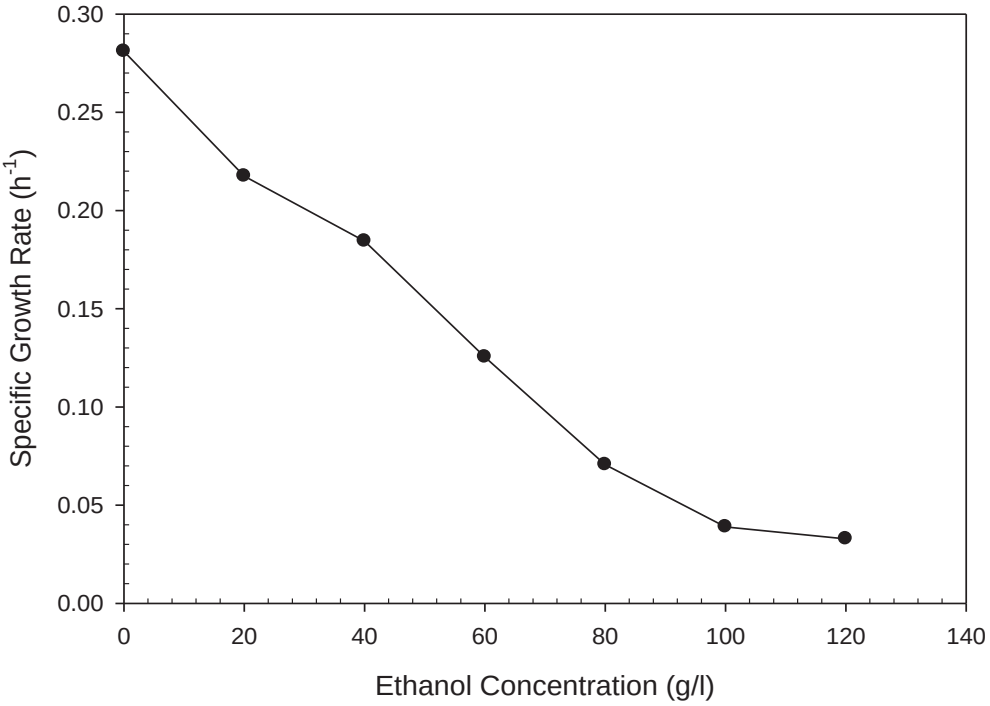


Figure 4.5 Correlation between specific growth rate and the added ethanol concentration. Data from Table 4.2.

Table 4.2 Effect of added ethanol on batch fermentation kinetics[a]

Kinetic parameter	Ethanol Concentration (g L^{-1})						
	Control	**20**	**40**	**60**	**80**	**100**	**120**
Maximum biomass productivity, P_x (g L^{-1} h^{-1})	0.131 ± 0.001	0.090 ± 0.002	0.056 ± 0.001	0.029 ± 0.005	0.010 ± 0.000	0.007 ± 0.000	0.004 ± 0.000
Maximum biomass yield on glucose, $Y_{x/s}$ (g g^{-1})	0.015 ± 0.000	0.013 ± 0.000	0.011 ± 0.000	0.007 ± 0.000	0.003 ± 0.000	0.002 ± 0.000	0.001 ± 0.000
Maximum ethanol productivity, P_E (g L^{-1} h^{-1})	4.067 ± 0.008	1.832 ± 0.042	1.300 ± 0.027	1.288 ± 0.022	1.186± 0.032	1.139 ± 0.080	0.586 ± 0.069
Maximum ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.463 ± 0.005	0.251 ± 0.004	0.245 ± 0.002	0.287 ± 0.002	0.325± 0.003	0.307 ± 0.005	0.247 ± 0.004
Maximum specific growth rate, μ (h^{-1})	0.281 ± 0.010	0.217 ± 0.019	0.181 ± 0.016	0.121 ± 0.010	0.071 ± 0.007	0.039 ± 0.000	0.033 ± 0.000

[a]Ethanol was added at the start of the fermentation. No ethanol was added to control fermentations. All data are based on three replicate fermentations.

Figure 4.6 shows the fermentation profiles for ethanol added at 10 h. An increased ethanol concentration had a measurable adverse impact on cell growth compared to the control culture. Growth inhibition effect of ethanol was concentration dependent (Figure 4.6)

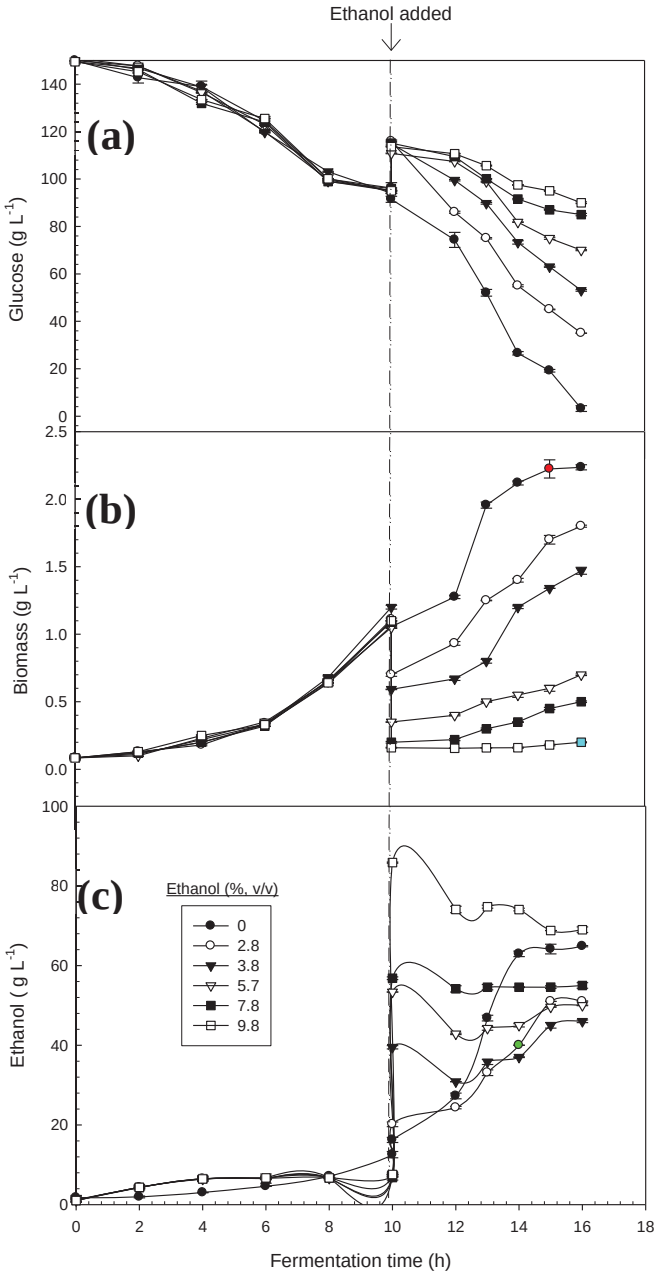


Figure 4.6 Inhibition by ethanol added at 10 hour of fermentation: (a) glucose utilization; (b) biomass concentration; and (c) ethanol concentration. Three replicate fermentations were performed for each concentration of initial added ethanol.

4.1.3 Effect of temperature on *Z. anaerobia*

Batch fermentation profiles of *Z. anaerobia* were sensitive to temperature as shown in Figure 4.7-4.9 for the temperature range of 30 to 40 °C. The effect of temperature on ethanol productivity is shown in Figure 4.10. All cultures had an initial glucose concentration of 150 g L^{-1}. The kinetics parameters of the fermentations (Figure 4.7-4.9) are shown in Table 4.3.

A close inspection of Figure 4.7 clearly shows a temperature of 35 °C to be optimal for growth. Lower temperature reduced growth rate but not the final biomass concentration (Figure 4.7). Temperature of ≥ 38 °C suppressed growth. Barely any growth was recorded at 40 °C. Glucose concentration data (Figure 4.8) were fully consistent with the growth profiles (Figure 4.7). Glucose was most rapidly consumed at 35 °C, the temperature that afforded the most rapid growth. The optimal temperature for growth was also optimal for ethanol production (Figure 4.9). These results are consistent with an earlier report (Kosaric *et al.,* 1982) on *Z. anaerobia* fermentation. Other reports have focused on other Zymomonas species (Bajpai and Margaritis, 1986b; Fieschko and Humphrey, 1983; Laudrin and Goma, 1982; Lee *et al.,* 1981a; Spangler and Emert, 1985).

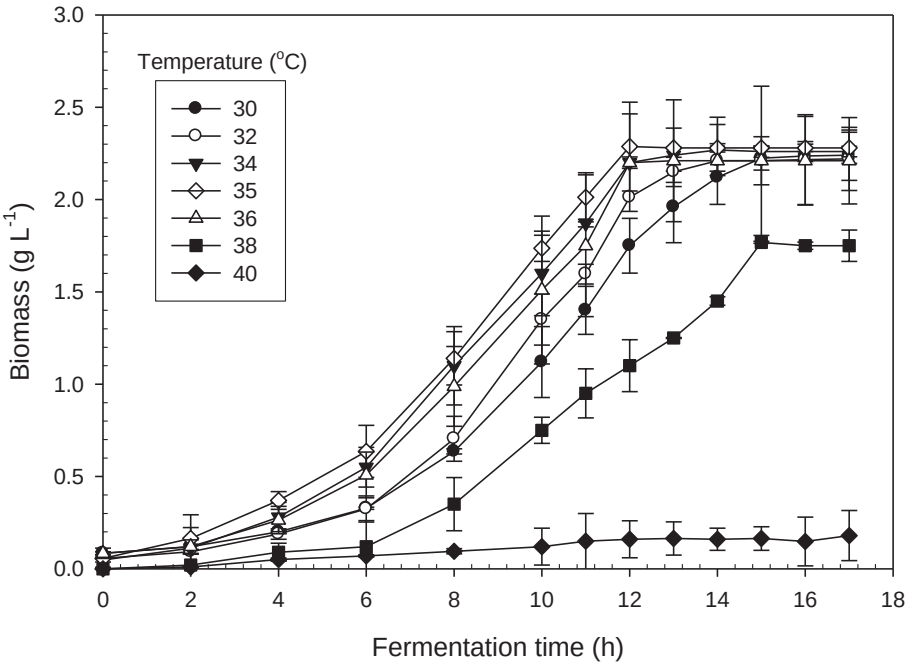


Figure 4.7 Biomass production profiles at various temperatures. Data are averaged values from three replicate fermentations at each temperature.

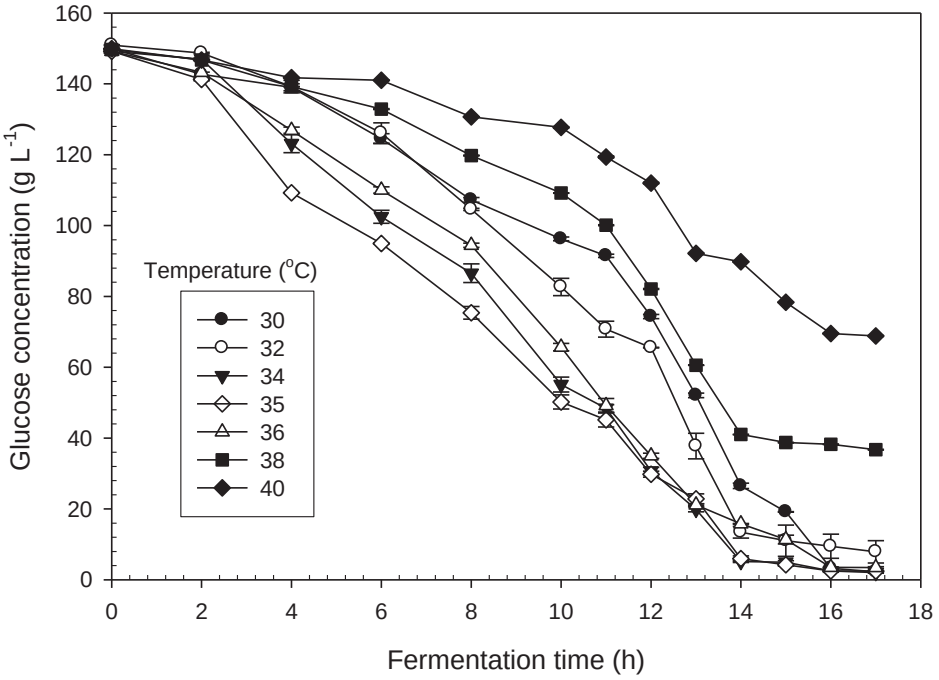


Figure 4.8 Glucose consumption profiles at various temperatures. Data are averaged values from three replicate fermentations at each temperature.

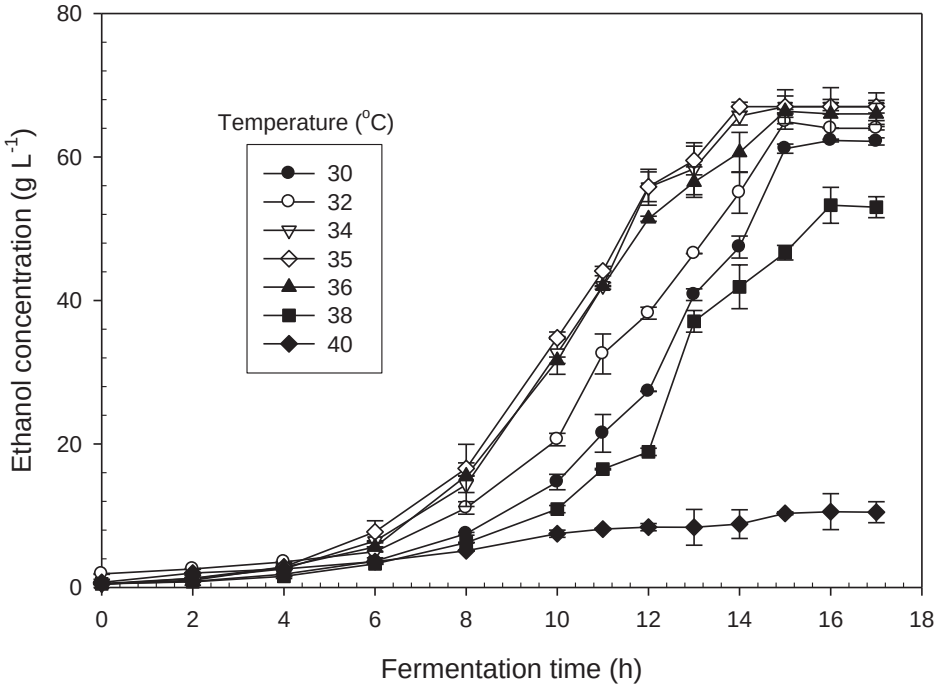


Figure 4.9 Ethanol production profiles at various temperatures. Data are averaged values from three replicate fermentations at each temperature.

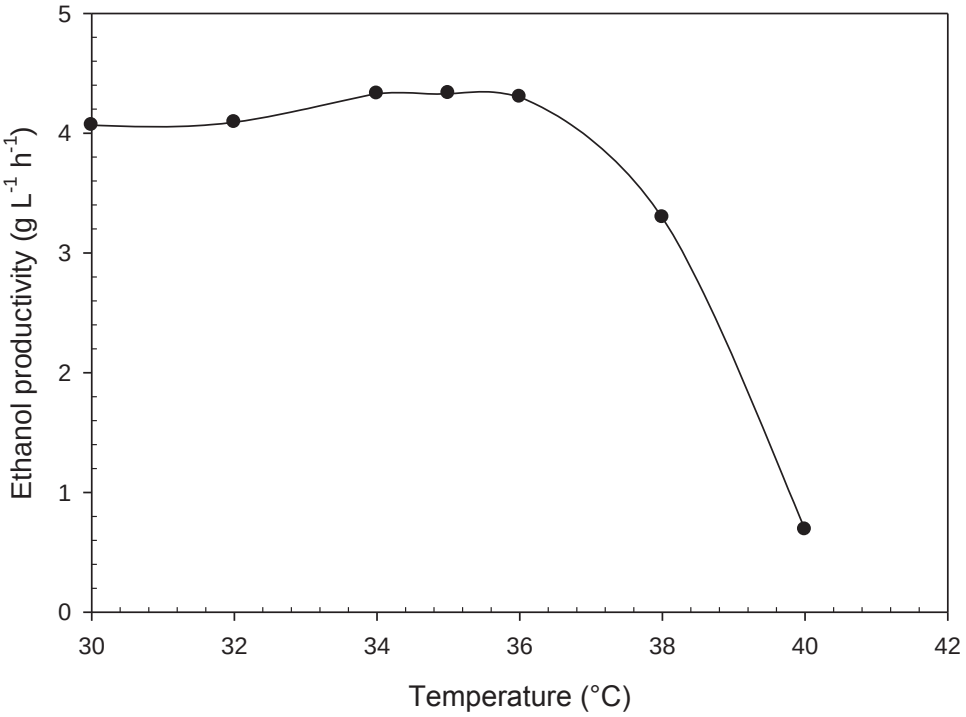


Figure 4.10 Effect of temperature on ethanol productivity in batch fermentation. Data from Table 4.3.

As shown in Table 4.3, values of all the main kinetic parameters of the fermentation were maximum at the optimal temperature of 35 °C.

Table 4.3 Effect temperature on batch fermentation kinetics

Kinetic parameter	Temperature (ºC)						
	30	**32**	**34**	**35**	**36**	**38**	**40**
Maximum biomass productivity, P_x (g L^{-1} h^{-1})	0.131 ± 0.001	0.135 ± 0.015	0.141 ± 0.002	0.142 ± 0.004	0.138± 0.016	0.100± 0.001	0.011± 0.008
Maximum biomass yield on glucose, $Y_{x/s}$ (g g^{-1})	0.015 ± 0.000	0.015 ± 0.000	0.015 ± 0.001	0.015 ± 0.001	0.015 ± 0.000	0.014 ± 0.000	0.002 ± 0.000
Maximum ethanol productivity, P_E (g L^{-1} h^{-1})	4.067 ± 0.008	4.091 ± 0.015	4.330 ± 0.015	4.333 ± 0.099	4.302 ± 0.016	3.297 ± 0.068	0.689 ± 0.014
Maximum ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.463 ± 0.005	0.464 ± 0.014	0.465 ± 0.016	0.468 ± 0.005	0.467 ± 0.004	0.418 ± 0.012	0.127 ± 0.018
Maximum specific growth rate, μ (h^{-1})	0.281 ± 0.010	0.285 ± 0.009	0.298 ± 0.006	0.304 ± 0.011	0.281 ± 0.003	0.234 ± 0.010	0.085 ± 0.020

Based on measurements from three replicate fermentations.

4.1.4 Impact of dilution rate on oscillations in continuous ethanol fermentations

Six different dilution rates (D = 0.05 – 0.30 h^{-1}) were used in a continuous steady-state fermentation. The experiment was conducted at a feed glucose concentration of 150 g L^{-1} at 35 °C. The continuous feed operation started after 10 h of a batch fermentation. The concentration profiles of the biomass, ethanol and glucose in the bioreactor are shown in Figure 4.11, Figure 4.12 and Figure 4.13, respectively.

At the lowest dilution rate, D = 0.05 h^{-1}, a steady state could not be established within 475 h (~20 days), or 20 residence times, of operation (Figure 4.11 - 4.13). Normally, in a well-mixed continuous culture a steady state is established within 4 residence times of operation. The fluctuation ranges of the biomass, residual glucose and ethanol were observed to be 0.83 – 1.306 g L^{-1}, 28.45 – 17.74 g L^{-1} and 28.12 – 52.12 g L^{-1}, respectively. The corresponding absolute fluctuation amplitudes were 0.48 g L^{-1}, 10.71 g L^{-1} and 24 g L^{-1}, respectively. The average of biomass, residual glucose and ethanol concentration at this dilution rate were 1.07 g L^{-1}, 23.1 g L^{-1} and 40.12 g L^{-1}, respectively. The oscillation period was observed to be around 71 hours. These periodic oscillations and the failure to establish a steady-state despite a prolonged operation at otherwise fixed conditions, suggest an underlying phenomenon that prevented a steady-state from being achieved. These results were consistent with similar ones as reported by Jarzebski (1992). Based on his study, strong oscillations take place when the feed substrate concentration is sufficiently high and the dilution rate is sufficiently small. Experiments of Jöbses *et al.* (1986a) and Lee *et al.* (1979) have clearly demonstrated such oscillations in fermentation of *Z. mobilis*.

At a dilution rate of 0.1 h^{-1}, the average biomass concentration increased to 1.47 g L^{-1} or 1.37-fold compared to the previous dilution rate (Figure 4.11), the average residual glucose concentration increased to 42.3 g L^{-1} (1.83-fold) (Figure 4.12) and the average ethanol concentration decreased to 38.76 g L^{-1} (1.04-fold) (Figure 4.13). The fermentation was still oscillatory until 132 hours after the switch to a dilution rate of 0.1 h^{-1}, but a stable steady state was eventually achieved (see circled data, Figure 4.11-4.13). At dilution rate of 0.1 h^{-1}, the oscillation ranges were 1.14 – 1.79 g L^{-1}, 68.16 – 16.43 g L^{-1} and 31.86 – 45.65 g L^{-1} for biomass, residual glucose, and ethanol concentrations. The corresponding values of the absolute

oscillation amplitudes were 0.65 g L^{-1}, 51.73 g L^{-1}, and 13.79 g L^{-1}, respectively. Compared with the oscillation profiles observed at the dilution rate of 0.05 h^{-1}, the amplitude of the oscillations of all the monitored parameters had decreased and the oscillation periods were shorter on average than at the dilution rate of 0.05 h^{-1}.

The dilution rate was then increased further to 0.15 h^{-1}. The average biomass concentration slightly increased to 1.54 g L^{-1} (1.05-fold compared to the previous dilution rate), the average residual glucose concentration decreased to 21.05 g L^{-1} and the average ethanol concentration increased to 41.25 g L^{-1}. The oscillation ranges were 1.36 – 1.72 g L^{-1}, 30.78 – 11.32 g L^{-1} and 39.53 – 42.96 g L^{-1} for the biomass concentration, the residual glucose concentration, and ethanol concentration. The corresponding absolute oscillation amplitudes were 0.36 g L^{-1}, 19.46 g L^{-1}, and 3.43 g L^{-1}, respectively. Compared with the oscillation profiles observed at the dilution rate of 0.1 h^{-1}, the oscillations of all the monitored parameters were apparently not significantly changed and the oscillation periods were observed to be around 36 h for all of these fermentation parameters. A relatively stable steady state was eventually attained.

The dilution rate was then increased to 0.2 h^{-1}. The average biomass concentration slightly decreased to 1.40 g L^{-1}, the average residual glucose concentration increased to 34.73 g L^{-1} (1.65-fold) and the average ethanol concentration slightly decreased to 40.1 g L^{-1}. The oscillation ranges were 1.32 – 1.47 g L^{-1}, 49.2 – 20.25 g L^{-1} and 33.18 – 47.0 g L^{-1} for biomass, residual glucose, and ethanol, respectively. The corresponding absolute oscillation amplitudes were 0.15 g L^{-1}, 28.95 g L^{-1}, and 13.82 g L^{-1}, respectively. Compared to the oscillation profiles observed at the dilution rate of 0.15 h^{-1}, the oscillations of all the monitored parameters were greatly damped the oscillation periods were shortened.

As the dilution rate was further increased to 0.25 h^{-1}, the average biomass concentration slightly decreased to 1.32 g L^{-1}, the average residual glucose concentration increased to 55.9 g L^{-1} (1.61-fold compared to the previous dilution rates) and the average ethanol concentration slightly decreased to 34.0 g L^{-1}. These fermentation parameters did not significantly oscillate with a clear period, but there were some fluctuations. The oscillation ranges were 1.24 – 1.39 g

L^{-1}, 63.30 – 48.4 g L^{-1} and 32.24 – 35.79 g L^{-1} for biomass concentration, the residual glucose concentration, and ethanol concentration. The corresponding absolute oscillation amplitudes were 0.15 g L^{-1}, 14.9 g L^{-1}, and 3.55 g L^{-1}, respectively.

The dilution rate was then raised close to the critical dilution rate of 0.3 h^{-1} (critical dilution rate is the value which if exceeded is expected to lead to a culture washout). The average biomass was not significantly changed at 1.34 g L^{-1}, the average residual glucose concentration decreased to 44.2 g L^{-1} and the average ethanol concentration slightly increased to 38.5 g L^{-1}. These fermentation parameters did not significantly oscillate with a defined period, but there were slight fluctuation. The ranges of fluctuations were 1.19 – 1.49 g L^{-1}, 70.0 – 18.47 g L^{-1} and 30.0 – 47.0 g L^{-1} for the biomass concentration, the residual glucose concentration, and the ethanol concentration. The corresponding absolute oscillation amplitudes of the fluctuation were 0.3 g L^{-1}, 51.3 g L^{-1}, and 17 g L^{-1}, respectively.

Figure 4.14 shows the effect of dilution rate on steady state parameters (i.e. averaged values of the data marked with circles in Figure 4.11-4.13) for the continuous fermentation. Each data point in Figure 4.14 was obtained by averaging three measurements at a given dilution rate. No steady state occurred at the low dilution rate of 0.05 h^{-1} hence this dilution rate is not included in Figure 4.14. The kinetic parameters of the fermentation at the various steady states are shown in Table 4.4. A dilution rate 0.15 h^{-1} appeared to be optimal (Figure 4.14) for attaining a high ethanol concentration. With this dilution rate, the ethanol concentration of 42.5 g L^{-1} was produced and 1.49 g L^{-1} of the biomass concentration was obtained. At this dilution rate, the ethanol yield on glucose and ethanol productivity values were 0.37 g g^{-1} and 6.38 g L^{-1} h^{-1}, respectively. Ethanol productivity, but not the ethanol concentration, was highest at a dilution rate of 0.3 h^{-1} (Table 4.4) although much residual glucose remained. Higher values of dilution rates tended to reduce the ethanol concentration in broth and dampen the oscillation (Ghommidh *et al.,* 1989).

Various hypotheses have been advanced to explain why oscillations occur during certain fermentations. Conventionally, oscillatory behavior is said to be linked directly to strong product inhibition (Jöbses *et al.,* 1986a; Mulchandani and Volesky, 1986). According to Lee *et al.* (1979), oscillations results from the inhibitory effect of ethanol. Once the ethanol concentration

becomes high cell growth and ethanol production are inhibited, but continuing dilution reduces the concentration of ethanol in the reactor. As a consequence, both the growth rate and the biomass specific ethanol production rate increase again to once again increase the ethanol concentration to an inhibitory level. This cycle repeats with a fairly well-defined frequency that depends on the dilution rate. Changes in ethanol concentration in the bioreactor also affect the bacterial physiology: the cells become filamentous or elongated in response to the stress environment of a high ethanol concentration (Daugulis *et al.,* 1985; Ghommidh *et al.,* 1989; Rogers *et al.,* 1979). Thus, there is a physiological component to the observed oscillations.

As this work is focused on preventing the oscillations during a continuous fermentation by continuously removing the ethanol from the broth, further studies focused on the low values of the dilution rate as at these dilution rates a steady state could not be achieved. If, therefore, a steady state operation can be achieved at these low dilution rates by implementing continuous ethanol removal, the hypothesis of inhibitory effect of ethanol causing the oscillations will be proved. Furthermore, a methodology (i.e. continuous ethanol removal) will become available for attaining steady states at low dilution rates in high gravity fermentations, and fermentations of other inhibitory products.

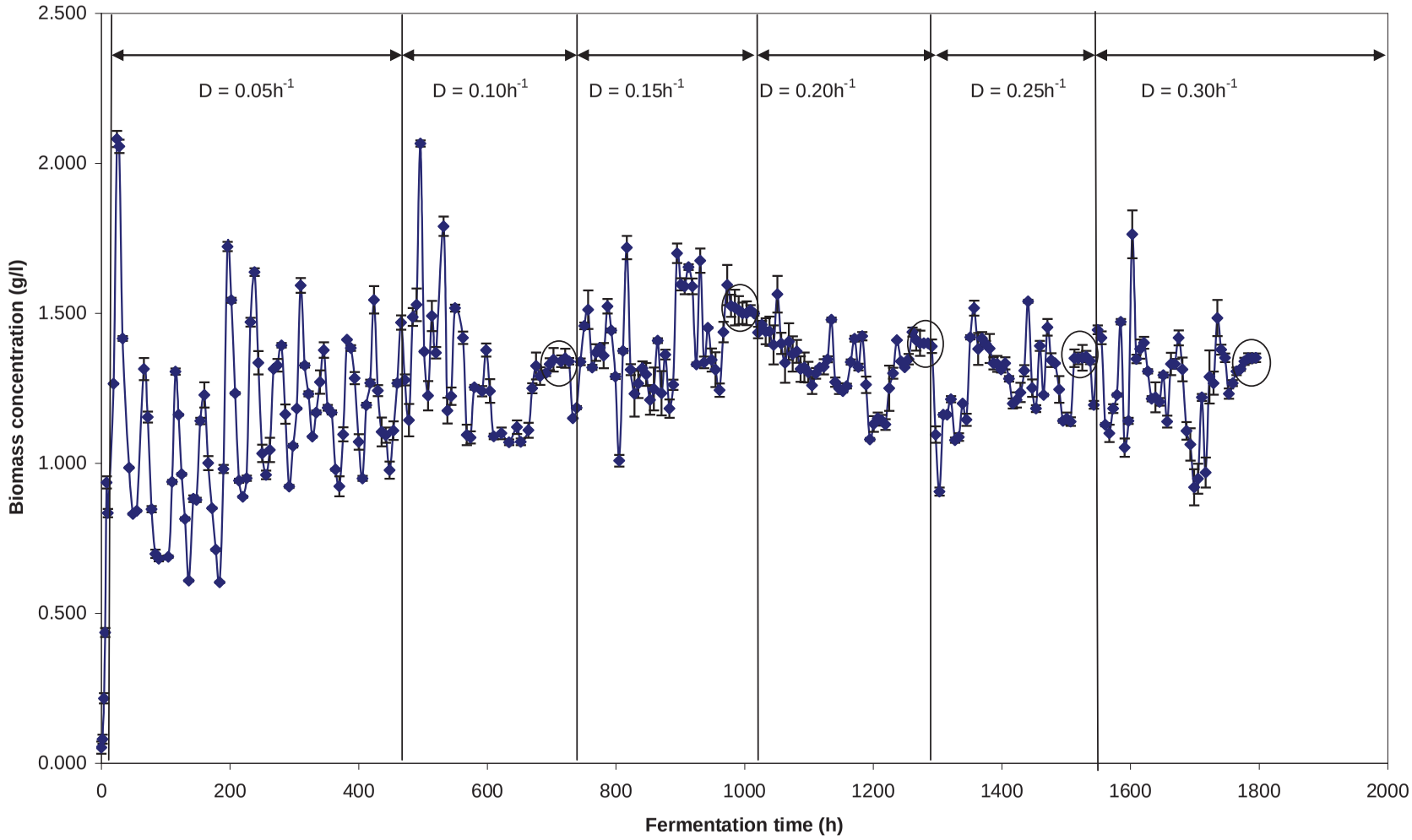


Figure 4.11 Impact of dilution rate (D) on the biomass concentration profile in continuous ethanol fermentation. Glucose concentration in the feed medium was 150 g L^{-1}. The working volume of the bioreactor was 1 L.

