PROLONGING A HIGH RATE OF SUCCINATE PRODUCTION IN DUAL-PHASE FERMENTATION USING *ESCHERICHIA COLI* AFP111

By

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(Under the direction of Mark A. Eiteman)

ABSTRACT

The commodity chemical succinic acid (succinate) is currently produced from petrochemicals. Succinate formed through microbial fermentation sequesters carbon dioxide and could reduce dependence on fossil fuels. This work aims to understand and prolong a high rate of succinate production in a dual-phase fermentation using a genetically modified *Escherichia coli*. Osmotic conditions resulting from high ionic strength influenced succinate production. Without any added osmoprotectant, 53 g succinate accumulated with a yield of 0.78 g/g and a productivity of 1.29 g/L•h. With 5mM betaine added to the medium succinate accumulated to 82.5 g with a 1.01 g/g yield and productivity of 2.01 g/L•h.

INDEX WORDS: Carbon dioxide sequestration, succinic acid, osmotic stress

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DEDICATION

To my parents, my brother and my guru

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CHAPTER 1

INTRODUCTION

Succinic acid (succinate) is a commodity chemical with a market currently more than 15,000 tonnes. It is predominantly produced chemically from butane through maleic anhydride. There has been a growing interest in fermentation-derived succinate which could supply over 2.7 $\times 10^8$ kg industrial products/year including 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, n-methylpyrrolidone and linear aliphatic esters (Zeikus et al., 1999). Improvements in process design and strain development may make succinate production by fermentation economically feasible (Schilling and Landucci, 1995). The four major existing markets for succinate in detergents, electroplating, and the food and pharmaceutical industries have a total value of $400 million/year. Furthermore, using glucose as a carbon source the greenhouse gas CO_2 is sequestered in the fermentation process to produce succinate. Succinate derived by this "green technology" could replace many commodities which are based on aromatic petrochemicals, resulting in a large reduction in pollution (Zeikus et al., 1999).

This project involves the biosynthesis of succinate from glucose as a carbon source, using *Escherichia coli* AFP111 through a dual-phase fermentation process. Specifically, the bacteria are subjected first to an aerobic growth phase followed by an anaerobic production phase. As growth and product formation are independent of each other, different operational conditions may be applied to each phase. Nevertheless, the enzymes that carry out the biotransformations in the production phase are largely expressed during the aerobic growth phase and many remain active during the production phase.

The goal of this project is to prolong a high rate of succinate formation and thereby accumulate a high concentration of succinate, and also gain an understanding of the physiology involved.

Microbial production of succinic acid

A linear saturated dicarboxylic acid, succinic acid is an intermediate in the metabolic pathway of several anaerobic and facultative microorganisms. Succinate accumulates in several anaerobic and facultative microorganisms. Propionate producing bacteria like *Propionibacterium* sp. form succinate from sugars or amino acids. Several rumen bacteria like *Bacteroides ruminicola, Succinimonas amylolytica, Ruminococcus flavefaciens* and *Bacteroides succinogenes* also produce succinate (Bryant et al., 1958; Scheifinger and Wolin, 1973). Gastrointestinal bacteria like *E. coli* and *Pectinatus* spp. also form succinic acid.

Anaerobiospirillum succiniciproducens can ferment whey directly into a succinate-rich product. In continuous culture the succinate productivity was 3 g/L•h, and the yield was 60%. In batch cultivation mode the yield was 80%. The highest succinate yield of 90% was obtained in a variable-volume fed-batch process (Samuelov et al., 1999). When non-treated whey and glucose were used as co-substrates in a batch fermentation, the succinate productivity and yield were 0.46 g/L•h and 95% respectively. Under continuous fermentation, a yield and productivity of 93% and 1.35 g/L•h respectively were obtained (Lee et al., 2000). Sucrose, maltose, lactose and fructose were efficiently utilized by *A. succiniciproducens* for succinate production (Lee et al., 1999). The optimum pH for the production of succinate by *A. succiniciproducens* was between 5.8 and 6.4, with lactate being generated as the main product at higher pH (Datta, 1992). Cells anaerobically cultured in a medium containing 6.5 g/L glycerol result in a high succinate yield (133%) while avoiding the formation of by-product acetic acid. The mass ratio of succinate to acetate generated was 26:1, which is 6.5 times higher than the ratio obtained using glucose as a carbon source (Lee et al., 2001). In a 2.5 L jar fermenter *A. succiniciproducens* fermented wood hydrolysate supplemented with corn steep liquor to 24 g/L succinate with a yield of 0.88 g/g (Lee et al., 2003).

In a repeat-batch fermentation with glucose as a carbon source, *Actinobacillus succinogenes* produced 35.1 g/L succinate in 48.2 h at a productivity of 0.7 g/L•h and yield of 0.68 g/g (Urbance et al., 2004). In fermentations carried out in a 1.8 L bioreactor in a semi-defined and wheat-derived media, *A. succinogenes* produced 35.6 g/L succinate with a productivity of 0.56 g/L•h and yield of 0.82 g/g in about 60 h (Du et al., 2007). *Actinobacillus* sp. 130Z ferments glucose principally to succinate, acetate, and formate with a minor amount of ethanol. Under CO_2-limiting conditions, less succinate and more ethanol is formed. In contrast, ethanol production decreases with an increase in CO_2 (Van der Werf et al., 1997). The fermentation product ratio remains constant in the pH range of 6.0 to 7.4. The addition of different amounts of CO_2 has demonstrated a direct relationship between CO_2 and succinate production.

The bovine rumen *Mannheimia succiniciproducens* MBEL55E fermented glucose to 13.5 g/L succinate in a 2.5 L jar fermenter (Lee et al., 2002). In a 6.6 L batch fermentation, *M. succinoproducens* LPK7 fermented glucose to 13.4 g/L succinate with an yield of 0.97 mol/mol and productivity 1.59 g/L•h. Fed batch culture with intermittent glucose feeding produced 52.4 g/L succinate with an yield of 1.16 mol/mol and a productivity of 1.8 g/L•h (Lee et al., 2006).

The anaerobic rumen bacterium *Fibrobacter succinogenes* S85 degrades lignocellulose substrates and can also use glucose, cellobiose, microcrystalline cellulose and pulped paper as substrates (Bibollet et al., 2000). The major end products by *F. succinogenes* from each of these substrates are succinate (69.5–83%) and acetate (16–30.5%). Maximum succinate productivity ranges from 14.1 mg/L•h for steam-exploded Yellow Poplar to 59.7 mg/L•h for pulped paper (Gokarn et al., 1997).

Escherichia coli

E. coli can generate succinate aerobically. An *E. coli* mutant HL27659k(pKK313) was recently developed with five pathways inactivated and which overexpressed *Sorghum vulgare* pyruvate carboxylase. In a fed-batch process on complex media this strain produced 58.3 g/L of succinate in 59 h under completely aerobic conditions (Lin et al., 2005).

Under anaerobic conditions wild-type *E. coli* ferments sugars to a mixture of products: lactate, ethanol, acetate, formate and succinate (Clark, 1989). Expression of plasmid-encoded phosphoenolpyruvate (PEP) carboxylase (Gokarn et al., 2000; Millard et al., 1996) or pyruvate carboxylase (Gokarn et al., 2000) results in increased succinate formation, but without additional mutations the highest yield of succinate was still less than 0.5 mol per mol of glucose (Millard et al., 1996). In a 10 L batch cultivation mode 17 g/L succinate was obtained in 30 h with a productivity of 0.56 g/L•h in a medium supplemented with 10% cane molasses. The optimal pH was determined to be 6.5 (Agarwal et al., 2006).

In order to accumulate significant succinate under anaerobic conditions, knockouts are necessary of key enzymes in those pathways generating by-products. *E. coli* NZN111 lacks activities for pyruvate-formate lyase and lactate dehydrogenase (Bunch et al., 1997). Unfortunately, these mutations prevent this strain from growing using glucose anaerobically. Expression of malic enzyme in this non-fermenting mutant restored glucose metabolism, and succinate was the major product of fermentation (Stols and Donnelly, 1997). *E. coli* AFP111 arose from a spontaneous chromosomal mutation of NZN111. The mutation was later identified in the *pts*G gene encoding for an enzyme of the phosphotransferase system (PTS). This mutation restored the ability to ferment glucose (slowly), and also resulted in a higher yield of succinate anaerobically (Chatterjee et al., 2001). The absence of a complete PTS causes AFP111 to rely on glucokinase for glucose uptake (Bunch et al., 1997; Vemuri et al., 2002a).

AFP111 ferments 1 mol of glucose to 1 mol of succinic acid and 0.5 mol each of acetic acid and ethanol (Chatterjee et al., 2001; Donnelly et al., 1998). When AFP111 was first grown aerobically and then subject to anaerobic conditions, the succinate yield was 0.99 g/g, and the productivity was 0.87 g/L•h (Nghiem et al., 1999). Such dual-phase fermentation is advantageous because it decouples growth and product formation. Unique operating conditions can be applied to each phase, and also enzymes that carry out the biotransformations in the second non-growth, production phase are largely expressed during the aerobic growth phase and remain active throughout the production phase (Vemuri et al., 2002a). In a dual-phase fermentation on 50% light steep water fermentation medium, AFP111 accumulated 51 g/L of succinate after 99 h at a productivity of 0.52 g/L•h (Nghiem et al., 1999). On complex media, AFP111/pTrc99A-*pyc* produced a final succinate concentration of 97.5 g/L in extended fed-batch dual-phase fermentation (Vemuri et al., 2002a).

Succinate producing pathways

The biochemical pathways leading to succinate formation are part of glycolysis and the TCA cycle. The pathways and their key enzymes are described below.

Glycolysis

Glycolysis is the sequence of reactions that convert glucose into pyruvate. For every mole of glucose consumed, 2 moles of pyruvate, ATP and NADH are produced. As the principal route to metabolize hexoses, glycolysis can be carried out both aerobically and anaerobically.

Glycolysis can be conceptually broken down into two parts. One mole of glucose is converted to two moles of glyceraldehyde-3-phosphate with the consumption of two moles of ATP. Subsequently, two moles of glyceraldehyde-3-phosphate is converted into two moles of pyruvate with the production of four moles of ATP and two moles of NADH.

Some important enzymes of glycolysis are:

Glucokinase

Glucokinase (EC 2.7.1.2) phosphorylates glucose using ATP to yield glucose-6-phosphate. In *E. coli* and most bacteria glucose transport is principally by the phosphotransferase system (PTS), and glucokinase only plays a minor role in glucose metabolism. Wild type *E. coli* can grow in the absence of PTS, and a mutation in *glk* gene for glucokinase is necessary to eliminate growth on glucose completely (Curtis and Epstein, 1975). As with other kinases, metal ions like Mg^{2+} and Mn^{2+} are essential components for *E. coli* glucokinase (Matte et al., 1998; Lunin et al., 2004). At a pH of 7.65, the K_M values for glucose and ATP are 0.78 mM and 3.76 mM respectively (Meyer et al., 1997).

Phosphofructokinase

Phosphofructokinase (PFK, EC 2.7.1.11) catalyzes the irreversible transfer of a phosphate from ATP to fructose-6-phosphate. PFK is a key regulatory enzyme in most organisms and tissues. The enzyme has complex regulatory properties, and the complexity is greatest in higher eukaryotes. At high concentration and alkaline pH, ATP acts as an inhibitor (Zheng and Kemp, 1992). In *E. coli*, ADP and other diphosphonucleosides act as activators and phosphoenol pyruvate (PEP) acts as an inhibitor (Blangy et al., 1968).

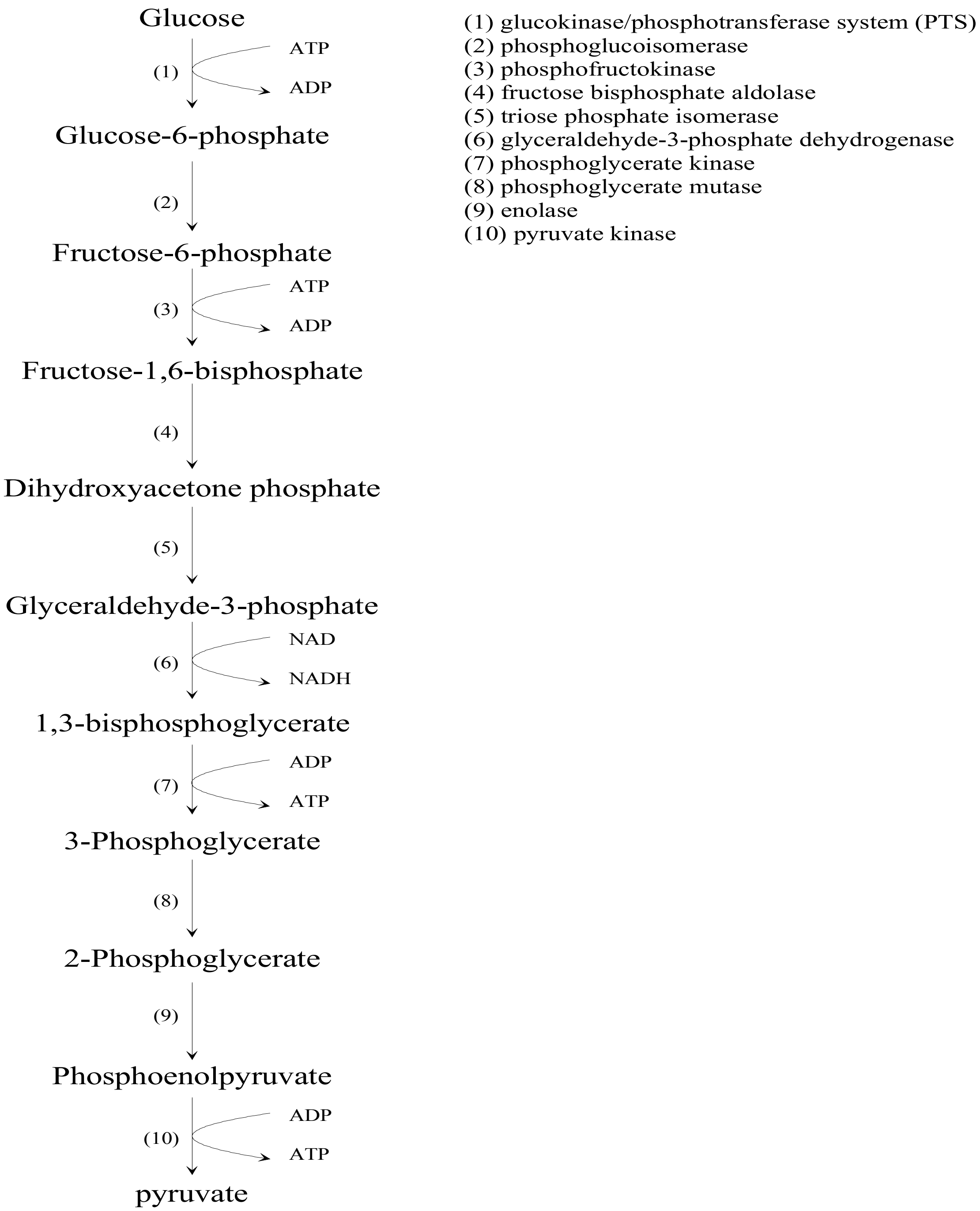

Figure 1.1. The glycolytic pathway

Glyceraldehyde-3-phosphate dehydrogenase

D-Glyceraldehyde-3-phosphate dehydrogenase (G3PDH, EC 1.2.1.12) is a key enzyme in the glycolytic conversion of glucose to pyruvic acid (Branlant et al., 1983). G3PDH plays an important role in glycolysis and gluconeogenesis (Huang et al., 1989) by reversibly catalysing the oxidation and phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. The quantification of control strength exerted by G3PDH on the overall activity of glycolytic pathway showed that G3PDH activity can be significantly rate limiting in non-growing cells (Poolman et al., 1987).

Pyruvate kinase

In the final energy-producing step of glycolytic pathway, pyruvate kinase (EC 2.7.1.40) catalyzes the conversion of PEP and ADP into pyruvate and ATP (Ponce et al., 1995). The reaction is essentially irreversible and has an absolute requirement for both a monovalent cation, usually K^+, and divalent cations, typically Mg^{2+} or Mn^{2+} (Mesecar and Nowak, 1997). Ca^{2+} is an activator of the enzyme at low Mg^{2+} and Ca^{2+} concentrations; otherwise it is an inhibitor (Boiteux et al., 1983).

TCA cycle

There are two pathways for the conversion of PEP or pyruvate generated by glycolysis to succinate: the reductive branch of TCA cycle and the glyoxylate shunt. The reductive branch of the TCA cycle converts oxaloacetic acid (OAA) to malate, fumarate, and then succinate. Four electrons and 1 mol of CO_2 are needed to convert 1 mol of PEP to 1 mol of succinate. The glyoxylate shunt operates as a cycle to convert acetyl coenzyme A (acetyl-CoA) to succinate. From 2 mol pyruvate, the cells generate 1 mol of succinate, 6 electrons and 2 mol of CO_2.

