Continuous Production of Citric Acid from Dairy Wastewater Using Immobilized *Aspergillus niger* ATCC 9142

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Abstract The continuous production of citric acid from dairy wastewater was investigated using calcium-alginate immobilized *Aspergillus niger* ATCC 9142. The citric acid productivity and yield were strongly affected by the culture conditions. The optimal pH, temperature, and dilution rate were 3.0, 30°C, and 0.025 h^{-1}, respectively. Under optimal culture conditions, the maximum productivity, concentration, and yield of citric acid produced by the calcium-alginate immobilized *Aspergillus niger* were 160 mg L^{-1} h^{-1}, 4.5 g/L, and 70.3%, respectively. The culture was continuously perfored for 20 days without any apparent loss in citric acid productivity. Conversely, under the same conditions with a batch shake-flask culture, the maximum productivity, citric acid concentration, and yield were only 63.3 mg L^{-1} h^{-1}, 4.7 g/L and 51.4%, respectively. Therefore, the results suggest that the bioreactor used in this study could be potentially used for continuous citric acid production from dairy wastewater by applying calcium-alginate immobilized *Aspergillus niger*.

Keywords: citric acid, dairy wastewater, *Aspergillus niger*, continuous production

INTRODUCTION

Modern dairies produce waste effluents in very high quantities in an order of magnitude of thousands of cubic meters per day [1]. Due to their very high concentration level of organic matter, these effluents can create serious problems by putting an organic burden on local municipal sewage treatment systems, as observed by Bough and Swientek [2]. Typically, dairy effluents are much more concentrated than domestic sewage and the main contributors of organic load to these effluents are lactose, fats, and proteins originated from milk.

Citric acid, with an estimated annual production of 500 thousand tons, is produced by the submerged fermentation of starch- or sucrose-based media using the filamentous fungus *Aspergillus niger* [3,4]. The food industry consumes the largest amount of citric acid, as much as 70% of the total production, followed by approximately 12% for the pharmaceutical industry and 18% for other applications [5]. There is an annual growth of 3.5-4.0% in the demand/consumption rate of citric acid.

In the past few years, the immobilization of microbial cells has received increasing attention, and immobilized cells have been used for the production of organic acids, amino acids, antibiotics, enzymes, alcohol, and other compounds [6,7]. The production of citric acid has already been investigated on a laboratory scale with *Aspergillus niger* immobilized in a calcium alginate gel [8,9], polyacrylamide gel [7,10], and polyurethane foam [11].

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The purpose of the current investigation was to examine the potential of dairy wastewater as a source for citric acid production by *Aspergillus niger* via a continuous reactor as well as to study the effect of various fermentation parameters, such as the dilution rate, pH, and temperature.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Aspergillus niger ATCC 9142 (American Type Culture Collection, Rockville, MA, USA) was used throughout this study. The strain was maintained on potato dextrose agar (Unipath Ltd., Cambridge, UK) slants at 4°C and subcultured at intervals of 1-2 months. For the inoculum preparation, the cultures were incubated on potato dextrose agar slants at 30°C and 200 rpm for 5 days to produce spores. These spores were then suspended in sterile-distilled water with a spore density of about 10^7 mL^{-1} and used as the inocula.

Medium

The dairy wastewater, obtained from the local dairy industry (Yangsan, Korea), contained about 2.5% reducing sugar mostly as lactose that had moisture rate of 97% (Table 1). Therefore, the total reducing sugar was concentrated up to 5% for the experiment, and sterilized at 121°C for 20 min. To remove protein and lipid, the initial pH was identified as 4.3 by 4 N of HCl, and carried out the activating charcoal treatment.

Table 1. Typical composition of dairy wastewater

Items	Dairy wastewater
pH	7.0-7.2
BOD_5[a] (mg/L)	20,000
COD[b] (mg/L)	17,000
Total reducing sugars (g/L)	10.0
Total nitrogen (mg/L)	575.0
Total phosphorus (mg/L)	2.1

[a] Biological oxygen demand, [b] Chemical oxygen demand.