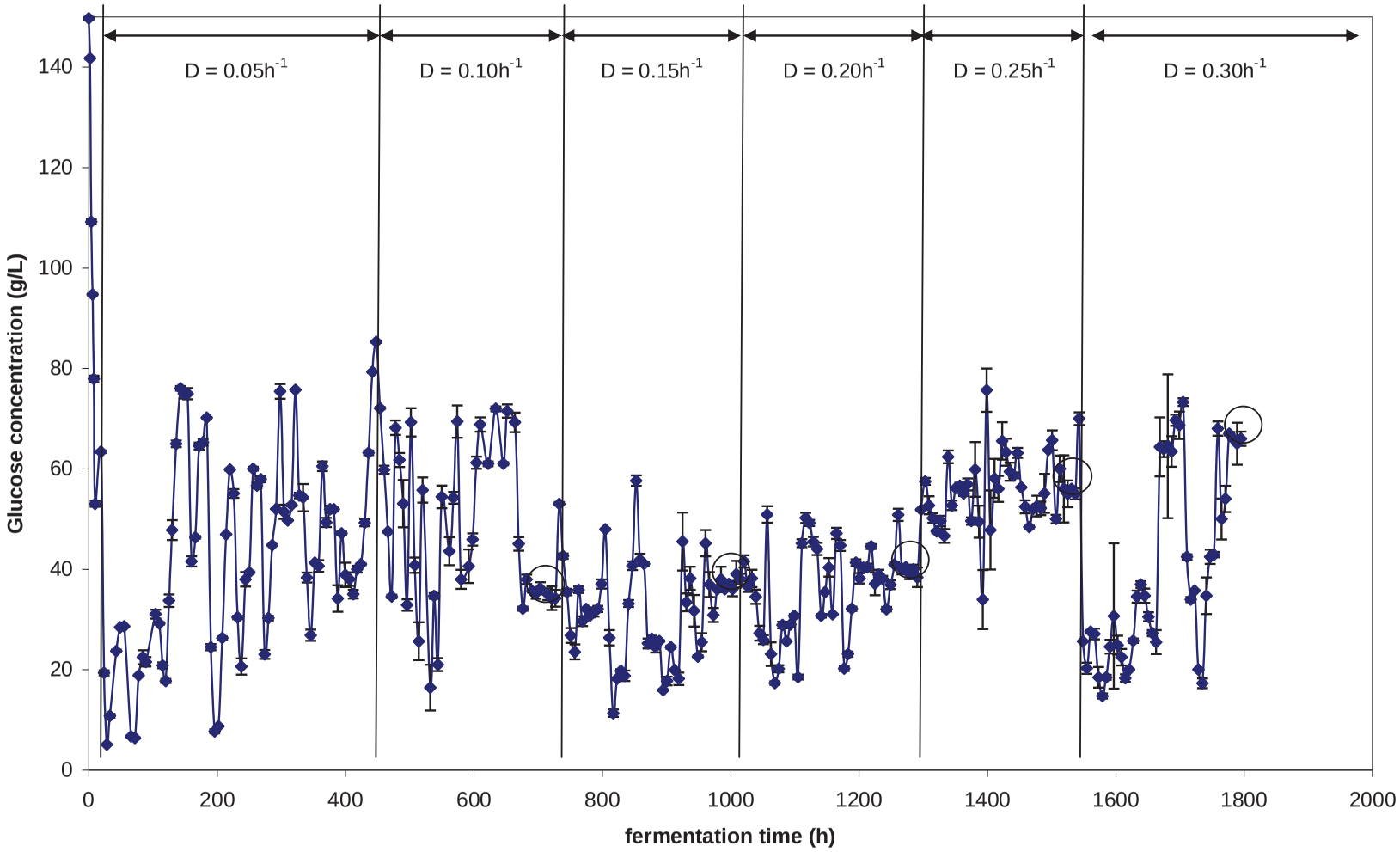


Figure 4.12 Impact of dilution rate (D) on the glucose concentration profile in continuous ethanol fermentation. Glucose concentration in the feed medium was 150 g L^{-1}. The working volume of the bioreactor was 1 L.

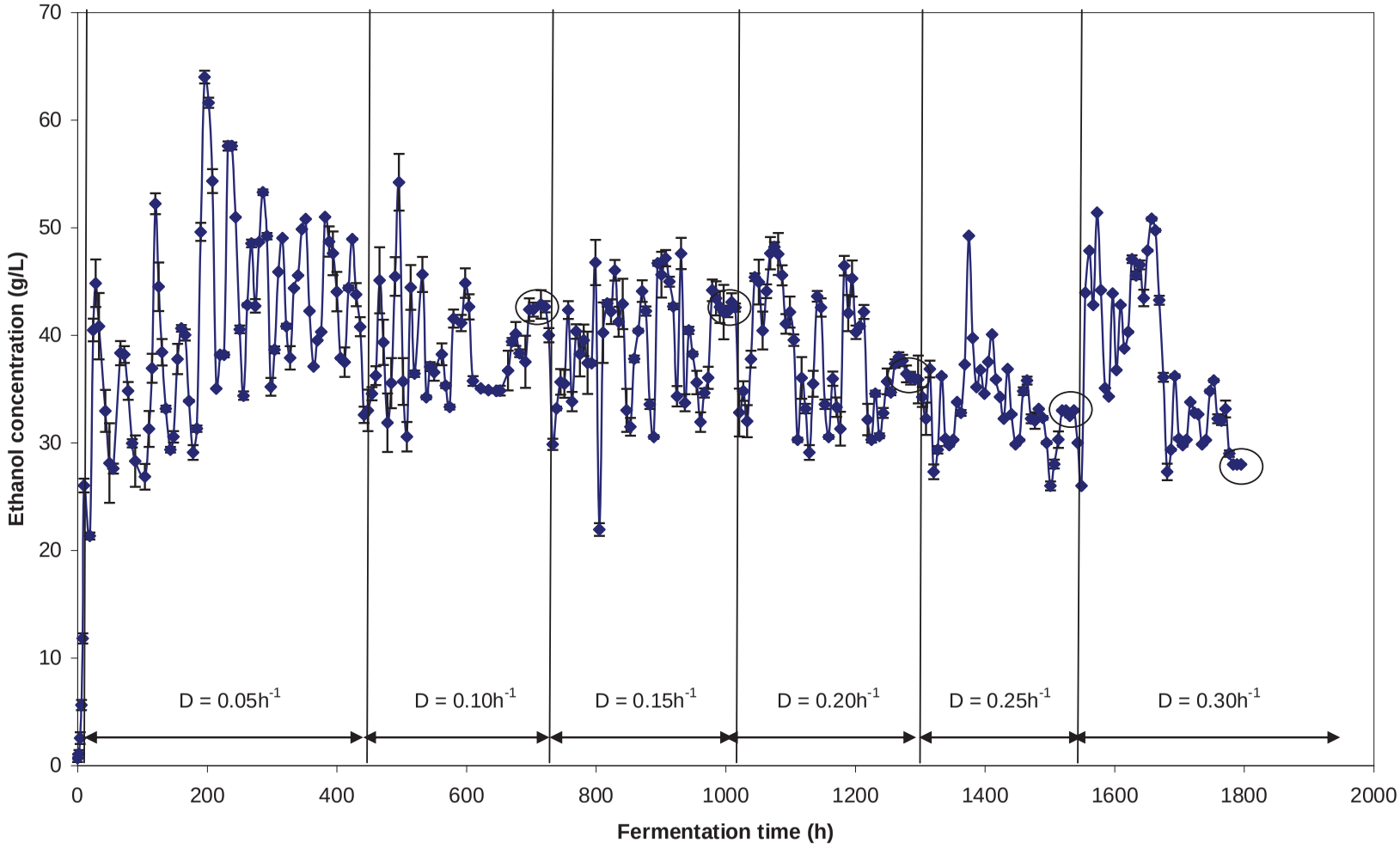


Figure 4.13 Impact of dilution rate (D) on the ethanol concentration profile in continuous ethanol fermentation. Glucose concentration in the feed medium was 150 g L^{-1}. The working volume of the bioreactor was 1 L.

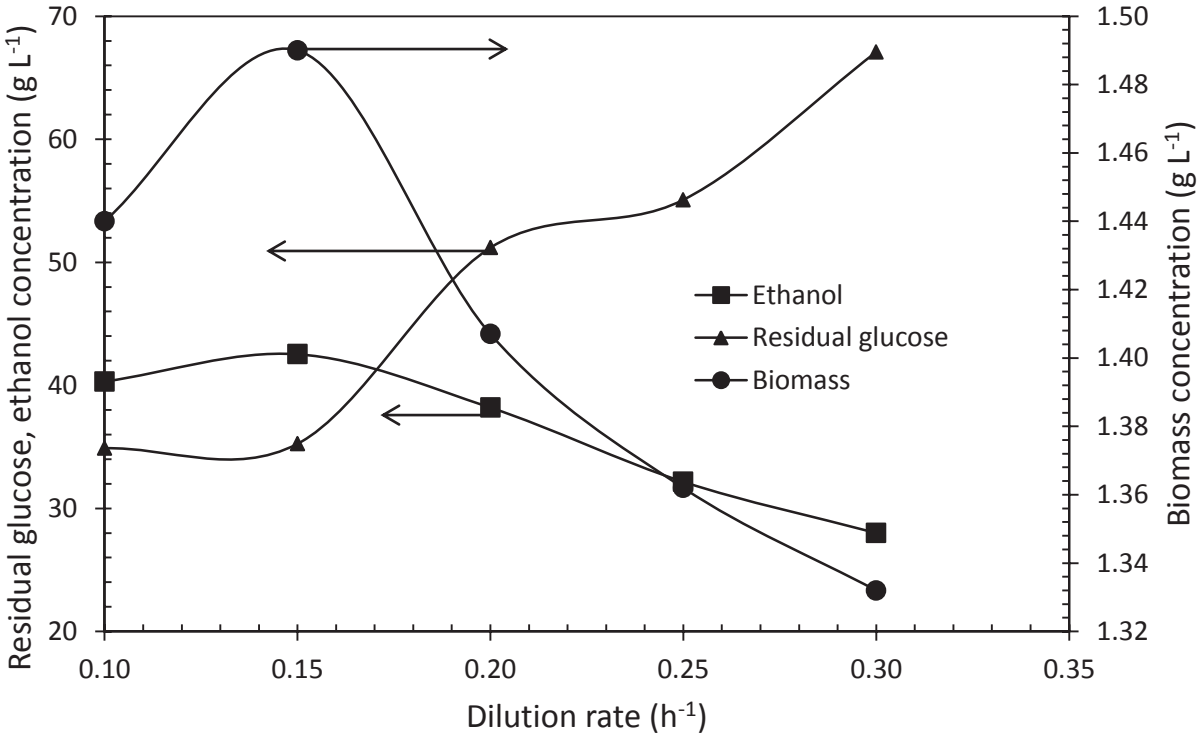


Figure 4.14 Steady state values of glucose concentration, ethanol concentration and biomass concentration in the bioreactor at various dilution rates. Glucose concentration in the feed medium was 150 g L^{-1}.

Table 4.4 Summarized steady state parameters from continuous culture of *Z. anaerobia* at feed glucose concentration of 150 g L^{-1}

	Kinetic parameter						
Dilution rate, D (h^{-1})	Glucose concentration, S (g L^{-1})	Biomass concentration, X (g L^{-1})	Ethanol concentration, E (g L^{-1})	Biomass yield on substrate, $Y_{x/s}$ (g g^{-1})	Biomass productivity, P_x (g L^{-1} h^{-1})	Ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	Ethanol productivity, P_E (g L^{-1} h^{-1})
0.10	34.89	1.44	40.31	0.01	0.15	0.35	4.03
0.15	35.25	1.49	42.53	0.01	0.22	0.37	6.38
0.20	51.19	1.41	38.19	0.01	0.28	0.39	7.64
0.25	55.08	1.36	32.16	0.01	0.34	0.34	8.04
0.30	67.09	1.33	28.00	0.02	0.40	0.34	8.40

4.1.5 Effect of feed glucose concentration on oscillations

As clear steady states could not be established at dilution rate values of 0.05 h^{-1} and 0.1 h^{-1} during continuous operation (see in Section 4.1.4) these dilution rates were selected for further study. The same bioreactor setup was used as in Section 4.1.4, but the glucose concentration in the feed medium was either 80 g L^{-1} or 100 g L^{-1}. The aim was to identify the minimum glucose concentration that fails to produce oscillations. Earlier studies (see Section 4.1.4) had shown that a feed glucose concentration of 150 g L^{-1} would cause oscillations at dilution rate values of ≤ 0.1 h^{-1}.

The results are shown in Figure 4.15 and Figure 4.16. Fermentations commenced as batches and were switched to continuous operation at 10 h. As can been seen in Figure 4.15 and Figure 4.16, clear and stable steady states were achieved at both the dilution rates and feed glucose concentrations. Thus a feed glucose concentration of $\geq$ 100 g L^{-1}, or at least 150 g L^{-1} (Section 4.1.4) was shown to be necessary for persistent oscillations. The steady state values of the fermentation parameters are shown in Table 4.5 and Table 4.6 for the feed glucose concentration values of 80 g L^{-1} and 100 g L^{-1}, respectively.

At any given feed glucose concentration, the ethanol productivity was higher at the higher dilution rate (Table 4.5, Table 4.6). Steady state ethanol concentration value was reduced by increasing dilution rate, but not the ethanol productivity as productivity is dilution rate multiplied by the steady state ethanol concentration. At a fixed dilution rate, the ethanol productivity value was distinctly higher at the higher feed glucose concentration (Table 4.5, Table 4.6). Clearly, therefore, operation at high glucose feed concentration is desired as this also enhances the ethanol yield on glucose (Table 4.5, Table 4.6). In a study of continuous glucose fermentation by *Z. mobilis,* Lee *et al.* (1979) reported that feed glucose concentration of 150 g L^{-1} and 200 g L^{-1} produced an oscillatory behavior, but not a feed glucose concentration of 100 g L^{-1} at a low dilution rate. Hence the data of the present study are consistent with similar observations for *Z. mobilis* (Lee *et al.*, 1979).

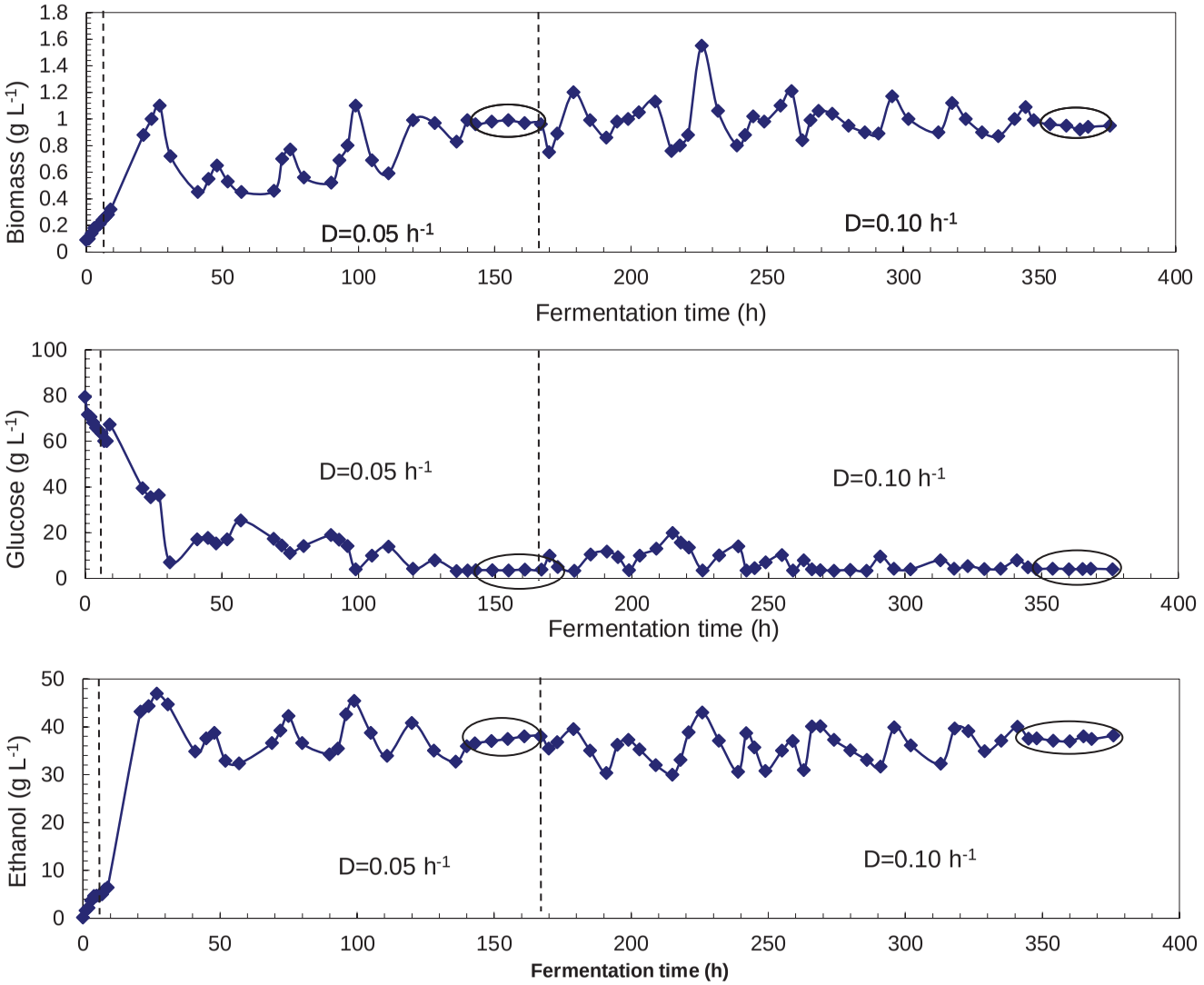


Figure 4.15 Continuous ethanol fermentation at feed glucose concentration of 80 g L^{-1}, D = 0.05 h^{-1} and D = 0.1 h^{-1}: a) biomass concentration profile; b) residual glucose concentration profile; and c) ethanol concentration profile. Dilution rate was changed at the instances marked by the dashed lines at 10 h and 170 h. The working volume of the bioreactor was 1 L.

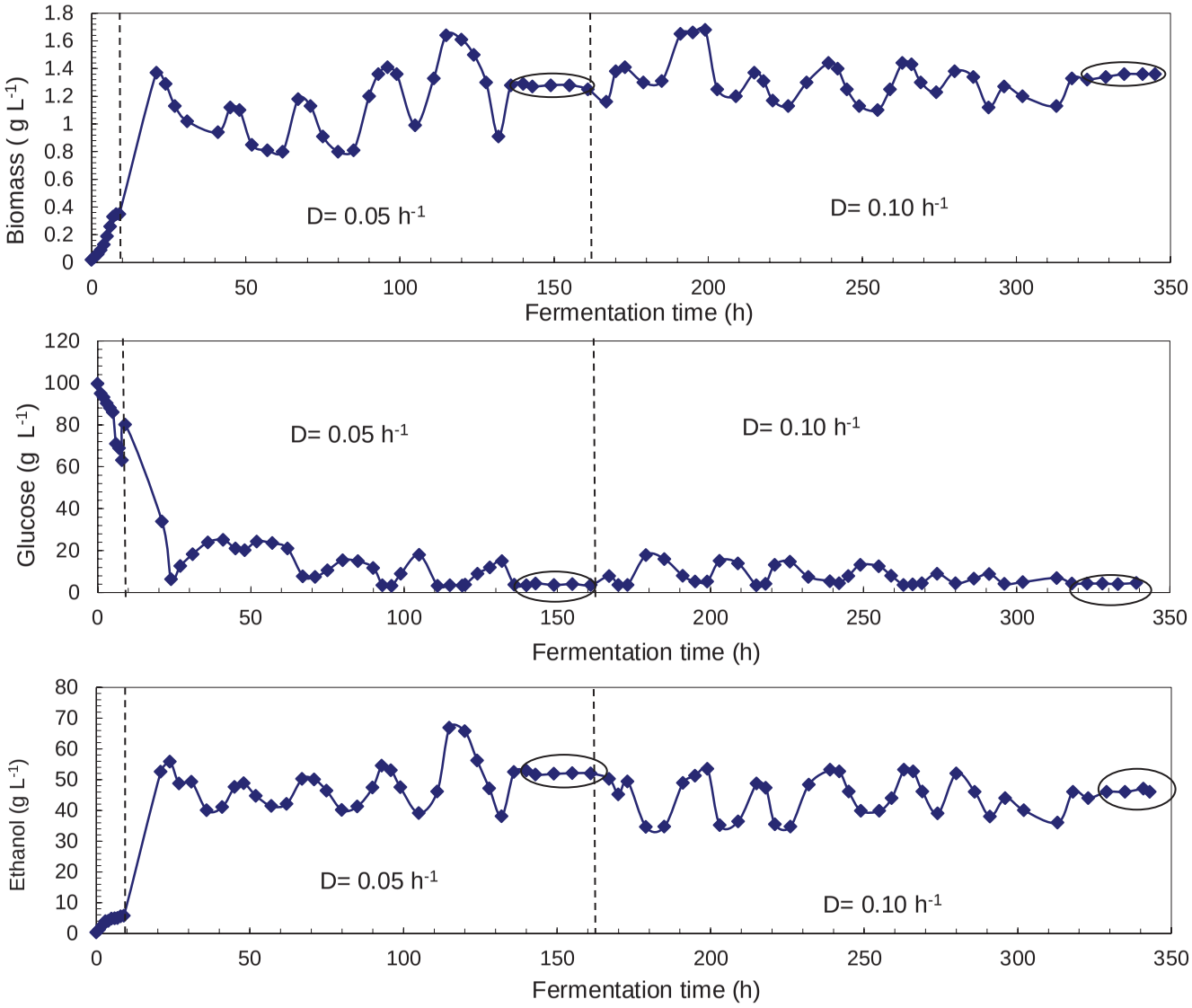


Figure 4.16 Continuous ethanol fermentation at feed glucose concentration of 100 g L^{-1}, D = 0.05 h^{-1} and D = 0.1 h^{-1}: a) biomass concentration profile; b) residual glucose concentration profile; and c) ethanol concentration profile. Dilution rate was changed at the instances marked by the dashed lines at 10 h and 160 h. The working volume of the bioreactor was 1 L.

Table 4.5 Steady state kinetic parameters for continuous culture of *Z. anaerobia* at feed glucose concentration of 80 g L^{-1}

Kinetic parameter						
Glucose concentration, S (g L^{-1})	Biomass concentration, X (g L^{-1})	Ethanol concentration, E (g L^{-1})	Biomass yield on substrate, $Y_{x/s}$ (g g^{-1})	Biomass productivity, P_x (g L^{-1} h^{-1})	Ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	Ethanol productivity, P_E (g L^{-1} h^{-1})
3.55	0.98	36.77	0.007	0.049	0.25	1.84
4.13	0.95	37.54	0.007	0.095	0.26	3.75

Table 4.6 Steady state kinetic parameters for continuous culture of *Z. anaerobia* at feed glucose concentration of 100 g L^{-1}

	Kinetic parameter						
Dilution rate, D (h^{-1})	Glucose concentration, S (g L^{-1})	Biomass concentration, X (g L^{-1})	Ethanol concentration, E(g L^{-1})	Biomass yield on substrate, $Y_{x/s}$ (g g^{-1})	Biomass productivity, P_x (g L^{-1} h^{-1})	Ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	Ethanol productivity, P_E (g L^{-1} h^{-1})
0.05	3.83	1.28	52.15	0.009	0.064	0.36	2.61
0.10	1.35	1.35	45.83	0.092	0.135	0.32	4.58

4.2 In situ extractive fermentation using solvents

This section discusses the in situ extractive fermentation via liquid-liquid extraction to control the oscillatory behavior discussed in the previous section. Compared to yeast, *Z. anaerobia* can produce ethanol twice as fast in an anaerobic fermentation, but accumulation of ethanol during the fermentation inhibits the bacterium. Product inhibition due to ethanol can be reduced by removing it from the fermentation broth as it is produced. This strategy is examined here using in-situ liquid-liquid extraction to remove the ethanol from the fermentation broth.

4.2.1 Biocompatibility of solvents (toxicity of solvents)

In order to realize the benefits of extractive fermentation, the water-immiscible organic solvent used for the extraction must be non toxic to the bacterium. Accordingly, several solvents were screened for their effect on *Z. anaerobia* growth, ethanol production and substrate consumption. The ease of the phase separation of the solvent and the broth was also checked during toxicity tests. Phase separation was considered good if the organic and aqueous phases separated cleanly and rapidly after the agitation was stopped. All of the eleven solvents tested (Table 4.7), readily separated from the fermentation broth to form an upper layer. These solvents (Table 4.7) were experimentally evaluated for biocompatibility with *Z. anaerobia* and the ability to extract ethanol during batch fermentations. Table 4.7 provides the measured partition coefficient of ethanol in the various solvents, and the values relative to control (i.e. no solvent added) of the biomass concentration attained, ethanol concentration attained, glucose consumed by 42 h in batch fermentation carried out as described in Section 3.14.

Table 4.7 Comparison of solvents

		Relative to control			
Solvents	Partition coefficient	Cell dry weight	Ethanol produced in broth	Glucose consumed	Cell viability
Soy oil	0.07	0.69 ± 0.02	0.68 ± 0.02	0.98 ± 0.04	1.78 ± 0.12
Castor oil	0.19	0.47 ± 0.01	0.89 ± 0.02	0.87 ± 0.03	1.19 ± 0.26
2-Octyl-1-dodecanol	0.20	1.0 ± 0.24	0.95 ± 0.017	0.98 ± 0.01	1.048 ± 0.07
Iso-octadecanol	0.27	1.21 ± 0.16	0.97 ± 0.37	0.98 ± 0.03	1.033 ± 0.18
Oleyl alcohol	0.30	0.71 ± 0.04	0.95 ± 0.09	1.01 ± 0.08	1.48 ± 0.4
Dodecanol	0.37	0.65 ± 0.01	0.72 ± 0.07	0.68 ± 0.03	0.75 ± 0.26
Etyhl butyrate	0.41	0.16 ± 0.11	0.19 ± 0.06	0.08 ± 0.06	0.002 ± 0.14
Butyl acetate	0.44	0.14 ± 0.04	0.19 ± 0.02	0.11 ± 0.05	0.006 ± 0.13
Polypropylene glycol 1200	0.51	0.64 ± 0.11	0.82 ± 0.05	1.00 ± 0.001	1.30 ± 0.03
Methyl isobutyl ketone	0.51	0.16 ± 0.02	0.19 ± 0.02	0.08 ± 0.07	0.009 ± 0.15
Etyhl hexanol	0.67	0.01 ± 0.02	0.09 ± 0.04	0.28 ± 0.01	0.37 ± 0.03

Oleyl alcohol, iso-octadecanol and 2-octyl-1-dodecanol were the three solvents that were found to be non-toxic (Table 4.7, highlighted in grey). With these solvents, the cell viability relative to control (i.e. no solvent added) was 1.48 ± 0.4, 1.03 ± 0.18 and 1.05 ± 0.07, respectively, after two days of exposure. Solvents that had a relatively high partition coefficient (Table 4.7) for ethanol were toxic to the bacterium and adversely affected the biomass concentration, glucose consumption and ethanol production relative to control. The cell dry weight, ethanol produced and glucose consumed relative to control also shown in Table 4.7. During extractive fermentations, accumulation of an extraction solvent in the cell membrane affects its integrity and metabolic function leading ultimately to cell death (Heipieper *et al*., 1994; Matsumoto *et al*., 2004; Taylor *et al.,* 2008).

These results generally agree with results obtained in other studies on solvent toxicity to *Zymomonas* species (Bruce and Daugulis, 1991a; Bruce and Daugulis, 1992; Malinowski and Daugulis, 1993). Among the non-toxic solvents, oleyl alcohol had the highest partition coefficient for ethanol and appeared to be suitable for its extraction.

4.2.2 In-situ batch extractive fermentation

The three solvents that were found to be non toxic (Table 4.7, highlighted in grey) to *Z. anaerobia* were used for in-situ batch extractive fermentations in a 3-L stirred bioreactor (1:1 by vol ratio of solvent and fermentation broth) with an initial glucose concentration of 150 g L^{-1}, 35 °C, and an agitation speed of 100 rpm. Solvents were added to the fermentation broth after ethanol production had began (i.e. after 10 h of fermentation). Each batch fermentation was run for 17 h.

