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**DISCLAIMER:**



**ABSTRACT:**

This report describes results toward developing a process to sequester $CO_2$ centered on the enzymes PEP carboxylase and pyruvate carboxylase. The process involves the use of bacteria to convert $CO_2$ and glucose as a co-substrate and generates succinic acid as a commodity chemical product.

The study reports on strain development and process development. In the area of strain development, knockouts in genes which divert carbon from the enzymatic steps involved in $CO_2$ consumption were completed, and were shown not to affect significantly the rate of CO2 sequestration and succinic acid generation. Furthermore, the *pyc* gene encoding for pyruvate carboxylase proved to be unstable when integrated onto the chromosome.

In the area of process development, an optimal medium, pH and base counterion were obtained, leading to a sequestration rate as great as 800 mg/Lh. Detailed studies of gas phase composition demonstrated that CO2 composition has a significant affect on CO2 sequestration, while the presence of "toxic" compounds in the gas, including $NO_2$, CO and $SO_2$ did not have a detrimental effect on sequestration. Some results on prolonging the rate of sequestration indicate that enzyme activities decrease with time, suggesting methods to prolong enzyme activity may benefit the overall process.

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## Introduction

Most research on the microbial sequestration of $CO_2$ has centered on *archaea* (Atom 2002, Shively et al. 1998) in liquid suspension reactor systems (Kodama 1996) or microalgal systems (Brown 1996, Watanabe and Hall 1996). Many of these microbial species require a photosynthetic reaction to generate ATP for subsequent $CO_2$ fixation, which severely limits their application for $CO_2$ sequestration due to scale-up problems, including the requirement for an extremely large reactor size (Zhang et al. 2002). In addition, many of the organisms proposed for $CO_2$ fixation have fastidious growth requirements, and have unacceptably low product yields and formation rates, both of which essentially eliminate industrial applications (Atom 2002, Shively et al. 1998). $CO_2$ is a gaseous substrate, and little research has centered on advanced reactor design configurations that significantly improve $CO_2$ utilization and continuously generate products of interest (Lamare and Legoy, 1993). In fact, a review of the literature shows no reports on the use of bacterial systems for $CO_2$ fixation in bioreactors; most of the research has centered on the use of microalgal systems that require light/dark cycles and reactors with large footprints (Brown 1996, Wantanabe and Hall 1996, Otsuki 2001). These microalgal reactors typically have extremely slow substrate consumption rates. For example, Otsuki (2001) reports a $CO_2$ utilization rate of 50 $g/m^2/day$ for a photobioreactor with a surface area to volume ratio of 6.2 $m^2/m^3$ or a volumetric rate of only 13 mg $CO_2$/L/hr. This number is the single most important parameter in assessing the viability of a biological $CO_2$ sequestration process. Substrate consumption and product formation rates are generally more than 100-fold greater in commercially relevant microbial based systems.

The general approach used in this project involves the biological incorporation of $CO_2$ into the backbone of another inexpensive organic compound to generate a $C_{X+1}$ compound. Promising examples of this strategy include using the enzymes malate oxidoreductases (EC 1.1.1.39, EC 1.1.1.83, etc.) and pyruvate carboxylase (EC 6.4.1.1). Malate oxidoreductases are enzymes which convert pyruvate ($C_3$) into malate ($C_4$), while pyruvate carboxylase converts pyruvate ($C_3$) into oxaloacetate ($C_4$). It must be understood that a microbial process which relies on a $CO_2$-fixing enzyme will use a co-substrate biochemically "upstream" of the $CO_2$-conversion step, while the ultimate product will be a compound biochemically "downstream" of the $CO_2$-conversion step. Thus, in practice pyruvate carboxylase could convert glycerol or glucose (upstream of $CO_2$ fixation) into succinic acid (downstream of the $CO_2$ fixation step).

The chemical product that will be the focus of this project is succinic acid. Cost analysis suggests that commercialization of succinic acid production by a biological route is feasible with improvements in strain and process design (Schilling 1995), and current economic models do not include benefits derived from carbon sequestration. Succinic acid would be used as a chemical feedstock for industrial chemicals such as polymers. As the cost for the chemical route increases in the coming years while improvements in the biological process are attained, a biological route will likely become the preferred route. The approach used in this research will furthermore be quite applicable to other biological processes which sequester $CO_2$, and we hope that other promising routes involving the use of $CO_2$ directly in the synthesis of organic $C_1$ compounds may be more fully developed, such as using formate oxidoreductases (EC 1.2.1.2 or EC 1.2.1.43) to generate formic acid or using urea amidohydrolase (EC 3.5.1.5) to generate urea.

Our research group has many years of experience developing microbial processes for the production of succinic acid and other biochemicals (see resumes of principal investigators). We have previously demonstrated that we can achieve succinic acid production at near theoretical yields and demonstrated the use of hydrogen gas as an example reducing agent to increase yield from glucose (Vemuri et al. 2002a, 2002b). More importantly, we estimate that the rate of $CO_2$ utilization by these fermentation routes would be about 45 kg/$m^3$day (16 metric tons/$m^3$year), about 150 times greater than the $CO_2$ utilization reported by Otsuki (2001) in a photobioreactor. This estimate is based on our projection of a 5 g/Lh volumetric succinate productivity in a bioreactor, a rate readily attainable by cell concentration step prior to the bioconversion step. In other words, a 1 $m^3$ vessel would consume 45 kg of $CO_2$ and 92 kg glucose per day to generate about 120 kg of succinic acid. (In practice a commodity chemical like this would be produced at the 1000 $m^3$ scale at one site.)

There has been limited research on the use of $CO_2$ as a substrate in high density microbial fermentations and advanced reactor designs to improve $CO_2$ utilization. Fortunately, there has been research in the area of synthesis gas fermentation processes. The composition of synthesis gas varies, but is composed primarily of CO and $H_2$, both poorly water-soluble substrates. The limited solubility of these gaseous substrates has led to the development of trickle-bed, airlift, and microbubble sparged reactors to enhance mass transfer and subsequently increase the rates of product formation (Bredwell 1999, Wolfrum and Watt 2002). In addition to mass transfer limitations, synthesis gas fermentations are limited by low product yields and concentrations, and by low rates of product formation primarily due to low cell densities (Worden 1997).

One bacterium that produces succinic acid from $CO_2$ is *Escherichia coli*, and it was the focus of this study. The key biochemical pathways involved in this process are shown in Figure 1. Glucose as a co-substrate is taken up by the cell through two enzymes (1) glucokinase and (2) the phosphotransferase system or "PTS" (numbers refer to paths in Figure 1). The PTS unfortunately also simultaneously converts PEP to pyruvate, which would tend to divert material away from some $CO_2$ sequestration. Therefore, the PTS has been genetically removed in the control strain (AFP111). Moreover, the overexpression of glucokinase might increase the uptake of glucose and therefore the rate of $CO_2$ sequestration. Some carbon may be lost via the pentose phosphate pathway (3), but during anaerobic $CO_2$ sequestration when microbial growth does not occur, this portion is believed to be negligible. Several by-products are generated naturally by *E. coli*, and some have been considered in this study. Specifically, lactate dehydrogenase (4) and pyruvate formate lyase (5) convert pyruvate respectively into lactate and formate. These enzymes are encoded by the *ldhA* and *pflAB* genes, which due to their significant drain on carbon, have been removed from the control strain. Acetate is another by-product, and its accumulation could be reduced by knocking out the genes encoding for pyruvate oxidase (6) or phosphoacetyltransferase (7). Other researchers have provided evidence that the latter knockout yields growth defects, and therefore only the former option was examined. That is, we tested the effect of knocking out the *poxB* gene encoding for pyruvate oxidase. *E. coli* also can accumulate ethanol (8) by the enzyme alcohol dehydrogenase encoded by the *adhE* gene. This path could particularly be detrimental to $CO_2$ sequestration because it consumes electrons which are needed for succinic acid accumulation. The key $CO_2$ sequestration enzyme is PEP carboxylase (9). A goal of this study was also to incorporate pyruvate carboxylase (10) into the genetic machinery

of *E. coli* to allow two paths for and therefore improve on the $CO_2$ sequestration. By virtue of these knockouts and/or operational conditions, carbon flow through other pathways is limited.

In addition to genetic perturbations, in the area of process development, operating parameters must be optimized such as pH and temperature, and the effect of other substances in the gas stream must be assessed. In the area of reactor design, $CO_2$ mass transfer resistance must be quantified and lead to complete $CO_2$ utilization. Ultimately, the process must produce succinic acid at high rates and yields. While large reductions in $CO_2$ levels are not anticipated using such a single process, the technology does provide a niche method for $CO_2$ transformation into commercial products, may lead to analogous technologies using other biological approaches (Creutz and Fujita 2000).

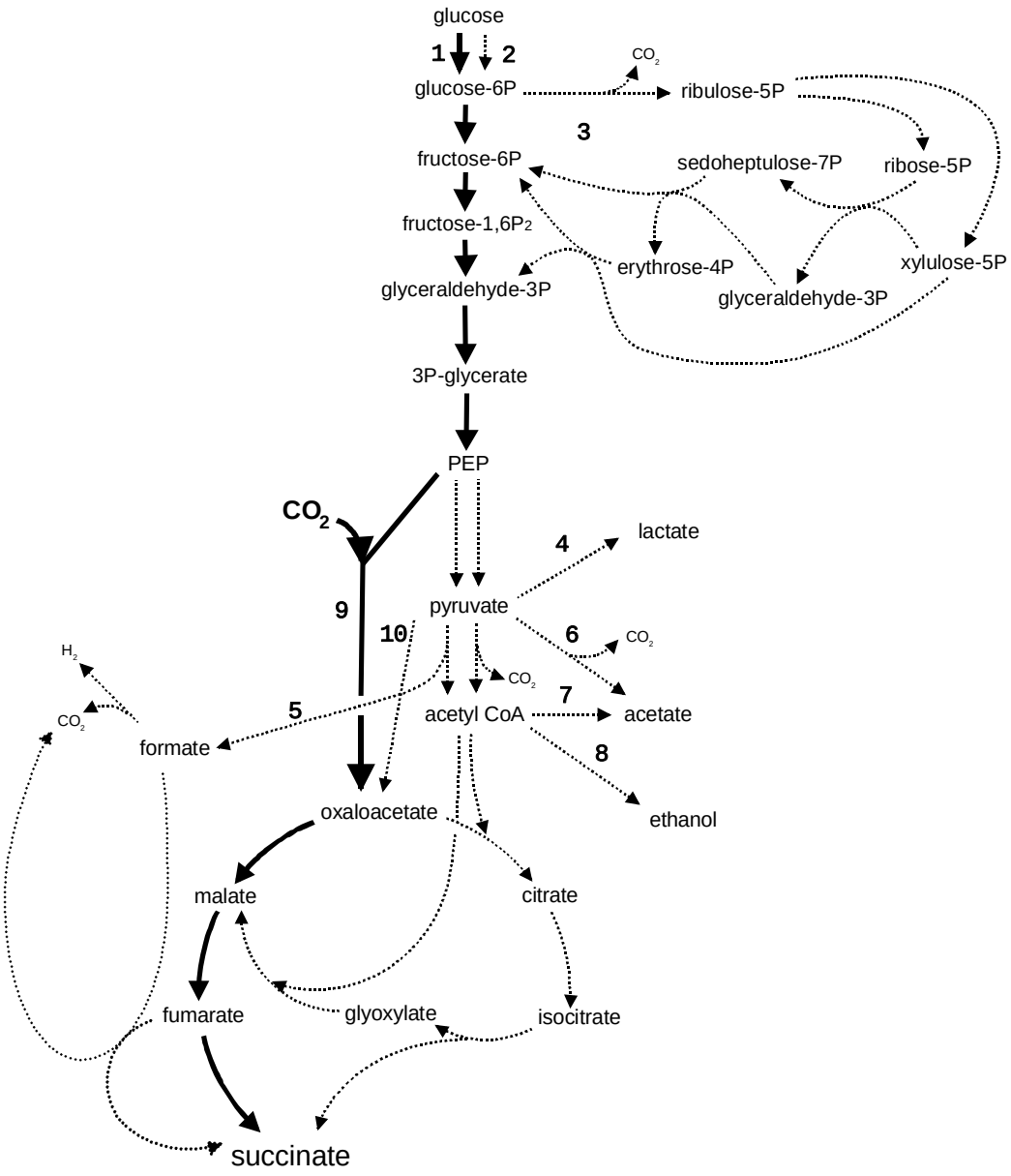


**Figure 1.** Biochemical pathways involved in microbial $CO_2$ sequestration. See text for information regarding reference numbers.

**Executive Summary**

This report describes results toward developing a process to sequester $CO_2$ using two $CO_2$-utilizating enzymes, PEP carboxylase and pyruvate carboxylase. The process involves the use of bacteria to convert $CO_2$ and glucose as a co-substrate and generates succinic acid as a commodity chemical product. The former enzyme occurs naturally in the bacterium studied, whereas the latter enzyme must be introduced into the bacterium.

Several strains were developed. The *adhE* knockout, *poxB* knockout, and strain overexpressing *glk* were developed. While some of these strains improved the yield, they generally did not enhance the rate of sequestration over the baseline strain to justify their use.

Numerous molecular procedures were explored to incorporate pyruvate carboxylase into the *E. coli* chromosome. We were successful in integrating the *pyc* gene into the chromosome. However, pyruvate carboxylase enzyme activity was observed to diminish after a few generations. Therefore, we were not successful at examining whether the rate of sequestration could be enhanced using this approach.

We achieved a $CO_2$ sequestration rate estimated to be as high as 800 mg/Lh. We were able to obtain consistent sequestration rates of over 600 mg/Lh. These numbers indicate, for example, that the contents of a 1000L vessel could consume 600 g/h $CO_2$.

We demonstrated that a defined medium could be used for the process, and that the process could be operated well in the pH range of 6.0 – 6.8, with a pH optimal of about 6.4.

The gas phase concentration of $CO_2$ was observed to affect the sequestration rate significantly. That is, the higher the concentration of $CO_2$ in the gas, the greater the rate of sequestration. A detailed analysis of the mass transfer and reaction rates involved does not provide an understanding of why this would be the case. The sequestration rate is therefore thought to be limited by the amount of PEP carboxylase that is present in the cell. It is unknown whether the amount of PEP carboxylase activity present in the cell is changing in response to $CO_2$ concentrations. It is likely that increasing enzyme activity of PEP carboxylase or pyruvate carboxylase would increase sequestration rate. The relationship between gas phase $CO_2$ concentration and sequestration rate does suggest that sequestration rate could be enhanced by pressurizing the gas, an approach which would require specialized equipment and therefore not examined.

The presence of trace amounts of impurities in the gas stream do not inhibit $CO_2$ sequestration and succinate generation. The gas impurities which either have no effect or promote sequestration slightly include $NO_2$, $SO_2$, CO and $O_2$. The process is sufficiently robust to use on a wide variety of $CO_2$ sources. This result warrants further study, as it suggests that some mechanism caused by these gases provides a physiological advantage to the cell.

The rate of sequestration falls with time during the process. The most important factor which diminishes $CO_2$ fixation appears to be the presence of a high ionic strength, which is a result of neutralizing the succinic acid generated as a product. This problem might be alleviated by

removing the product, succinic acid, as it is being generated by the microorganisms. $CO_2$ fixation may also be prolonged by intermittent supply of oxygen.