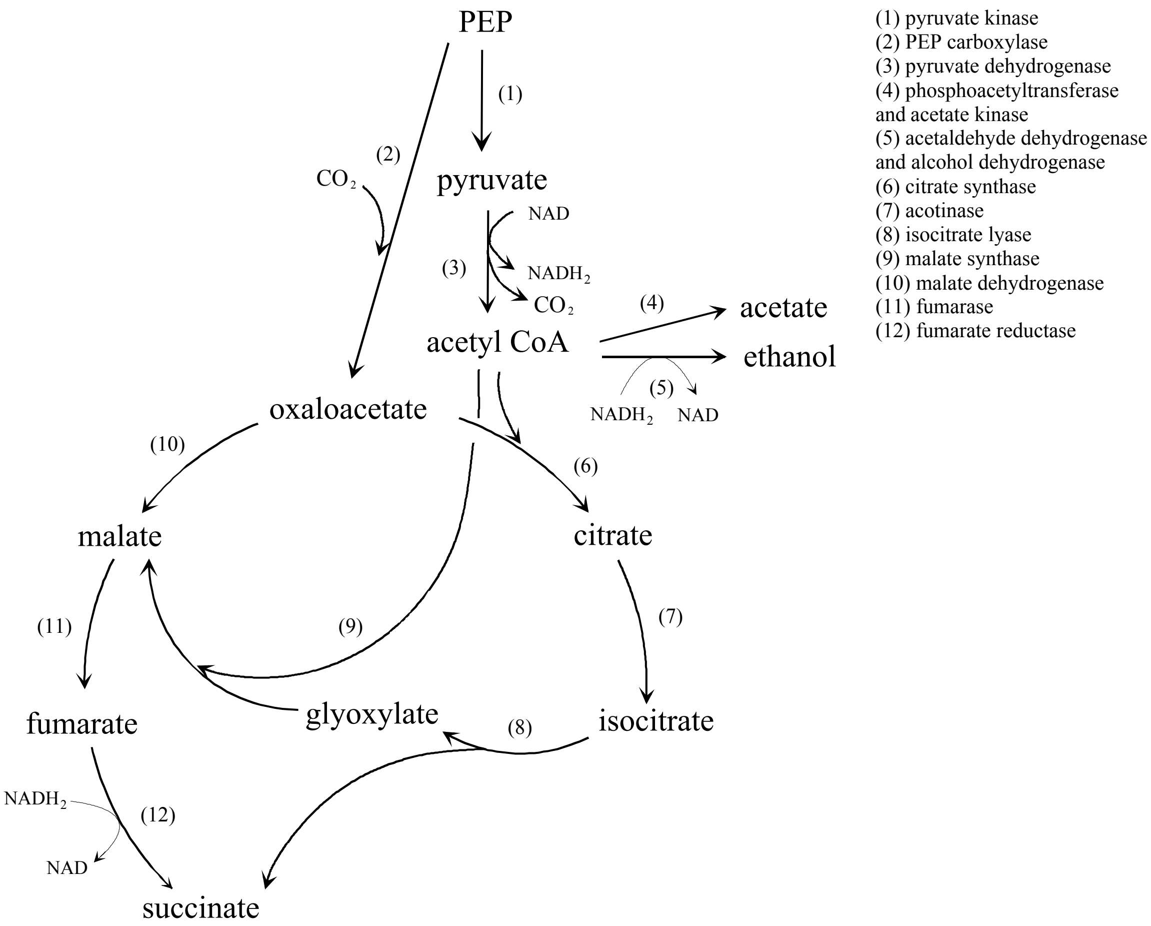

Figure 1.2. The metabolic pathway to form succinate from PEP and/or pyruvate (modified from Vemuri et al., 2002b).

The maximum possible succinate yield based only on carbon balance occurs when all succinate is produced via the TCA cycle with a mass yield of 1.31 based on glucose (Stols and Donnelly, 1997). The maximum possible succinate yield based on the additional constraint of redox balance is 1.714 mol of succinate from 1 mol of glucose, with a mass yield of 1.12 (Vemuri et al., 2002b). In the absence of additional electron donor, for maximum theoretical yield of succinate, 71.4% of carbon must flow through OAA and 28.6 % of carbon must flow through acetyl-coA. Hence the glyoxylate shunt is essential for maximum succinate accumulation (Vemuri et al., 2002b).

The key enzymes of TCA cycle are:

PEP carboxylase

PEP carboxylase [EC 4.1.1.31] is widely present in all plants and many bacteria. The enzyme performs anaplerotic functions by replenishing C_4-dicarboxylic acids for the synthesis of various cellular constituents and for the maintenance of the citric acid cycle. PEP carboxylase catalyzes the HCO_3^- dependent/biotin-independent carboxylation of PEP in the presence of divalent cations like Mg^{2+} or Mn^{2+} to form oxaloacetate and phosphate (Matsumura et al., 2002). In *E. coli* PEP carboxylase is the only enzyme that directs carbon towards OAA for succinate production, and hence this enzyme is vital for succinate production. Overexpression of PEP carboxylase causes a significant increase in the amount of succinate formed (Millard et al., 1996). Acetyl-CoA catalytically stimulates the rate of oxaloacetate formation by increasing the apparent affinity of the enzyme for PEP (Canovas and Kornberg, 1966). PEP carboxylase is inhibited by malate (Corwin and Fanning, 1968), aspartate and chloride ion (Izui et al., 1970).

Isocitrate lyase

Isocitrate lyase (ICL, EC 4.1.3.1) was not observed in anaerobically grown AFP111 and NZN111, but observed after aerobic growth (Vemuri et al., 2002b). ICL activity was found to be essential in high cell density aerobic fermentation with *E. coli* (Kleman and Strohl, 1994; Walle and Shiloach, 1998). It is unique to the glyoxylate shunt system and diverts isocitrate to glyoxylate and succinate. ICL is strongly inhibited by hydroxylmalonate. Oxalate and malonate, analogs of glyoxylate, are linear competitive inhibitors of ICL with respect to isocitrate and respectively have K_i values of 5.1 μM and 580 μM. Succinate is a linear noncompetitive inhibitor of ICL with respect to isocitrate, with a K_i value of 290 μM. Itaconate, a succinate analog, is an uncompetitive inhibitor of ICL with respect to isocitrate with a K_i value of 5.3 μM.

There is no detectable ICL activity in the absence of exogenous cations. Mg^{2+} is the most effective cation followed by Mn^{2+}, Ni^{2+} and Sr^{2+} (Hoyt et al., 1988). In *E. coli* cells grown at 37°C and assayed at 30°C the half-lives of ICL were: 7.8 min (extract), 77.5 min (extract + 0.4 M KCl), 137 min (extract + 10mM $MgCl_2$), and 137 min (extract + 0.1 M $CaCl_2$) (Griffiths and Sundaram, 1973).

Malate synthase

In a second step of the glyoxylate shunt, the glyoxylate formed by ICL is combined with acetyl-CoA to form malate and CoA by the action of malate synthase (EC 2.3.3.9). Malate synthase in cell extract has a half-life of 25 min at 45°C and 3 min at 50°C. It exhibited a gradual decrease in thermostability at 45°C when the pH was increased from 7.5 to 9.5 (Chell and Sundaram, 1978). The optimum pH for malate synthase from *Mycobacterium tuberculosis* is 7.5. The highest activity was found with 5 mM Mg^{2+}. Mn^{2+} was able to replace Mg^{2+}, yielding 40% of the activity obtained with Mg^{2+} (Smith et al., 2003). Peroxisomal malate synthase from *Candida tropicalis* showed the optimal activity at pH 8.0 and absolutely required Mg^{2+} for enzymatic activity. The K_M values for Mg^{2+}, acetyl-CoA and glyoxylate were 4.7 mM, 80 μM and 1.0 mM respectively (Okada et al., 1986).

Fumarate reductase

Fumarate reductase (EC 1.3.1.6) catalyzes the reduction of fumarate to succinate with NADH as a cofactor. In *Bacteroides amylophilus* Zn^{2+} is a strong inhibitor. The oxidation of NADH by fumarate was stimulated by low concentrations of Na^+ (Wetzstein and Gottschalk, 1985). In *Leishmania major* and *L. donovani* promastigotes, Ca^{2+} and Mg^{2+} are not needed for the enzyme activity. Both Zn^{2+} and Cu^{2+} inhibited the activity of fumarate reductase at relatively low concentrations of 25 μM and 50 μM, respectively (Chen et al., 2001).

Effect of aerobic and anaerobic conditions on *E. coli*

Some enzymes related to succinate are not induced under anaerobic conditions. The DNA binding protein FNR and the two component regulatory system ArcAB play a major physiological role during switching between aerobic and anaerobic growth conditions in *E. coli*. FNR is active only under anaerobic conditions, while ArcA/ArcB functions under both aerobic and anaerobic conditions (Gunsalus and Park, 1994). FNR regulates 297 genes contained within 184 operons (Kang et al., 2005). Under anaerobic conditions the ArcAB system represses 17 operons and activates 9 operons (Lynch and Lin, 1996). Under anaerobic growth conditions, genes involved in anaerobic respiration, fermentation and acid resistance are expressed and genes involved in aerobic respiration are repressed (Kang et al., 2005).

Aerobic conditions provide a greater energetic advantage than anaerobic conditions (Unden et. al., 2002), and *E. coli* adapts to varying levels of oxygen by varying the expression of respiratory enzymes. However, oxygen also causes production of reactive oxygen species (ROS) like the superoxide anion radical, hydrogen peroxide and hydroxyl radical (Imlay, 2002). ROS damages DNA, proteins, lipids and other biological molecules. The protection to oxidative stress is conferred by SoxRS (responding to superoxide) and OxyR (responding to peroxide) (Storz and Zheng, 2000). On switching to aerobic conditions from anaerobic conditions SoxS and OxyR transcripts increased in abundance, and OxyR is most active during the early stages of adaptation to aerobic environment (Partridge et al., 2006). Switching to aerobic metabolism from anaerobic metabolism creates additional demand for *de novo* iron-sulfur cluster synthesis to satisfy the needs of new iron-sulfur proteins, and to replace damaged iron-sulfur proteins that are required under both aerobic and anaerobic conditions (Partridge et al., 2006).

In one study, a 1.8 L anaerobic chemostat culture of *E. coli* MG1655 was disturbed by aeration. The transcript profiling revealed that the switch from fermentative to aerobic metabolism occurred mostly within 5 minutes. However, 2-oxoglutarate dehydrogenase complex and succinyl-CoA synthetase (*suc*ABCD) responded only after 10 minutes of aeration. Also fermentation products like lactate and succinate could be used as a respiratory substrate during aeration (Partridge et al., 2006).

Osmoregulation in *E. coli*

E. coli adapts to changes in external osmolarity and can grow in medium of external osmolarity which is even 100-fold higher than the intracellular osmolarity. *E. coli* responds to these changes by adjusting the concentration of cytoplasmic K^+ (Epstein and Schultz, 1965; Richey et al., 1987), cytoplasmic water, and other ionic solutes (Capp et al., 1996; Cayley et al., 1991).

Under non-growing conditions *E. coli* responds passively to changes in external osmolarity by losing or gaining water to alter the concentration of cytoplasmic solutes (Cayley et al., 1992; Cayley et al., 1991). In growing cells an increase in external osmolarity causes a decrease in cytoplasmic water (Cayley et al., 1991; Larsen et al., 1987; Richey et al., 1987) and the uptake of K^+. Accumulation of a high intracellular K^+ concentration is deleterious to cells. Hence at higher external osmolarity, the cells start accumulating proline and glycine betaine, or more simply "betaine" (Lucht and Bremer, 1994; Sutherland et al., 1986). The net rate of biosynthesis of other osmolytes like glutamate and trehalose increases thereafter (Dinnbier at al., 1988; McLaggan et al., 1994). This increase in cytoplasmic solutes causes the net influx of water and exponential growth resumes.

Addition of osmoprotectants to cells growing at high osmolarity causes an increase in cytoplasmic water and hence cytoplasmic volume without a corresponding increase in the amount of cytoplasmic osmolytes (Cayley et al., 1992). Betaine is an osmoprotectant that is not catabolized by *E. coli* (Perroud and Le Rudulier, 1985). Usually betaine is accumulated *E. coli* from the extracellular environment, although some strains can also synthesize betaine from choline (Conska and Epstein, 1996). Betaine transport in *E. coli* is energy driven, mainly through the proton gradient generated through respiration (Perroud and Le Rudulier, 1985), and its uptake in *E. coli* is stimulated by external osmotic pressure and not by any particular solute in the extracellular environment like KCl, NaCl, or K_2SO_4 (Perroud and Le Rudulier, 1985).

Addition of betaine to the growth medium can provide cells with a growth advantage. Addition of 10 mM betaine to osmotically stressed *E. coli* greatly helped in recombinant protein production. More than 95% functional protein was accumulated compared to less than 10% without the addition of betaine (Barth et. al., 2000). A concentration of 0.7 M NaCl in the medium inhibited cell growth. Addition of betaine resulted in cell growth even at 1 M NaCl concentration (Chambers et al., 1987). Under severe osmotic stress proline was the prominent compatible solute in growing *Corynebacterium glutamicum* MH20-22B cells. Availability of betaine in the medium reduced the concentration of proline from 750 to 300 mM indicating that uptake of compatible solutes is preferred to synthesis (Ronsch et al., 2003). The addition of 0.65 M NaCl to to *E. coli* K-10 growing in a minimal media decreased the growth rate of *E. coli* K-10 from 0.96 h^{-1} to 0.14 h^{-1}. Addition of 1 mM betaine alleviated this inhibitory effect of NaCl. This effect of betaine was also observed with KCl, K_2SO_4, K_2HPO_4, NaH_2PO_4 and sucrose (Perroud and Le Rudulier, 1985). Apart from these advantages, betaine also acts as a thermoprotectant in *E. coli* (Caldas et al., 1999).

The advantage of supplementing betaine depends on the dosage. The addition of betaine to *E. coli* KO11 during xylose fermentation improved the cell mass and volumetric productivity in a dose-dependent manner (Underwood et al., 2004). Supplementing mineral salts medium with betaine resulted in a dose-dependent increase in the cell yield by extending the growth phase. The production of glutamate and trehalose were insufficient as protective osmolytes during the fermentation of 10% (w/v) glucose or sucrose to D-lactate by *E. coli* SZ132. The addition of 1 mM betaine doubled cell yield, increased specific productivity of D-lactate and glycolytic flux by 50% and tripled volumetric productivity (Zhou et al., 2006). Excessive accumulation of betaine can negatively impact the cells (Umenishi et al., 2005).

In summary, an optimal concentration of the osmoprotectant betaine may aid in succinate production by alleviating the negative effects of osmotic stress caused by the increasing counter ion concentration in the fermentation medium.

Effect of sodium on growth and succinate production

In one set of fermentations using AFP111 with 2M NaOH to control pH, 23.9 g/L succinate was obtained after 72 h with a productivity of 0.33 g/L•h. In contrast when 15% NH_4OH used as base, the succinate concentration reached 33.4 g/L for a productivity of 0.46 g/L•h (Nghiem et al., 1999). This result suggests that Na^+ negatively impacts succinate production. Cell growth and succinate production in *A. succiniciproducens* is affected by presence of sodium ion in medium (Datta, 1992). Maximum cell and succinate concentrations were obtained in medium containing 4 g/L NaCl. When NaCl concentration exceeded 4 g/L, the maximum cell concentration decreased, probably due to increasing osmolarity of the medium. Glucose was not consumed in sodium ion-deficient medium, suggesting that sodium ion affects glucose transport in *A. succiniciproducens* (Lee et al., 1999). Sodium is also required for glucose transport in *F. succinogenes*. The glucose transport rate was maximal when the sodium concentration was 80 mM. However, the rate decreased when Na^+ was increased to 100 mM (Chow and Russell, 1992). The growth rate of the *E. coli* wild-type strain W3110 fermenting glucose under anaerobic conditions decreased with increasing NaCl concentration in the medium. The inhibition in growth rate was less significant in aerobically grown cells. The decrease in growth rate by 0.5 M NaCl was threefold higher than 0.5 M KCl or potassium glutamate. Thus, the inhibition in anaerobic growth rate by NaCl appears not to be entirely due to an increase in osmolarity but also is due to the chemical nature of Na^+ itself (Trchounian and Kobayashi, 1999) These results suggest that Na^+ above a threshold concentration can reduce glucose transport which in turn may decrease succinate productivity by AFP111. Furthermore, when cells are growing, reduced glucose transport may impact cell growth and viability. Also NaCl decreases cell viability in a dose-dependent manner (Umenishi et al., 2005). For AFP111, because the cells are not growing during the anaerobic production of succinate, Na^+ may affect only glucose transport or this ion might also reduce the viability of the non-growing cells.

Methods for measuring cell viability

Several common methods exist for quantifying viable cells. In the culture-based method, the number of viable cells in a sample is determined by pouring or spreading the sample in a solid medium, and counting the number of colony forming units (CFU) after incubation for about 24-48 h. Direct viability count involves incubation of bacterial sample with yeast extract and the antibiotic nalidixic acid which causes viable cells to elongate. Total cells and viable cells are counted microscopically (Kogure et al., 1979) with difficulty due to variation in size of bacterial population (Yokomaku et al., 2000). Trypan Blue exclusion method only stains the damaged cells blue (Black and Berenbaum, 1964) and significantly overestimates cell viability (Altman et al., 1999). 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) in its native oxidized form is colorless and when reduced fluoresces red. A dehydrogenase enzyme present in the electron transport chain (Hatzinger et al., 2003) of metabolically active bacteria (Bhupathiraju et al., 1999; McDaniels et al., 2005) in aerobic, facultative, and anaerobic conditions will reduce CTC. CTC staining is sometimes combined with DAPI or SYTO 22 (which give green fluorescence for non-viable cells) counterstaining for measuring total cell count. Differential staining is a dual-staining method which allows effective separation between viable and dead cells (Boulos et al., 1999). Dyes like 6-carboxyfluorescein diacetate (6CFDA), PI, CTC, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), TO-PRO-1, SYTOX green, DiBAC4(3), and Rhodamine 123 have been used to differentially stain viable and non-viable cells (López-Amorós et al., 1997; Mortimer et al., 2000; Tanaka et al., 2000; McDaniels et al., 2005). Each approach has its own advantages and disadvantages. Except for the culture based method, these methods require instrumentation or reagents or both.