The profiles of the in-situ batch extractive fermentations are shown in Figure 4.17 (biomass and glucose concentration profile) and Figure 4.18 (ethanol concentration profile) respectively. All solvents tested improved biomass concentration, glucose consumption and ethanol concentration relative to control, but iso-octadecanol was clearly the most effective solvent. Using iso-octadecanol, the final ethanol concentration of 75 ± 3.07 g L^{-1} was nearly 1.25-fold that of the control fermentation and slightly higher compared to extractive fermentation which used oleyl alcohol and 2-octa-1-dodecanol for extraction. For iso-octadecanol, the ethanol yield on glucose was 0.485 ± 0.005 g g^{-1} compared to a yield of 0.468 ± 0.005 g g^{-1} for the control culture (Table 4.8).

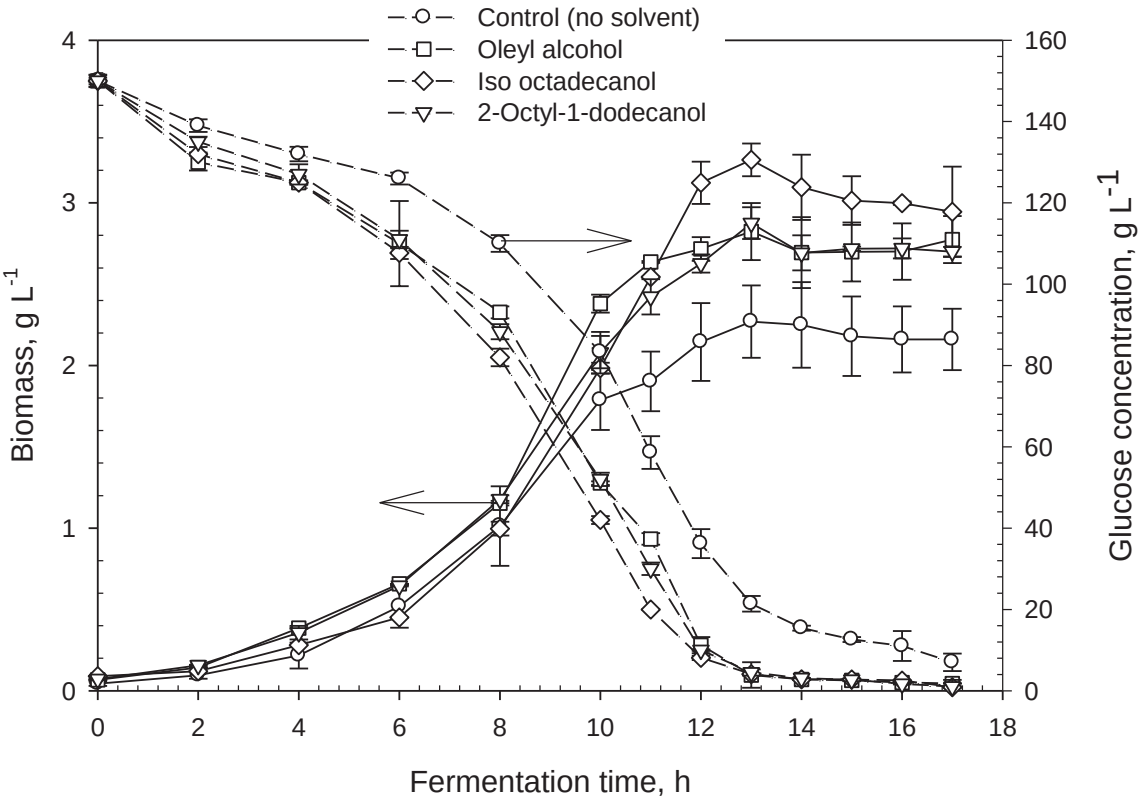


Figure 4.17 Effects of solvents on biomass and glucose concentration profiles in batch extractive fermentations. Each curve is based on three replicate fermentations.

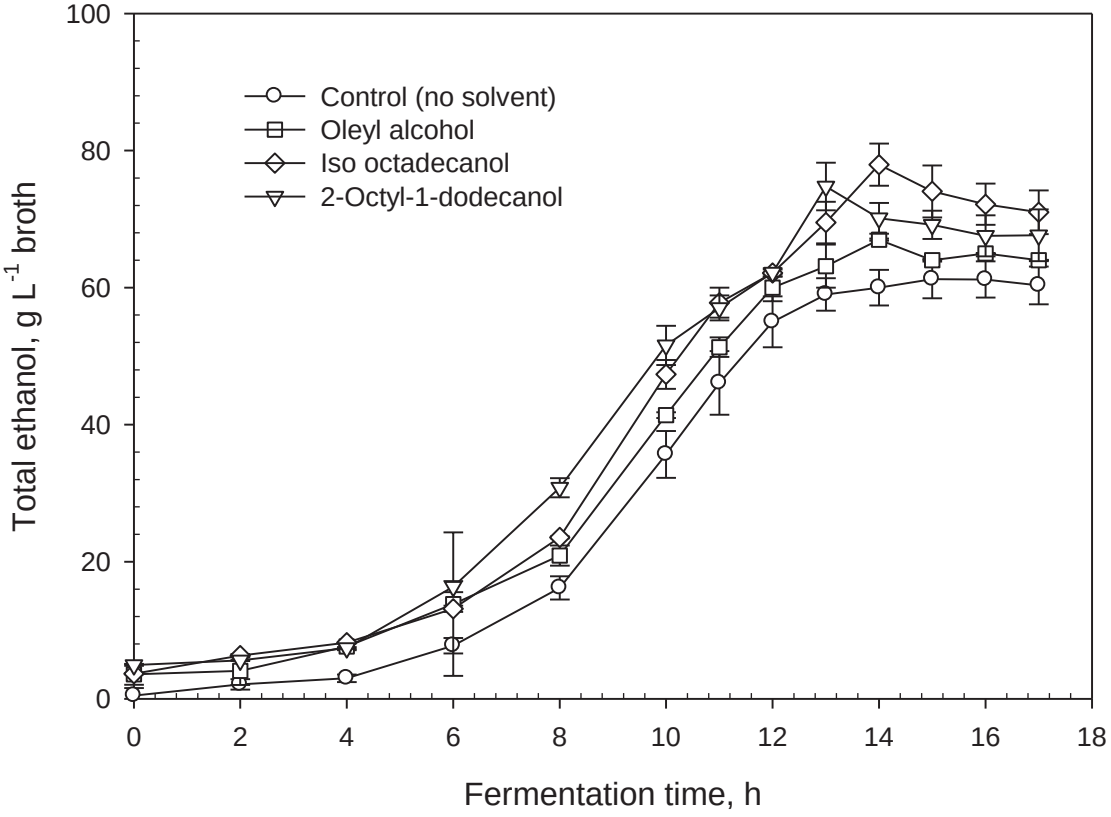


Figure 4.18 Effects of solvents on total ethanol production in batch extractive fermentations. Each curve is based on three replicate fermentations.

Because the ethanol being produced was being removed in the extractant phase, all the extractive fermentations consumed glucose faster than in the control fermentation (Figure 4.17). Also, glucose consumption in the extractive fermentations was essentially complete by 14 h (Figure 4.17), but the control fermentation had significant residual glucose at this time. Clearly, iso-octadecanol was the best solvent, but all solvents were distinctly superior to the control fermentation.

The kinetic parameters for the various batch fermentations (Figure 4.17, Figure 4.18) are compared in Table 4.8. Using iso-octadecanol, the best solvent, the extractive fermentation was substantially superior to the control culture. For example, compared to control, the biomass yield on glucose was 33% greater for the iso-octadecanol culture; the maximum biomass concentration was 37% greater; the maximum biomass productivity was 28% greater; the maximum ethanol

yield on glucose was 1.04-fold greater; the maximum ethanol concentration was 1.25-fold greater; and the maximum ethanol productivity was 1.16-fold greater (Table 4.8).

Table 4.8 Comparison of fermentation kinetics of in-situ extractive batch fermentations

		Solvent		
Kinetic parameter	**Control**	**Oleyl alcohol**	**Iso octadecanol**	**2-Octyl-1-dodecanol**
Maximum biomass productivity, P_x (g L^{-1} h^{-1})	0.142 ± 0.004	0.169 ± 0.004	0.182 ± 0.004	0.166 ± 0.004
Maximum biomass yield on glucose, $Y_{x/s}$ (g g^{-1})	0.015 ± 0.001	0.020 ± 0.001	0.020 ± 0.001	0.02 ± 0.001
Maximum ethanol productivity, P_E (g L^{-1} h^{-1})	4.333 ± 0.099	4.701 ± 0.099	5.025 ± 0.099	4.648 ± 0.099
Maximum ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.468 ± 0.005	0.431 ± 0.005	0.485 ± 0.005	0.443 ± 0.005
Maximum specific growth rate, μ (h^{-1})	0.304 ± 0.011	0.328 ± 0.005	0.336 ± 0.004	0.321 ± 0.009

Based on three replicate fermentations. No solvent was added to control fermentations.

Because ethanol was removed from the cell environment as it was produced in extractive fermentation, product inhibition was reduced and the fermentor productivity improved. Cells remained viable throughout the extraction as evidenced by a continued production of the biomass and substrate consumption (Figure 4.17). A good extractive fermentation system requires a solvent that is a good extractant for ethanol, but allows easy recovery of ethanol from the solvent. In this work, ethanol was recovered from the extractants by using the simplest method of distillation. The solvent containing ethanol and a small amount of entrained aqueous medium was removed from the fermentor. The 2-L separatory funnel was used to separate the solvent and the aqueous medium. A rotary evaporator was then used to evaporate the ethanol. The vapour was condensed to ethanol and collected from the condensate-collecting flask at the bottom of the condenser. The residual solvent was centrifuged at 18000 × g for 20-min at room temperature in order to remove biomass particles. The solvent was reused in future fermentations. In a batch fermentation, the extraction process may be further improved by improving mass transfer between aqueous and the organic phase (e.g. by better agitation). Also, the extraction solvent may be fed and removed continuously so that the ethanol concentration in it does not rise to a high value. As iso-octadecanol proved to be the best solvent, it was used in continuous flow extraction studies in continuous culture of *Z. anaerobia*.

4.2.3 Continuous extractive fermentation

As a dilution rate of 0.05 h^{-1} was found to produce nonpreventable regular oscillations in continuous fermentation of *Z. anaerobia* (see Section 3.11) this dilution rate was selected for in situ continuous extractive fermentation in a stirred bioreactor with a working volume of 3-L (1:1 ratio vol/vol of solvent and fermentation broth). Fermentation conditions were identical to those used previously (Section 3.10) an initial glucose concentration of 150 g L^{-1}, 35 °C, and an agitation speed of 140 rpm (to maintain good contact of the fermentation broth and solvent in the bioreactor). No pH control was used but the initial pH of the broth was set at 5.5. The bioreactor was equipped with various pumps and accessories to accommodate extractive fermentation involving the aqueous phase as the bottom phase and a stagnant solvent phase as the top phase (Figure 3.4). The best solvent (i.e. iso-octadecanol) that had given the highest ethanol productivity in in-situ batch extractive fermentation was used throughout this study.

The fermentation dilution rate is based on the aqueous phase. Different solvent flow rates (at above specified constant volume) were used ranging from 100 mL h^{-1} to 400 mL h^{-1}. The continuous fermentation and extraction operations started after 10 h of a batch fermentation (without solvent extraction). The solvent was introduced at the bottom of the aqueous phase and dispersed through the holes of the feed tube located beside the impeller shaft. The solvent then rose through the aqueous phase to form a separate layer on top. This arrangement was necessary in order to reduce the possibility of the solvent forming a stable emulsion with the fermentation broth and to maintain a relatively stagnant solvent layer in the upper part of the bioreactor, allowing for continuous withdrawal of only the solvent through the overflow. Samples from fermentation broth were taken at regular intervals and analysed for biomass, ethanol and glucose. Samples from the solvent phase were analysed for ethanol concentration only.

Figures 4.19-4.21 show the time course of the fermentation. The value of the solvent flow rate (Fs) used at different times are shown on the Figures. As discussed earlier in Section 4.1.4, at a dilution rate of 0.05 h^{-1}, a steady state operation could not be established within a duration of 475 h (~20 days). Use of continuous in situ extraction with a solvent flow rate of 100 mL h^{-1} allowed a steady state (circled data, Figure 4.19-4.21) to be achieved by 166 h of fermentation. Similarly, steady states were achieved at progressively higher biomass concentration (Figure 4.19), lower residual glucose concentration (Figure 4.20) and higher ethanol concentration (Figure 4.21) as the steady-state solvent flow rate was increased from 100 mL h^{-1} in steps to 400 mL h^{-1}.

At a fixed dilution rate at a steady-state, an increase in solvent flow rate caused the concentration (glucose, biomass, ethanol) to fluctuate until a new steady-state was established. As the solvent flowrate rose, the ethanol removal in the solvent increased (Figure 4.21) and this reduced ethanol inhibition. At a solvent flow rate of $\geq$ 300 mL h^{-1} essentially all glucose in the fermentation broth was consumed at steady-state (Figure 4.20). Clearly, extractive fermentation effectively eliminated the oscillations and allowed steady-state operations to be established. Cell growth and ethanol production and glucose consumption were improved by continuous in situ extraction relative to the control case (Section 4.1.4). Essentially complete utilization of glucose could be achieved by suitable selection of the flow rate of the extraction solvent. The steady state data for the extractive fermentation shown in Figure 4.19-4.21 was used to calculate the kinetic parameters shown in Table 4.9.

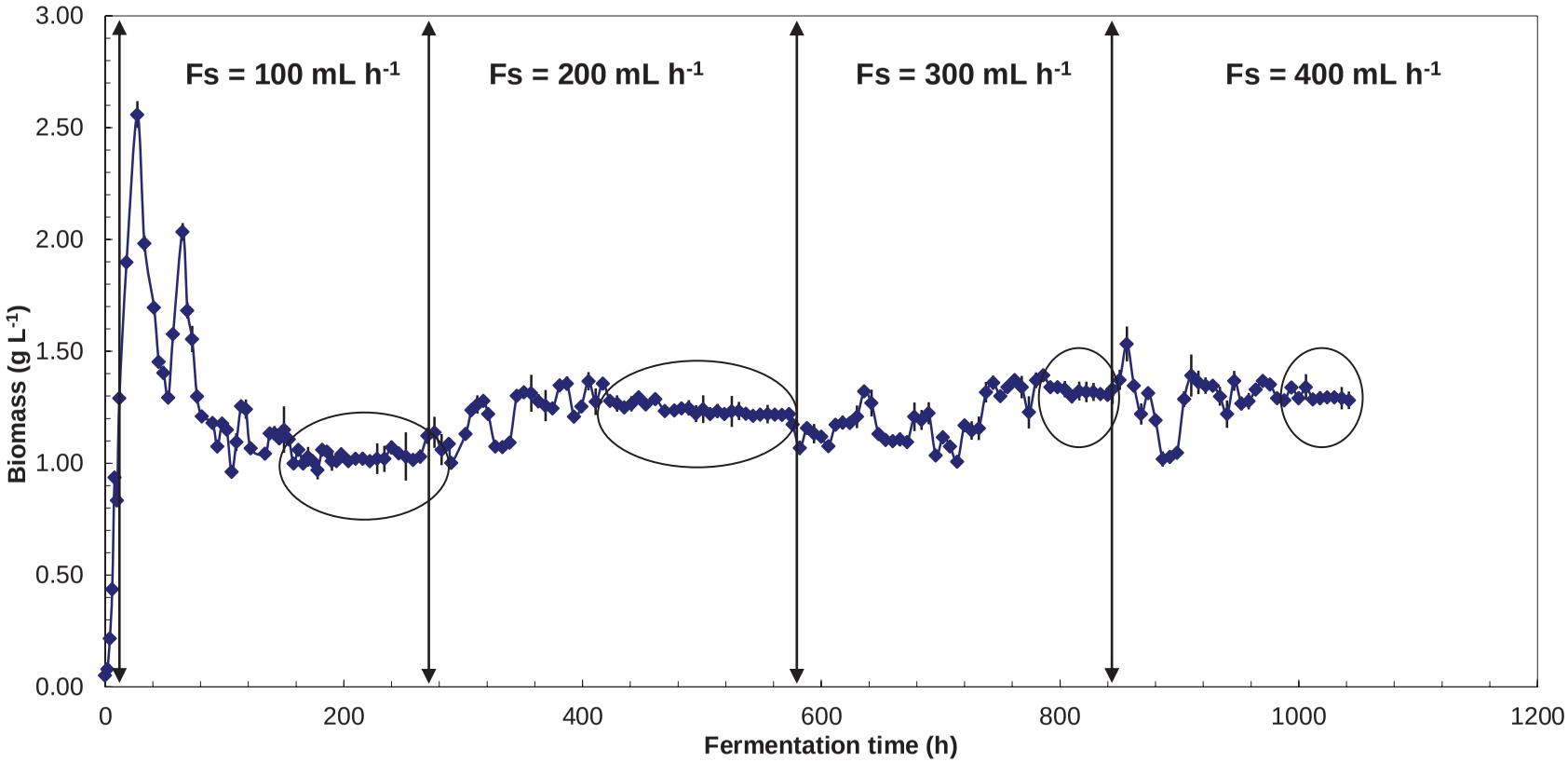


Figure 4.19 Biomass concentration in the aqueous phase of continuous extractive fermentation (feed glucose concentration 150 g L^{-1}, dilution rate 0.05 h^{-1}, 35 °C, agitation rate of 140 rpm). Fs is the flow rate of solvent. The bioreactor had a constant working volume of 1 L of culture broth and 1 L of solvent.

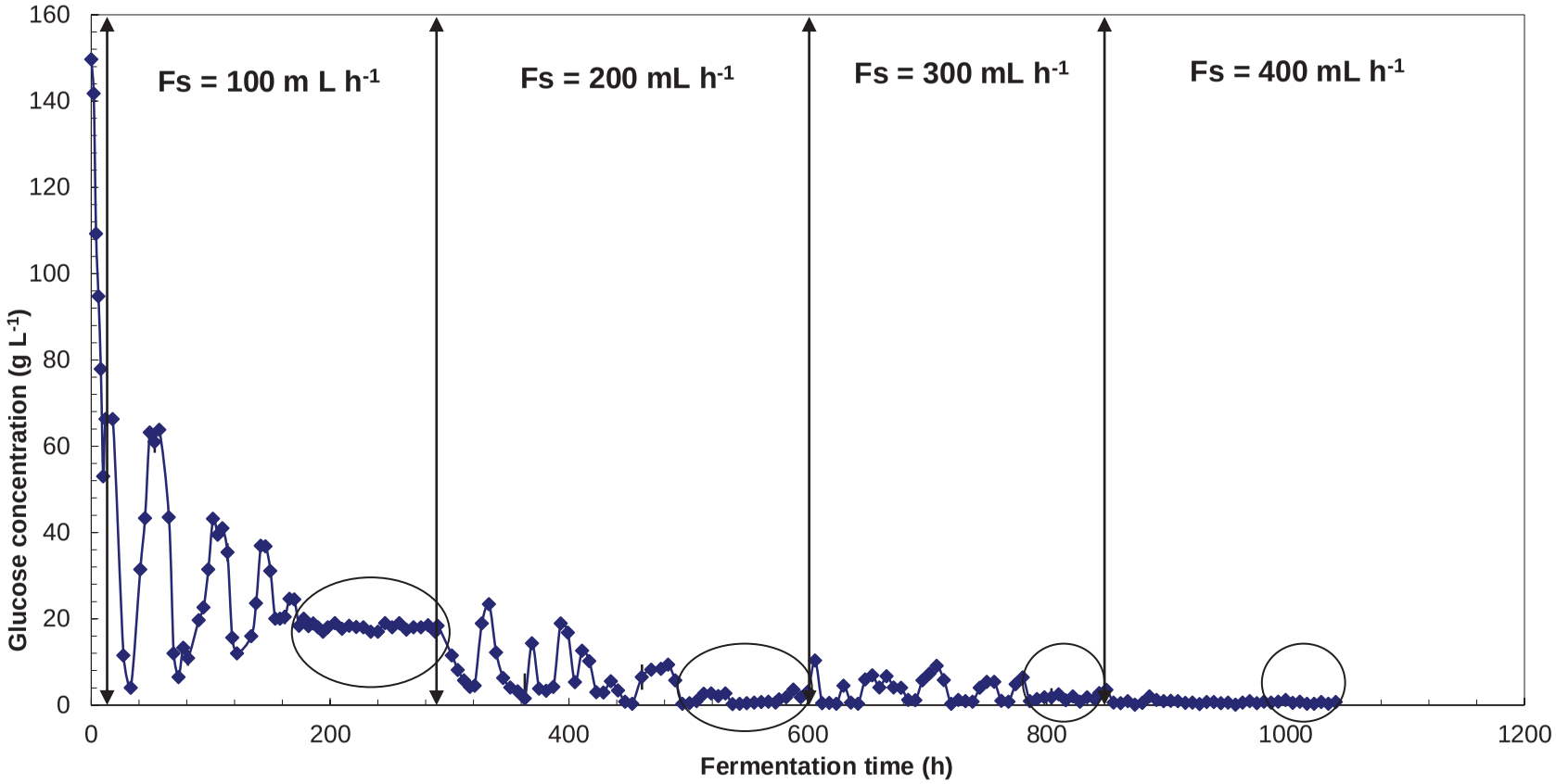


Figure 4.20 Glucose concentration in the aqueous phase of continuous extractive fermentation (feed glucose concentration = 150 g L^{-1}, dilution rate 0.05 h^{-1}, 35 °C, agitation rate of 140 rpm). Fs is the flow rate of the solvent. The bioreactor had a constant working volume of 1 L of culture broth and 1 L of solvent.

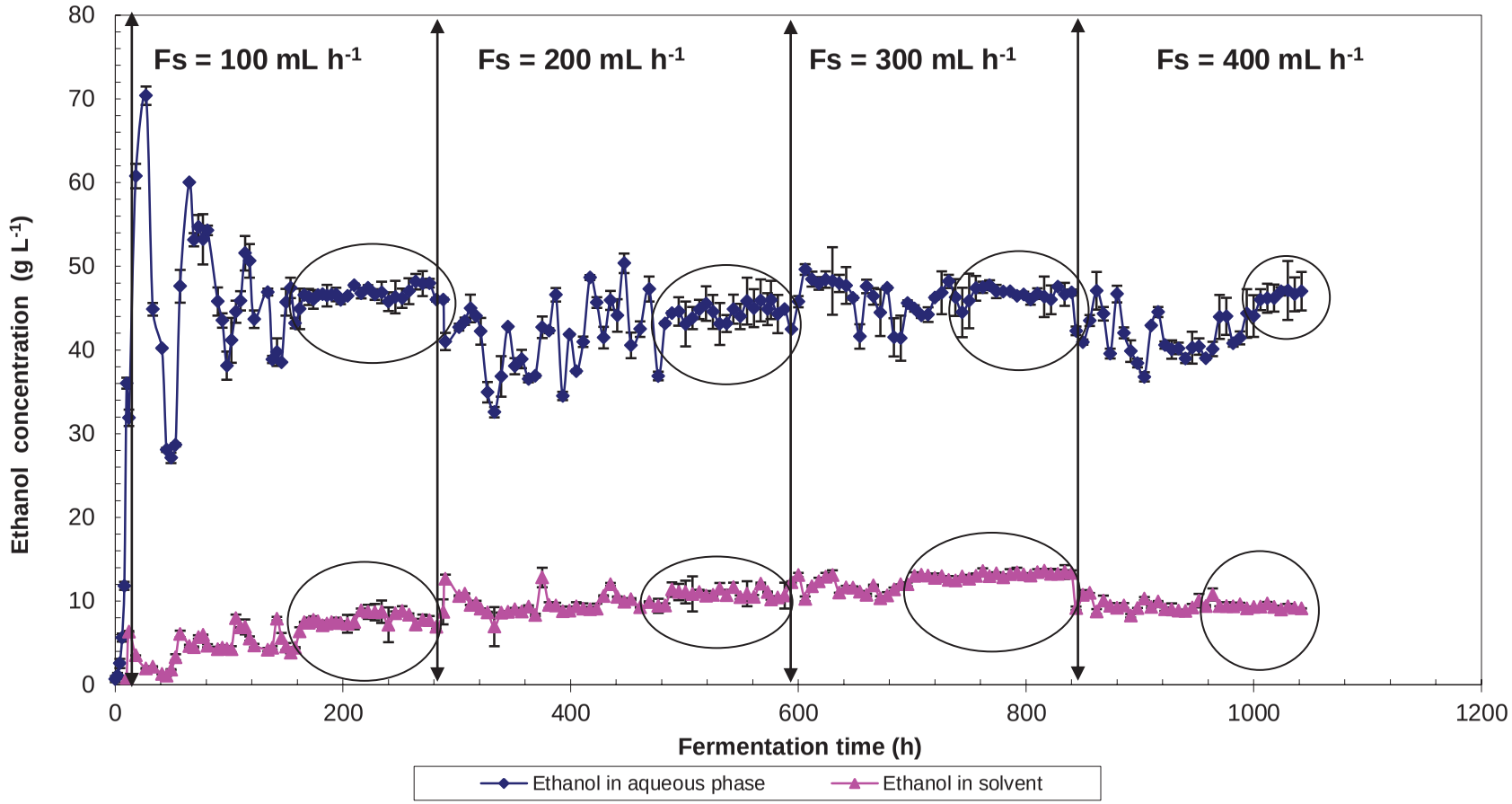


Figure 4.21 Ethanol concentration in the aqueous phase and the solvent phase of continuous extractive fermentation (feed glucose concentration 150 g L^{-1}, dilution rate 0.05 h^{-1}, 35 °C, agitation rate of 140 rpm). Fs is the flow rate of the solvent. The bioreactor had a constant working volume of 1 L of culture broth and 1 L of solvent.

Table 4.9 Steady state kinetic parameters at different solvent flowrates and a constant medium flowrate (dilution rate = 0.05 h^{-1})

Kinetic parameter	Solvent flowrate (mL h^{-1})			
	100	200	300	400
Average biomass productivity, P_x (g L^{-1} h^{-1})	0.054 ± 0.002	0.061 ± 0.001	0.066 ± 0.002	0.065 ± 0.001
Average biomass yield on glucose, $Y_{x/s}$ (g g^{-1})	0.008 ± 0.000	0.008 ± 0.000	0.009 ± 0.000	0.009 ± 0.000
Average biomass concentration, X (g L^{-1})	1.080 ± 0.040	1.220 ± 0.006	1.310 ± 0.030	1.290 ± 0.020
Average ethanol productivity in aqueous phase, P_{EA} (g L^{-1} h^{-1})	2.361 ± 0.046	2.254 ± 0.050	2.335 ± 0.020	2.335 ± 0.060
Average ethanol productivity in solvent, P_{ES} (g L^{-1} h^{-1})	0.770 ± 0.077	2.230 ± 0.100	4.020 ± 0.050	3.710 ± 0.090
Total ethanol productivity, P_{ET} (g L^{-1} h^{-1})	1.560 ± 0.060	2.240 ± 0.080	3.180 ± 0.040	3.020 ± 0.070
Average ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.474 ± 0.012	0.608 ± 0.016	0.856 ± 0.007	0.809 ± 0.008
Average ethanol concentration in aqueous phase, E_A (g L^{-1})	47.22 0± 0.920	45.080 ± 0.950	46.690 ± 0.470	46.690 ± 0.380
Average ethanol concentration in solvent, E_S (g L^{-1})	7.660 ± 0.690	11.130 ± 0.520	13.400± 0.170	9.280 ± 0.150
Average glucose concentration, S (g L^{-1})	18.00 ± 0.770	2.500± 0.990	1.600 ± 0.530	0.510 ± 0.290
Average partition coefficient, K_d	0.162 ± 0.010	0.247 ± 0.010	0.287 ± 0.050	0.199 ± 0.007

At a dilution rate of 0.05 h^{-1}, a solvent flow rate of 300 mL h^{-1} provided the highest total ethanol productivity and ethanol yield on glucose (Tables 4.9) while keeping the solvent use to a minimum.

Previously, in continuous fermentation of *Z. mobilis* at feed glucose concentration of ≥ 150 g L^{-1} oscillations were reported by Lee *et al.* (1979). Ghommidh *et al*. (1989) reported a failure to eliminate such oscillations in extended operation of 200 – 400 h. Jöbses *et al.* (1986a) observed for continuous cultures of *Z. mobilis* that oscillations occurred if the ethanol concentration exceeded 55 g L^{-1}. In situ extraction (Figure 4.21) did not allow the ethanol concentration in this study to build up to 50 g L^{-1}. The continuous extractive principle demonstrated here to suppress oscillations in a continuous fermentation subject to product inhibition may be effectively used for other similar fermentations. For example, oscillations have been reported in continuous production of reuterin in by *Lactobacillus reuteri* (Rasch *et al.*, 2002) and ethanol fermentation of *Z. mobilis* (Daugulis *et al.,* 1997; McLellan *et al.,* 1999). High-gravity continuous ethanol fermentations of *S. cerevisiae* (Beuse *et al.,* 1998; Borzani, 2001; Chen and McDonald, 1990b; Chen *et al*., 1990a; Duboc *et al*., 1996; Patnaik, 2003; Satroutdinov *et al*., 1992; Shen *et al.,* 2010) are also known to experience oscillations.

4.3 In situ removal of ethanol from fermentation broth by solid-sorbents method

This section discusses the results of in-situ removal of ethanol from the fermentation broth by adsorption on the various polymer resins (Section 3.3). The objective was continuous removal of ethanol by selective adsorption to minimize product inhibition of the fermentation. To do this, fermentation broth from the bioreactor was recirculated through a packed bed adsorption column containing the adsorbent particles. Prior to this, experiments were done to characterize the selective adsorption potential of the various adsorbents and assess their biocompatibility with the cells.

