Appl Biochem Biotechnol (2010) 162:1915–1928
DOI 10.1007/s12010-010-8969-1

Integration of Succinic Acid and Ethanol Production With Potential Application in a Corn or Barley Biorefinery

Nhuan P. Nghiem · Kevin B. Hicks · David B. Johnston

Received: 30 October 2009 / Accepted: 11 April 2010 /
Published online: 3 June 2010

Abstract Production of succinic acid from glucose by *Escherichia coli* strain AFP184 was studied in a batch fermentor. The bases used for pH control included NaOH, KOH, NH_4OH, and Na_2CO_3. The yield of succinic acid without and with carbon dioxide supplied by an adjacent ethanol fermentor using either corn or barley as feedstock was examined. The carbon dioxide gas from the ethanol fermentor was sparged directly into the liquid media in the succinic acid fermentor without any pretreatment. Without the CO_2 supplement, the highest succinic acid yield was observed with Na_2CO_3, followed by NH_4OH, and lowest with the other two bases. When the CO_2 produced in the ethanol fermentation was sparged into the media in the succinic acid fermentor, no improvement of succinic acid yield was observed with Na_2CO_3. However, several-fold increases in succinic acid yield were observed with the other bases, with NH_4OH giving the highest yield increase. The yield of succinic acid with CO_2 supplement from the ethanol fermentor when NH_4OH was used for pH control was equal to that obtained when Na_2CO_3 was used, with or without CO_2 supplementation. The benefit of sparging CO_2 from ethanol fermentation on the yield of succinic acid demonstrated the feasibility of integration of succinic acid fermentation with ethanol fermentation in a biorefinery for production of fuels and industrial chemicals.

Keywords Succinic acid · *Escherichia coli* AFP184 · CO_2 fixation · Ethanol fermentation · Biorefinery

Introduction

Succinic acid is a four-carbon compound that has high value since it can be used as a precursor for many important industrial chemicals and consumer products [1]. Traditionally,

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

N. P. Nghiem (✉) · K. B. Hicks · D. B. Johnston
U.S. Department of Agriculture, Eastern Regional Research Center, Agricultural Research Service,
600 East Mermaid Lane, Wyndmoor, PA 19038, USA
e-mail: John.Nghiem@ars.usda.gov

succinic acid found applications in food additives, soldering fluxes, and pharmaceutical products [2]. The range of potential succinic acid applications has been widened by the development of several catalytic processes, in which succinic acid is a direct intermediate in the production of important industrial chemicals and solvents such as 1,4-butanediol (BDO), tetrahydrofuran (THF), γ-butyrolactone (GBL), *N*-methyl pyrrolidinone (NMP), and 2-pyrrolidinone (2P) [3, 4]. Recently, noncorrosive deicers based on succinic acid derivatives have been developed [5, 6], which expanded the potential total market value of succinic acid-based products even further.

Succinic acid currently is produced commercially by chemical processes for a small market volume [2]. Interest in production of industrial chemicals from renewable resources has led to the development of several microorganisms that could produce succinic acid at concentrations sufficiently high to make the development of commercial fermentation processes economically feasible and attractive [7–13]. At the US Department of Energy's National Laboratories, the effort of the Alternative Feedstocks Program (AFP) was focused on *Escherichia coli*. Among the many succinic acid-producing *E. coli* strains developed, AFP111 and AFP184 were the two most promising [9, 10]. Both strains carried mutations in the glucose-specific phosphotransferase transport gene (*ptsG*), the pyruvate formate lysase gene (*pfl*), and the lactate dehydrogenase gene (*ldh*). Between the two, AFP184 gave higher yield of succinic acid from glucose and also had the capability of utilizing both glucose and xylose simultaneously for succinic acid production [10]. This capability allowed the strain to be used for production of succinic acid using sugar hydrolysates derived from lignocellulosic biomass as feedstock [10]. The fermentation process developed for succinic acid production consisted of two stages. In the first stage, the cells were grown under aerobic conditions until a critical cell mass was achieved. Aeration then was suspended in the second stage to introduce anaerobic conditions, which initiated succinic acid synthesis at high yields and rates. The process was initially developed for AFP111 [9] and subsequently was also shown to be applicable to AFP184 [10]. An integrated process for production of biologically derived succinic acid and its subsequent conversion to industrial solvents by chemical catalysis also has been developed [14].

One of the key steps in the succinic acid biosynthetic pathway in *E. coli* is the one catalyzed by the enzyme phosphoenolpyruvate (PEP) carboxylase [15]. This step involves incorporation of a molecule of carbon dioxide into the conversion of the three-carbon compound PEP to oxaloacetate (OAA), which is a four-carbon compound. Therefore, sufficient CO_2 supply was accomplished by carbon dioxide gas sparging into the medium to achieve high yields in succinic acid fermentations using the AFP strains [9, 10, 16–18]. In commercial production of succinic acid, however, the use of purified carbon dioxide gas may be cost inhibitive. Thus, an inexpensive source of carbon dioxide is highly desirable for industrial succinic acid production. Ethanol fermentation could serve as such source. In industrial ethanol fermentation by the yeast *Saccharomyces cerevisiae*, two moles of carbon dioxide and two moles of ethanol are produced for every mole of glucose fermented. The carbon dioxide byproduct normally is vented, or in some cases, recovered and sold at relatively low prices to bottling facilities [19]. If the carbon dioxide produced in an industrial ethanol fermentor can be used for succinic acid production in an adjacent fermentor, it will improve the economics of the succinic acid fermentation process and also will help the environment since it will result in reduced quantities of this green house gas released into the atmosphere. The feedstock for ethanol production in the United States is almost exclusively corn starch [19]. Efforts have been made at the Eastern Regional Research Center (ERRC) of the US Department of Agriculture's Agricultural Research Service to develop fermentation process for ethanol production from winter barley, an

alternate nonfood starch-based feedstock. Commercial ethanol production from winter barley feedstock is expected to begin soon [20]. In this report, we present results that demonstrate for the first time the feasibility of direct use of carbon dioxide produced in either a corn or barley ethanol fermentor, without any pretreatment, for succinic acid production.

