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Principal Author: Mark A. Eiteman

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Name and Address of Submitting Organization:

University of Georgia
Faculty of Engineering
c/o Mark A. Eiteman, Ph.D.
Driftmier Engineering Center
Athens, GA 30602
(706) 542-0833
eiteman@engr.uga.edu

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ABSTRACT:

This report describes results toward developing a process to sequester CO_2 centered on the enzyme 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 first phase of this research has focused on strain development and on process development.

Progress in strain development has been made in three areas. The gene encoding for alcohol dehydrogenase has been "knocked out" of the bacteria, and thereby eliminating the synthesis of the by-product ethanol. The gene for glucokinase has been overexpressed in the production strain with the goal of faster utilization of glucose (and hence CO_2). Efforts have continued toward integrating pyruvate carboxylase gene (*pyc*) onto the *E. coli* chromosome.

Progress in process development has come in conducting several dozen fermentation experiments to find a defined medium that would be successful for the growth of the bacteria, while permitting a high rate of CO_2 utilization in a subsequent prolonged production phase. Using this defined medium, the strains that continue to be constructed are being compared for CO_2 utilization, so that we may understand the factors that govern the biological sequestration process.

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INTRODUCTION:

Most research on the microbial sequestration of CO_2 has centered on *bacteria* and *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 *bacteria* and *archaea* 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^3day (16 metric tons/m^3year), 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).

The bacteria that produce succinic acid from CO_2 are *Escherichia coli*, the name of the specific strain is AFP111/*pyc*, and it is well characterized genetically and biochemically. Our technology centers on incorporating pyruvate carboxylase enzyme activity into *E. coli* by overexpressing the *pyc* gene that this organism does not normally have. This enzyme uses biotin very effectively and specifically to fix CO_2 onto an organic backbone. The general process involves growing the cells aerobically to a high cell density, and then subjecting them to a prolonged CO_2-rich anaerobic non-growth phase for succinic acid production. Once generated, the cells can remain productive for many cycles. The current barriers in the proposed process for the implementation of this technology include: 1) strain development, 2) process development and 3) reactor design. In the area of strain development, the needs are to knockout genes which lead to by-product formation, overproduce glucose uptake genes, and incorporate pyruvate carboxylase onto the chromosome of *Escherichia coli*. 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, and is part of DOE's vision or roadmap for CO_2 sequestration (Creutz and Fujita 2000).

EXECUTIVE SUMMARY:

This report describes results toward developing a process to sequester CO_2 centered on the enzyme 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 first phase of this research has focused on strain development and on process development.

Several strains have been developed. To construct the *adhE* 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. Amplified DNA was electroporated into DY330, one of the strains used for Lambda Red mediated recombination. Ultimately, the *adhE*::tet knockout was moved by P1 transduction into MG1655 and the production strain AFP111. An experiment was completed in which anaerobic ethanol production was compared in MG1655 with production in MG1655 *adhE*. In duplicate experiments with MG1655, we found an ethanol yield of 0.103 g/g with an alcohol dehydrogenase activity of about 14 U/g dry cell weight (DCW). In contrast, we could not detect alcohol dehydrogenase activity in MG1655 *adhE*, and no ethanol was generated. These experiments confirm that the ability to generate the by-production ethanol has been removed.

To construct a plasmid containing the glucokinase-encoding gene, *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 *E. coli*. Overproduction of glucokinase was initially verified by SDS-PAGE analysis. Glucokinase assays demonstrated a 80-fold increase in specific glucokinase activity in the transformed production strain.

Various procedures were explored to incorporate pyruvate carboxylase into the *E. coli* chromosome. We were successful in integrating the *pyc* gene into the chromosome. However, no pyruvate carboxylase enzyme activity was detected. We have recently begun to clone the *pyc* gene into new plasmids that have much stronger promoters in order to increase the expression of pyruvate carboxylase that can be obtained from a single chromosomal copy of the gene.