CHAPTER 2

HYPOTHESES

E. coli AFP111 and the two-phase process have some advantages for the biosynthesis of succinate. In addition to being able to generate a high succinate concentration, because the growth and product formation is decoupled, the operational conditions of the process are very flexible. Since succinate is produced in a non-growth phase, maintaining cell activity in order to prolong the succinate formation is vital. Succinate production is likely influenced by the rate of glycolysis. G3PDH is an important enzyme as its activity is directly related to the glycolytic metabolism. Succinate production is also related to the ability of cells to maintain their redox environment. ICL is a key enzyme in this regard as its activity allows cells to bypass oxaloacetate and fumarate reduction. ICL is not produced during the anaerobic production phase. Introducing aeration during the anaerobic production phase may replenish key enzymes and influence succinate production. However introduction of aeration can cause oxidative stress, and succinate could be consumed as a substrate in the TCA cycle. Hence a judicious choice of duration and interval of aeration is required. Culture based methods of enumerating viable cells are preferred because this method does not require any additional instrumentation or reagents. Maintaining pH at 6.5 during the anaerobic production may be beneficial for succinate production.

Using NaOH to maintain the pH during the bioprocess may be suboptimal for succinate production due to the influence of Na^+ ions. The influence of Na^+ on succinate production can be quantitatively determined by comparing the succinate production using other alkali metal bases.

Using $Ca(OH)_2$ to maintain pH may aid in minimizing the osmotic stress by reducing the accumulation of any cation by generating calcium succinate, which has a low solubility and hence precipitates removing calcium from the medium. Addition of osmoprotectant betaine may also reduce the deleterious effects of certain ions such as Na^+.

Based on the inferences from the review of the literature the following hypotheses are stated:

1. Compared to a pH of 7.0 during the ananerobic production phase, pH of 6.5 will benefit succinate production in *E. coli* AFP111.
2. Increasing counter ion concentration (e.g., Na^+) during the course of the bioprocess will negatively impact succinate production due to increasing osmotic stress.
3. Introducing aeration during the anaerobic production phase will influence succinate production.
4. Addition of osmoprotectant will enhance succinate formation.

CHAPTER 3

MATERIALS AND METHODS

The overall goal of this work is to prolong a high rate of succinate formation during the anaerobic phase in dual phase fermentation with *E. coli* AFP111. The detailed materials and methods elaborated in this chapter will be used for testing the hypotheses stated in the previous chapter.

Strain:

Escherichia coli AFP111 (F+λ- *rpo*S396(Am) *rph*-1 *ldh*A::Kan Δ(*pflAB*::Cam) *pts*G) was used in the study, and has key mutations in the *pfl*, *ldhA*, and *ptsG* genes. (Chatterjee et al., 2001).

Medium:

A defined medium with the following composition (per L): 40.00 g glucose, 3.0 g $Na_2HPO_4 \cdot 7H_2O$, 8.00 g KH_2PO_4, 8.00 g $(NH4)\ {}_2HPO_4$, 0.20 g NH_4Cl, 0.75 g $(NH_4)\ {}_2SO_4$, 1.00 g $MgSO_4 \cdot 7H_2O$, 10.00 mg $CaCl_2 \cdot 2H_2O$, 0.5 mg $ZnSO_4 \cdot 7H_2O$, 0.25 mg $CuCl_2 \cdot 2H_2O$, 2.5 mg $MnSO_4 \cdot H_2O$, 1.75 mg $CoCl_2 \cdot 6H_2O$, 0.12 mg H_3BO_3, 1.77 mg $Al_2(SO_4)3.xH_2O$, 0.5 mg $Na_2MoO_4 \cdot 2H_2O$, 16.1 mg Fe(III) citrate, 20 mg thiamine·HCl, 2 mg biotin.

Fermentations:

Duplicate 1.2 L dual-phase batch fermentations were conducted in a 2.5 L Bioflow II fermenter (New Brunswick Scientific Instruments, NJ, USA). During the aerobic growth phase cells were grown to an optical density (OD) of about 20, and then switched to a 25 h anaerobic production phase (unless otherwise specified). At the time of switch and subsequently when glucose decreased to 5–10 g/L, 40 g glucose was added. During growth the pH was maintained at 7.0, the air flowrate was 1 L/min, and the agitation was gradually increased from 200 to 1000 rpm during growth phase in all the fermentations. During production phase different operational conditions were applied. Unless specified, the CO_2 flowrate was maintained at 0.2 L/min and the agitation was 200 rpm during the production phase, and samples were collected every 3–4 h. The samples were analyzed for glucose and succinate concentration, G3PDH and ICL enzyme activity, and the number of colony forming units.

Analytical techniques:

The cell density was estimated by measuring OD at 600 nm using a DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). The glucose and succinate content was analyzed by High Performance Liquid Chromatography (HPLC) (Eiteman and Chastain, 1997). G3PDH activity (Branlant et al., 1983) and ICL activity (Griffiths and Sundaram, 1973) was measured as previously described.

The experiments performed to test the hypotheses are classified as follows:

Study 1: Effect of pH on succinate production

Fermentations were performed with NaOH and KOH as bases at pH values of 6.5 and pH 7.0.

Study 2: Effect of various counter ions on succinate production

Fermentations with NaOH, KOH, K_2CO_3 and $(NH_4)_2CO_3$ as counter ions were performed at a pH of 6.5. No carbon dioxide was sparged into the vessel during the production phase when K_2CO_3 and $(NH_4)_2CO_3$ were used.

Study 3: Effect of aeration during production phase on succinate production

With K_2CO_3 as counter ion at a pH of 6.5, aeration for 10 minutes was introduced after every 3 h during anaerobic production phase,. During these brief aeration, the agitation was increased from 200 rpm to 400 rom , and the air flowrate was 1 L/min.

Study 4: Effect of osmotic stress on succinate production

Fermentations were performed with $Ca(OH)_2$ as counter ion at a pH of 6.5. During the growth phase, NaOH was used as the counter ion. To study the effect of osmoprotectant on succinate production, fermentations were performed with the growth media supplemented with 5 mM betaine. K_2CO_3 was used as counter ion at a pH of 6.5.

CHAPTER 4

RESULTS AND DISCUSSION

Study 1: Effect of pH on succinate production

In order to confirm the pH optimum for succinate production, two pH levels were compared. Figure 4.1 and Figure 4.2 show the effect of pH on succinate production using two different bases, NaOH and KOH. Based on the F statistics associated with the effect of bases and pH on succinate production, there is no significant difference between the bases used ($F = 0.92$, $p = 0.3693$, $\alpha = 0.1$), but there is a significant difference between the pH ($F = 21.48$, $p = 0.0024$, $\alpha = 0.1$). With 5M NaOH as the base to maintain a pH of 7.0, AFP111 accumulated 32 g succinate in 25 h of the anaerobic phase. The succinate yield was 0.48 g/g, productivity 0.75 g/L•h and glucose consumption was 1.56g/L•h. With 5M KOH at a pH of 7.0, the 26 g succinate accumulated with yield of 0.68 g/g, and a productivity of 0.66 g/L•h. A pH of 6.5 improved succinate production (Figure 4.1).

At a pH of 6.5 using 5M NaOH, 49 g succinate accumulated with a yield of 0.86 g/g and a productivity of 1.21 g/L•h. Using 5M KOH, 47 g succinate accumulated with a yield of 0.84 g/g and a productivity of 1.16 g/L•h. A pH of 6.5 resulted in more succinate accumulation, and this pH also increased succinate yield and productivity. This observation is consistent with recent literature (Agarwal et al., 2006) which showed that a pH of 6.5 is optimum for succinate production by *E. coli*. As shown in Figure 4.2, no less counter ion were added at pH 6.5 compared to pH 7.0.

Figure 4.3 illustrates the decrease in succinate productivity and glucose consumption with an increase in the Na^+ concentration in the medium during the course of the fermentation. This result is consistent with data that have already been reported (Chow and Russell, 1992; Datta, 1992; Lee et al., 1999; Nghiem et al., 1999). A similar result was observed in fermentations with 5M KOH.

During the anaerobic phases for these KOH and NaOH processes, samples were withdrawn to analyze the activity of two key enzymes, G3PDH and ICL (Figure 4.4 and Figure 4.5) Decreased activity of key enzymes G3PDH and ICL also correlated with increasing counter ion concentration (Na^+). This observation suggests that increasing osmotic stress from the addition of counter ion also affected the key glycolytic enzyme (Poolman et al., 1987) G3PDH activity as well as ICL. The higher enzyme activity at pH 6.5 (Figure 4.5) correlates with the increased succinate production when compared with pH 7.0 during the production phase. In any event, the decreasing activities of these enzymes correlated with decreased succinate productivity and glucose consumption rates (Figure 4.3).

Figure 4.6 shows an example bioprocess during the anaerobic production phase when 5M NaOH was used as the base. The succinate productivity and glucose consumption rate decreased with time, along with the G3PDH and ICL activity. At the onset of the anaerobic phase and again at 10 h, a pulse of glucose was added to the fermenter. The rate of glucose consumption after the second glucose pulse (at 10 h) was lower than after the first pulse. As already described, the amount of counter ion added increased due to the production of succinate (not shown).

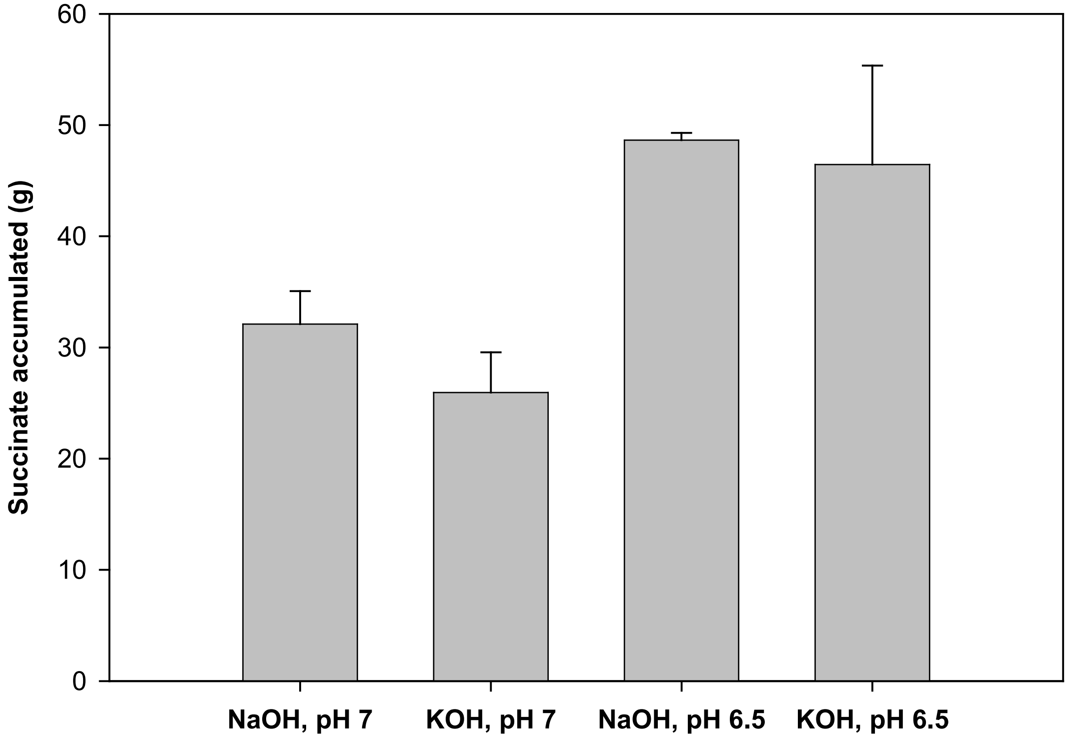

Figure 4.1. Effect of pH 7.0 and pH 6.5 on succinate accumulation.

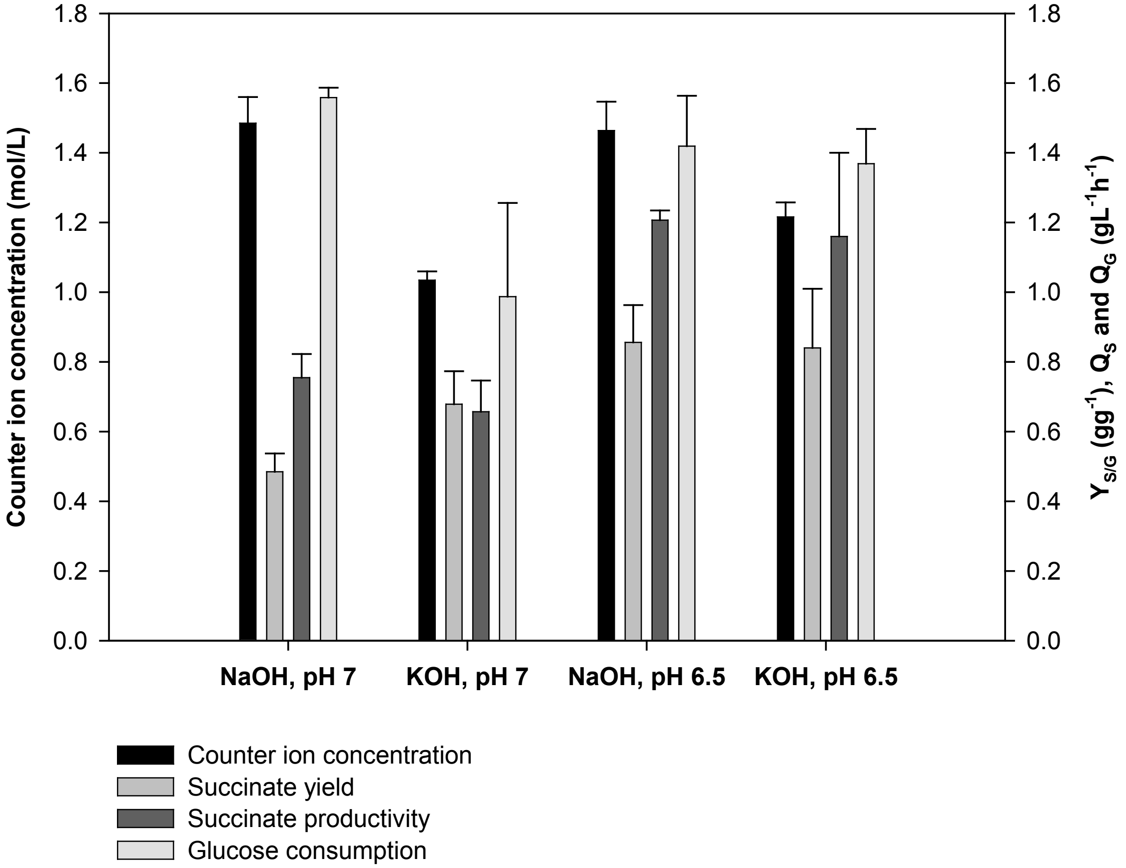

Figure 4.2. Effect of pH 7.0 and pH 6.5 on amount of counter ion added, overall succinate yield, succinate productivity and glucose consumption during the production phase.

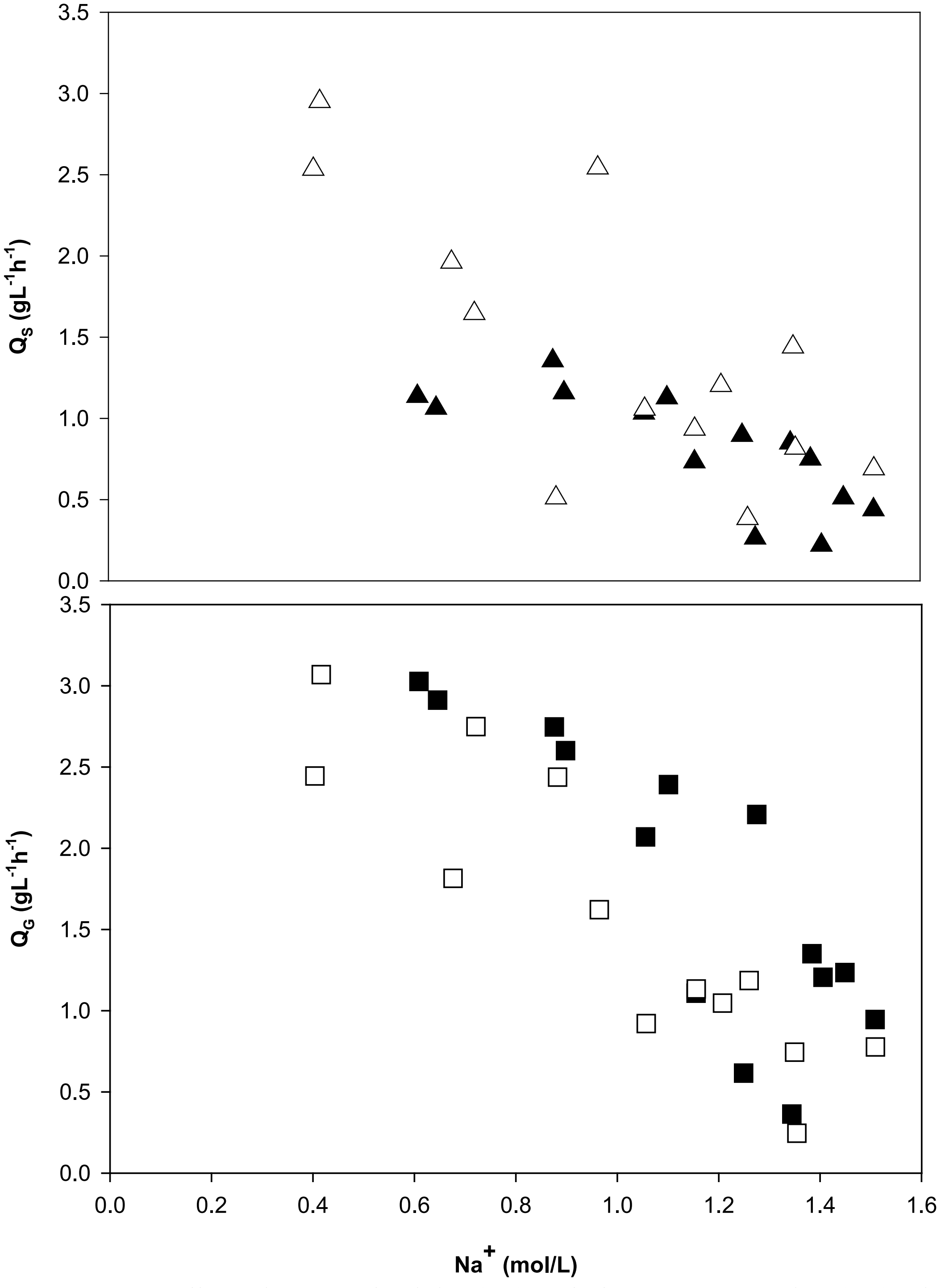

Figure 4.3. Overall succinate productivity (Q_S) and glucose consumption (Q_G) with 5M NaOH at pH 6.5 and 7.0. ▲ Succinate productivity at pH 7.0, ■ Glucose consumption at pH 7.0, △ Succinate productivity at pH 6.5, □ Glucose consumption at pH 6.5.