Materials and Methods

Microbial Strains

E. coli AFP184 was obtained from the American Type Culture Collection (Manassas, VA). The freeze-dried culture was reconstituted in LB medium [21]. The reconstituted culture was grown in 50-mL medium in a 250-mL shakeflask. Following incubation at 37 °C and 250 rpm for 16 h, two volumes of the broth were mixed with one volume of sterile glycerol, and the stock cultures were stored at −70 °C. Active dry ethanol red *S. cerevisiae* was purchased from Lesaffre Yeast Corporation (Milwaukee, WI). The dry yeast powder was kept refrigerated at 4 °C.

Materials

Shelled feed corn was purchased from Davis Feed of Bucks County (Perkasie, PA). The starch content of the corn was determined as 69.1% on a dry basis (see the section on analytical techniques). Thoroughbred hulled barley harvested in 2008 was obtained from the Virginia Foundation Seed Center, Mt. Holly, Virginia. The starch and β-glucan contents of the barley were determined as 59.6% and 3.6% on a dry basis, respectively (see "Analytical Methods"). All chemicals used were of reagent grades and purchased from various sources. Corn steep liquor used for complex nutrient source was purchased from Sigma-Aldrich (St. Louis, MO). All enzymes used in ethanol fermentation were kindly provided by Genencor, a Danisco Division.

Experimental Procedures

Ethanol fermentation Ethanol fermentations were performed in a 7.5-L Bioflow 110 fermentor (New Brunswick Scientific, Edison, NJ). The quantity of mash used in each experiment was 5 kg. The mash contained 30% dry total solids by weight. The procedures that have been used routinely in our laboratory were used to prepare the mash for ethanol fermentations. Thus, barley and corn were first ground in a Wiley mill, and fractions that passed through a 1-mm screen were collected for use in the experiments. The corn mash was prepared in several batches. Each batch consisted of 750 g dry total solids and deionized water sufficient to make a 30% by weight corn slurry. The corn/water mixture was placed in a 4-L glass beaker. The slurry was stirred with a mechanical agitator. The pH of the slurry was adjusted to 5.2 with 5 N sulfuric acid. A volume of 239 μL of SPEZYME Xtra, which is a thermostable α-amylase, was added to liquefy the starch in the ground corn. This enzyme dosage was equivalent to 0.35 kg/metric ton (MT) total dry solids. The beaker was heated on a hot plate. The temperature of the slurry was brought to 60 °C and maintained at that level for 1 h. The temperature then was increased to 85–90 °C and maintained at that level for 2 h before it was cooled to 32 °C. The beaker was weighed before and after the liquefaction, and water was added to compensate for the evaporation

loss. The pH of the cooled mash was adjusted to 4.2 with 5 N sulfuric acid. A volume of 518 μL of FERMENZYME L-400, which is a glucoamylase, then was added for saccharification of the solubilized starch. This enzyme dosage was equivalent to 0.76 kg/MT total dry solids. Urea also was added to a final concentration of 0.4 g/L. The batches of mash were transferred to the fermentor to a total working volume of 5 L. The fermentor content was stirred at 250 rpm and was maintained at 10 °C overnight. The temperature of the mash was increased to 32 °C the next day before inoculation. The inoculum consisted of 2.5 g dry yeast that had been rehydrated in 50 mL deionized water for 30 min. In the first experiment in which KOH was used for pH control in the corresponding succinic acid fermentor, inoculation of the ethanol fermentor was performed at the same time with inoculation of the corresponding succinic acid fermentor, i.e., 6 h before the start of the anaerobic stage in the succinic acid fermentor (see below). In subsequent experiments, which included the duplicated experiment using KOH and experiments where other bases were used for pH control in the corresponding succinic acid fermentors, inoculation of the ethanol fermentors was performed 4 h after inoculation of the succinic acid fermentors, i.e., 2 h before the start of the anaerobic stage in the succinic acid fermentors, to allow more CO_2 produced in ethanol fermentations to flow into the succinic acid fermentors.

The procedure used for preparation and inoculation of the barley mash was similar to the one used for the corn mash with the following exceptions: (a) there was no preliquefaction period at 60 °C; (b) SPEZYME Xtra was used at 205 μL per batch (0.30 kg/MT total dry solids); (c) OPTIMASH BG, a β-glucanase, also was added during the liquefaction at 89 μL per batch (0.13 kg/MT total dry solids); (d) FERMENZYME L-400 was used at 443 μL per batch (0.65 kg/MT total dry solids); and (e) an experimental β-glucosidase also was added after the mash had been cooled to 32 °C at 415 μL per batch (0.61 kg/MT total dry solids). The two enzymes β-glucanase and β-glucosidase were added to hydrolyze the β-glucans in barley to glucose, which subsequently was fermented by the yeast to produce additional ethanol.