We also had several goals in the development of a process. One goal has been to determine the effect of pH and temperature during the growth phase on the sequestration of CO_2 of the biocatalyst. That is, does the biocatalyst need to be prepared under a certain set of conditions in order for it to be "primed" for CO_2 utilization? We conducted these experiments using AFP111 pTc99A-*pyc* and complex media with three levels of pH and three levels of temperature 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 results have shown a limited, but statistically significant effect of pH and temperature and yield, but within the range of the experimental variable no effect on CO_2 utilization was observed.

A second process-related goal for the project was to develop a defined medium that would support microbial growth and subsequent CO_2 sequestration. A great deal of effort was spent during the first year on examining numerous media combinations. Ultimately, a medium was

found that can readily achieve cell concentrations of about 10 g/L, which will be sufficient for detailed comparison of the strains constructed. Specifically, we have completely replicate experiments on three strains: AFP111, AFP111 *adhE*, AFP111 *poxB*. The strain containing pyruvate carboxylase has not yet been studied under these medium conditions. For AFP111, the rate of succinate production averaged 1.84 g/Lh, corresponding to a CO_2 sequestration rate of 700 mg/Lh, but as high as about 800 mg/Lh. The succinate yield was 0.62 g/g. When the *adhE* gene was knocked out, the rate of succinate production averaged only 0.55 g/Lh (Q_{CO2} = 210 mg/Lh), although the yield increased to 0.75 g/g. For the strain carrying the *poxB* knock out, the rate of succinate production was 0.62 g/Lh (Q_{CO2} = 230 mg/Lh) but the succinate yield was only 0.55 g/g. Although the *adhE* knockout was successful at removing the by-product ethanol as we had hoped, this knockout also resulted in a markedly slower rate of production/sequestration. We anticipate that including the plasmid pTrc99A-*pyc* in AFP111 *adhE* would afford the system a means of diverting that pyruvate into succinate, with the simultaneous additional utilization of CO_2.

A third process-related goal for the project is to determine the effect of production phase conditions on CO_2 sequestration. As the first year of the project draws to a close, we have constructed the experimental device and are implementing the experiments.

EXPERIMENTAL:

For all experiments, the control strain was *Escherichia coli* AFP111 [ATCC 202021, F+λ-*rpoS396*(Am) *rph*-1 *ldhA*::kan Δ(*pflAB*::cam)] (Donnelly et al., 1998; Chatterjee et al. 2001). Transformations were conducted as described in the Results Section.

For preliminary experiments an "unoptimized" growth medium was used. This 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, as described in the Results and Discussion Section.

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. During this aerobic phase, 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. During the production phase Na_2CO_3 was used for pH control. In general a feed solution of 40 g/100 mL glucose was added at the initiation of the production phase. Also, in this phase CO_2 was sparged into fermenter at 0.15 L/min.

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). Of course, 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. Strain Development

A major portion of the first year of this project was devoted to strain development. As noted in the introduction, the three specific goals were to knockout the *adhE* gene expressing alcohol

dehydrogenase, to overexpress the *glk* gene expressing glucokinase, and to integrate the *pyc* gene into the chromosome.

During the first year we were successful in constructing an alcohol dehydrogenase gene knockout. 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. An experiment was completed in which anaerobic ethanol production was compared in MG1655 with production in MG1655 *adhE*. In duplicate experiments with MG1655, we found an ethanol yield of 0.103 g/g with an alcohol dehydrogenase activity of about 14 U/g dry cell weight (DCW). In contrast, we could not detect alcohol dehydrogenase activity in MG1655 *adhE*, and no ethanol was generated. These experiments confirm that the ability to generate the by-production ethanol has been removed. In section 2 we will show results of succinate production using this strain (see Figure 1).

During this first year we were also successful in the overproduction of glucokinase through the construction of 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 a 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. No additional experiments have yet been conducted on AFP111 pTrc99A-*glk*. However, soon we will determine whether this strain has in increased rate of glucose consumption and hence increased rate of CO_2 utilization.

