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Production of Fumaric Acid with Immobilized Biocatalysts

HELENA KAUTOLA* AND YU-YEN LINKO

Laboratory of Biotechnology and Food Engineering, Helsinki University of Technology, SF-02150 Espoo, Finland

ABSTRACT

Immobilization of *Rhizopus arrhizus* mycelium improved fumaric acid production. The optimum conditions for fumaric acid production with immobilized cells were investigated using a statistical experimental design. Substrate concentration, carbon:nitrogen ratio, and residence time were chosen as independent variables. In the repeated batch shake flask fermentation, the fumaric acid yield from xylose was as much as 3.5 times higher with immobilized mycelium than with free mycelium. Polyurethane foam cubes, in this case, gave better results than nylon net cubes as a carrier.

Index Entries: Fumaric acid fermentation; immobilized biocatalysts; *Rhizopus arrhizus.*

INTRODUCTION

Fumaric acid has become of great interest because of its large use in the manufacture of sizing resins for paper and polyester resins, and its nonhygroscopic and practically nontoxic characteristics as an acidulant in foods and pharmaceutical preparations (*1*). Although fumaric acid was produced biotechnically in the US in the 1940s in commercial quantities from glucose, and to a lesser extent from sucrose, starch, and molasses, it is today produced economically only by chemical synthesis (*2*). The fungi *Rhizopus nigricans, R. arrhizus, R. oryzae, Aspergillus fumaricus, Mucor* sp., and some *Candida* yeasts have been used in the biotechnical production of fumaric acid (*3*).

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

In order to utilize the biomass, we have investigated the pentose utilization for chemical productions (4,5) for several years. With its double bond and two carboxylic groups, fumaric acid attracted our attention as a potentially valuable product. The fumaric acid production from xylose both with free (6) and immobilized mycelium (7) has been recently reported by our laboratory. In this paper, we report further details on the optimization of the production of fumaric acid with immobilized *Rhizopus arrhizus* mycelium from xylose in repeated batch shake flask fermentations. Also, different carriers and carbon sources were investigated.

METHODS

Culture of Microorganism

Rhizopus arrhizus TKK 204-l-la fungus used in this investigation was maintained on agar slants containing 2% xylose or glucose, 2% malt extract, 0.1% peptone, and 2% agar (6). To obtain an inoculum, spores from a fresh 6-d-old slant were transferred with 5 mL of sterilized distilled water to a 250 mL Erlenmeyer flask with 50 mL growth medium containing 0.236% ammonium sulfate, 0.03% potassium dihydrogenphosphate, 0.025% magnesium sulfate, 0.0066% zinc sulfate, 0.001% iron(III)sulfate, 0.3% corn steep liquor, 5% of either xylose, glucose, sucrose, fructose, galactose, or maltose, and the possible carrier. After cultivating for 20 h at 33°C with shaking at 250 rpm, the free or immobilized mycelium formed was washed with sterilized water and transferred to the production medium.

Immobilization

Rhizopus arrhizus mycelium was immobilized on 1 cm polyurethane foam (Espe, Espoo, Finland) or nylon net (3M, St. Paul, MN) cubes. Washed 30 pieces of cubes per flask were autoclaved in the growth medium, and spores from a 6-d-old slant were added into 250 mL Erlenmeyer flasks. After cultivation for 20 h, cubes with attached mycelium were washed with sterilized water and transferred to fresh production medium.

Production Medium

Fumaric acid production was carried out with the medium containing 0.03% potassium dihydrogenphosphate, 0.04% magnesium sulfate, 0.0044% zinc sulfate, 0.001% iron(III)sulfate, 0.05% corn steep liquor, 5% calcium carbonate, 0.0688–0.2261% ammonium sulfate, and 5–15% xylose or 6% glucose, sucrose, fructose, galactose, or maltose.