During the course of the ethanol fermentation, the fermentor contents were stirred at 500 rpm and maintained at 32 °C. The progress of the fermentation was followed by measuring the total carbon dioxide produced with a METRIS M250 gas meter (Actaris Metering Systems, Owenton, KY), which used positive displacement of an internal liquid for gas volume measurements. The gas volumes were measured in liters. The meter was calibrated with air and displayed a sensitivity of one tenth of a liter. The ethanol fermentations were carried out in parallel with the corresponding succinic acid fermentations and were terminated when the succinic acid fermentations were stopped. At the end of the fermentations, samples were taken for determination of the final concentrations of soluble starch, glucose, ethanol, and other metabolites such as glycerol, succinic acid, lactic acid, and acetic acid.

Succinic acid fermentation Succinic acid fermentations also were performed in a 7.5-L Bioflow 110 fermentor located adjacent to the ethanol fermentor. The carbon dioxide produced in the ethanol fermentor was allowed to flow through the gas meter before it entered the medium in the succinic acid fermentor through the sparger, which delivered the gas underneath the bottom impeller. The fermentation medium contained 100 g/L glucose, 1.4 g/L K_2HPO_4, 0.6 g/L KH_2PO_4, 3.3 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4.7H_2O$, 15 g/L corn steep liquor, and 1 mL/L antifoam A-204 (Sigma-Aldrich). In preparation of the fermentation medium, the quantities of all the ingredients, except glucose, that were required to make 4 L were dissolved in 3 L of deionized water. The solution was adjusted to pH 6.5 and placed in the fermentor. The fermentor and its content were sterilized by

autoclaving at 121 °C for 20 min. A solution of 400 g/L glucose also was sterilized by autoclaving under the same conditions. After the fermentor was allowed to cool to about 35 °C, 1 L of the glucose solution was added. The inoculum medium had the same pH and composition as the fermentation medium except glucose was used at 5 g/L. The inoculum medium with all of its components dissolved in deionized water was sterilized by autoclaving as described previously. For each fermentation experiment, four inoculum flasks were made. Each flask contained 50 mL medium and was inoculated with 0.2 mL of thawed stock culture. The flasks were incubated at 37 °C and shaken at 250 rpm in an incubator shaker for 16 h. All four flasks were used to inoculate the fermentor. After inoculation, the aerobic growth stage was maintained for 6 h. During this time, the air flow was kept at 4.2 L/min or 1 volume per volume per minute, and the dissolved oxygen concentration was maintained at 20% of air saturation. Samples were taken every 2 h for measurements of absorbance at 660 nm. At the end of the aerobic stage, the air supply was cut off to introduce anaerobic conditions, which would initiate succinic acid production. During both the aerobic and anaerobic stages, the succinic acid fermentor was maintained at 37 °C and pH 6.5. Four bases were used for pH control. These included 5 M NaOH, 5 M KOH, 7.75 M NH_4OH, and 2.5 M Na_2CO_3. In experiments that were performed to study the effect of using carbon dioxide produced in ethanol fermentation for succinic acid production, immediately after the air supply was suspended, the outlet from the gas meter that was used to measure carbon dioxide production in the adjacent ethanol fermentor was connected to the sparger on the succinic acid fermentor. The carbon dioxide stream from the ethanol fermentor was filter-sterilized with a 0.2-μ gas filter prior to entering the succinic acid fermentor. All fermentors using the four bases described previously for pH control received carbon dioxide produced in the barley ethanol fermentors. In a separate experiment, a fermentor that used NH_4OH for pH control received carbon dioxide from an adjacent corn ethanol fermentor. Succinic acid fermentation experiments were performed for 72 h (including both aerobic growth stage and anaerobic production stage). During the anaerobic stage, samples were taken at intervals for determination of glucose, succinic acid, and acetic acid. At the end of the experiments, the final volumes of the broth were measured and used for succinic acid yield calculations. The succinic acid yield was calculated as follows:

Succinic acid yield (g succinic acid/g glucose consumed) = $SA_F \times V_F \div [(G_{AN} \times 4.1) - (G_F \times V_F)]$ where SA_F is final succinic acid concentration (g/L), G_F is final glucose concentration (g/L), G_{AN} is glucose concentration at the start of the anaerobic production stage (g/L), 4.1 is the broth volume in the fermentor at the start of the anaerobic production stage (L), and V_F is the final broth volume (L).

All succinic acid fermentation experiments (and the accompanied ethanol fermentation experiments) were performed in duplicate.

Analytical Methods

For starch analysis, barley and corn samples were ground in a cyclone mill fitted with a 0.5-mm screen (Udy, Ft. Collins, CO), and the flours were analyzed using a starch determination kit (Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland; ICC Standard Method No. 168; International Association for Cereal Science and Technology). The method was modified by use of a YSI 2700 Analyzer (YSI Incorporated, Yellow Springs, OH) fitted with a YSI 2710 turntable for automated glucose determination of enzymatically hydrolyzed starch containing samples. Barley

β-glucan was analyzed using a kit obtained from Megazyme International Ireland Ltd. according to ICC Standard Method 168 (International Association for Cereal Science and Technology) and instructions for the "streamlined method" provided by the manufacturer. This method conforms with standard methods (AOAC 995.16; Association of Official Agricultural Chemists, and AACC 32-33; American Association of Cereal Chemists).