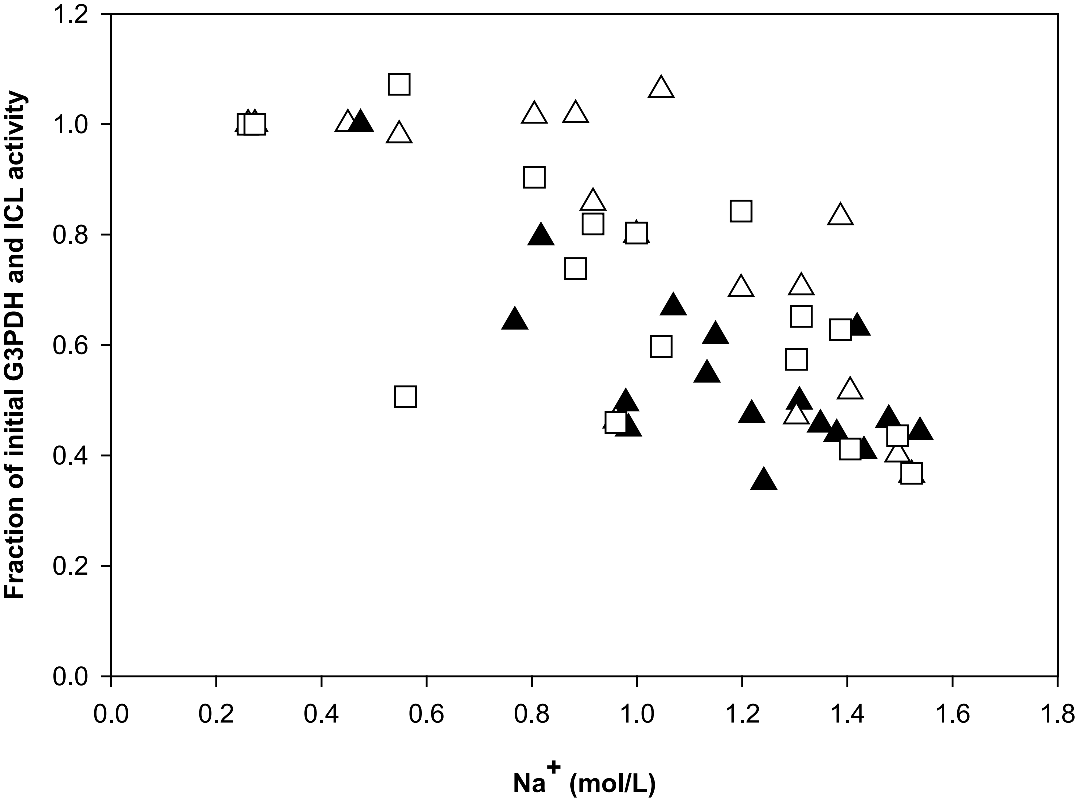

Figure 4.4. G3PDH and ICL activity at pH 7.0 and pH 6.5 with 5M NaOH. ▲G3PDH activity at pH 7.0, △ G3PDH activity at pH 6.5, □ ICL activity at pH 6.5.

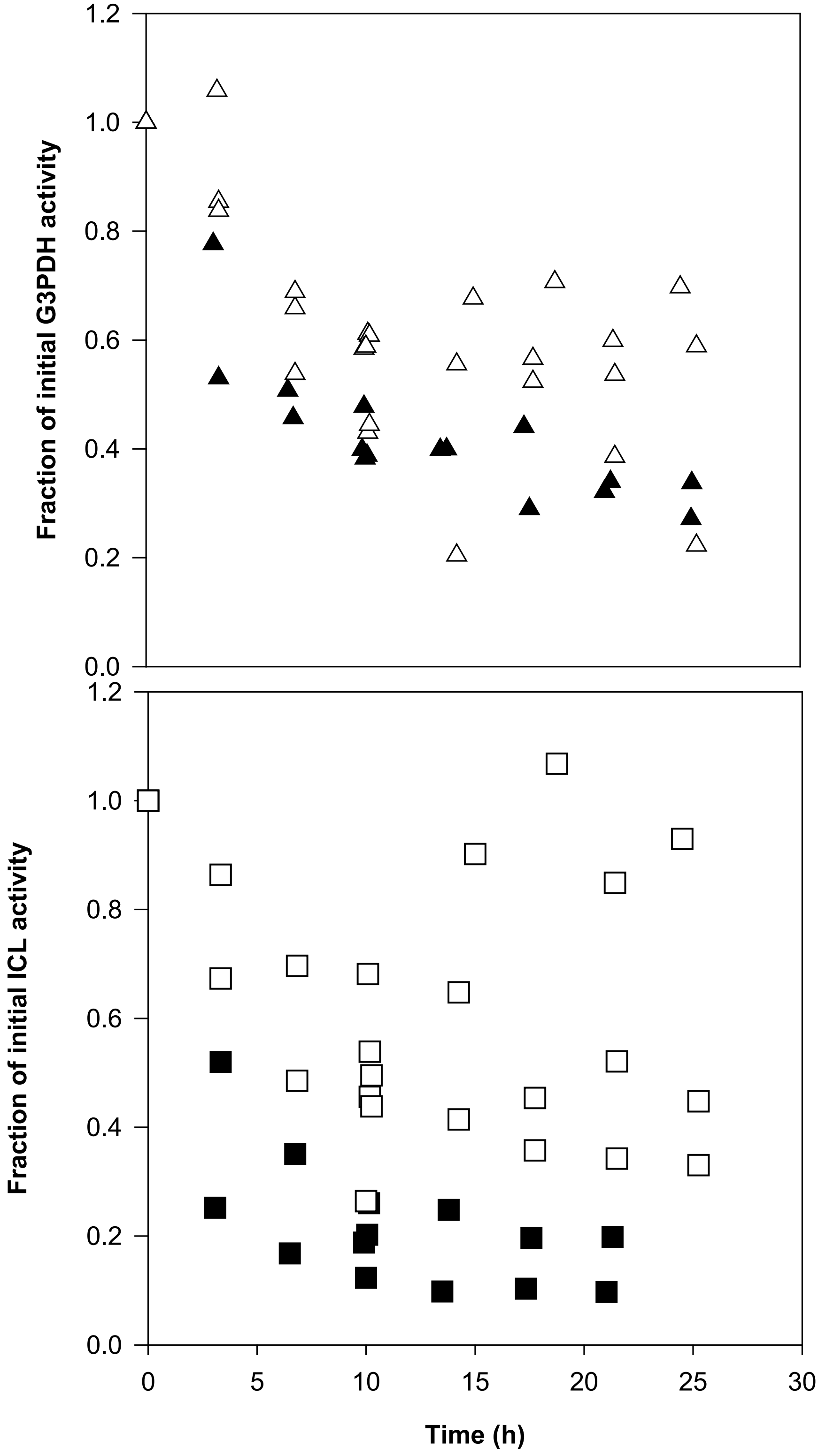

Figure 4.5. G3PDH and ICL activity at pH of 7.0 and 6.5 with 5M KOH. ▲G3PDH activity at pH 7.0, △ICL activity at pH 7.0, ■ G3PDH activity at pH 6.5, □ ICL activity at pH 6.5.

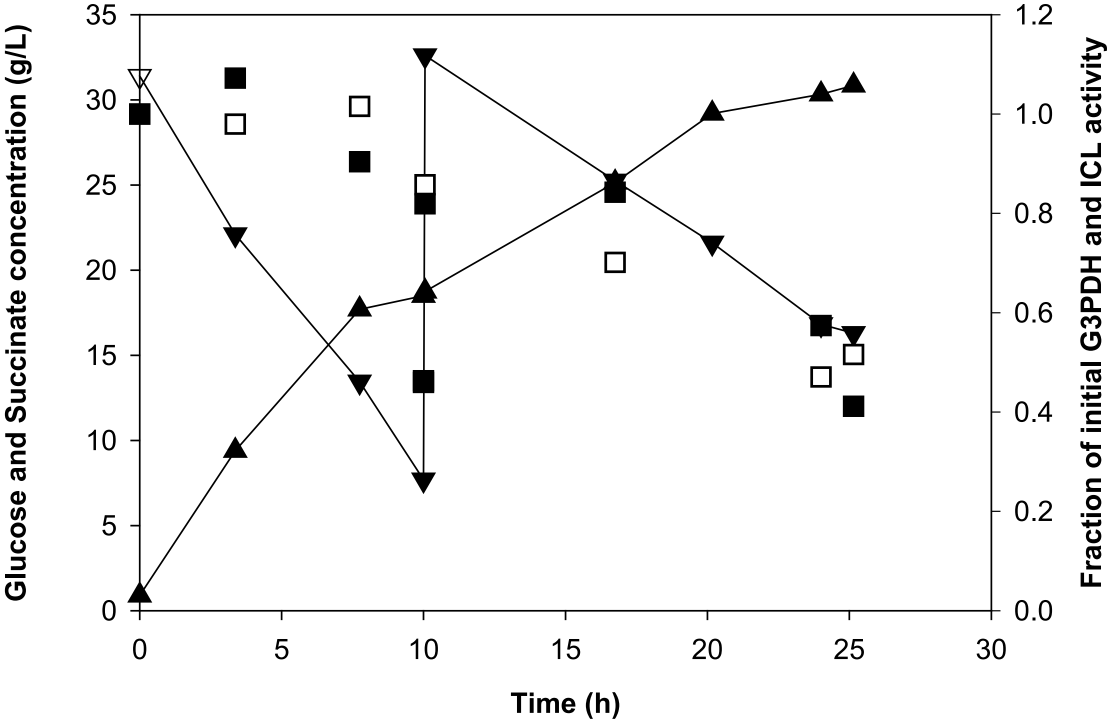

Figure 4.6. Biochemical changes during the anaerobic production phase at pH 6.5 with 5M NaOH. ▼ Glucose, ▲Succinate, □ G3PDH, ■ ICL.

Study 2: Effect of various counter ions on succinate production

Carbon dioxide is sequestered in the biosynthesis of succinate. CO_2 was sparged at a flowrate of 0.2 L/min in the production phase when NaOH or KOH was used as the base for pH control When 2.5 M K_2CO_3 or 2.5M $(NH_4)_2CO_3$ were used as the base, CO_2 was not sparged since the base contained the bicarbonate ion. When compared to KOH, K_2CO_3 decreased the amount of counter ion added during the anaerobic phase to 53% (Figure 4.8) and resulted in a 14% increase in succinate accumulation (Figure 4.7). However, based on the F statistics for the various counter ions used there is no significant advantage of one counter ion over the other in succinate accumulation ($F = 0.32$, $p = 0.8108$, $\alpha = 0.1$).

When 2.5M $(NH_4)_2CO_3$ was used as counter ion, 49 g of succinate accumulated (Figure 4.7). At the pH of 6.5, 46–53 g of succinate was accumulated by AFP111. As seen from Figure 4.8 and Figure 4.7, succinate production is not directly proportional to the concentration of counter ions. This vindicates a previous study (Trchounian and Kobayashi, 1999) that apart from osmolarity, the chemical nature of cations also influence the physiology.

Figure 4.9 shows the effect of 2.5M $(NH_4)_2CO_3$ on succinate productivity and glucose consumption. As observed with other counter ions, increasing concentration of NH_4^+ correlates with the decreasing succinate productivity and glucose consumption during fermentation. This result was also observed when 2.5 M K_2CO_3 was used to maintain pH (data not shown). Perroud and Le Rudulier (1985) suggested that osmotic stress is not caused by any particular solute in the extracellular environment.

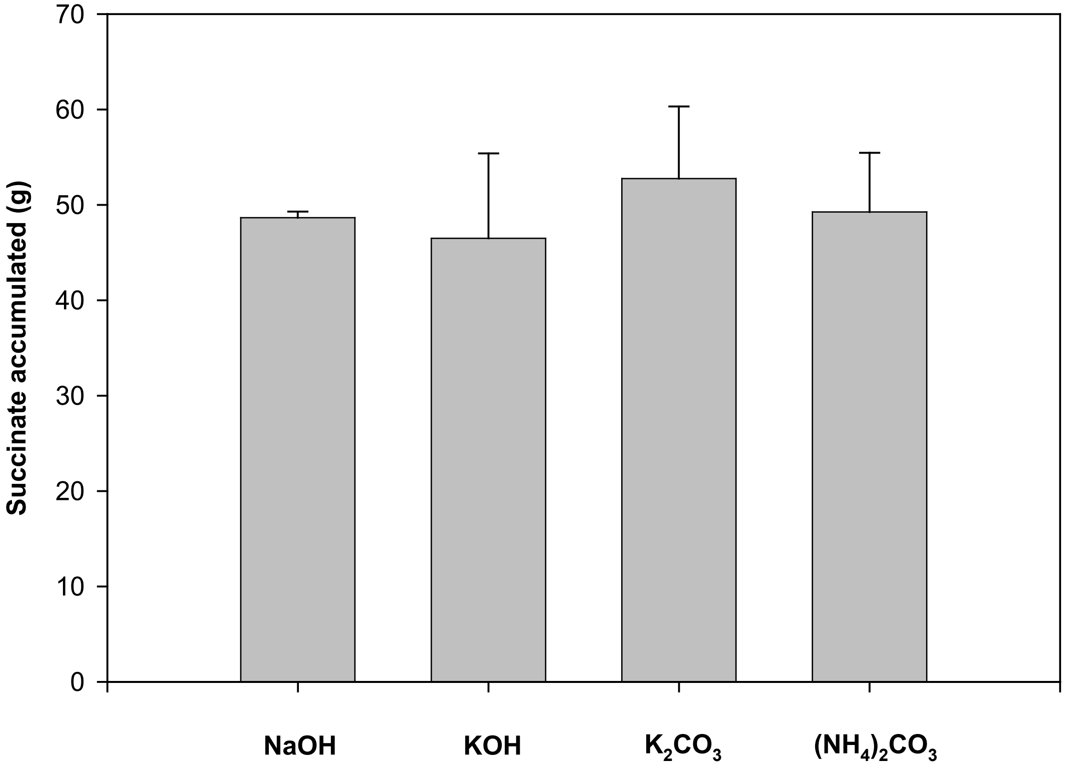

Figure 4.7. Effect of various counter ions on succinate accumulation at pH 6.5.

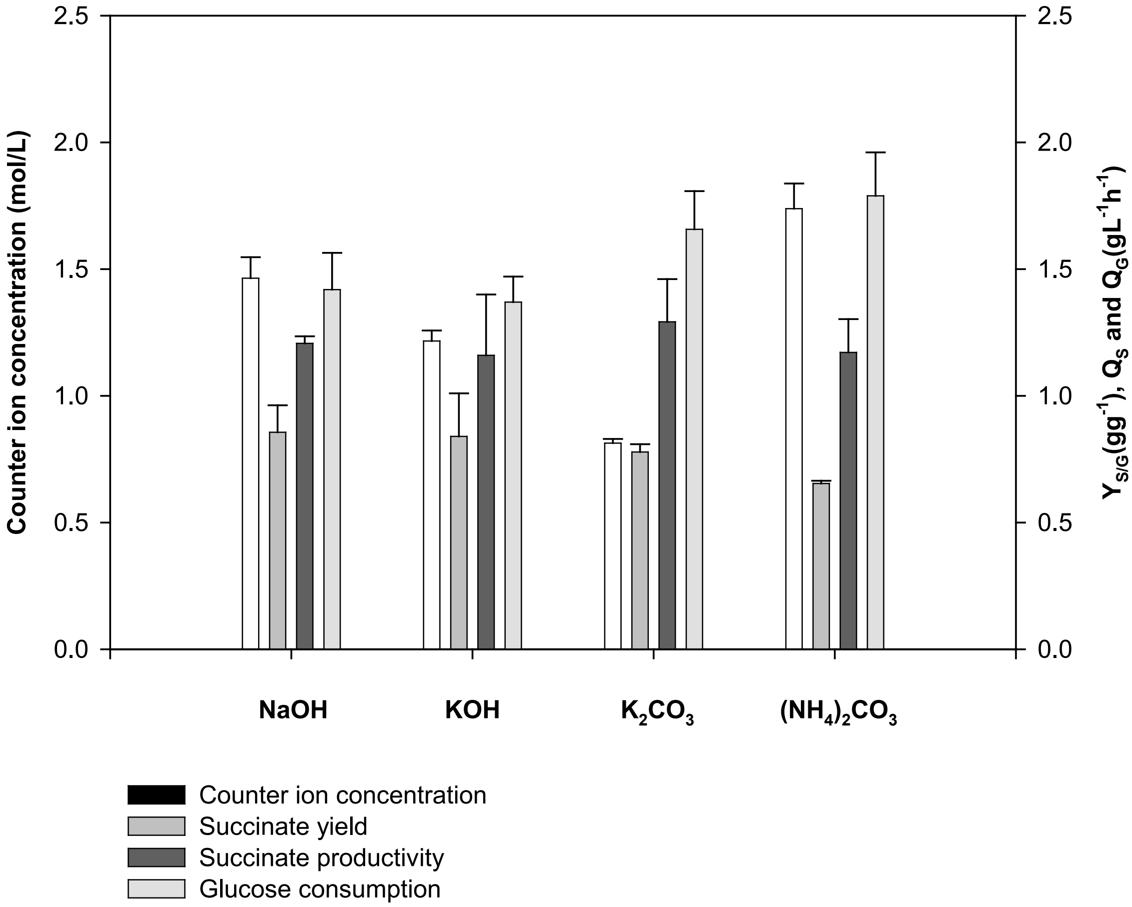

Figure 4.8. Effect of various counter ions at pH 6.5 on amount of counter ion added, overall succinate yield, succinate productivity and glucose consumption during the production phase.