The third goal for the first year of this project was to incorporate pyruvate carboxylase into the *E. coli* chromosome. We had previously constructed pTrc99A-*pyc* derivative plasmids which contained the *tetA* gene immediately downstream of *pyc*. PCR primers were designed which could amplify the *pyc* gene and tetracycline resistance from the pTrc99A-*pyc*-*tetA* plasmid bracketed by the first and last 50 bases of the *xylB* gene which encodes for xylulokinase. The amplified DNA was electroporated into DY330 and tetracycline resistant recombinants were selected. The introduction of *pyc* into the *E. coli* chromosome was verified using primers designed to amplify the wild-type *pyc* gene. Pyruvate carboxylase enzyme assays were performed, however no activity was detected. This is not an unanticipated result. We routinely

obtain pyruvate carboxylase activities of 1 unit per mg of total cellular protein from *E. coli* strains that contain the pTrc99A-*pyc* plasmid. The copy number of the pTrc99A plasmid is about 100. Therefore, by incorporating the gene into the chromosome it is possible to obtain a 100 fold reduction in the amount of pyruvate carboxylase activity, which would be undetectable. As discussed in the proposal, we anticipated this possible outcome. We have recently begun to clone the *pyc* gene into new plasmids that have much stronger promoters in order to increase the expression of pyruvate carboxylase that can be obtained from a single chromosomal copy of the gene.

2. Process Development

During the first year of the project, we had several goals in the development of a process. Because we did not completed the strain development phase of the research, we conducted many of these experiments using the preliminary control strains such as AFP111, in anticipation that many of the process conditions will be applicable to any of the other strains developed.

One goal has been to determine the effect of pH and temperature during the growth phase on the sequestration of CO_2 of the biocatalyst. That is, does the biocatalyst need to be prepared under a certain set of conditions in order for it to be "primed" for CO_2 utilization? We conducted these experiments using AFP111 pTc99A-*pyc* and complex media (because at the onset we had not yet generated the strain carrying *pyc* on the chromosome and because we had not yet "optimized" a defined media). Three levels of pH (6.2, 6.6, 7.0) and three levels of temperature (34°C, 37°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 requires 18 total experiments, which were to be conducted in a fermenter at the 1.5 liter scale. After 12 experiments were completed, a 4 month break from this experimentation was taken (so that the graduate student could complete Ph.D. examination requirements). After this break, 3 addition experiments were completed. However, the results of those experiments indicated a shift in the rate of CO_2 sequestration (but not in the yields). We have not been able to determine the cause for the shift in these numbers, but suspect an instability in the strain (which contains the pTrc99A-*pyc* plasmid). However, we have not completed this pH-temperature study because of this shift. Presently, we can make some inferences in the effect of pH and temperature on yield (since 15 out of 18 experiments in the design were completed). We cannot make any inferences on the effect of pH and temperature on the rate of CO_2 sequestration.

The yield at five hours (Y5) is a measure of how much of the co-substrate glucose is being converted into the product succinic acid. From the ANOVA table obtained (Table 1), we observed that the interaction term (pH × temperature) had no significant contribution, and this term was therefore removed from the model. The resulting model (data not shown) showed that both pH and temperature had significant effects on Y5, with p-values of 0.059, and 0.006, respectively. The pH of 6.2 resulted in the greatest response, while pH levels of 6.6 and 7.0 were not statistically different. For temperature, the Y5 at 37°C was significantly different than that at 40°C (p=0.0022) with the higher response at 40°C. The normal probability plot showed that the analyzed data were normally distributed; and the plots of residuals versus fitted values, pH, and temperature indicated that they had constant variances, therefore our inference was reasonable.

Table 1. ANOVA table of 15 experiments (incomplete design) at three levels of temperature and pH using Box-Behnken design.

Dependent Variable: Y5

Source	DF	Sum Squares	Mean Square	F Value	Pr > F
Model	6	0.19123000	0.03187167	4.28	0.0313
Error	8	0.05953000	0.00744125		
Corrected Totall	14	0.25076000			

R-Square	Coeff Var	Root MSE	Y5 Mean
0.762602	12.21851	0.086263	0.706000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pH	2	0.04630231	0.02315115	3.11	0.1001
Temp	2	0.08792987	0.04396493	5.91	0.0266
pH*Temp	2	0.00344692	0.00172346	0.23	0.7984

The yield at ten hours (Y10) is an estimation of the longer term yield of succinic acid from glucose. A similar method was used to analyze the effects of pH and temperature on Y10. The results indicate that only temperature had a significant effect on Y10, with the higher response at a temperature of 37°C, significantly different than the value of Y10 at 40°C ($p=0.0185$). For the Y10 results, the normal probability plot showed that the data were normally distributed; and the plots of residuals versus fitted values, pH and temperature indicated that they had constant variances, therefore our inference was reasonable.