Batch and Repeated Batch Fermentations

Free and immobilized mycelium separated from growth medium were transferred to respective 250 mL Erlenmeyer flasks with 100 mL of the

Table 1
Independent Variables and Results of the Experimental Design for the Optimization of the Repeated Batch Fermentations[a]

Experiment	x_1, %	x_2,	x_3, d	Y_1, g/L	Y_2, mg/L/h	Y_3, %
1	−1	−1	−1	3.956	55	6.1
2	1	−1	−1	0.168	2	0.12
3	−1	1	−1	3.963	55	6.1
4	−1	−1	1	11.798	55	18.2
5	1	1	−1	2.271	32	1.7
6	−1	1	1	12.117	56	18.7
7	1	−1	1	0.032	0.1	0.02
8	1	1	1	0.007	0.03	0.005
9	−1.415	0	0	7.477	52	15.0
10	1.415	0	0	0.005	0.03	0.003
11	0	−1.415	0	2.803	19	2.8
12	0	1.415	0	4.306	30	4.3
13	0	0	−1.415	3.287	78	3.3
14	0	0	1.415	12.947	53	12.9
15	0	0	0	3.480	24	3.5
16	0	0	0	8.075	56	8.1
17	0	0	0	3.651	25	3.7
18	0	0	0	7.078	49	7.1

[a] x_1=xylose concentration (%); x_2=carbon:nitrogen molar ratio; x_3=residence time (d); Y_1=fumaric acid concentration (g/L); Y_2=volumetric productivity of fumaric acid (mg/L/h); Y_3=yield based on the initial xylose concentration (%); and $\alpha=1.415$.

production medium. The fumaric acid production was carried out at 250 rpm (rotational), 33°C, and residence time of 1.75 to 10.25 d. In repeated batch fermentations, both free and immobilized mycelium of each batch were washed once with sterilized water.

Experimental Design with the Immobilized Mycelium

A 2^3-factorial experimental design with six star points and four replicates at the center point was employed in the optimization of fumaric acid production by immobilized mycelium (*8*). Initial sugar concentration (X_1: 5, 6.47, 10, 13.53, and 15% w/v), carbon:nitrogen molar ratio (X_2: 120, 131.7, 160, 188.3, and 200), and residence time (X_3: 1.75, 3, 6, 9, and 10.25 d) were chosen as independent variables when α was 1.415 (Table 1). The levels chosen for the independent variables were based on unpublished results obtained from the experiments with free cells in shake flask fermentations (*6*). Second degree polynomials were used to estimate the responses Y, which values were always taken as average values from repeated batches after reaching a steady state. The responses determined were the average fumaric acid concentration of repeated batches (Y_1, g/L), the average volumetric productivity (Y_2, mg/L/h), and average yield based on initial sugar concentration (Y_3, %) as the dependent variable. All terms in the equations, regardless of their significance, were included in the final equations.

Analytical Assays

Fumaric acid was determined using HPLC Varian 5000 (Varian Associates, Inc., Walnut Creek, CA) with a 30 cm Aminex HPX-87 ion exchange column (Bio-Rad Laboratories, Richmond, CA) and 0.4 m*M* sulfuric acid as the mobile phase at 75°C. Samples were boiled for 20 min and filtered before analyzing.

RESULTS AND DISCUSSION

Optimization with Models in Repeated Batch Fermentations

The average production of fumaric acid on xylose with the polyurethane foam immobilized *Rhizopus arrhizus* TKK 204-1-1a mycelium in repeated batch fermentations was optimized using statistical experimental design and empirical modeling. The regression equations calculated for average fumaric acid product concentration (Y_1, g/L), average volumetric productivity (Y_2, mg/L/h), and average yield based on initial xylose concentration (Y_3, %) as functions of xylose concentration (X_1, %), carbon:nitrogen molar ratio (X_2) and residence time (X_3, d) are given in Eq. (1–3)

$$\begin{aligned} Y_1 = & -47.3379 + 1.81491X_1^{***} + 0.494035X_2 + 1.9417X_3^{**} \\ & +0.00219221X_1X_2 - 0.217139X_1X_3^{**} - 0.00267373X_2X_3 \\ & -0.0902543X_1^2 - 0.00152056X_2^2 + 0.11788X_3^2 \end{aligned} \quad (1)$$

