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Citric Acid Production from Concentrated Milk-wastewater by *Aspergillus niger*

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Abstract

The possible use of milk-wastewater as a fermentation media for the production of citric acid by *Aspergillus niger* has been investigated. The addition of Mn^{2+}, Fe^{2+} and Cu^{2+} to the medium promoted citric acid production while only Mg^{2+} decreased citric acid production. The concentrations of citric acid produced were marked up to 7.2g/ℓ and 16.5g/ℓ in a batch bioreactor by *Aspergillus niger* ATCC 9142 with 50g/ℓ and 100g/ℓ of reducing sugar concentration in milk-wastewater, respectively. A mathematical model was developed and simulating the predictability of cell growth, citric acid production and substrate consumption rate, and gave good agreement results with experimental data.

Key words : citric acid production, milk-wastewater, Aspergillus niger ATCC 9142

Introduction

Recently, and along with the growing popularity of instant foods consumption of alkaline foods(e.g. milk, potato etc.) has increased rapidly. Mass production of these foods in factories causes water pollution and the treatment of water pollution is becoming a social issue. In the processing of milk, large amounts of waste, as well as some sugars and proteins, are released into the waste stream. The high organic load in milk processing wastewater creates a serious pollution problem and poses a threat to water quality when discharged to rivers and lakes[1]. With the increasing cost of pollution abatement, there is a need for information on ways to reduce the strength of effluent from the processing of milk.

The conversion of different organic wastes by means of microorganisms has been the subject of many research works[2]. Most suitable substrates are food industry waste materials.

Citric acid is widely used in the food and beverage industries as an acidulant, preservative, precursor for soluble aspirins and for the stabilization of ascorbic acid. Commercial citric acid is produced by submerged fungal fermentation of a glucose, sucrose, cane or molasses medium etc[3]. However, several investigators have reported on citric acid production using *Aspergillus niger* and different sources of carbohydrates[4)~11)].

Recently, research has been directed towards finding ways of utilizing wastewaters. Milk-wastewater is a significant source of high-strength waste in the dairy industry.

For any fermentation process based on milk-wastewater it is advantageous if the microorganism concerned is capable of utilizing all of the sugars present in the substrate, both to maximize product yield and to minimize

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any waste disposal problem resulting from residual sugars. In spite of this, little information has been published on the use of milk-wastewater as a substrate.

The purpose of this investigation was to explore the possible use of milk-wastewater instead of sugars for the production of citric acid. Also, a mathematical model was developed and tested for the predictability of cell growth, citric acid production and substrate consumption rates.

Materials and Methods

Organisms and medium

A citric acid producing strain of *Aspergillus niger* ATCC 9142 was obtained from the Genetic Engineering Center in Korea. It was grown on a potato dextrose agar slant at 30℃ for 7 days, and stored at 4℃. The agar slant was renewed every two months.

Milk-wastewater used in this study was obtained from the dairy industry. It contained 1.2%~3.5% reducing sugar mostly as lactose and had a moisture of 97%. So it was concentrated to 5% of total reducing sugar for our experiment.

Inoculum

A 0.2% inoculum, containing $10^5 \sim 10^6$ spores/mℓ, in distilled water was used to inoculate the 100mℓ culture medium.

Fermentation studies

Shake-flask culture experiments were performed in 250mℓ Erlenmeyer flasks containing 100mℓ of medium. All media and equipment were sterilized at 121℃ for 15min. The flasks were incubated at 30℃ on a rotary shaking incubator Model KMC-8480SF(Vision Scientific Co., Korea) operating at a speed of 180rpm.

Fermentation experiments were performed in a batch reactor(working volume 1ℓ). The aeration rate was lvvm during the fermentation time and agitation speed was set at 400rpm. The temperature was controlled at 30℃ but the pH was not controlled.

Analytical methods

Mycelial dry weight was determined by filtering, washing with distilled water and drying at 105℃ overnight. Reducing sugar was measured by the method of DNS[12], and citric acid measured by the method of Marier and Boulet[13].

Results and Discussion

Flask experiments

Effect of Initial pH on the Production of Citric Acid, Cell Mass and Residual Sugars

The effect of initial pH on the production of citric acid is shown in Fig. 1(A). Citric acid concentration increased steadily between pH 3 and pH 7.2, reaching a maximum concentration of 3.6g/ℓ at pH 3. At pH 2 and pH values greater than pH 3 a loss of production occurred i.e., 1.12g/ℓ at pH 2, 2.8g/ℓ at pH 4 and 2.5 g/ℓ at pH 7.2. These findings are similiar to those observed by Kristiansen and Sinclair[14] who found that *Aspergillus foetidus* produced a maximum concentration of 6g/ℓ citric acid at pH 3.4.

