Simultaneous Production and Recovery of Fumaric Acid from Immobilized *Rhizopus oryzae* with a Rotary Biofilm Contactor and an Adsorption Column

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An integrated system of simultaneous fermentation-adsorption for the production and recovery of fumaric acid from glucose by *Rhizopus oryzae* was investigated. The system was constructed such that growing *Rhizopus* mycelia were self-immobilized on the plastic discs of a rotary biofilm contactor during the nitrogen-rich growth phase. During the nongrowth, production phase, the biofilm was alternately exposed to liquid medium and air upon rotation of the discs in the horizontal fermentation vessel. The product of fermentation, fumaric acid, was removed simultaneously and continuously by a coupled adsorption column, thereby moderating inhibition, enhancing the fermentation rate, and sustaining cell viability. Another beneficial effect of the removal of fumaric acid is release of hydroxyl ions from a polyvinyl pyridine adsorbent into the circulating fermentation broth. This moderates the decrease in pH that would otherwise occur. Polyvinyl pyridine and IRA-900 gave the highest loading for this type of fermentation. This fermentation system is capable of producing fumaric acid with an average yield of 85 g/liter from 100 g of glucose per liter within 20 h under repetitive fed-batch cycles. On a weight yield basis, 91% of the theoretical maximum was obtained with a productivity of 4.25 g/liter/h. This is in contrast to stirred-tank fermentation supplemented with calcium carbonate, whose average weight yield was 65% after 72 h with a productivity of 0.9 g/liter/h. The immobilized reactor was operated repetitively for 2 weeks without loss of biological activity.

Fumaric acid is a naturally occurring four-carbon dicarboxylic acid that is finding increasing use as a food acidulant and beverage ingredient. Because of its double bond and two carboxylic groups, fumaric acid has many potential industrial applications, ranging from the manufacture of synthetic resins and biodegradable polymers (16, 19) to the production of intermediates for chemical syntheses (10). Fumaric acid is derived exclusively from petroleum-based materials, since large-scale biological production of fumaric acid is too expensive to compete with the synthetic route (4).

Many species of organisms produce small amounts of fumaric, malic, and other organic acids as metabolic byproducts during oxidative metabolism. In some instances, certain mycelial fungi are capable of producing significant quantities of fumaric acid from glucose and carbon dioxide with high yield (3, 9, 15). For maximal productivity, fumarate must be produced via reductive tricarboxylic acid cycle reactions, emanating from pyruvate via pyruvate carboxylase (8, 11–13, 17).

By limiting the nitrogen source, *Rhizopus* growth can be minimized. During this nongrowth stage, fumaric acid can be accumulated with a maximal yield of 2 mol/mol of glucose consumed or 1.29 g of fumarate per g of glucose consumed on a weight basis (4, 5). In reality, however, the reductive bypass requires supply of NADH from the tricarboxylic acid cycle. Therefore, the obtainable yield is about 1.45 mol of fumarate per mol of glucose (as much as 0.93 g/g of glucose consumed) (14).

In a typical fumaric acid fermentation with *Rhizopus oryzae*, the presence of a neutralizing agent is required not only to

neutralize the acid but also to remove fumaric acid produced from fermentation broth (14). Recovery of calcium fumarate and regeneration of free acid from fumarate are complicated, tedious, and expensive (4, 14). One technique to overcome this difficulty is the use of adsorbents such as polyvinyl pyridine (PVP) to remove acid while it is being produced (2, 6, 18). PVP resin is selective in that it adsorbs free acid but not salt (20). There have been no reports regarding the biological production of fumaric acid with a simultaneous production-recovery system.

The purpose of this report is to describe the production of fumaric acid by a *Rhizopus* strain from glucose in a rotary biofilm contactor (RBC) coupled with an adsorption column.

MATERIALS AND METHODS

Microorganism and inoculum. *R. oryzae* ATCC 20344, purchased from the American Type Culture Collection (Rockville, Md.), was chosen for this study because this strain was shown to have the best specific productivity from glucose (unpublished observation). The culture was maintained on potato dextrose agar (Difco) slants and propagated by growth in Erlenmeyer flasks with potato dextrose agar to obtain sporangiospores. For a fermentation study, spores were obtained by washing spore-bearing cultures with sterile water, filtered through a sterile Kendall milk filter (The Kendall Co., Agricultural Products, Boston, Mass.), and collected as a spore suspension.