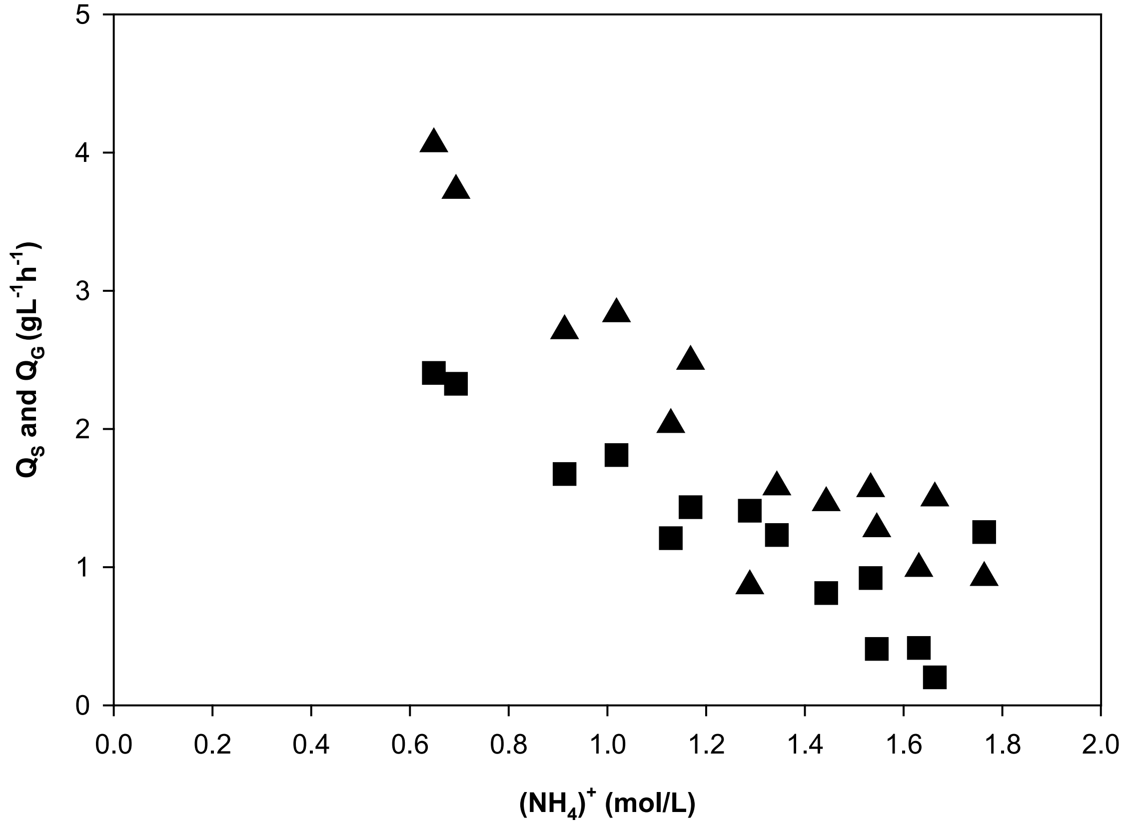

Figure 4.9. Effect of 2.5M $(NH_4)_2CO_3$ on succinate productivity and glucose consumption at pH 6.5. ■ Succinate productivity, ▲ Glucose consumption

Study 3: Effect of aeration during production phase on succinate production

Some key enzymes in succinate production like ICL are induced only under aerobic conditions (Vemuri et al., 2002b). In order to determine if introducing aerobic conditions during the anaerobic production phase aided succinate formation by restoring the activity of key enzymes, during the production phase a 10 minute aerobic burst was introduced after every 3 h of anaerobic conditions. In *E. coli*, the switch from fermentative to aerobic metabolism occurs mostly within 5 minutes (Partridge et al., 2006). However, this approach did not increase succinate accumulation (Figure 4.10). Based on F statistics, there is no significant advantage in introducing aerobic bursts (F = 0.44, p = 0.5765, α = 0.1) The overall glucose consumption was higher with aeration, but the succinate yield was lower (Figure 4.11).

The lower succinate yield observed in the process using intermittent aeration may be due to intermittent succinate consumption, since succinate can be consumed as a respiratory substrate during aeration (Partridge et al., 2006). Aeration also induces oxidative stress which is detrimental to the cells (Imlay, 2002; Storz and Zheng, 2000). Hence introducing aeration appears not to be advantageous for succinate formation.

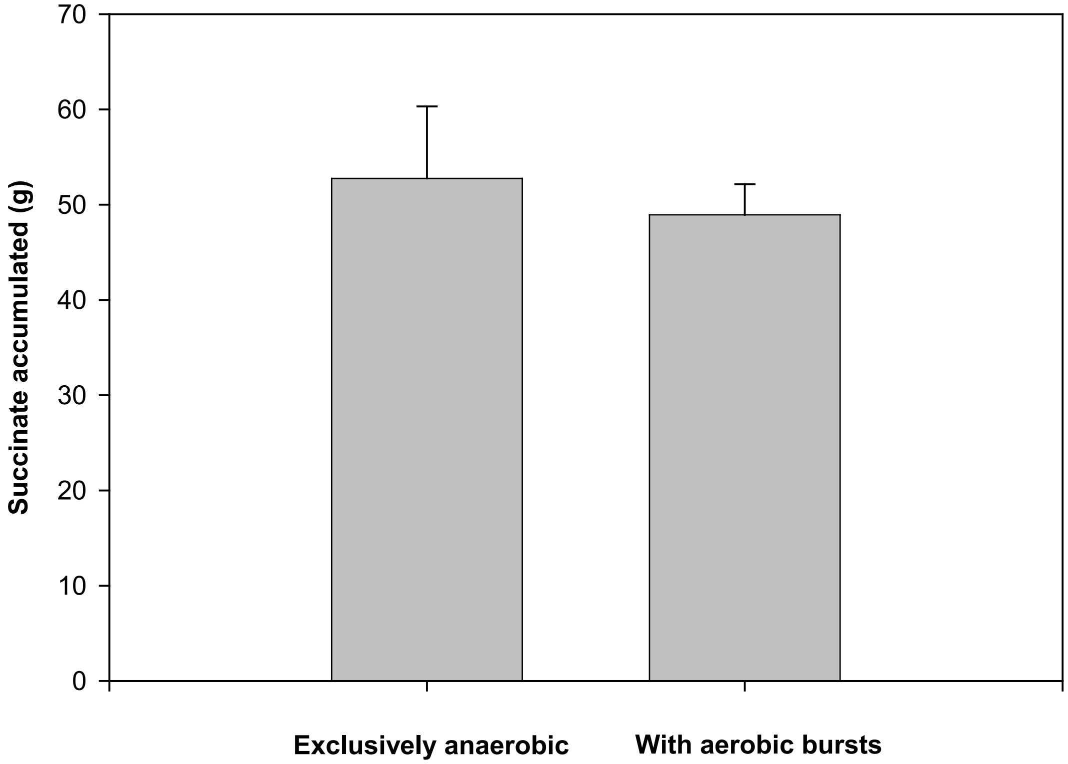

Figure 4.10. Effect of brief and intermittent aeration on succinate production at pH 6.5 with 2.5 M K_2CO_3 as base.

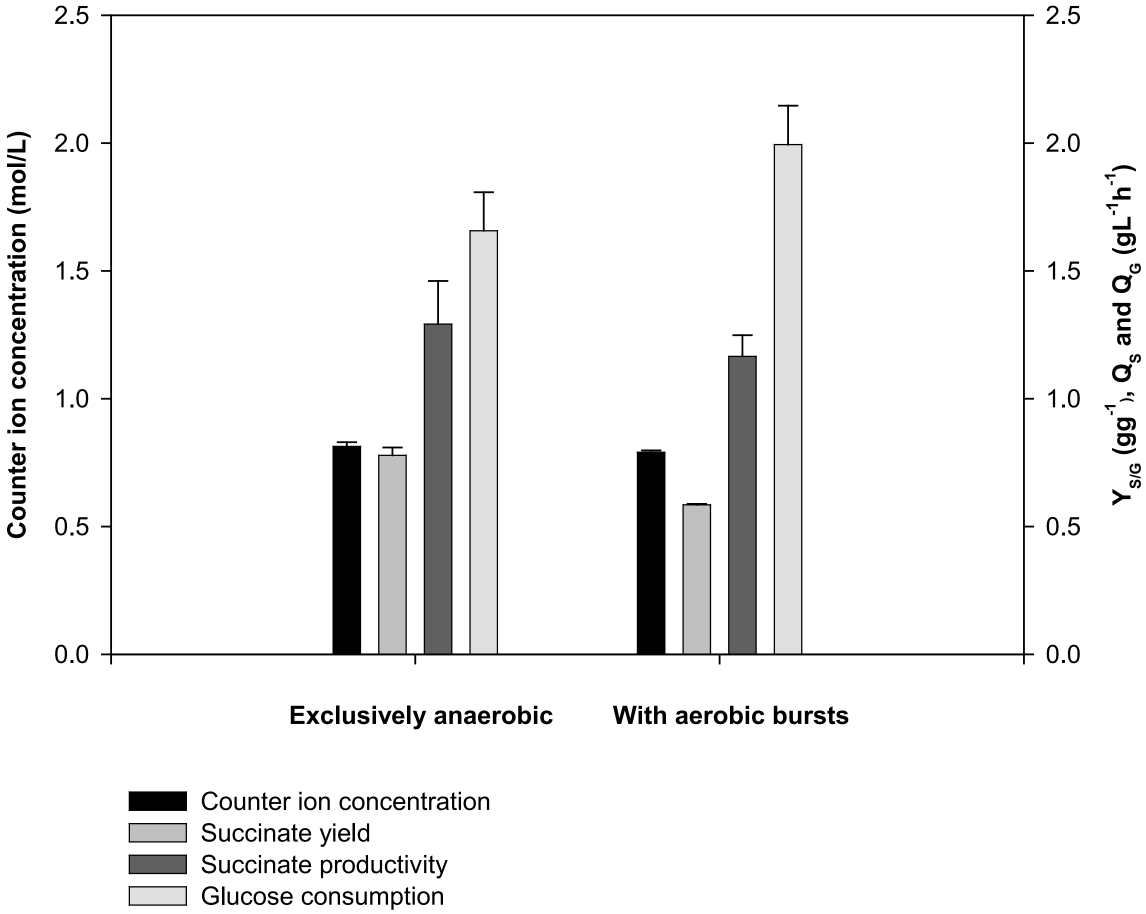

Figure 4.11. Effect of introducing brief and intermittent aeration during fermentation with 2.5 M K_2CO_3 as counter ion at pH 6.5 on amount of counter ion added, overall succinate yield, succinate productivity and glucose consumption during the production phase.

Study 4: Effect of osmotic stress on succinate production

E. coli responds to changes in external osmolarity by synthesizing proline, betaine, trehalose and glutamate (Dinnbier at al., 1988; Lucht and Bremer, 1994; McLaggan et al., 1994; Sutherland et al., 1986). However, under anaerobic conditions which serve as the production phase of *E. coli* AFP111, there is no cell growth and hence no presumably limited protein synthesis. Osmoprotectants like trehalose, proline and glutamate are probably not generated by the cells in response to increasing osmolarity. With the osmoprotectant mechanism thus hampered, cells may be particularly vulnerable to osmotic stress caused by the accumulation of cations during succinate production. Osmotic stress and inadequate levels of protective osmolytes decreased the productivity in *E. coli* KO11 during xylose fermentation (Underwood et al., 2004).

Two different approaches to reducing the ionic strength were examined. First, because calcium succinate has a low solubility in water, using $Ca(OH)_2$ as a base would reduce calcium ion accumulation as a result of its precipitation. (Use of this base might be undesirable in a commercial process because of the need to handle this precipitate.) Using $Ca(OH)_2$, 71 g succinate accumulated compared with 53 g obtained with K_2CO_3 (Figure 4.12). Though 34% more succinate accumulated, no significant improvement in the succinate yield was observed (Figure 4.13). However, the overall succinate productivity and glucose consumption increased by over 20% (Figure 4.13). Osmotic stress caused by the accumulation of counter ions in the media may be responsible for curtailing succinate production.

The second approach examined was to add an osmoprotectant into the medium. Specifically, the medium was supplemented with an inert osmoprotectant 5 mM betaine, and 2.5 M K_2CO_3 was used for pH control. Addition of betaine resulted in 83 g succinate accumulation, a 56% increase compared to this base without the addition of betaine. The overall succinate productivity was 2 g/L•h and the yield was 1.01 g/g, the highest values observed (Figure 4.13). Interestingly, the use of betaine allowed more succinate formation, which in turn necessitated the use of more base and lead to a higher K^+ concentration. Based on F statistics, there is a significant advantage in using $Ca(OH)_2$ ($F = 7.68$, $p= 0.0695$, $\alpha = 0.1$) and medium supplemented with 5 mM betaine ($F = 20.56$, $p = 0.0201$, $\alpha = 0.1$) over K_2CO_3. However there is no significant advantage between $Ca(OH)_2$ and K_2CO_3 supplemented with betaine ($F = 3.11$, $p = 0.1761$, $\alpha = 0.1$).

Another way to observe the benefit of betaine is to compare succinate productivity as a function of K^+ concentration. The succinate productivity was prolonged during the fermentation containing betaine in the medium (Figure 4.14). Consistent with previous results, addition of betaine helps in alleviating the undesirable effect of osmotic stress on cells (Chambers et al., 1987; Perroud and Le Rudulier, 1985). Inspite of addition of betaine, the rate of drop in succinate productivity with increasing counter ion concentration did not decrease (Figure 4.14). Anaerobic conditions create an energy disadvantage for betaine uptake by the cells. Under non-growing anaerobic conditions in the production phase, there is no nett synthesis of biomoleculaes including protective osmolytes. Limited growth under anaerobic conditions results in inadequate flux into the synthesis of glutamate, a protective osmolyte in *E. coli* KO11 (Underwood et al.,2002a; Underwood et al., 2002b). The *E. coli* AFP111 cells in the anaerobic production phase are highly vulnerable to increasing osmolarity as there could be very limited or no uptake, or synthesis of protective osmolytes. This indicates that betaine uptaken during the aerobic growth phase provided the beneficial effect on succinate production during the anaerobic production phase.

Key parameters from this study are summarized in Table 4.1. Addition of osmoprotectant betaine proved to be vital in prolonging a high rate of succinate production. During the course of fermentation the number of viable cells as defined by the ability to form CFU in the fermenter decreased (Table 4.2). Osmotic stress can lead cells to a state in which they are viable but nonculturable (VBNC) (Oliver, 2005; Oliver 1993). *E. coli* in VNBC state does not grow on agar media (Xu et al., 1982). VNBC cells have low metabolic activity and become culturable on resuscitation (Oliver, 2000). The increased osmotic stress towards the end of the bioprocess could explain the decrease in CFUs. However, the role of viable or active, but nonculturable cells in succinate production is unknown from this study.

