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Production of Fumaric Acid by Immobilized Rhizopus Using Rotary Biofilm Contactor

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ABSTRACT

Rotary biofilm contactor (RBC) is a reactor consisting of plastic discs that act as supports for micro-organisms. The discs are mounted on a horizontal shaft and placed in a medium-containing vessel. During nitrogen-rich growth phase, mycelia of *Rhizopus oryzae* ATCC 20344 grew on and around the discs and formed the "biofilm" of self-immobilized cells on the surface of the plastic discs. During the fermentation phase, the discs are slowly rotated, and the biofilms are exposed to the medium and the air space, alternately. With RBC, in the presence of $CaCO_3$, *Rhizopus* biofilm consumes glucose and produces fumaric acid with a volumetric productivity of 3.78 g/L/h within 24 h. The volumetric productivity is about threefolds higher with RBC than with a stirred-tank fermenter with $CaCO_3$. Furthermore, the duration of fermentation is one-third of the stirred-tank system. The immobilized biofilm is active for over a 2-wk period with repetitive use without loss of activity.

Index Entries: Biofilm; calcium carbonate; calcium fumarate; immobilized *Rhizopus*; fumaric acid; *Rhizopus oryzae*; rotary biofilm contactor (RBC).

INTRODUCTION

Fumaric acid, a naturally occurring four-carbon dicarboxylic acid, is a normal intermediate in cell metabolism. Because of its double bond and two carboxylic groups, fumaric acid has many potential industrial uses;

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such as in the manufacturing of synthetic resins and biodegradable polymers *(1,2)*. It can also serve as an intermediate for chemical syntheses *(3)*. In addition to its use as feedstock for chemical synthesis, fumaric acid is commonly used as a food ingredient and beverage acidulant. Many studies show that fumaric acid reduces processing costs and improves the quality of many food products. There are only two major processes for fumaric acid production: chemical synthesis via the catalytic isomerization of petroleum-derived maleic anhydride or maleic acid *(3)*, and the biological production through the direct fermentation of glucose *(4,5)*. Currently, fumaric acid is produced exclusively using the synthetic route. Recently, however, the microbial production of fumaric acid has received increased attention because of its increased use in food industry.

Many species of micro-organisms produce small amounts of fumaric and other organic acids extracellularly as metabolic by-products during oxidative metabolism. In some cases, certain mycelial fungi are capable of producing significant quantities of fumaric acid from glucose. The most noticeable is the group of fungi belonging to the genus *Rhizopus (6)*. A few selected strains of *Rhizopus oryzae* (*R. arrhizus*) produce, almost exclusively, fumaric acid in high yield from glucose under specific cultural conditions *(7,8)*. The high yield of fumaric acid from glucose is believed to be a result of the ability of the fungi to fix CO_2 during the acid production stage *(9,10)*.

Currently, biological production of fumaric acid is too expensive to compete with the synthetic route *(11)*. Also, biological production is hindered by some technical drawbacks. For example, when *Rhizopus* produces fumaric acid, the pH of the fermentation broth decreases to a threshold point where the organism stops producing the acid and the metabolism shifts to produce ethanol and other products *(12)*. Final concentrations of free acid in the fermentation broth are typically very low. The most common approach to overcome the low concentration of acid in the fermentation broth is to continuously neutralize the acid produced by adding a base such as calcium carbonate. Consequently, the product that accumulates in the fermentation broth is calcium fumarate instead of fumaric acid. The recovery of calcium fumarate and the generation of free acid from calcium fumarate is difficult. Furthermore, when the fermentation is carried out with calcium carbonate as the neutralizing agent, calcium fumarate will precipitate because of its low solubility. It also causes an increase in liquid viscosity resulting in the stoppage of the agitator and thus, the entire fermentation operation *(8)*. If, however, sodium bicarbonate or other neutralizing agents were used in place of calcium carbonate, less fumarate is formed. This is because the accumulated sodium fumarate exhibited an inhibitory effect on fumaric acid production *(8,11)*. Another problem encountered is the need for a continuous supply of antifoam agent because of the tendency of excessive foaming during stirred tank fermentation.

In this research, self-immobilized *Rhizopus oryzae* mycelia were used to carry out repetitive fumaric acid fermentation in a rotary biofilm contactor (RBC) with the supplement of $CaCO_3$. The characteristics of biofilm during fermentation are also described.

