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Production of Succinic Acid by *Anaerobiospirillum succiniciproducens*

NHUAN P. NGHIEM,* BRIAN H. DAVISON, BRUCE E. SUTTLE, AND GERALD R. RICHARDSON

Chemical Technology Division Oak Ridge National Laboratory P.O. Box 2008, MS-6226 Oak Ridge, TN 37831-6226

ABSTRACT

The effect of an external supply of carbon dioxide and pH on the production of succinic acid by *Anaerobiospirillum succiniciproducens* was studied. In a rich medium containing yeast extract and peptone, when the external carbon dioxide supply was provided by a 1.5*M* Na_2CO_3 solution that also was used to maintain the pH at 6.0, no additional carbon dioxide supply was needed. In fact, sparging CO_2 gas into the fermenter at 0.025 L/L-min or higher rates resulted in significant decreases in both production rate and yield of succinate. Under the same conditions, the production of the main by-product acetate was not affected by sparging CO_2 gas into the fermenter. The optimum pH (pH 6.0) for the production of succinic acid was found to be in agreement with results previously reported in the literature. Succinic acid production also was studied in an industrial-type inexpensive medium in which light steep water was the only source of organic nutrients. At pH 6.0 and with a CO_2 gas sparge rate of 0.08 L/L-min, succinate concentration reached a maximum of 32 g/L in 27 h with a yield of 0.99 g succinate/g glucose consumed.

Index Entries: Succinic acid; fermentation; renewable resources; corn sugars; *Anaerobiospirillum succiniciproducens*.

INTRODUCTION

Succinic acid has been used for applications in many areas, including agriculture, food, medicine, plastics, cosmetics, textiles, plating, and waste-gas scrubbing *(1)*. Catalytic processes have recently been developed for the conversion of succinic acid to a number of industrially important chemicals

*Author to whom all correspondence and reprint requests should be addressed.

that include 1,4-butanediol, tetrahydrofuran and gamma-butyrolactone *(2,3)*. This new development has rendered the market for succinic acid much larger.

Succinic acid currently is produced commercially by chemical processes *(1)*. A fermentation process for its production is of great interest because in such processes, renewable resources such as corn-derived glucose can be used as starting material. There is not a current biological process for the commercial production of succinic acid, although a number of patents have been issued on the production of succinic acid by micro-organisms *(4–8)*.

Succinic acid is an intermediate of the tricarboxylic acid cycle and also is a product of anaerobic metabolism *(9)*. As such, its accumulation in fermentation broth has been observed with a number of micro-organisms, which were both aerobes and anaerobes *(4–8,10–12)*. The anaerobic bacterium, *Anaerobiospirillum succiniciproducens*, is considered among the best succinic acid producers. It has been observed that the main products of fermentation of this organism included succinic and acetic acids; other products included lactic acid and ethanol. Based on these observations, a biochemical pathway for the synthesis of succinic acid by *A. succiniciproducens* was proposed. The proposed pathway involved the conversion of phosphoenolpyruvate (PEP) to oxaloacetate by a carbon dioxide-fixing enzyme, PEP-carboxykinase *(13)*. It has been shown that extracellular supply of carbon dioxide was needed for succinic acid synthesis *(7,13)*.

Our efforts to develop a biological process for the production of succinic acid by *A. succiniciproducens* have focused on the establishment of process conditions and the development of an inexpensive fermentation medium. Some of the conditions for the production of succinic acid by *A. succiniciproducens* have been reported by Datta *(7)*. The author studied succinic acid production in a one-L fermenter and reported on the effects of pH and CO_2 gas sparge. However, only end-point results were reported. In addition, only one CO_2 gas sparge rate of 0.01 mL/min was examined. Therefore, it was decided to re-examine the effects of pH and CO_2 gas sparge rates during the course of the fermentations. In this investigation, wider CO_2 gas sparge rates were used. The use of light steep water, which is an inexpensive source of organic nutrients, in the fermentation medium was also investigated. The development of the light steep water medium was still in its very early stage, and therefore, only preliminary results are reported.