The results of steady-state cell and residual sugar concentrations as a function of time are shown in Fig.1 (B)(C). Between pH 2.0 and 7.0, the cell mass increased to a maximum of 13.4g/ℓ at pH 2.0. As shown in Fig.1(B) the cell mass at pH 2.0 was 65% higher than that of cell mass at pH 7.2, cell mass is, therefore, also dependent on pH. As expected the concentration of residual sugars decreased between pH 2.0 and 7.2 coinciding with an increase in cell and citric acid production.

Effect of addition ammonium nitrate on citric acid production

Ammonium nitrate was used as a nitrogen source. pH is known to be very important in citric acid fermentation and can be maintained at a low level by using ammonium nitrate as a nitrogen source[15,16].

A series of flask cultures were tested with different initial ammonium nitrate concentrations from 0 to 3g/ℓ.

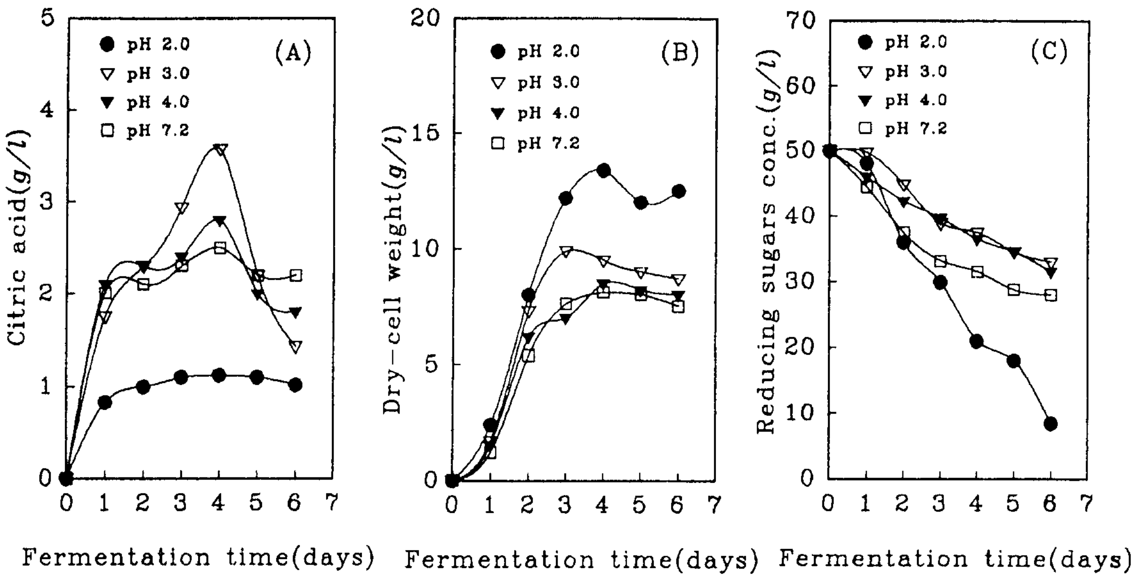

Fig. 1. Effect of initial pH on citric acid production, mycelial growth and total reducing sugars.

The affect of the addition of ammonium nitrate on citric acid conversion from the milk-wastewater is shown in Fig. 2. It is apparent that in no case did the added ammonium nitrate increase the formation of citric acid. These results show that citric acid yield is inversely related to the nitrogen content of the culture medium. As the nitrogen content increases, so does the amount of sugar consumption(data not shown), but this appears to be converted to storage carbohydrate, and possibly carbon dioxide, rather than to citric acid. In general, the data supports the observation of Kristansen *et al.*[17] that an excess level of nitrogen affects citric acid production.

Effect of addition metal ions on citric acid production

Metal ions are the micronutrients which modulate the biochemical conversion, and direct the sequence of metabolic conversion and result in the overproduction of desired secondary metabolites. *Aspergillus niger* accumulates large amounts of citric acid extracellularly when grown on a minimal salts medium, but an overabundance of the metal ions leads to excessive vegetative growth at the expense of citrate accumulation[18].

The concept of enhancing citric acid production by the optimization of different trace metal levels was addressed next. Separate runs were carried out with various levels of Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} in milk-wastewater. It was observed that the yield of citric acid is enhanced by higher concentrations of Mn^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}(Table 1). By contrast, the Mg^{2+} ion at higher concentrations, shows an inhibitory effect on citric acid production. The optimum concentrations of metal ions were taken as the value above which no significant increase in the yield of citric acid was observed.