$R = 0.95^{**}$, $R^2 = 0.90$

(P: $^{***} \leq 0.001$, $^{**} \leq 0.01$, $^{*} \leq 0.05$
R = multiple correlation coefficient
R^2 = coefficient of determination)

$$\begin{aligned} Y_2 = & -237.122 + 4.2113X_1^{***} + 3.73921X_2 - 5.86173X_3 \\ & +0.0361991X_1X_2 - 0.411591X_1X_3 - 0.0428003X_2X_3 \\ & -0.683941X_1^2 - 0.0115861X_2^2 + 1.24423X_3^{2*} \end{aligned} \quad (2)$$

$R = 0.94^{**}$, $R^2 = 0.89$

$$\begin{aligned} Y_3 = & -30.4465 - 1.25976X_1^{***} + 0.471838X_2 + 2.91414X_3^{***} \\ & +0.00133255X_1X_2 - 0.312736X_1X_3^{***} - 0.00161213X_2X_3 \\ & +0.0653524X_1^2 - 0.00144739X_2^2 + 0.123697X_3^2 \end{aligned} \quad (3)$$

$R = 0.98^{***}$, $R^2 = 0.97$

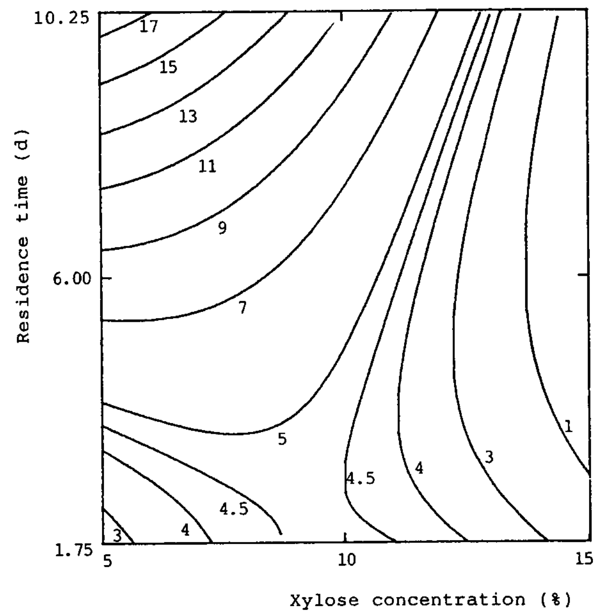

Fig. 1. The average fumaric acid product concentration (g/L) during repeated batch fermentations with immobilized mycelium as a function of xylose concentration and residence time at the center point of a carbon:nitrogen ratio of 160, shown as response surfaces.

According to the Fig. 1 (Eq. 1), the average fumaric acid product concentration reached under the investigated conditions is at its highest expected value of 18 g/L at the xylose concentration of 5%, the carbon:nitrogen molar ratio at the center point of 160, and residence time of 10.25 d. In the actual fermentations, the highest reached average fumaric acid concentration of repeated batches was 12.9 g/L at the xylose concentration of 10%, the carbon:nitrogen ratio of 160, and the residence time of 10.75 d. Under these conditions, the value 11 g/L, given by the Eq. (1), agreed well with the result obtained by fermentation. Consequently, the maximum product concentration was obtained at the lowest xylose level (5%) with the longest residence time (10.75 d) in the experimental plan.