Cell growth during the aerobic stage was monitored by measuring absorbance at 660 nm (A_{660}) using a Spectronic 20 D + (Thermo Electron Corporation, Madison WI). The growth stopped during the anaerobic stage [10], and hence, the A_{660} was not measured. Glucose, soluble starch oligomers (maltose, maltotriose, maltotetraose), succinic acid, ethanol, and other metabolites (acetic acid, lactic acid, glycerol) were determined by high-performance liquid chromatography. The system was an ISCO model 2350 using 0.5% sulfuric acid as solvent at 0.6 mL/min combined with an Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, CA) operated at 60 °C and an HP 1047A refractive index (RI) detector (Hewlett Packard, Palo Alto, CA). The injection volume was 5 μL. The software used for data analysis was Chrom Perfect Spirit version 4 build 17 (Justice Laboratory Software, Auchtermuchty, Fife, UK).

Results and Discussion

Ethanol fermentation The time courses of CO_2 production in the ethanol fermentors are plotted in Fig. 1, and the ethanol fermentation results are summarized in Table 1. The results plotted in Fig. 1 shows that CO_2 production practically stopped after 48 to 52 h. Thus, the succinic acid fermentors received CO_2 from the corresponding ethanol fermentors for only 2 days. During this period, the average rate of CO_2 allowed to bubble into the media in the succinic acid fermentors was 0.08 L/min or 0.02 volume per volume per minute. Since the system was always at nonsteady state, it was not possible to calculate the degree of CO_2 saturation in the liquid phase in the succinic acid fermentors during the course of the fermentations. Assuming 5% of the available glucose was used by the yeast for cell growth and maintenance (previous results, not shown), the average theoretical ethanol yield in the barley fermentations and the theoretical ethanol yield in the corn fermentation were calculated to be 81% and 91%, respectively. Lower theoretical ethanol yield was observed for barley mainly because of incomplete starch hydrolysis in the barley mash, which was indicated by the maltose, maltotriose, and maltotetraose peaks on the high-performance liquid chromatography chromatograms. Starch hyrolysis was practically complete for the corn mash (95% conversion).

Succinic acid fermentation The average specific growth rate during the aerobic stage was about 0.3 h^{-1}. The average A_{660} at the end of this stage was about 8–9. Very little accumulation of succinic acid was observed during the aerobic stage, typically less than 0.5 g/L. High rates of succinic acid production were observed only after aeration was discontinued. The glucose consumption and succinic acid production profiles obtained when different bases were used for pH control are plotted in Figs. 2, 3, 4, 5, 6. The final results are summarized in Table 2. Figure 7 shows the succinic acid biosynthetic pathway in *E. coli* AFP184 [17]. It can be seen that when NaOH, KOH, and NH_4OH were used for pH control, significant improvement of succinic acid production was observed when the carbon dioxide produced in the adjacent ethanol fermentor was sparged into the succinic acid fermentor. The improvement included both higher final succinic acid concentrations and

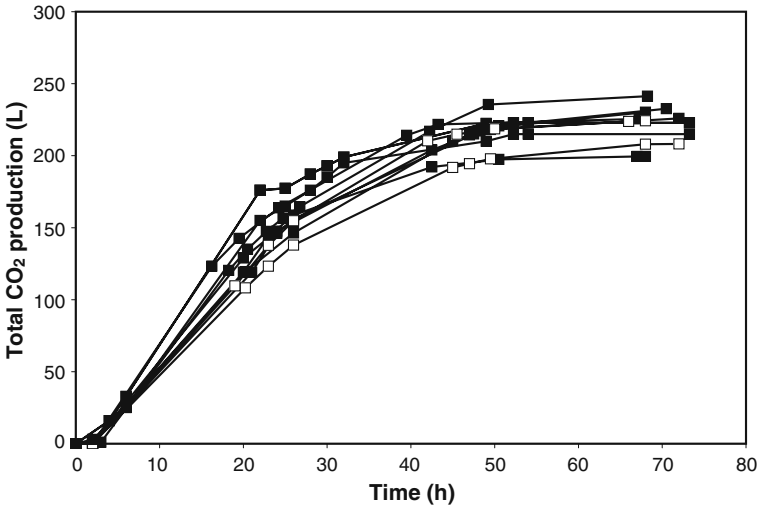

Fig. 1 Total CO_2 production in barley and corn ethanol fermentation experiments. ■, Barley as feedstock; □, corn as feedstock

higher yields (grams of succinic acid produced per gram of glucose consumed). When Na_2CO_3 was used for pH control, succinic acid production was the same without and with CO_2 sparging. As shown in Fig. 7, there were two pathways contributing to succinic acid synthesis in *E. coli* AFP184. The first pathway involved the enzyme PEP carboxylase (PEPC), which required bicarbonate to convert PEP to OAA [21]. The second pathway proceeded via the nonreductive branch of the citric acid cycle, i.e., citrate → isocitrate → succinate, which did not require bicarbonate. The fact that significant improvement of succinic acid production was obtained with external supply of carbon dioxide when NaOH, KOH, and NH_4OH were used for pH control indicated that the PEPC pathway was the more important one. When Na_2CO_3 was used for pH control, the bicarbonate provided by the base probably was sufficient to meet the demand of the enzyme PEPC, and hence, no improvement of succinic acid was observed with CO_2 sparging. External supply of CO_2 also increased the rates of glucose consumption in the experiments where the noncarbonate bases were used. This indicated that the PEPC was a rate-limiting step of glucose utilization

Table 1 Total CO_2 production and final ethanol concentrations in barley and corn fermentation experiments.