In summary of our efforts toward the goal of determining the effect of pH and temperature: 1) an insufficient number of experiments were completed to make any inferences concerning the effect of pH and temperature on the rate of CO_2 sequestration; 2) pH and temperature were important factors for Y5 with a pH of 6.2 and temperature of 40°C maximizing this response; 3) temperature was a factor for Y10, with a maximum occurring at a temperature of 37°C. These inferences were made with a suboptimal number of results (15 out of a complete statistical design of 18).

A second process-related goal for the project was to develop a defined medium that would support microbial growth and subsequent CO_2 sequestration. A great deal of effort was spent during the first year on examining numerous media combinations. This process is iterative in that a medium is necessary prior to the development of the ultimate strain. However, a strain to be studied requires an optimal media to demonstrate that the strain is optimal. So, currently we need only a medium which is very good, but not necessarily the one which is the "optimal." We anticipate that we will continue improving both the strains and the media in the next year. The medium that we selected contains the components listed in Table 2.

Table 2. JS Medium 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

With this medium we have begun to study the various strains using the procedure outlined in the Experimental Section. Presently, we have completely replicate experiments on three strains: AFP111, AFP111 *adhE*, AFP111 *poxB*. The latter strain is one we constructed in order to determine if we could reduce the level of acetate production. The strain containing pyruvate carboxylase has not yet been studied under these medium conditions. Based on the results, we anticipate transforming some of the knockout strains with the pTrc99A-*pyc* plasmid encoding for pyruvate carboxylase. Example results obtained on CO_2 sequestration using these strains is shown in Figures 1-3.

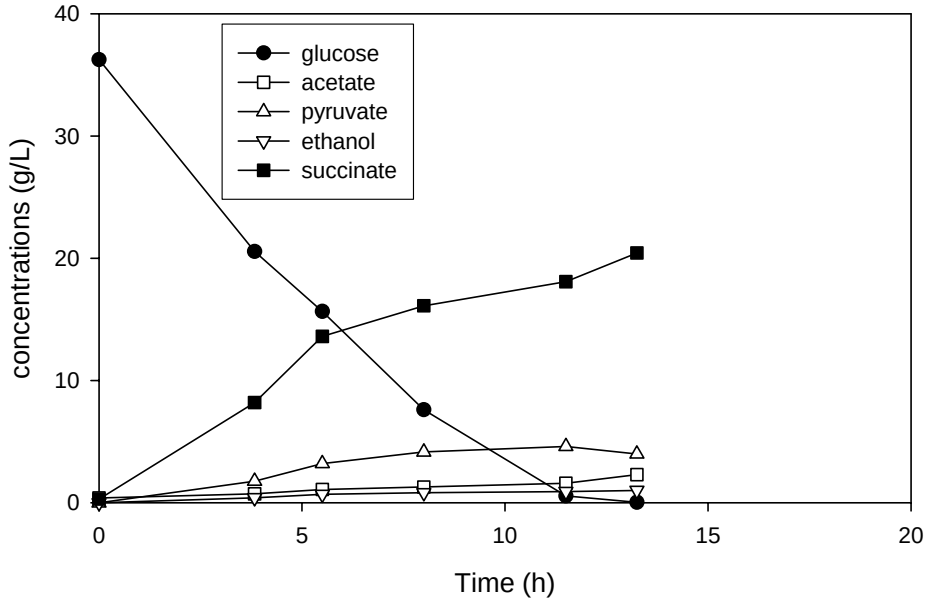

Figure 1: Conversion of glucose to succinate in AFP111 after growth in JS Medium. Note that 1 mol of succinate generation sequesters 1 mol of CO_2.

