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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, although 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 a 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 article, we show that higher yield of succinic acid could be achieved by supplementing the fermentation media with biotin, as a potential nutrient supplement representative.

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MATERIALS AND METHODS

The culture of *A. succiniciproducens* (ATCC 53488) was provided by Michigan Biotechnology Institute (MBI, Lansing, MI). To prepare a stock culture for subsequent experiments, the original MBI culture was grown in a 100-mL serum bottle and then used to inoculate a 2-L fermentor following the procedure described below. At 24 h, when the culture in the fermentor was in its logarithmic growth phase, 1-mL aliquots were removed from the fermentor and injected into vials containing an 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 $Na_2S{\cdot}9H_2O$ were added, and 20 min 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 fermentor when the residual glucose dropped to about 9 g/L. This normally took about 14–16 h.

All fermentation experiments were batched and performed in 2-L Virtis Omni fermentors. 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 deionized water, autoclaved, allowed to cool, and aseptically transferred to the fermentor. To the fermentor 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, 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 fermentor.

In the experiment for which the media were supplemented with biotin, the initial volume of deionized water used to dissolve the ingredients was 675 mL. Three hundred milliliters of deionized water were boiled to remove dissolved gases, which included oxygen, and allowed to cool to about 50°C. Two hundred milliliters of this were then used to dissolve 50 mg biotin. The biotin solution was filter sterilized and aseptically added to the fermentor.

After all the required solutions were added to the fermentors, the CO_2 sparge (100% CO_2) 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 fermentors then were inoculated with 45 mL broth from the same 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.

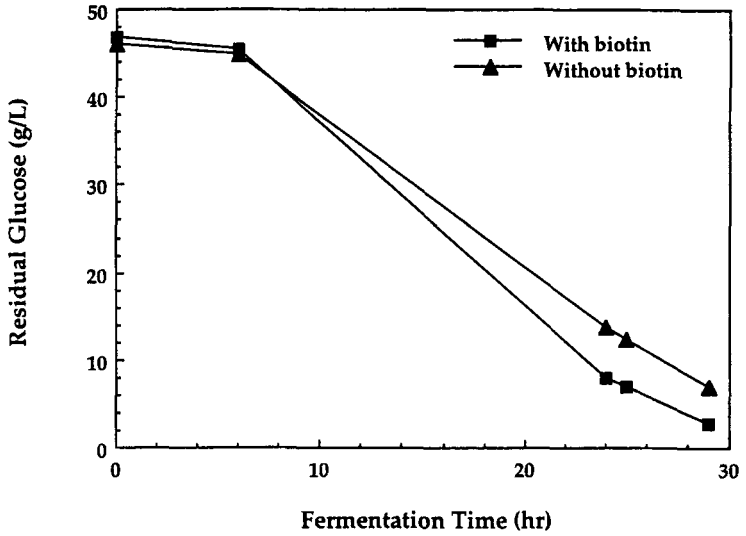

Fig. 1. The effect of biotin on glucose consumption.

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 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. 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 16% increase in glucose consumption was observed. In the case of cell growth, the increase was more modest at 7%. The effect of biotin on the production of succinic and acetic acids, which were the main products of *A. succiniciproducens* fermentation at pH 6.0 *(4)*, is shown in Fig. 3. Biotin also increased the production of both acids. Maximum concentration of succinic acid was increased by 17% and that of acetic acid was increased by 30%. The molar ratio of succinate:acetate vs 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 of glucose from 81 to 92%. Total succinic acid production was increased from 33–40 g, which represented a 20% improvement. In other side-by-side fermentations performed under the same conditions, in which equal volumes of inoculum from one single serum bottle were used to inoculate the two fermentors, the variation of the total production of suc-

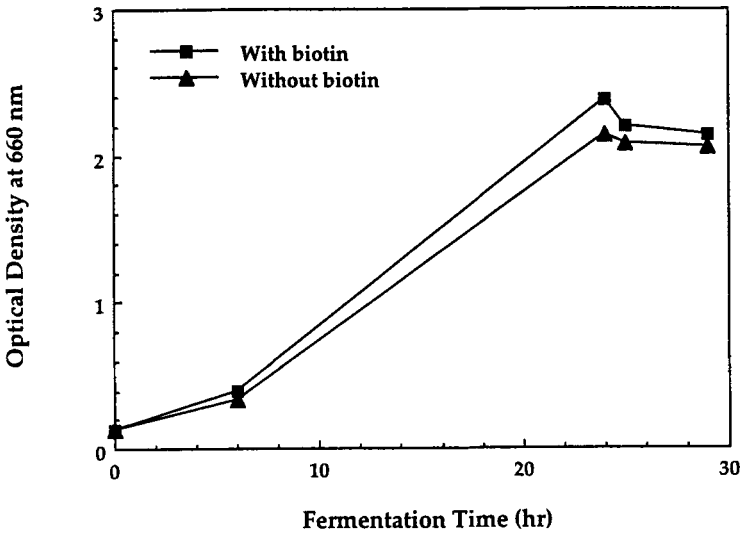

Fig. 2. The effect of biotin on cell growth.