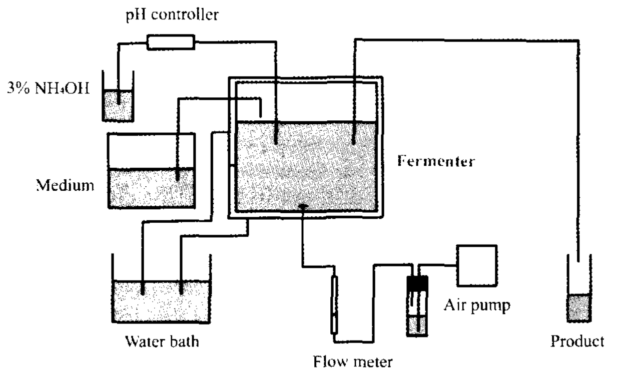

Fig. 1. Schematic digram of bioreactor to produce continuously citric acid from dairy wastewater by immobilized *Aspergillus niger* ATCC 9142.

Cell Immobilization

The cell immobilization for the continuous flask culture was achieved by putting 4% sodium alginate (cell : alginate = 1 : 10, w/w) into ATCC 9142 strain growing in a 500 mL flask at 30°C for 72 h, then the cell-alginate mixture was extruded into a 2% $CaCl_2$ solution through a needle. The Ca-alginate beads with entrapped cells of *Aspergillus niger* exhibited a typical shrinking behavior, and the sizes of the beads were from 2.5 mm to 3.5 mm particle diameter.

Bioreactor and Fermentation

The experimental set-up for the continuous production of citric acid is shown in Fig. 1. The bioreactor was constructed of glass, and its total working volume was 250 mL (300 mm length and 25 mm diameter). The Ca-alginate beads entrapped with immobilized cells were packed up to 30% in the column. The air-flow rate and pH were measured and controlled by an air-flow controller and a pH controller, as well as the operation temperature by pumping temperature-controlled water through a jacket. In addition, a positive displacement peristaltic pump (EYELA, model SA-1, Japan) was used to vary the liquid feed flow rate.

The shake-flask experiments were performed in 500 mL Erlenmeyer flasks containing 150 mL of the medium mixture (bead volume : medium volume = 1 : 2). All media and equipment were sterilized at 121°C for 20 min. The flasks were incubated at 30°C on a rotary shaking incubator (Vision Scientific Co., Model KMC-8480SF, Korea), and the operation speed was 200 rpm.

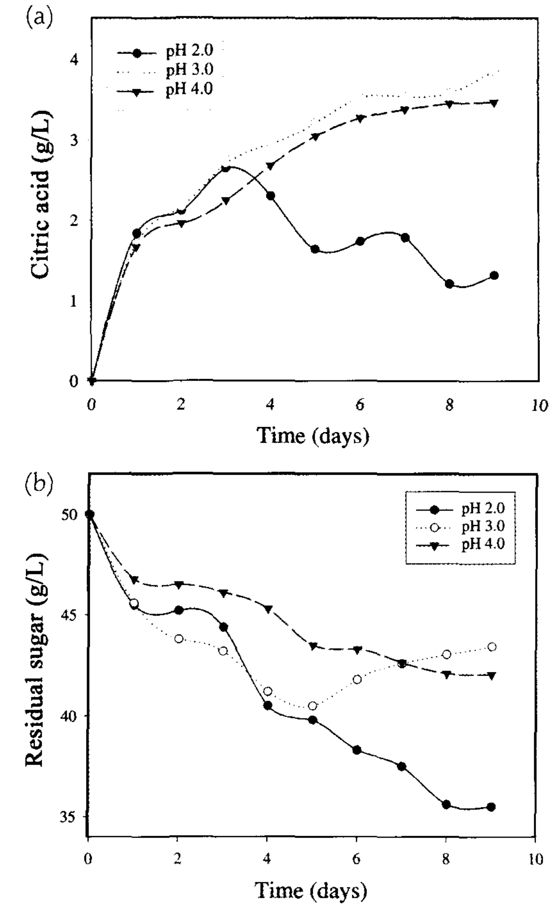

Fig. 2. Effect of pH on continuous citric acid production (a) and amount of residual reducing sugar (b) at 30°C and 0.450 h^{-1} dilution rate from dairy wastewater by immobilized *Aspergillus niger* ATCC 9142.

Analytical Techniques

The citric acid was determined using the colorimetric method of Marrier and Boulet [12]. The reduced sugar was assayed by applying a colorimetric method in which 3,5-dinitrosalicylic acid (DNS) was used as the color reagent, as described by Miller [13]. All analyses were performed in duplicate.