Although the rate of sequestration was high, and the process appears very amenable to $CO_2$ streams containing trace amounts of toxic and other gases, it is unlikely that the process is economically feasible. The requirement for significant base for neutralization and the reduction in sequestration rate over time are two factors which are significant impediments to the use of this approach and which need further development.

## Methodology

Two-phase fed-batch experiments were carried out in 2.0 L or 2.5 L bioreactors (Bioflow 2000 or Bioflow III, New Brunswick Scientific Co. Edison, NJ, USA) initially containing 1.5 L media. In the growth phase, air and $O_2$ were mixed at 1.0 L/min total flow rate at 400 rpm constant agitation to maintain a dissolved oxygen concentration (DO) above 40% of saturation. Also, the pH was controlled at a pH of 7.0 using 20% (w/v) NaOH, and the temperature was controlled at 37°C. When the OD reached about 20-25, depending on the specific experiment, the second anaerobic experimental phase was initiated. During this phase, conditions of temperature and pH were selected according to the experimental design. Various substances were examined to control the pH, such as $Na_2CO_3$, NaOH, KOH or $Ca(OH)_2$. The carbon backbone was provided by glucose, and a couple methods were used to introduce glucose into the fermenter. Often, a feed solution of 40 g/100 mL glucose was added at the initiation of the production phase. Also, during the anaerobic sequestration phase, $CO_2$ was sparged into fermenter at 0.15 – 0.5 L/min as indicated, sometimes with other gases as impurities as described.

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth, and this value was correlated to dry cell mass. The final concentrations of soluble organics compounds were determined by liquid chromatography as previous described (Eiteman and Chastain, 1997).

Various enzyme assays were completed. The alcohol dehydrogenase assay was based on the NAD-dependent oxidation of ethanol (Blandino et al. 1997). Glucokinase phosphorylates glucose into glucose 6-phosphate using ATP as the phosphate group donor. The assay used is based on the NADPH-dependent reduction of glucose 6-phosphate (Pakoskey et al. 1965). Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate using ATP and carbonate as co-substrates. The assay used is a coupled enzyme assay (Payne and Morris, 1969) in which the oxaloacetate generated by the action of pyruvate carboxylase reacts with acetyl CoA via citrate synthase (both in excess). The free CoA generated by this second reaction is the species that actually causes the signal by its reaction with the chemical DTNB.

## Results and Discussion

### 1. Construction of Microbial Strains

Table 1 summarizes all the strains used in this study.

For all experiments, the control strain was *Escherichia coli* AFP111 [ATCC 202021, F+λ-*rpoS396*(Am) *rph*-1 *ldhA*::kan Δ(*pflAB*::cam) *ptsG*] (Donnelly et al., 1998; Chatterjee et al. 2001). This strain has key mutations in the *ptsG*, *ldhA* and *pflAB* genes encoding respectively for the PTS-glucose transport system, lactate dehydrogenase, and pyruvate formate lyase (see Figure 1). A significant effort was directed toward the construction of a strain in which the *pyc* gene encoding for pyruvate carboxylase was integrated on the chromosome. Several other strains were also constructed: AFP111 *adhE*, AFP111 *poxB* and AFP111 pTrc99A-*glk* as described below.

The pyruvate carboxylase protein from *Rhizobium etli* was initially cloned into the pTrc99A vector to enable the expression of this anaplerotic enzyme by our laboratory (Gokarn et al., 2001). The pTrc99A-*pyc* construct has been utilized in a variety of metabolic engineering approaches which rely on pyruvate carboxylase, an enzyme that does not naturally occur in wild-type *Escherichia coli* (March et al., 2002; Vemuri et al., 2002; Vemuri et al., 2005).

We proposed to utilize pTrc99A-*pyc* derivatives we had constructed in which the kanamycin resistance gene (encodes aminoglycoside-3'-O-phosphotransferase) from Tn903 had been inserted just downstream of the *pyc* gene to introduce *R. etli pyc* into the chromosome. The *pyc* and kanamycin resistance genes were amplified using the polymerase chain reaction (PCR) and the resulting fragment was recombined into the *E. coli* chromosome using the lambda Red recombination system (Datsenko and Wanner, 2000; Yu et al., 2000). The resulting *E. coli pyc* strain did not express any pyruvate carboxylase activity. This result was not surprising and had been anticipated in the proposal since moving the pTrc99A-*pyc* construct into the chromosome effectively reduced a 200 copy plasmid to a single copy.

To express pyruvate carboxylase at sufficient levels from a single copy of the gene in *E. coli*, we needed a stronger promoter. Two new expression vectors were constructed for this purpose that could accommodate the relatively large 3,500 kb *pyc* gene. The pBR322.P*rrnA* and pBR322.P*ompA* expression vectors were constructed using the strong promoter regions from ribosomal *rrnA* and the outer membrane protein A. Each of these promoter regions is unusual. The promoter for the *rrnA* gene consists of four distinct promoters which function collectively to form a very strong promoter (deBoer et al., 1979) while the promoter for the *ompA* gene consists of one strong promoter that is operationally linked to four independent Shine-Dalgarno ribosome binding sites (Movva et al., 1980). The *R. etli pyc* gene was cloned into each of these vectors. In the case of the pBR322.P*rrnA* vector, *pyc* was cloned using four different Shine-Dalgarno ribosome binding sites of varying lengths in order to select the clone that expressed maximal amounts of *pyc*. Pyruvate carboxylase activities were determined for all the clones and the clone that expressed the highest levels of pyruvate carboxylase, which was one of the pBR322.P*rrnA*.*pyc* constructs, was selected. The tetracycline resistance gene (encodes tetracycline efflux pump protein) from Tn10 or the kanamycin resistance gene from Tn903 were cloned into pBR322.P*rrnA*.*pyc* just downstream of the *pyc* gene. These pBR322.P*rrn*A.*pyc* Tet(R) and pBR322.P*rrn*A.*pyc* Kan(R) constructs were used to PCR amplify *pyc* Tet(R) and *pyc* Kan(R) cassettes to introduce *pyc* into the *E. coli* chromosome. The resulting *pyc* chromosomal insertions with either tetracycline or kanamycin resistance expressed pyruvate carboxylase at the same level as an isogenic *E.coli* strain that contained the pTrc99A-*pyc* reference plasmid.

While the lambda Red recombination system is routinely utilized to insert 1 – 3 kb DNA fragments into the *E. coli* chromosome, the use of this system is problematic for the insertion of larger 4 – 6 kb DNA fragments as required by this research proposal. A cursory examination of the lambda Red recombination vectors that have been developed revealed that the expression of the lambda Beta, Exo and Gamma proteins that constitute the lambda Red recombination system may not be optimal. Based on our laboratories experience with maximizing gene expression in *E. coli*, we decided that an expression vector that maximally expressed the lambda Beta, Exo and Gamma proteins might be developed. Using a couple of different strategies a pTrc99A derivative that expresses maximal amounts of the lambda Beta, Exo and Gamma proteins was

developed. In our preliminary assessment this vector enables recombination to occur at rates that are 10 fold higher than the rates that are achievable by other lambda Red vectors. Our new pTrc99A derivative is also easier to use. To construct a gene knockout or "knockin" in the final destination strain using vectors developed by other researchers requires 10 days because the initial recombinants must be maintained at 30°C. To construct a gene knockout or knockin using our new pTrc99A derivative which can be maintained at 37°C only requires 4 days.

We have thoroughly investigated *E. coli* strains that have had *pyc* cassettes incorporated into the chromosome. While the plasmids that harbor these gene cassettes are quite stable and produce high levels of pyruvate carboxylase enzyme that are equal to or higher than the pTrc99A-*pyc* reference plasmid (Gokarn et al., 2001), when the cassettes are introduced into the chromosome, pyruvate carboxylase enzyme levels begin to diminish and disappear after several generations of growth. Interestingly, the drug resistance is still present and the *pyc* region is intact and can be amplified by the polymerase chain reaction. Thus chromosomal integration of the *pyc* gene does not appear to be a viable means of providing companies with excess levels of pyruvate carboxylase enzyme to enhance product production.

AFP111 *adhE* contains a knockout in the *adhE* gene encoding for alcohol dehydrogenase (see Figure 1). To accomplish this knockout, primers were designed which could amplify the *tetA* (tetracycline resistance) gene from the Tn10 transposon bracketed by the first and last 50 bp of the *adhE* gene. The polymerase chain reaction (PCR) was performed using these primers and Tn10 DNA. The resulting amplified DNA was electroporated into DY330, one of the strains used for Lambda Red mediated recombination. Tetracycline resistant recombinants were selected and by employing PCR with primers designed to amplify the wild-type *adhE* gene, it was verified that the tetracycline resistant recombinants were indeed *adhE*::tet knockouts where the coding sequence of *adhE* had been removed except for the first and last 50 bp. The *adhE*::tet knockout was moved by P1 transduction into MG1655 and the production strain AFP111. This strain was designated ALS942.

AFP111 *poxB* contains a knockout in the *poxB* gene encoding for pyruvate oxidase. To accomplish this knockout, an approach was used which was completely analogous to what had been completed for the *adhE* knockout as described above. This strain was designated ALS943.

A strain which overproduced glucokinase was generated by first constructing the plasmid pTrc99A-*glk*. Primers were designed which could PCR amplify the *glk* gene from *Escherichia coli* and introduce varying length Shine-Dalgarno (SD) ribosome-binding sites in order to maximize the overproduction of glucokinase. The amplified DNA was cloned into the plasmid pTrc99A and transformed into an R-M+ *E. coli* strain. Overproduction of glucokinase was initially verified by SDS-PAGE analysis. Clones with SD lengths of 4 – 6 bp expressed glucokinase at 2 - 5% of total cellular protein while clones with a SD length of 7 bp expressed glucokinase at 5 - 10% of total cellular protein. Glucokinase assays were then performed. Compared to a control *E. coli* strain which had 0.3 units per mg of total cellular protein (U/mg) of specific glucokinase activity, *E. coli* strains which contained the pTrc99A-*glk* SD4, pTrc99A-*glk* SD5, pTrc99A-*glk* SD6, and pTrc99A-*glk* SD7 had specific glucokinase activities of 6.1, 5.4, 9.8, and 23.7 U/mg, respectively. The pTrc99A-*glk* SD7 plasmid was transformed into AFP111,

and the activity was measured to be about 25 U/mg. This strain, AFP111 pTrc99A-*glk*, was designated ALS984 and was used for subsequent experiments.

Two additional strains were studied. Strain ALS874 was AFP111 transformed with the pTrc99A-*pyc* plasmid as previously described (Gokarn et al., 2001). Strain ALS1000 was ALS942 transformed with pTrc99A-*pyc*.

**Table 1.** The strains used in this study.

| Strain name | Key Genotype Information |
|---|---|
| AFP111 | *rpoS396 rph ldhA pflAB ptsG* |
| ALS942 | *rpoS396 rph ldhA pflAB ptsG adhE* |
| ALS943 | *rpoS396 rph ldhA pflAB ptsG poxB* |
| ALS874 | *rpoS396 rph ldhA pflAB ptsG* pTrc99A-*pyc* |
| ALS984 | *rpoS396 rph ldhA pflAB ptsG* pTrc99A-*glk* |
| ALS1000 | *rpoS396 rph ldhA pflAB ptsG adhE* pTrc99A-*pyc* |

## 2. Medium Development

For preliminary experiments an "unoptimized" growth medium was used. This medium (referred to as "complex" medium) contained (per L): 40.0 g glucose, 20 g tryptone, 10 g yeast extract, 5.0 g $NH_4SO_4$, 2.0 g $KH_2PO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 1 mg thiamine·HCl, 1 mg biotin. During the course of our research, a wide variety of other media compositions were examined, and not all results reported in herein use the same medium composition.

Generic medium compositions are well-known for the high cell growth of *Escherichia coli*. We began by using the formulations of Horn et al. (1996) and Causey et al. (2003). In the "Horn medium" in shake flask studies we consistently observed poor growth, while the "Causey medium" fared much better. We therefore focused on the Causey medium as our baseline.

We completed a series of Plackett-Burman experiments to determine medium components which affected cell growth. The Plackett-Burman experiment is a fairly imprecise but simple way to determine which of numerous factors appear to be the most important determinants. The results indicated that $MnCl_2 \cdot 4H_2O$ (2.0-10.0mg/L) had a significant effect on the cell growth rate at >70% confidence level, and $CaCl_2 \cdot 2H_2O$ (2.0-10.0 mg/L) had an effectat above 80% confidence level. Because of this result, we designed a modified L8($2^7$) matrix to investigate the effects of three variables ($Mn^{2+}$, $Ca^{2+}$, and trace elements) on the cell growth. From these experiments we determined that $Ca^{2+}$ had no significant effect and the highest concentration of $Mn^{2+}$ (within our exerpimental design) had a positive impact on cell growth. We therefore developed a "modified Causey Medium" which used higher levels of $Mn^{2+}$ and trace elements. When we used this medium in the fermenter, the addition of NH4OH to control pH during cell growth resulted in gradual precipitation. We therefore reduced the concentration of $MgSO_4.7H_2O$ and $KH_2PO_4$ in the medium. The final medium selected which both prevented the formation of precipitate during the course of the growth and which yielded the highest rate of succinate formation during the production phase because referred to as "JSM" (Table 2).

**Table 2.** JSM used for comparing the strains for $CO_2$ sequestration.

| | |
|---|---|
| glucose | 40.00 g/L |
| citric acid | 3.0 g/L |
| $Na_2HPO_4 \cdot 7H_2O$ | 3.00 g |
| $KH_2PO_4$ | 8.00 g |
| $(NH_4)_2HPO_4$ | 8.00 g |
| $NH_4Cl$ | 0.20 g |
| $(NH_4)_2SO_4$ | 0.75 g |
| $MgSO_4 \cdot 7H_2O$ | 1.00 g/L |
| $CaCl_2 \cdot 2H_2O$ | 10.0 mg/L |
| $ZnSO_4 \cdot 7H_2O$ | 0.5 mg/L |
| $CuCl_2 \cdot 2H_2O$ | 0.25 mg/L |
| $MnSO_4 \cdot H_2O$ | 2.5 mg/L |
| $CoCl_2 \cdot 6H_2O$ | 1.75 mg/L |
| $H_3BO_3$ | 0.12 mg/L |
| $Al_2(SO_4)_3 \cdot xH_2O$ | 1.77 mg/L |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.5 mg/L |
| Fe(III) citrate | 16.1 mg/L |
| thiamine·HCl | 20 mg/L |
| biotin | 2 mg/L |

### 3. Sequestration by Different Strains

With the JSM medium, the six strains listed in Table 1 were compared for $CO_2$ sequestration and succinate yield. Each strain was studied with multiple fermentations, and an example for each process is shown in Figures 2-7. Note that in these figures to assist comparison, the scales for the coordinate axes are identical, regardless of the quantity of materials consumed and generated and the rate at which the process occurs. As described in the Methodology section, the cell density for each process was approximately 8 g/L (OD=20), and therefore it is appropriate to compare volumetric rates as shown. Figure 2 shows the results with the control strain AFP111. Using this strain, three by-products were formed: pyruvate, ethanol and acetate. The ca. 40 g/L glucose added had been converted (and thus $CO_2$ consumed) within 13 hours.