4.3.1 Equilibrium adsorption of ethanol and glucose on polymer resins

The equilibrium adsorption of ethanol on Dowex Optipore L-493, Amberlite XAD-16, poly(styrene-co-divinylbenzene) and poly(4-vinylpyridine) was examined in separate experiments at 35 °C. Adsorption isotherms of ethanol on the tested resins are shown in Figure 4.22. The specific loading of ethanol on the resins increased with increasing initial concentration of ethanol in solution. Adsorption solutions consisted of various different ethanol concentrations in the range of 1 – 5 % (w/v) and the experiments were carried out as noted in Section 3.15.1. Dowex Optipore L-493 was clearly the best adsorption resin in view of its high specific loading for ethanol. The second best resin was poly(styrene-co-divinylbenzene). For all resins, the amount of ethanol adsorbed increased almost linearly with increasing concentration of ethanol in the concentration range of 1 – 5 % (w/v). Data on percent ethanol removal by adsorption on a given mass of the resins from a given volume of an aqueous solution of ethanol of a given initial concentration, at equilibrium are shown in Figure 4.23. Under the conditions used, Dowex Optipore L-493 had the greatest ethanol removal capability. Once the initial ethanol concentration reached 50 g L^{-1}, percent ethanol removal declined (except for Amberlite XAD 16) compared the data for solutions with initial ethanol concentrations of $\leq$ 40 g L^{-1} (Figure 4.23). This was because all the resins (except for Amberlite XAD 16) were becoming saturated with ethanol.

The equilibrium adsorption of glucose on the various resins was investigated from aqueous glucose solution of initial glucose concentrations in the range of 50 – 250 g L^{-1}. The methodology used was as explained in Section 3.15.2. All resins showed some adsorption of glucose (Figure 4.24). In all cases, adsorption increased with increasing initial concentration of glucose in solution. Amberlite XAD-16 was the best adsorbent for glucose (Figure 4.24). On the basis of these results, Amberlite XAD-16 is not a suitable adsorbent for in situ removal of ethanol from fermentation broths. Amberlite resin is a weakly basic adsorbent and has been used for recovery of carboxylic acids from aqueous solutions and fermentation broths (Uslu, 2009; Uslu *et al.*, 2010).

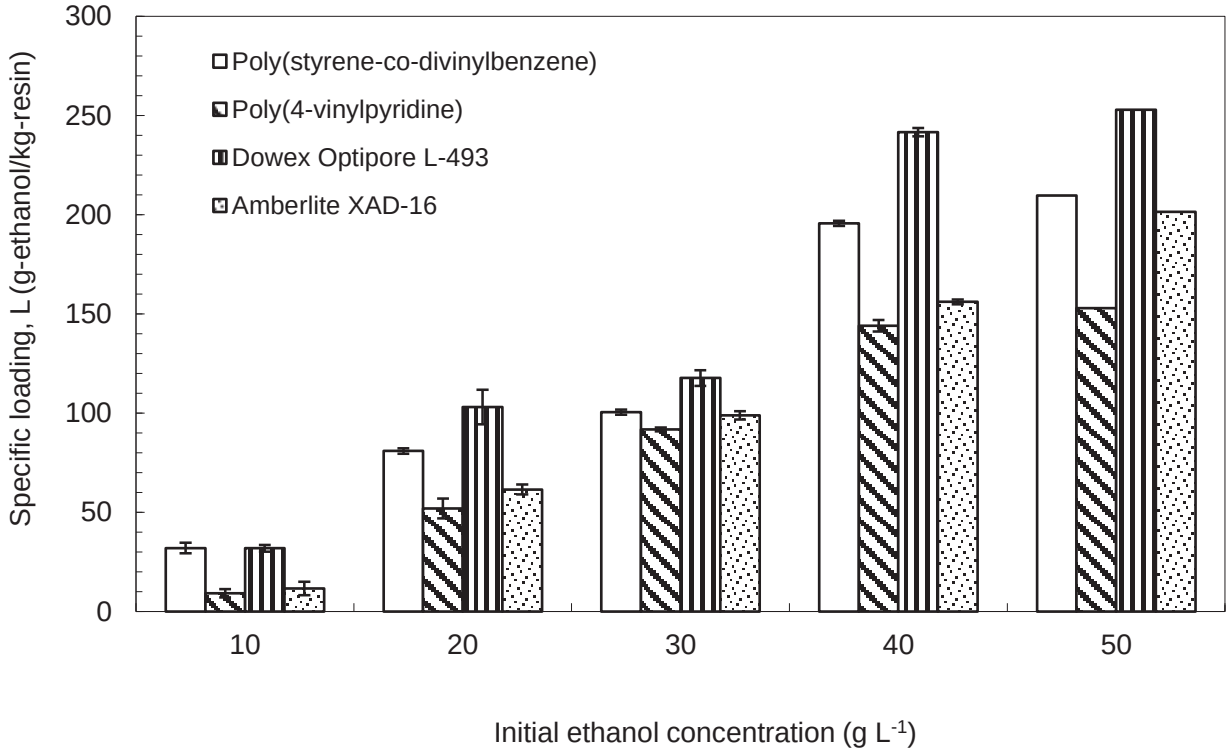


Figure 4.22 Adsorption isotherms of ethanol on solid-sorbents materials. Based on three replicates in each case.

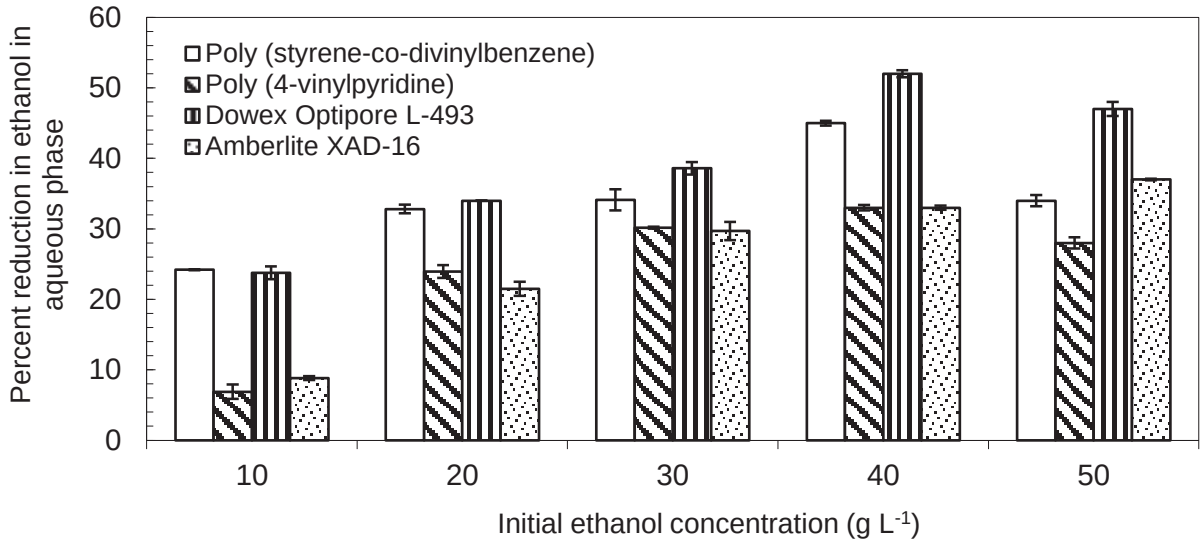


Figure 4.23 Percent reduction of ethanol in the aqueous phase (10 mL) in equilibrium with 1 g of the resin. Based on three replicates in each case.

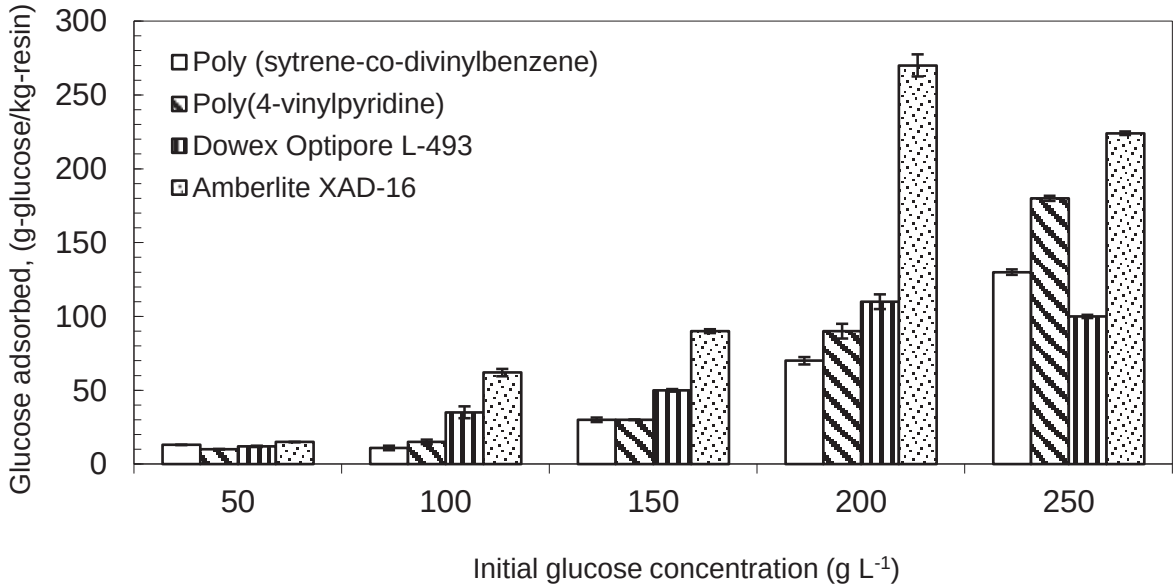


Figure 4.24 Adsorption isotherms of glucose on the solid adsorbents. Based on three replicates in each case.

4.3.2 Adsorption isotherms

Empirical adsorption isotherms for ethanol on the various resins at 35 °C are shown in Figure 4.25 based on the data in Figure 4.22. In Figure 4.25, q_e (g kg^{-1}) is the equilibrium adsorption capacity of the resins for ethanol and C_e (g L^{-1}) is the equilibrium concentration of ethanol in the solution in equilibrium with the resins.

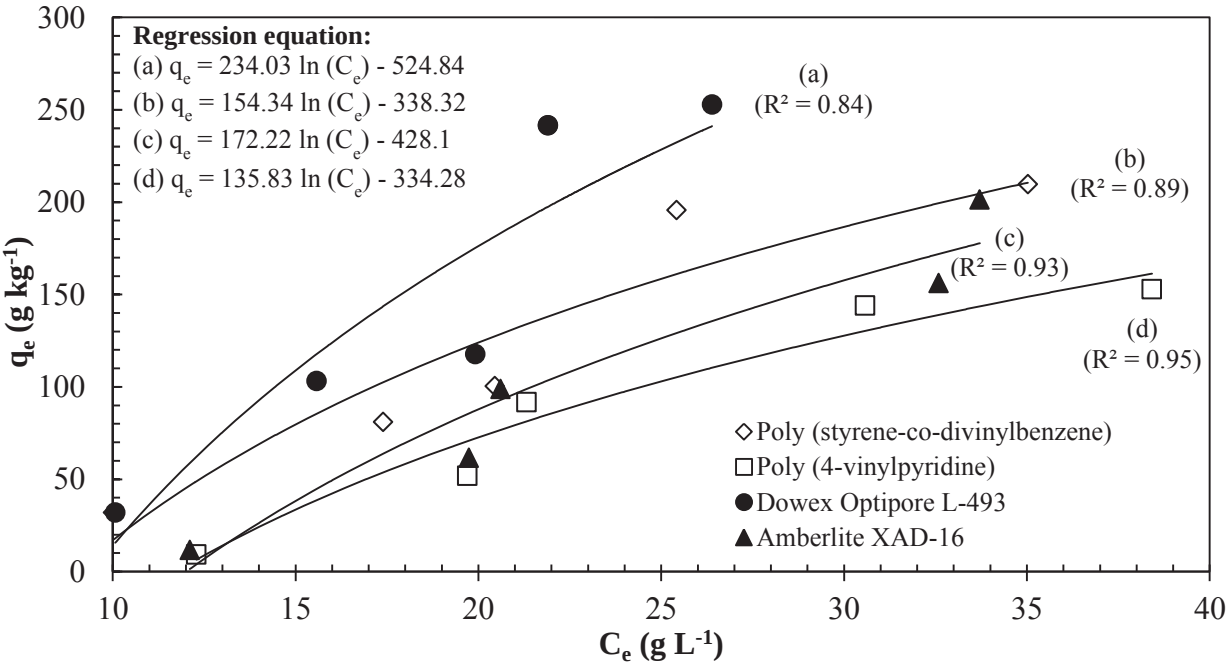


Figure 4.25 Equilibrium adsorption data for ethanol on various resins at 35 °C. The lines are empirical best fit curves (Equation a-d) for the data.

Attempts were made to fit the data in Figure 4.25 with the Langmuir isotherm and Freundlich isotherm (Garcia-Delgado *et al.*, 1992; Li *et al.*, 2002; Li *et al.*, 2004; Nielsen *et al.*, 2010). The equation for Langmuir isotherm is as follows:

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \qquad Equation\ 4.1$$

where C_e is the equilibrium concentration of ethanol in solution (g L^{-1}), q_e is the equilibrium adsorption capacity of ethanol on the adsorbent (g kg^{-1}), q_m is the maximum adsorption capacity of ethanol on the adsorbent (g kg^{-1}), and K_L is a constant. Equation 4.1 can be rearranged to the following:

$$\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{q_m K_L} \qquad Equation\ 4.2$$

Thus, for a system conforming to the Langmuir adsorption model a plot of C_e/q_e versus C_e can be used to estimate q_m from the slope (slope = $1/q_m$) and K_L from y-intercept (y-intercept = $1/q_m K_L$). Figure A1 (Appendix 2I) shows Langmuir plots (Equation 4.2) for the data.

The Freundlich isotherm can be expressed as follows:

$$q_e = K_F \times C_e^{\frac{1}{n}} \qquad Equation\ 4.3$$

where K_F is a characteristic parameter and an indicator of the adsorption capacity, the constant n is an indicator of the intensity of adsorption. Equation 4.3 can be linearized to the following:

$$\log q_e = logK_F + \frac{1}{n} logC_e \qquad Equation\ 4.4$$

Figure A2 (Appendix 2I) shows Freundlich plots for the data (Equation 4.4).

Thus, for a system conforming to Freundlich adsorption, the coefficient n and K_F can be estimated from a plot of log q_e versus log C_e. The slope of such a plot provides $1/n$ and the y-intercept provides K_F (y-intercept = log K_F).

The adsorption data in Figure 4.25 were fitted to the Langmuir equation (Eq. 4.2) and the Freundlich equation (Eq. 4.4). The regression equations, the correlation coefficients R^2 for the fits and the calculated parameters (q_m, K_L, K_F, n) are summarized in Table 4.10 and Table 4.11. In view of the low values of the regression coefficients in Table 4.10, the data did not conform to

Langmuir adsorption. The negative q_m values in Table 4.10 and K_L values are therefore completely meaningless. The Langmuir adsorption model assumes that the molecules of adsorbate adsorb at energetically identical well-dispersed sites without interacting with each other. In a porous adsorption matrix such as the polymer beads used in this work the adsorbate molecules must diffuse into pores. Adsorption therefore occurs first at the adsorption sites near to the entrance of the pores and later at sites located deeper in a pore. Therefore, the adsorption sites cannot be considered all identical and this likely explains a failure of the data to fit this model. The data did fit the Freundlich adsorption model well (regression coefficient value ≥ 0.96 in Table 4.11). The values of K_F in the Freundlich model are a relative indicator of adsorption capacity of a resin (Raja, 2006). The high K_F value (Table 4.11) for Dowex Optipore L-493 and poly (styrene-co-divinylbenzene) indicate higher adsorption capacity of these resins compared to the other resins.

From the equilibrium data, the adsorption Gibbs free energy (ΔG) for ethanol on the various resins can be calculated as follows (Garcia-Delgado *et al.*, 1992):

$$\Delta G = -nRT$$

where ΔG is the adsorption Gibbs free energy (kJ/mol), n is the characteristic constant in Freundlich isotherm, R is the ideal gas constant, and T is the absolute temperature. The calculated ΔG values are presented in Table 4.12. The ΔG values are all negative, which indicates that the adsorption is exothermic (Raja, 2006). Based on negative ΔG values, adsorption leads to a decrease in the surface energy of the adsorbent.

Table 4.10 Regression equations of C_e/q_e vs C_e for Langmuir isotherms at 35 °C

Adsorbent	Regression equation	q_m	K_L	R^2
Dowex Optipore L-493	$\frac{C_e}{q_e} = -0.013\,C_e + 0.403$	−79.365	−0.031	0.769
Amberlite XAD-16	$\frac{C_e}{q_e} = -0.031\,C_e + 1.127$	−32.258	−0.028	0.604
Poly (styrene-co-divinylbenzene)	$\frac{C_e}{q_e} = -0.006\,C_e + 0.335$	−166.07	−0.017	0.664
Poly (4-vinylpyridine)	$\frac{C_e}{q_e} = -0.034\,C_e + 1.308$	−29.586	−0.026	0.510

Table 4.11 Regression equations of log q_e vs log C_e for Freundlich isotherms at 35 °C

Adsorbent	Regression equation	K_F	n	R^2
Dowex Optipore L-493	$\log q_e = 2.191 \log C_e - 0.667$	0.215	0.456	0.940
Amberlite XAD-16	$\log q_e = 2.576 \log C_e - 1.599$	0.025	0.388	0.923
Poly (styrene-co-divinylbenzene)	$\log q_e = 1.602 \log C_e - 0.077$	0.837	0.624	0.954
Poly (4-vinylpyridine)	$\log q_e = 2.475 \log C_e - 1.559$	0.028	0.404	0.878

Table 4.12 Adsorption Gibbs free energy parameters of ethanol on the resins at 35 °C

Adsorbent	- ΔG= nRT (kJ/mol)
Dowex Optipore L-493	1.971
Amberlite XAD-16	1.946
Poly (styrene-co-divinylbenzene)	1.357
Poly (4-vinylpyridine)	2.074

4.3.3 Batch solid-sorbent extractive fermentation

The biocompatibility of the various resins with *Z. anaerobia* was investigated by culturing the organism with the resins at 35 °C, 100 rpm agitation rate, for 24 h (Section 3.15.1). The ratio of the culture broth to resin was 10:1 by weight. No resin was added to the control culture. No harmful effects on growth were seen with any of the resins (Figure 4.26) with the possible exception of Amberlite XAD-16. The cells tended to accumulate around the resin particles so care was taken to vigorously shake each culture flask prior to sampling for biomass growth. The results (based on 4 independent culture flasks for each resin and the controls) were quite reproducible (Figure 4.26). Biomass growth and the final yield were low in flasks which contained Amberlite XAD 16. This may have been partly because this resins tended to remove glucose from solution by adsorption (see Figure 4.24). Similarly, poly (4-vinylpyridine) adsorbed glucose from solution (Figure 4.24) and therefore reduced biomass growth (Figure 4.26). Poly (4-vinylpyridine) did not reduce growth as much as Amberlite XAD 16 likely because it was less effective than Amberlite XAD 16 in removing glucose.

Glucose concentration profiles for the fermentations shown in Figure 4.26 are shown in Figure 4.27. A substantial amount of residual glucose remained in the broth supplemented with Amberlite XAD 16. This suggests that glucose removed by the resin as discussed above cannot account for the reduced growth and the low final yield seen with this resin (Figure 4.26). This resin may have removed other trace nutrients from the broth or may have had a direct growth suppressory effect. Dowex Optipore L-493 actually improved biomass growth and the final yield

relative to control (Figure 4.26). This effect was linked to its ability to remove ethanol from solution (Figure 4.23) and thereby reduce the inhibitory effect of ethanol. Also, Dowex Optopire L-493 had a low adsorption capacity for glucose (Figure 4.24) and therefore did not deprive the microorganism of this essential growth substrate.

Note that the cell mass that attached to the adsorbents may not have been measured fully and therefore the data for some of the resins in Figure 4.26 may have been an underestimate relative to control.

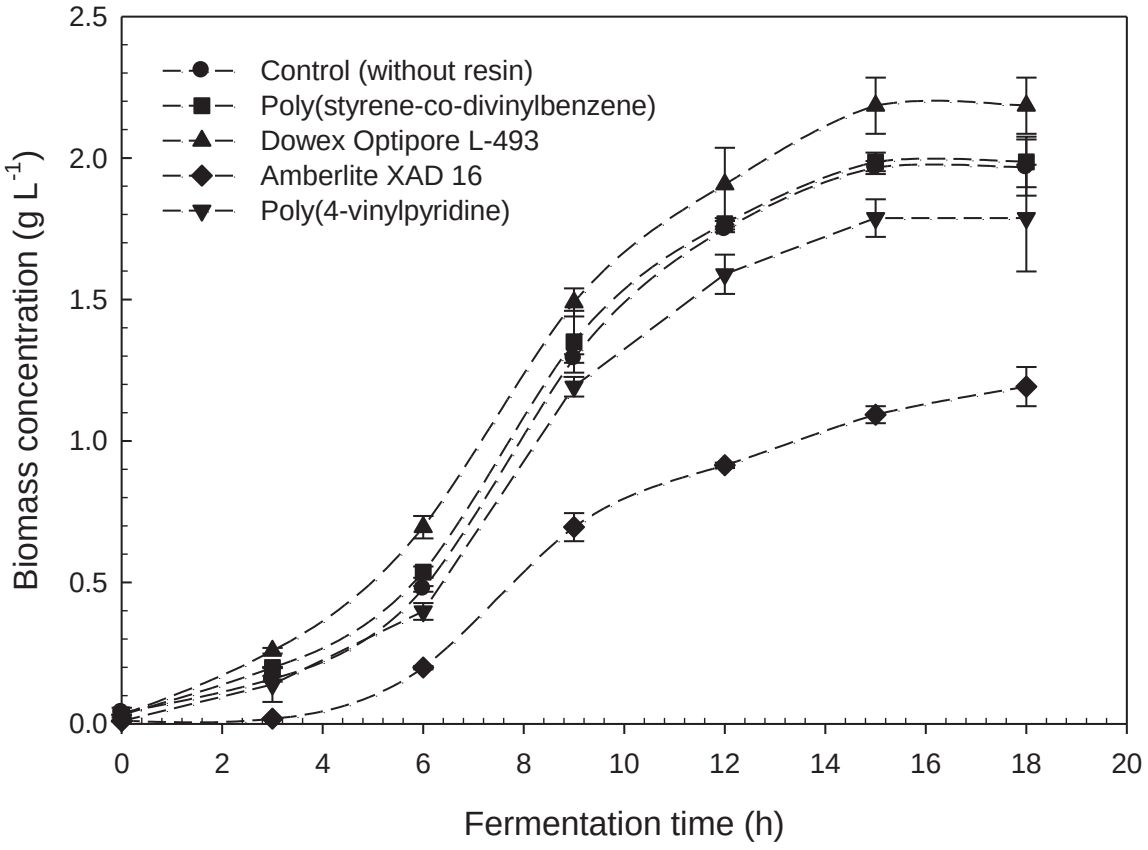


Figure 4.26 Biomass concentration profiles in fermentations involving adsorption resins. The initial glucose concentration was 150 g L^{-1}. Data were measured in three replicate fermentations for each resin.

Glucose consumption of the fermentations conducted with Dowex Optipore L-493 was comparable to that of the control (Figure 4.27).

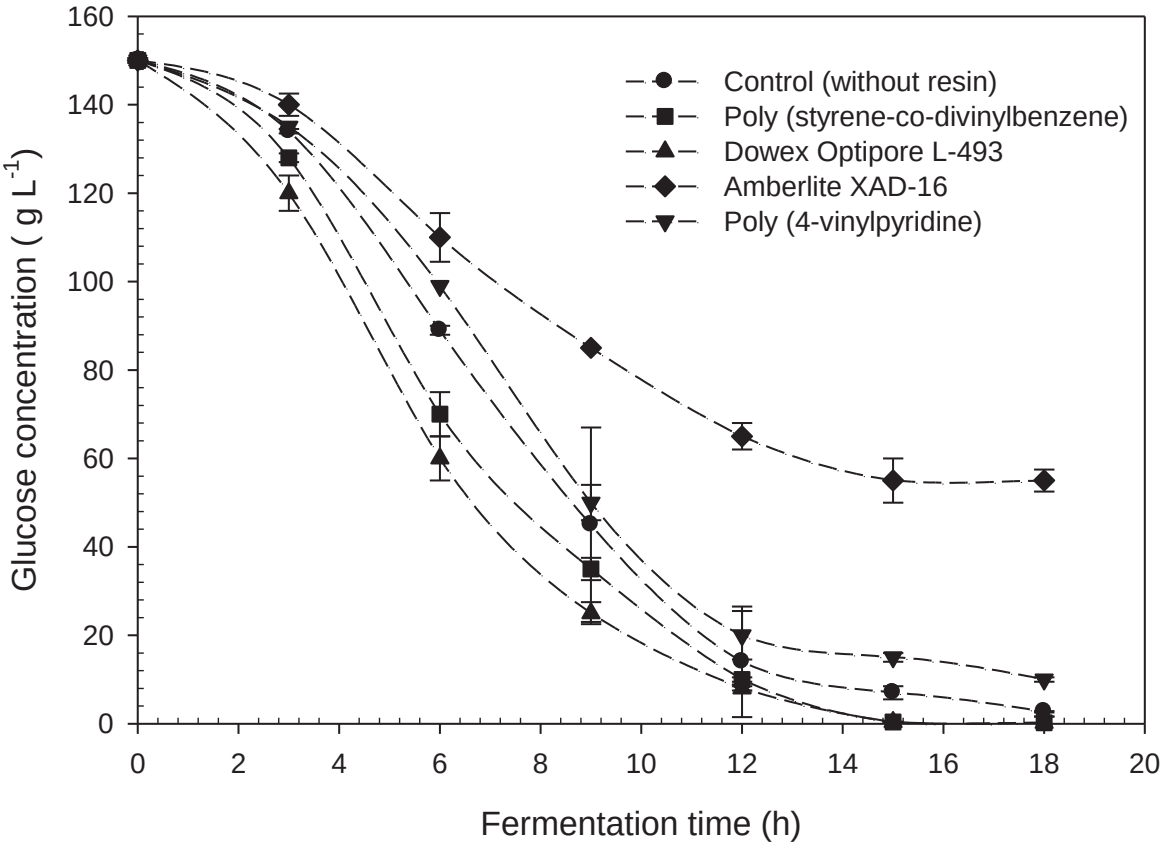


Figure 4.27 Residual glucose concentration profiles in the fermentations involving adsorption resins. The initial glucose concentration was 150 g L^{-1}. Data were measured in three replicate fermentations for each resin.

The effects of in situ adsorption of ethanol on its production are shown in Figure 4.28. The ethanol concentration shown includes the amount adsorbed on the resin phase. This was measured by desorption after the fermentation as explained in Section 3.16. Based on Figure 4.28, Dowex Optipore L-493 appeared to be somewhat better relative to control. With this resin, a final ethanol concentration of 63.3 ±1.035 g L^{-1} was obtained, or nearly 1.2-fold that of the control fermentation.

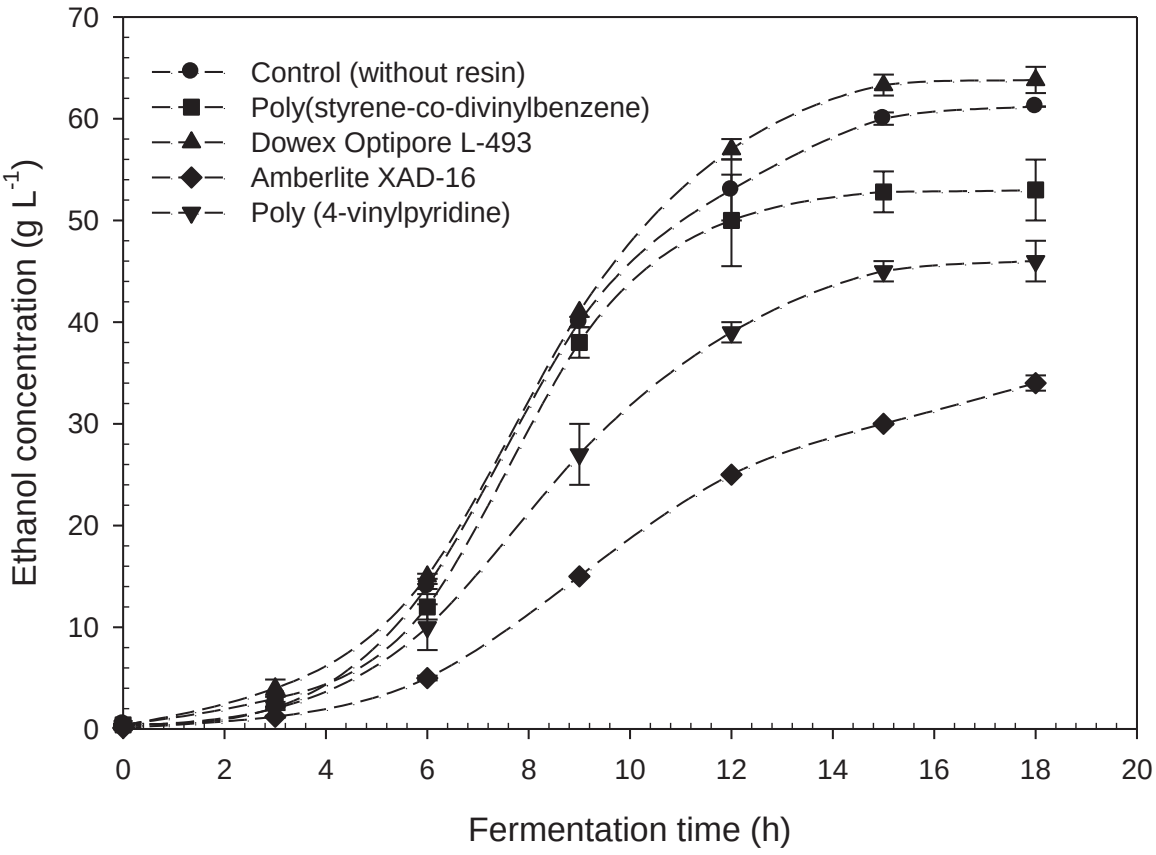


Figure 4.28 Ethanol concentration profiles in the fermentations involving adsorption resins. The initial glucose concentration was 150 g L^{-1}. Data were measured in three replicate fermentations for each resin.