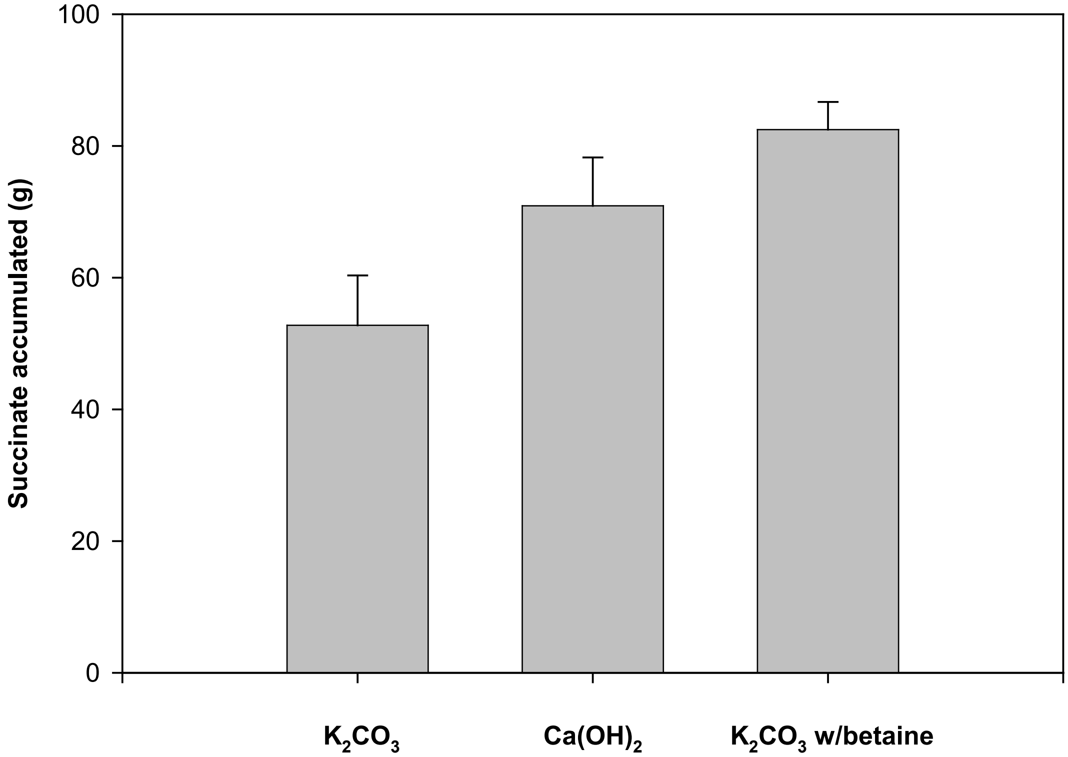

Figure 4.12. Effect of 2.5 M K_2CO_3, 2.7 M $Ca(OH)_2$, and 2.5 M K_2CO_3 + 5 mM betaine on succinate accumulation at pH 6.5.

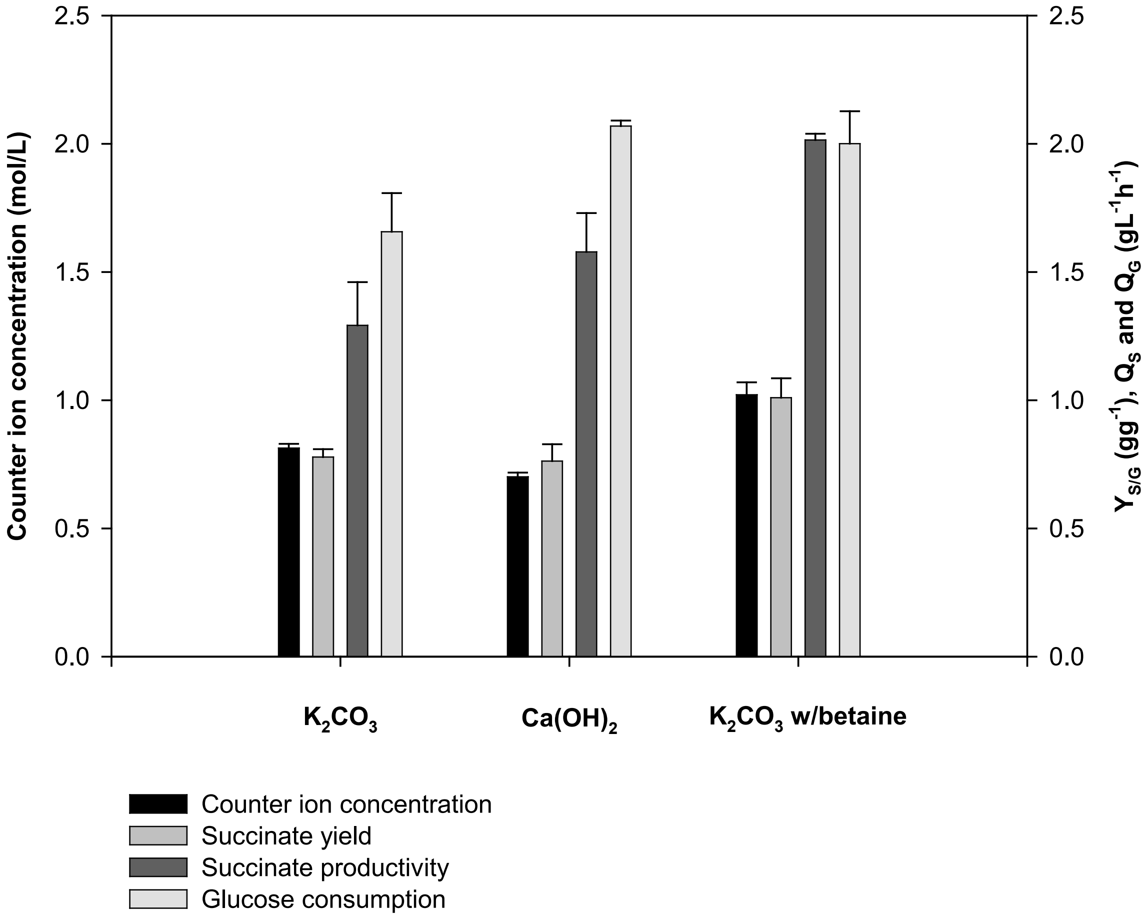

Figure 4.13. Effect of 2.5 M K_2CO_3, 2.7 M $Ca(OH)_2$, and 2.5 M K_2CO_3 + 5 mM betaine on amount of counter ion added, overall succinate yield, succinate accumulation and glucose consumption at pH 6.5 during the production phase.

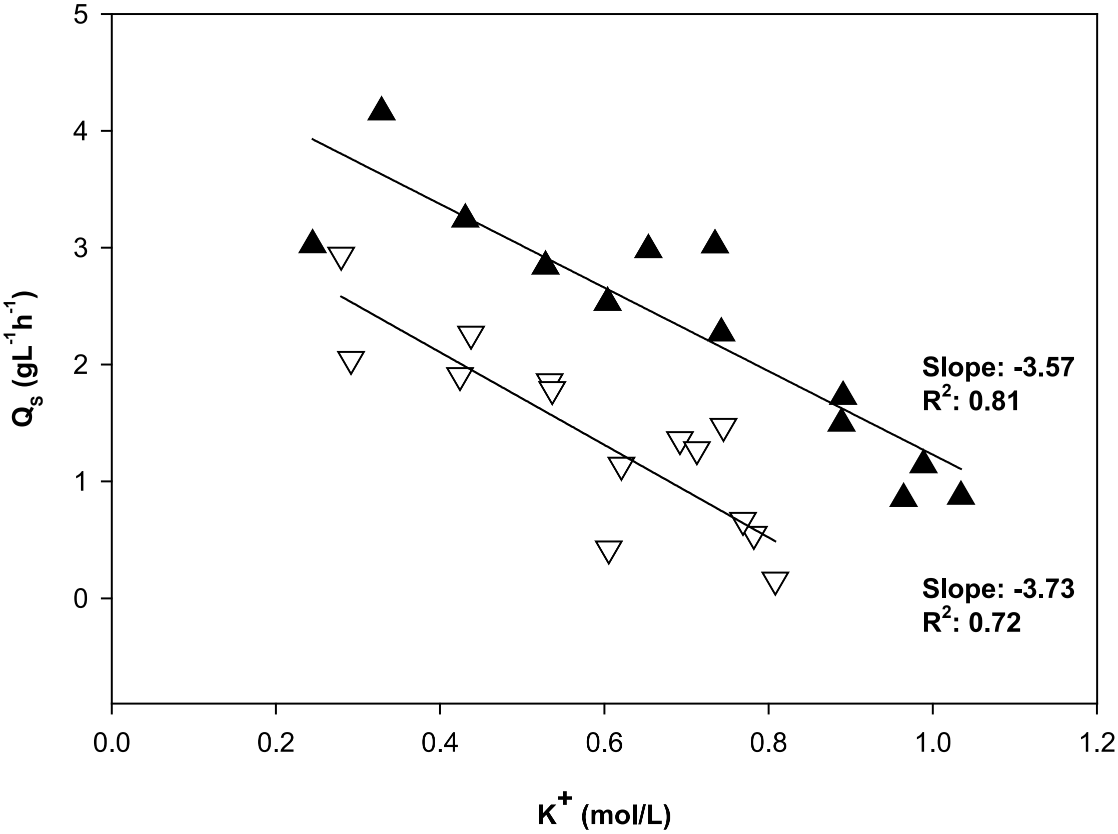

Figure 4.14. ▽ Succinate productivity, ▲ succinate productivity after addition of 5 mM betaine.

Table 4.1. Summary of the fermentations

Base	pH	Succinate accumulated (g)	Counter ion concentration (mol/L)	$Y_{S/G}$ (g/g)	Q_S (g/L•h)	Q_G (g/L•h)
5M NaOH	7.00	32.10 (2.97)	1.48 (0.08)	0.48 (0.05)	0.75 (0.07)	1.56 (0.03)
5M KOH	7.00	25.95 (3.61)	1.03 (0.02)	0.68 (0.09)	0.66 (0.09)	0.99 (0.27)
5M NaOH	6.50	48.65 (0.64)	1.46 (0.08)	0.86 (0.11)	1.21 (0.03)	1.42 (0.14)
5M KOH	6.50	46.45 (8.90)	1.22 (0.04)	0.84 (0.17)	1.16 (0.24)	1.37 (0.10)
2.5M K_2CO_3	6.50	52.75 (7.57)	0.81 (0.02)	0.78 (0.03)	1.29 (0.17)	1.66 (0.15)
2.5M $(NH_4)_2CO_3$	6.50	49.24 (6.22)	1.74 (0.10)	0.65 (0.01)	1.17 (0.13)	1.79 (0.17)
2.7M $Ca(OH)_2$	6.50	70.90 (7.34)	0.70 (0.02)	0.76 (0.07)	1.58 (0.15)	2.07 (0.02)
With 10 minutes aeration at 3 hr interval						
2.5M K_2CO_3	6.50	48.92 (3.22)	0.79 (0.01)	0.58 (0.00)	1.17 (0.08)	1.99 (0.15)
With the addition of 5mM betaine						
2.5M K_2CO_3	6.50	82.47 (4.20)	1.02 (0.05)	1.01 (0.08)	2.01 (0.02)	2.00 (0.13)

Table 4.2. Summary of CFUs during the course of fermentation

Counter ion	Fermentation	CFUs (10^{10}) in fermenter	
		Start of anaerobic phase	End of anaerobic phase
KOH		6867 (542)	3199 (167)
K_2CO_3		4121 (724)	2289 (644)
K_2CO_3		3453 (808)	3088 (369)
$(NH_4)_2CO_3$		4649 (508)	2589 (269)
$(NH_4)_2CO_3$		3980 (341)	2535 (35)
K_2CO_3	with betaine	4650 (423)	2977 (322)
K_2CO_3	with betaine	3891 (550)	3130 (250)
K_2CO_3	With aeration	7227 (2370)	2091 (351)
K_2CO_3	With aeration	6399 (426)	2576 (627)

CHAPTER 5

CONCLUSIONS

The objective of the research was to prolong a high rate of succinate formation and gain a better understanding of the physiological factors that affect succinate production. In this direction four hypotheses were proposed and experimentally verified. pH 6.5 is more favorable than pH 7.0 for succinate production. Identifying the optimum pH for favorable succinate production by *E. coli* AFP111 is a future prospect. Osmotic conditions play a major role in succinate production. The osmotic stress was independent of the counter ion used, and reduced succinate production and cell viability. Apart from the osmolarity, the chemical nature of counter ions also had an influence on the physiology. Introducing aeration during the anaerobic production phase did not show any significant advantage. In addition to reducing the succinate yield without benefiting succinate productivity, aeration does not address what appeaers to be the principal limitation to prolonged succinate formation — osmotic stress. Addition of osmoprotectant betaine greatly enhanced succinate formation. This observed beneficial effect of betaine could be due to betaine uptaken in the aerobic growth phase considering that betaine uptake might not be energetically favorable in the anaerobic phase. Understanding betaine and other osmolyte uptake mechanism and biosynthesis under non-growing anaerobic conditions is crucial for process optimization. As the effect of betaine is dose-dependent (Umenishi et al., 2005; Zhou et al., 2006) optimizing the dosage of betaine for prolonging a high rate of succinate formation may be required. The cost of supplementing osmoprotectant vs. improvement in succinate production could be a critical factor in industrial production. Along with supplementing osmoprotectant, removing counter ions periodically may be an effective strategy. The role of active or viable, but nonculturable cells in succinate production has not been addressed in this study. Research in this direction will aid in understanding the physiological changes that occur in the cells during the course of bioprocess for succinate production.

Additional research and development will be required to make biosynthesis of succinate a viable alternative to current petrochemical production. Prolonging a high rate of succinate formation in fermentation process is a key area for research and development. This presented research indicates that osmotic conditions have a major impact on succinate production and addition of osmoprotectant is vital to prolong a high rate of succinate formation.

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APPENDIX

Experiment date:	6/7/2007
Experiment No:	Book 9 - J
Strain:	AFP111
Media:	JSM
Fermenter Volume:	1.201 L
Inoculum Volume:	50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	7.0
base:	200 g/L NaOH	200 g/L NaOH
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	Na^+ (mol/L)
16.0	3.1	32.0	0.0	3.4	0.0	21575	68	104	1.4	0.45
19.1	7.5	21.4	3.5	2.4	0.0	13862	123	104	1.4	0.77
22.6	12.3	11.2	8.1	0.0	0.8	9677	163	104	1.5	0.98
26.1	15.8	2.5	11.8	1.9	1.3	10209	209	104	1.5	1.22
26.2	15.0	23.0	11.0	1.9	1.2	13293	211	208	1.6	1.15
30.3	17.9	19.9	14.5	2.0	1.2	9832	255	208	1.7	1.35
33.7	19.5	15.2	16.8	2.1	1.4	13624	271	208	1.7	1.42
37.5	20.7	10.3	18.7	2.1	1.3	10020	285	208	1.7	1.48
40.9	21.4	7.0	20.0	2.2	0.0	9537	299	208	1.7	1.54

Notes:
At 16 h switched to anaerobic and added 40 g glucose in solution
At 26.1 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	16.0	26.1	43.9	3.8	0.0	17.9	0.45	1.17	2.63
2.0	26.2	40.9	37.3	12.0	17.9	34.2	0.64	0.65	1.01
overall	24.9		65.5		34.2		0.52	0.80	1.54

Experiment date:	6/7/2007		aerobic	anaerobic
Experiment No:	Book 9 - K	Temperature (°C):	37	37
Strain:	AFP111	pH:	7.0	7.0
Media:	JSM	base:	200 g/L NaOH	200 g/L NaOH
Fermenter Volume:	1.201 L	gas phase:	air	CO_2
Inoculum Volume:	50 mL	gas flowrate (L/min):	1	0.2
		Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	Na^+ (mol/L)
15.2	3.0	33.9	0.0	5.3	0.9	20832	72	104	1.4	0.47
18.6	7.7	22.5	3.6	4.0	1.6	16560	132	104	1.4	0.82
22.0	11.8	13.2	7.5	3.2	2.1	10293	162	104	1.5	0.98
25.4	15.4	5.9	10.9	2.8	2.4	11384	192	104	1.5	1.13
25.6	15.0	30.2	10.5	2.6	2.3	13914	194	208	1.6	1.07
29.8	17.5	24.8	13.4	2.5	2.4	7335	231	208	1.6	1.24
33.2	16.0	17.2	14.1	1.4	1.5	10361	246	208	1.7	1.31
37.0	19.9	15.6	17.2	2.0	2.0	9134	262	208	1.7	1.38
40.4	20.2	11.4	17.9	1.8	2.3	8485	274	208	1.7	1.43

Notes:
At 15.2 h switched to anaerobic and added 40 g glucose in solution
At 25.4 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	15.2	25.4	46.7	8.8	0.0	16.3	0.43	1.06	2.47
2.0	25.6	40.4	48.4	19.2	16.8	30.0	0.45	0.53	1.17
overall	25.3		67.1		30.0		0.45	0.71	1.58

Experiment date:	6/29/2007		aerobic	anaerobic
Experiment No:	Book 9 - L	Temperature (°C):	37	37
Strain:	AFP111	pH:	7.0	7.0
Media:	JSM	base:	280.55 g/L KOH	280.55 g/L KOH
Fermenter Volume:	1.201 L	gas phase:	air	CO_2
Inoculum Volume:	50 mL	gas flowrate (L/min):	1	0.2
		Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K+ (mol/L)
17.5	2.7	44.6	0.0	3.4	0.0	25796	4763	60	79	1.3	0.37
20.8	6.7	37.7	4.3	2.8	0.7	13681	2476	110	79	1.4	0.62
24.3	9.2	29.3	7.7	2.6	1.0	11788	1670	136	79	1.4	0.74
27.6	11.6	22.8	11.2	2.5	1.2	9877	964	156	79	1.4	0.83
27.6	10.9	52.5	10.3	2.0	1.1	10011	1241	156	158	1.5	0.79
31.3	12.2	50.0	12.4	1.9	1.2	10306	1181	182	158	1.5	0.89
35.1	12.0	40.9	13.1	1.8	1.2	7480	935	198	158	1.6	0.96
38.8	14.5	40.1	16.7	1.9	1.1	8760	947	209	158	1.6	1.00
42.5	15.4	38.0	18.0	1.7	1.0	7000		222	158	1.6	1.05

Notes:
At 17.5 h switched to anaerobic and added 40 g glucose in solution
At 27.55 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	17.5	27.6	59.8	32.8	0.0	16.0	0.59	1.11	1.87
2.0	27.6	42.5	79.6	60.0	15.6	28.5	0.66	0.55	0.83
overall	25.0		46.5		28.5		0.61	0.72	1.18