MATERIALS AND METHODS

The culture of *A. succiniciproducens* (ATCC 53488) was provided by Michigan Biotechnology Institute. The stock culure was prepared and stored in 25% glycerol at –70°C as described earlier *(14)*.

To prepare inoculum for fermentation experiments, one glycerol vial was used to inoculate a serum bottle containing 100 mL medium. The inoculum medium that has been described by Datta *(7)* contained 20 g/L

glucose, 10 g/L polypeptone(Difco), 5 g/L yeast extract (Difco), 3 g/L K_2HPO_4, 1 g/L NaCl, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2{\cdot}2H_2O$, and 0.2 g/L $MgCl_2{\cdot}6H_2O$. The glucose-free medium was heat-sterilized and allowed to cool to ambient temperature before 1 mL of 0.03*M* Na_2CO_3 and 0.15 mL of 0.18*M* H_2SO_4 were added. Glucose then was added as a 20% solution to bring its concentration to 20 g/L. Finally, 0.5 mL of a solution containing 0.25 g/L cystein.HCl and 0.25 g/L $Na_2S{\cdot}9H_2O$ was added and 20 min was allowed for the reduction of the medium before the serum bottle was inoculated with the entire contents of the glycerol vial. The glucose, sodium carbonate, sulfuric acid, and cystein-sodium sulfide solutions were all heat-sterilized. The serum bottle was incubated with gentle shaking at 39°C. The contents of the serum bottle were used to inoculate the fermenter when the residual glucose dropped to about 10 g/L; this normally took about 14 to 16 h. In each experiment, 45 mL broth from the serum bottle was used for inoculation.

All fermentations were batch and performed in 1-L Virtis Omni fermenters. Two fermentation media were used. The composition of the medium that was used to study the effect of pH and the level of external carbon dioxide supply was a slight modification of the one described by Datta (*7*). With the exception of 50 g/L glucose and 5 g/L $(NH_4)_2SO_4$, and the addition of 5 mg/L $FeSO_4{\cdot}7H_2O$, other components were the same as described in the previous paragraph for the inoculum medium. All the ingredients, except glucose and the iron salt, were dissolved in 875 mL deionized water, transferred to the fermenter, autoclaved, and allowed to cool to ambient temperature. To the fermenter then were added 100 mL of 50% glucose, 1 mL of 0.5 g/L $FeSO_4{\cdot}7H_2O$, 20 mL of 1.5*M* Na_2CO_3, 1.5 mL concentrated H_2SO_4, and 5 mL of 0.25 g/L cystein HCl, and 0.25 g/L $Na_2S{\cdot}9H_2O$. All these solutions were heat-sterilized prior to being added to the fermenter. In the light steep water medium, both yeast extract and peptone were replaced by 100 mL of light steep water that was obtained from the A. E. Staley corn processing plant in Loudon, TN.

The temperature was maintained at 39°C, which was the temperature used by Datta (*7*). The pH was controlled by adding a 1.5*M* Na_2CO_3 solution on demand. This solution also served as an external source of carbon dioxide. In the experiments performed to study the effect of additional external carbon dioxide supply, pure CO_2 gas was sparged into the fermenter at 0.025, 0.05, and 0.1 L/min. The pH in these experiments was maintained at 6.0. The effect of pH on succinic acid production was studied at five pH values, which were 5.0, 5.5, 6.0, 6.5, and 7.0. CO_2 gas was not sparged into the fermenter in these experiments, therefore, the 1.5*M* Na_2CO_3 solution added for pH control was the only source of external carbon dioxide. Samples were withdrawn at intervals and analyzed for cell growth, residual glucose, succinate and acetate concentrations.