Stirred-tank fermentation. A 6.5-liter stirred tank (with a 5-liter working volume) equipped with a pH controller was employed to produce fumaric acid from glucose with addition of calcium carbonate. The spore suspension was inoculated into 2.0-liter Erlenmeyer flasks containing 600 ml of growth medium that consisted of 10 g of glucose, 2.0 g of urea, 0.6 g of KH_2PO_4 , 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.088 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 liter of distilled water. The production medium consisted of 100 g of glucose, 0.6 g of KH_2PO_4 , 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.088 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 liter of distilled water. Media were sterilized by autoclaving at 15 lb/in² for 15 min. Nutrients and sugar solutions were autoclaved separately. Growth was carried out at 30°C and 215 rpm in a gyratory incubator-shaker for 2 days. After cultivation, the mycelial pellets were harvested by filtration and transferred into a fermentor containing production medium. Sterile CaCO_3 was added whenever needed to maintain the pH at 5. The fermentation was operated with an aeration rate of 2.0 liter/liter/min at 31°C and 600 rpm for 84 h. Dissolved oxygen was not controlled, nor was

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FIG. 1. Schematic diagram of an RBC coupled with an adsorption column. A, RBC; B, sample port; C, pump; D, motor; E, filter; F, flow meter; G, adsorption column; H, feed; I, medium; T, temperature probe.

it monitored. Samples were taken periodically for high-performance liquid chromatography (HPLC) analysis.

RBC. Figure 1 is a schematic diagram of the RBC. The RBC is a 2-liter fermentor with a 0.9-liter working liquid volume that was fabricated locally. The reactor is 100 mm in diameter and 260 mm long. The reactor contains six plastic discs (125 cm² per disc) that act as supports for microorganisms. The discs were made from a semiglossy polysulfone sheet (thickness, 5 mm; Lafayette Plastics, Lafayette, Ind.) with a total surface area of 750 cm², mounted on a horizontal shaft, and placed in the fermentor containing sterilized growth medium. Mycelial fungi have a strong tendency to grow on and around the solid plastic surfaces of the discs and form a biofilm which is immobilized on the surfaces of the plastic discs. In operation, compressed air flowed into and out of the upper portion of the air space at a flow rate of 1.0 liter/liter/min. No aeration or CO₂ was applied to the liquid medium. Dissolved oxygen was not controlled, nor was it monitored. The discs were rotated at 22 rpm, and the biofilms were exposed alternately to the fermentation medium and the air space. Clear fermentation broth, without any treatment, was pumped directly into the adsorption column. A pH monitor with a sterile probe was used to monitor pH changes at the outlet of adsorption resins. Resins in the column were considered saturated when the pH of the column outlet was lowered and the column was switched. Adsorption columns were reused after product removal and regeneration. No fungal growth, and therefore no clogging of the column, was observed after extended operation.

Preparation of *Rhizopus* biofilms. The thickness of the biofilm affects the rate of diffusion of nutrients and products in the fermentor. The key controlling factors for biofilm thickness include the amount of nitrogen added, the pH, and the initial sporangiospore density in the growth medium. In this experiment, the spore suspension (100 ml with a spore concentration of 10⁶/ml) was inoculated into an RBC containing 800 ml of sterilized growth medium that consisted of 20 g of glucose, 2.0 g of urea, 0.6 g of KH₂PO₄, 0.25 g of MgSO₄ · 7H₂O, and 0.088 g of ZnSO₄ · 7H₂O in 1 liter of distilled water. The pH during the growth stage was maintained at 5 with a small amount of CaCO₃. After 60 h of incubation at 35°C with a rotating speed of 14 rpm, a biofilm was grown on and around the surfaces of the plastic discs. The broth in the reactor was then changed from growth medium to nitrogen-limited production medium. A series of three resin columns (60 by 4.6 cm) were connected to replace calcium carbonate for product recovery.

Adsorption of fumaric acid by anion-exchange and PVP resins. Strong- and weak-base anion-exchange resins (Amberlite IRA-900 and IRA-94, purchased from Sigma Chemical Co.) in hydroxyl form and PVP resins (Reillex PVP-425; Reilly Industries, Indianapolis, Ind.) were used for this study.

(i) Batch adsorption. Composite adsorption isotherms were determined by using a 1:10 (wt/vol) ratio of dry resins to starting solution with the equilibrium method. An aqueous fumarate solution with concentrations ranging from 5 to 70 mg/ml at different pH values (adjusted with ammonium hydroxide) was used as the starting solution. The flasks containing the adsorbents and fumarate were mixed and incubated at 35°C in an incubator-shaker (New Brunswick Scientific) and allowed to equilibrate for at least 48 h. The pH of the bulk solution at equilibrium was measured, and the fumarate concentrations were determined by HPLC.