Experiment date:	6/29/2007
Experiment No:	Book 9 - M
Strain:	AFP111
Media:	JSM
Fermenter Volume:	1.201 L
Inoculum Volume:	50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	7.0
base:	280.55 g/L KOH	280.55 g/L KOH
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K+ (mol/L)
17.7	2.9	39.7	0.0	3.2	0.0	24386	10140	59	79	1.3	0.37
20.8	6.1	32.4	3.2	2.4	0.7	18941	2558	108	79	1.4	0.61
24.2	8.7	25.2	7.0	2.3	1.1	12381	1707	131	79	1.4	0.72
27.6	10.8	20.2	9.3	2.0	1.0	9718	1905	150	79	1.4	0.80
27.7	10.2	48.7	8.5	1.8	1.2	11650	1249	150	158	1.5	0.76
31.2	11.3	45.8	10.6	1.7	1.3	9722	998	170	158	1.5	0.84
35.0	12.6	42.6	12.1	1.6	1.2	10751	1047	185	158	1.5	0.91
38.7	13.5	38.9	13.4	1.5	1.4	7838	987	202	158	1.6	0.97
42.7	14.5	36.0	14.9	1.5	1.4	8229		213	158	1.6	1.02

Notes:
At 17.7 h switched to anaerobic and added 40 g glucose in solution
At 27.6 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	17.7	27.6	43.3	28.9	0.0	13.4	0.92	0.94	1.02
2.0	27.7	42.7	73.4	56.6	12.8	23.4	0.63	0.45	0.71
overall	25.0		31.3		23.4		0.75	0.59	0.80

Experiment date:	8/24/2007
Experiment No:	Book 9 - P
Strain:	AFP111
Media:	JSM
Fermenter Volume:	1.201 L
Inoculum Volume:	50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	280.55 g/L KOH	280.55 g/L KOH
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K+ (mol/L)
19.5	2.6	34.3	0.8	2.4	1.1	26700	4089	57	80	1.3	0.36
22.8	7.3	20.3	8.7	1.7	2.1	22806	2753	113	80	1.4	0.63
26.3	8.8	11.4	13.8	1.8	2.2	17588	1984	153	80	1.4	0.81
29.7	9.6	5.9	16.8	1.9	2.5	16341	1864	181	80	1.5	0.94
29.7	8.8	30.3	15.2	1.7	2.4	16246	1792	203	160	1.6	0.98
33.7	10.0	28.9	18.5	1.9	2.4	14831	1695	217	160	1.6	1.03
37.2	10.2	24.9	19.4	1.9	2.4	15109	1462	231	160	1.6	1.09
41.0	10.7	22.5	20.9	2.0	2.6	10305	1400	243	160	1.6	1.13
44.7	11.4	20.7	23.0	2.2	2.5	5944	1351	255	160	1.6	1.18

Notes:
At 19.5 h switched to anaerobic and added 40 g glucose in solution
At 29.7 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	19.5	29.7	45.9	8.6	1.0	24.6	0.63	1.59	2.51
2.0	29.7	44.7	47.4	33.5	23.8	37.1	0.95	0.55	0.58
overall	25.2		51.2		37.1		0.72	0.91	1.26

Experiment date:	8/24/2007		aerobic	anaerobic
Experiment No:	Book 9 - Q	Temperature (°C):	37	37
Strain:	AFP111	pH:	7.0	6.5
Media:	JSM	base:	280.55 g/L KOH	280.55 g/L KOH
Fermenter Volume:	1.201 L	gas phase:	air	CO_2
Inoculum Volume:	50 mL	gas flowrate (L/min):	1	0.2
		Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K+ (mol/L)
19.5	2.3	32.8	0.4	4.1	2.2	24550	3693	62	80	1.3	0.38
22.8	5.9	19.6	7.7	3.3	3.4	20568	3191	112	80	1.4	0.63
26.3	7.1	9.7	12.7	3.2	3.9	13210	2572	152	80	1.4	0.81
29.7	8.3	3.9	16.9	3.6	4.2	10558	1990	180	80	1.5	0.93
29.7	8.1	30.7	16.2	3.3	4.4	10913	1828	182	160	1.5	0.89
33.7	8.1	24.2	17.7	3.2	3.8	5019	2392	210	160	1.6	1.00
37.2	9.0	21.7	21.0	3.5	4.5	12853	1676	230	160	1.6	1.08
41.0	9.1	17.6	22.4	3.6	4.3	13168	1924	250	160	1.6	1.16
44.7	9.9	15.4	25.0	3.9	4.3	14453	1653	262	160	1.6	1.20

Notes:
At 19.5 h switched to anaerobic and added 40 g glucose in solution
At 29.7 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	19.5	29.7	44.1	5.7	0.6	24.8	0.63	1.63	2.58
2.0	29.7	44.7	47.4	25.1	25.0	40.6	0.70	0.64	0.92
overall	25.2		60.7		40.6		0.67	0.99	1.48

Experiment date: 9/4/2007
Experiment No: Book 9 - R
Strain: AFP111
Media: JSM
Fermenter Volume: 1.201 L
Inoculum Volume: 50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	200 g/L NaOH	200 g/L NaOH
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	Na+ (mol/L)
26.0	1.7	31.4	0.9	0.0	0.0	16827	10858	36	68	1.3	0.26
29.4	3.1	22.1	9.4	0.3	1.1	16486	11642	82	68	1.4	0.55
33.8	4.1	13.5	17.7	0.8	1.6	17085	9816	126	68	1.4	0.80
36.0	3.7	7.7	18.5	1.0	1.5	7792	4991	154	68	1.4	0.96
36.1	4.0	32.6	18.8	1.0	1.7	14431	8894	154	136	1.5	0.92
42.8	4.3	25.3	25.2	1.4	2.1	11801	9149	210	136	1.5	1.20
46.2	5.0	21.6	29.2	1.5	2.1			214	136	1.6	1.22
50.0	5.1	16.8	30.3	1.7	2.3	7918	6234	232	136	1.6	1.30
51.2	4.9	16.3	30.9	1.7	1.9	8679	4468	254	136	1.6	1.41

Notes:
At 26 h switched to anaerobic and added 40 g glucose in solution
At 36 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	26.0	36.0	41.0	11.0	1.2	26.3	0.84	2.62	2.81
2.0	36.1	51.2	48.6	25.9	28.0	49.1	0.93	0.88	0.94
overall	25.2		52.7		49.1		0.93	1.23	1.32

Experiment date: 9/4/2007
Experiment No: Book 9 - S
Strain: AFP111
Media: JSM
Fermenter Volume: 1.201 L
Inoculum Volume: 50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	200 g/L NaOH	200 g/L NaOH
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	Na+ (mol/L)
25.8	1.9	34.3	0.9	0.0	0.0	13944	12337	38	68	1.3	0.27
29.3	3.5	22.6	11.0	0.4	0.9	30928	6244	84	68	1.4	0.56
33.6	3.4	9.7	17.7	0.9	1.3	14176	9110	140	68	1.4	0.88
35.9	4.0	5.9	23.1	1.3	1.6	14806	7370	170	68	1.4	1.05
35.9	3.7	29.3	21.4	1.2	1.3	11147	9905	170	136	1.5	1.00
42.6	3.8	20.5	26.8	1.4	1.4	9823	8043	234	136	1.6	1.31
46.0	4.6	17.8	31.4	1.6	1.8	11591	7741	250	136	1.6	1.39
49.8	4.2	12.5	29.2	1.5	1.6	5600	5374	274	136	1.6	1.50
50.9	4.3	11.6	29.8	1.6	1.5	5090	4538	280	136	1.6	1.52

Notes:
At 25.8 h switched to anaerobic and added 40 g glucose in solution
At 35.9 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	25.8	35.9	44.8	8.4	1.2	33.2	0.88	2.22	2.52
2.0	35.9	50.9	44.1	18.7	32.2	48.2	0.63	0.66	1.05
overall	25.1		61.8		48.2		0.78	1.19	1.52

Experiment date:	10/16/2007		aerobic	anaerobic
Experiment No:	Book 9 - T	Temperature (°C):	37	37
Strain:	AFP111	pH:	7.0	6.5
Media:	JSM	base:	280.55 g/L KOH	280.55 g/L KOH
Fermenter Volume:	1.201 L	gas phase:	air	CO_2
Inoculum Volume:	50 mL	gas flowrate (L/min):	1	0.2
		Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
24.2	1.6	30.4	1.0	0.0	1.8	11900	4935	46	92	1.3	0.30
27.4	3.5	19.2	9.6	0.5	1.6	12595	6996	98	92	1.4	0.56
31.0	3.9	9.3	17.0	1.1	1.9	8195	8309	142	92	1.4	0.76
34.2	4.2	2.8	21.6	1.6	2.3	6953	1303	182	92	1.5	0.93
34.3	3.8	22.9	18.3	1.3	2.3	7001	3363	183	184	1.6	0.88
39.2	5.2	21.1	27.0	1.8	2.8	8052	4452	230	184	1.6	1.07
42.9	5.5	16.1	29.2	2.0	3.0	8410	5270	260	184	1.6	1.18
45.6	5.6	13.4	30.3	2.0	2.9	7126	4190	275	184	1.7	1.23
48.7	6.0	10.8	32.0	2.1	3.2	8298	4589	287	184	1.7	1.27

Notes:
At 24.2 h, switched to anaerobic phase and added 40 g glucose in solution
At 34.3 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	24.2	34.2	40.7	4.1	1.3	31.8	0.83	2.06	2.48
2.0	34.3	48.7	35.8	18.0	28.6	53.5	1.39	0.83	0.60
overall	24.5		54.4		53.5		0.98	1.31	1.33

Experiment date:	10/16/2007
Experiment No:	Book 9 - U
Strain:	AFP111
Media:	JSM
Fermenter Volume:	1.201 L
Inoculum Volume:	50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	280.55 g/L KOH	280.55 g/L KOH
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
24.3	1.6	32.5	1.2	0.0	0.7			56	92	1.3	0.35
27.7	3.0	21.5	10.1	0.5	1.4	7894	10930	98	92	1.4	0.56
31.3	3.3	12.3	17.3	1.0	1.5	8278	7896	142	92	1.4	0.76
34.2	3.4	6.8	21.2	1.4	1.9	8395	8869	170	92	1.5	0.88
34.3	3.2	31.6	19.3	1.2	1.8	7978	5268	172	184	1.6	0.84
39.3	3.9	25.8	26.0	1.5	2.2	8713	5895	218	184	1.6	1.02
42.0	4.1	21.1	28.2	1.6	2.3	7434	6477	242	184	1.6	1.11
44.7	4.2	17.9	28.6	1.7	2.4	6577	4983	256	184	1.6	1.16
47.6	4.8	17.3	33.0	1.9	2.5	9648	4934	270	184	1.7	1.21

Notes:

At 24.3 h, switched to anaerobic phase and added 40 g glucose in solution

At 34.3 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	24.3	34.2	43.8	9.9	1.6	30.9	0.87	2.03	2.34
2.0	34.3	47.6	49.1	28.6	30.0	54.6	1.20	1.12	0.93
overall	23.3		54.4		54.6		1.00	1.41	1.41

Experiment date: 10/21/2007
Experiment No: Book 9 - V
Strain: AFP111
Media: JSM
Fermenter Volume: 1.201 L
Inoculum Volume: 50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	345.525 g/L K_2CO_3	345.525 g/L K_2CO_3
gas phase:	air	CO_2
gas flowrate (L/min):	1	0
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
26.3	1.9	40.4	0.8	0.1	0.0	17103	10379	29	85	1.3	0.16
30.8	3.1	22.3	13.9	0.8	1.8	9487	9119	95	85	1.4	0.40
33.2	3.2	15.2	19.0	1.2	2.2	6669	10336	121	85	1.4	0.48
36.7	3.2	7.5	25.0	1.9	3.0	10585	7653	155	85	1.4	0.59
36.7	3.0	34.8	23.4	1.8	2.9	16801	10352	155	170	1.5	0.55
41.3	2.7	23.1	24.8	1.9	3.1	11200	11782	191	170	1.6	0.66
46.1	3.2	19.3	30.9	2.4	3.6	8040	11011	217	170	1.6	0.73
48.8	3.5	17.4	34.7	2.7	3.9	11418	11574	230	170	1.6	0.76
51.8	3.6	14.1	36.0	2.8	3.1	10847	9282	245	170	1.6	0.80

Notes:
At 26.3 h, switched to anaerobic phase and added 40 g glucose in solution
At 36.7 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	26.3	36.7	53.1	10.8	1.0	36.0	0.83	2.35	2.84
2.0	36.7	51.8	53.1	22.7	35.7	58.1	0.74	0.97	1.32
overall	25.5		72.7		58.1		0.80	1.41	1.76

Experiment date:	10/21/2007
Experiment No:	Book 9 - W
Strain:	AFP111
Media:	JSM
Fermenter Volume:	1.201 L
Inoculum Volume:	50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	345.525 g/L K_2CO_3	345.525 g/L K_2CO_3
gas phase:	air	CO_2
gas flowrate (L/min):	1	0
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
26.5	2.5	30.8	0.6	0.0	0.0	18951	20744	42	85	1.3	0.21
30.4	8.2	21.0	8.6	0.7	2.1	14981	15200	88	85	1.4	0.37
32.7	9.5	13.6	12.8	0.9	2.3	11002	14440	120	85	1.4	0.48
36.4	10.9	4.7	19.0	1.4	2.4	11865	14777	158	85	1.4	0.60
36.5	9.9	29.2	17.1	1.2	2.6	12330	14784	158	170	1.5	0.56
41.0	11.0	22.3	21.8	1.5	3.0	10541	11169	199	170	1.6	0.68
45.6	12.7	17.3	27.4	2.0	2.5	9701	15052	224	170	1.6	0.75
48.4	13.0	13.6	29.0	2.1	2.7	10403	12844	241	170	1.6	0.79
51.4	12.7	9.8	29.2	2.1	2.6	10205	13311	254	170	1.6	0.82

Notes:

At 26.5 h, switched to anaerobic phase and added 40 g glucose in solution

At 36.4 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	26.5	36.4	40.8	6.8	0.8	27.5	0.78	1.87	2.38
2.0	36.5	51.4	44.7	16.0	26.2	47.4	0.74	0.88	1.18
overall	24.9		62.7		47.4		0.76	1.17	1.55

Experiment Date: 12/9/2007
Experiment No: Book 9 - Z1
Strain: AFP111
Media: JSM
Fermenter Volume: 1.201 L
Inoculum Volume: 50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	240 g/L $(NH_4)_2CO_3$	240 g/L $(NH_4)_2CO_3$
gas phase:	air	CO_2
gas flowrate (L/min):	1	0
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	G3PDH (U in ferm)	ICL (U in ferm)	Base Acc (mL)	Total Feed (mL)	Volume (L)	NH_4^+ (mol/L)
17.9	3.4	35.4	0.9	4.75	1.65	12604	7303	59	81	1.3	0.46
22.6	6.4	15.7	11.7	4.5	2.8	11344	7850	153	81	1.4	0.93
25.8	6.7	6.3	17.1	4.9	3.2	11513	8111	189	81	1.5	1.11
28.0	6.5	0.6	20.1	5.2	3.45	11152	7677	213	81	1.5	1.23
28.1	6.4	27.4	19.3	5	3.35	10637	7698	213	162	1.6	1.23
32.8	6.4	19.3	24.5	5.6	3.75	9878	8808	259	162	1.6	1.46
36.2	6.2	13.6	27.2	5.8	3.9	7548	6474	289	162	1.7	1.61
39.4	5.6	8.5	27.5	5.65	3.8	8647	7795	311	162	1.7	1.72
43.0	5.8	5.1	31.7	6.55	4.15	5889	6135	329	162	1.7	1.81

Notes:
At 17.9 h, switched to anaerobic phase and added 40 g glucose in solution
At 28.4 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	17.9	28.0	47.4	0.9	1.1	30.0	0.62	1.91	3.09
2.0	28.1	43.0	43.2	8.6	30.4	53.6	0.67	0.92	1.37
overall	25.1		81.1		53.6		0.66	1.26	1.91

			aerobic	anaerobic
Experiment date:	12/9/2007			
Experiment No:	Book 9 - Z2	Temperature (°C):	37	37
Strain:	AFP111	pH:	7.0	6.5
Media:	JSM	base:	240 g/L $(NH_4)_2CO_3$	240 g/L $(NH_4)_2CO_3$
Fermenter Volume:	1.201 L	gas phase:	air	CO_2
Inoculum Volume:	50 mL	gas flowrate (L/min):	1	0
		Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	Base Acc (mL)	Total Feed (mL)	Volume (L)	NH_4^+ (mol/L)
25.4	2.4	30.5	0.5	7.5	1.8	75	81	1.4	0.54
28.0	5.0	19.0	6.7	6.9	2.6	119	81	1.4	0.76
32.5	6.4	6.0	13.9	7.0	3.1	181	81	1.5	1.07
35.3	6.4	0.2	17.1	7.2	3.3	205	81	1.5	1.19
35.4	6.3	26.8	16.6	6.9	3.3	205	162	1.6	1.19
39.3		22.7	21.7	7.5	3.6	245	162	1.6	1.39
43.1		16.9	24.5	7.7	3.8	267	162	1.6	1.50
47.1	7.0	11.6	25.8	7.6	3.7	286	162	1.6	1.59
50.4	6.7	8.2	27.0	7.6	3.7	301	162	1.7	1.67

Notes:
At 25.4 h, switched to anaerobic phase and added 40 g glucose in solution
At 35.3 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	25.4	35.3	41.3	0.2	0.6	25.4	0.60	1.68	2.79
2.0	35.4	50.4	41.9	13.6	26.0	44.8	0.66	0.75	1.13
overall	25.0		69.4		44.8		0.65	1.08	1.67