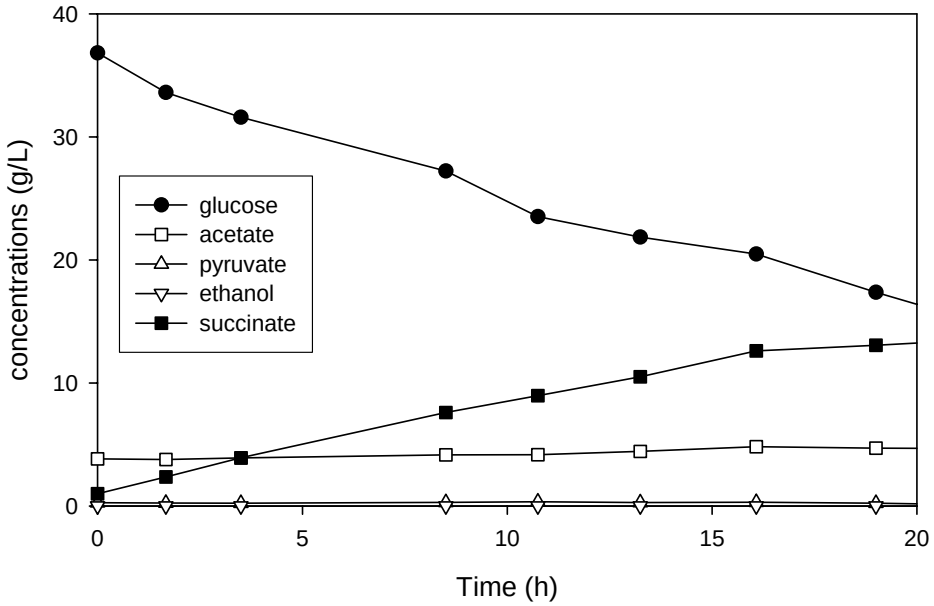

Figure 2: Conversion of glucose to succinate in AFP111 *adhE* after growth in JS Medium. Note that 1 mol of succinate generation sequesters 1 mol of CO_2.

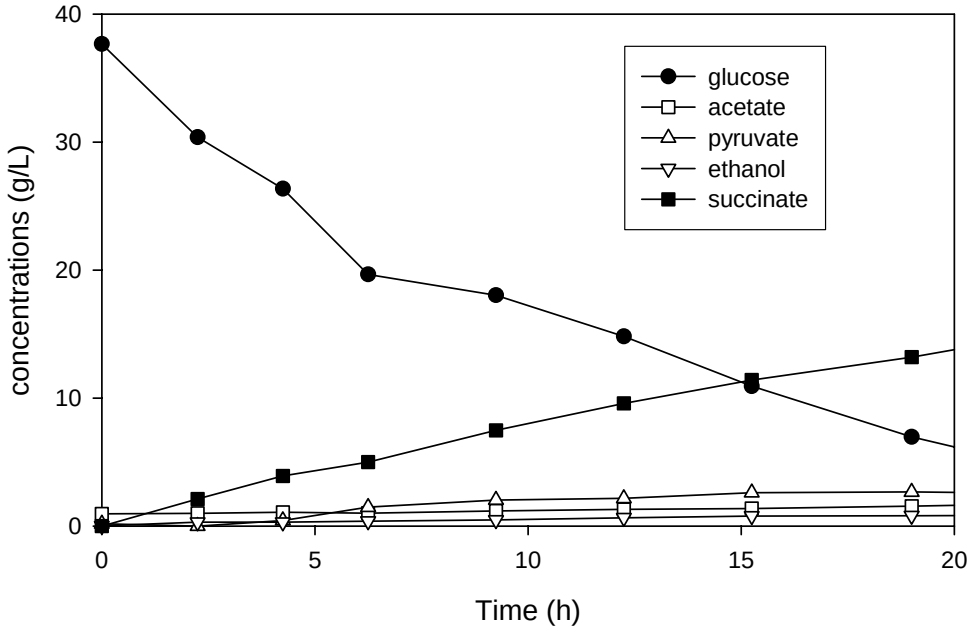

Figure 3: Conversion of glucose to succinate in AFP111 *poxB* after growth in JS Medium. Note that 1 mol of succinate generation sequesters 1 mol of CO_2.