RESULTS AND DISCUSSION

Effect of pH on Citric Acid Production

The pH of the substrate is an important factor that affects the performance of dairy wastewater fermentation. In the current experiment, the optimum pH of the dairy wastewater for citric acid production is shown in

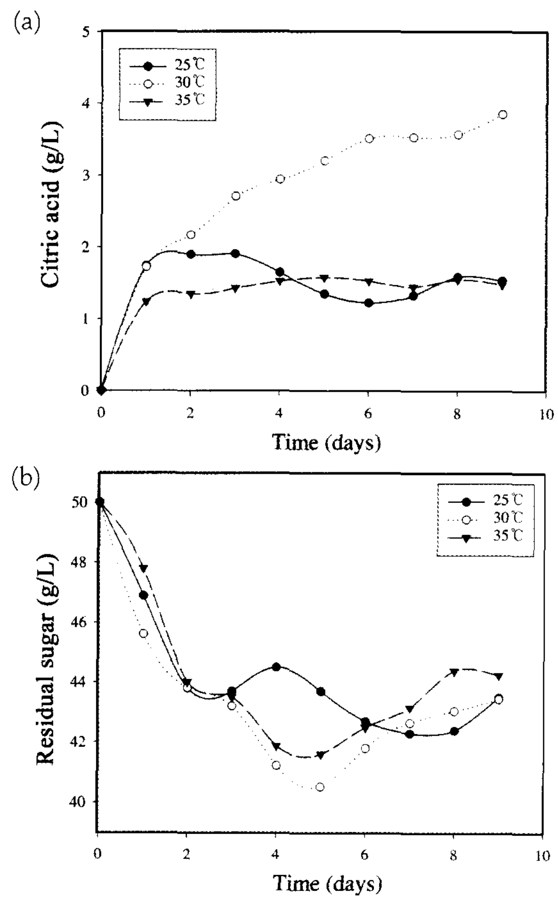

Fig. 3. Effect of temperature on continuous citric acid production (a) and amount of residual reducing sugar (b) at pH 3.0 and 0.450 h^{-1} dilution rate from dairy wastewater by immobilized *Aspergillus niger* ATCC 9142.

Fig. 2. The citric acid concentration and yield were highest at pH 3.0 (Fig. 2(a)), yet the consumption of sugar was highest at pH 2.0 (Fig. 2(b)). These results agree with those of Kim *et al.* [14] who studied the effects of pH on citric acid production from concentrated milk-wastewater by *Aspergillus niger*. This would seem to suggest that the pH did not directly influence the citric acid production mechanism, but rather affected the enzymes which were active in degrading the substrate and/or the permeability of the cell membrane of the substrate and product.

In general, a low pH is essential for achieving the maximum production of citric acid [15]. Shadafza *et al.* [16] reported that a low initial pH has the advantage of checking contamination and inhibiting oxalic acid formation. At pH 4 or above, the formation of oxalic acid is accelerated in a medium with a high buffering capacity [17]. A pH of 2.2 has been reported to be the optimum for the growth of a mould in addition to the production of citric acid [18], whereas a higher pH (5.4 and 6.0-6.5) has been found to be the optimum level for citric acid production in a molasses medium [19]. In addition, Kristiansen and Sinclair [20] reported that the maximum production of citric acid by *Aspergillus foetidus* in a single-stage continuous culture was at a pH of around 3.4.