MATERIALS AND METHODS

Micro-organism and Inoculum

Rhizopus oryzae ATCC 20344 was purchased from American Type Culture Collection (Rockville, MD). The culture was maintained on potato dextrose agar (Difco) slants and propagated by growing in Erlenmeyer flasks with potato dextrose agar to obtain sporangiospores. For fermentation studies, spores were obtained by washing spore-bearing cultures with sterile water, filtered through sterile filter paper and collected as spore suspension.

Medium

Growth medium contained: 10 g glucose, 2.0 g urea, 0.6 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, and 0.088 g $ZnSO_4 \cdot 7H_2O$ in one L distilled water *(6)*. The fermentation medium consisted of 100 g glucose, 1.0 g yeast extract, 0.6 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.088 g $ZnSO_4 \cdot 7H_2O$, in 1 L distilled water. $CaCO_3$ was added whenever necessary to maintain the pH at 5.0 *(6)*.

Rotating Biofilm Contactor (RBC)

The reactor is a 2 L fermentor with 0.9 L working liquid volume (100 mm in diameter and 260 mm in length). The reactor contains six plastic discs (125 cm^2/disc) that act as supports for the micro-organism. The discs, with a total surface area of 750 cm^2, are mounted on a horizontal shaft and placed in the fermentor containing growth medium. Mycelial fungi showed a strong tendency to grow on and around the solid plastic surface of the discs and to form the "biofilm" and immobilized on the surface of the plastic discs. During the operation, air was flowed into and out of the upper portion of air space at an air-flow rate of 1.0 L/L/min. The discs were rotated at 22 rpm and the biofilms were exposed alternately to the fermentation medium and the air space. Figure 1 shows the schematic diagram of the RBC.

Growth of *Rhizopus* Biofilm

The spore suspension (100 mL with spore concentration of 1×10^6/mL) was inoculated into RBC containing 800 mL of the growth medium. The pH during growth stage was maintained at 5 with a small addition of $CaCO_3$. After 60 h of incubation at 35°C with a rotating speed of 14 rpm, biofilm was grown on and around the surface of the plastic

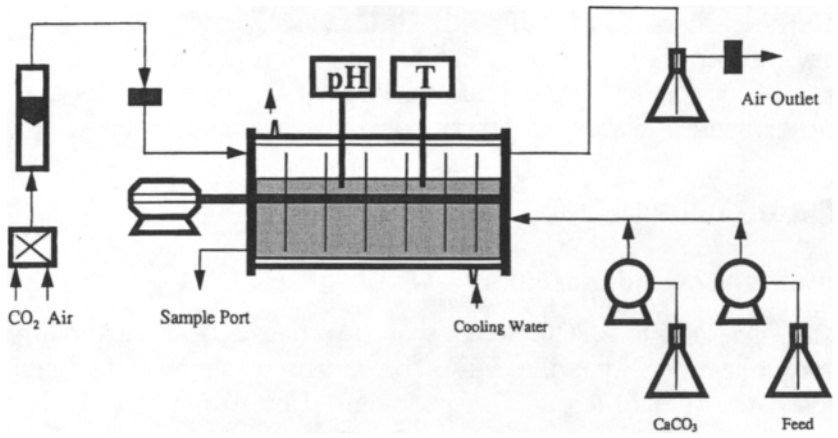

Fig. 1. Schematic diagram of rotary biofilm contactor.

discs. Then the broth in the reactor was changed from growth to nitrogen-deprived fermentation medium.

Analytical Methods

Fumaric acid, L-malic acid, succinic acid, and carbohydrates were determined and quantified by high-performance liquid chromatography (Hitachi Instrument, Tokyo, Japan, L-6200A) using a BioRad Aminex HPX-87H ion exclusion column (300 × 7.8 mm) with a refractive index Detector (Hitachi Instrument, L-3350 RI). The column was eluted with dilute sulfuric acid (0.005*M*) at a column temperature of 80°C and a flow rate of 0.8 mL/min over a 13-min period.

RESULTS AND DISCUSSION

Fumaric acid fermentation by *Rhizopus oryzae* can be separated into cell growth and product formation stages. During the aerobic growth in the presence of growth medium, *R. oryzae* grows with extended hyphae and forms large size pellets or mycelial aggregates. Once the cells are fully grown, the medium is changed to fermentation medium to encourage cells to produce fumaric acid from glucose. When fermentation was carried out in the conventional stirred tank fermentor, the growing mycelia adsorbed onto the heat exchanger and propellers and formed mycelial clumps. The fermentation time was long, and unwanted by-products, particularly ethanol, were formed. The conversion yield was low because of localized anaerobic condition and the limitation of mass transfer. In addition, when $CaCO_3$ is added, it will intermingle with mycelial aggregates and further complicate the problem. One method that can alleviate this problem is to grow the cells in the specially formulated growth medium to prevent large mycelial pellet formation before

subjecting the cells to the nongrowth fermentation medium *(6)*. Another method is to use reactors, such as RBC.