These results show several noteworthy features. In each case, we simply fed 40 g/L of glucose at the onset of succinate production and permitted that concentration to decrease (i.e., without feeding more). For AFP111, the rate of succinate production averaged 1.84 g/Lh, corresponding to a CO_2 sequestration rate of 700 mg/Lh (the highest rate we have observed was about 800 mg/Lh). The succinate yield was 0.62 g/g. When the *adhE* gene was knocked out, the rate of succinate production averaged only 0.55 g/Lh (Q_{CO2} = 210 mg/Lh), although the yield increased to 0.75 g/g (presumably because some of the carbon which would have been converted to ethanol was converted to succinate). For the strain carrying the *poxB* knock out, the rate of succinate production was 0.62 g/Lh (Q_{CO2} = 230 mg/Lh) but the succinate yield was only 0.55 g/g. Although the *adhE* knockout was successful at removing the by-product ethanol as we had hoped, this knockout also resulted in a markedly slower rate of production/sequestration. Furthermore, knocking out *adhE* resulted in an increase in acetate production (although not as much acetate as ethanol was reduced). This result is not unexpected. AFP111 *adhE* would not have a means of converting pyruvate into succinate, and therefore would generate another product biochemically downstream of pyruvate, acetate. We anticipate that including the plasmid pTrc99A-*pyc* in AFP111 *adhE* would afford the system a means of diverting that pyruvate into succinate, with the simultaneous additional utilization of CO_2. In the next year we will examine the CO_2 sequestration in the following strains: AFP111 pTrc99A-*pyc*, AFP111 pTrc99A-*glk*, AFP111 *adhE* pTrc99A-*pyc* and other strains that are constructed (for example, when *pyc* gene is integrated onto the chromosome.)

A third goal for the project is to determine the effect of production phase conditions on CO_2 sequestration. In particular, we wish to complete batch fermentations in which the gas phase

during the production phase is composed of a range of gas mixtures which correspond to flue gas composition. We plan to examine the effect of CO_2 concentrations in the gas phase, with an emphasis on determining what minimum composition of CO_2 is necessary before its concentration limits the rate of sequestration. We have developed a GC technique to measure CO_2 down to a concentration of about 100 ppm. The technique uses a HayesSep C column and a Thermal Conductivity detector. We are currently examining the analysis of SO_2 and NO_2. Also, we plan to examine the effect of potentially inhibitory gases, including O_2, SO_2 and NO_2. As the first year of the project draws to a close, we have constructed the experimental apparatus shown in Figure 4. However, we do not yet have the mass flow controllers used to supply the gas to the system (a special order by virtue of the corrosive gas requirements). In the next year we will examine the CO2 sequestration rate using gas phase compositions of 0-50% CO_2, 0 – 1000 ppm NO_2, 0-1000 ppm SO_2, 0-5% O_2.