Feedstock	Total CO_2 production (L)[a]	Final ethanol concentration (g/L)[a]	Base used in corresponding succinic acid fermentor
Barley	223	111	NaOH
Barley	228	123	KOH
Barley	226	116	NH_4OH
Barley	220	116	Na_2CO_3
Corn	216	122	NH_4OH

[a] Averages of results obtained in duplicated experiments

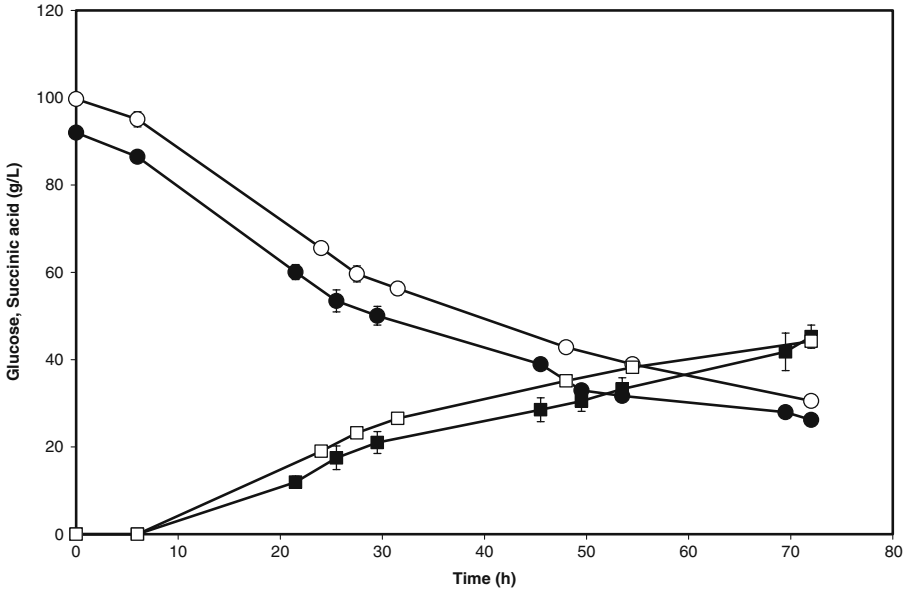

Fig. 2 Glucose consumption and succinic acid production in experiments using Na_2CO_3 for pH control. ●, Glucose consumption—control; ○, glucose consumption with CO_2 supplied by the barley ethanol fermentor; ■, succinic acid production—control; □, succinic acid production with CO_2 supplied by the barley ethanol fermentor

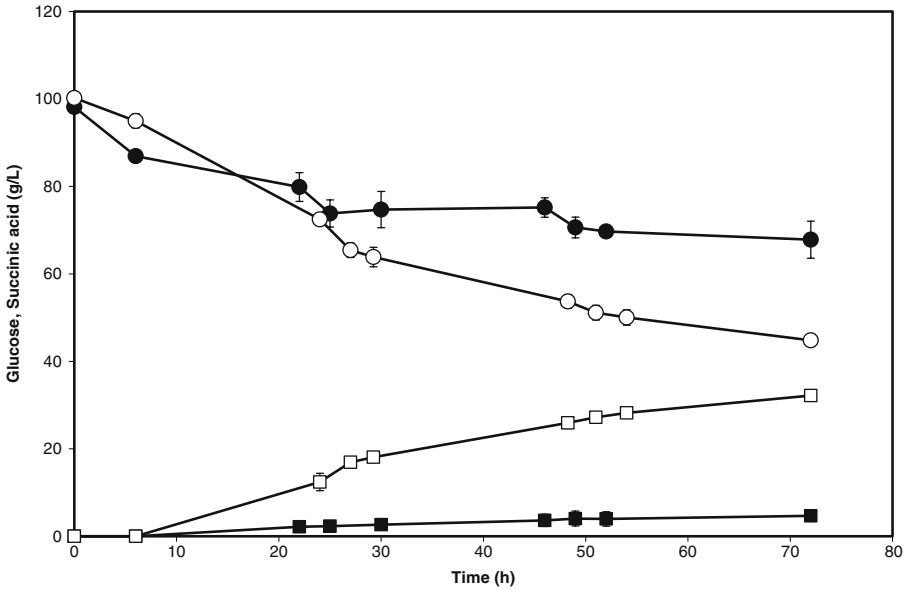

Fig. 3 Glucose consumption and succinic acid production in experiments using NaOH for pH control. ●, Glucose consumption—control; ○, glucose consumption with CO_2 supplied by the barley ethanol fermentor; ■, succinic acid production—control; □, succinic acid production with CO_2 supplied by the barley ethanol fermentor