The biofilm formation in the RBC was conducted after inoculating *Rhizopus* sporangiospores into liquid growth medium. During incubation, with slow rotation of the plastic discs, the germinated spores with hyphae became attached to the surface of the plastic discs upon contact. They grew on and around the discs and eventually covered the entire surface of the discs (Fig. 2A, B) and formed a mycelial film of not more than 2 mm in thickness (Fig. 2C, D). Typically, it takes from 48 to 72 h for growing mycelia to cover the entire surface of the discs. A small amount of $CaCO_3$ was added during the growth stage to maintain the pH of the medium at about 5.0. After the completion of the formation of biofilm, the fermentation medium was introduced to replace the growth medium in order to carry out fumaric acid production. During the fermentation stage, biofilm was exposed to sterile air in the head space of RBC that enhances oxygen exposure and opportunity for CO_2 fixation by biofilm.

Typical results of RBC fermentation are shown in Fig. 3. During the operation, the discs were rotated at 22 rpm, slightly faster than during growth stage. The slower rotating speed used during the biofilm formation stage was to allow the mycelia to attach itself to the disc without too much friction. In the absence of $CaCO_3$, the fumaric acid concentration reached to a level of 6 g/L, which is the solubility of fumaric acid at the incubation temperature of 35°C. At this concentration, the pH of the broth was at 2.9. In the presence of $CaCO_3$, the pH of the broth was at 5.0 and the soluble calcium fumarate concentration reached 30 g/L. The excess calcium salt was crystallized as calcium fumarate and settled to the bottom of RBC. After about 24 h of operation, the glucose present was consumed entirely to produce approx 75.7 g/L of calcium fumarate. During fermentation, ethanol was produced and reached a maximum of 12g/L and stayed more or less unchanged throughout the entire course of operation. A small amount of glycerol, approx 5 g/L, was produced by this strain of *R. oryzae*. Different strains of *Rhizopus* produced different amounts of glycerol. *R. oryzae* ATCC 12702 produced twice as much glycerol as *R. oryzae* ATCC 20344 under identical fermentation conditions *(13)*.

An average weight yield of about 75% (74 g/L fumaric acid) was obtained from an initial average glucose concentration of 98.7 g/L. This is much higher than those in the stirred tank fermentation with $CaCO_3$. The weight yield of stirred tank fermentation was 65%. The time required for the completion of fermentation is 24 h, which is one-third the time required for the stirred tank fermentation. The most significant advantage of RBC fermentation is the increase of volumetric productivity and the weight yield of fumaric acid. The volumetric productivity (3.78 g/L/h) achieved using RBC is almost five times higher than that achieved using a stirred tank system (0.94 g/L/h) with neutralizing agents. Likewise, the weight yield is much higher in RBC than in a traditional fermentor (65%) with