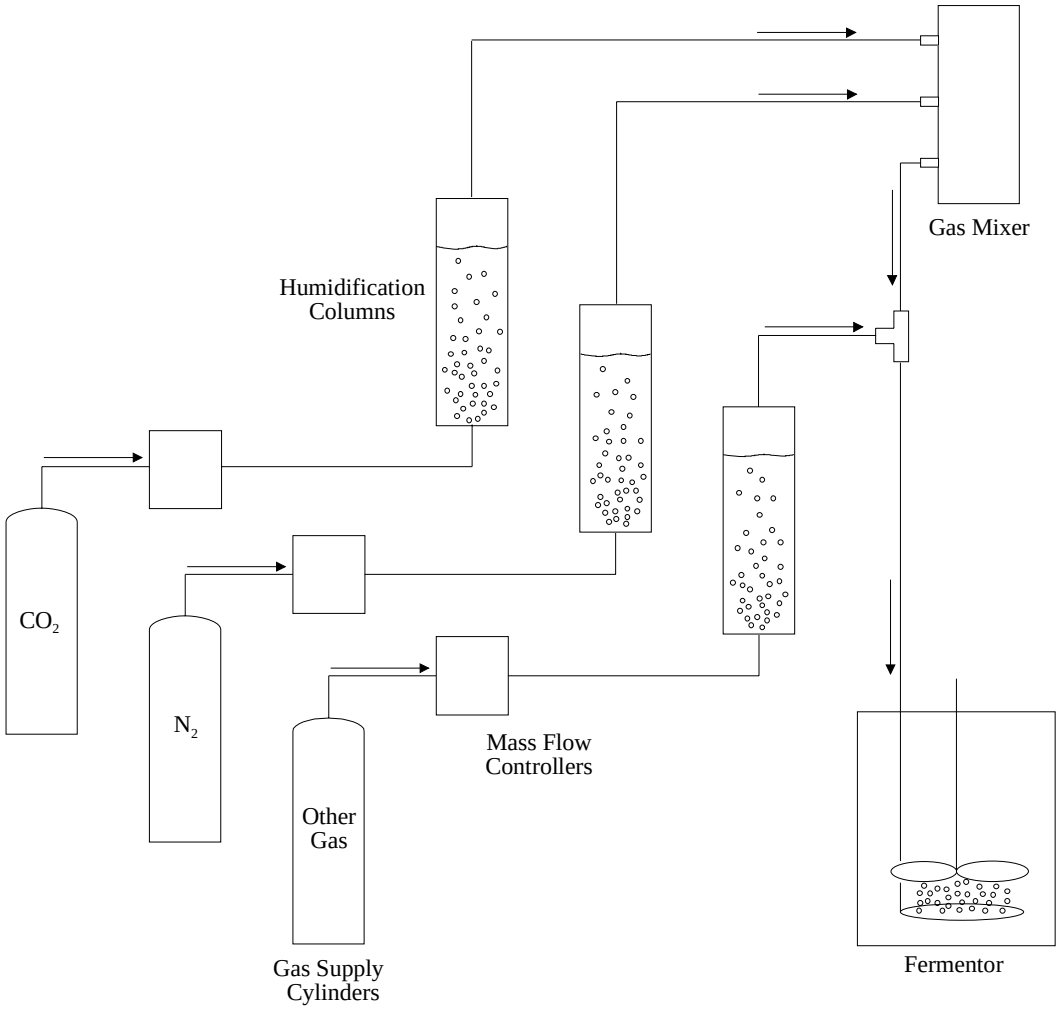

Figure 4: System to determine the effect of gas phase composition on succinate production and CO_2 sequestration.

CONCLUSIONS:

We have had no difficulty in generating strains containing the *adhE* knockout or which overexpresses the *glk* gene encoding for glucokinase. The *adhE* knockout by itself does not improve CO_2 sequestration. However, additional study is required using pyruvate carboxylase in combination with the *adhE* knockout to determine whether these genetic perturbations positively impact CO_2 sequestration rate. The integration of pyruvate carboxylase onto the chromosome has been problematic. Significant time has been used toward constructing a new promoter with greater "activity" that will permit sufficient *pyc* expression when there is only one copy on the chromosome.

Using a complex media, we have shown minimal statistical difference between three levels of pH and temperature. This result suggests that the process has a certain degree of robustness, and would be sufficiently flexible to accommodate at least a slight variation in process conditions. Additional studies will be necessary with our second generation medium to determine whether growth conditions affect the quality of the biocatalyst.

A good defined medium was developed which allowed us to perform studies comparing the various strains. This on-going work has demonstrated that, although they each do reduce by-product formation, the *adhE* and *poxB* are not by themselves beneficial for CO_2 sequestration. Future experiments will determine whether the inclusion of the CO_2 sequestering enzyme, pyruvate carboxylase, improves the rate of CO_2 sequestration while still maintaining low by-product formation.

We have been able to demonstrate CO_2 sequestration at a rate of about 800 mg/Lh, which exceeds the rate of CO_2 sequestration previously attained (in a photosynthetic system) by a factor of more than 50.

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