According to Eq. (2) (Fig. 2a), the average volumetric productivity of fumaric acid, in this case, reached 54 mg/L/h near the xylose concentration of 5% and the center point of carbon:nitrogen ratio 160 at the center point of the residence time of 6 d, agreeing well with the actual experimental result of 52 mg/L/h. The highest average productivity of 78 mg/L/h with the product concentration of 3.3 g/L and yield of 3.3% was obtained in the actual fermentations at the center point of xylose concentration (10%) and carbon:nitrogen ratio (160) with 1.75 d residence time, close to the 71 mg/L/h given by Eq. (2) (Fig. 2b). Figure 2b shows that, in the investigated area, the highest calculated productivity was 77 mg/L/h at the xylose concentration of 5%, the carbon:nitrogen ratio of 160 at the center

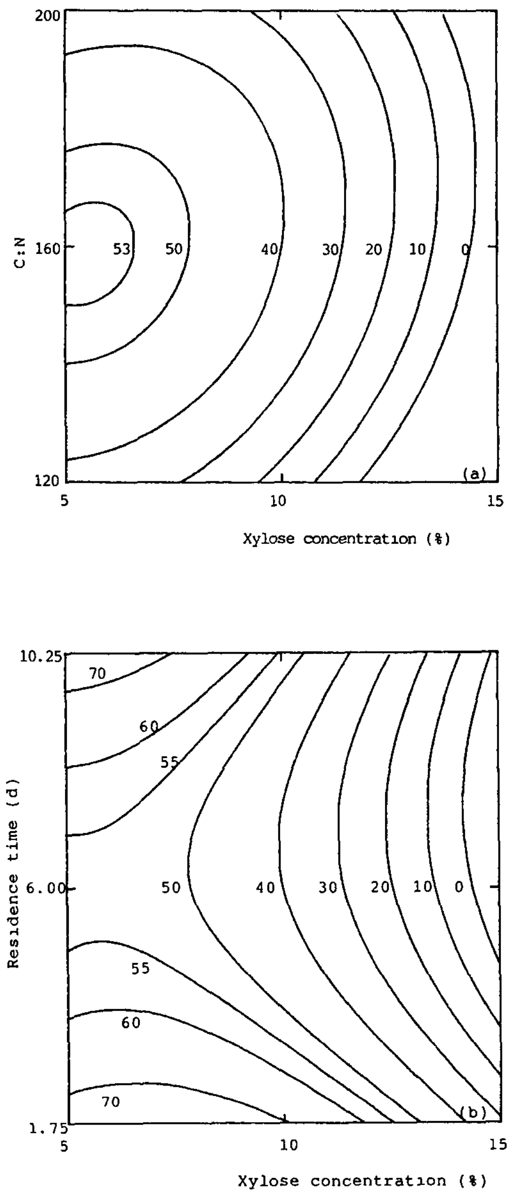

Fig. 2. The average fumaric acid productivity (mg/L/h) during repeated batch fermentations with immobilized mycelium as a function of xylose concentration and carbon:nitrogen ratio at the center point of 6 h residence time (a) or residence time at the center point of C:N of 160 (b), shown as response surfaces.

point, and the residence time of 10.25 d. In this case, the calculated average fumaric acid yield (Eq. 3) also reached its highest value of 29% (Fig. 3) under equal conditions as the maximum expected fumaric acid concentration of 18 g/L (Fig. 1).

Consequently, the optimization with polynomial models obtained in repeated batch fermentations with xylose suggested, for optimum condi-

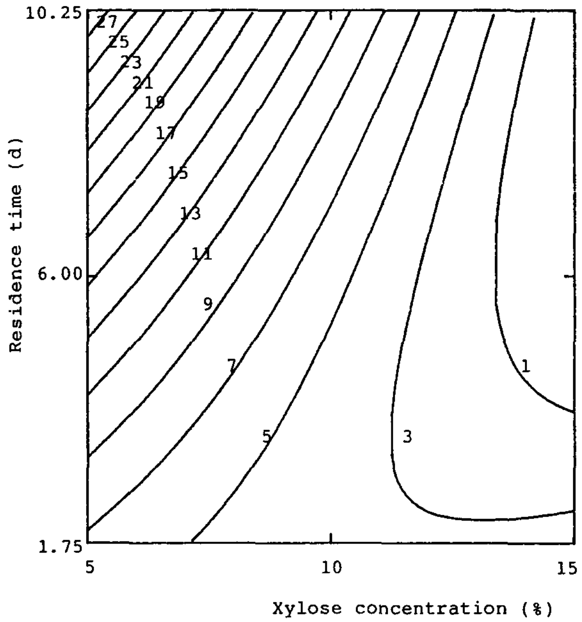

Fig. 3. The average fumaric acid yield based on initial xylose concentration (%) during repeated batch fermentations with immobilized mycelium as a function of xylose concentration and residence time at the center point of carbon:nitrogen ratio 160, shown as response surfaces.