Growth was monitored by measuring optical density at 660 nm with a Milton Roy Spectronic 21D. Glucose was measured with a Yellow

Springs Instrument 2700 Select glucose analyzer. Succinic and acetic acids were determined by gas chromatography using the method developed by Playne *(15)*. A Varian 3700 gas chromatograph equiped with a flame ionization detector and a Chromosorb 101 column maintained at 200°C was used. The carrier gas was helium flowing at 50 mL/min. The injector and detector were maintained at 250°C. Sample was prepared by mixing 500 µL fermentation broth with 100 µL 25% metaphosphoric acid; the mixture then was centrifuged on an Eppendorf microcentrifuge at 12000 rpm for 2 min and the supernatant used for analysis. The injection volume was 2 µL. The integrator was a Hewlett Packard 3396 Series II.

RESULTS AND DISCUSSION

In a previous report on the preliminary results of the effect of biotin on the production of succinic acid by *A. succiniciproducens (14)*, it was shown that the final concentration of succinic acid in a fermentation medium containing polypeptone and yeast extract as organic nitrogen sources could be improved by 17% by adding 50 mg/L biotin. It was also pointed out that this was not the optimal concentration. Since the optimal biotin concentration had not been determined yet, it was decided to omit biotin from the polypeptone-yeast extract fermentation medium used in the present investigation.

During the course of all fermentations, the broth volumes in the fermenters increased owing to Na_2CO_3 addition for pH control. The final volumes were from 1.2 to 1.3 L. These volumes were used in the calculations of the yield of succinic acid and acetic acid.

The effect of CO_2 sparge rates is shown in Fig. 1 and summarized in Table 1. The rate of glucose consumption did not seem to be affected by sparging CO_2 gas into the fermenter at 0.025 L/min (Fig. 1A). However, at higher CO_2 sparge rates, the rate of glucose consumption was significantly decreased. In the experiment having the CO_2 sparge rate set at 0.025 L/min, all of the initial glucose was depleted in 23 h; in the control experiment (no CO_2 sparge), although the consumption of glucose was not complete, the glucose concentration remaining at 23 h was only 2.3 g/L. When the CO_2 sparge rates were increased to 0.05 and 0.1 L/min, the glucose concentrations at 23 h were 10.9 and 27.5 g/L, respectively. The authors' results were not in agreement with Datta's results *(7)*. Datta found that when CO_2 gas was sparged into the fermenter at 0.01 L/min, complete utilization of glucose occured at 38 h, whereas in the control experiment (no CO_2 gas sparge), only 71% of the initial glucose was consumed at 40 h. However, in his control experiment, the initial glucose concentration was 54 g/L, whereas in the other experiment it was 47.5 g/L. The high glucose concentration used initially might have caused a long lag before the fermentation took off.

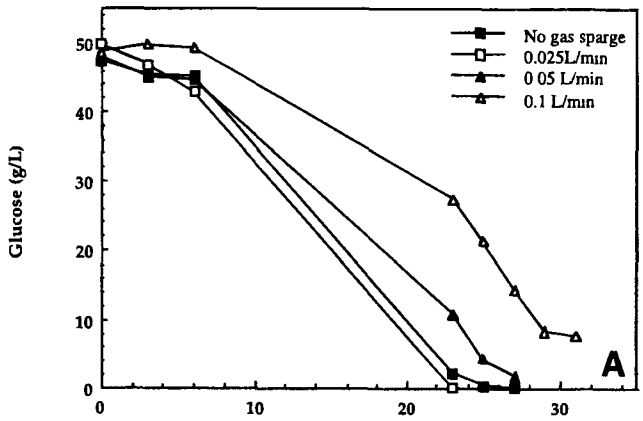

Fig. 1A. The effect of CO_2 gas sparge rates on glucose consumption at pH 6.0 in yeast extract-polypeptone medium.