(ii) Fixed-bed adsorption. The activated resins were transferred into jacked columns (30 by 2.5 cm) equipped with adjustable plungers. Air trapped in the resin bed was removed, and the plunger was lowered to the top of the resin bed. The feed solution, containing 4.0 to 8.0 mg of fumarate per ml at a specified pH, was introduced into the column by a peristaltic pump at different flow rates. A constant-temperature circulator was used to maintain the desired temperature of

FIG. 2. Fumaric acid production by *R. oryzae* in a stirred-tank fermentor. Symbols: ●, fumaric acid; ■, glucose; ○, ethanol.

TABLE 1. Fumaric acid adsorption capacities of selected adsorbents

Adsorbent	Maximum adsorption capacity (g/g of dry wt)
Duolite A-7	0.13
Amberlite IRA-35	0.15
Dianion WA-30	0.20
Amberlite IRA-900	0.22
Amberlite IRA-94	0.32
PVP-425	0.31

water in the jacket. Samples were collected from the column, and the fumaric acid concentration was analyzed to monitor the breakthrough point. The resins were considered saturated when the fumaric acid concentration in the effluent was at least 95% of that in the feed. The fumaric acid concentration and pH of each fraction were determined, and the adsorption capacity was expressed as grams of fumaric acid adsorbed per gram of dry resin.

(iii) **Desorption.** The adsorbed fumaric acid was separated from adsorption resins by using NaCl (0.4 M) for IRA-900-packed columns and NaOH (0.4 M) for IRA-94- or PVP-packed columns with downflow elution. The recovered fumaric acid was then crystallized and collected in a dilute acid solution.

(iv) **Regeneration of resins.** Downflow washing was conducted with 3 bed volumes of 5% HCl, 5 bed volumes of deionized water, 3 bed volumes of 4% NaOH, and another 5 bed volumes of deionized water. The flow rate was adjusted to allow at least 30 min of contact time between the resins and washing solution. The final water rinse was performed until the effluent pH was less than 8.

Analytical methods. Fumaric acid, L-malic acid, succinic acid, and carbohydrates were determined and quantified by HPLC (Hitachi instrument L-6200A) with a Bio-Rad Aminex HPX-87H ion-exclusion column (300 by 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

Stirred-tank fermentation. Figure 2 shows the time courses of glucose utilization and fumaric acid production by *R. oryzae* in a 6.7-liter stirred-tank fermentor in the presence of calcium carbonate. Calcium carbonate maintained the pH at approximately 5.0 throughout the entire course of fermentation. The glucose utilization rate during incubation was 1.25 g/liter/h, and the estimated fumaric acid production rate was about 0.9 g/liter/h. The calcium fumarate concentration of the ferment-

FIG. 3. Fumaric acid-fumarate adsorption time courses of different resins. The initial pH was 4.8, and the temperature was 35°C. Symbols: ●, IRA-94 resin; ○, IRA-900 resin; ■, PVP resin.

FIG. 4. Fumaric acid-fumarate adsorption isotherms of different resins at different pHs. The temperature was 35°C. Symbols: ●, IRA-94 resin; ○, IRA-900 resin; ■, PVP resin.

tation broth at its peak was 30 g/liter. The glycerol concentration was less than 3 g/liter throughout fermentation. The final fumaric acid concentration recovered was 65 g/liter after all of the glucose was consumed.

Adsorption of fumaric acid. The capacities of six adsorbents including PVP for fumaric acid-fumarate adsorption are shown in Table 1. PVP-425 and ion-exchange resin IRA-94 exhibited the greatest fumaric acid adsorption capacities. Figure 3 shows the fumaric acid-fumarate time courses of IRA-94, IRA-900, and PVP-425 resins. On the basis of the results of adsorption experiments, most of the fumaric acid uptake occurred during the first 20 min of contact with the resins. The rate of adsorption is an important criterion for choosing an appropriate adsorbent for a fermentation-recovery process, since the goal is to remove the product from the fermentation broth as soon as it is produced. If the adsorption rate is too slow, more adsorbent is needed to achieve the desired reduction of the product inhibition in a given time. The results indicated that a minimum retention time of 20 min was necessary for optimal resin adsorption of fumaric acid in the coupled operations.