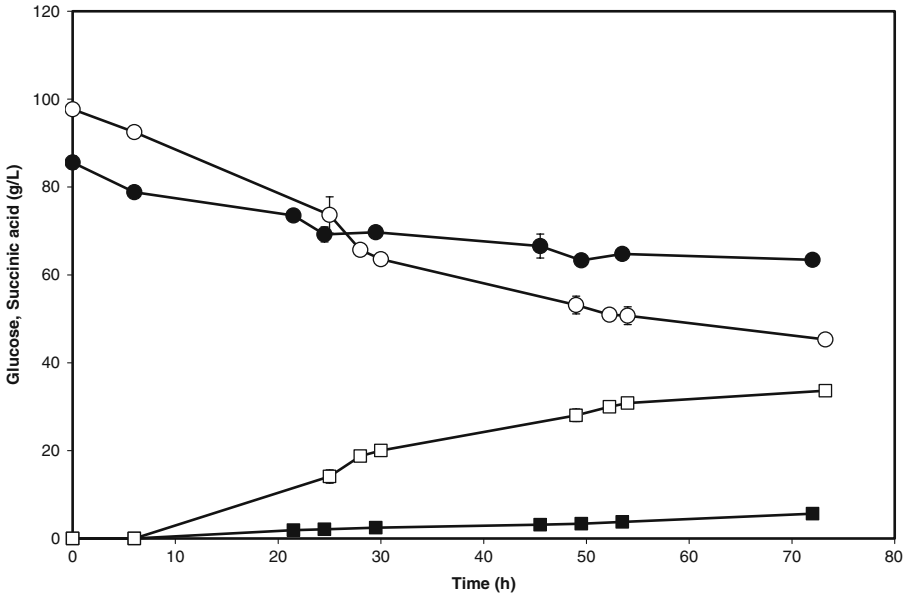

Fig. 4 Glucose consumption and succinic acid production in experiments using KOH for pH control. ●, Glucose consumption—control; ○, glucose consumption with CO_2 supplied by the barley ethanol fermentor; ■, succinic acid production—control; □, succinic acid production with CO_2 supplied by the barley ethanol fermentor

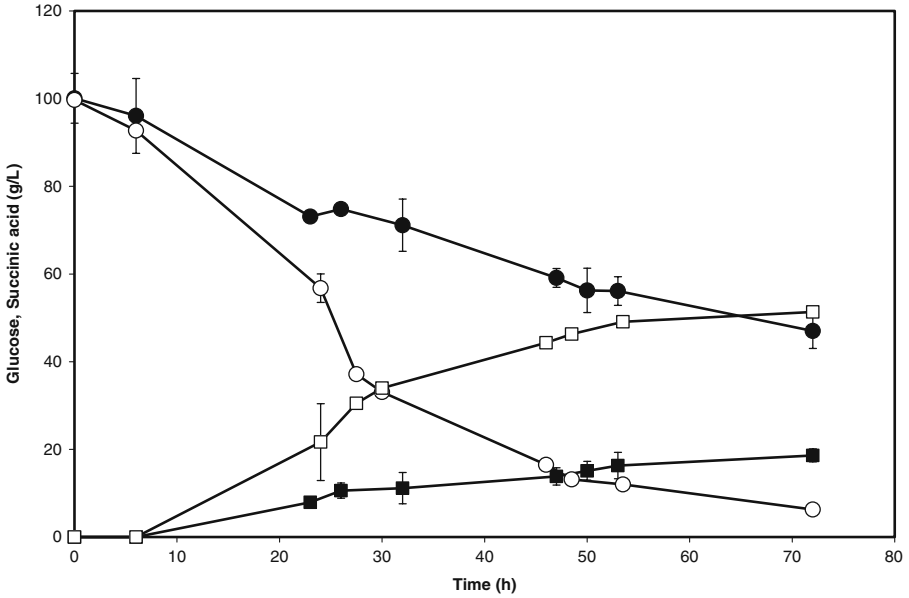

Fig. 5 Glucose consumption and succinic acid production in experiments using NH_4OH for pH control. ●, Glucose consumption—control; ○, glucose consumption with CO_2 supplied by the barley ethanol fermentor; ■, succinic acid production—control; □, succinic acid production with CO_2 supplied by the barley ethanol fermentor

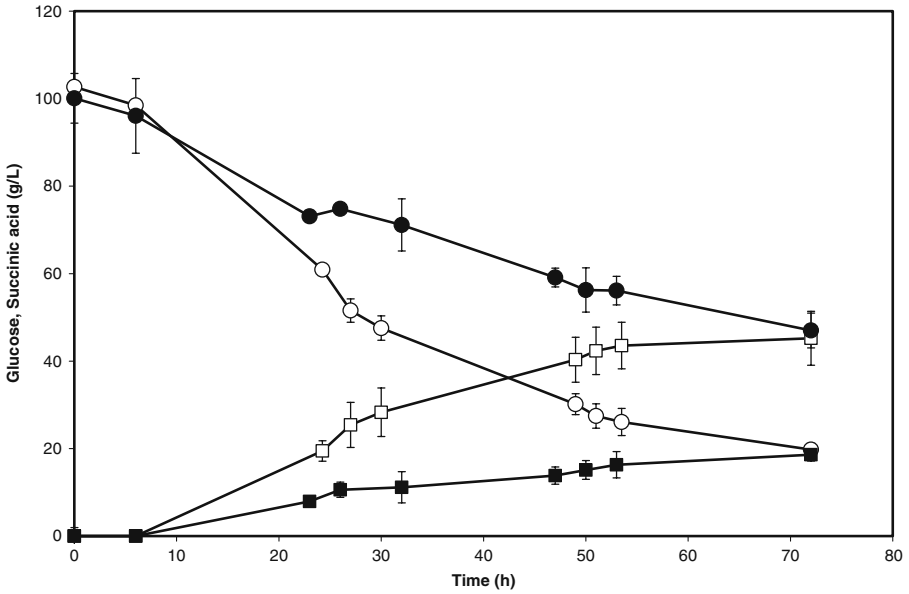

Fig. 6 Glucose consumption and succinic acid production in experiments using NH_4OH for pH control. ●, Glucose consumption—control; ○, glucose consumption with CO_2 supplied by the corn ethanol fermentor; ■, succinic acid production—control; □, succinic acid production with CO_2 supplied by the corn ethanol fermentor