From the results obtained with AFP111, it seems reasonable to pursue a study using strains in which the ability to generate one or more of the by-products has been prevented. Therefore, we generated one strain in which the *adhE* gene expressing alcohol dehydrogenase was knocked out (ALS942) and a second strain in which the *poxB* gene expressing pyruvate oxidase had been knocked out (ALS943). We then grew these strains in identical processes as we had for AFP111 (described above) in order to compare their ability to consume $CO_2$ and generate succinate. Several striking results were obtained with strain ALS942 (Figure 3). First, as expected in a strain lacking the enzyme to generate ethanol, the process using ALS942 does not accumulate any ethanol. Moreover, the process does not accumulate any pyruvate. However, the process accumulated about twice as much acetate compared to the process using AFP111, and the process was much slower. This reduction is rate is readily observable by comparing Figures 2

and 3; whereas the glucose was consumed in 13 hours for AFP111, for ALS942 after 20 hours only a little more than half of that quantity of glucose had been consumed. For ALS943, the acetate yield was reduced from 0.05 g/g to 0.02 g/g, but the rate of succinate formation and thus $CO_2$ consumption was markedly reduced compared to the control strain AFP111 (Figure 4).

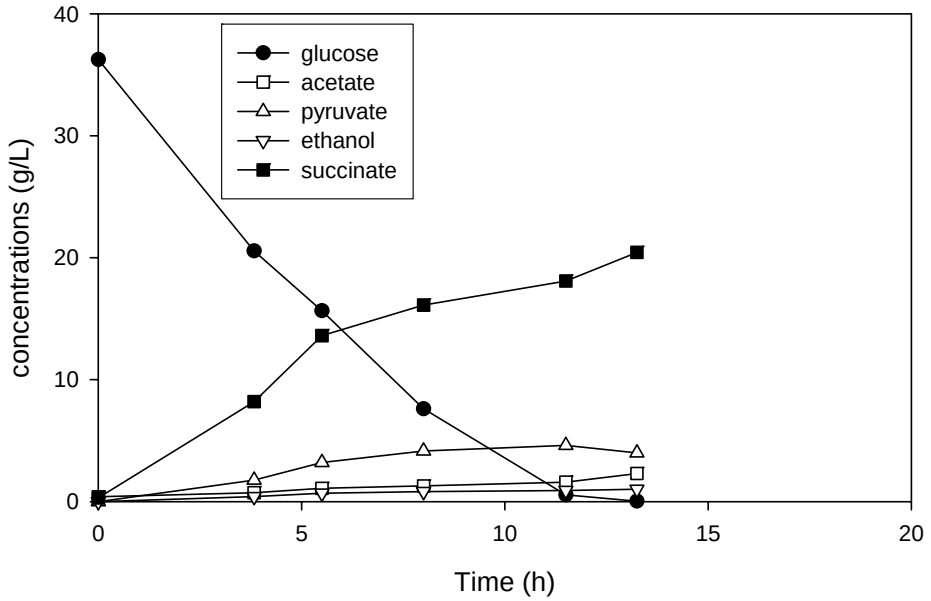


**Figure 2.** Conversion of glucose to succinate in AFP111 after growth in JSM. Note that 1 mol of succinate generation sequesters 1 mol of $CO_2$.

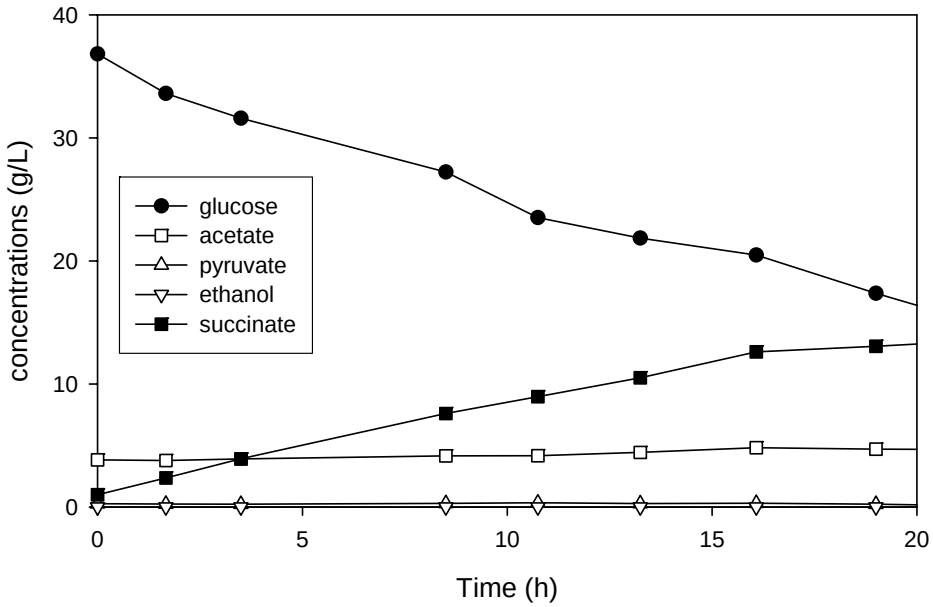


**Figure 3**. Conversion of glucose to succinate in ALS942 (AFP111 *adhE*) after growth in JSM. Note that 1 mol of succinate generation sequesters 1 mol of $CO_2$.

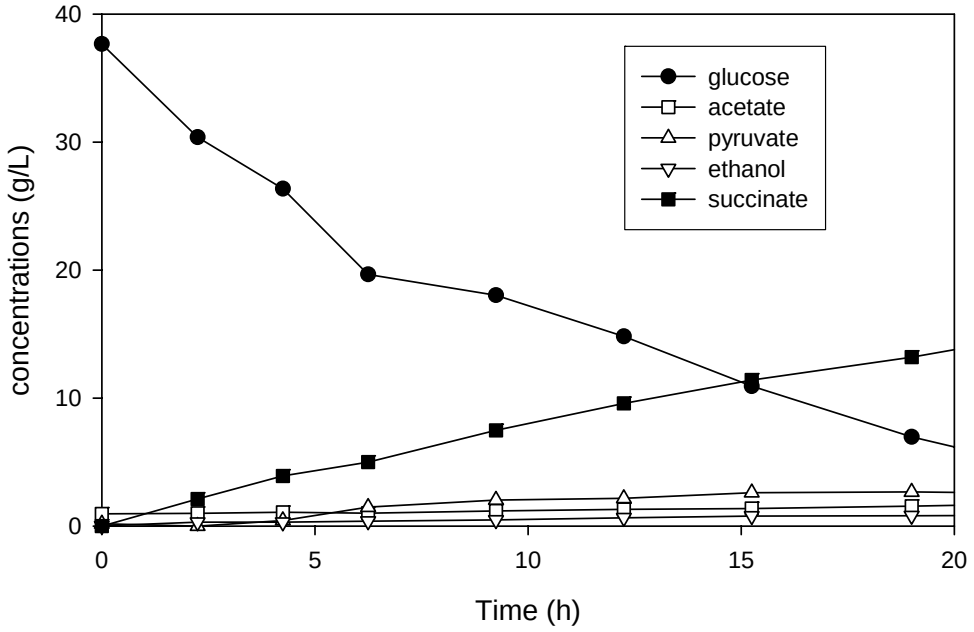


**Figure 4**. Conversion of glucose to succinate in ALS943 (AFP111 *poxB*) after growth in JSM. Note that 1 mol of succinate generation sequesters 1 mol of $CO_2$.

A fourth strain that we examined was AFP111 containing the gene encoding for the enzyme pyruvate carboxylase on a plasmid. A goal of our project was to incorporate the *pyc* gene on the chromosome of AFP111. As described elsewhere, we were not successful in accomplishing this goal. However, we did examine the expression of pyruvate carboxylase from a multi-copy plasmid pTrc99A under the *trc* promoter. Use of a plasmid is not optimal because of the increased metabolic burden placed on a cell to maintain a plasmid. Nevertheless, lacking the strain containing *pyc* on the chromosome, we examined how strain ALS874 (AFP111 with *pyc*) performed in our $CO_2$ sequestration experiment. Figure 5 shows an example of the results. Processes with ALS874 fared similar to AFP111. One difference was the ALS874, counter-intuitively, accumulated more pyruvate than AFP111. One other difference was that ALS874 was very inconsistent (over the course of four runs). Although we observed the highest $CO_2$ sequestration rate (1030 mg/Lh) in one of the runs with ALS874, the mean was lower than the mean observed for AFP111.

We next examined a strain in which the rate of glucose consumption would presumably be increased by overexpressing the *glk* gene encoding for glucokinase. Glucokinase converts glucose into glucose-6-phosphate (Figure 1). Like ALS874, we used the multicopy pTrc99A plasmid. This strain, ALS984, was grown and provided with $CO_2$ using the same procedure, and an example of our results is shown in Figure 6. Although surprisingly no pyruvate and little ethanol were generated, as well as a higher yield of succinate, the process was actually slower than the control strain AFP111.

Finally, we examined a combination of two features. We took the strain lacking activity in alcohol dehydrogenase, ALS942, and transformed it with the *pyc* gene on the pTrc99A plasmid. This new strain, ALS1000, therefore both contains the *pyc* gene and has a knockout in the *adhE* gene. Figure 7 shows an example of the results obtained using strain ALS1000. In this case, like ALS942 no ethanol was generated. However, the pyruvate accumulation was significant, and the rate of succinate formation was very low.

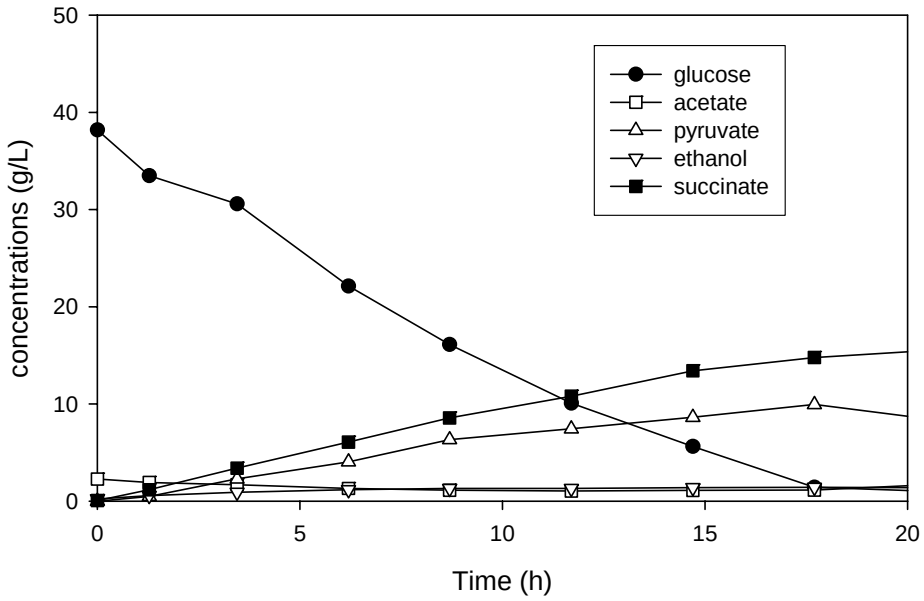


**Figure 5**. Conversion of glucose to succinate in ALS874 (AFP111 pTrc99A-*pyc*) after growth in JSM. Note that 1 mol of succinate generation sequesters 1 mol of $CO_2$.

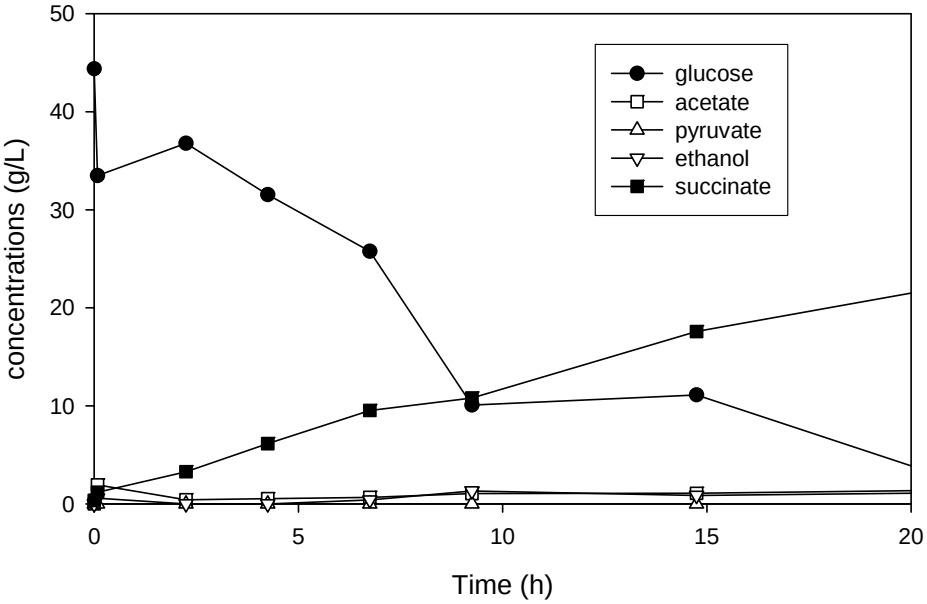


**Figure 6**. Conversion of glucose to succinate in ALS984 (AFP111 pTrc99A-*glk*) after growth in JSM. Note that 1 mol of succinate generation sequesters 1 mol of $CO_2$.

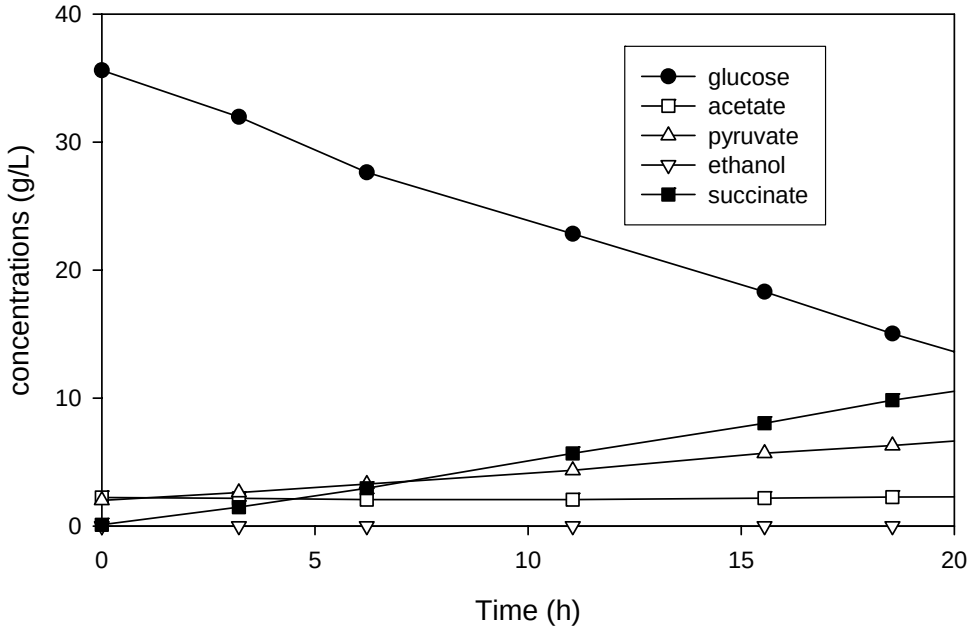


**Figure 7**. Conversion of glucose to succinate in ALS1000 (AFP111 *adhE* pTrc99A-*pyc*) after growth in JSM. Note that 1 mol of succinate generation sequesters 1 mol of $CO_2$.