The kinetic parameters of the fermentations (Figure 4.26-4.28) with adsorption resins and the control fermentation are summarized in Table 4.13. Use of Dowex Optipore L-493 improved: the maximum biomass productivity by about 13% relative to control; the maximum ethanol productivity by about 9% relative to control; the maximum yield on substrate by about 7% relative to control. As Dowex Optipore L-493 resin measurably improved the fermentation performance relative to control, this resin alone was further tested in the continuous fermentation studies described in the next section (Section 4.3.4).

Table 4.13 Comparison of the kinetic parameters of batch fermentations with added adsorbents

Kinetic parameter	Control	Resins			
		Poly (styrene-co-divinylbenzene)	Dowex Optipore L-493	Amberlite XAD-16	Poly (4-vinylpyridine
Maximum biomass productivity, P_x (g L^{-1} h^{-1})	0.120 ± 0.006	0.122 ± 0.031	0.135 ± 0.006	0.074 ± 0.001	0.111 ± 0.012
Maximum biomass yield on glucose, $Y_{x/s}$ (g g^{-1})	0.013 ± 0.001	0.013 ± 0.000	0.014 ± 0.001	0.012 ± 0.000	0.013 ± 0.001
Maximum ethanol productivity, P_E (g L^{-1} h^{-1})	3.721 ± 0.066	3.474 ± 0.061	4.041 ± 0.034	2.128 ± 0.48	2.919 ± 0.125
Maximum ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.404 ± 0.001	0.371 ± 0.007	0.432 ± 0.005	0.367 ± 0.043	0.334 ± 0.014
Maximum specific growth rate, μ (h^{-1})	0.972 ± 0.081	0.271 ± 0.022	0.306 ± 0.010	0.152 ± 0.011	0.280 ± 0.017

Based on three replicate fermentations for each case. No adsorbent was added to control fermentations.

4.3.4 Continuous solid-sorbent extractive fermentation

This section discusses the continuous solid-sorbent extractive fermentation conducted using Dowex Optipore L-493 resin. The relevant experimental procedure was described in Section 3.17. The column filled with Dowex Optipore L-493 resin (100g) was sterilized at 121 °C for 15 min. *Z. anaerobia* broth was recirculated through the column from a 3 L fermentor (Section 3.17). A 150 g L^{-1} glucose medium was used to feed the bioreactor. Feeding and recirculation through the adsorption column began a 10 h after the batch fermentation phase. The recirculation flow rate was 0.22 L min^{-1}. The dilution rate was 0.05 h^{-1}.

The fermentation profiles are shown in Figure 4.29 and Figure 4.30. Under the conditions used, a steady state was achieved by 180 h (Figure 4.29, Figure 4.30). No oscillations occurred during the steady state. Under otherwise identical conditions, but without adsorptive removal of ethanol, a continuous fermentation had completely failed to attain steady state (Figure 4.10, Figure 4.11, Figure 4.12) despite being run for 475h. Thus, compared to control (Figures 4.10-4.12), adsorptive removal of ethanol with Dowex Optipore L-493 allowed steady state operation (Figures 4.29-4.30). The values of the various fermentation parameters at the steady state are shown in Table 4.14. At steady state, an ethanol productivity of 2.627 ± 0.039 g ethanol L^{-1} h^{-1} was obtained with an 89% substrate utilization (Table 4.14).

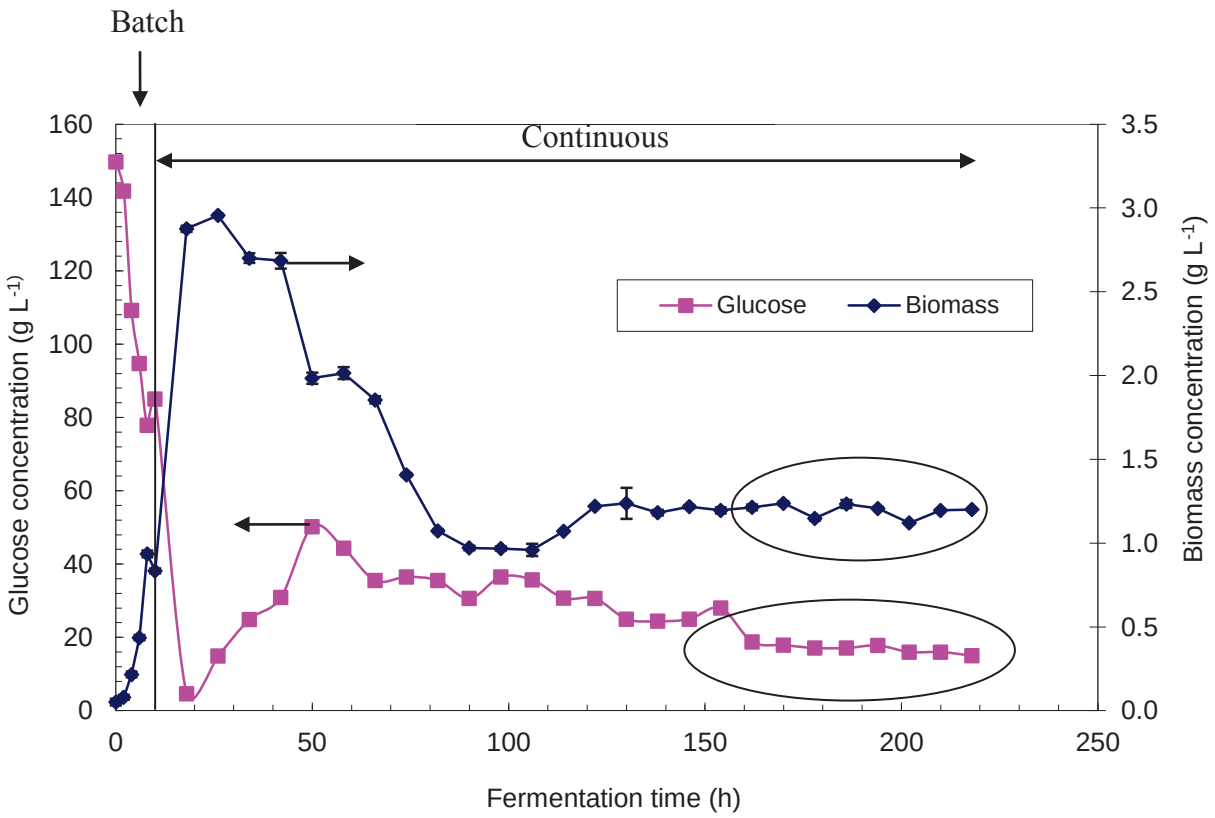


Figure 4.29 Biomass and glucose concentration profiles in continuous fermentation with ethanol extraction in a fluidized bed. The working volume of the bioreactor was 1 L.

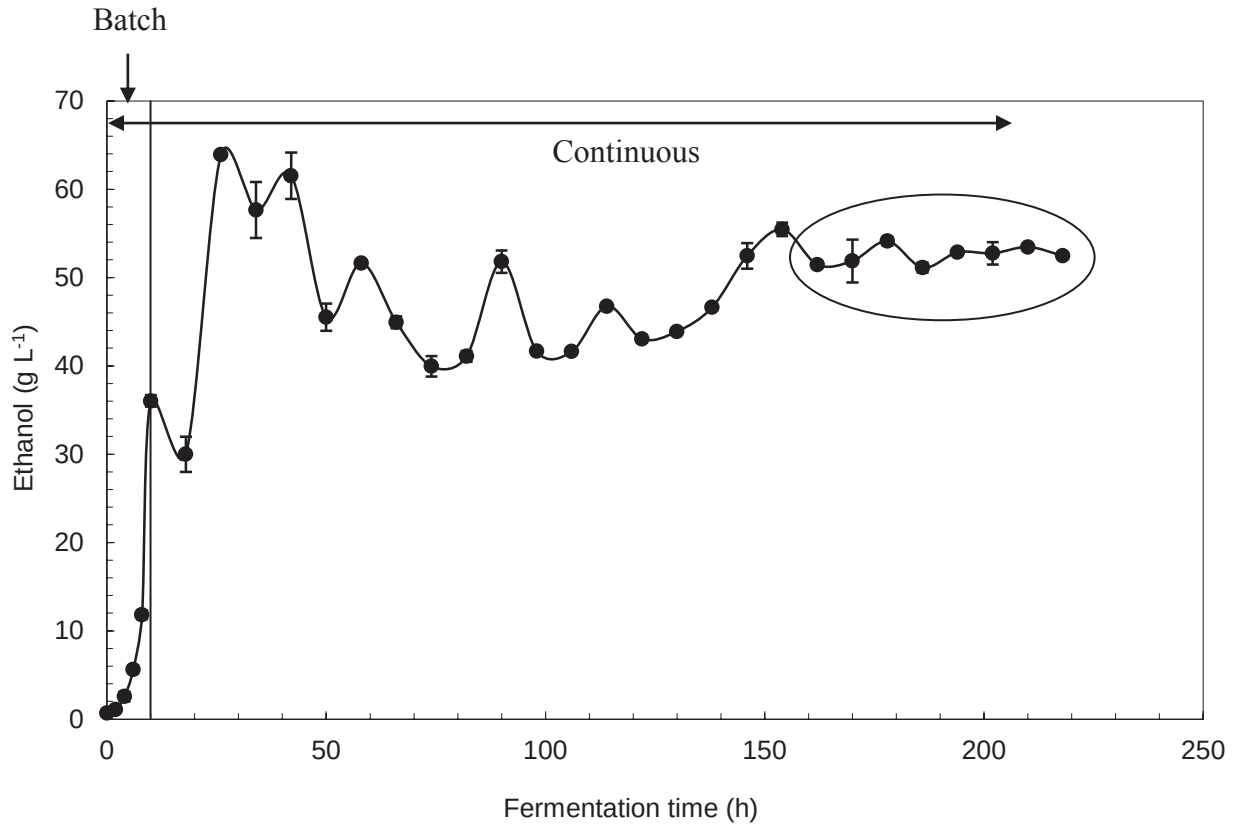


Figure 4.30 Ethanol concentration profile in continuous fermentation with ethanol extraction in a fluidized bed. The working volume of the bioreactor was 1 L.

Table 4.14 Steady state kinetic parameters of the adsorptive continuous fermentation at dilution rate of 0.05 h^{-1} and column recirculation flowrate of 0.22 L min^{-1}

Kinetic parameters	Value
Residual glucose concentration, S (g L^{-1})	16.00 ± 0.970
Biomass concentration, X (g L^{-1})	1.300 ± 0.037
Ethanol concentration, P(g L^{-1})	52.53 ± 0.770
Biomass yield on substrate, $Y_{x/s}$ (g g^{-1})	0.010 ± 0.000
Biomass productivity, P_x (g L^{-1} h^{-1})	0.065 ± 0.002
Ethanol yield on substrate, $Y_{p/s}$ (g g^{-1})	0.392 ± 0.009
Ethanol productivity, P_E (g L^{-1} h^{-1})	2.627 ± 0.039

CHAPTER 5

CONCLUDING REMARKS AND RECOMMENDATIONS

5.1 Conclusions

This work aimed to investigate the effects of a high substrate concentration on ethanol production by *Zymomonas anaerobia* ATCC 29501. Use of a concentrated substrate was desired to enhance ethanol productivity. Attempts were made to at least partly relieve product inhibition and achieve steady state operation in continuous fermentation.

Based on the results of this study, the following conclusions could be drawn:

1. In a batch fermentation conducted at optimal temperature (35 °C), the maximum permissible initial glucose concentration is 150 g L^{-1} and this allows an average productivity of 4.33 ± 0.099 g L^{-1} h^{-1} to be achieved.

2. Ethanol added to fermentation broth strongly inhibits *Z. anaerobia* growth as well as further production of ethanol and, therefore, in situ ethanol removal is useful for this fermentation.

3. In high gravity continuous fermentation, an oscillatory behaviour is observed and a steady state operation cannot be achieved especially at a low value of the dilution rate as a consequence of the inhibitory effect of ethanol.

4. In continuous high-gravity fermentations that failed to attain a steady state due to the above mentioned periodic oscillations, a steady state operation can be achieved by in situ removal of ethanol.

5. A screening of 11 solvents for in situ extraction of ethanol revealed iso-octadecanol to be best: this solvent was nontoxic to *Z. anaerobia* and could extract a significant amount of ethanol. Use of this solvent allowed a 1.2 fold increase in ethanol productivity compared to control fermentation. All the nontoxic solvents tested could be recovered and reused without substantial deterioration.

6. In extractive continuous fermentation using the best solvent, iso-octadecanol, a steady-state operation could be achieved at a dilution rate of 0.05 h^{-1} after 166 h of fermentation at a solvent recirculation rate of 100 mL h^{-1}. Without in situ extraction, a steady state operation proved to be impossible at the same dilution rate. By increasing the solvent recirculation rate to 400 mL h^{-1}, the total ethanol productivity could be increased to nearly 2-fold relative to the value at the lower solvent recirculation rate.

7. Of the four polymer resins evaluated for in situ removal of ethanol, Dowex Optipore L-493 was found to be the best. This resin reduced the aqueous ethanol concentration by 70% while achieving a specific loading of ethanol of 253 g/kg-resin at an initial ethanol concentration of 5% (w/v). Dowex Optipore L-493 was also found to not adsorb any measurable amount of glucose in the glucose concentration range of 50 – 250 g L^{-1} in the liquid.

8. For all adsorption resins tested, the adsorption isotherms were consistent with Freundlich adsorption model but not with Langmuir adsorption. This suggested that the ethanol adsorption sites on a given resin were not energetically identical.

9. In batch in situ adsorptive fermentations, Dowex Optipore L-493 was found to increase the cell growth by at least partly relieving ethanol inhibition and was clearly the most effective resin. Using Dowex Optipore L-493, a final ethanol concentration of 63.3±1.035 g L^{-1} was obtained, or nearly 1.2-fold that of the control fermentation. With Dowex Optipore L-493, the maximum ethanol productivity and maximum ethanol yield on substrate were 4.041 ± 0.034 g L^{-1} h^{-1} and 0.432 ± 0.005 g g^{-1}, respectively. Both these values were greater than for the control fermentation.

10. The use of Dowex Optipore L-493 in in-situ adsorptive continuous fermentation reduced the time needed to achieve a steady state by up to 54% compared to the control. An ethanol concentration of 52.53 ± 0.77 g L^{-1} could be obtained with 89% substrate utilization at a dilution rate of 0.05 h^{-1} and a flow rate through the fluidized bed column of 0.2 L min^{-1}.

5.2 Recommendations

Even though this work conclusively showed an improved ethanol productivity and an ability to attain steady state operation in high gravity fermentations with in situ removal of ethanol, the productivity is likely to be further enhanced if *Z. anaerobia* cells are retained in the bioreactor instead of being allowed to bleed in the harvest stream of continuous culture. This strategy is recommended for investigation in a possible future study.

LIST OF PUBLICATIONS

This work has been published or presented in part as follows:

1. Azilah Ajit, Ahmad Ziad Sulaiman, Yusuf Chisti, Extractive fermentation for enhanced productivity of bioethanol by Z. anaerobia, Presented at 14th International Symposium of Biotechnology and Exhibition (IBS 2010), Rimini, Italy, 14-16th September 2010. Abstract published in Journal of Biotechnology, Volume 150, Suplement 1, November 2010, Pages 135-136.

2. Azilah Ajit, Ahmad Ziad Sulaiman, Yusuf Chisti, High gravity fermentation for ethanol production by Zymomonas anaerobia. Presented at 3rd International Conference on Chemical and Bioprocess Engineering (ICCBPE 2010), Sabah, Malaysia, 16-18th August 2009.

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APPENDIX 1

The equations used in calculating the standard deviation of kinetic parameters for all experiments. (http://www.ecs.umass.edu/cee/reckhow/courses/572/572bk23/572BK23.html)

Addition or subtraction of two numbers (x_1,x_2) with significant errors (s_1, s_2)

$$(x_1 \pm s_1) + (x_2 \pm s_2) = (x_1 + x_2) \pm \sqrt{s_1^2 + s_2^2}$$

$$(x_1 \pm s_1) - (x_2 \pm s_2) = (x_1 - x_2) \pm \sqrt{s_1^2 + s_2^2}$$

Multiplication or division by a constant

$$n(x \pm s) = nx \pm ns$$

Where n is a constant

Multiplication or division of two numbers (x_1,x_2) with significant errors (s_1, s_2)

$$(x_1 \pm s_1)(x_2 \pm s_2) = (x_1 \times x_2) \pm (x_1 \times x_2)\sqrt{\left(\frac{s_1}{x_1}\right)^2 + \left(\frac{s_2}{x_2}\right)^2}$$

$$\frac{(x_1 \pm s_1)}{(x_2 \pm s_2)} = \frac{x_1}{x_2} \pm \left(\frac{x_1}{x_2}\right)\sqrt{\left(\frac{s_1}{x_1}\right)^2 + \left(\frac{s_2}{x_2}\right)^2}$$

APPENDIX 2

A) Batch fermentation for effect of high substrate concentration experiment

1.0 An initial glucose concentration of 50 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.05	0.01
1	0.06	0.01
2	0.10	0.01
3	0.16	0.01
4	0.25	0.02
5	0.36	0.02
6	0.64	0.01
7	0.90	0.01
8	1.25	0.02
9	1.43	0.02
10	1.49	0.07

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.98	0.10
1	1.12	0.09
2	1.40	0.08
3	2.04	0.25
4	2.60	0.07
5	4.01	0.45
6	5.97	0.06
7	10.71	0.10
8	13.19	0.34
9	18.28	1.87
10	23.00	1.73

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	49.65	0.22
1	48.85	0.91
2	44.68	0.28
3	38.72	0.25
4	32.92	0.18
5	31.92	0.22
6	28.37	0.19
7	24.87	0.22
8	14.30	0.10
9	7.84	0.16
10	1.24	0.04

2.0 An initial glucose concentration of 100 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.05	0.01
2	0.09	0.01
4	0.17	0.01
6	0.29	0.01
7	0.41	0.02
8	0.59	0.01
9	0.79	0.03
10	1.20	0.01
11	1.70	0.01
12	1.80	0.03
13	1.90	0.02
14	2.00	0.02

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.21	0.10
2	0.67	0.11
4	1.64	0.26
6	3.28	0.35
7	6.56	0.32
8	9.54	0.17
9	14.20	0.18
10	23.01	1.59
11	39.22	1.62
12	43.99	0.57
13	45.56	0.09
14	46.24	0.18

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	100.00	0.70
2	96.31	0.48
4	85.19	0.31
6	81.82	0.33
7	72.40	0.22
8	60.92	0.43
9	52.53	0.32
10	36.24	0.63
11	12.51	0.27
12	9.43	0.23
13	3.23	0.29
14	2.50	0.20

3.0 An initial glucose concentration of 150 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.02	0.01
2	0.04	0.03
4	0.06	0.01
6	0.18	0.02
8	0.32	0.01
10	0.53	0.03
11	0.71	0.01
12	0.94	0.00
13	1.26	0.01
14	2.12	0.00
15	2.22	0.01
16	2.24	0.01
17	2.24	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.51	0.03
2	0.84	0.01
4	1.84	0.07
6	3.67	0.38
8	7.48	0.25
10	14.69	0.08
11	21.47	0.12
12	27.33	0.00
13	40.80	0.62
14	47.44	0.13
15	61.16	0.12
16	62.29	0.19
17	62.16	0.05

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.93	0.17
2	141.49	2.25
4	140.99	2.29
6	121.73	1.91
8	105.89	1.72
10	95.17	2.24
11	90.55	1.26
12	75.51	3.18
13	50.74	1.44
14	28.00	0.73
15	19.31	0.56
16	2.85	1.22
17	2.48	1.25

4.0 An initial glucose concentration of 200 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.03	0.01
3	0.08	0.03
12	0.40	0.01
14	0.59	0.02
16	0.88	0.01
18	1.17	0.03
20	1.50	0.01
22	2.00	0.00
24	2.20	0.01
26	2.45	0.00
28	2.50	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.95	0.30
3	3.75	0.06
12	12.50	0.04
14	37.00	0.40
16	54.29	0.17
18	65.00	0.49
20	83.36	0.41
22	88.42	0.27
24	90.00	0.27
26	94.00	0.27
28	94.00	0.47

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	198.97	1.81
3	190.00	2.10
12	144.86	0.17
14	126.63	1.95
16	102.81	2.09
18	95.76	0.42
20	74.25	1.49
22	46.00	0.70
24	36.82	2.31
26	16.53	0.26
28	9.00	0.97

5.0 An initial glucose concentration of 250 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.00	0.01
3	0.01	0.01
6	0.02	0.01
9	0.07	0.01
18	0.25	0.02
20	0.37	0.01
22	0.57	0.03
24	0.70	0.01
26	0.90	0.01
28	1.29	0.03
30	1.53	0.02
32	1.67	0.02
34	1.74	0.03
36	1.82	0.05

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.22	0.00
3	1.93	0.25
6	2.84	0.05
9	4.95	0.53
18	25.00	1.23
20	35.00	0.28
22	55.02	0.60
24	80.00	1.25
26	85.00	0.51
28	90.00	0.03
30	92.00	3.91
32	94.00	1.19
34	94.00	0.02
36	94.10	0.02

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	250.00	2.92
3	240.00	0.00
6	235.00	7.13
9	220.00	10.94
18	128.72	10.62
20	119.95	1.00
22	101.20	0.50
24	96.94	0.50
26	86.78	0.50
28	73.20	0.50
30	56.98	0.50
32	45.09	1.50
34	39.74	1.00
36	26.64	0.64

6.0 An initial glucose concentration of 300 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.00	0.01
5	0.01	0.05
25	0.05	0.06
40	0.25	0.02
42	0.35	0.01
44	0.45	0.05
46	0.50	0.02
50	0.65	0.09
52	0.75	0.07
53	0.85	0.08
54	0.90	0.06
56	1.05	0.09

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.59	0.02
5	3.06	0.39
25	11.74	0.14
40	18.82	1.42
42	45.02	3.07
44	49.00	2.65
46	63.00	0.76
50	80.00	0.55
52	85.00	2.37
53	89.00	0.20
54	90.00	0.43
56	94.00	0.27

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	300.00	1.00
5	290.00	0.59
25	250.00	0.73
40	230.00	2.22
42	98.65	1.93
44	81.75	1.66
46	70.47	0.26
50	66.00	0.33
52	57.83	0.93
53	50.00	0.49
54	46.76	0.44
56	46.18	0.50

B) Batch fermentation for effect of ethanol added at 0 hour

1.0 Control (no ethanol added)

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.02	0.01
2	0.04	0.03
4	0.06	0.01
6	0.18	0.02
8	0.32	0.01
10	0.53	0.03
11	0.71	0.01
12	0.94	0.00
13	1.26	0.01
14	2.12	0.00
15	2.22	0.01
16	2.24	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.51	0.03
2	0.84	0.01
4	1.84	0.07
6	3.67	0.38
8	7.48	0.25
10	14.69	0.08
11	21.47	0.12
12	27.33	0.00
13	40.80	0.62
14	47.44	0.13
15	61.16	0.12
16	62.29	0.19

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.93	0.17
2	141.49	2.25
4	140.99	2.29
6	121.73	1.91
8	105.89	1.72
10	95.17	2.24
11	90.55	1.26
12	75.51	3.18
13	50.74	1.44
14	28.00	0.73
15	19.31	0.56
16	2.85	1.22

2.0 Ethanol concentration of 20 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.10	0.02
2	0.12	0.01
4	0.18	0.01
6	0.26	0.01
8	0.30	0.01
10	0.34	0.01
11	0.42	0.01
12	0.67	0.01
13	0.94	0.00
14	1.06	0.01
15	1.34	0.03
16	1.62	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	18.12	0.67
2	21.05	0.63
4	22.06	0.70
6	24.41	0.33
8	25.61	0.05
10	27.48	0.27
11	28.08	0.58
12	32.18	0.30
13	35.10	0.71
14	39.07	0.00
15	41.07	0.01
16	47.43	0.12

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	151.08	0.58
2	140.00	0.39
4	135.00	0.73
6	121.53	0.39
8	110.00	0.19
10	97.56	0.22
11	83.27	0.58
12	78.02	0.44
13	70.00	0.29
14	65.63	0.44
15	50.00	0.15
16	33.25	0.02

3.0 Ethanol concentration of 40 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.09	0.00
2	0.10	0.00
4	0.14	0.00
6	0.17	0.01
8	0.27	0.01
10	0.33	0.01
11	0.41	0.00
12	0.51	0.01
13	0.64	0.01
14	0.75	0.01
15	0.86	0.00
16	0.99	0.03

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	38.25	0.28
2	39.22	0.11
4	41.49	0.54
6	43.75	0.15
8	45.02	0.04
10	47.05	0.01
11	48.39	0.41
12	49.53	0.02
13	51.03	0.64
14	54.06	0.09
15	56.27	0.69
16	59.05	0.33

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	151.67	0.88
2	145.00	0.58
4	140.00	0.85
6	131.00	0.37
8	120.00	0.22
10	110.00	0.15
11	99.75	0.00
12	90.00	0.29
13	85.00	0.37
14	81.00	0.73
15	70.00	0.24
16	65.00	0.34

4.0 Ethanol concentration of 60 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.09	0.01
2	0.11	0.01
4	0.15	0.00
6	0.18	0.01
8	0.21	0.02
10	0.25	0.01
11	0.28	0.00
12	0.29	0.00
13	0.30	0.00
14	0.37	0.02
15	0.49	0.02
16	0.57	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	58.46	0.34
2	62.15	0.44
4	65.24	0.13
6	66.75	0.16
8	66.92	0.02
10	67.09	0.30
11	68.07	0.00
12	70.01	0.17
13	72.36	0.50
14	76.07	0.41
15	77.06	0.00
16	79.06	0.08

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	151.67	0.49
2	145.00	0.24
4	142.00	0.12
6	135.00	0.24
8	125.00	0.10
10	115.00	0.07
11	110.00	0.15
12	105.00	0.15
13	98.44	0.22
14	93.48	0.07
15	87.21	0.44
16	78.31	0.22

5.0 Ethanol concentration of 80 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.08	0.01
2	0.09	0.00
4	0.10	0.00
6	0.12	0.00
8	0.13	0.00
10	0.16	0.01
11	0.17	0.01
12	0.18	0.01
13	0.19	0.01
14	0.19	0.00
15	0.20	0.01
16	0.24	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	78.89	0.10
2	81.63	0.11
4	82.45	0.12
6	84.70	0.82
8	84.89	0.06
10	85.96	0.45
11	87.67	0.24
12	91.27	0.56
13	93.06	0.48
14	95.48	0.07
15	96.54	0.24
16	99.05	0.50

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	151.08	0.19
2	147.00	0.29
4	145.64	0.39
6	141.94	0.49
8	135.00	0.39
10	125.00	0.29
11	120.00	0.78
12	115.00	0.29
13	110.00	0.07
14	100.00	0.29
15	92.00	0.04
16	88.52	0.51

6.0 Ethanol concentration of 100 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.07	0.00
2	0.08	0.00
4	0.10	0.01
6	0.11	0.00
8	0.12	0.00
10	0.13	0.00
11	0.14	0.00
12	0.15	0.00
13	0.16	0.00
14	0.18	0.00
15	0.19	0.01
16	0.20	0.00

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	99.82	1.29
2	101.26	0.27
4	102.99	0.37
6	103.50	0.35
8	104.25	0.50
10	106.05	0.55
11	108.53	0.16
12	111.02	0.81
13	113.41	0.38
14	114.52	0.33
15	115.02	0.03
16	118.05	0.71

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	151.86	0.49
2	149.53	0.68
4	148.17	0.10
6	147.58	0.19
8	140.00	0.10
10	135.00	0.10
11	130.00	0.15
12	125.00	0.15
13	115.00	0.22
14	110.00	0.07
15	100.00	0.07
16	98.00	0.03

7.0 Ethanol concentration of 120 g L^{-1}

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.07	0.00
2	0.08	0.00
4	0.08	0.00
6	0.09	0.00
8	0.10	0.00
10	0.10	0.00
11	0.11	0.00
12	0.12	0.00
13	0.13	0.00
14	0.13	0.00
15	0.14	0.00
16	0.15	0.00

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	119.67	0.49
2	119.90	0.31
4	120.00	0.73
6	120.12	0.23
8	123.48	0.01
10	124.42	0.42
11	124.15	0.09
12	125.63	0.44
13	126.49	0.02
14	126.52	0.35
15	128.05	0.49
16	129.05	0.99

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	150.00	0.39
2	149.00	0.37
4	148.00	0.58
6	147.00	0.00
8	145.00	0.10
10	138.00	0.15
11	135.00	0.39
12	132.00	0.19
13	128.00	0.37
14	125.00	0.07
15	115.00	0.58
16	112.00	0.05

C) Batch fermentation for the effect of ethanol added at 10 hour

1.0 Control (no ethanol added)

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.02	0.01
2	0.04	0.03
4	0.06	0.01
6	0.18	0.02
8	0.32	0.01
10	0.53	0.03
11	0.71	0.01
12	0.94	0.00
13	1.26	0.01
14	2.12	0.00
15	2.22	0.01
16	2.24	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.51	0.03
2	0.84	0.01
4	1.84	0.07
6	3.67	0.38
8	7.48	0.25
10	14.69	0.08
11	21.47	0.12
12	27.33	0.00
13	40.80	0.62
14	47.44	0.13
15	61.16	0.12
16	62.29	0.19