in *E. coli* AFP184. External supply of CO_2 also favored the synthesis of succinic acid over the major byproduct acetic acid when NaOH and KOH were used. When the CO_2 produced in the adjacent ethanol fermentor was sparged into the succinic acid fermentor, the molar ratio of succinate:acetate was doubled in the case of KOH and increased by 30% in the other case (Table 2). This was expected since, with sufficient bicarbonate supplied to the PEPC to allow this enzyme to function at high efficiency, more carbon would move toward succinic acid synthesis and less toward acetic acid. Another possibility was the feedback inhibition exerted by the excess CO_2 on the pyruvate dehydrogenase complex, which converted pyruvate to acetyl-CoA and CO_2. When NH_4OH was used for pH control, external supply of CO_2 caused increases in production of both succinic acid and acetic acid, with the net result being lower molar ratio of succinate/acetate. It should be noted also that glucose consumption with external supply of CO_2 when NH_4OH was used was much higher than in the cases of NaOH and KOH. It was probable that in the case of NH_4OH, the carbon flux through the upper section of the glycolytic pathway was so high that the PEPC was saturated with its substrate, and the excess PEP was diverted toward acetic acid synthesis via pyruvate kinase.

From a practical point of view, several important observations could be made from the results reported here. The use of carbon dioxide produced in ethanol fermentation for succinic acid production has been suggested [22, 23], but to the best of our knowledge, this concept has not been demonstrated before. It has been shown here for the first time that the carbon dioxide gas produced in an ethanol fermentor could be used directly without any pretreatment to achieve multifold increases of succinic acid production in an adjacent fermentor using noncarbonate bases for pH control. The results obtained in experiments where NH_4OH was used for pH control also demonstrated that the concept was applicable

Table 2 Summary of succinic acid fermentation results without and with carbon dioxide from the adjacent ethanol fermentors.

Base for pH control	CO_2 from ethanol fermentor	Ethanol feedstock	Final succinic acid (SA) concentration (g/L)[a]	Final acetic acid (AA) concentration (g/L)[a]	SA/AA (mol/mol)	Total SA production (g)	Total glucose consumption (g)	SA yield (g SA/g glucose consumed)
Na_2CO_3	NO	-	45.3	3.9	5.9	199.3	240.1	0.83
Na_2CO_3	YES	Barley	44.2	2.8	8.0	200.5	233.1	0.86
NaOH	NO	-	4.7	0.64	3.7	18.3	107.6	0.17
NaOH	YES	Barley	32.2	3.4	4.8	146.2	172.0	0.85
KOH	NO	-	5.6	0.74	3.8	22.4	93.3	0.24
KOH	YES	Barley	33.6	2.3	7.4	148.0	170.1	0.87
NH_4OH	NO	-	18.6	1.2	7.9	75.4	228.4	0.33
NH_4OH	YES	Barley	51.4	8.1	3.2	230.6	324.8	0.71
NH_4OH	YES	Corn	45.2	5.7	4.0	196.7	269.5	0.73

[a] Averages of results obtained in duplicated experiments

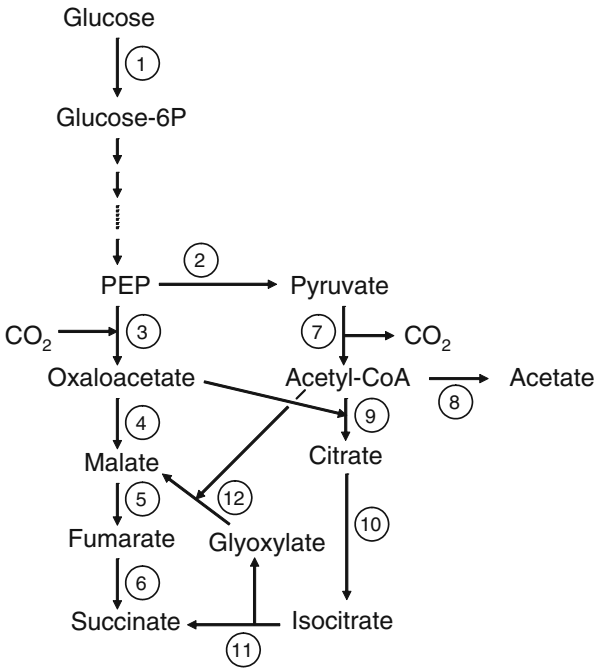

Fig. 7 Succinic acid biosynthetic pathway in *E. coli* AFP184 [7]. Not all steps of glycolysis are shown. Key enzymes in the pathway are as follows: [1] glucokinase, [2] pyruvate kinase, [3] phosphoenolpyruvate (PEP) carboxylase, [4] malate dehydrogenase, [5] fumarase, [6] fumarate reductase, [7] pyruvate dehydrogenase complex, [8] phosphoacetyl transferase, [9] citrate synthase, [10] aconitase, [11] isocitrate lyase, and [12] malate synthase