The studies involving the comparison of these six strains are summarized in Table 3. The control strain showed a mean $CO_2$ sequestration rate of 660 mg/Lh. All other strains showed a lower rate of $CO_2$ sequestration. The strain containing the *pyc* gene on a plasmid showed a statistically identical rate of 630 mg/Lh. However, this latter strain was the most inconsistent of all the strains studied. This observation itself suggests that the process would be improved significantly by "stabilizing" the strain, for example, by incorporating this gene onto the chromosome with sufficient expression.

The other measure of "success" is the yield of succinate generated for the amount of glucose consumed. ALS942 showed the highest succinate yield of 0.75 g/g, presumably because some of the carbon which would have been converted to ethanol was converted to succinate.

**Table 3.** Comparison of strains for $CO_2$ consumption and succinate yield. See Table 1 for genetic information on strains.

| Strain name | $CO_2$ consumption (mg/L·h) | succinate yield (g/g) |
|---|---|---|
| AFP111 | 660 | 0.59 |
| ALS942 | 200 | 0.75 |
| ALS943 | 290 | 0.49 |
| ALS874 | 630 | 0.54 |
| ALS984 | 450 | 0.67 |
| ALS1000 | 200 | 0.55 |

## 4. The Effect of pH, Temperature and Counterion

One goal was to optimize the process for $CO_2$ sequestration. This issue has multiple facets. One question is how growth pH and temperature impact sequestration of $CO_2$ of the biocatalyst. These initial studies occurred earlier in our research and were therefore conducted using "complex" medium. Moreover, these studies were conducted using ALS874. A range of pH (6.2-7.0) and temperature (34°C-40°C) were examined using a Box-Behnken response surface experimental design. Three responses were examined: yield at five hours, yield at ten hours and rate of $CO_2$ sequestration. The design required 18 total experiments, and these were comleted in a fermenter at the 1.5 liter scale. The conclusions of these experiments are 1) the temperature and pH within these ranges did not significantly affect the rate of $CO_2$ sequestration. The yield of succinate is optimal at either 37°C or 40°C, and pH did not have a significant effect in the range of 6.2-7.0.

Although these results were obtained with ALS874 and with complex medium, we used them for subsequent studies which exclusively involved AFP111 and the "best" JSM medium.

Whereas in the first series of experiments on the growth phase the temperature and pH were simultaneously considered, we next examined the effect of pH only on succinate formation and $CO_2$ consumption, during the sequestration phase. We controlled the pH at a constant level in the range of 5.8–7.0 using 25% $Ca(OH)_2$ as the base. The temperature was maintained at 37°C throughout the process. Figure 8 shows the generation of several products during the anaerobic process when the pH was maintained at 6.4. Over the course of 14-16 h, succinate was generated to 25-30 g/l, pyruvate to 7-10 g/l, ethanol to about 1 g/l and acetate to a lower concentration. For each pH studied, the volumetric succinate productivity (Qs), specific succinate productivity ($q_s$) and mass product yields of succinate, acetate, ethanol and pyruvate were calculated during 14 h of the anaerobic phase, and the values reported are the means of 2-3 experiments (Table 4). The $CO_2$ utilization rate ($Q_{CO2}$) was calculated based on the formation of 1 mole succinate using 1 mole $CO_2$ (Figure 9a). Fermentations in which the pH was controlled at 6.4 or 6.6 resulted in the highest specific succinate productivity although the succinate yield was not significantly different in the pH range of 5.8–6.8. Moreover, the volumetric succinate productivity at a pH of 6.4 or 6.6 remained high throughout the course of the anaerobic production phase (about 1.2-1.8 g/L·h), resulting in the highest mean succinate productivity (Table 4). For a pH above 6.4, the productivity declined over the course of the anaerobic phase (from about 2.0 to 0.5 g/L·h), but the yield of pyruvate decreased. Since pure (acidic) $CO_2$ was sparged into the fermenter during the anaerobic phase and three acid products were formed, base was required to maintain the pH (Figure 9b). Indeed, an unacceptably large quantity of base was needed above a pH of about 6.6. Because of these results, a pH controlled at 6.4 during the anaerobic production phase was selected for the subsequent study.

Using a pH of 6.4 and 100% $CO_2$ in the gas phase, we next examined the effect of the type of base used on succinate production. Three different bases were compared: 25% KOH, 25% NaOH and 25% $Ca(OH)_2$. When 25% $Ca(OH)_2$ was used for pH control, the volumetric succinate productivity remained high during 14 h of an anaerobic production phase (about 1.2-1.8 g/l·h), resulting in the highest mean succinate productivity and yield (Table 5). For both KOH and NaOH the productivity declined during the anaerobic phase (from about 1.7 to 0.3

g/l·h). The small reduction in succinate productivity over the course of the anaerobic phase for $Ca(OH)_2$ can be attributed to a dilution of fermenter contents (i.e., adding base to a non-growing population).

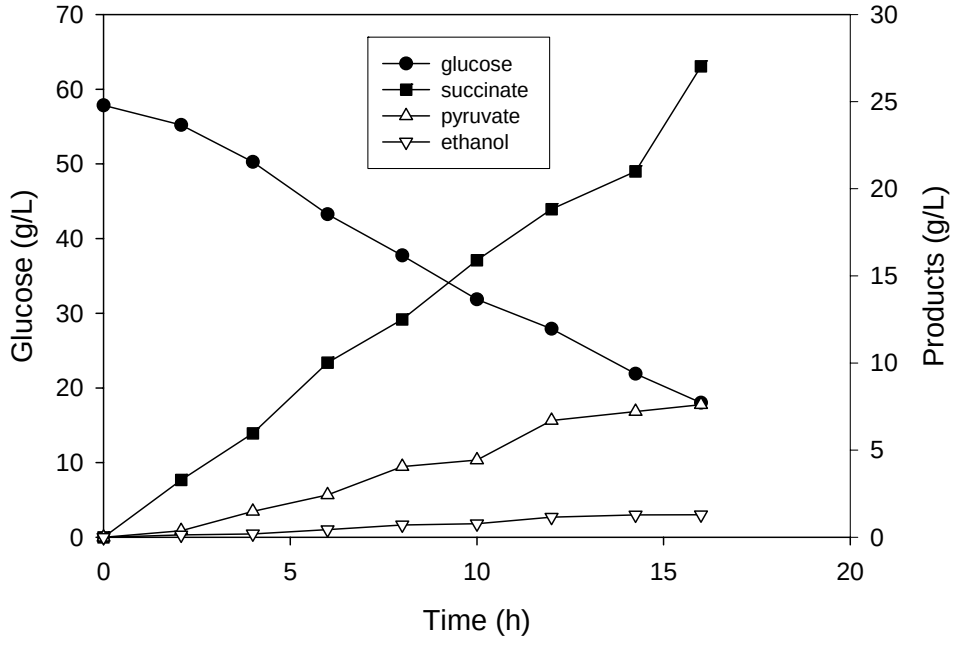


**Figure 8.** Conversion of glucose to succinate in AFP111 after growth in JSM. Note that 1 mol of succinate generation sequesters 1 mol of $CO_2$. This experiments used 25% $Ca(OH)_2$ for pH control at 6.4.

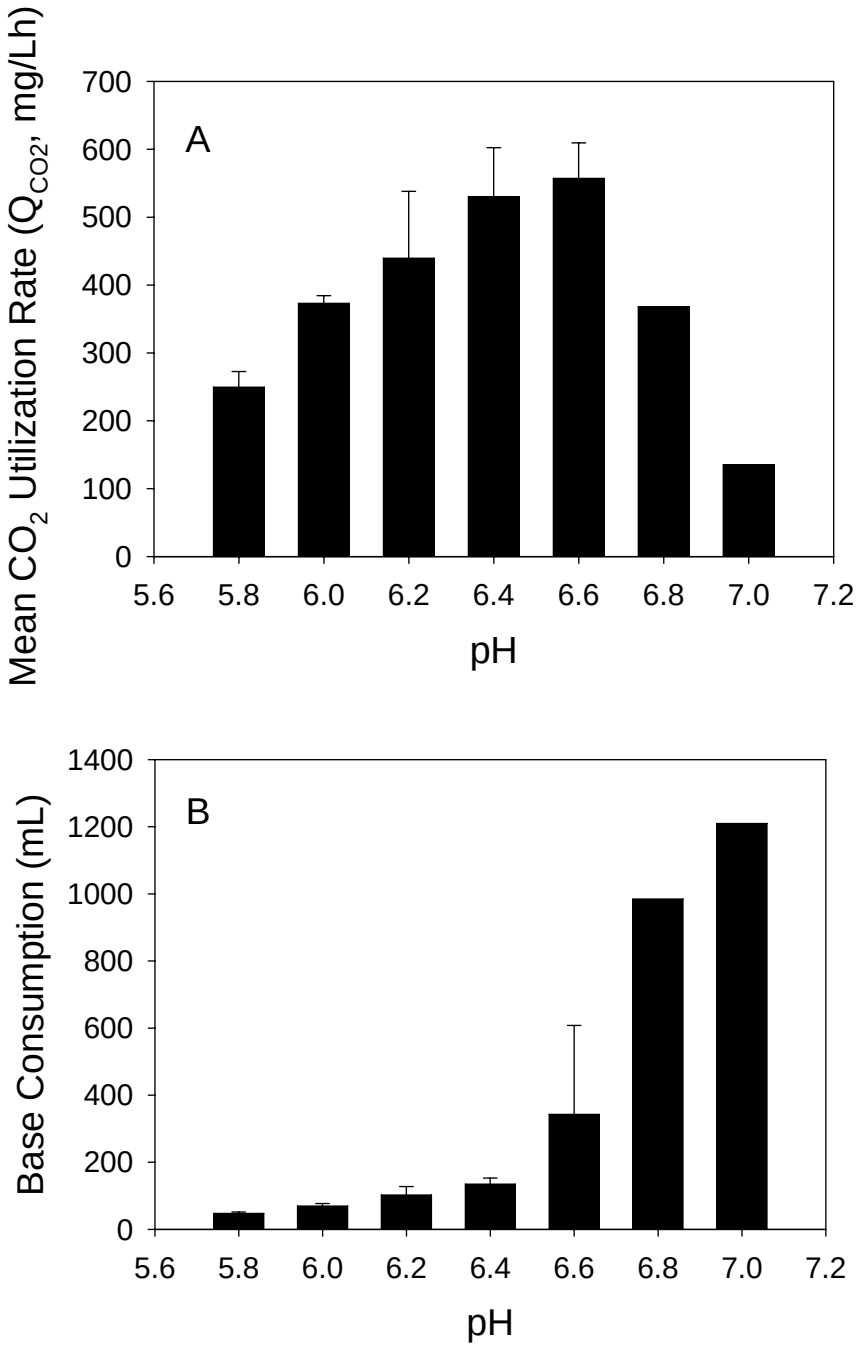


**Figure 9.** Effect of pH on succinate production by *E. coli* AFP111. A) Mean $CO_2$ utilization rate and B) volume of 25% $Ca(OH)_2$ consumption during 14 h of an anaerobic non-growth production phase.

**Table 4.** Various process parameters as a function of pH. The succinate volumetric (Q) and specific productivity (q) and mass yield of products (Y) during 14 h of an anaerobic non-growth phase when $Ca(OH)_2$ was used as the base to control pH.

| pH | $Q_S$ (g/l·h) | $q_S$ (mg/g·h) | $Y_S$ (g/g) | $Y_A$ (g/g) | $Y_P$ (g/g) | $Y_E$ (g/g) |
|---|---|---|---|---|---|---|
| 5.8 | 0.67 | 76.5 | 0.71 | 0.01 | 0.24 | 0.00 |
| 6.0 | 1.02 | 126.0 | 0.67 | 0.01 | 0.21 | 0.02 |
| 6.2 | 1.18 | 125.7 | 0.65 | 0.02 | 0.24 | 0.02 |
| 6.4 | 1.42 | 174.3 | 0.61 | 0.00 | 0.23 | 0.02 |
| 6.6 | 1.49 | 169.3 | 0.66 | 0.00 | 0.16 | 0.03 |
| 6.8 | 0.99 | 119.4 | 0.72 | 0.01 | 0.19 | 0.03 |
| 7.0 | 0.36 | 47.4 | 0.55 | 0.06 | 0.05 | 0.02 |

Subscripts: S: succinate, A: acetate, P: pyruvate, E: ethanol

**Table 5.** Various process parameters with different base counterions. The succinate volumetric productivity ($Q_S$), specific productivity ($q_S$), volumetric $CO_2$ utilization rate ($Q_{CO2}$), specific $CO_2$ utilization rate ($q_{CO2}$), and mass yield ($Y_S$) during 14 h of an anaerobic non-growth phase at a controlled pH of 6.4.

| Base (25% w/v) | $Q_S$ (g/l·h) | $q_S$ (mg/g·h) | $Q_{CO2}$ (mg/l·h) | $q_{CO2}$ (mg/g·h) | $Y_S$ (g/g) |
|---|---|---|---|---|---|
| $Ca(OH)_2$ | 1.42 (0.19)* | 174 (40) | 531 (121) | 65 (15) | 0.61 (0.13) |
| KOH | 0.88 (0.07) | 95 (6) | 328 (25) | 36 (2) | 0.54 (0.08) |
| NaOH | 0.99 (0.19) | 102 (24) | 367 (69) | 38 (9) | 0.57 (0.04) |

* Data in parentheses were standard deviation from 2-3 experiments

The enzyme phosphoenolpyruvate carboxylase (PPC) uses bicarbonate as the form of $CO_2$ (O'Leary 1982). A relationship exists between pH and forms of dissolved $CO_2$. At 37°C and a pH of 7.0 only 0.3% of dissolved $CO_2$ exists as $H_2CO_3$ and almost none as $CO_3^{2-}$ (Stumm and Morgan 1996; Frahm et al. 2002). The useful pH range for *E. coli* fermentation is approximately 6.0–7.0, and therefore the principal forms of $CO_2$ in the culture are dissolved $CO_2$ and $HCO_3^-$. As constrained by Henry's Law, the concentration of dissolved $CO_2$ is constant for the constant gas composition (100% $CO_2$) used in this study. With increasing pH the total quantity of $CO_2$ (i.e., $CO_2$ plus $HCO_3^-$) increases as a consequence of the bicarbonate equilibrium.

In this portion of our project we showed that the succinate formation rate did not increase indefinitely with increasing pH. The rate of succinate formation increased within a range at low values of pH, but decreased above a pH of 6.4. Of course, many other factors affect the rate of succinate formation, including the activities of the numerous other enzymes in the glucose to succinate metabolic pathways. Also, because the quantity of buffer required to maintain the pH

increased with increasing pH (Figure 8, the ionic strength increased significantly during fermentations in which the pH was above 6.4, a factor which may be detrimental to succinate formation. So, two mechanisms may exist which result in the observed optimum pH: a bicarbonate effect which dominates at low pH to increase succinate formation with increasing pH, and an ionic strength or other effect which reduces the succinate formation rate at higher pH. It is noteworthy that the optimal pH for succinate productivity observed in this study (6.4 – 6.6) corresponds closely with the pK of the carbonic acid/bicarbonate equilibrium (6.35). Our optimal pH for succinate production by *E. coli* AFP111 near 6.4 is consistent with other succinate-producing bacteria including *A. succiniciproducens* at a pH of 6.2 (Samuelov et al. 1991).