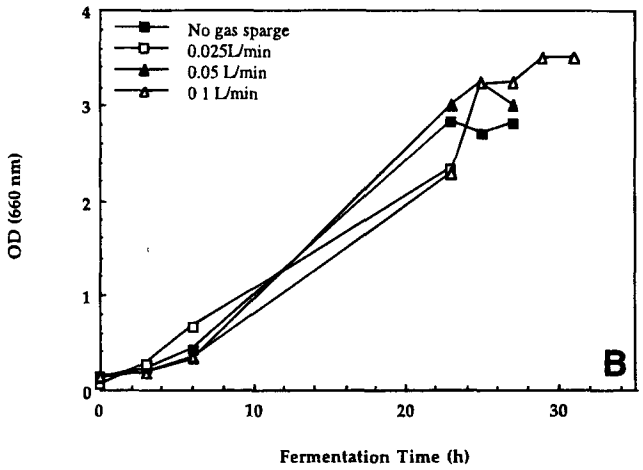

Fig. 1B. The effect of CO_2 gas sparge rates on cell growth at pH 6.0 in yeast extract-polypeptone medium.

The sparge of CO_2 gas into the fermenter did not affect the cell growth rate (Fig. 1B). The cell yield also was not affected by the CO_2 sparge rates up to 0.05 L/min. However, an increase in cell yield was observed when the CO_2 sparge rate was increased to 0.1 L/min.

The sparge of CO_2 gas into the fermenter adversely affected the rate of succinic acid production (Fig. 1C). The succinic acid concentration obtained in the control experiment at 23 h was 34.9 g/L. When CO_2 gas was sparged into the fermenter at 0.025, 0.05, and 0.1 L/min, the succinic acid concentrations at 23 h were 28.0, 22.1, and 12.5 g/L, respectively. The sparge of CO_2 had a slightly different effect on the yield of succinic acid. The succinic acid yield calculated at the exhaustion of glucose in the control experiment was 0.93 g/g glucose consumed. When CO_2 was

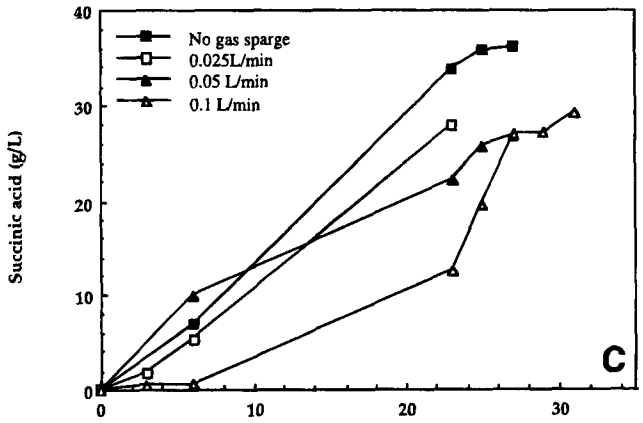

Fig. 1C. The effect of CO_2 gas sparge rates on succinic acid production at pH 6.0 in yeast extract-polypeptone medium.

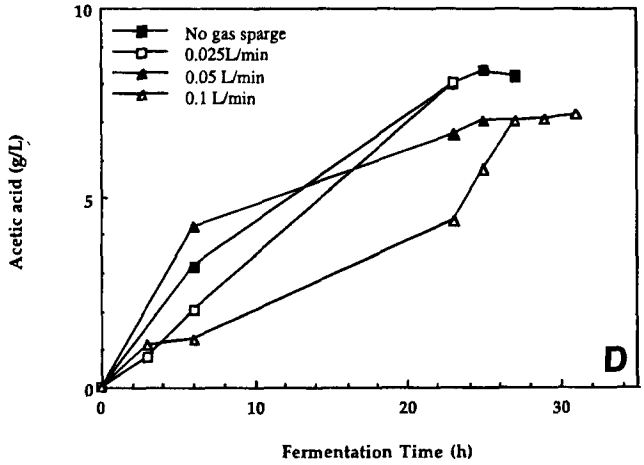

Fig. 1D. The effect of CO_2 gas sparge rates on acetic acid production at pH 6.0 in yeast extract-polypeptone medium.

sparged into the fermenter at 0.025 and 0.05 L/min, the succinic acid yields at glucose exhaustion dropped to 0.69 and 0.71 g/g glucose consumed, respectively. However, the succinic acid yield when the CO_2 sparge rate was increased to 0.1 L/min increased to 0.88 g/g glucose consumed. It should be pointed out that this yield result was calculated at the end of the experiment when the residual glucose concentration was still 7.7 g/L.

