Scientific Note

The Effect of Biotin on the Production of Succinic Acid by
*Anaerobiospirillum succiniciproducens**

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INTRODUCTION

Succinic acid is an intermediate of the tricarboxylic acid (TCA) cycle, and therefore, is found in almost all plant and animal cells, albeit at very low concentrations. It has a very wide usage range, which includes applications in agriculture, food, medicine, plastics, cosmetics, textiles, plating and waste-gas scrubbing (1).

Succinic acid currently is produced commercially by chemical processes. A fermentation process for its production is of great interest because in such process, 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.

Extensive efforts have been devoted to the isolation and screening of succinic acid-producing microorganisms. The anaerobic bacterium, *Anaerobiospirillum succiniciproducens*, is considered among the best direct succinic acid producers. A number of patents concerning the production of succinic acid by this organism have been issued (2-5).

Our first attempt to develop a biological process for the production of succinic acid by *A. succiniciproducens* involved fermentation media improvement, in particular the use of supplemented nutrients. In this note, we show that higher yield of succinic acid could be achieved by supplementing the fermentation media with biotin, as a potential nutrient supplement representative.

MATERIALS AND METHODS

The culture of *A. succiniciproducens* was provided by Michigan Biotechnology Institute (MBI). To prepare stock culture for subsequent experiments, the original MBI culture was grown in a 100-mL serum bottle and then used to inoculate a two-liter fermenter following the procedure which is described below. At 24 h, when the culture in the fermenter was in its logarithmic growth phase, one milliliter aliquots were removed from the fermenter and injected into vials containing equal volume of 50% sterile and oxygen-free glycerol. The vials were shaken gently to mix their contents and then stored in a -70°C freezer.

To prepare inoculum for fermentation experiments, one glycerol vial was removed from the freezer, thawed, and then used to inoculate a serum bottle containing 100 mL media. The inoculum media which have been described by Datta (4) contained 20 g/L glucose, 10 g/L polypeptone, 5 g/L yeast extract, 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 media were 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 minutes were allowed for the reduction of the media before the serum bottle

was inoculated with the full contents of the thawed 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 9 g/L. This normally took about 14 to 16 h.

All fermentation experiments were batch and performed in two-liter Virtis Omni fermenters. The composition of the fermentation media was a slight modification of the one described by Datta (4). The fermentation media contained 50 g/L glucose, 10 g/L polypeptone, 5 g/L yeast extract, 1 g/L K_2HPO_4 , 1 g/L NaCl, 5 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2 \cdot 2H_2O$, 0.2 g/L $MgCl_2 \cdot 6H_2O$, and 5 mg/L $FeSO_4 \cdot 7H_2O$. All the ingredients, except glucose and the iron salt, were dissolved in 875 mL de-ionized water, autoclaved, allowed to cool and aseptically transferred to the fermenter. 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 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 experiment for which the media were supplemented with biotin, the initial volume of de-ionized water used to dissolve the ingredients was 675 mL. Three hundred milliliters of de-ionized water was boiled to remove dissolved gases which included oxygen and allowed to cooled to about 50°C. Two hundred milliliters of this then was used to dissolve 50 mg biotin. The biotin solution was filter-sterilized and aseptically added to the fermenter.

After all the required solutions were added to the fermenters, the C sparge (100% C) was turned on and the flow rate was set at 0.25 L/min. The temperature was set at 39°C and pH at 6.0. The fermenters then were inoculated with 45 mL broth from the serum bottle. The pH was

maintained between 6.0 and 6.2 by addition of a 1.5 M Na_2CO_3 solution using a pH controller. Samples were withdrawn with a hypodermic syringe 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 (6). A Varian 3700 gas chromatograph equipped with an FID 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. Samples were prepared by mixing 500 μL with 100 μL 25% metaphosphoric acid. The injection volume was 2 μL . The integrator was a Hewlett Packard 3396 Series II.

RESULTS AND DISCUSSION

The effect of biotin on glucose consumption and cell growth is shown in Figs. 1 and 2, respectively. The base case results generally confirmed those previously reported (2-5). It can be seen that by supplementing the fermentation media with 50 mg/L biotin, a significant increase in glucose consumption and growth was observed. The effect of biotin on the production of succinic and acetic acids, which were the main products of *A. succiniciproducens* fermentation at pH 6 (7), is shown in Fig. 3. Biotin also increased the production of both acids. The molar ratio of succinate:acetate versus fermentation time is plotted in Fig. 4. The succinate:acetate ratio increased with time in both cases and reached a value of 2.3 at 29 h.

The fermentation results obtained at 29 h are summarized in Table 1. The results indicate that biotin improved the conversion efficiency of glucose from 81 to 92%. Total succinic acid production was increased from 33 g to 40 g, which represented a 20% improvement. During the

course of all fermentations, the broth volumes in the fermenters increased due to Na_2CO_3 addition for pH control. The final volume in the base case was 1.25 liters. Using this volume, the specific productivity was calculated to be 0.92 g/L·h. The final volume in the biotin-supplemented experiment was 1.30 liters. The specific productivity in this case was 1.07 g/L·h, which represented a 16% improvement over the base case. The overall yield, expressed as gram carbon in products (succinate and acetate) per gram carbon in glucose consumed, was higher than one in both cases. This was also observed by other investigators and attributed to the ability of the organism to incorporate externally supplied carbon dioxide into the fermentation products (7).

It should be noted that the biotin concentration used in the experiment for which the results are discussed was by no means the optimal concentration. This concentration has yet to be determined. The improvement of succinic acid production by biotin strongly suggests that further improvements are possible from optimization of the media. To make the commercial process economically feasible, a low-cost organic nitrogen source which contains biotin, for example, corn steep liquor (8) should be used to replace peptone and yeast extract in the fermentation media. The use of corn steep liquor is being investigated in our laboratory.

CONCLUSIONS

The effect of biotin on the production of succinic acid by *A. succiniciproducens* has been studied. Biotin improved glucose consumption, cell growth and succinic acid production. The specific productivity of succinic was improved by 16%. Biotin also increased the production of the by-product acetic acid. The molar ratio of succinate:acetate was not changed by biotin.

Table 1. Summary of fermentation results obtained at 29 hours.

	With Biotin	Without Biotin
Total succinate produced (g)	40.23	33.47
Total acetate produced (g)	9.47	7.33
Total glucose consumed (g)	43.10	37.23
Y/S (g succinate/ g glucose consumed)	0.93	0.90
Y/A (g acetate/ g glucose consumed)	0.22	0.20
Y/T (g carbon in products/g carbon in glucose consumed)	1.18	1.12
Glucose conversion efficiency (%)	92.1	81.0

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Fig. 1. The effect of biotin on glucose consumption

Fig. 2. The effect of biotin on cell growth

Fig. 3. The effect of biotin on succinate and acetate production

Fig. 4. The variation of succinate:acetate molar ratio with fermentation time

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