tions, a low xylose concentration of 5%, a carbon:nitrogen ratio of 160, and a high residence time of 10.25 d. Xylose concentration had the greatest effect on average fumaric acid production, productivity, and yield in repeated batch fermentations, according to the Eq. (1–3). Residence time also had some effect on fumaric acid production and yield. The effect of the carbon:nitrogen ratio under the conditions investigated did not seem to be very important.

Comparison of Fumaric Acid Yield with Immobilized or Free Mycelium in Repeated Batch Fermentations

In repeated batch fermentations, the fumaric acid yields, based on the initial xylose concentration with immobilized and free cells, are compared in Fig. 4. The immobilization increased the fumaric acid yield remarkably. At the residence time of 6 d, the increase was 1.7 times, whereas at 10.25 d, it was about as much as 3.5 times. At 10% xylose concentration and a carbon:nitrogen ratio of 160, the fumaric acid yields reached at the residence time of 6 d were 7.3% with the immobilized mycelium and 4.3% with free mycelium, and at the residence time of 10.25 d, 13.5% and 4%, respectively. The yield of fumaric acid on xylose is relatively low, as could be expected from the poor microbial utilization of xylose in general. No

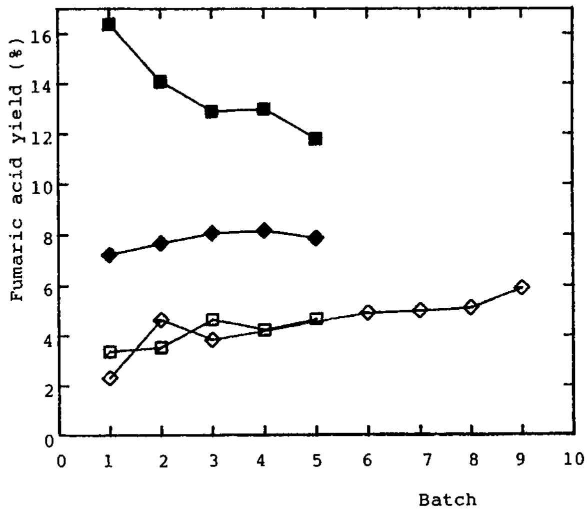

Fig. 4. The fumaric acid yield based on initial xylose concentration in repeated batch fermentations (10% xylose, C:N = 160) with immobilized mycelium (◆, ■) and free mycelium (◇, □), at the residence time of 6 (◆, ◇) and 10.25 d (■, □).

traces of other acids could be detected during the production of fumaric acid, and the rest of the xylose remained unutilized in the repeated batch fermentations. We have also observed similar results in our earlier work with polyurethane foam cube immobilized *Aspergillus terreus* mycelium for itaconic acid production. In that case, itaconic acid production with the immobilized fungus was two times higher than with free mycelium in 14 d (*9*).

Comparison of Carriers in Fumaric Acid Production

Two different carriers were tested in the production of fumaric acid with immobilized mycelium in shake flask fermentations at 6% sugar concentration and a carbon:nitrogen ratio of 188. Figure 5 shows that polyurethane foam cubes with the fumaric acid concentration of 16.8 g/L after 7 d were 1.6 times better as carrier than nylon net cubes with 10.4 g/L for fumaric acid production from glucose. The difference 9 to 3.6 g/L, respectively, when using xylose as substrate was obtained after 8 d. This is contrary, for example, to lignin peroxidase production with nylon net immobilized *Phanerochaete chrysosporium*, which gives more than twice the enzyme activity than the polyurethane carrier system (*10*). Although the actual immobilized biomass concentration could not be determined, because of the presence of calcium carbonate, no marked differences in the degree of immobilization could be noticed.