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.93	0.17
2	141.49	2.25
4	140.99	2.29
6	121.73	1.91
8	105.89	1.72
10	95.17	2.24
11	90.55	1.26
12	75.51	3.18
13	50.74	1.44
14	28.00	0.73
15	19.31	0.56
16	2.85	1.22

2.0 Added ethanol concentration in flask of 1.9%

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.02	0.01
2	0.04	0.03
4	0.06	0.01
6	0.18	0.02
8	0.32	0.01
10	0.53	0.03
11	0.71	0.01
12	0.94	0.00
13	1.26	0.01
14	2.12	0.00
15	2.22	0.01
16	2.24	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.22	0.67
2	4.30	0.63
4	6.48	0.70
6	6.55	0.33
8	6.78	0.05
10	7.30	0.27
11	20.16	0.58
12	24.34	0.30
13	33.11	0.71
14	40.00	0.00
15	51.00	0.01
16	51.00	0.12

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	150.92	0.58
2	147.70	0.39
4	137.00	0.73
6	120.00	0.39
8	99.64	0.19
10	95.00	0.22
11	115.87	0.58
12	86.00	0.44
13	75.00	0.29
14	55.00	0.44
15	45.00	0.15
16	35.00	0.02

3.0 Added ethanol concentration in flask of 3.8%

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.08	0.00
2	0.11	0.00
4	0.22	0.00
6	0.33	0.01
8	0.68	0.01
10	1.20	0.01
11	0.59	0.00
12	0.67	0.01
13	0.80	0.01
14	1.20	0.01
15	1.34	0.00
16	1.47	0.03

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.12	0.28
2	4.34	0.11
4	6.49	0.54
6	6.65	0.15
8	6.82	0.04
10	7.66	0.01
11	39.49	0.41
12	30.86	0.02
13	35.79	0.64
14	37.00	0.09
15	45.00	0.69
16	46.00	0.33

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.43	0.88
2	146.70	0.58
4	138.81	0.85
6	119.75	0.37
8	103.06	0.22
10	94.00	0.15
11	114.83	0.00
12	99.49	0.29
13	89.76	0.37
14	73.28	0.73
15	63.00	0.24
16	53.00	0.34

4.0 Added ethanol concentration in flask of 5.8%

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.08	0.01
2	0.10	0.01
4	0.23	0.00
6	0.35	0.01
8	0.65	0.02
10	1.05	0.01
11	0.35	0.00
12	0.40	0.00
13	0.50	0.00
14	0.55	0.02
15	0.60	0.02
16	0.70	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.02	0.34
2	4.34	0.44
4	6.40	0.13
6	6.55	0.16
8	6.62	0.02
10	7.13	0.30
11	53.36	0.00
12	42.84	0.17
13	44.25	0.50
14	45.00	0.41
15	49.57	0.00
16	50.00	0.08

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.93	0.49
2	147.70	0.24
4	136.43	0.12
6	123.00	0.24
8	99.04	0.10
10	95.00	0.07
11	110.81	0.15
12	107.38	0.15
13	98.89	0.22
14	81.82	0.07
15	75.00	0.44
16	70.00	0.22

5.0 Added ethanol concentration in flask of 7.6%

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.08	0.01
2	0.12	0.00
4	0.20	0.00
6	0.32	0.00
8	0.66	0.00
10	1.09	0.01
11	0.20	0.01
12	0.22	0.01
13	0.30	0.01
14	0.35	0.00
15	0.45	0.01
16	0.50	0.01

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.02	0.14
2	4.27	0.16
4	6.51	0.17
6	6.53	1.16
8	6.70	0.08
10	6.79	0.63
11	56.81	0.34
12	54.15	0.79
13	54.57	0.68
14	54.57	0.09
15	54.58	0.34
16	55.00	0.71

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	150.67	0.19
2	146.21	0.29
4	131.86	0.39
6	124.00	0.49
8	98.89	0.39
10	96.00	0.29
11	115.13	0.78
12	109.47	0.29
13	100.09	0.07
14	91.55	0.29
15	87.00	0.04
16	85.00	0.51

6.0 Added ethanol concentration in flask of 9.5%

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.08	0.00
2	0.13	0.00
4	0.25	0.01
6	0.33	0.00
8	0.64	0.00
10	1.10	0.00
11	0.16	0.00
12	0.16	0.00
13	0.16	0.00
14	0.16	0.00
15	0.18	0.01
16	0.20	0.00

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.02	1.29
2	4.33	0.27
4	6.43	0.37
6	6.69	0.35
8	6.70	0.50
10	7.56	0.55
11	85.83	0.16
12	74.07	0.81
13	74.76	0.38
14	74.05	0.33
15	68.78	0.03
16	69.00	0.71

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.43	0.49
2	145.21	0.68
4	133.45	0.10
6	125.50	0.19
8	100.09	0.10
10	95.00	0.10
11	113.64	0.15
12	110.66	0.15
13	105.65	0.22
14	97.50	0.07
15	95.00	0.07
16	90.00	0.37

D) Batch fermentation for effect of temperature

1.0 Temperature at 30 °C

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.08	0.01
2	0.12	0.00
4	0.20	0.01
6	0.33	0.01
8	0.64	0.01
10	1.12	0.19
11	1.40	0.13
12	1.75	0.15
13	1.96	0.19
14	2.12	0.00
15	2.22	0.06
16	2.24	0.00
17	2.24	0.14

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.51	0.03
2	0.84	0.01
4	1.84	0.07
6	3.67	0.38
8	7.48	0.25
10	14.69	0.08
11	21.47	0.12
12	27.33	0.00
13	40.80	0.62
14	47.44	0.13
15	61.16	0.12
16	62.29	0.19
17	62.16	0.05

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.93	0.17
2	141.49	2.25
4	140.99	2.29
6	121.73	1.91
8	105.89	1.72
10	95.17	2.24
11	90.55	1.26
12	75.51	3.18
13	50.74	1.44
14	28.00	0.73
15	19.31	0.56
16	2.85	1.22
17	2.48	1.25

2.0 Temperature at 32 °C

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.06	0.02
2	0.09	0.01
4	0.19	0.03
6	0.32	0.07
8	0.70	0.12
10	1.35	0.14
11	1.60	0.05
12	2.01	0.00
13	2.15	0.08
14	2.21	0.06
15	2.21	0.13
16	2.22	0.24
17	2.22	0.17

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	1.89	0.06
2	2.58	0.06
4	3.56	0.06
6	4.98	0.05
8	11.07	0.87
10	20.61	0.87
11	32.54	2.79
12	38.22	0.83
13	46.53	0.01
14	55.02	2.88
15	64.93	1.05
16	64.00	0.02
17	64.00	0.23

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	150.92	0.12
2	148.69	0.25
4	139.21	1.79
6	126.10	2.88
8	104.55	0.30
10	82.66	2.46
11	70.75	2.23
12	65.53	0.08
13	37.73	3.62
14	13.40	1.64
15	11.02	4.39
16	9.46	3.41
17	7.89	3.16

3.0 Temperature at 34 °C

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.05	0.00
2	0.11	0.18
4	0.28	0.08
6	0.55	0.11
8	1.10	0.21
10	1.60	0.23
11	1.87	0.02
12	2.20	0.03
13	2.24	0.15
14	2.27	0.14
15	2.26	0.03
16	2.26	0.05
17	2.26	0.03

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.41	0.41
2	1.25	0.42
4	2.60	0.72
6	6.45	0.06
8	14.32	3.05
10	32.65	2.96
11	42.01	0.44
12	55.77	2.51
13	58.35	3.62
14	65.71	1.27
15	67.00	2.36
16	67.00	0.55
17	67.00	1.93

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.18	0.53
2	146.95	0.35
4	123.12	2.53
6	102.47	1.83
8	86.53	2.63
10	55.11	2.11
11	48.40	1.05
12	30.68	1.05
13	19.86	0.70
14	5.16	0.28
15	4.97	0.42
16	2.48	0.35
17	2.02	0.18

4.0 Temperature at 35 °C

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.06	0.00
2	0.16	0.06
4	0.37	0.05
6	0.64	0.14
8	1.14	0.14
10	1.74	0.07
11	2.01	0.13
12	2.29	0.24
13	2.28	0.00
14	2.28	0.02
15	2.28	0.01
16	2.28	0.02
17	2.28	0.09

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.52	0.17
2	1.26	0.44
4	2.85	0.24
6	7.75	1.55
8	16.58	3.36
10	34.77	0.18
11	44.10	0.65
12	55.84	2.07
13	59.50	1.99
14	67.00	0.63
15	67.00	1.49
16	67.00	1.02
17	67.00	0.88

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.18	0.35
2	141.24	0.35
4	109.22	0.00
6	94.92	0.14
8	75.36	1.79
10	50.19	2.00
11	45.13	2.00
12	29.79	0.84
13	22.84	1.40
14	5.96	0.77
15	4.22	0.35
16	2.47	0.03
17	2.43	0.22

5.0 Temperature at 36 °C

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.08	0.03
2	0.12	0.04
4	0.26	0.07
6	0.51	0.12
8	0.99	0.22
10	1.51	0.40
11	1.75	0.38
12	2.20	0.26
13	2.21	0.33
14	2.21	0.24
15	2.21	0.40
16	2.21	0.24
17	2.21	0.23

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.47	0.00
2	1.00	0.01
4	2.82	0.09
6	5.64	0.02
8	15.55	0.04
10	31.66	0.45
11	42.00	0.07
12	51.38	0.36
13	56.48	2.13
14	60.63	2.79
15	66.37	0.24
16	66.00	3.66
17	66.00	1.46

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.18	0.00
2	143.23	0.00
4	126.89	0.89
6	110.01	0.89
8	94.43	0.60
10	65.68	1.04
11	49.15	2.01
12	35.00	0.75
13	21.15	0.30
14	15.79	0.10
15	11.37	1.23
16	3.49	0.06
17	2.40	0.25

6.0 Temperature at 38 °C

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.00	0.00
2	0.02	0.06
4	0.09	0.05
6	0.12	0.14
8	0.35	0.14
10	0.75	0.07
11	0.95	0.13
12	1.10	0.14
13	1.25	0.00
14	1.45	0.02
15	1.77	0.01
16	1.75	0.02
17	1.75	0.09

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.50	0.00
2	0.80	0.01
4	1.53	0.05
6	3.27	0.02
8	6.23	0.06
10	10.92	0.50
11	16.48	0.10
12	18.90	0.50
13	37.09	1.50
14	41.89	3.05
15	46.65	1.02
16	46.00	2.50
17	46.00	1.46

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.68	0.00
2	146.70	0.06
4	139.21	0.05
6	132.85	0.14
8	119.75	0.14
10	109.17	0.07
11	100.09	0.13
12	82.06	0.14
13	60.57	0.00
14	41.01	0.02
15	38.72	0.01
16	38.23	0.02
17	36.74	0.09

7.0 Temperature at 40 °C

Fermentation time (h)	Average biomass concentration (g L^{-1})	Standard deviation
0	0.00	0.01
2	0.01	0.00
4	0.05	0.01
6	0.07	0.01
8	0.10	0.01
10	0.12	0.10
11	0.15	0.15
12	0.16	0.10
13	0.16	0.09
14	0.16	0.06
15	0.16	0.06
16	0.15	0.13
17	0.18	0.14

Fermentation time (h)	Average ethanol concentration (g L^{-1})	Standard deviation
0	0.69	0.00
2	2.00	0.01
4	2.57	0.05
6	3.59	0.02
8	5.12	0.06
10	7.49	0.50
11	8.13	0.10
12	8.40	0.50
13	8.38	2.50
14	8.82	2.00
15	10.33	0.20
16	10.56	2.50
17	10.48	1.46

Fermentation time (h)	Average glucose concentration (g L^{-1})	Standard deviation
0	149.93	0.01
2	146.70	0.00
4	141.74	0.01
6	140.99	0.01
8	130.67	0.01
10	127.69	0.19
11	119.35	0.29
12	112.00	0.20
13	92.14	0.19
14	89.76	0.06
15	78.34	0.06
16	69.50	0.13
17	68.81	0.14

E) Continuous fermentation for effect of dilution rate using an initial glucose concentration of 150 g L^{-1}

1.0 Dilution rate of 0.05 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
0	0.05	0.02	149.68	0.25	0.69	0.35
2	0.08	0.02	141.74	0.20	1.08	0.35
4	0.22	0.02	109.22	0.50	2.56	0.54
6	0.44	0.02	94.72	0.25	5.63	0.48
8	0.94	0.02	77.89	0.64	11.83	0.48
10	0.83	0.01	53.02	0.57	26.03	0.64
19	1.27	0.00	63.41	0.25	21.34	0.31
24	2.08	0.03	19.35	0.52	40.48	1.05
28	2.06	0.02	5.09	0.02	44.83	2.21
33	1.42	0.01	10.81	0.38	40.84	3.09
43	0.99	0.00	23.73	0.24	32.96	1.95
49	0.83	0.00	28.45	0.04	28.12	3.70
55	0.84	0.00	28.61	0.06	27.60	0.46
66	1.31	0.04	6.69	0.13	38.34	1.12
72	1.15	0.02	6.37	0.12	38.20	0.78
78	0.85	0.01	18.85	0.16	34.83	0.82
84	0.70	0.01	22.49	1.40	29.96	0.38
89	0.68	0.01	21.55	0.93	28.30	2.39
104	0.69	0.00	31.05	0.89	26.85	1.20
110	0.94	0.01	29.21	0.23	31.29	1.67
115	1.31	0.01	20.85	0.56	36.93	1.34
120	1.16	0.00	17.74	0.41	52.22	0.96

125	0.96	0.00	33.73	1.24	44.51	2.27
130	0.81	0.01	47.79	1.99	38.42	1.23
136	0.61	0.00	64.97	0.68	33.17	0.28
143	0.88	0.01	76.02	0.47	29.36	0.26
148	0.88	0.01	74.97	0.96	30.56	0.53
154	1.14	0.01	74.97	1.12	37.78	1.41
160	1.23	0.04	41.57	1.01	40.63	0.31
166	1.00	0.02	46.34	0.26	40.02	0.53
172	0.85	0.00	64.54	0.70	33.89	0.11
178	0.71	0.00	65.27	0.66	29.11	0.68
184	0.60	0.00	70.17	0.12	31.32	0.29
190	0.98	0.01	24.49	0.61	49.60	0.84
196	1.72	0.02	7.71	0.41	64.00	0.60
202	1.54	0.01	8.74	0.08	61.62	0.47
208	1.23	0.00	26.31	0.29	54.33	1.10
214	0.94	0.01	46.92	0.12	35.04	0.02
220	0.89	0.00	59.84	0.19	38.18	0.16
226	0.95	0.01	55.07	0.86	38.18	0.23
232	1.47	0.02	30.38	0.32	57.59	0.43
238	1.64	0.01	20.60	1.62	57.59	0.33
244	1.34	0.04	37.98	1.43	50.98	0.04
250	1.03	0.03	39.39	0.20	40.55	0.36
256	0.96	0.01	60.01	0.38	34.38	0.39
262	1.05	0.04	56.66	0.53	42.80	0.18
268	1.31	0.00	57.92	0.61	48.53	0.35
274	1.33	0.02	23.04	0.89	42.73	0.63
280	1.39	0.01	30.25	0.46	48.64	0.01
286	1.16	0.03	44.78	0.00	53.29	0.26
292	0.92	0.01	51.98	0.00	49.21	0.29
298	1.06	0.01	75.41	1.45	35.21	0.83

304	1.18	0.00	51.43	1.41	38.65	0.32
310	1.59	0.02	49.71	0.00	45.88	0.00
316	1.33	0.01	52.79	0.23	49.03	0.03
322	1.23	0.01	75.73	0.05	40.84	0.23
328	1.09	0.00	54.66	0.56	37.90	1.08
334	1.17	0.01	54.26	2.72	44.38	0.01
340	1.27	0.04	38.33	0.98	45.57	0.00
346	1.38	0.03	26.86	1.10	49.85	0.03
352	1.19	0.01	41.31	0.07	50.81	0.02
358	1.17	0.01	40.66	1.16	42.25	0.00
364	0.98	0.00	60.52	0.98	37.08	0.01
370	0.92	0.03	49.30	0.92	39.52	0.02
376	1.10	0.02	51.93	0.71	40.32	0.03
382	1.41	0.00	51.93	0.32	50.99	0.01
388	1.38	0.01	34.18	2.64	48.71	1.41
394	1.28	0.02	47.11	0.39	47.61	2.03
400	1.07	0.03	38.87	2.42	44.05	1.83
406	0.95	0.01	38.02	1.34	37.86	0.08
412	1.19	0.01	35.00	0.86	37.50	1.38
418	1.27	0.01	40.00	0.68	44.39	0.22
424	1.55	0.05	41.00	0.21	48.95	0.05
430	1.24	0.02	49.25	0.70	43.78	1.08
436	1.10	0.05	63.20	0.46	40.78	0.89
442	1.09	0.03	79.28	0.19	32.60	0.76
448	0.98	0.03	85.29	0.19	33.01	1.94
454	1.11	0.03	72.09	0.21	34.57	0.62
460	1.27	0.01	59.78	0.81	36.24	0.88
466	1.47	0.02	47.46	0.19	45.10	3.07
472	1.28	0.02	34.57	0.35	39.34	2.11

2.0 Dilution rate of 0.1 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
478	1.14	0.05	68.16	1.44	31.86	2.72
484	1.49	0.03	61.76	1.40	35.55	2.33
490	1.53	0.05	53.07	4.72	45.45	1.80
496	2.07	0.01	32.87	1.15	54.22	2.64
502	1.37	0.00	69.26	2.81	35.69	2.17
508	1.23	0.05	40.81	1.52	30.57	1.38
514	1.49	0.05	25.67	3.76	44.44	2.08
520	1.37	0.02	55.75	2.51	36.43	0.30
532	1.79	0.03	16.43	4.57	45.65	1.64
538	1.18	0.04	34.65	0.55	34.22	0.18
544	1.22	0.03	21.00	1.29	37.08	0.42
550	1.52	0.01	54.41	2.25	36.54	0.78
562	1.42	0.02	43.59	2.79	38.22	1.02
568	1.09	0.03	54.21	1.16	35.32	0.24
574	1.09	0.02	69.40	3.22	33.33	0.23
580	1.25	0.01	37.98	1.89	41.54	0.84
592	1.24	0.02	40.61	3.34	41.14	0.75
598	1.38	0.02	45.92	1.22	44.85	1.36
604	1.24	0.04	61.21	1.23	42.64	1.17
610	1.09	0.01	68.81	1.40	35.75	0.45
622	1.10	0.02	61.00	0.49	35.06	0.09
634	1.07	0.01	71.94	0.49	34.89	0.18
646	1.12	0.02	61.00	0.31	34.83	0.22
652	1.07	0.01	71.49	1.31	34.85	0.46
664	1.11	0.02	69.26	1.95	36.72	1.85

670	1.25	0.02	45.03	1.34	39.42	0.33
676	1.32	0.05	32.12	0.39	40.12	1.11
682	1.29	0.03	37.98	0.92	38.32	0.35
691	1.30	0.01	35.61	0.25	37.53	2.42
697	1.44	0.02	35.21	1.10	42.38	1.05
703	1.45	0.04	36.21	1.18	42.38	0.65
715	1.44	0.02	35.03	1.16	42.86	1.33
721	1.45	0.03	34.22	2.35	42.65	0.52
727	1.44	0.01	34.22	1.68	40.00	0.66

3.0 Dilution rate of 0.15 h^{-1}

Time (h)	Average biomass (gL^{-1})	Standard deviation	Average glucose (gL^{-1})	Standard deviation	Average ethanol (gL^{-1})	Standard deviation
733	1.15	0.00	53.02	0.32	29.85	0.52
739	1.19	0.01	42.60	0.53	33.20	0.16
745	1.34	0.01	35.45	0.63	35.66	1.19
751	1.46	0.01	26.81	1.47	35.48	1.30
757	1.51	0.06	23.53	1.48	42.33	0.83
763	1.32	0.01	35.89	0.63	33.82	0.90
769	1.37	0.03	29.64	0.97	40.35	0.67
775	1.38	0.01	32.07	0.19	38.26	2.10
781	1.36	0.04	30.78	0.39	39.53	1.46
787	1.52	0.02	31.48	0.93	37.43	2.89
793	1.44	0.01	32.07	0.60	37.40	0.22
799	1.29	0.01	37.04	0.91	46.76	2.11
805	1.01	0.02	47.96	0.12	21.93	0.60
811	1.37	0.01	26.36	1.52	40.24	2.81
817	1.72	0.04	11.32	0.74	42.96	0.30
823	1.31	0.02	18.17	0.21	42.19	1.14
829	1.23	0.08	19.76	0.37	46.03	0.96
835	1.27	0.05	18.77	1.04	41.25	1.40
841	1.32	0.02	33.16	0.67	42.91	2.35
847	1.30	0.04	40.71	0.86	33.02	2.00
853	1.21	0.05	57.64	1.05	31.50	0.83
859	1.25	0.07	41.85	1.22	37.80	0.30
865	1.41	0.01	41.06	0.39	40.41	0.18
871	1.23	0.07	25.22	0.98	44.10	1.01
877	1.36	0.02	26.16	0.60	42.24	0.43

883	1.18	0.03	24.52	1.12	33.58	0.44
889	1.26	0.02	25.72	0.14	30.55	0.17
895	1.70	0.03	15.90	0.14	46.68	0.24
901	1.60	0.02	17.72	0.85	45.63	2.13
907	1.59	0.03	24.48	0.31	47.17	0.76
913	1.65	0.01	19.91	0.07	44.98	0.46
919	1.59	0.03	18.17	1.23	42.67	0.26
925	1.33	0.01	45.52	5.78	34.33	0.95
931	1.68	0.04	33.41	1.77	47.60	1.46
937	1.34	0.02	38.18	2.31	33.72	0.80
943	1.45	0.00	31.72	3.12	40.45	0.34
949	1.34	0.04	22.59	0.25	38.26	0.20
955	1.31	0.06	25.47	1.79	35.60	1.09
961	1.24	0.02	45.18	2.60	31.93	0.90
967	1.44	0.03	36.94	2.50	34.64	0.43
973	1.59	0.07	30.88	1.38	36.05	1.03
979	1.53	0.04	35.99	0.14	44.17	1.00
985	1.52	0.06	37.95	2.53	43.43	1.58
991	1.51	0.05	36.01	0.14	42.62	1.49
997	1.50	0.04	37.05	0.61	42.16	2.54
1003	1.50	0.04	36.02	1.43	42.21	0.52
1009	1.51	0.02	39.00	2.65	43.07	0.82
1015	1.50	0.01	38.05	1.03	42.58	0.37

4.0 Dilution rate of 0.20 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
1021	1.44	0.02	41.55	1.22	32.81	2.22
1027	1.46	0.01	36.54	1.05	34.81	0.92
1033	1.44	0.04	38.18	1.72	32.01	1.50
1039	1.44	0.05	34.50	1.41	37.78	0.78
1045	1.39	0.07	27.26	1.46	45.40	0.36
1051	1.56	0.06	25.96	0.90	44.95	2.08
1057	1.40	0.04	50.89	1.65	40.44	1.77
1063	1.33	0.07	23.09	2.35	44.09	0.64
1069	1.41	0.06	17.33	0.35	47.62	1.50
1075	1.36	0.06	20.16	0.70	48.20	0.46
1081	1.37	0.04	28.89	0.64	47.55	1.96
1087	1.31	0.04	25.67	0.07	45.57	0.94
1093	1.32	0.05	28.99	0.43	41.06	0.91
1099	1.30	0.03	30.73	0.35	42.15	1.43
1105	1.26	0.03	18.52	0.46	39.54	0.52
1111	1.30	0.02	45.18	0.79	30.28	0.20
1117	1.32	0.00	50.24	1.01	36.03	1.95
1123	1.32	0.03	49.20	0.62	33.18	0.43
1129	1.35	0.01	45.48	0.94	29.10	0.68
1135	1.48	0.01	44.04	1.22	35.48	1.21
1141	1.27	0.02	30.73	0.35	43.61	0.50
1147	1.25	0.02	35.40	0.14	42.58	0.85
1153	1.24	0.01	40.36	1.84	33.58	0.44
1159	1.26	0.01	31.03	0.07	30.55	0.17
1165	1.34	0.01	47.11	1.12	35.95	0.66

1171	1.42	0.01	44.73	1.10	33.31	0.90
1177	1.32	0.01	20.21	0.49	31.31	1.57
1183	1.42	0.01	23.09	0.53	46.46	0.92
1189	1.26	0.03	32.12	0.43	42.05	1.68
1195	1.08	0.00	41.30	0.67	45.27	1.68
1201	1.13	0.03	38.18	1.03	40.27	0.63
1207	1.15	0.02	40.26	0.83	40.84	0.14
1213	1.14	0.02	40.41	0.67	42.17	0.65
1219	1.13	0.02	44.53	0.49	32.16	1.45
1225	1.25	0.08	37.09	2.25	30.29	0.27
1231	1.30	0.02	38.92	0.98	34.57	0.27
1237	1.41	0.00	38.13	0.53	30.65	0.24
1243	1.34	0.00	32.02	0.44	32.78	0.47
1249	1.32	0.00	36.89	0.86	35.70	1.19
1255	1.35	0.02	40.96	0.44	34.68	0.26
1261	1.44	0.02	50.84	1.22	37.36	0.39
1267	1.41	0.03	40.00	1.05	37.96	0.45
1273	1.40	0.04	40.41	0.37	37.64	0.53
1279	1.40	0.01	39.00	0.97	36.41	0.77
1285	1.40	0.01	40.00	0.88	36.00	0.59
1291	1.39	0.02	38.38	1.94	36.00	0.24

5.0 Dilution rate of 0.25 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
1297	1.10	0.03	51.83	0.21	35.89	2.22
1303	0.91	0.01	57.49	0.68	34.27	0.92
1309	1.16	0.01	52.72	1.81	32.24	1.50
1315	1.16	0.00	50.19	0.93	36.86	0.78
1321	1.21	0.01	47.51	0.32	27.30	0.68
1327	1.08	0.01	49.60	1.05	29.35	0.35
1333	1.09	0.01	46.62	1.38	36.20	0.10
1339	1.20	0.00	62.40	1.27	30.39	0.12
1345	1.15	0.02	52.72	0.94	29.79	0.12
1351	1.42	0.01	56.15	0.25	30.28	0.05
1357	1.52	0.03	56.60	0.62	33.79	0.06
1363	1.38	0.05	55.11	0.53	32.79	0.27
1369	1.42	0.02	56.89	1.19	37.28	0.02
1375	1.40	0.01	49.60	0.74	49.23	0.01
1381	1.38	0.05	59.87	5.44	39.75	0.01
1387	1.33	0.02	49.45	3.20	35.18	0.01
1393	1.33	0.03	33.96	5.88	36.76	0.02
1399	1.31	0.01	75.66	4.32	34.57	0.00
1405	1.33	0.02	47.81	7.89	37.53	0.01
1411	1.28	0.01	58.09	3.87	40.07	0.01
1417	1.20	0.02	56.00	2.61	35.89	0.08
1423	1.21	0.03	65.53	3.72	34.27	0.01
1429	1.24	0.03	63.30	2.68	32.24	0.02
1435	1.31	0.02	59.43	1.79	36.86	0.02
1441	1.54	0.01	58.68	0.57	32.66	0.06

1447	1.25	0.03	63.15	0.98	29.86	0.10
1453	1.18	0.01	56.30	0.00	30.25	0.08
1459	1.39	0.02	52.43	1.31	34.79	0.35
1465	1.23	0.00	48.40	0.24	35.79	0.37
1471	1.45	0.03	51.98	0.21	32.24	0.42
1477	1.34	0.02	52.57	2.07	32.07	0.77
1483	1.33	0.00	52.13	1.34	33.14	0.08
1489	1.25	0.04	55.11	3.88	32.32	0.16
1495	1.14	0.01	63.74	0.14	30.01	0.13
1501	1.15	0.02	65.68	1.96	26.00	0.41
1507	1.14	0.01	50.00	0.85	28.02	0.42
1513	1.35	0.03	60.00	2.65	30.30	0.77
1519	1.35	0.02	56.00	6.67	33.00	0.08
1525	1.35	0.04	55.00	2.65	33.00	0.16
1531	1.36	0.02	56.00	0.46	32.50	0.13
1537	1.34	0.01	55.00	1.08	33.01	0.13

6.0 Dilution rate of 0.30 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
1543	1.19	0.01	70.00	1.27	30.00	0.03
1549	1.44	0.01	25.62	0.14	26.00	0.01
1555	1.42	0.02	20.26	1.12	44.00	0.01
1561	1.13	0.01	27.55	0.32	48.00	0.00
1567	1.10	0.03	27.11	1.08	43.00	0.01
1573	1.18	0.01	18.47	2.05	51.00	0.01
1579	1.23	0.01	14.74	0.49	44.00	0.01
1585	1.47	0.01	18.47	0.49	35.00	0.00
1591	1.05	0.03	24.57	1.35	34.00	0.02
1597	1.14	0.01	30.68	14.46	44.00	0.01
1603	1.76	0.08	24.87	1.89	37.00	0.03
1609	1.35	0.01	22.49	1.66	43.00	0.05
1615	1.38	0.03	18.32	0.71	39.00	0.01
1621	1.40	0.02	20.00	0.15	40.00	0.08
1627	1.31	0.01	25.77	0.45	47.00	0.35
1633	1.22	0.01	34.55	1.19	46.00	0.37
1639	1.22	0.05	36.94	0.67	47.00	0.42
1645	1.21	0.01	34.70	1.41	43.00	0.77
1651	1.29	0.01	30.53	0.89	48.00	0.08
1657	1.14	0.02	27.26	0.60	51.00	0.16
1663	1.33	0.04	25.47	2.38	50.00	0.13
1669	1.33	0.02	64.34	5.88	43.00	0.41
1675	1.42	0.02	63.89	1.56	36.00	0.42
1681	1.31	0.04	64.49	14.30	27.00	0.77
1687	1.11	0.03	63.45	3.05	29.00	0.08