Effect of Temperature on Citric Acid Production

Temperature is also an important factor for citric acid production. The effect of temperature on the citric acid production and residual sugar from the dairy wastewater when using immobilized *Aspergillus niger* ATCC 9142 is shown in Fig. 3(a) and (b). The maximal citric acid production was obtained at 30°C. The yields and productivities of citric acid at 25, 30, and 35°C were 23.7, 58.9, and 25.7%, and 100, 250 and 96 mg/L·h, respectively (data not shown). Kopper *et al.* [21] recommended that a temperature between 25 and 30°C helps to obtain high yields and rapid rates of accumulation of citric acid. At higher temperatures, the fermentation process is very rapid and abundant mycelial growth occurs, consuming large amounts of sugar and thus lowering the yield of citric acid. Conversely, at lower temperatures, higher yields of citric acid are possible, by prolonging the fermentation process [9]. Eikmeier and Rehm [8], and Roukas and Kotzekidou [22] reported that the optimal condition for cirtic acid production by immobilized *Aspergillus niger* was 30°C. In addition, Roukas [23] reported the citric acid concentration increased significantly with an increase in the fermentation temperature from 25 to 30°C and then decreased above 30°C, plus Szewczyk and Myszka [24] studied the effect of temperature on *Aspergillus niger* growth in solid-state fermentation and found that temperature did not have strong a affect on the growth rate within a range of 28-34°C. In the current study, the optimal temperature for citric acid production was found to be 30°C when the fermentation was with immobilized conidia. This result agrees with that of Hang and Woodams [25] who studied the effect of temperature on citric acid production from grape pomace by solid-state fermentation, and Roukas [23] who studied the effect of temperature on citric acid production from carob pods by solid-state fermentation.

Effect of Dilution Rate on Citric Acid Production

The dilution rate is the medium flow rate per effective reactor volume. Fig. 4 shows the effect of the dilution rate on the citric acid production from the dairy wastewater when using immobilized *Aspergillus niger*. The optimum citric acid productivity and yield were 160 mg L^{-1} h^{-1} and 70.3% at a dilution rate of 0.025 h^{-1} (Fig. 4 and Table 2). However, the yield of citric acid decreased when the dilution rate fell below 0.010 h^{-1} because the sugar consumption rate decreased with a dilution rate. These results are similar to those reported by Rymowicz *et al.* [26] who studied the effect of the dilution rate on citric acid production from glucose by calcium-alginate immobilized *Yarrowia lipolytica* A-101 yeast in continuous air-lift cultivations. Kristiansen and Sinclair [20] reported that the maximum productivity

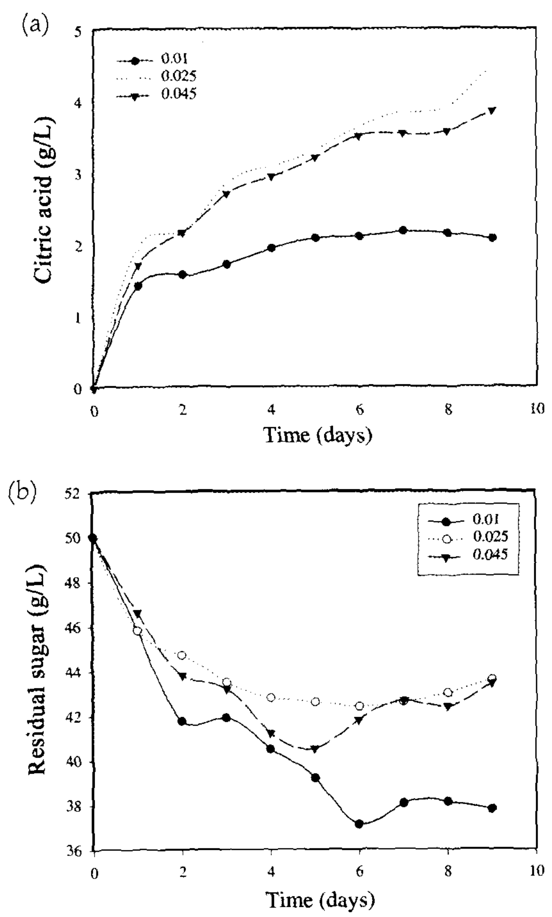

Fig. 4. Effect of dilution rate on continuous citric acid production (a) and amount of residual reducing sugar (b) at 30°C and pH 3.0 from dairy wastewater by immobilized *Aspergillus niger* ATCC 9142.

Table 2. Effect of dilution rate on yield of citric acid and productivity from dairy wastewater using immobilized *Aspergillus niger* at 30°C and pH 3.0 after 9 days

Dilution rate (h^{-1})	Yield (%)	Productivity (mg L^{-1} h^{-1})
0.010	29.5	31
0.025	70.3	160
0.450	58.9	250

of citric acid by *Aspergillus foetidus* in a single-stage con-tinuous culture was 0.43 kg/L·h at a dilution rate of 0.075 h^{-1}, and Dawson *et al.* [27] reported that the optimal dilution rate was 0.017 h^{-1}. Gupta and Sharma [28] were reported that the optimal dilution rate for the continuous production of citric acid from sugarcane molasses was 0.015 h^{-1}.