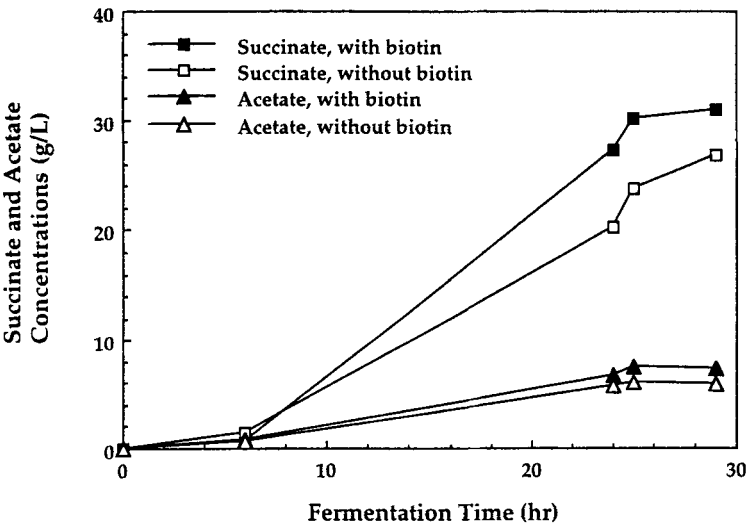

Fig. 3. The effect of biotin on succinate and acetate production.

cinic and acetic acids was <5% (results not shown). Therefore, the 20% improvement in the total production of succinic acid obtained when biotin was used was quite significant. During the course of all fermentations, the broth volumes in the fermentors increased owing to Na_2CO_3 addition for pH control. The final volume in the base case was 1.25 L. 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 L. 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 plus acetate) per gram carbon in glucose consumed, was higher than one in both cases. This could be very likely owing to the contribution of other carbon sources, such as yeast extract and peptone, in the fermentation medium.

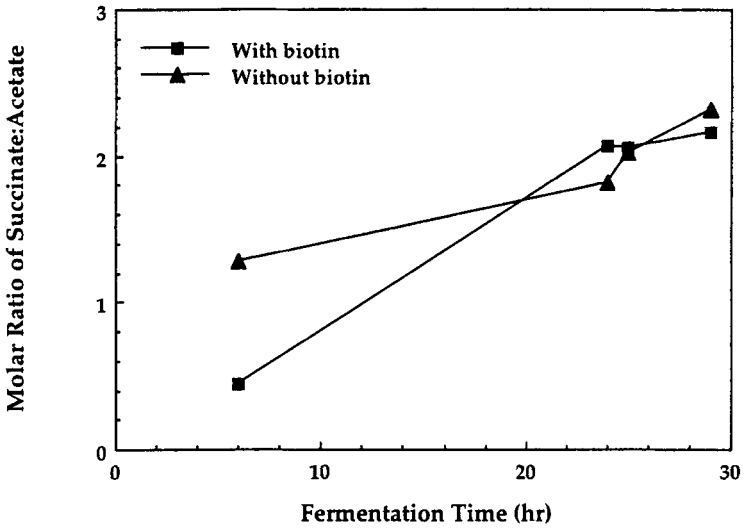

Fig. 4. The variation of succinate:acetate molar ratio with fermentation time.

Table 1
Summary of Fermentation Results Obtained at 29 h

Fermentation results	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 succinate and acetate/g glucose consumed	1.18	1.12
Glucose conversion, %	92.1	81.0

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. If the biotin concentration required can be reduced to the 100 μg/L range, a low-cost organic nitrogen source, containing biotin (e.g., corn steep liquor *[7]*), could be used as a source of biotin and to replace peptone and yeast extract in the fermentation media. Corn steep liquor typically contains biotin at about 1 mg/kg. If used at 10% (v/v), corn steep liquor would provide sufficient biotin, or at least, makes quite a significant contribution to the biotin

requirement. The only practical problem concerning the use of corn steep liquor as a source of biotin in a commercial fermentation process is that it will have to be sterilized by filtration instead of heating, since biotin may be destroyed in such a sterilization process. The use of corn steep liquor is being investigated in our laboratory.

CONCLUSION

The effect of biotin at 50 mg/L 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 acid was improved by 16%. Biotin also increased the production of the byproduct acetic acid. The molar ratio of succinate:acetate was not changed by biotin.

ACKNOWLEDGMENT

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REFERENCES

1. Winstrom, L. O. (1978), *Kirk-Othmer Encyclopedia of Chemical Technology*, vol. 21, Wiley, New York, pp. 848–864.
2. Lemme, C. J. and Datta, R. (1987), Eur. Pat. Appl. 249–773.
3. Glassner, D. A. and Datta, R. (1990), Eur. Pat. Appl. 389–103.
4. Datta, R. (1992), US Pat. 5143833.
5. Glassner, D. A. and Datta, R. (1992), US Pat. 5143834.
6. Playne, M. J. (1985), *J. Sci. Food Agriculture* **36**, 638–644.
7. Atkinson, B. and Mavituna, F. (1991), *Biochemical Engineering and Biotechnology Handbook*, Stockton, New York, p. 69.