The results demonstrate that $Ca^{2+}$ is superior to $Na^{+}$ or $K^{+}$ as a base counterion to control the pH during the fermentation. Although the specific cause for this difference is unknown, the calcium succinate has a solubility of only 11.8 g/l at 40°C (Miczynski 1886) which is far lower than sodium succinate or potassium succinate. Thus, the use of $Ca(OH)_2$ would remove both the cation and succinate from the solution. At the end of the processes studies, the concentration of $Na^{+}$ or $K^{+}$ was about 500 mmol/l, while the concentration of dissociated $Ca^{2+}$ would have been about 180 mmol/l. High cation strength leads to high osmotic pressure, and under aerobic conditions *E. coli* can export $Na^{+}$ as a response to the high extracellular concentration of $Na^{+}$ (Sakuma et al. 1998). However, under our anaerobic conditions, $Na^{+}$ extrusion activity may decrease (Trchounian and Kobayashi 1999). A recent study on lactate generation similarly demonstrated that monovalent cations reduce acid formation by *E. coli* compared to $Ca^{2+}$ (Zhu et al. 2007). Gouesbet et al. (1993) found that about 0.24 M KCl (osmolarity of 820 mosM) had inhibitory and repressive effects on anaerobic enzymes and the corresponding genes in *E. coli* due to osmotic stress. Finally, the removal of succinate by the precipitation of calcium succinate would relieve the inhibition of PPC (Corwin and Fanning 1968) and isocitrate lyase (MacKintosh and Nimmo 1988), two key enzymes in succinate formation (Vemuri et al. 2002b).

### 5. The Effect of $CO_2$

The concentration (or partial pressure) of $CO_2$ varies widely in the man-made or natural sources that might serve as "substrate" material for this sequestration process. An important issue to address is what the effect of that $CO_2$ composition is on the rate of consumption and the yield of succinate based on glucose consumed.

The effect of $CO_2$ was studied using methodology analogous to that described above for previous studies. Like before, when the culture optical density (OD) reached about 20, the aerobic growth phase was terminated by switching the inlet gas composition to the particular $CO_2/N_2$ mixture desired. The total flowrate was reduced to 500 mL/min (dry basis, 0°C and 1 atm), the pH setpoint was reduced to 6.40, the agitation reduced to 200 rpm, and 120 mL 550 g/L glucose was added. 25% $Ca(OH)_2$ was used as base control during this second phase (reference previous article). The proportion of $CO_2$ and $N_2$ flowrates in the influent gas was controlled using mass flow controllers (Unit Instruments, Inc., Yorba Linda, CA, USA) Gases were pre-humidified in 300 mm×50 mm glass columns (Ace Glass Inc., Vineland, NJ, U.S.A.) before entering the fermenter. Fig. 10 shows the setup for the anaerobic production phase.

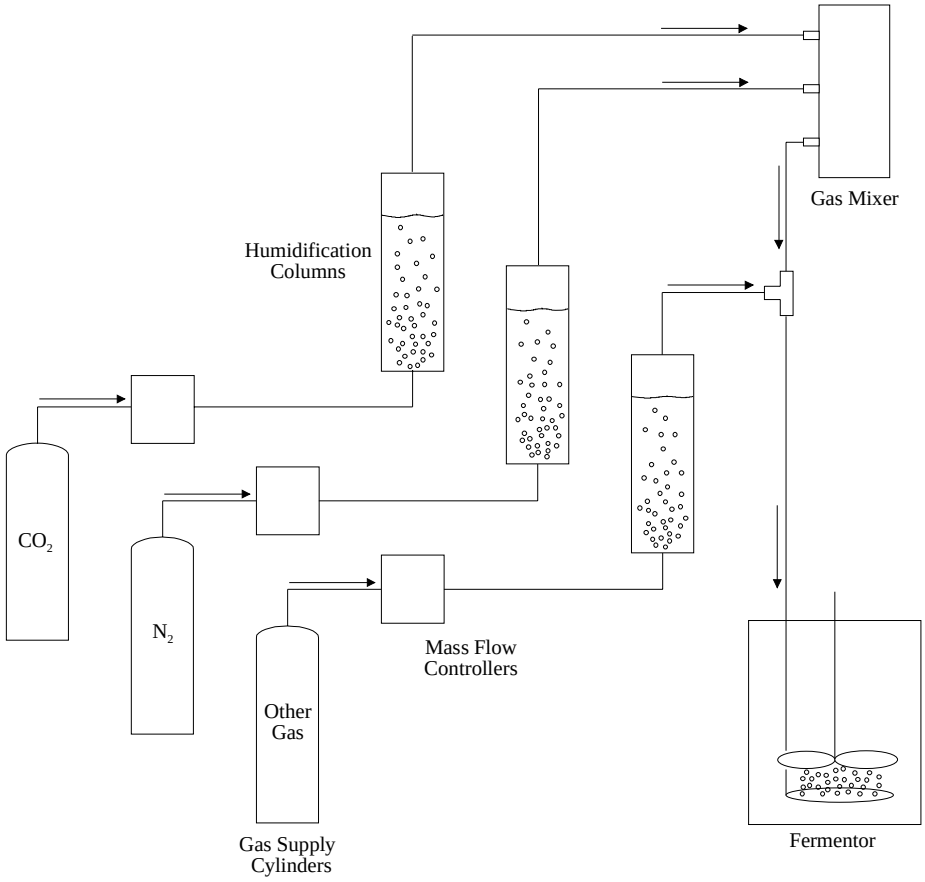


**Figure 10.** System to supply gas mixtures into fermentor in order to determine the effect of gas phase composition on succinate production and $CO_2$ sequestration.

Using a constant agitation rate (200 rpm), total gas flowrate (0.5 L/min) and temperature (37°C), the process was conducted with the gas phase $CO_2$ composition set at a value in the range of 10-100% with the balance $N_2$. A direct, proportional correlation between $CO_2$ and succinate specific productivity ($q_s$) was observed although the yield ($Y_s$) did not significant vary in this range of $CO_2$ concentrations (Fig. 11). When $CO_2$ partial pressure increased by 10 fold (from 10% to 100%), the succinate formation rate increased to 6 times. The formation of other by-products was not significantly affected by $CO_2$ composition (data not shown).

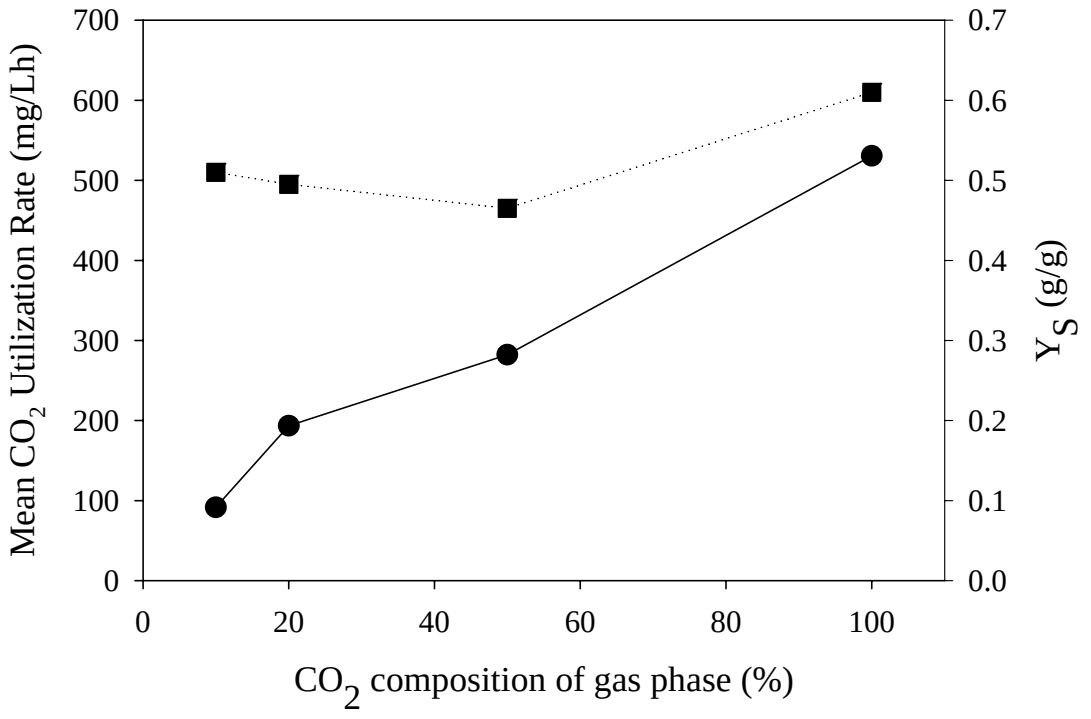


**Figure 11.** Effect of gas phase $CO_2$ composition on succinate production in AFP111: specific rate of production, $q_S$ (●) and mass yield (■).

In *E. coli* AFP111, $HCO_3^-$ is the actual substrate for PPC, the key enzyme in the conversion of glucose and $CO_2$ into succinate. Succinate can be formed through two pathways: the reductive arm of the tricarboxylic acid cycle and through the anaplerotic enzyme PPC or through the glyoxylate shunt (Vemuri et al., 2002b). We assume that succinate formation occurs mainly through the PPC pathway, which sequesters $CO_2$, and this must be the principal way for carbon to flow in order for an electron (redox) balance to be maintained by the cell. Four separate processes therefore occur in the incorporation of gas phase $CO_2$ and its enzymatic utilization (Fig. 12): 1) the transport of $CO_2$ from the gas phase to the bulk liquid phase, 2) the transport of $CO_2$ from the bulk across the cell membrane into the cytoplasm, 3) the intracellular conversion of dissolved $CO_2$ into bicarbonate, 4) the diffusion of bicarbonate and reaction catalyzed by the enzyme PPC.

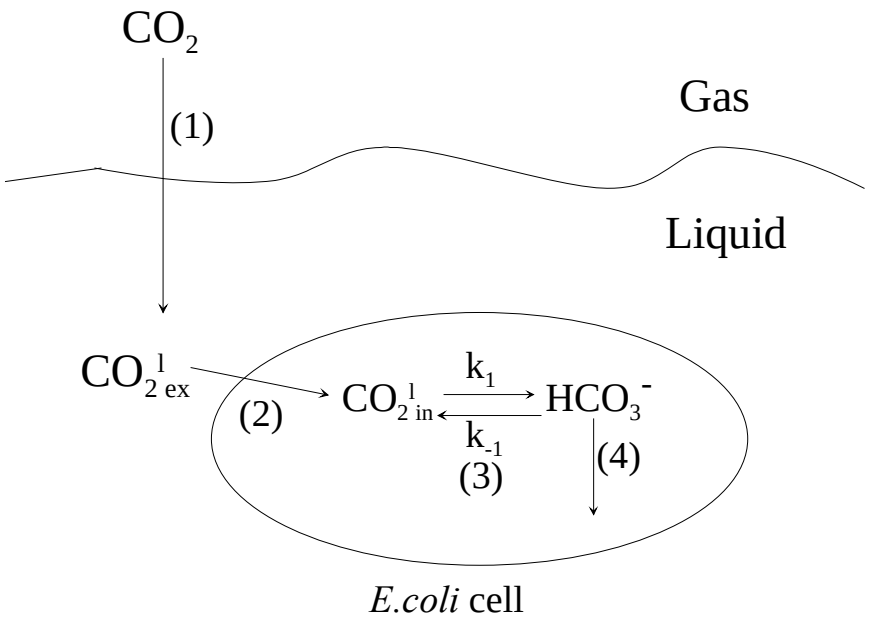


**Figure 12.** Explicit Model of $CO_2$ utilization.

One possible explanation for the observation that higher gas phase $CO_2$ concentration leads to higher succinate productivity is that the intracellular $CO_2$ ($HCO_3^-$) concentration limits the sequestration rate. We examined two possible limitations in the process. First, an identical experiment with 10% $CO_2$ was completed, except that instead of using an agitation of 200 rpm for the impeller, we used an impeller speed of 1000 rpm to increase the $CO_2$ mass transfer rate (which is process 1 in Figure 12). An agitation of 1000 rpm resulted in a mean sequestration rate of 350 mg/Lh compared to the mean rate of 91 mg/Lh observed when 200 rpm was the agitation rate, about a 4-fold increase. Despite this increase, the variability of the numeric results was high, and because the statistical significance of the difference between these two numbers is only moderate, the effect of increasing mass transfer on this process remains unclear. The succinate yield was approximate 0.53 g/g for both 200 rpm and 1000 rpm, while the generation of all other by-products was not significantly different (data not shown). Thus, the yield did not change with respect to $CO_2$ concentration, but there is evidence that the low agitation (of 200 rpm) may have limited the $CO_2$ utilization rate, particularly at low $CO_2$ concentrations.

A second possible explanation for the observation that higher $CO_2$ gas phase leads to higher succinate productivity is that the rate of conversion between $CO_2$ and bicarbonate is limiting (which is process 3 in Figure 12). We next examined the effect of overexpression of carbonic anhydrase (CA) on succinate production and $CO_2$ sequestration. This enzyme catalyzes the conversion of $CO_2$ into $HCO_3^-$, which as noted above is the actual substrate used in the carboxylation of PEP to oxaloacetate by the enzyme PPC. If the rate of oxaloacetate formation (and thus subsequently succinate formation) is limited by the availability of $HCO_3^-$, then overexpressing CA should increase the rate of succinate formation. We repeated the anaerobic process under identical conditions (with 10% $CO_2$ in the gas phase) using AFP111/pTrc99A-*yadF*. In this case, the rate of $CO_2$ sequestration for AFP111/pTrc99A-*yadF* was about 2 fold of

the rate observed using AFP111 (202 versus 91 mg/Lh). Moreover, the process with overexpressed CA did not accumulate significant pyruvate.

Each of the four steps (shown in Figure 12) involved in $CO_2$ sequestration, from dissolving $CO_2$ in the liquid phase to the enzymatic incorporation of $HCO_3^-$ into the carbon backbone, can be modeled mathematically. At steady-state, with no accumulation of $CO_2$ within any subsystems, the four rates must be equal. In this analysis the *specific* rate of $CO_2$ consumption is used. The values of this rate were 10.8 mg/gh for 10% $CO_2$ and 200 rpm, 65.0 mg/gh for 100% $CO_2$ and 200 rpm, and 43.2 mg/gh for 10% $CO_2$ and 1000 rpm. This rate was observed to be 24.3 mg/gh for the 200 rpm 10% $CO_2$ processes using carbonic anhydrase.

The specific rate of $CO_2$ transport from the gas (bubble) sparged into the fermenter to the liquid phase ($q^1_{CO_2}$) is proportional to the mass transfer coefficient:

$$q^1_{CO_2} = \frac{d[CO_2]_{ex}}{Xdt} = \frac{k_L a([CO_2^*] - [CO_2^l]_{ex})}{X} = \frac{k_L a \Delta[CO_2]}{X} \quad (1)$$

where $k_L a$ is the volumetric $CO_2$ mass transfer coefficient ($h^{-1}$), $[CO_2^*]$ is the molar $CO_2$ concentration in equilibrium with the gas phase, $[CO_2^l]_{ex}$ is the molar (extracellular) liquid phase $CO_2$ concentration, and X is dry cell density (g/L). For 10% $CO_2$ and 200 rpm, the value for $q^1_{CO_2}$ was 10.8 and for 10% $CO_2$ and 1000 rpm, the value for $q^1_{CO_2}$ was 32.2 mg/gh. For both cases the value for X was always 8-9 g/L. For 200 rpm, the value of $k_La$ was measured to be 29 $h^{-1}$, while for 1000 rpm the value of $k_La$ was measured to be 61 $h^{-1}$. From Equation 1, one can therefore calculate the value for $\Delta CO_2$ to be 3.2 mg/L and 4.5 mg/L for 200 rpm and 1000 rpm, respectively. Carroll (1991) reported values of $[CO_2^*]$ of about 0.004 mol/L when the 10% gas is in equilibrium with water. This concentration is equivalent to about 170 mg/L, and therefore the external $CO_2$ concentration $[CO_2^l]^{ex}$ is equal to about 166-167 mg/L, about the same as the concentration at saturation (i.e., 170 mg/L). Therefore, for either 200 rpm or 1000 rpm and 10% $CO_2$ in the gas phase, the transport of $CO_2$ should not be limiting $CO_2$ sequestration by this process. The calculations predict no difference in results between 200 rpm and 1000 rpm, despite our observations suggesting a difference.