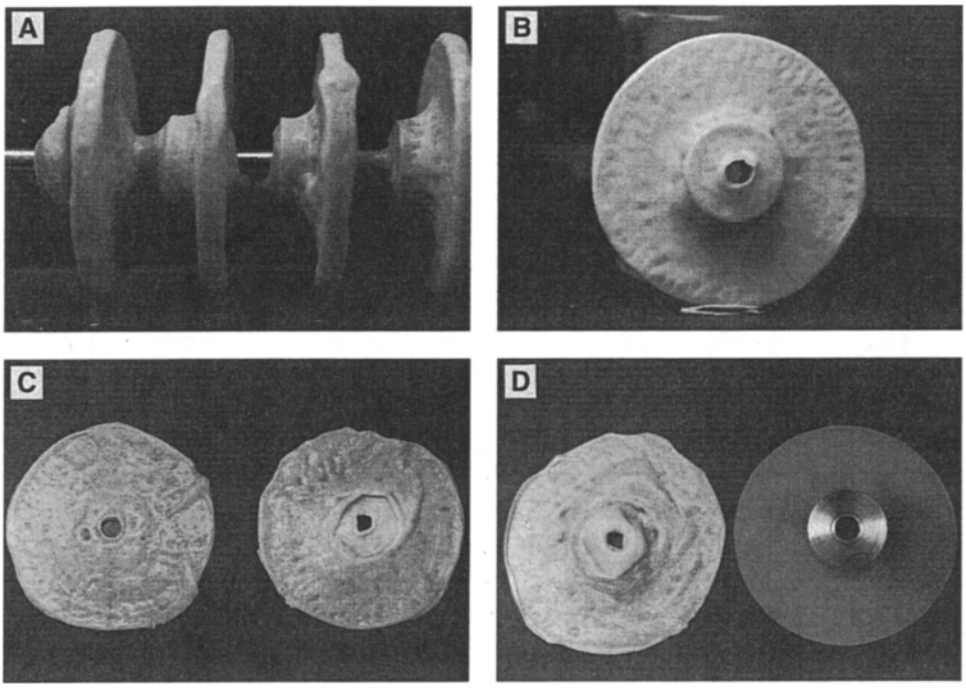

Fig. 2. *Rhizopus* biofilm. **a.** Fully grown biofilm on the discs that were attached to the horizontal shaft. **b.** Close-up of biofilm on a single disc. **c.** Peeled off biofilms. **d.** The naked disc with biofilm.

neutralizing agents *(8)*. Other significant advantages of RBC fermentation are the avoidance of foaming and the reusability of fungal mycelia. In stirred tank fermentation, the organisms can be used only once *(8)*.

Self-immobilized *Rhizopus* biofilm is stable during extended RBC operations. There is no observable deterioration of biofilm after 10 cycles of operation. Figure 4 shows the glucose consumption and fumaric acid production by biofilm after the repetitive fed-batch cycles. The glucose utilization rates were similar for the first three cycles of operation. During the fourth operation cycle, the glucose utilization activity was reduced by about 20%. The activity was restored to its full capacity after incubating the biofilm in growth medium. The biofilm activity after growth reactivation was shown in the fifth through seventh cycle. The same procedure was repeated with similar results.

The uses of RBC or the similar fermentor configuration for the biological applications have been reported. The similar reactor was described by Sudo and Aiba *(14)* in the treatment of polluted water with naturally occurring microflora, mainly protozoa. Likewise, Sublette et al. *(15)* described the treatment of munition waste water with RBC to reduce the concentrations of trinitrotoluene and cyclotrimethylene trinitramine using a mycelial fungus, *Phanerochaete chrysosporium*, as the source of biofilm.

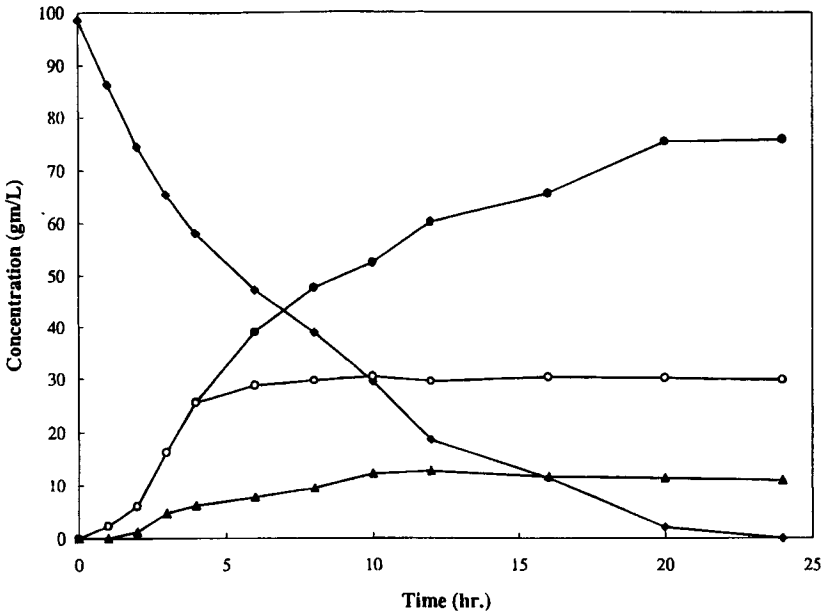

Fig. 3. Kinetics of fumaric acid fermentation by *Rhizopus oryzae* ATCC 20344 in rotary biofilm contactor. -◆- Glucose; -●- Fumaric acid, total; -○- Fumaric acid, in solution; -▲- Ethanol.