Similar to other organic acid recovery operations, pH is one of the important factors determining the adsorption capacity of resins. The fumaric acid adsorption isotherms of different resins at pHs of 3.2, 4, and 5.3 are shown in Fig. 4. While the capacity of strong-base ion-exchange resin IRA-900 was inde-

FIG. 5. Fumaric acid-fumarate adsorption breakthrough curves of different resins in the column. The initial pH was 4.0, and the temperature was 35°C. Symbols: ●, IRA-94 resin; ○, IRA-900 resin; ■, PVP resin.

FIG. 6. Kinetics of fumaric acid fermentation by *R. oryzae* in an RBC coupled with an IRA-900 adsorption column. Symbols: ●, fumaric acid; ■, glucose; ○, ethanol.

pendent of pH, the capacities of IRA-94 and PVP decreased with increasing pH, although the effect was more pronounced for PVP. The pH range of 3.8 to 4.5 was chosen since the adsorption capacities of all three materials were similar. Figure 5 shows that the operational capacity, as measured by determining the breakthrough curve of fumaric acid, was highest for IRA-900 and lowest for PVP-425.

Simultaneous fermentation and adsorption. Fumaric acid fermentation by *R. oryzae* is strongly inhibited by the presence of fumaric acid and, to a lesser extent, by its salt, which is formed when chemicals are added to control the pH during fermentation (4, 14). It should be beneficial to integrate fermentation and product recovery to remove fumaric acid from the reactor while it is being formed. However, for this type of fermentation, the reactor performance will be highly dependent on the pH during the production-recovery process. A pH range of 5.8 to 6.2 favors fermentation (14), while a lower pH was detrimental to fermentation but favored adsorption by PVP (Fig. 4). When IRA-900 was the adsorbent during the coupled fermentation-recovery process (Fig. 6), the pH of the

FIG. 7. Kinetics of fumaric acid fermentation by *R. oryzae* in an RBC coupled with a PVP-425 adsorption column. Symbols: ●, fumaric acid; ■, glucose; ○, ethanol.

FIG. 8. Kinetics of glucose consumption by *R. oryzae* during repetitive fumaric acid fermentation in an RBC coupled with an IRA-900 adsorption column. Symbols: ●, 1st cycle (production medium); □, 4th cycle (growth medium); ▲, 5th cycle (production medium); ○, 7th cycle (growth medium); △, 10th cycle (production medium).

fermentation broth was maintained at 4.5 after an initial drop to approximately 3.5. This was due to continuous replacement of the hydroxyl group of IRA-900 by the adsorbed fumaric acid. At this pH, the fumaric acid adsorption capacity of IRA-900 was also at its high value of 0.22 g of fumaric acid per g of dry resin.

A fumaric acid yield of 85% by weight was obtained with an initial glucose concentration of 109 g/liter. This is higher than that obtained by stirred-tank fermentation with CaCO_3 . The time required for complete fermentation was 24 h, compared with 72 h for stirred-tank fermentation. Fumaric acid was adsorbed as fumarate in the column packed with IRA-900. It was desorbed by 0.4 M NaCl, and fumaric acid was recovered in its pure acid form and crystallized out of the solution upon acidification because of its low solubility of 6 g/liter.

When the fermentation was carried out in conjunction with an adsorption column packed with PVP resin (Fig. 7), the fumaric acid fermentation rate was also increased. The final

FIG. 9. Accumulation of fumaric acid by *R. oryzae* during repetitive fumaric acid fermentation in an RBC coupled with an IRA-900 adsorption column. The 1st, 2nd, 5th, and 10th cycles were in production medium, and the 4th and 7th cycles were in growth medium. Yield is expressed as grams of fumaric acid produced per gram of glucose consumed.

TABLE 2. Comparison of fumaric acid production processes using *R. oryzae*

Process	Neutralizing agent	Initial glucose concn (g/liter)	pH or pH range	Fermentation time (h)	Air flow rate (liters/liter/min)	Volumetric productivity (g/liter/h)
RBC	OH ⁻	100	4.5	20	1.0	4.25
Stirred tank used in this study	CaCO ₃	100	5–5.5	72	2.0	0.92
Stirred tank used by Rhodes et al. (14)	CaCO ₃	130	5.5	72	0.5	0.944
Stirred tank used by Gangl et al. (4)	Na ₂ CO ₃	102	5.5	147	2.5	0.49

fumaric acid concentration also reached 86 g/liter. This is equivalent to an indicated theoretical yield approaching 91%. Fumaric acid was recovered from a PVP-packed column with NaOH (0.4 M) as the eluent.