The effect of CO_2 sparge on the rate of acetic acid production followed a pattern similar to the one observed for the rate of glucose consumption, i.e., the gas sparge rates had to be increased above 0.025 L/min before a significant decrease in the production rate of acetic acid could be seen (Fig. 1D). The sparge of CO_2 gas into the fermenter, how-

Table 1
Effect of CO_2 Gas Sparge Rates on Succinic Acid and Acetic Acid Production at pH 6.0

CO_2 gas sparge rate (L/min)	0	0.025	0.05	0.1
Succinic acid yield (g/g glucose consumed)	0.93	0.69	0.71	0.88
Acetic acid yield (g/g glucose consumed)	0.21	0.20	0.19	0.21
Succinic acid:acetic acid (mole:mole)	2.23	1.77	1.94	2.08

Note: The results for the CO_2 gas sparge rate of 0.1 L/min were calculated at the end of the experiment when the residual glucose concentration was 7.7 g/L. All other results were calculated at the exhaustion of glucose.

ever, did not affect the acetic acid yield; in all four cases, the yield was unchanged at about 0.2 g/g glucose consumed. The net result of the negative effect on the succinic acid yield and the no-effect on the acetic acid yield was the highest molar ratio of succinic acid:acetic acid of 2.2 obtained in the control experiment.

The effect of pH is shown in Fig. 2 and summarized in Table 2. Highest glucose consumption rate was obtained at pH 6.0. At pH values both above and below 6.0, the rate of glucose consumption was significantly decreased. At pH 5.0, glucose was not consumed at all (Fig. 2A). The growth of cells followed the same pattern (Fig. 2B). Similar results were obtained for the production of succinic and acetica acids (Figs. 2C, D). For both products, the highest production rate was observed at pH 6.0. Maximum succinic acid yield also was obtained at this pH. At pH values above and below 6.0, the succinic acid yield was significantly decreased. Our results are in agreement with those of Datta's patent *(7)*, in which it was reported that the succinic acid yield was significantly lowered at pH below and above 6.0. Samuelov et al. *(13)* also observed that the production of succinic acid was significantly lower at pH 7.2 than at pH 6.2. However, Datta *(7)* reported significant production of lactic acid (more than 20 g/L) at pH values above 6. In our study, very little lactic acid (less than 5 g/L) was produced. pH did not seem to affect the acetic acid yield. At all pH values studied, except pH 5.0 at which no glucose consumption

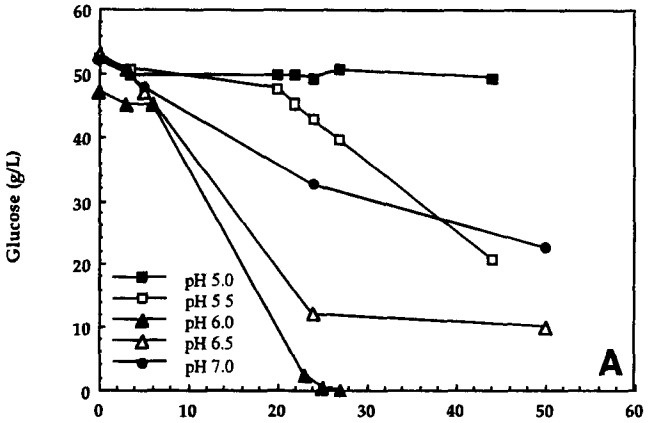

Fig. 2A. The effect of pH on glucose consumption in yeast extract-polypeptone medium without CO_2 gas sparge.