Experiment date:	12/27/2007		aerobic	anaerobic
Experiment No	Book 9 - Z5	Temperature	37C	37C
Strain:	AFP111	pH	7	6.5
Media:	JSM	base	345.525 g/L K_2CO_3	345.525 g/L K2CO3
Fermenter Volume:	1.201 L	gas phase	air	CO_2
Inoculum Volume:	50 mL	gas flowrate (L/min)	1	0
		Agitation:	200 - 1000 rpm	200 rpm

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
21.9	3.7	37.6	0.9	2.2	1.1	46	75	1.3	0.23
24.9	8.8	21.2	11.4	1.6	2.3	101	75	1.4	0.42
25.1	9.4	19.5	11.6	1.6	2.3	103	75	1.4	0.42
28.1	10.3	8.3	17.9	2.2	2.6	148	75	1.4	0.57
28.3	10.8	6.9	18.4	2.2	2.7	148	75	1.4	0.57
29.0	10.9	5.2	19.2	2.4	2.8	150	75	1.4	0.58
29.1	10.6	35.5	18.8	2.3	2.7	152	150	1.5	0.55
31.2	11.1	30.0	22.4	2.6	3.0	182	150	1.5	0.64
31.3	10.8	27.4	21.4	2.5	2.9	182	150	1.5	0.64
34.2	10.3	21.0	22.5	2.7	2.9	198	150	1.5	0.68
34.3	11.4	22.1	24.5	3.0	3.0	198	150	1.5	0.68
37.2	11.8	18.1	28.2	3.6	3.4	214	150	1.6	0.73
37.3	11.9	17.4	28.0	3.5	3.5	214	150	1.6	0.73
40.2	11.8	12.9	29.9	4.0	3.6	228	150	1.6	0.77
40.3	12.2	12.6	30.1	4.0	3.7	228	150	1.6	0.77
42.1	11.4	9.3	29.0	4	3.5	236	150	1.6	0.79
42.2	11.6	39.0	29.9	4.1	3.4	236	220	1.7	0.75
43.2	11.3	38.1	29.9	4.1	3.4	242	220	1.7	0.77
43.3	11.7	38.5	30.2	4.2	3.5	242	220	1.7	0.77
46.2	11.5	34.4	31.2	4.4	3.5	252	220	1.7	0.80
46.3	11.9	34.5	31.6	4.5	3.6	252	220	1.7	0.80
46.9	11.4	32.5	30.6	4.2	3.5	252	220	1.7	0.80

Notes:

Between 24.9-25.1, 28.1-28.3, 31.2-31.3, 34.2-34.3, 37.2-37.3, 40.2-40.3, 43.2-43.3, 46.2-46.3 h,
aerobic conditions were introduced for 10 minutes and agitation was increased to 400 rpm from 200 rpm during that time.
At the completion of 10 minutes, the condition was returned to anaerobic and agitation was decreased to 200 rpm.
Added 40g glucose in solution at 21.9, 29 & 42.1 h
At 21.9h switched to anaerobic phase

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	21.9	29.0	49.6	7.4	1.1	27.4	0.6	2.6	4.2
2.0	29.1	42.1	53.4	18.0	28.2	45.9	0.5	0.9	1.8
3.0	42.2	46.9	64.6	54.3	49.5	51.2	0.2	0.2	1.3
overall	25.0		87.9		51.2		0.58	1.22	2.10

Experiment date:	12/27/2007		aerobic	anaerobic
Experiment No:	Book 9 - Z6	Temperature (°C):	37	37
Strain:	AFP111	pH:	7.0	6.5
Media:	JSM	base:	345.525 g/L K2CO3	345.525 g/L K_2CO_3
Fermenter Volume:	1.201 L	gas phase:	air	CO_2
Inoculum Volume:	50 mL	gas flowrate (L/min):	1	0
		Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
21.9	4.7	38.4	0.7	1.9	1.1	48.0	75.0	1.3	0.2
24.9	9.8	21.7	9.8	1.3	2.3	101.0	75.0	1.4	0.4
25.1	10.7	20.6	10.3	1.3	2.5	101.0	75.0	1.4	0.4
28.1	10.3	9.3	14.3	1.7	2.7	138.0	75.0	1.4	0.5
28.3	10.6	8.0	14.6	1.7	2.7	138.0	75.0	1.4	0.5
29.2	10.7	6.1	15.3	1.8	2.8	142.0	75.0	1.4	0.6
29.3	10.4	33.9	14.6	1.7	2.7	142.0	150.0	1.5	0.5
31.3	10.8	28.6	17.2	1.9	2.7	170.0	150.0	1.5	0.6
31.4	11.6	28.5	18.4	2.0	3.0	170.0	150.0	1.5	0.6
34.3	11.6	23.0	20.1	2.2	3.1	186.0	150.0	1.5	0.7
34.4	12.2	22.6	20.7	2.4	3.1	186.0	150.0	1.5	0.7
37.2	11.9	16.6	22.1	2.6	3.1	206.0	150.0	1.6	0.7
37.3	12.5	16.3	23.1	2.7	3.6	206.0	150.0	1.6	0.7
40.2	12.4	11.5	24.2	2.9	3.4	222.0	150.0	1.6	0.8
40.3	13.1	11.5	25.5	3.1	3.7	222.0	150.0	1.6	0.8
41.9	13.2	9.2	26.3	3.2	3.7	228.0	150.0	1.6	0.8
42.0	12.5	34.0	24.6	3.0	3.7	228.0	220.0	1.6	0.7
43.3	12.2	32.4	24.7	3.0	3.6	236.0	220.0	1.7	0.8
43.4	12.9	33.3	25.7	3.1	3.7	236.0	220.0	1.7	0.8
46.4	12.7	28.9	26.3	3.3	3.8	248.0	220.0	1.7	0.8
46.6	13.1	29.1	27.0	3.4	4.0	248.0	220.0	1.7	0.8
47.2	13.3	29.0	28.0	3.4	4.0	248.0	220.0	1.7	0.8

Notes:
Between 24.9-25.1, 28.1-28.3, 31.2-31.3, 34.2-34.3, 37.2-37.3, 40.2-40.3, 43.-43.4 & 46.4-46.6 h,
aerobic conditions were introduced for 10 minutes and agitation was increased to 400 rpm from 200 rpm during that time.
At the completion of 10 minutes, the condition was returned to anaerobic and agitation was decreased to 200 rpm.
Added 40g glucose in solution at 21.9 ,29.2 & 41.9 h
At 21.9 h switched to anaerobic phase

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
1.0	21.9	29.2	50.8	8.6	0.9	21.6	0.5	2.0	4.1
2.0	29.3	41.9	50.5	20.8	21.7	41.4	0.7	1.0	1.6
3.0	42.0	47.2	56.0	48.4	40.5	46.6	0.8	0.7	0.9
overall	25.3		79.5		46.6		0.59	1.11	1.89

Experiment date:	2/10/2008
Experiment No:	Book 9 - Z11
Strain:	AFP111
Media:	JSM
Fermenter Volume:	1.201 L
Inoculum Volume:	50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	200 g/L NaOH	200 g/L $Ca(OH)_2$
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	Base Acc (mL)	Total Feed (mL)	Volume (L)	Ca^{2+} (mol/L)
35.0	2.0	38.9	0.5	0	0	36	70	1.3	0.1
39.2	5.4	21.4	10.3	0.1	0.8	136	70	1.4	0.3
41.9	7.1	15.0	14.9	0.4	1.8	161	70	1.4	0.3
42.1	6.5	40.3	14.2	0.4	1.6	171	140	1.5	0.3
46.5	8.6	28.8	22.0	0.5	1.2	236	140	1.6	0.5
49.0	8.3	21.0	24.4	0.6	1.2	281	140	1.6	0.6
50.5	8.7	18.0	26.6	0.6	1.5	286	140	1.6	0.5
50.6	8.6	42.6	26.0	0.6	1.4	286	210	1.7	0.6
56.9	9.0	31.1	35.2	0.7	1.8	336	210	1.7	0.6
60.1	8.5	23.4	36.9	0.8	1.5	371	210	1.8	0.7

Notes:

At 35 h switched to anaerobic phase

At 35, 41.9 & 50.5 h, added 40 g glucose in solution

Ca2+ concentration is amount of free calcium ion after binding with succinate

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
I	35.0	41.9	50.9	21.4	0.7	21.3	0.7	2.1	3.0
II	42.1	50.5	61.0	29.2	21.5	43.3	0.7	1.6	2.3
III	50.6	60.1	72.3	41.7	44.1	65.7	0.7	1.3	1.8
overall	25.1		91.8		65.7		0.72	1.47	2.05

Experiment date: 2/10/2008
Experiment No: Book 9 - Z12
Strain: AFP111
Media: JSM
Fermenter Volume: 1.201 L
Inoculum Volume: 50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	200 g/L NaOH	200 g/L $Ca(OH)_2$
gas phase:	air	CO_2
gas flowrate (L/min):	1	0.2
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	Base Acc (mL)	Total Feed (mL)	Volume (L)	Ca^{2+} (mol/L)
34.5	2.0	36.6	0.7	0.2	0.0	36	70	1.3	0.1
39.2	5.0	17.3	12.5	0.5	0.8	151	70	1.4	0.3
41.5	5.8	9.9	17.4	0.8	0.9	171	70	1.4	0.3
41.6	5.6	35.2	16.8	0.7	1.0	181	140	1.5	0.3
46.2	6.5	22.5	25.8	1.0	1.2	246	140	1.6	0.4
49.0	6.4	14.7	29.6	1.2	1.2	296	140	1.6	0.5
50.2	7.1	12.4	33.1	1.4	1.9	296	140	1.6	0.5
50.3	6.5	34.2	30.5	1.2	1.8	296	210	1.7	0.5
56.9	6.9	22.9	39.2	1.5	2.1	371	210	1.8	0.7
59.5	6.4	17.2	42.1	1.5	1.8	396	210	1.8	0.7

Notes:
At 34.5 h switched to anaerobic phase
At 34.5, 41.6 & 50.2 h, added 40 g glucose in solution
Ca2+ concentration is amount of free calcium ion after binding with succinate

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
I	34.5	41.5	47.8	14.3	0.9	25.0	0.7	2.4	3.3
II	41.6	50.2	53.5	20.3	25.6	54.2	0.9	2.0	2.3
III	50.3	59.5	58.5	31.2	52.1	76.1	0.9	1.4	1.6
overall	25.0		94.1		76.1		0.81	1.69	2.08

Experiment date:	2/13/2008
Experiment No:	Book 9 - Z13
Strain:	AFP111
Media:	JSM
Fermenter Volume:	1.201 L
Inoculum Volume:	50 mL

	aerobic	anaerobic
Temperature (°C):	37	37
pH:	7.0	6.5
base:	345.525 g/L K_2CO_3	345.525 g/L K_2CO_3
gas phase:	air	CO_2
gas flowrate (L/min):	1	0
Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
22.5	0.9	37.0	1.1	4.5	1.1	45.0	70.0	1.3	0.2
25.9	0.3	20.3	15.3	4.7	1.8	105.0	70.0	1.4	0.4
29.8	0.0	6.5	25.6	4.6	2.1	165.0	70.0	1.4	0.6
30.8	0.0	3.7	28.2	4.9	2.2	185.0	70.0	1.5	0.7
30.8	0.0	25.0	23.4	3.9	1.9	185.0	140.0	1.5	0.7
35.9	0.0	17.7	37.9	4.8	2.4	245.0	140.0	1.6	0.8
40.9	0.0	8.0	45.2	5.3	2.6	303.0	140.0	1.6	1.0
44.5	0.0	3.6	48.7	5.7	2.7	323.0	140.0	1.7	1.0
47.5	0.0	0.6	50.8	5.9	2.7	341.0	140.0	1.7	1.1

Notes:
Added 5mM betaine to the minimal media before inoculating the fermenter
At 22.5 h, switched to anaerobic phase and added 40 g glucose in solution
At 30.8 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
I	22.5	30.8	48.7	5.4	1.5	41.0	0.9	3.3	3.6
II	30.8	47.5	38.1	1.1	35.7	85.4	1.3	1.8	1.3
overall	25.0		80.4		85.4		1.06	2.03	1.91

Experiment date:	2/13/2008		aerobic	anaerobic
Experiment No	Book 9 - Z14	Temperature (°C):	37	37
Strain:	AFP111	pH:	7.0	6.5
Media:	JSM	base:	345.525 g/L K_2CO_3	345.525 g/L K_2CO_3
Fermenter Volume:	1.201 L	gas phase:	air	CO_2
Experiment No:	50 mL	gas flowrate (L/min):	1	0
		Agitation (rpm):	200 - 1000	200

Time (h)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Acetate (g/L)	Ethanol (g/L)	Base Acc (mL)	Total Feed (mL)	Volume (L)	K^+ (mol/L)
19.0	1.6	45.6	0.5	0.0	0.0	26.0	70.0	1.3	0.2
22.4	1.3	30.6	10.8	1.0	1.1	76.0	70.0	1.3	0.3
25.8	1.0	17.8	21.4	1.4	0.8	134.0	70.0	1.4	0.5
29.3	0.9	8.5	29.5	1.9	0.8	184.0	70.0	1.5	0.7
29.4	0.8	33.7	28.3	1.8	0.8	184.0	140.0	1.5	0.6
34.7	0.7	21.6	39.2	2.2	1.0	252.0	140.0	1.6	0.8
39.9	0.6	13.8	45.9	2.7	1.0	294.0	140.0	1.6	0.9
43.1	0.6	9.1	48.1	2.9	1.0	312.0	140.0	1.7	1.0

Notes:

Added 5mM betaine to the minimal media before inoculating the fermenter

At 19 h, switched to anaerobic phase and added 40 g glucose in solution

At 29.3 h added 40 g glucose in solution

	time		glucose		succinate				
Phase	start (h)	end (h)	start (g)	end (g)	start (g)	end (g)	$Y_{S/G}$ (g/g)	Q_S (g/Lh)	Q_G (g/Lh)
I	19.0	29.3	59.2	12.4	0.6	43.0	0.91	2.82	3.11
II	29.4	43.1	51.5	15.0	43.1	79.5	1.00	1.61	1.61
overall	24.1		83.2		79.5		0.96	2.00	2.09

STUDY 1: SAS OUTPUT

```
                         The GLM Procedure

Dependent Variable: Succinate

                                Sum of
Source                  DF        Squares    Mean Square   F Value   Pr > F

Model                    2     826.700286     413.350143     10.76   0.0073

Error                    7     268.915714      38.416531

Corrected Total          9    1095.616000


          R-Square     Coeff Var      Root MSE    Succinate Mean

          0.754553      15.52632      6.198107          39.92000


Source                  DF      Type I SS    Mean Square   F Value   Pr > F

Base                    1      1.3801667      1.3801667      0.04   0.8550
pH                      1    825.3201190    825.3201190     21.48   0.0024


Source                  DF    Type III SS    Mean Square   F Value   Pr > F

Base                    1     35.3601190     35.3601190      0.92   0.3693
pH                      1    825.3201190    825.3201190     21.48   0.0024
```

STUDY 2: SAS OUTPUT

```
The GLM Procedure

Dependent Variable: Succinate

                                 Sum of
Source                  DF      Squares     Mean Square    F Value    Pr > F

Model                    3    53.5600000     17.8533333       0.32    0.8108

Error                    6   334.1400000     55.6900000

Corrected Total          9   387.7000000


          R-Square     Coeff Var      Root MSE    Succinate Mean

          0.138148      15.32356      7.462573          48.70000


Source                  DF     Type I SS     Mean Square    F Value    Pr > F

Base                    3    53.56000000    17.85333333       0.32    0.8108


Source                  DF   Type III SS     Mean Square    F Value    Pr > F

Base                    3    53.56000000    17.85333333       0.32    0.8108
```

STUDY 3: SAS OUTPUT

```
The GLM Procedure

Dependent Variable: Succinate

                          Sum of
Source             DF       Squares    Mean Square   F Value   Pr > F

Model               1   14.82250000    14.82250000      0.44   0.5765

Error               2   67.82500000    33.91250000

Corrected Total     3   82.64750000


        R-Square    Coeff Var     Root MSE    Succinate Mean

        0.179346     11.45783     5.823444          50.82500


Source             DF      Type I SS    Mean Square   F Value   Pr > F

Base               1   14.82250000    14.82250000      0.44   0.5765


Source             DF    Type III SS    Mean Square   F Value   Pr > F

Base               1   14.82250000    14.82250000      0.44   0.5765
```

STUDY 4: SAS OUTPUT

```
The GLM Procedure

Dependent Variable: Succinate

                            Sum of
Source              DF     Squares    Mean Square   F Value   Pr > F

Model                2    896.610000    448.305000    10.45   0.0445

Error                3    128.730000     42.910000

Corrected Total      5   1025.340000


         R-Square    Coeff Var     Root MSE    Succinate Mean

         0.874451     9.535040     6.550572          68.70000


Source              DF     Type I SS    Mean Square   F Value   Pr > F

Base               2    896.6100000    448.3050000     10.45   0.0445


Source              DF    Type III SS    Mean Square   F Value   Pr > F

Base               2    896.6100000    448.3050000     10.45   0.0445


Contrast            DF    Contrast SS    Mean Square   F Value   Pr > F

K2CO3 Vs Ca(OH)2          1    329.4225000    329.4225000     7.68   0.0695
```

```
K2CO3 Vs Betaine             1    882.0900000    882.0900000     20.56   0.0201
Ca(OH)2 Vs Betaine           1    133.4025000    133.4025000      3.11   0.1761


                                   Standard
 Parameter                Estimate          Error    t Value    Pr > |t|

 K2CO3 Vs Ca(OH)2          -18.1500000      6.55057249      -2.77      0.0695
 K2CO3 Vs Betaine          -29.7000000      6.55057249      -4.53      0.0201
 Ca(OH)2  Vs Betaine       -11.5500000      6.55057249      -1.76      0.1761
```