The second process is the diffusion of dissolved $CO_2$ into the cells through the cell membrane (step 2 in Figure 12). Permeation of $HCO_3^-$ through lipid membrane is limited (Gutknecht et al., 1977). For rod-shaped cells, the $CO_2$ diffusion rate ($q^2_{CO_2}$) is given by (Berg, 1983a):

$$q^2_{CO_2} = \frac{4\pi D a [CO_2^l]_{ex}}{m \ln(2a/b)} \quad (2)$$

and *E. coli* has a width (b) of about $10^{-4}$ cm and a length (a) of $2 \times 10^{-4}$ cm (Berg, 1983b). D is $CO_2$ diffusion coefficient, which has a value of $1.78 \times 10^{-9}$ $m^2/s$ (Mark et al., 1978). The parameter *m* is the average dry weight of an *E. coli* cell, and it has a value of $3 \times 10^{-13}$ g. This equation can be used to estimate the (maximum) possible diffusion rate for the cells, taking the

value of $[CO_2^l]_{ex}$ to be that obtained in considering step 1 above (e.g., 166 mg/L). Using all these parameters, the maximum diffusion rate for $CO_2$ is 640 mg/gh. This value is about 10 times greater than the 65.0 mg/gh observed for 100% $CO_2$ and 200 rpm. Therefore, the sequestration rate does not appear to be limited by diffusion across the cell membrane, and we would expect the internal concentration of $CO_2$ to be about 90% of the external value.

The third process is the intracellular hydration of $CO_2$ to $HCO_3^-$, which occurs both by chemical hydration and by enzymatic hydration catalyzed by CA (step 3 in Figure 12). The first chemical mechanism is described by the following equilibria:

$$CO_2 + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} H_2CO_3 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} HCO_3^- + H^+$$

In the first equilibrium, $k_1 = 0.1\ s^{-1}$ at 38°C (Mills and Urey, 1940). In the second equilibrium, the reaction is considered to occur instantaneously (Gutknecht et al., 1977). Like all enzymatic reactions, the second biochemical mechanism can be represented by the Michaelis-Menten Equation: $\frac{V_{CA}[CO_2^l]_{in}}{X(K_M+[CO_2^l]_{in})}$ where $V_{CA}$ is the maximum rate (largely determined by the amount of enzyme present), and $K_M$ is the Michaelis constant for the reaction of $CO_2$ by CA. Accordingly, the $CO_2$ conversion rate is the sum of the rates of the two reactions:

$$q^3_{CO_2} \approx \frac{k_1[CO_2^l]_{in}}{X} + \frac{V_{CA}[CO_2^l]_{in}}{X(K_M+[CO_2^l]_{in})} \qquad (3)$$

The carbonic anhydrase activity measurement is very difficult to quantify, and first the chemical reaction only will be considered. Since diffusion across the membrane does not limit the overall process and the value for the maximum diffusion rate is 10-20 times greater than the sequestration rate observed, the value for $[CO_2^l]_{in}$ was taken to be no less than 90% of the value for $[CO_2^l]_{ex}$. Therefore, we take $[CO_2^l]_{in}$ to be 150 mg/L (and X to be 8.5 g/L, and $k_1$ to be 0.1 $s^{-1}$). The reaction rate (of the chemical reaction only) is therefore calculated to be 6400 mg/gh, about 100 times greater than the sequestration rate we observed. Therefore, we conclude that the rate of $CO_2$ sequestration is not limited by carbonic anhydrase or by the conversion of $CO_2$ into $HCO_3^-$. We would expect the overproduction of CA not to have any tangible benefit to $CO_2$ sequestration. Moreover, we would expect that CO2 is in equilibrium with bicarbonate. Since the equilibrium constant (for the chemical reactions above) is $4.47 \times 10^{-7}$ mol/L, we calculate that for a pH of 7.0 the $HCO_3^-$ concentration in equilibrium with 150 mg/L (3.4 mmol/L) $CO_2$ is 15.2 mmol/L. At a pH of 6.4 this concentration is 3.8 mmol/L.

The fourth process is the utilization of $HCO_3^-$ by the enzyme PPC. If the majority of succinate is generated in the cell via the reductive arm of the tricarboxylic acid cycle, then the rate of $HCO_3^-$ utilization by PPC ($q^4_{CO2}$) is equal to the rate of succinate production measured during the anaerobic phase. Does the concentration of bicarbonate limit succinate formation and therefore

does it limit the rate of $CO_2$ sequestration? In equilibrium with 150 mg/L $[CO_2^l]_{in}$ the concentration of bicarbonate is 3.8 mmol/L at a pH of 6.4. This value is the "worst-case" concentration since actually the intercellular environment maintains an intracellular pH near neutrality. The $K_M$ (with respect to $HCO_3^-$) for PPC is about 0.1 mmol/L (Kai et al., 1999). The $HCO_3^-$ concentration is about 40 times greater than the value of $K_M$, and therefore the enzyme should be operating near its maximum reaction rate even when the concentration of $CO_2$ in the gas phase is only 10%. Therefore, we conclude that the activity of the enzyme PPC does not limit the reaction.

What does limit $CO_2$ sequestration? Our analysis did not, and could not, include other potential sinks of $CO_2$ within the system. The results suggest that a significant exchange of $CO_2$ is not accounted for, and this exchange may be a strong function of $CO_2$ concentration. Also, although the activity of PPC does not limit the reaction, this presumes that the quantity of this and other enzymes present in the cell are not affected by the gas phase concentration of $CO_2$. It is possible, for example, that the intracellular concentration of pyruvate is a function of the $CO_2$ concentration. If this were the case, the other substrate (pyruvate) could be the limiting factor in the sequestration of $CO_2$ at lower $CO_2$ concentrations.

**6. The Effect of Gas Impurities**

As noted elsewhere some sources of $CO_2$ such as flue gas have trace quantities of other gaseous substances, including $NO_2$, $SO_2$, CO and $O_2$. The effect of these flue gas components was examined by controlling the proportion of $CO_2$, particular gas (e.g. 600 ppm $NO_2$ balanced in $N_2$ gas) and $N_2$ flowrates in the influent gas using mass flow controllers as shown in Figure 10. Gases except $NO_2$ and $SO_2$ were pre-humidified in 300 mm×50 mm glass columns (Ace Glass Inc., Vineland, NJ, U.S.A.) before entering the fermentor.

We first examined the effect of $NO_2$ on succinate formation by maintaining 50% $CO_2$ in the gas phase and by comparing three $NO_2$ levels in the range of 0-200 ppm. 50% $CO_2$ was selected because a higher $CO_2$ concentration may cover the potential influence of small amount of flue gas components. For the fermentations with 50% $CO_2$, succinate reached about 25 g/L with a yield of 0.5 g/g within 36 h anaerobic production phase, and acetate and pyruvate were major by-products. Figure 13 shows the substrate consumption and products formation versus time in a typical batch fermentation. When 50 ppm or 200 ppm $NO_2$ was in the gas stream, it appears that $NO_2$ had no deleterious effect on succinate production; specific succinate productivity ($q_s$) and mass product yields (Ys) did not decrease (Fig. 14), and no significant difference was observed in all other by products (data not shown). Indeed, the trend observed was that $NO_2$ *improved* $CO_2$ sequestration.

$NO_2$ is very reactive and thought to be toxic to microorganism. It has been reported that $NO_2$ caused chromosome aberrations in cultured Chinese hamster (Tsuda et al., 1981) and mutations in *Salmonella typhimurium* (Victorin and Stahlberg, 1988). One group (Kosaka et al., 1986) bubbled a $NO_2$ gas into 5 mL fractions for 30 min at a flowrate of 0.1 L/min and found wild type *E. coli* was resistant to 90 ppm $NO_2$. This effect was because $NO_2$ induced the expression of *umuC* gene, which induces SOS functions including increased DNA repair capacity. When the

$NO_2$ concentration was increased to 180 ppm, the lag phase of the induction of *umuC* gene expression was delayed. Similar repair mechanism may have functioned in our succinate-production system which prevents cell death. The major impact of $NO_2$ during the production phase was to make fermentation environment more acidic rather than influence products formation, and this is supported by the fact that the addition of 200 ppm $NO_2$ resulted in more base consumption (148 mL vs. 103 mL for control).

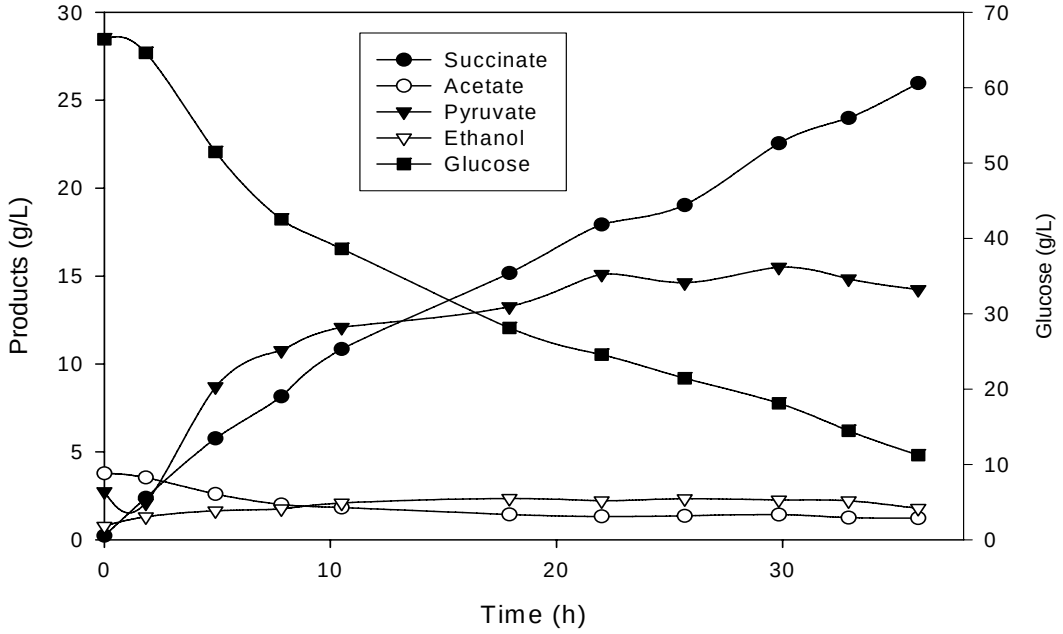


**Figure 13.** Conversion of glucose to succinate in AFP111 using 50% $CO_2$ containing 200 ppm $NO_2$. This experiments used 25% $Ca(OH)_2$ for pH control at 6.4.

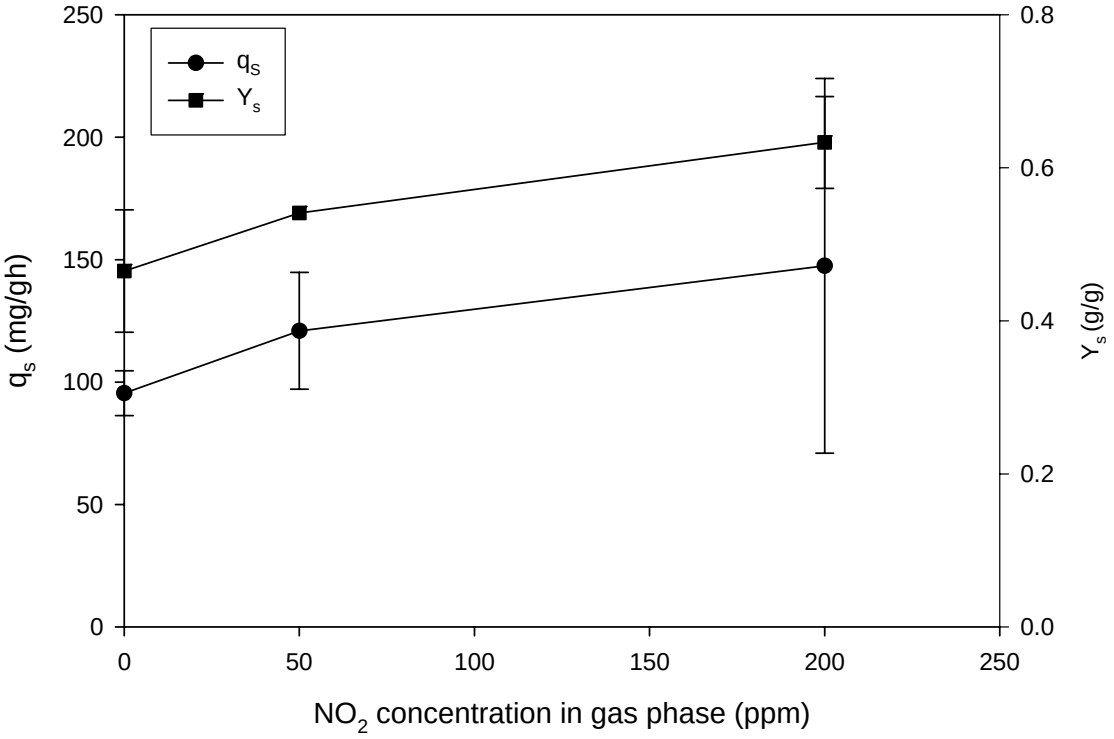


**Figure 14**. Effect of $NO_2$ on succinate production in AFP111: specific rate of production, $q_S$ (●) and mass yield (■).

A similar set of experiments were completed to study the effects of $SO_2$ in the range of 0-300 ppm, which is a typical range in the industry. Similarly, $SO_2$ did not have a negative effect on succinate formation and $CO_2$ sequestration (Fig. 15). Also, a small quantity of $SO_2$ (50 ppm) seemed to show a slight positive effect, but statistically no significant difference was observed between any concentration.

$SO_2$ (bisulfite) has been used as a preservative in foods and beverages because it can inhibits the growth of a variety of microorganisms. *E. coli* stops RNA synthesis and the synthesis of total protein almost immediately upon addition of 2 mM bisulfite (or higher concentrations). These functions resume after a lag whose duration is proportional to the concentration of bisulfite added (Robakis et al., 1983a; Robakis et al., 1983b). Another evidence, however, suggests bisulfite itself is not mutagenic to *E. coli* but acts as a comutagen with UV irradiation (Mallon and Rossman, 1981). In our system cells did not grow during the second phase, and therefore synthesis of macromolecules was not necessary. $SO_2$ may have had no deleterious effect on succinate formation since $SO_2$ only inhibits cell growth. Also, the concentration of $SO_2$ (bisulfite) in our system was comparatively low: 300 ppm $SO_2$ is about 0.78 mg/L, and using a gas flowrate of 0.5 L/min the $SO_2$ concentration would not reach 2 mM until 7 h of anaerobic fermentation.