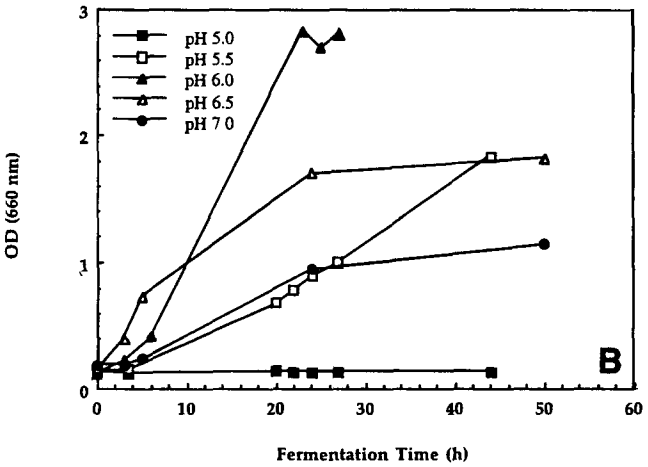

Fig. 2B. The effect of pH on cell growth in yeast extract-polypeptone medium without CO_2 gas sparge.

was observed, the acetic acid yield was unchanged ai about 0.2 g acetic acid/g glucose consumed.

The results of succinic acid fermentation in the light steep water medium are shown in Fig. 3. In this study, a CO_2 sparge rate of 0.08 L/min, which was an intermediate value of the two highest CO_2 sparge rates used in the study of succinic acid production in the polypeptone-yeast extract medium, was used. Good production of succinic acid was observed. At 23 h, 24.5 g/L succinic acid was produced. This compared favorably with the succinic acid concentration of 22.1 and 12.5 g/L obtained in the polypeptone-yeast extract medium when the CO_2 sparge rates were 0.025 and 0.5 L/min, respectively. A maximum succinic acid concentration of 32.2 g/L was obtained at 27 h. This was equivalent to a productivity of 1.2 g/L-h.

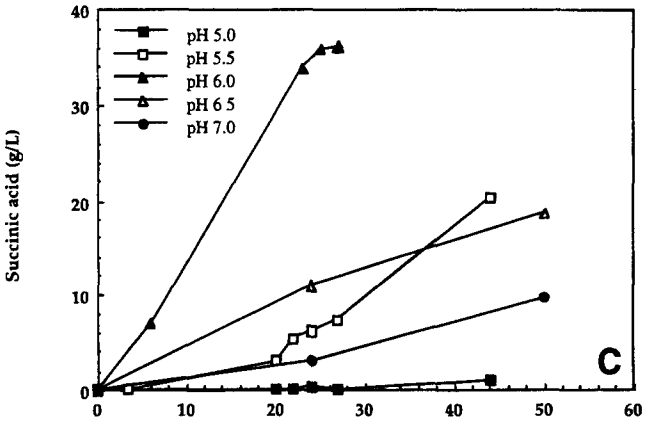

Fig. 2C. The effect of pH on succinic acid production in yeast extract-polypeptone medium without CO_2 gas sparge.

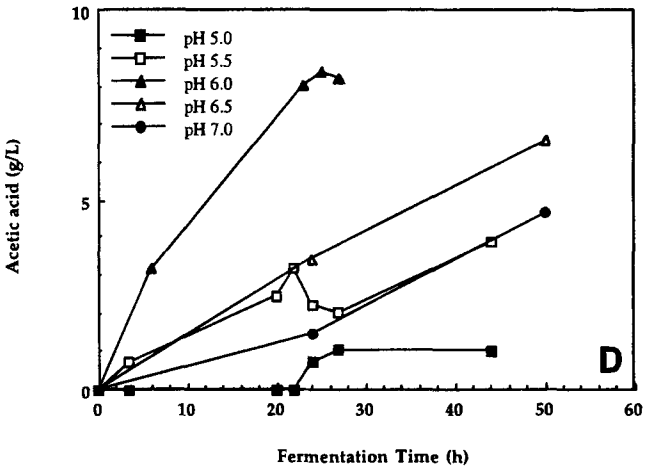

Fig. 2D. The effect of pH on acetic acid production in yeast extract-polypeptone medium without CO_2 gas sparge.