Stability of biofilm. Figures 8 and 9 show biofilm activity after repetitive fed-batch cycles. The glucose utilization rates (Fig. 8) and final fumaric acid concentrations (Fig. 9) were similar for the first three cycles of operation. At the fourth operation cycle, the activity was reduced by 20%. The activity was restored to its original level after incubation of the biofilm with growth medium. The biofilm activity after growth reactivation was shown in the fifth through seventh cycles. The procedure was repeated, and similar results were obtained.

DISCUSSION

Like many other mycelial fungi, *R. oryzae* grows aerobically with extended hyphae that tend to form large pellets or aggregates. These cause a dramatic increase in oxygen and mass transfer resistance (20). In a conventional stirred-tank fermentor, the growing mycelia anchor onto the inside elements of the reactor, such as the baffles, propellers, and heat exchanger, and exacerbate the problem. The fermentation time is long; unwanted byproducts, particularly ethanol, are formed; and the conversion yield is low because of the localized anaerobic condition and the limitation of oxygen transfer. To circumvent the problems, an RBC was used for fumaric acid production. With the RBC, germinated spores with hyphae attached to, grew on, and were immobilized on the surfaces of plastic discs and formed a mycelial film not more than 2 mm thick. During the production stage, the biofilm was exposed to the sterile air in the headspace of the RBC; this enhanced oxygen exposure and the opportunity for CO₂ fixation by the biofilm. This type of self-immobilization of fungal mycelia is easier to operate in the reactor and easier to scale up because no special technique or immobilization medium is required and no antifoam is needed.

Since fumaric acid has a very low solubility of 5 g/liter or less at 25°C, recovery of adsorbed acid from the adsorption column is relative easy. In our laboratory, we used NaCl for desorption of the adsorbed acid from IRA-900 resin. Upon acidification with HCl, pure fumaric acid is crystallized out of solution with a purity approaching 100%. In a stirred-tank fermentation with CaCO₃, fumaric acid recovery is more complicated. It requires heating to dissolve the calcium fumarate, followed by addition of sulfuric acid to form fumaric acid and calcium sulfate. The heating process deactivates fungal activity that cannot be recycled and reused. The insoluble CaSO₄ (gypsum) formed as a result has no commercial value and must be disposed of. In addition, the residual CaCO₃ releases CO₂ upon exposure to acidic conditions. This further complicates the recovery process (14).

There are no other detectable organic acids throughout the entire course of fermentation except a trace amount of L-malic acid. Other by-products include ethanol (10 g/liter) and glycerol (3 g/liter). Ethanol production is most likely the result of an insufficient supply of dissolved oxygen. Ethanol formation

should be eliminated once the construction of a new RBC equipped with an air sparger is completed. With the completion of a new RBC, the effects of dissolved oxygen and CO₂ on the fermentation rate and productivity can be studied and compared with those in the current system.

The overall performance of the system is very favorable compared with that of the traditional stirred-tank–CaCO₃ system or the stirred-tank–Na₂CO₃ system. Table 2 compares the results of fermentation by this method with those of other methods. The media in these examples were all similar. Likewise, the organisms used belonged to the same species, *R. oryzae*. The species names are different in the published reports, but according to the American Type Culture Collection catalog, all of the strains are classified as *R. oryzae*. The most significant improvements of the simultaneous fermentation-recovery system are the fumaric acid volumetric productivity and weight yield. The volumetric productivity of the coupled system is almost five times greater than those of stirred-tank systems with neutralizing agents. Likewise, the weight yield is much higher in the RBC-coupled reactor than in traditional fermentors with neutralizing agents. Another significant advantage of RBC fermentation is the reusable fungal mycelia. In stirred-tank fermentation, the organisms can be used only once.

Conclusions. Metabolic feedback inhibition is a general phenomenon, even though the inhibitory effects vary widely from metabolite to metabolite. Interest in simultaneous removal of the metabolite during the biosynthesis process as a way to minimize the effect of metabolic feedback inhibition has been strong (1, 7). In the fungal fermentation system using *R. oryzae* to produce fumaric acid, the coupled fermentation and column adsorption system is particularly useful. Our study indicated that fumaric acid can be produced and recovered as free acid and the immobilized *Rhizopus* biofilm can be maintained for long periods without loss of biological activity. The stability of the biofilm over prolonged periods of operation is currently under investigation.

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