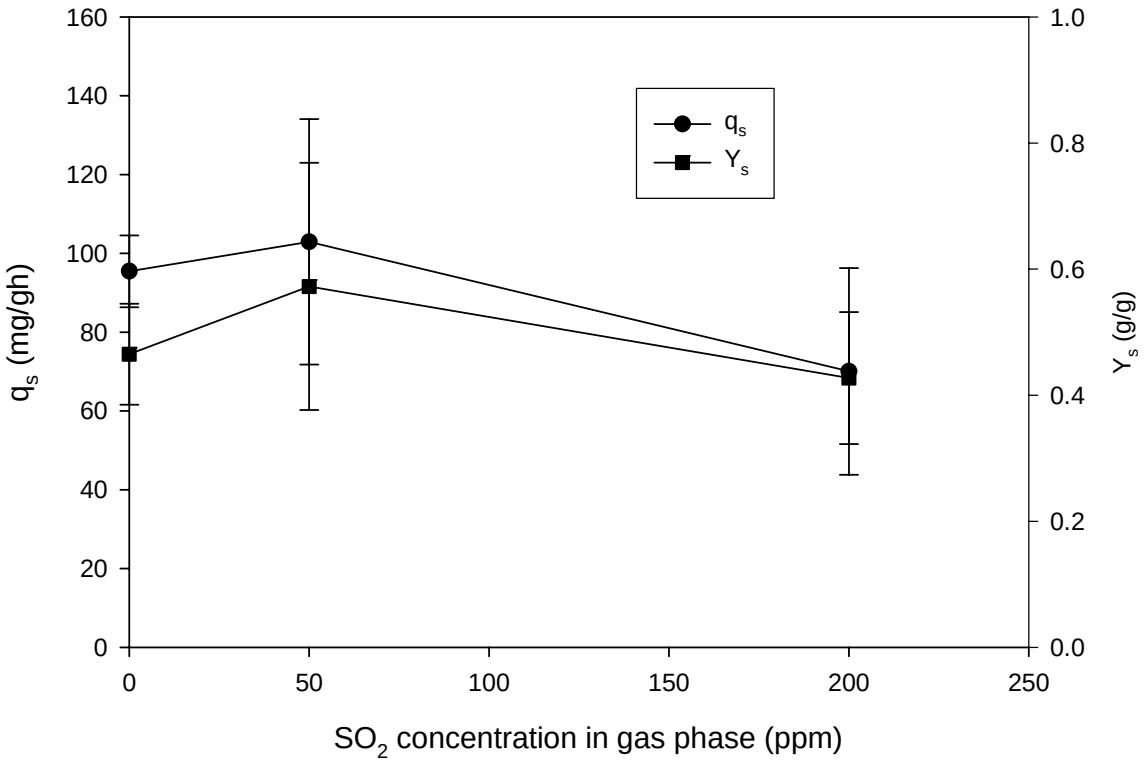


**Figure 15**. Effect of $SO_2$ on succinate production in AFP111: specific rate of production, $q_S$ (●) and mass yield (■).

Some $O_2$ invariably exists in $CO_2$ sources. Since the formation of succinate and concomitant sequestration of $CO_2$ is intrinsically an anaerobic process, the presence of $O_2$ would ultimately be detrimental to the process. But how much O2 is necessary to affect the process? An experiment was completed with 1% $O_2$ in the $CO_2$ stream. Succinate formation and other products were not significantly affected by this concentration of $O_2$ (Fig. 16). Additional experiments using higher concentrations of $O_2$ will determine what level of this gas is harmful to the $CO_2$ sequestration process.

For facultative anaerobic *E. coli* the availability of $O_2$ has many consequences for the function and synthesis of catabolic and anabolic pathways. Becker et al. (1996; 1997) found that at $O_2$ tension value ($pO_2$) greater than10 mbar, aerobic respiration appears to be most efficient, and below 10 mbar it is replaced by anaerobic respiration. The synthesis of fermentation products of *E. coli* is known to depend on the $pO_2$ in the medium. The 1% $O_2$ used in the gas composition is just about the switching concentration (10 mbar), so its influence on cells needs further study. In addition, succinate formation requires reducing equivalents. Like other oxidants such as $NO_2$ and $SO_2$, addition of $O_2$ could change the reductive environment.

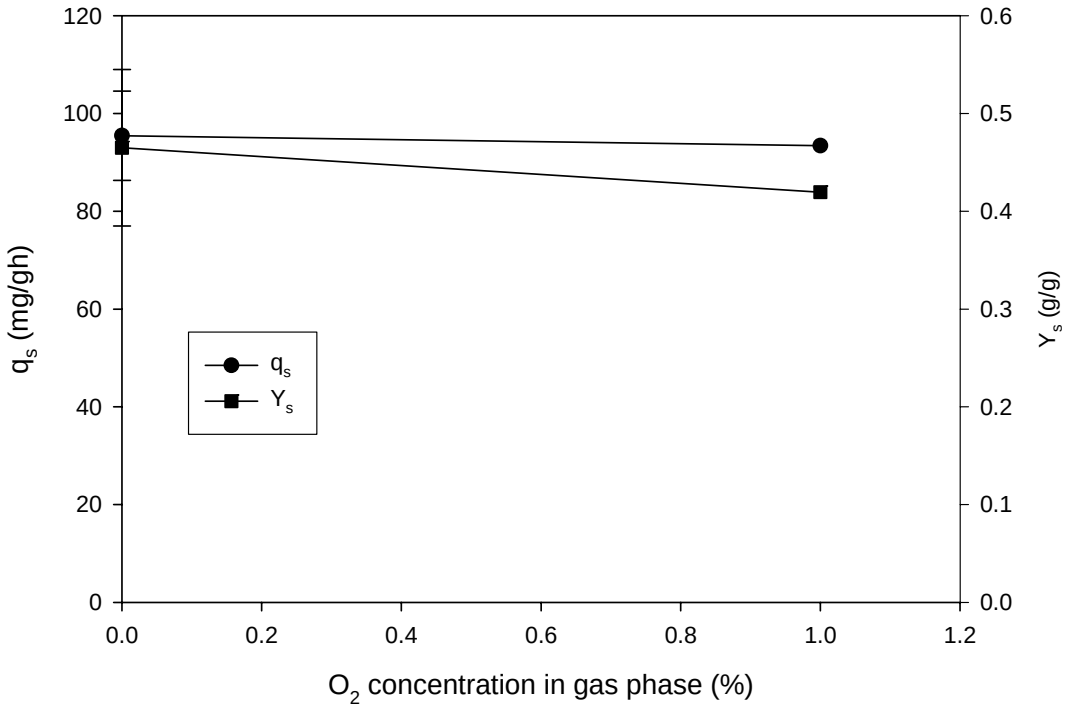


**Figure 16**. Effect of $O_2$ on succinate production in AFP111: specific rate of production, $q_S$ (●) and mass yield (■).

There may be a trace amount of CO in a source of $CO_2$ used for this process. The properties of CO are different from the other three gases studied because it is a reductive gas. In an experiment with 100 ppm CO, the specific succinate productivity and yield improved (Fig. 17) as a result of CO, pyruvate accumulation slight reduced, and the formation of other by-products had no significant change (data not shown). Additional experiments using higher concentrations of CO will clarify the extent of this improvement and whether this improvement in $CO_2$ sequestration is statistically significant.

Succinate is a highly reduced fermentation product using four electrons per molecule formed (Clark, 1989). Van der Werf et al. (1997) reported that $H_2$, acting as an electron donor, increased succinate formation due to the increased availability of reducing equivalents. CO here may assume a similar role: encouraging more electron flow to reduce fumurate to succinate. If this effect is indeed the case, greater $CO_2$ sequestration and succinate accumulation by AFP111 might also occur when another electron donor is provided such as NO, formate or $H_2$. Also, CO may be converted into $CO_2$ after it donates electrons, which provides another source of substrate for PPC.

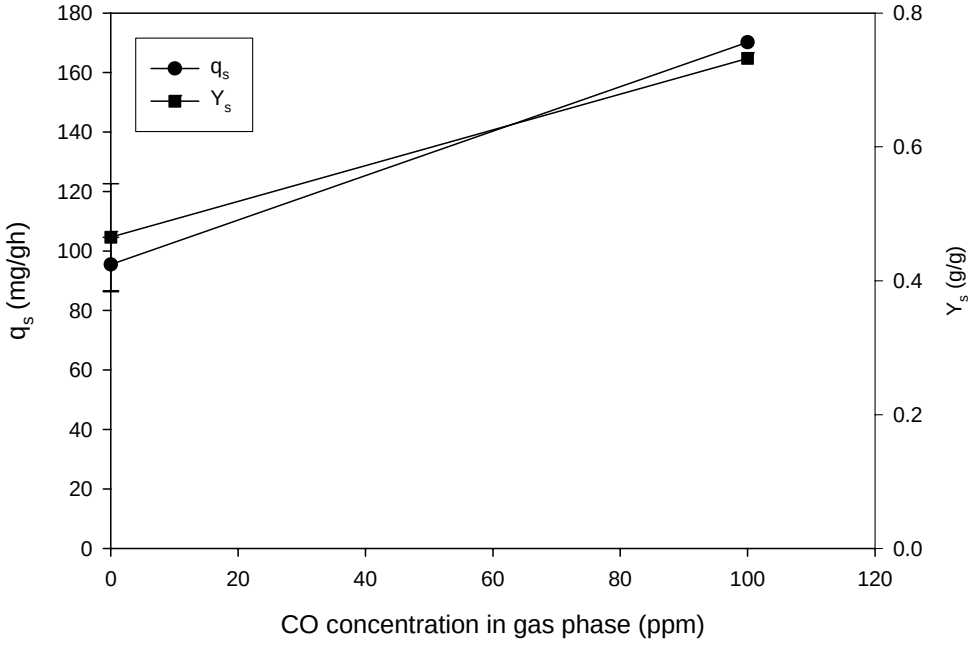


**Figure 17**. Effect of CO on succinate production in AFP111: specific rate of production, $q_S$ (●) and mass yield (■).

## 7. Prolonging $CO_2$ Sequestration

Although we have demonstrated that $CO_2$ can be sequestered by bacteria at a rate exceeding 600 mg/Lh while generating the product succinic acid, the process is limited in that the rate will diminish significantly after 24 hours. Under such a circumstance, the microbial cells would have to be re-grown, a process which consumes additional nutrients and itself diminishes the overall rate of sequestration. We therefore have been interested in understanding what physiological changes occur over the course of the process which are correlated to the reduction in rate. It is believed that an understanding of the underlying causes of this reduction can lead to an improved process.

As a first examination of the underlying causes for the diminishing rate, we conducted fermentations and measured activities of two key enzymes, isocitrate lyase (ICL) and glyceraldehyde 3-phosphate dehydrogenase (G3PDH). ICL converts isocitrate into glyoxylate and succinate (see Figure 1) while glyceraldehyde 3-phosphate dehydrogenase converts glyceraldehydes 3 phosphate into 3-phosphoglycerate. We observed a market decrease in the enzyme activities of these two enzymes soon after the sequestration non-growth phase commenced. This activity loss correlated with a decrease in sequestration rate from over 800 mg/Lh to 400 mg/Lh. Moreover, the concentration of Na+ increased during the course of the anaerobic process to about 1.7 M. This high concentration results from the us of NaOH to neutralize the succinic acid generated during the process, and as noted previously, results in a lower sequestration rate than $Ca(OH)_2$.

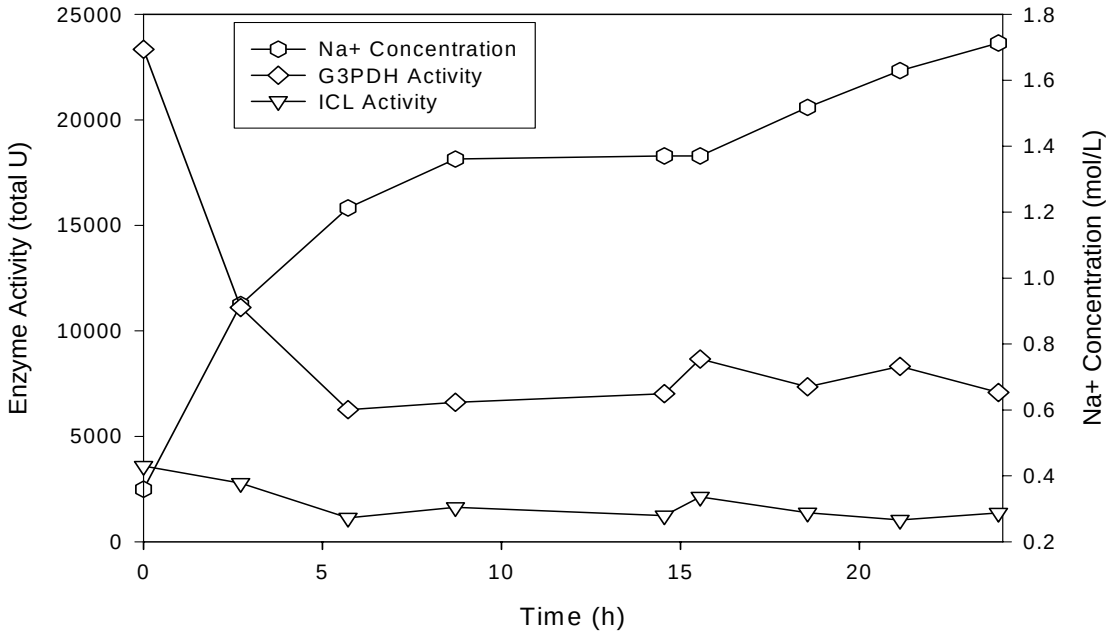


**Figure 18**. Enzyme activity and Na+ ion concentration during the course of $CO_2$ sequestration. ICL = isocitrate lyase and G3PDH = glyceraldehyde 3-phosphate dehydrogenase.

These results do not provide any specific guidance as to how to prolong the rate of sequestration, but they do provide some clues about the cause of the rate loss. They suggest the rate loss is due to a gradual deterioration of biocatalyst activity. It may be possible to prolong the rate by providing an environment more conducive to biocatalysis activity, such as for example by increasing the oxidizing environment or removing the product succinate as it is being generated.

## Conclusions

We have been able to demonstrate $CO_2$ sequestration at a rate as high as 800 mg/Lh, and consistently over 600 mg/Lh, which exceeds the rate of $CO_2$ sequestration previously attained (in a photosynthetic system) by a factor of 40-50.

We have demonstrated that $CO_2$ sequestration is not mass transfer limited. Converting the 800 mg/Lh sequestration rate to a rate on a per bacterial cell basis yields about $10^{-19}$ mol/s. The maximum rate of mass transfer of $CO_2$ (per cell) when pure $CO_2$ is used for the gas phase is about $10^{-12}$ mol/s. Thus, the rate of mass transfer is potentially 10 million times greater than the rate of sequestration. This conclusion is supported by evidence that $CO_2$ sequestration rate is unaffected by agitation or other mechanical means to increase mass transfer. Mass transfer should only begin to become an issue when the concentration of $CO_2$ is about 1000 times less concentrated than pure $CO_2$, or when the $CO_2$ concentration in the gas phase is about 0.1% or less. There would appear to be no difficulty in supplying $CO_2$ to cells under conditions of high cell density, or fairly low $CO_2$ concentration.