The yield calculated at maximum succinic acid concentration was 0.99 g succinic acid/g glucose consumed and the molar ratio of succinic acid:acetic acid was 1.9. Since the inoculum was raised in the polypeptone-yeast extract medium, there obviously were questions on the effect of nutrient carry-over. The concentrations of polypeptone and yeast extract carried over from the inoculum into the light steep water medium in the fermenter were 0.5 and 0.25 g/L, respectively. In our early study of *A. succiniciproducens* fermentation, it was observed that even with 20 g/L glucose, succinic acid could not be produced beyond 5 g/L at those levels of polypeptone and yeast extract. Therefore, the contribution of nutrients by these two organic nitrogen sources toward succinic acid production in the light steep water medium was insignificant.

Table 2
Effect of pH on Succinic Acid and Acetic Acid Production Without CO_2 Gas Sparge

pH	5.5	6.0	6.5	7.0
Succinic acid yield (g/g glucose consumed)	0.74	0.93	0.53	0.41
Acetic acid yield (g/g glucose consumed)	0.15	0.21	0.19	0.20
Succinic acid:acetic acid (mole:mole)	2.65	2.23	1.45	1.05

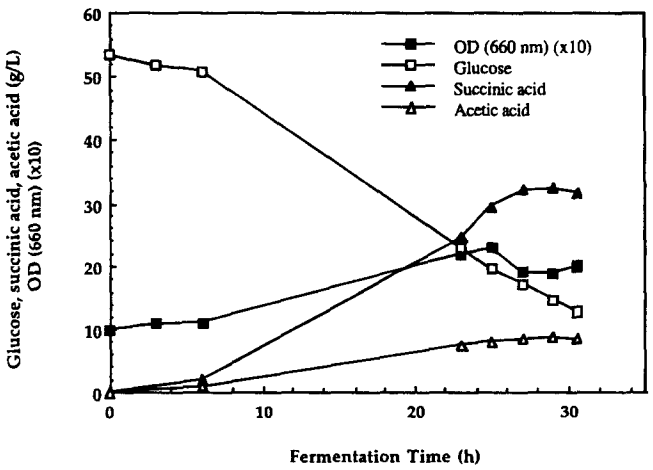

Fig. 3. Concentration profiles of OD(660), glucose, succinic acid, and acetic acid in light steep water medium at pH 6.0 and CO_2 gas sparge rate of 0.08 L/min.

The results showed that light steep water could replace yeast extract and polypeptone in the production of succinic acid by *A. succiniciproducens*. Light steep water is a waste product in a corn processing plant and normally is available at no cost. However, its use in a succinic acid fermentation process will be beneficial only if the succinic acid manufacturing plant is located next to the corn processing plant. The transportation costs of light steep water to a distant location will add significantly to the manufacturing costs of succinic acid. Its use for the production of succinic acid in this case will not be economical.

CONCLUSION

The effect of external supply of carbon dioxide and pH on the production of succinic acid by *A. succiniciproducens* have been studied. The following conclusions can be made:

1. In the yeast extract-peptone medium, when the external carbon dioxide suppy was provided by a 1.5*M* Na_2CO_3 solution that also was used to maintain the pH at 6.0, no additional carbon dioxide supply was needed. In fact, sparging CO_2 gas into the fermenter at 0.025 L/min or higher rates resulted in significant decreases in both production rate and yield of succinate.
2. Under the same conditions, the production of acetate was not affected by sparging CO_2 gas into the fermenter.
3. The optimum pH (pH 6) for the production of succinic acid was found to be in agreement with previously reported results.
4. Succinic acid could be produced in an industrial-type inexpensive medium in which light steep water was the only source of organic nutrients. Under the conditions studied, succinate concentration reached a maximum of 32.2 g/L in 27 h with a yield of 0.99 g succinate/g glucose consumed.

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The light steep water was a gift from the A. E. Staley corn processing plant in Loudon, TN.

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