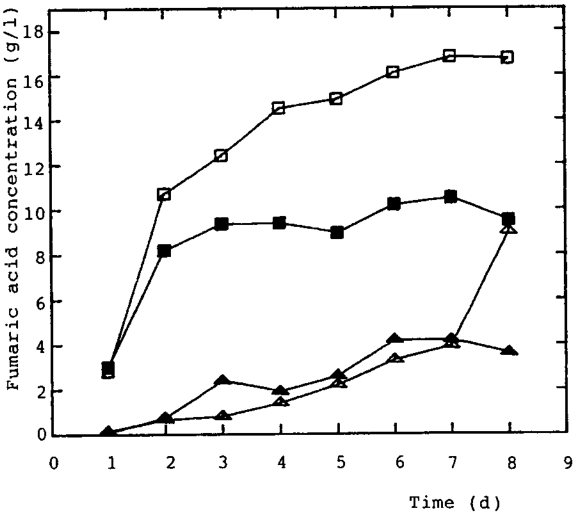

Fig. 5. The fumaric acid production (g/L) with polyurethane foam (△, □) or nylon net (▲, ■) cubes immobilized mycelium from glucose (□, ■) or xylose (△, ▲) in batch fermentations.

Fumaric Acid Production with Immobilized Mycelium from Different Substrates

Different carbon sources were tested in producing fumaric acid with the polyurethane foam immobilized mycelium in 250 mL shake flask fermentations. At 6% carbon source concentration and a carbon:nitrogen ratio 188, Fig. 6 shows that the best substrates were glucose, sucrose, and fructose, giving about 15 g/L fumaric acid in 5 d. Production was much slower from xylose, as could be expected (*4,5*). Also, maltose and galactose could be used for fumaric acid production (13.5 and 6.7 g/L after 5 d, respectively). The ability of utilizing these carbon sources also as a mixture makes the production of fumaric acid a potentially useful way of utilizing biomass sources obtained as byproducts from food and forest industries.

CONCLUSION

The optimum conditions for fumaric acid production from xylose by immobilized mycelium in the investigated area was 5% xylose, carbon: nitrogen ratio of 160, and residence time of 10.25 d. Immobilization of mycelium increased fumaric acid yield as much as 3.5-fold in comparison with the free mycelium.

Polyurethane foam cubes gave better results than nylon net cubes used as the carrier in the immobilization of mycelium for fumaric acid production. Glucose, sucrose, and fructose gave the highest fumaric acid

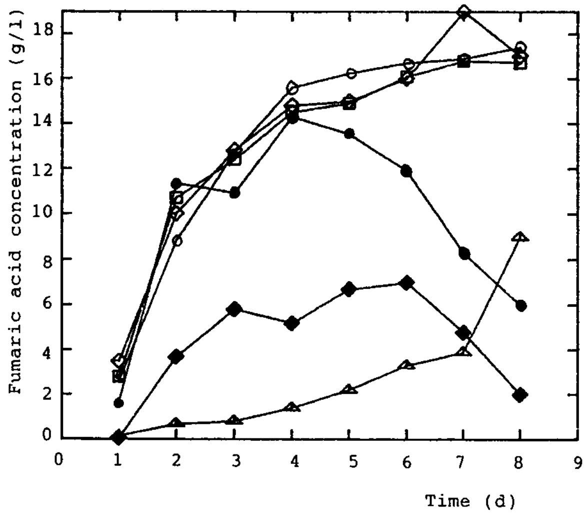

Fig. 6. The fumaric acid production (g/L) with polyurethane foam cubes-immobilized mycelium from glucose (□), sucrose (◇), fructose (○), xylose (△), maltose (●), and galactose (◆) in batch fermentations.

concentrations in 5 d. The immobilized mycelium used xylose at a much slower rate than hexoses in fumaric acid production.

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