1693	1.06	0.05	69.70	1.12	36.00	0.16
1699	0.92	0.06	68.66	2.76	30.00	0.13
1705	0.95	0.05	73.28	0.74	30.00	0.13
1711	1.22	0.01	42.45	0.52	30.00	0.02
1717	0.97	0.05	33.96	0.52	34.00	0.00
1723	1.29	0.09	35.74	0.07	33.00	0.01
1729	1.27	0.04	20.00	0.00	33.00	0.01
1735	1.48	0.06	17.28	0.97	30.00	0.08
1741	1.38	0.02	34.70	3.65	30.00	0.01
1747	1.35	0.01	42.45	1.41	35.00	0.16
1753	1.23	0.02	42.89	0.52	36.00	0.13
1759	1.27	0.01	68.00	1.41	32.00	0.41
1765	1.31	0.01	50.00	4.10	32.00	0.42
1771	1.31	0.01	54.00	2.61	33.00	0.77
1777	1.34	0.01	67.00	0.37	29.00	0.30
1783	1.35	0.02	66.00	0.30	28.00	0.03
1789	1.35	0.01	65.00	4.17	28.00	0.03
1795	1.35	0.01	66.00	1.41	28.00	0.03

F) Continuous fermentation for effect of initial glucose concentration on oscillation

1.0 An initial glucose concentration of 80 g L^{-1} at dilution rate of 0.05 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
0	0.09	0.00	79.43	1.38	0.15	0.92
1	0.10	0.00	71.69	1.27	1.59	1.50
2	0.14	0.02	70.70	0.94	2.14	0.78
3	0.18	0.02	68.31	0.25	3.65	0.68
4	0.19	0.03	66.03	0.62	4.67	0.35
5	0.21	0.04	64.94	0.53	4.74	0.10
6	0.24	0.01	63.45	1.19	4.74	0.12
7	0.26	0.01	60.07	0.74	5.01	0.12
8	0.28	0.02	60.15	5.44	6.02	0.05
9	0.32	0.03	67.40	3.20	6.41	0.06
21	0.88	0.01	39.50	5.88	43.19	0.27
24	1.00	0.01	35.50	4.32	44.34	0.02
27	1.10	0.00	36.50	7.89	46.92	0.01
31	0.72	0.01	7.10	3.87	44.66	0.01
41	0.45	0.01	17.15	2.61	34.87	0.01
45	0.55	0.01	17.70	3.72	37.55	0.02
48	0.65	0.00	15.35	2.68	38.74	0.00
52	0.53	0.02	17.05	1.79	32.89	0.01
57	0.45	0.01	25.35	0.57	32.32	0.01
69	0.46	0.03	17.35	0.98	36.59	0.08
72	0.70	0.05	14.45	0.00	39.21	0.01
75	0.77	0.02	11.10	1.31	42.28	0.02
80	0.56	0.01	14.20	0.24	36.59	0.02
90	0.52	0.05	19.00	0.21	34.22	0.06

93	0.69	0.02	17.00	2.07	35.43	0.10
96	0.80	0.03	14.20	1.34	42.59	0.08
99	1.10	0.01	4.00	3.88	45.43	0.35
105	0.69	0.02	10.00	0.14	38.70	0.37
111	0.59	0.01	13.90	1.96	33.94	0.42
120	0.99	0.02	4.30	0.85	40.78	0.77
128	0.97	0.03	8.00	2.65	35.02	0.08
136	0.83	0.03	3.20	6.67	32.70	0.16
140	0.99	0.02	3.50	2.65	35.93	0.13
143	0.96	0.01	3.60	0.46	36.54	0.41
149	0.98	0.03	3.60	1.08	37.03	0.42
155	0.99	0.01	3.50	1.27	37.46	0.77
161	0.97	0.02	3.90	0.14	37.99	0.08
167	0.96	0.00	3.85	1.12	37.99	0.16

2.0 An initial glucose concentration of 80 g L^{-1} at dilution rate of 0.10 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
170	0.75	0.03	10.00	0.32	35.45	0.13
173	0.89	0.02	5.00	1.08	36.79	0.13
179	1.20	0.00	3.35	2.05	39.52	0.03
185	0.99	0.04	10.50	0.49	35.01	0.01
191	0.86	0.01	11.75	0.49	30.32	0.01
195	0.98	0.02	9.40	1.35	36.22	0.00
199	1.00	0.01	3.65	14.46	37.24	0.01
203	1.05	0.03	10.00	1.89	35.27	0.01
209	1.13	0.02	13.00	1.66	32.00	0.01
215	0.76	0.04	19.95	0.71	30.00	0.00
218	0.80	0.02	15.65	0.15	33.11	0.02
221	0.88	0.01	13.60	0.45	38.83	0.01
226	1.55	0.01	3.55	1.19	42.98	0.03
232	1.06	0.01	10.10	0.67	37.09	0.05
239	0.80	0.02	14.00	1.41	30.56	0.01
242	0.88	0.01	3.55	0.89	38.68	0.08
245	1.02	0.03	4.45	0.60	35.69	0.35
249	0.98	0.01	7.00	2.38	30.75	0.37
255	1.10	0.01	10.20	5.88	35.00	0.42
259	1.21	0.01	3.45	1.56	37.00	0.77
263	0.84	0.02	8.00	14.30	30.95	0.08
266	0.99	0.01	3.95	3.05	40.06	0.16
269	1.06	0.01	3.60	1.12	40.10	0.13
274	1.04	0.05	3.40	2.76	37.25	0.41
280	0.95	0.01	3.70	0.74	35.09	0.42

286	0.90	0.01	3.35	0.52	33.09	0.77
291	0.89	0.02	9.60	0.52	31.69	0.08
296	1.17	0.04	4.25	0.07	39.89	0.16
302	1.00	0.02	3.95	0.00	36.09	0.13
313	0.90	0.02	8.00	0.97	32.29	0.13
318	1.12	0.04	4.25	3.65	39.62	0.02
323	1.00	0.03	5.35	1.41	39.07	0.00
329	0.90	0.05	4.10	0.52	34.88	0.01
335	0.87	0.06	4.25	1.41	37.09	0.01
341	1.00	0.05	8.00	2.10	40.03	0.08
345	1.09	0.01	4.90	2.61	37.50	0.01
348	0.99	0.05	4.10	0.37	37.59	0.16
354	0.96	0.09	4.30	0.30	37.09	0.13
360	0.95	0.04	4.05	2.12	36.99	0.41
365	0.92	0.06	4.10	1.41	37.98	0.42
368	0.94	0.02	4.20	1.00	37.47	0.77
376	0.95	0.01	4.00	1.00	38.17	0.30

3.0 An initial glucose concentration of 100 g L^{-1} at dilution rate of 0.05 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
0	0.02	0.02	99.69	0.25	0.33	0.35
1	0.04	0.02	95.02	0.20	1.25	0.35
2	0.06	0.02	93.13	0.50	2.50	0.54
3	0.09	0.02	90.26	0.25	3.90	0.48
4	0.13	0.02	88.07	0.64	4.05	0.48
5	0.19	0.01	86.09	0.57	4.81	0.64
6	0.26	0.00	70.99	0.25	4.86	0.31
7	0.33	0.03	68.91	0.52	4.94	1.05
8	0.35	0.02	63.15	0.02	5.51	2.21
9	0.35	0.01	80.10	0.39	5.74	3.09
21	1.37	0.00	34.00	0.24	52.59	1.95
24	1.29	0.00	6.40	0.04	55.85	3.70
27	1.13	0.00	12.70	0.06	48.69	0.46
31	1.02	0.04	18.30	0.13	49.27	1.11
36	0.94	0.02	24.00	0.12	40.00	0.78
41	1.12	0.01	25.15	0.16	41.02	0.82
45	1.10	0.01	21.00	1.40	47.59	0.38
48	0.85	0.01	20.30	0.93	48.85	2.39
52	0.81	0.00	24.40	0.89	44.72	1.20
57	0.80	0.01	23.60	0.23	41.39	1.67
62	1.18	0.01	21.00	0.56	42.12	1.34
67	1.13	0.00	7.80	0.41	50.24	0.96
71	0.91	0.00	7.45	1.29	50.05	2.27
75	0.80	0.01	10.60	1.99	46.41	1.22
80	0.81	0.00	15.45	0.68	40.09	0.28

85	1.20	0.01	14.98	0.47	41.25	0.26
90	1.36	0.01	11.80	0.96	47.43	0.53
93	1.41	0.01	3.50	1.11	54.54	1.41
96	1.36	0.04	3.30	1.01	53.03	0.31
99	0.99	0.02	9.02	0.26	47.50	0.53
105	1.33	0.00	18.00	0.70	39.08	0.11
111	1.64	0.00	3.30	0.66	46.09	0.68
115	1.61	0.00	3.50	0.12	66.89	0.29
120	1.50	0.01	3.40	0.61	65.69	0.84
124	1.30	0.02	3.80	0.41	56.23	0.60
128	0.91	0.01	9.00	0.08	47.09	0.47
132	1.28	0.00	12.00	0.29	38.09	1.10
136	1.29	0.01	15.00	0.12	52.42	0.02
140	1.27	0.00	3.80	0.19	52.96	0.16
143	1.28	0.01	3.50	0.86	51.60	0.23
149	1.28	0.01	4.40	0.32	51.86	0.43
155	1.25	0.01	3.60	1.62	52.09	0.33
161	1.16	0.04	4.10	1.43	51.99	0.04
167	1.38	0.03	3.60	0.20	50.26	0.36

4.0 An initial glucose concentration of 100 g L^{-1} at dilution rate of 0.10 h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})	Standard deviation
170	1.41	0.01	7.90	0.38	45.19	0.39
173	1.30	0.04	3.60	0.52	49.34	0.18
179	1.31	0.00	3.60	0.61	34.56	0.35
185	1.65	0.02	17.90	0.89	34.72	0.63
191	1.66	0.01	16.00	0.46	48.89	0.01
195	1.68	0.03	8.00	0.00	51.27	0.26
199	1.25	0.00	5.30	0.00	53.45	0.29
203	1.20	0.01	5.30	1.45	35.20	0.83
209	1.37	0.00	15.10	1.41	36.39	0.32
215	1.31	0.02	14.00	0.00	48.69	0.00
218	1.17	0.01	3.50	0.23	47.32	0.03
221	1.13	0.01	4.20	0.05	35.41	0.23
226	1.30	0.00	13.20	0.56	34.72	1.08
232	1.44	0.01	14.80	2.72	48.38	0.01
239	1.40	0.04	7.50	0.98	53.18	0.00
242	1.25	0.03	5.50	1.10	52.63	0.03
245	1.13	0.01	4.50	0.07	46.07	0.13
249	1.10	0.01	7.90	1.16	39.87	0.41
255	1.25	0.00	13.20	0.98	39.87	0.42
259	1.44	0.03	12.50	0.92	44.00	0.77
263	1.43	0.02	8.10	0.71	53.18	0.35
266	1.30	0.00	3.70	0.32	52.63	1.44
269	1.23	0.01	3.90	2.64	46.07	1.40
274	1.38	0.02	4.50	0.39	39.00	4.72
280	1.34	0.03	9.00	2.42	52.00	1.15

286	1.12	0.01	4.45	1.34	46.00	2.80
291	1.27	0.01	6.60	0.86	38.00	1.52
296	1.20	0.01	8.90	0.68	44.00	3.76
302	1.13	0.05	4.20	0.21	40.00	2.51
313	1.33	0.02	5.00	0.70	36.00	1.50
318	1.32	0.05	7.00	0.46	46.00	0.55
323	1.34	0.03	4.40	0.19	44.00	1.29
329	1.36	0.03	4.30	0.19	46.00	0.41
335	1.36	0.03	4.30	0.21	46.00	0.42
341	1.36	0.01	4.10	0.81	47.00	0.77
343	1.35	0.02	4.60	0.19	46.00	0.30

G) In situ batch extraction fermentation

Fermentation time (h)	Control		Oleyl alcohol		Iso oxtadecanol		Octyl-1-dodecanol	
	Average biomass ($g\ L^{-1}$)	Standard deviation	Average biomass ($g\ L^{-1}$)	Standard deviation	Average biomass ($g\ L^{-1}$)	Standard deviation	Average biomass ($g\ L^{-1}$)	Standard deviation
0	0.06	0.00	0.07	0.00	0.09	0.00	0.07	0.00
2	0.16	0.06	0.14	0.00	0.12	0.00	0.16	0.00
4	0.37	0.05	0.39	0.00	0.28	0.00	0.36	0.04
6	0.64	0.14	0.66	0.00	0.45	0.00	0.65	0.00
8	1.14	0.14	1.15	0.01	1.00	0.04	1.18	0.02
10	1.74	0.07	2.38	0.06	1.98	0.03	2.08	0.13
11	2.01	0.13	2.64	0.01	2.54	0.00	2.42	0.11
12	2.29	0.24	2.72	0.07	3.12	0.13	2.63	0.06
13	2.28	0.00	2.82	0.18	3.26	0.10	2.88	0.10
14	2.28	0.02	2.80	0.22	3.10	0.20	2.78	0.11
15	2.28	0.01	2.79	0.18	3.01	0.15	2.72	0.01
16	2.28	0.02	2.77	0.17	3.00	0.01	2.72	0.06
17	2.28	0.08	2.77	0.14	2.94	0.28	2.70	0.03

Fermentation time (h)	Control		Oleyl alcohol		Iso oxtadecanol		Octyl-1-dodecanol	
	Average ethanol ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation
0	0.50	0.20	3.58	1.52	3.65	0.09	4.93	0.11
2	1.30	0.40	4.07	2.05	6.30	0.24	5.59	0.07
4	2.90	0.20	7.64	0.53	8.19	0.12	7.42	0.12
6	7.70	1.60	13.81	10.48	13.13	0.45	16.37	0.80
8	16.60	3.40	20.90	1.47	23.55	0.15	30.80	1.40
10	34.80	0.20	41.40	0.42	47.34	2.10	51.58	2.86
11	44.10	0.70	51.31	1.43	57.80	2.18	57.04	1.82
12	55.80	2.10	60.00	2.00	62.15	0.49	62.20	0.61
13	59.50	2.00	63.13	3.15	69.50	3.03	70.00	3.47
14	67.00	0.60	67.00	0.15	75.00	3.07	70.11	2.25
15	67.00	1.50	64.00	0.20	74.04	3.79	69.16	2.07
16	67.00	1.00	65.00	0.01	72.17	3.00	67.54	2.99
17	67.00	0.90	64.00	0.05	71.00	3.18	67.63	3.78

Fermentation time (h)	Control		Oleyl alcohol		Iso oxtadecanol		Octyl-1-dodecanol	
	Average glucose ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation
0	149.18	0.35	0.07	0.00	0.09	0.00	0.07	0.00
2	141.24	0.35	0.14	0.00	0.12	0.00	0.16	0.00
4	109.22	0.00	0.39	0.00	0.28	0.00	0.36	0.04
6	94.92	0.14	0.66	0.00	0.45	0.00	0.65	0.00
8	75.36	1.79	1.15	0.01	1.00	0.04	1.18	0.02
10	50.19	2.00	2.38	0.06	1.98	0.03	2.08	0.13
11	45.13	2.00	2.64	0.01	2.54	0.00	2.42	0.11
12	29.79	0.84	2.72	0.07	3.12	0.13	2.63	0.06
13	22.84	1.40	2.82	0.18	3.26	0.10	2.88	0.10
14	5.96	0.77	2.80	0.22	3.10	0.20	2.78	0.11
15	4.22	0.35	2.79	0.18	3.01	0.15	2.72	0.01
16	2.47	0.03	2.77	0.17	3.00	0.01	2.72	0.06
17	2.43	0.22	2.77	0.14	2.94	0.28	2.70	0.03

H) In situ continuous extraction fermentation

1.0 Media dilution rate of 0.05 h^{-1} and solvent recirculation at 100 mL h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})			
					Medium	Standard deviation	Solvent	Standard deviation
0	0.05	0.02	149.68	0.21	0.69	0.35		
2	0.08	0.02	141.74	0.25	1.08	0.35		
4	0.22	0.02	109.22	0.20	2.56	0.54		
6	0.44	0.02	94.72	0.50	5.63	0.48		
8	0.94	0.02	77.89	0.25	11.82	0.48		
10	0.83	0.01	53.02	0.64	36.03	0.64	0.82	0.03
12	1.29	0.01	66.30	0.57	31.89	0.98	6.34	0.09
18	1.90	0.03	66.30	0.40	60.78	1.45	3.52	0.00
27	2.56	0.06	11.50	0.15	70.39	1.10	1.94	0.07
33	1.98	0.03	4.00	0.40	44.86	0.74	2.14	0.07
41	1.70	0.02	31.40	0.15	40.22	0.09	1.29	0.03
45	1.45	0.02	43.30	0.50	28.08	0.25	1.08	0.06
49	1.40	0.03	63.20	0.65	27.12	0.62	1.80	0.07
53	1.29	0.01	61.00	2.55	28.67	0.19	3.28	0.36
57	1.58	0.02	63.80	0.70	47.61	1.97	6.09	0.39

65	2.03	0.04	43.50	1.25	60.02	0.04	4.63	0.14
69	1.68	0.04	12.00	0.30	53.17	0.79	4.50	0.07
73	1.56	0.06	6.50	0.10	54.71	1.41	5.71	0.21
77	1.30	0.02	13.30	0.45	53.22	3.00	5.97	0.01
81	1.21	0.02	10.90	1.80	54.30	0.57	4.67	0.20
90	1.18	0.03	19.70	0.10	45.80	1.68	4.26	0.29
94	1.07	0.01	22.60	0.10	43.53	0.86	4.44	0.28
98	1.18	0.02	31.40	1.45	38.13	1.69	4.28	0.26
102	1.15	0.01	43.20	0.70	41.16	2.68	4.25	0.36
106	0.96	0.02	39.50	0.20	44.58	1.33	7.96	0.45
110	1.09	0.04	41.00	0.20	45.87	1.15	6.85	0.01
114	1.26	0.01	35.40	2.10	51.57	2.05	6.92	0.90
118	1.24	0.04	15.60	0.20	50.65	2.03	5.52	0.26
122	1.07	0.01	12.00	0.95	43.68	1.03	4.69	0.21
134	1.04	0.00	16.00	0.15	46.92	0.40	4.19	0.21
138	1.13	0.02	23.60	0.65	38.87	0.43	4.43	0.19
142	1.13	0.03	36.90	0.55	39.75	1.64	7.91	0.20
146	1.11	0.03	36.80	0.45	38.52	0.01	5.59	0.58
150	1.15	0.10	31.10	1.25	45.70	1.60	4.58	0.41
154	1.11	0.03	20.00	0.00	47.43	1.22	3.83	0.08
158	1.00	0.02	20.00	0.00	43.21	0.62	4.42	0.08
162	1.06	0.01	20.40	1.65	44.92	2.42	6.37	0.51

166	1.00	0.01	24.60	0.05	46.55	0.02	7.48	0.06
170	1.03	0.04	24.50	0.10	46.33	0.96	7.41	0.60
174	1.01	0.02	18.40	0.05	46.05	1.12	7.76	0.42
178	0.97	0.04	20.00	0.10	46.56	0.40	7.57	0.07
182	1.06	0.01	18.30	1.20	46.66	0.19	7.11	0.31
186	1.05	0.02	18.90	0.45	46.48	1.29	7.32	0.34
190	1.01	0.04	18.00	0.00	46.60	0.29	7.48	0.06
194	1.01	0.01	17.00	0.10	46.63	0.81	7.41	0.60
198	1.04	0.03	18.00	0.40	45.98	0.50	7.37	0.24
204	1.01	0.01	19.00	0.35	46.47	0.11	7.28	1.07
210	1.02	0.01	17.70	0.10	47.73	0.19	7.50	0.85
216	1.02	0.03	18.40	0.15	46.78	0.61	8.88	0.24
222	1.01	0.02	18.10	0.40	47.44	0.30	8.72	0.85
228	1.02	0.07	18.00	1.00	46.64	0.64	8.69	0.99
234	1.02	0.06	17.00	0.40	46.86	1.29	8.74	1.35
240	1.07	0.03	17.00	0.20	45.76	1.12	7.17	2.09
246	1.04	0.01	19.00	0.15	46.32	1.94	8.55	0.24
252	1.03	0.11	18.00	0.50	46.17	1.15	8.83	0.57
258	1.01	0.02	19.00	0.25	47.03	1.54	8.36	0.15
264	1.03	0.03	17.50	0.30	48.17	0.92	7.21	0.65
270	1.02	0.04	18.00	0.10	47.95	1.50	7.76	0.64
276	1.04	0.07	18.00	0.65	47.98	0.51	7.74	0.50

282	1.06	0.07	18.50	0.70	46.01	0.22	6.92	0.22
288	1.09	0.02	17.00	0.50	46.01	0.22	8.71	1.49

2.0 Media dilution rate of 0.05 h^{-1} and solvent recirculation at 200 mL h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})			
					Medium	Standard deviation	Solvent	Standard deviation
290	1.00	0.03	18.40	0.70	290.00	41.01	1.03	12.68
302	1.13	0.00	11.50	0.40	302.00	42.73	0.40	10.61
307	1.24	0.01	8.10	1.05	307.00	43.45	0.43	10.90
312	1.26	0.04	5.70	0.10	312.00	44.97	1.64	9.55
317	1.28	0.01	4.20	0.25	317.00	44.06	0.01	9.75
321	1.22	0.02	4.50	1.20	321.00	42.24	1.60	9.11
327	1.07	0.00	18.90	0.25	327.00	34.94	1.22	8.66
333	1.07	0.03	23.40	1.35	333.00	32.58	0.62	6.96
339	1.09	0.01	12.20	0.50	339.00	36.85	2.42	8.61
345	1.30	0.01	6.30	0.35	345.00	42.77	0.02	8.71
351	1.32	0.00	4.10	0.75	351.00	38.07	0.96	9.03
357	1.31	0.08	3.20	0.35	357.00	38.92	1.12	8.76
363	1.28	0.03	1.53	5.79	363.00	36.53	0.40	9.34
369	1.26	0.07	14.30	0.55	369.00	36.90	0.19	8.35
375	1.25	0.01	3.80	0.15	375.00	42.73	1.29	12.83
381	1.35	0.01	3.30	0.10	381.00	42.30	0.29	9.53

387	1.36	0.03	4.20	0.10	387.00	46.61	0.81	9.46
393	1.21	0.02	18.90	0.15	393.00	34.50	0.50	8.80
399	1.25	0.02	16.80	0.05	399.00	41.87	0.11	8.87
405	1.37	0.04	5.30	0.50	405.00	37.47	0.19	9.38
411	1.28	0.06	12.60	0.05	411.00	40.97	0.61	9.09
417	1.36	0.03	10.20	0.10	417.00	48.66	0.30	9.04
423	1.28	0.01	3.00	0.70	423.00	45.68	0.64	9.11
429	1.27	0.04	2.90	0.75	429.00	41.48	1.29	10.60
435	1.25	0.00	5.50	0.40	435.00	45.93	1.12	12.01
441	1.27	0.03	3.40	0.05	441.00	44.13	1.94	10.51
447	1.29	0.03	0.80	0.05	447.00	50.36	1.15	9.93
453	1.26	0.03	0.30	0.10	453.00	40.55	1.54	10.23
461	1.29	0.01	6.50	2.93	461.00	42.50	0.92	9.26
469	1.23	0.01	8.20	0.70	469.00	47.28	1.50	9.90
477	1.24	0.01	8.40	0.05	477.00	36.88	0.51	9.44
483	1.25	0.03	9.40	0.05	483.00	43.18	0.22	9.48
489	1.25	0.03	5.70	0.60	489.00	44.39	0.22	11.39
495	1.22	0.04	0.30	0.10	495.00	44.61	1.69	11.09
501	1.24	0.06	0.40	0.15	501.00	43.10	2.68	11.09
507	1.22	0.02	0.90	0.15	507.00	43.80	1.33	10.85
513	1.23	0.03	2.60	0.05	513.00	44.86	1.15	11.09
519	1.22	0.03	2.70	0.20	519.00	45.54	2.05	10.64

525	1.23	0.07	2.10	0.20	525.00	44.56	2.03	10.76
531	1.23	0.04	2.70	0.05	531.00	43.12	2.51	11.50
537	1.22	0.02	0.20	0.05	537.00	43.16	1.02	10.71
543	1.21	0.02	0.30	0.10	543.00	44.87	1.73	11.69
549	1.22	0.02	0.40	0.05	549.00	44.01	1.52	10.46
555	1.22	0.04	0.60	0.10	555.00	45.83	2.81	10.89
561	1.22	0.01	0.70	0.05	561.00	44.99	2.27	10.51
567	1.22	0.01	0.80	0.05	567.00	45.92	2.50	12.08
573	1.22	0.00	0.60	0.35	573.00	44.87	1.91	11.07

3.0 Media dilution rate of 0.05 h^{-1} and solvent recirculation at 300 mL h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})			
					Medium	Standard deviation	Solvent	Standard deviation
576	1.17	0.02	1.30	0.35	45.97	2.27	10.18	0.01
582	1.07	0.03	1.80	0.10	44.30	2.29	10.45	0.13
588	1.16	0.01	3.60	0.05	44.89	0.00	10.67	1.54
594	1.13	0.04	1.80	0.20	42.46	0.01	12.26	0.12
600	1.12	0.00	3.10	0.00	45.77	0.68	13.11	0.27
606	1.08	0.01	10.30	0.85	49.63	0.63	10.26	0.31
612	1.17	0.02	0.40	0.10	48.46	0.88	11.77	0.12
618	1.18	0.03	0.50	0.10	47.95	0.76	12.37	0.42
624	1.18	0.02	0.30	0.10	48.42	0.99	12.91	0.46
630	1.21	0.05	4.50	0.10	48.24	4.03	13.10	0.57
636	1.32	0.02	0.60	0.25	47.96	1.02	11.02	0.00
642	1.27	0.06	0.30	0.05	47.71	2.20	11.65	0.19
648	1.13	0.02	5.90	0.10	46.23	0.20	11.61	0.16
654	1.10	0.00	6.90	0.15	41.62	1.46	11.13	0.06
660	1.10	0.02	4.10	0.00	47.59	0.79	10.75	0.15
666	1.11	0.03	6.70	0.25	46.41	1.00	11.86	0.10
672	1.09	0.02	4.10	0.15	44.48	2.80	10.34	0.24

678	1.21	0.06	4.00	0.00	47.42	0.19	10.67	0.41
684	1.19	0.04	1.20	0.05	41.51	2.28	11.41	0.20
690	1.22	0.05	1.10	0.05	41.42	2.70	12.00	0.01
696	1.03	0.02	5.70	0.75	45.60	0.34	12.00	0.25
702	1.12	0.02	7.30	0.10	44.96	0.39	13.00	0.45
708	1.07	0.03	9.10	0.00	44.11	0.28	13.15	0.05
714	1.01	0.02	5.80	0.50	44.23	0.88	13.04	0.13
720	1.17	0.02	0.30	0.10	46.25	0.18	12.76	0.56
726	1.15	0.04	1.20	0.00	46.84	2.55	12.98	0.02
732	1.16	0.05	0.90	0.00	48.19	0.81	12.54	0.46
738	1.32	0.05	0.80	0.05	46.27	2.21	12.47	0.08
744	1.36	0.03	4.00	0.10	44.49	2.95	12.95	0.01
750	1.30	0.02	5.40	0.40	45.86	3.27	12.65	0.49
756	1.34	0.00	5.40	0.45	47.38	1.48	13.00	0.30
762	1.37	0.02	1.00	0.00	47.49	0.66	13.61	0.30
768	1.34	0.05	0.80	0.35	47.75	0.58	12.96	0.09
774	1.23	0.07	4.80	0.15	47.00	0.79	13.34	0.12
780	1.37	0.04	6.40	0.00	46.99	0.10	12.84	0.36
786	1.39	0.02	0.90	0.40	47.00	0.10	13.21	0.59
792	1.34	0.01	1.40	0.05	46.45	0.20	13.43	0.57
798	1.34	0.02	1.80	0.65	46.65	0.30	13.17	0.11
804	1.33	0.04	1.80	2.10	46.02	0.62	13.04	0.30

810	1.30	0.03	2.50	0.10	46.73	0.63	13.47	0.35
816	1.32	0.04	1.10	0.20	46.35	2.42	13.63	0.19
822	1.32	0.05	2.00	0.00	46.00	1.73	13.26	0.58
828	1.32	0.04	0.80	0.30	47.56	0.10	13.25	0.50
834	1.31	0.02	1.80	0.05	46.64	1.37	13.49	0.83
840	1.30	0.01	1.40	0.05	46.87	0.34	13.34	0.33

4.0 Media dilution rate of 0.05 h^{-1} and solvent recirculation at 400 mL h^{-1}

Time (h)	Average biomass (g L^{-1})	Standard deviation	Average glucose (g L^{-1})	Standard deviation	Average ethanol (g L^{-1})			
					Medium	Standard deviation	Solvent	Standard deviation
844	1.33	0.07	2.80	0.20	42.28	0.52	9.16	0.21
850	1.37	0.05	3.50	1.45	40.95	0.31	10.74	0.58
856	1.53	0.08	0.60	0.15	43.51	0.65	10.98	0.00
862	1.35	0.01	0.40	0.15	47.08	2.26	8.76	0.29
868	1.22	0.05	0.90	0.05	44.34	0.81	10.06	0.60
874	1.31	0.02	0.10	0.15	39.57	0.58	9.45	0.31
880	1.19	0.02	0.50	0.05	46.68	1.01	9.21	0.00
886	1.02	0.03	2.00	0.40	42.04	0.65	9.48	0.29
892	1.03	0.03	1.20	0.15	39.88	1.29	8.26	0.20
898	1.05	0.00	0.90	0.05	38.43	0.51	9.17	0.00
904	1.29	0.04	1.00	0.05	36.79	0.54	10.29	0.19
910	1.39	0.09	0.90	0.05	42.92	0.12	9.29	0.15
916	1.36	0.05	0.50	0.05	44.53	0.60	9.95	0.07
922	1.35	0.04	0.60	0.00	40.59	0.43	9.04	0.22
928	1.35	0.01	0.20	0.05	40.06	1.11	9.04	0.71
934	1.30	0.04	0.70	0.05	40.18	0.68	8.86	0.05