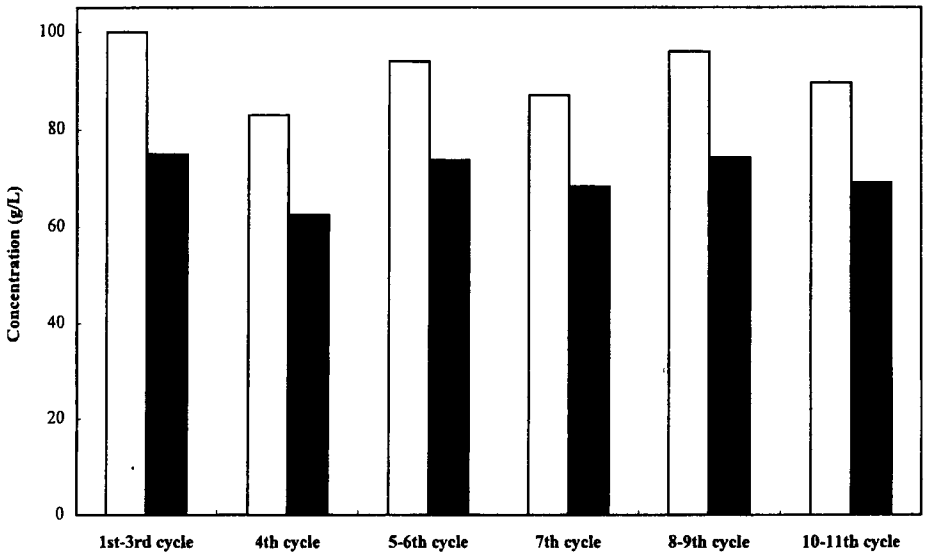

Fig. 4. Stability of *Rhizopus* biofilm during repetitive fumaric acid fermentation in RBC. ❑ Glucose utilized, ■ Fumaric acid produced.

Unfortunately, no detailed description of the reactor configuration was provided. Another example is the utilization of mixed-culture biofilm reactor for lactic acid production *(16)*. In this case, a biofilm forming bacterium, *Streptomyces viridosporus*, was used for the biofilm formation on lignocellu-

losic materials. Another bacterium, *Lactobacillus casei*, was used for the lactic acid production. The system is more or less similar to the cell immobilization system. So far, we are not aware of any reports using RBC for the biological production of fumaric acid.

CONCLUSION

This fermentation system has several advantages including:

1. No direct physical contact of mycelia with $CaCO_3$, thus avoiding the mingling of $CaCO_3$ with mycelia that caused localized mass transfer interference;
2. The mycelia that formed the biofilm can be reused and revitalized *in situ*, whenever necessary, for reuse;
3. By periodically removing the calcium fumarate formed and by continuous feeding of fresh glucose, the production process can be kept almost continuously; and
4. Higher volumetric productivity and product yield.

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REFERENCES

1. Robinson, W. D. and Mount R. A. (1981), in *Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 14*, Grayson, M. and Eckroth, D., eds., Wiley, New York, NY, p. 770.
2. Tao, W. Y., Collier, J. R., Collier, B. J., and Negulescu, I. I. (1993), *Textile Res. J.* **63,** 162–170.
3. Ng, T. K., Hesser, R. J., Stieglitz, B., Griffiths, B. S., and Ling, L. B. (1986), *Biotechnol. Bioeng. Symp.* **17,** 355–363.
4. Foster, J. W. and Waksman, S. A. (1939), *J. Am. Chem. Soc.* **61,** 127–135.
5. Margulies, M. and Vishniac, W. (1981), *J. Bacteriol.* **81,** 1–9.
6. Yang, C. W., Lu, J. and Tsao, G. T. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 57–71.
7. Rhodes, R. A., Moyer, A. J., Smith, M. L. and Kelley, S. E. (1959), *Appl. Microbiol.* **7,** 74–80.
8. Rhodes, R. A., Lagoda, A. A., Misenheimer, T. J., Smith, M. L., Anderson, R. F., and Jackson, R. W. (1962), *Appl. Microbiol.* **10,** 9–15.
9. Kenealy, W., Zaady, E., Du Preez, J. C., Stieglitz, B., and Goldberg, I. (1986), *Appl. Environ. Microbiol.* **52,** 128–133.
10. Osmani, S. A. and Scrutton, M. C. (1985), *Eur. J. Biochem.* **147,** 119–128.
11. Gangl, I. C., Weigand, W. A., and Keller, F. A. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 663–677.
12. Romano, A. H., Bright, M. M., and Scott, W. E. (1967), *J. Bacteriol.* **93,** 600–604.
13. Lu, Z, J., Yang. C. W., and Tsao, G. T. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 83–95.
14. Sudo, R. and Aiba, S. (1984), *Adv. Biochem. Eng. Biotechnol.* **29,** 117–141.
15. Sublette, K. L., Ganapathy, E. V., and Schwartz, S. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 709–723.
16. Demirci, A., Pometto, A. L. III., and Johnson, K. E. (1993), *Appl. Environ. Microbiol.* **59,** 203–207.