However, over the range of 10%-100% $CO_2$ gas concentration, the sequestration rate is linear. In other words, the rate of sequestration does depend on the concentration of $CO_2$ supplied. Since mass transfer does not appear to limit the rate of the overall process, the sequestration must be limited by either a cellular transport process (e.g., the diffusion of $CO_2$ across the cell membrane) or by a reaction (e.g., the conversion of $CO_2$ into bicarbonate). We therefore have explored the latter possibility by overexpressing carbonic anhydrase in our strains, and enzyme which converts $CO_2$ into bicarbonate used by the cell. Although we have to confirm the presence of this enzyme, initial experiments indicate that the presence of carbonic anhydrase does not increase the rate of sequestration. So, what limits sequestration remains a mystery. The results indicating $CO_2$ sequestration rates are enhanced proportionately by elevating the gas composition suggest that pressurizing the $CO_2$ could benefit the sequestration rate. We can not account for the increased rate of sequestration resulting from increased $CO_2$ (partial) pressure, and we do not have facilities capable of pressurizing bioreactors to determine if this trend continues.

The presence of trace amounts of impurities in the gas stream generally promotes $CO_2$ sequestration and succinate generation. The gas impurities which do not inhibit and may promote sequestration include $NO_2$, $SO_2$, CO and $O_2$. This result warrants further study, as it suggests that some mechanism caused by these gases provides a physiological advantage to the cell. Some results suggest a redox effect. If it were better understood, the effect might be used to enhance sequestration, or prolong it.

We obtained some information what does not prolong $CO_2$ fixation. The most important factor which diminishes $CO_2$ fixation appears to be the presence of high ionic strength. We therefore want to ensure that a process will not have high salt concentrations. The best means to accomplish this would be to remove the product, succinic acid, as it is being generating by the microorganisms. This approach has not been studied in this project. One factor, other than merely excluding high salt concentrations, which may prolong $CO_2$ fixation is the intermittent supply of oxygen. As evidenced by the sharp decrease in enzyme activity which occurs during sequestration, the biocatalytic ability to fix $CO_2$ appears to require recharging every several hours, and the best way to accomplish this may be to supply brief periods of oxygen. This approach is also supported by evidence that low concentrations of oxidizing gases such as $NO_2$ may enhance sequestration.

## REFERENCES


Agarwal L, Isar J, Meghwanshi GK, Saxena RK (2006) A cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by *Escherichia coli*. J Appl Microbiol 100:1348-1354

Atom H. (2002) Microbial Enzymes Involved in Carbon Dioxide Fixation. *J Biosci. Bioeng.* 94(6):497-505

Berg, H.C. 1983a. Diffusion: Macroscopic theory, p. 29. Random walks in biology. Princeton University Press, Princeton, New Jersey.

Berg, H.C. 1983b. Movement of self-propelled objects, p. 81. Random walks in biology. Princeton University Press, Princeton, New Jersey.

Blandino, A., I. Caro, D. Cantero (1997) "Comparative Study of Alcohol Dehydrogenase Activity in Flor Yeast Extracts, Biotechnology Letters, 19(7), 651-654.

Brown L.M., Uptake of Carbon Dioxide from Flue Gas by Microalgae (1996) *Energy Convers. Mgmt*. 37(6-8): 1363-1367.

Bredwall M.D., P. Srivastava, R.M. Worden (1999) Reactor Design Issues for Synthesis-Gas Fermentations. *Biotechnol. Prog*. 15:834-844.

Carroll, J., J. Slupsky, and A. Mather. 1991. The solubility of carbon dioxide in water at low pressure. J. Phys. Chem. Ref. Data. 20:1201-1209.

Causey, T. B., S. Zhou, K. T. Shanmugam, and L. O. Ingram. (2003). Engineering the metabolism of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products. Homoacetate production. *Proc. Natl. Acad. Sci.* 100(3):825–832.

Chatterjee, R. Millard, C. S., Champoin, K., Clark, D. P., Donnelly, M. I. (2001) Mutation of the *ptsG* gene results in increased production of succinate in fermentation of glucose by *Escherichia coli*, Appl Environ Microbiol 67:148-151.

Corwin LM, Fanning GR (1968) Studies of parameters affecting the allosteric nature of phosphoenolpyruvate carboxylase of *Escherichia coli*. J Biol Chem 243:3517-3525

Creutz, C.; Fujita, E. (2000) Carbon dioxide as feedstock**.** Brookhaven National Laboratory, Report, BNL (BNL-68111), 1-11.

Datsenko, K. A. and Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci. USA **97**:6640-6645.

de Boer, H. A., Gilbert, S. F. and Nomura, M. 1979. DNA sequences of promoter regions for rRNA operons rrnE and rrnA in *E. coli*. Cell. **17**:201-209.

Donnelly MI, Millard CS, Clark DP, Chen MJ, Rathke JW (1998a) A novel fermentation pathway in an *Escherichia coli* mutant producing succinic acid, acetic acid, and ethanol. Appl Biochem Biotechnol 70-72:187-198

Eiteman MA, Chastain MJ (1997) Optimization of the ion-exchange analysis of organic acids from fermentation. Anal Chim Acta 338:69-75

Frahm B, Blank H-C, Cornand P, Oelssner W, Guth U, Lane P, Munack A, Johannsen K, Portner R (2002) Determination of dissolved $CO_2$ concentration and $CO_2$ production rate of mammalian cell suspension culture based on off-gas measurement. J Biotechnol 99:133-148

Gokarn, RR, Eiteman MA, Altman E (2000) Metabolic analysis of *Escherichia coli* in the presence and absence of carboxylating enzymes phosphoenolpyruvate carboxylase and pyruvate carboxylase. Appl Environ Microbiol 66:1844-1850.

Gokarn, R. R., M. A. Eiteman, E. Altman (2002) Metabolically engineered organism for enhanced production of oxaloacetate-derived biochemicals, U. S. Patent 6,455,284.

Gokarn, R. R., Evans, J. D., Walker, J. R., Martin, S. A., Eiteman, M. A., and Altman, E. 2001. The physiological effects and metabolic alterations caused by the expression of *Rhizobium etli* pyruvate carboxylase in *Escherichia coli*. Applied Microbiology and Biotechnology. **56**:188-195

Gouesbet G, Abaibou H, Wu LF, Mandrand-Berthelot MA, Blanco C (1993) Osmotic repression of anaerobic metabolic systems in *Escherichia coli*. J Bacteriol 175:214–221

Guettler MV, Jain MK, Soni BK (1998) Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US patent 5,723,322, 3 March 1998

Guettler MV, Rumler D, Jain MK (1999) *Actinobacillus succinogenes sp.* nov., a novel succinic-acid-producing strain from the bovine rumen. Int J Syst Bacteriol 49:207-216

Horn, U., W. Strittmatter, A. Krebber, U. Knupfer, M. Kujau, R. Wenderoth, K. Muller, S. Matzku, A. Pluckthun, and D. Riesenberg. 1996. High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. *Appl. Microbiol. Biotechnol.* 46:524–532.

Isar J, Agarwal L, Saran S, Saxena RK (2006) Succinic acid production from *Bacteroides fragilis*: Process optimization and scale up in a bioreactor. Anaerobe 12:231-237

Kai, Y., H. Matsumura, T. Inoue, K. Terada, Y. Nagara, T. Yoshinoaga, A. Kihara, K. Tsumura, K. Izui. (1999) Three-dimensional structure of phosphoenolpyruvate carboxylase: A proposed mechanism for allosteric inhibition. *Proc. Natl. Acad. Sci. USA* 96(3):823-828.

Kodama, T. 1996. Microbial $CO_2$ fixation and its application. *Microbial Utilization of Renewable Resources*, 9: 39-45.

Kosaka H., Yamamoto K., Oda Y., Uozumi M. 1986. Induction of SOS functions by nitrogen dioxide in *Escherichia coli* with different NDA-repair capacities. Mutat Res.162:1-5.

Lamare, S; Legoy, M. (1993) Biocatalysis in the gas phase. *Trends in Biotechnology* 11(10), 413-18.

Lee PC, Lee WG, Kwon S, Lee SY, Chang HN (2000) Batch and continuous cultivation of *Anaerobiospirillum succiniciproducens* for the production of succinic acid from whey. Appl Microbiol Biotechnol 54:23-27

Lee PC, Lee WG, Lee SY, Chang HN (2001) Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. Biotechnol Bioeng 72:41-48

Lee SJ, Song H, Lee SY (2006) Genome-based metabolic engineering of Mannheimia succiniciproducens for succinic acid production. Appl Environ Microbiol 72:1939-1948

Lin H, Bennett GN, San K-Y (2005a) Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate. Biotechnol Bioeng 89:148-156

Lin H, Bennett GN, San K-Y (2005b) Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. Metab Eng 7:116-127

MacKintosh C, Nimmo HG (1988) Purification and regulatory properties of isocitrate lyase from *Escherichia coli* ML308. Biochem J 250:25-31

Mallon R., Rossman T. 1981. Bisulfite (sulfur dioxide) is a comutagen in *E. coli* and in Chinese hamster cells. Mutat Res. 88:125-133.

Mark, H.F., D.F. Othmer, C.G. Overberger, G.T. Seaborg. 1978. Kirk-Othmer: Encyclopedia of Chemical Technology., 3rd edition. Volume 1. Wiley-Interscience New York.

March, J. C., Eiteman, M. A., and Altman, E. 2002. Expression of anaplerotic enzyme pyruvate carboxylase improves recombinant protein expression in *Escherichia coli*. Applied and Environmental Microbiology. **68**:5620-5624.

Matsuda, Tomoko; Watanabe, Kazunori; Kamitanaka, Takashi; Harada, Tadao; Nakamura, Kaoru. (2003) Biocatalytic reduction of ketones by a semi-continuous flow process using supercritical carbon dioxide. *Chemical Communications*. 10:1198-1199.

Miczynski ZN (1886) Uber die Bestimmung der Loslichkeit einiger Sauren und Salze der Oxalsaurereihe in Wasser bei verschiedenen Temperaturen. Monatshefte für Chemie 7:255-272

Movva N. R., Nakamura, K., and Inouye, M. 1980. Regulatory region of the gene for the ompA protein, a major outer membrane protein of *Escherichia coli.* Proc Natl Acad Sci U S A. **77**:3845-3849.

Munson M, Predki PF, Regan L. 1994. ColE1-compatible vectors for high-level expression of cloned DNAs from the T7 promoter. Gene.**144**:59-62.

O'Leary MH (1982) Phosphoenolpyruvate carboxylase: an enzymologist's view. Annu Rev Plant Physiol 33:297– 315

Otsuki, T. (2001) A study for the biological $CO_2$ fixation and utilization system. *The Science of the Total Environment*. 277:21-25.

Pakoskey, A. M., E. C. Lesher, D. B. M. Scott (1965) Hexokinase of *Escherichia coli*. Assay of enzyme activity and adaptation to growth in various media. *J. Gen. Microbiol.* 38:73-80.

Payne, J., Morris, J.G., (1969) Pyruvate carboxylase in *Rhodopseudomonas spheroides*, J. Gen. Microbiol., 59, 97-101.

Robakis N., Rossman T., Shapiro R., Szer W. 1983a. The effects of bisulfite on growth and macromolecular synthesis in *Escherichia coli*. Chem Biol Interact 43:289-298.

Robakis N., Shapiro R., Szer W. 1983b. Reversal of growth inhibition in *Escherichia coli* by bisulfite. Chem Biol Interact. 43:299-311.

Sakuma T, Yamada N, Saito H, Kakegawa T, Kobayashi H (1998) pH dependence of the sodium ion extrusion systems in *Escherichia coli*. Biochim Biophys Acta 1363:231–237

Samuelov N, Lamed R, Lowe S, Zeikus JG (1991) Influence of $CO_2^{-}$-$HCO_3^{-}$ levels and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*. Appl Environ Microbiol 57:3013-3019

Sanchez AM, Bennett GN, San K-Y (2005) Efficient succinic acid production from glucose through overexpression of pyruvate carboxylase in an *Escherichia coli* alcohol dehydrogenase and lactate dehydrogenase mutant. Biotechnol Prog 21:358-365

Schilling, L. B. (1995) Chemicals from alternative feedstocks in the United States. *FEMS Microbiology Reviews.* 16:101-110.

Shively, Jessup M.; Van Keulen, Geertje; Meijer, Wim G. (1998) Something from almost nothing: carbon dioxide fixation in chemoautotrophs. *Annual Review of Microbiology*. 52:191-230.

Stumm W, Morgan J (1996). Dissolved carbon dioxide. In: Aquatic chemistry, John Wiley, New York, p192

Trchounian A, Kobayashi H (1999) Fermenting *Escherichia coli* is able to grow in media of high osmolarity, but is sensitive to the presence of sodium ion. Curr Microbiol 39: 109-114

Tsuda H., Kushi A., Yoshida D., Goto E. 1981. Chromosomal aberrations and sister-chromatid exchanges induced by gaseous nitrogen dioxide in cultured Chinese hamster cells. Mutat Res. 89:303-309.

Urbance S, Pometto A 3rd, Dispirito A, Denli Y (2004) Evaluation of succinic acid continuous and repeat-batch biofilm fermentation by *Actinobacillus succinogenes* using plastic composite support bioreactors. Appl Microbiol Biotechnol 65:664-670

Van der Werf MJ, Guettler MV, Jain MK, Zeikus JG (1997) Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus sp*. 130z. Arch Microbiol 167:332-342

Vemuri GN, Eiteman MA, Altman E (2002a) Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J Ind Microbiol Biotechnol 28:325-332

Vemuri GN, Eiteman MA, Altman E (2002b) Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol 68:1715-1727

Vemuri, G. N., Minning, T. A., Altman, E., and Eiteman, M. A. 2005. Physiological response of central metabolism in *Escherichia coli* to deletion of pyruvate oxidase and introduction of heterologous pyruvate carboxylase. Biotechnology and Bioengineering. **90**:64-76.

Victorin K., Stahlberg M. 1988. A method for studying the mutagenicity of some gaseous compounds in *Salmonella typhimurium*. Environ Mol Mutagen. 11:65-77.

Warren JW, Walker JR, Roth JR, Altman E. 2000. Construction and characterization of a highly regulable expression vector, pLAC11, and its multipurpose derivatives, pLAC22 and pLAC33. Plasmid. 44:138-151.

Watanabe, Y.; D.O. Hall. Photosynthetic $CO_2$ Conversion Technologies Using a Photobioreactor Incorporating Microalgae-Energy and Material Balances (1996) *Energy Convers. Mgmt.* 37(6-8): 1321-1326.

Wolfrum, E.J.; Watt, A.S. (2002) Bioreactor design studies for a hydrogen-producing bacterium. *Applied Biochemistry and Biotechnology*, 98-100 (Biotechnology for Fuels and Chemicals), 611-625.

Worden, R. M.; Bredwell, M. D.; Grethlein, A. J. (1997) Engineering issues in synthesis-gas fermentations. ACS Symposium Series, 666 (Fuels and Chemicals from Biomass), 320-335.

Yu, D., Ellis, H. M., Lee, E. C., Jenkins, N. A., Copeland, N. G. and Court, D. L. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **97**:5978-83.

Zeikus JG, Jain MK, Elankovan P (1999) Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biotechnol 51:545-552

Zhang, K.; Miyachi, S.; Kurano, N. (2001) Evaluation of a vertical flat-plate photobioreactor for outdoor biomass production and carbon dioxide bio-fixation: effects of reactor dimensions, irradiation and cell concentration on the biomass productivity and irradiation utilization efficiency. *Applied Microbiology and Biotechnology.* 55(4): 428-433.

Zhu Y, Eiteman MA, DeWitt K, Altman E (2007) Homolactate fermentation by metabolically engineered *Escherichia coli* strains. Appl Environ Microbiol 73:456-464.