to both barley and corn ethanol fermentation. In the experiments performed by other investigators, purified CO_2 gas was used. In our experiments, we used the CO_2 produced in an ethanol fermentor, which might contain low levels of impurities such as moisture, oil, acetaldehyde, and others. Although we have demonstrated that the CO_2 could be used directly, experiments may need to be performed to investigate whether the use of the CO_2 from an ethanol fermentor that has gone through some cleaning process would result in even further improvement over its direct use. In our experiments, because of the experimental setup, the CO_2 was provided to the succinic acid fermentors for only the first 48 h. In a typical dry-grind ethanol plant using corn at 30% total dry solids, a batch fermentation process normally lasts approximately that long. However, commercial ethanol fermentation is a continuous operation. Thus, CO_2 would be available throughout the course of succinic acid fermentation. Sparging CO_2 into the succinic acid fermentor throughout the entire course of the fermentation may result in even better improved succinic acid production. In addition to the economic benefits toward production of an important industrial chemical in a biorefinery, the use of CO_2 produced in an ethanol fermentor for succinic acid production also has significant environmental impacts. The incorporation of CO_2 into succinic acid synthesis will help remove significant quantities of this greenhouse gas from escaping to the atmosphere. It has been shown that 61% of the PEP partitioned to OAA and 39% partitioned to pyruvate during succinate synthesis in *E. coli* strain AFP111 [24]. Assuming this would also be true in the case of strain AFP184, which had many physiological and biochemical characteristics similar to strain AFP111, it could be

calculated that about 8% of the CO_2 sparged into the succinic acid fermentor using NH_4OH for pH control was incorporated into succinic acid synthesis. In an ethanol plant producing 50 million gallons per year, this will be equivalent to about 11,000 MT per year of carbon dioxide being removed from the atmosphere.

Conclusion

It has been demonstrated that carbon dioxide produced in an ethanol fermentor can be used directly for succinic acid production in an adjacent fermentor without any treatment required for removal of impurities. The experiments were performed with corn and barley as feedstocks for ethanol fermentation. It is expected that similar results also can be obtained with other feedstocks. The results indicated the feasibility of an integrated biorefinery where production of both fuels (ethanol) and industrial chemicals (succinic acid) can be implemented with a byproduct in one process being used to benefit another process. Additional research is still needed to obtain data for process development and optimization at bench scale and for scale-up demonstration before commercial implementation is considered.

Acknowledgement The authors would like to thank Gerard Senske and John Minutolo for skillfully performing the fermentation experiments and Michael Kurantz for the compositional analysis of the barley and corn used in this study. The many invaluable comments and suggestions of Dr. Susanne Kleff of MBI International, Lansing, MI, given during the review of the first draft of the manuscript also are greatly appreciated.

References

1. Werpy, T., & Petersen, G. (2004). *Top value added chemicals from biomass*, U.S. Department of Energy.
2. Fumagalli, C. (1997). In J. I. Kroschwitz & M. Howe-Grant (Eds.), *Kirk-Othmer encyclopedia of chemical technology Vol. 22* (pp. 1072–1102). New York, NY: Wiley.
3. Rao, V. N. M. (1988). U.S. Patent 4,782,167.
4. Mabry, M. A. (1985). U.S. Patent 4,550,185.
5. Berglund, K. A., Dunuwila, D. D., Alizadeh, H. (2003). U. S. Patent 6,623,657.
6. Berglund, K. A., Dunuwila, D. D., Alizadeh, H. (2003). U.S. Patent 6,635,188.
7. Glassner, D. A., & Datta, R. (1992). U.S. Patent 5,143,834.
8. Guettler, M. V., Jain, M. K., Soni, B. K. (1996). U.S. Patent 5,504,004.
9. Nghiem, N. P., Donnelly, M., Millard, C. S. (1999). U.S. Patent 5,869,301.
10. Donnelly, M., & Nghiem, N. P. (2004). U.S. Patent 6,743,610.
11. Lin, H., Bennett, G. N., & San, K. Y. (2005). *Biotechnology and Bioengineering, 90*, 775–779.
12. Lee, S. J., Song, H., & Lee, S. Y. (2006). *Applied and Environmental Microbiology, 72*, 1939–1948.
13. Okino, S., Inui, M., & Yukawa, H. (2005). *Applied Microbiology and Biotechnology, 68*, 475–480.
14. Nghiem, N. P., Davison, B. H., Donnelly, M. I., Tsai, S. -P., Frye, J. G. (2000). Chemicals and materials from renewable resources. In J. J. Bozell (Ed.), American chemical society symposium series 784:160–173.
15. Millard, C. S., Chao, Y.-P., Liao, J. C., & Donnelly, M. I. (1996). *Appl Environmental Microbiology, 62*, 1808–1810.
16. Vemuri, G. N., Eiteman, M. A., & Altman, E. (2002). *Journal of Industrial Microbiology & Biotechnology, 28*, 325–332.
17. Anderson, C., Hodge, D., Berglund, K. A., & Rova, U. (2007). *Biotechnology Progress, 23*, 381–388.
18. Anderson, C., Helmerius, J., Hodge, D., Berglund, K. A., & Rova, U. (2009). *Biotechnology Progress, 25*, 116–123.

19. Drapcho, C. M., Nghiem, N. P., & Walker, T. H. (2008). *Biofuels engineering process technology Chapter 5* (pp. 105–195). New York: McGraw-Hill.
20. Schill, S. R. (2008). *Ethanol producer magazine*, October Issue.
21. Gottschalk, G. (1986). *Bacterial metabolism, Chapter 5* (2nd ed., pp. 127–129). New York: Springer-Verlag.
22. Zeikus, J. G., Jain, M. K., & Elankovan, P. (1999). *Applied and Environmental Microbiology, 51*, 545–552.
23. McKinlay, J. B., Vieille, C., & Zeikus, J. G. (2007). *Applied and Environmental Microbiology, 76*, 727–740.
24. Lu, S., Eiteman, M. A., & Altman, E. (2009). *Journal of Industrial Microbiology & Biotechnology, 36*, 1101–1109.