940	1.22	0.06	0.70	0.10	38.99	0.54	8.83	0.13
946	1.37	0.04	0.40	0.15	40.29	1.91	9.23	0.42
952	1.27	0.03	0.60	0.05	40.41	0.97	10.05	0.81
958	1.28	0.04	0.10	0.10	39.00	0.15	9.39	0.15
964	1.33	0.01	0.60	0.10	40.12	0.86	10.79	0.73
970	1.37	0.01	0.90	0.35	43.96	2.63	9.40	0.00
976	1.35	0.02	0.40	0.10	44.02	2.22	9.39	0.47
982	1.29	0.02	0.80	0.00	40.80	0.39	9.38	0.42
988	1.28	0.02	0.60	0.05	41.45	0.76	9.62	0.19
994	1.34	0.02	0.80	0.05	44.35	2.90	9.14	0.12
1000	1.29	0.03	1.20	0.30	44.06	2.49	9.32	0.05
1006	1.34	0.06	0.50	0.00	46.02	1.25	9.32	0.04
1012	1.29	0.02	0.80	0.00	46.19	1.74	9.71	0.20
1018	1.29	0.03	0.30	0.10	46.19	1.62	9.30	0.15
1024	1.29	0.02	0.30	0.05	47.00	0.50	9.00	0.03
1030	1.29	0.01	0.70	0.05	47.10	3.52	9.39	0.26
1036	1.29	0.05	0.30	0.05	46.66	2.03	9.21	0.02
1042	1.28	0.04	0.70	0.00	47.02	2.30	9.12	0.01

I) Adsorption isotherm

1.0 Specific loading, L (g-ethanol/kg-resin)

Resins	Ethanol concentration (g L^{-1})									
	10	Standard deviation	20	Standard deviation	30	Standard deviation	40	Standard deviation	50	Standard deviation
Poly (styrene-co-divinylbenzene)	32.01	2.68	80.94	2.04	100.50	1.72	195.68	1.60	209.70	3.43
Poly (4-vinylpyridine)	9.22	1.37	52.00	5.00	91.81	8.70	144.09	1.71	152.94	2.45
Dowex Optipore L-493	31.93	1.26	103.15	1.01	117.70	3.96	241.67	2.35	252.91	2.10
Amberlite XAD 16	11.67	1.35	61.55	2.95	98.93	2.15	156.13	2.69	201.42	1.20

2.0 Partitioning coefficient, K_r

Resins	Ethanol concentration (g L^{-1})									
	10	Standard deviation	20	Standard deviation	30	Standard deviation	40	Standard deviation	50	Standard deviation
Poly (styrene-co-divinylbenzene)	3.19	0.27	4.66	0.11	4.91	0.08	8.12	0.06	5.30	0.12
Poly (4-vinylpyridine)	0.75	0.11	3.14	0.25	4.30	0.87	5.06	0.01	3.90	0.22
Dowex Optipore L-493	3.13	0.03	5.31	0.00	6.61	0.23	11.03	0.11	9.50	0.38
Amberlite XAD 16	0.96	0.03	3.11	0.15	4.80	0.20	4.30	0.30	5.93	0.03

3.0 Percent reduction of ethanol in solution at equilibrium

Resins	Ethanol concentration (g L^{-1})									
	10	Standard deviation	20	Standard deviation	30	Standard deviation	40	Standard deviation	50	Standard deviation
Poly (styrene-co-divinylbenzene)	24.20	0.07	32.82	0.60	34.12	1.50	45.00	0.34	34.00	0.80
Poly (4-vinylpyridine)	6.90	1.02	23.94	0.90	30.17	0.13	33.00	0.37	28.00	0.80
Dowex Optipore L-493	23.77	0.90	34.00	0.02	38.59	0.90	52.00	0.50	47.00	1.00
Amberlite XAD 16	8.83	0.26	21.48	1.00	29.70	1.30	33.00	0.30	37.00	0.12

4.0 Adsorption isotherm of glucose (g-glucose adsorbed per kg resin)

Resins	Glucose concentration (g L^{-1})									
	50		100		150		200		250	
	Glucose adsorbed	Standard deviation	Glucose adsorbed	Standard deviation	Glucose adsorbed	Standard deviation	Glucose adsorbed	Standard deviation	Glucose adsorbed	Standard deviation
Poly (styrene-co-divinylbenzene)	13.00	0.10	11.00	1.50	30.00	1.50	70.00	2.50	130.00	1.80
Poly (4-vinylpyridine)	10.00	0.20	15.00	1.50	30.00	0.13	90.00	5.00	180.00	1.80
Dowex Optipore L-493	12.00	0.50	35.00	4.00	50.00	0.90	110.00	5.00	100.00	1.00
Amberlite XAD 16	15.00	0.10	62.00	2.50	90.00	1.30	270.00	7.50	224.00	1.20

5.0 Equilibrium adsorption of ethanol

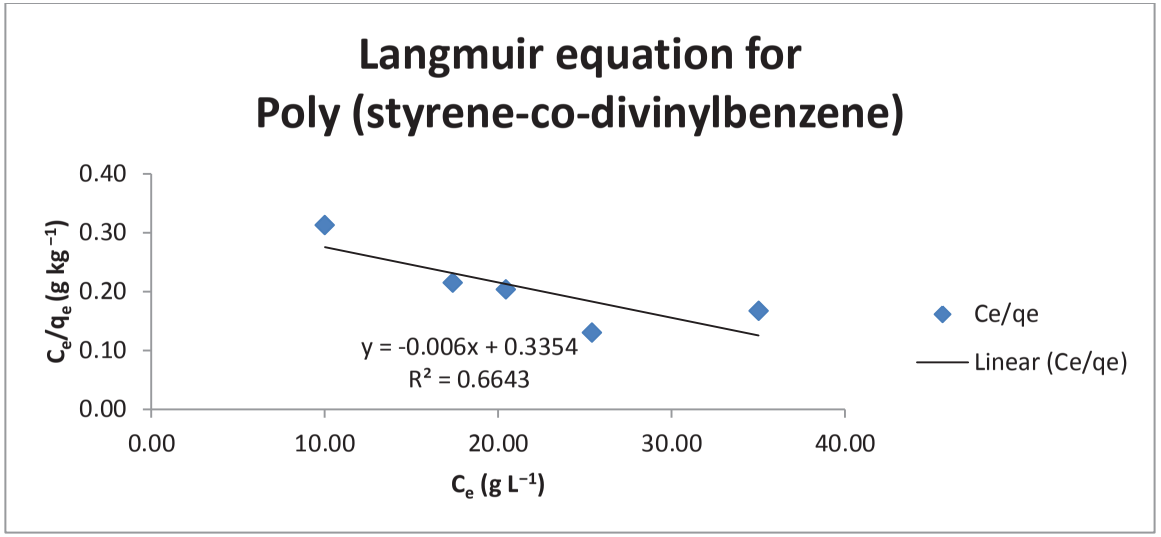
Poly (styrene-co-benzene)		Poly (4-vinylpyridine)		Dowex Optipore L-493		Amberlite XAD-16	
C_e	q_e	C_e	q_e	C_e	q_e	C_e	q_e
10.01	32.01	12.29	9.22	10.07	31.93	12.11	11.67
17.40	80.94	19.70	52.00	15.58	103.15	19.74	61.55
20.45	100.50	21.32	91.81	19.92	117.70	20.61	98.93
25.42	195.68	30.58	144.09	21.90	241.67	32.59	156.13
35.03	209.70	38.42	152.94	26.39	252.91	33.71	201.42

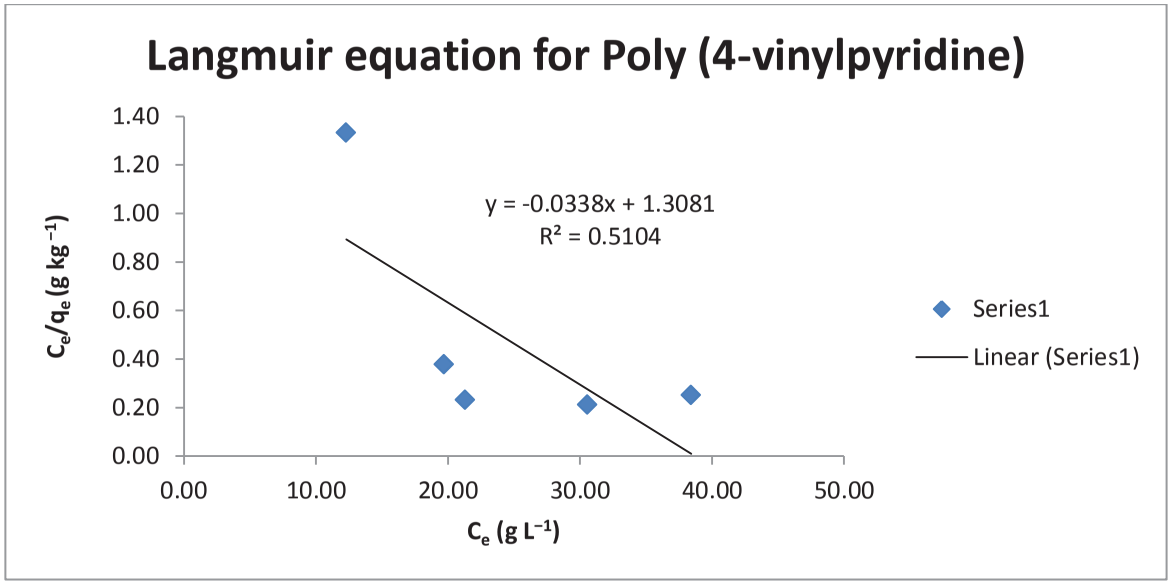
***C_e is in g L^{-1}, q_e is in g kg^{-1}**

5.1 Langmuir regression

Poly (styrene-co-benzene)		Poly (4-vinylpyridine)		Dowex Optipore L-493		Amberlite XAD-16	
C_e	C_e/q_e	C_e	C_e/q_e	C_e	C_e/q_e	C_e	C_e/q_e
10.01	0.31	12.29	1.33	10.07	0.32	12.12	1.04
17.40	0.21	19.70	0.38	15.58	0.15	19.74	0.32
20.45	0.20	21.32	0.23	19.92	0.17	20.61	0.21
25.42	0.13	30.58	0.21	21.90	0.09	32.59	0.21
35.03	0.17	38.42	0.25	26.39	0.10	33.71	0.17

***C_e is in g L^{-1}, q_e is in g kg^{-1}**


Langmuir equation for
Poly (styrene-co-divinylbenzene)
0.40
0.30
0.20
0.10
0.00
C_e/q_e (g kg^{-1})
y = -0.006x + 0.3354
R^2 = 0.6643
0.00
10.00
20.00
30.00
40.00
C_e (g L^{-1})
Ce/qe
Linear (Ce/qe)


Langmuir equation for Poly (4-vinylpyridine)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
C_e/q_e (g kg^{-1})
y = -0.0338x + 1.3081
R^2 = 0.5104
0.00
10.00
20.00
30.00
40.00
50.00
C_e (g L^{-1})
Series1
Linear (Series1)

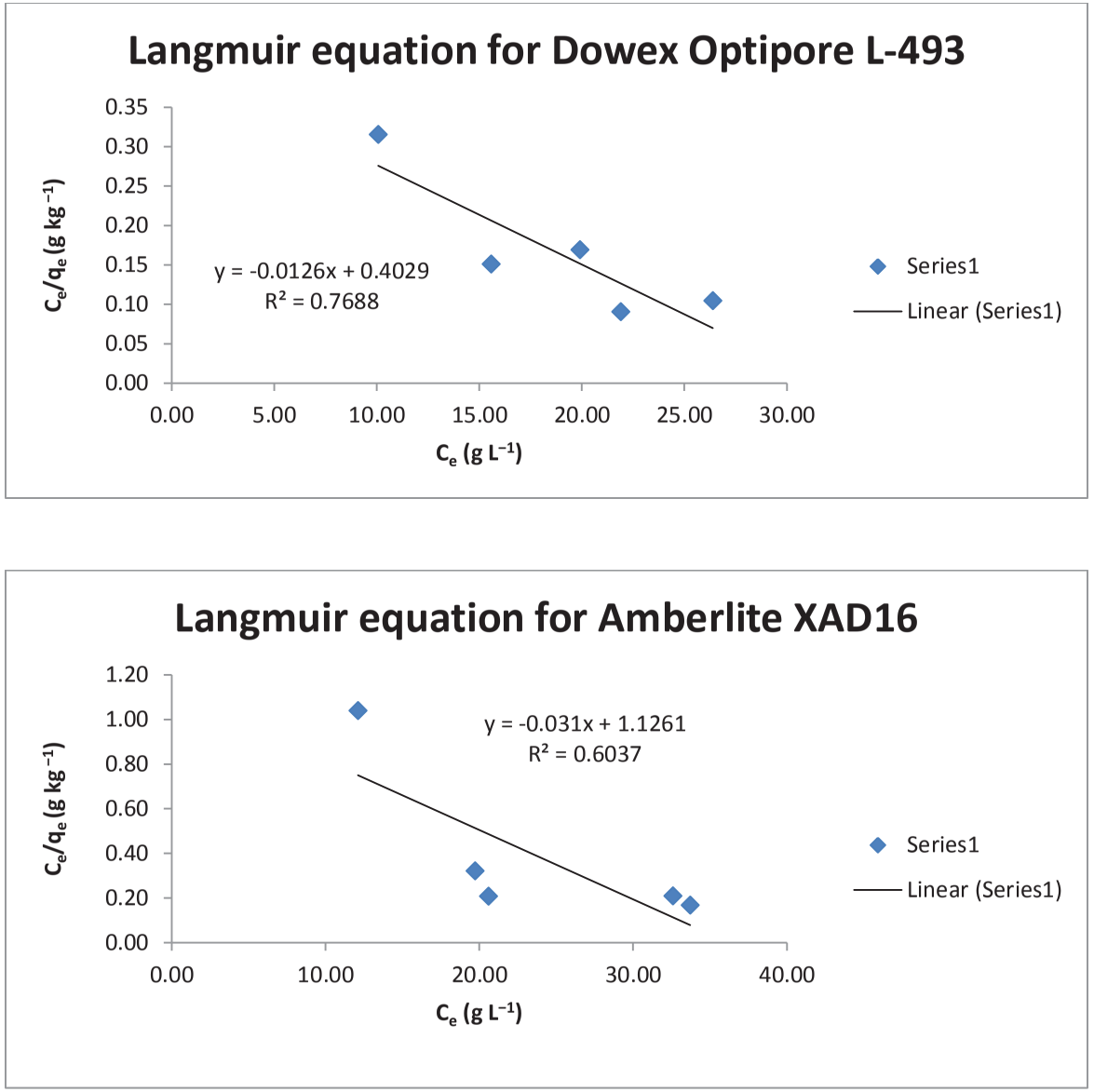
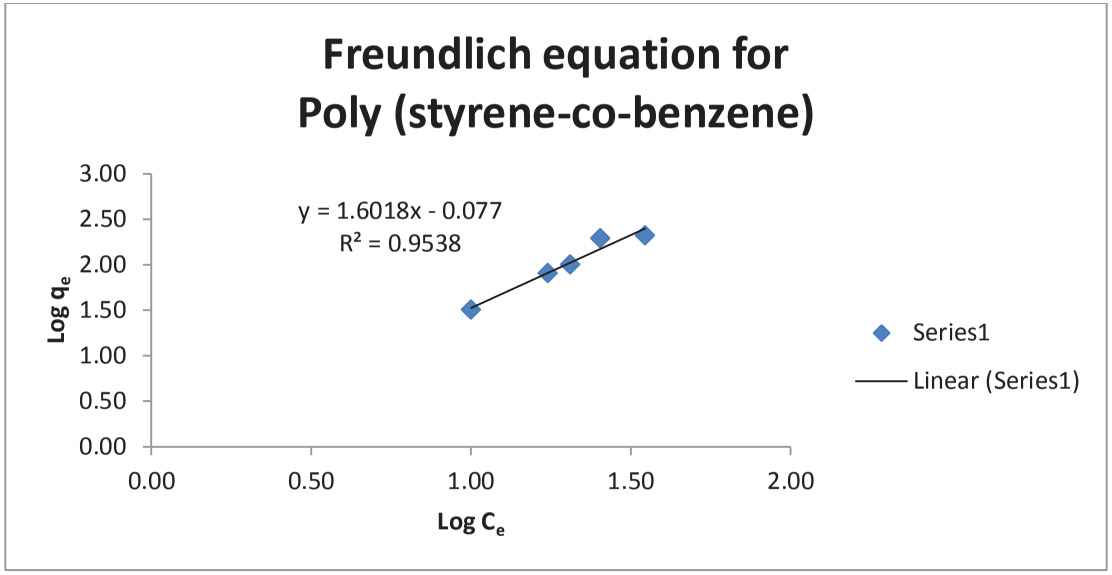


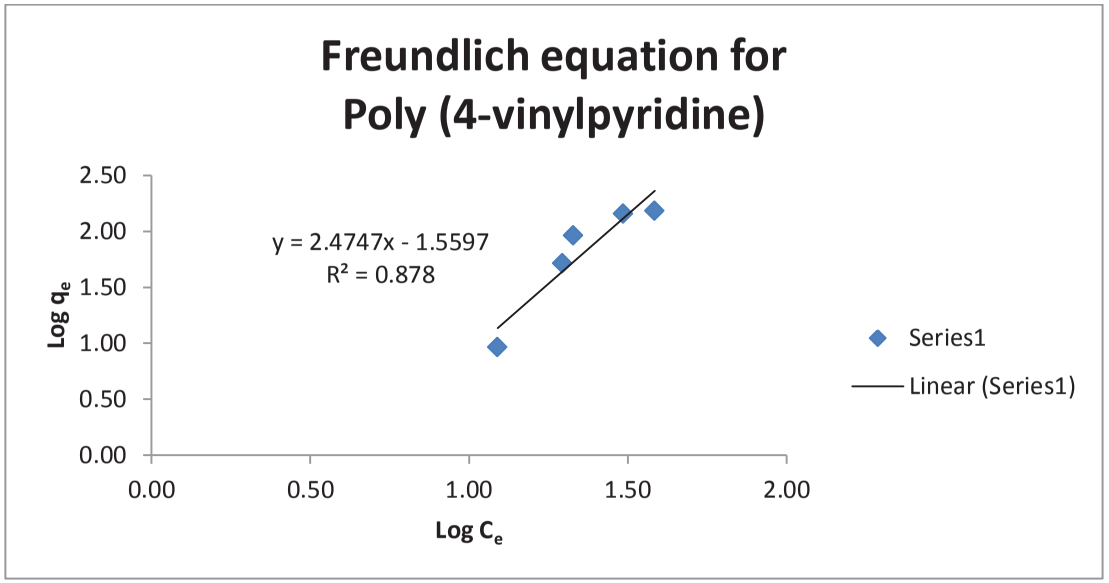
Figure A1. Adsorption data for various resins plotted according to Langmuir isotherm Eq 4.2.

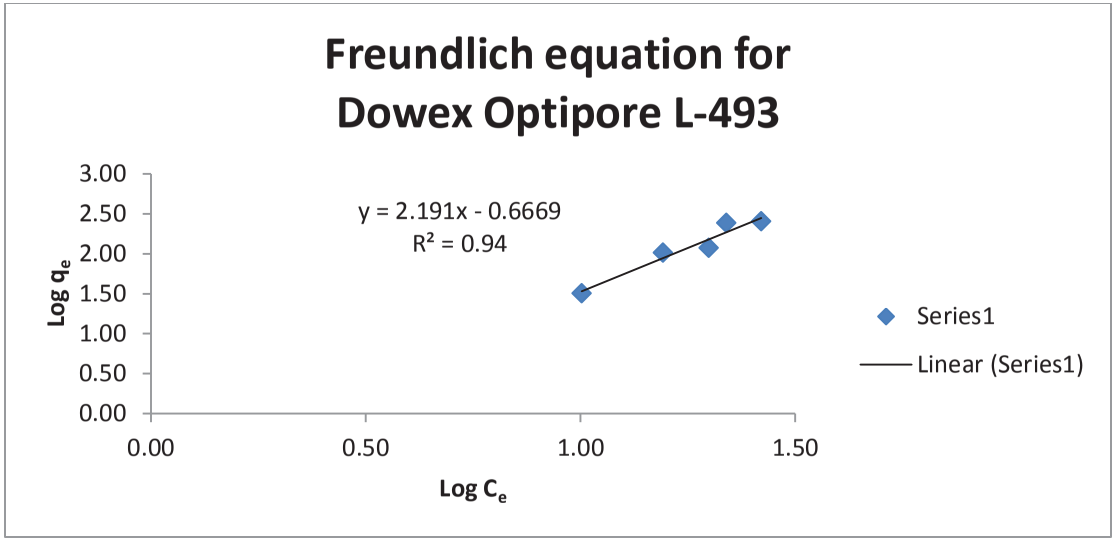
5.2 Freundlich Regression

Poly (styrene-co-benzene)		Poly (4-vinylpyridine)		Dowex Optipore L-493		Amberlite XAD-16	
log q_e	log C_e	log q_e	log C_e	log q_e	log C_e	log q_e	log C_e
1.00	1.51	1.09	0.96	1.00	1.50	1.08	1.07
1.24	1.91	1.29	1.72	1.19	2.01	1.30	1.79
1.31	2.00	1.33	1.96	1.30	2.07	1.31	2.00
1.41	2.29	1.49	2.16	1.34	2.38	1.51	2.19
1.54	2.32	1.58	2.18	1.42	2.40	1.53	2.30

*** C_e is in g L^{-1}, q_e is in g kg^{-1}**


Freundlich equation for
Poly (styrene-co-benzene)
y = 1.6018x - 0.077
R² = 0.9538
Log qe
Log Ce
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.50
1.00
1.50
2.00
Series1
Linear (Series1)


Freundlich equation for
Poly (4-vinylpyridine)
y = 2.4747x - 1.5597
R² = 0.878
Log qe
Log Ce
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.50
1.00
1.50
2.00
Series1
Linear (Series1)



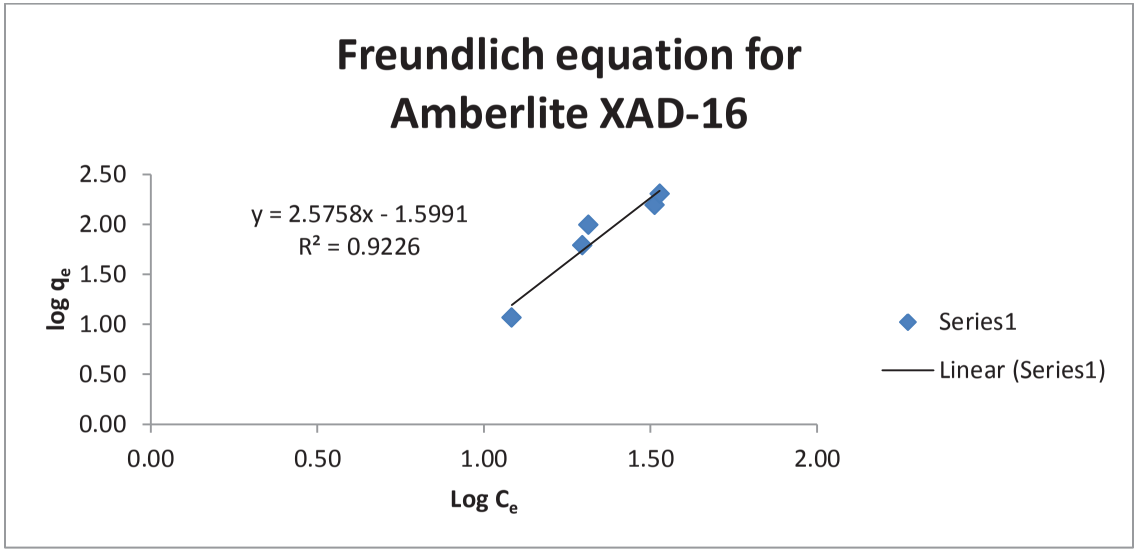


Figure A2. Adsorption data for various resins plotted according to Freundlich isotherm Eq 4.2.

6.0 In situ batch solid sorbent extractive fermentation

6.1 Biomass concentration profiles

Time (h)	Control		Poly (styrene-co-divinylbenzene		Dowex Optipore L-493		Amberlite XAD 16		Poly (4-vinylpyridine)	
	Average biomass ($g\ L^{-1}$)	Standard deviation	Average biomass ($g\ L^{-1}$)	Standard deviation	Average biomass ($g\ L^{-1}$)	Standard deviation	Average biomass ($g\ L^{-1}$)	Standard deviation	Average biomass ($g\ L^{-1}$)	Standard deviation
0	0.04	0.02	0.03	0.00	0.03	0.00	0.01	0.00	0.01	0.00
3	0.16	0.01	0.20	0.00	0.26	0.01	0.02	0.00	0.14	0.06
6	0.48	0.01	0.54	0.02	0.70	0.04	0.20	0.01	0.40	0.03
9	1.29	0.01	1.35	0.11	1.49	0.05	0.70	0.05	1.19	0.03
12	1.75	0.01	1.77	0.02	1.91	0.13	0.91	0.01	1.59	0.07
15	1.97	0.02	1.99	0.03	2.18	0.10	1.09	0.03	1.79	0.07
18	1.97	0.10	1.99	0.09	2.18	0.10	1.19	0.07	1.79	0.19

6.2 Ethanol concentration profiles

Time (h)	Control		Poly (styrene-co-divinylbenzene		Dowex Optipore L-493		Amberlite XAD 16		Poly (4-vinylpyridine)	
	Average ethanol ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation
0	0.47	0.02	0.40	0.02	0.35	0.05	0.10	0.18	0.30	0.07
3	2.10	0.10	3.00	0.35	4.00	0.85	1.20	0.10	2.00	0.15
6	14.00	0.25	12.00	1.25	15.00	0.25	5.00	0.22	10.00	2.25
9	40.00	0.50	38.00	1.50	41.00	0.00	15.00	0.01	27.00	3.00
12	53.00	3.00	50.00	4.50	57.00	1.00	25.00	0.01	39.00	1.00
15	60.00	0.62	52.80	2.02	59.40	0.92	30.00	0.06	45.00	1.00
18	61.19	0.01	52.98	2.99	60.00	0.60	34.00	0.75	46.00	2.00

6.3 Glucose concentration profiles

Time (h)	Control		Poly (styrene-co-divinylbenzene		Dowex Optipore L-493		Amberlite XAD 16		Poly (4-vinylpyridine)	
	Average glucose ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation
0	150.00	0.50	150.00	0.50	150.00	0.50	150.00	0.50	150.00	0.50
3	134.00	0.50	128.00	0.10	120.00	0.20	140.00	1.00	135.00	1.50
6	89.00	0.20	70.00	0.15	60.00	0.26	110.00	5.00	99.00	0.00
9	45.00	0.25	35.00	0.14	25.00	0.02	85.00	0.50	50.00	4.00
12	14.00	0.25	10.00	0.50	8.00	0.50	65.00	0.50	20.00	0.50
15	7.00	0.15	0.40	0.05	0.40	0.05	55.00	0.50	15.00	1.00
18	2.70	0.15	0.21	0.04	0.21	0.01	55.00	2.50	10.00	0.50

7.0 In situ continuous solid sorbent extractive fermentation

Time (h)	Average biomass ($g\ L^{-1}$)	Standard deviation	Average glucose ($g\ L^{-1}$)	Standard deviation	Average ethanol ($g\ L^{-1}$)	Standard deviation
0	0.05	0.02	149.68	0.21	0.69	0.35
2	0.08	0.02	141.74	0.25	1.08	0.35
4	0.22	0.02	109.22	0.20	2.56	0.54
6	0.44	0.02	94.72	0.50	5.63	0.48
8	0.94	0.02	77.89	0.25	11.82	0.48
10	0.83	0.01	85.00	0.64	36.03	0.64
18	2.88	0.02	4.62	0.15	30.00	33.99
26	2.96	0.01	14.90	0.00	63.91	63.24
34	2.70	0.03	24.90	0.13	57.64	51.31
42	2.69	0.05	30.90	0.00	61.54	66.81
50	1.98	0.03	50.20	0.07	45.52	48.61
58	2.01	0.04	44.30	0.25	51.64	51.00
66	1.86	0.02	35.50	0.10	44.94	46.27
74	1.41	0.01	36.50	0.05	39.95	37.65
82	1.07	0.01	35.50	0.05	41.07	39.83
90	0.97	0.01	30.60	0.05	51.81	49.27
98	0.97	0.01	36.50	0.00	41.66	41.79

106	0.96	0.04	35.70	0.05	41.62	41.62
114	1.07	0.00	30.70	0.20	46.76	46.76
122	1.22	0.00	30.60	0.15	43.06	43.93
130	1.24	0.09	25.00	0.10	43.90	43.90
138	1.18	0.02	24.40	0.05	46.63	46.63
146	1.22	0.01	25.00	0.00	52.46	55.36
154	1.20	0.02	28.00	0.05	55.44	56.97
162	1.21	0.02	18.80	0.05	51.47	52.24
170	1.24	0.01	17.90	0.05	51.88	56.72
178	1.15	0.01	17.10	0.05	54.12	55.16
186	1.23	0.02	17.10	0.05	51.12	49.99
194	1.21	0.01	17.80	0.10	52.86	52.86
202	1.12	0.01	16.00	0.05	52.75	55.26
210	1.20	0.00	16.00	0.15	53.46	53.61
218	1.20	0.00	15.00	0.10	52.46	53.10