Comparison of Citric Acid Production between Shake-Flask and Continuous Fermentation

The major objective of cell immobilization technology is to achieve a reactor system with a high productivity that can be maintained for extended time periods. In addition, the immobilization material should be inexpensive while the actual immobilization procedure and subsequent reactor operation should be simple [29]. The current study attempted to improve the citric acid productivity by a continuous fermentation process using calcium-alginate immobilized cells of *Aspergillus niger* in a bioreactor. In a batch shake-flask fermentation, the productivity (63.3 mg L^{-1} h^{-1}) and yield (51.4%) of citric acid reached a maximum after 3 days, and the citric acid concentration and residual sugar concentration after 20 days were 1.38 g/L and 8.85 g/L, respectively. Meanwhile, the yield (70.3%) of continuous cit-

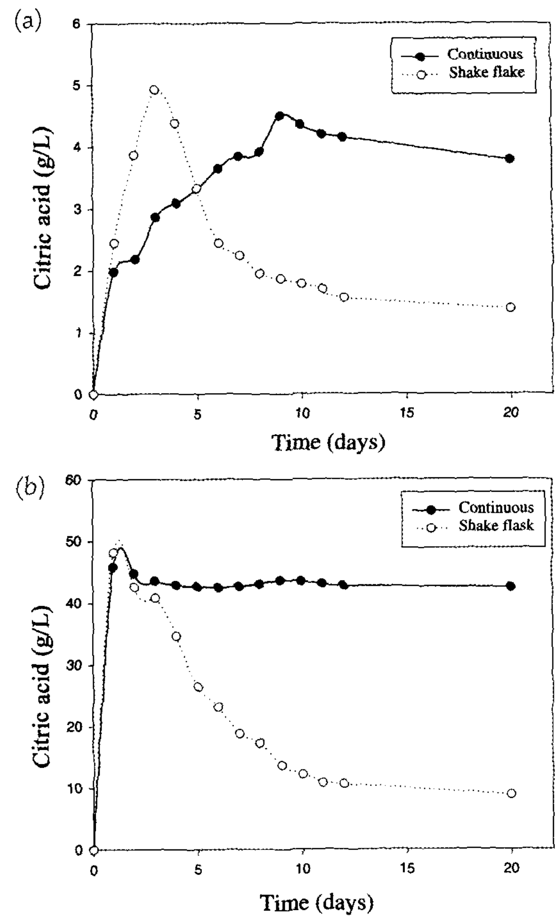

Fig. 5. Comparison of continuous and shake flask culture after 9 and 3 days for citric acid production (a) and amount of residual sugar (b).

Table 3. Comparison of citric acid production between shake flask after 3 days and continuous fermentation after 9 days under optimal conditions

Fermentation	Yield (%)	Productivity (mg L^{-1} h^{-1})
Shake-flask fermentation	51.4	63.3
Continuous fermentation	70.3	160.0

ric acid production when using immobilized *Aspergillus niger* reached a maximum level after 9 days (Fig. 5). In addition, the productivity value (160.0 mg $L^{-1} h^{-1}$) was twice that of the batch shake-flask culture (Table 3).

Dawson *et al.* [27] reported that the production of citric acid by *Aspergilus niger* in a continuous culture increased 2-fold when compared to a batch process. Chung and Chang [30] reported that the yield of continuous citric acid production in a dual hollow fiber bioreactor increased about 40% when compared to batch fermentation. In addition, Rymowicz *et al.* [26] reported that the productivity of citric acid produced by immobilized *Yarrowia lipolytica* achieved 350 mg/L·h, which was about a 2.5-fold improvement when compared to the productivity obtained in a batch processs.

In conclusion, the current results reveal some important aspects of citric acid production from dairy wastewater by immobilized *Aspergillus niger*. The optimum conditions were pH, 3.0; temperature, 30°C; and dilution rate, 0.025 h^{-1}. In addition, the productivity of citric acid by the immobilized *Aspergillus niger* in a continuous reactor was more than two-fold higher than that in a the shake-flask culture. Accordingly, the results suggest that the bioreactor used in the current study could be potentially used for continuous citric acid production from dairy wastewater by calcium-alginate immobilized *Aspergillus niger*.

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