Bioreactor experiments

Effect of substrate concentration on citric acid production

Milk-wastewater contains about 12~35g/ℓ of reducing sugar depending on the process of milk production used. In order to raise concentrations of citric acid, reducing sugars were used at different levels in batch bioreactor. In these experiments milk-wastewater with 50, 70 and 100g/ℓ of reducing sugar concentrations were

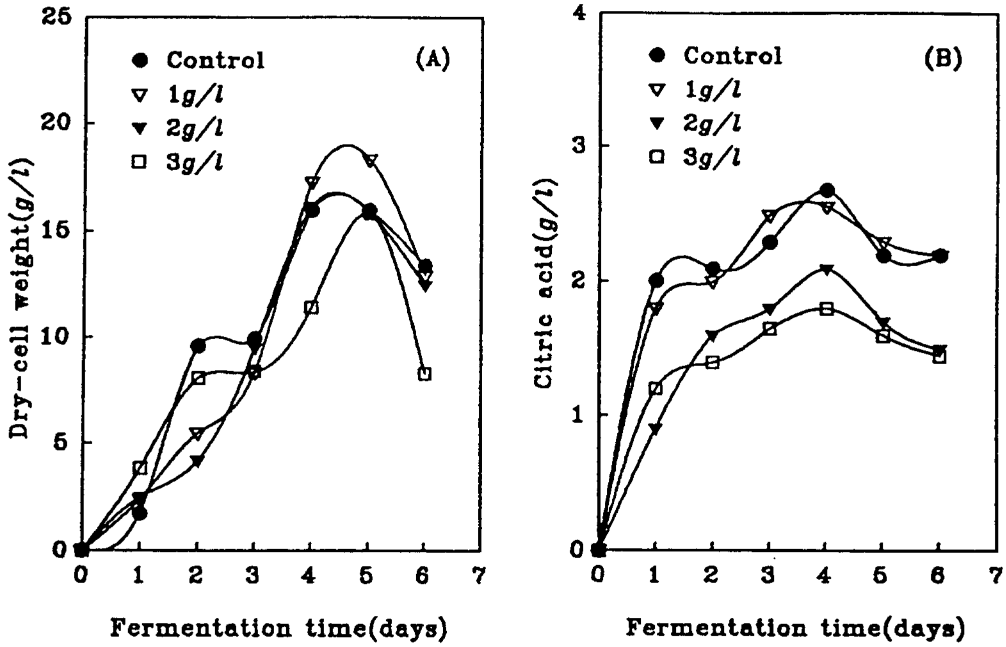

Fig. 2. Effect of nitrogen source as NH_4NO_3 on citric acid production and mycelial growth.

Table 1. Data of addition some metal ions

Metal ions	Addition concentration (g/ℓ)	Mycelial dry weight (g/ℓ)	Reducing sugar consumption (g/ℓ)	Reducing sugar consumption (%)	Yield of citric acid (g/ℓ)	Yield of citric acid (%)
Mn^{2+}	0.00	9.1	25.5	51.0	2.7	10.6
	0.01	9.3	21.0	42.0	3.5	16.7
	0.02	9.0	24.0	48.0	3.6	15.0
	0.03	12.3	25.8	51.6	3.9	16.3
Mg^{2+}	0.00	8.1	25.5	51.0	2.7	10.6
	0.01	5.1	28.5	57.0	2.3	8.1
	0.02	4.1	26.9	53.8	2.3	8.6
	0.03	3.3	29.7	59.4	2.2	7.4
Zn^{2+}	0.00	8.1	25.5	51.0	2.7	10.6
	0.01	6.5	9.8	19.6	2.3	23.5
	0.02	6.0	7.9	15.8	2.7	34.2
	0.03	4.8	13.0	26.0	3.2	24.6
Fe^{2+}	0.00	8.1	25.5	51.0	2.7	10.6
	0.01	8.7	30.5	61.0	2.9	9.5
	0.02	11.7	28.6	57.2	3.4	11.9
	0.03	12.3	27.5	55.0	3.0	10.9
Cu^{2+}	0.00	8.1	25.5	51.0	2.7	10.6
	0.01	9.9	9.5	19.0	3.8	40.0
	0.02	10.5	15.0	30.0	4.2	28.0
	0.03	13.1	17.0	34.0	4.4	25.9

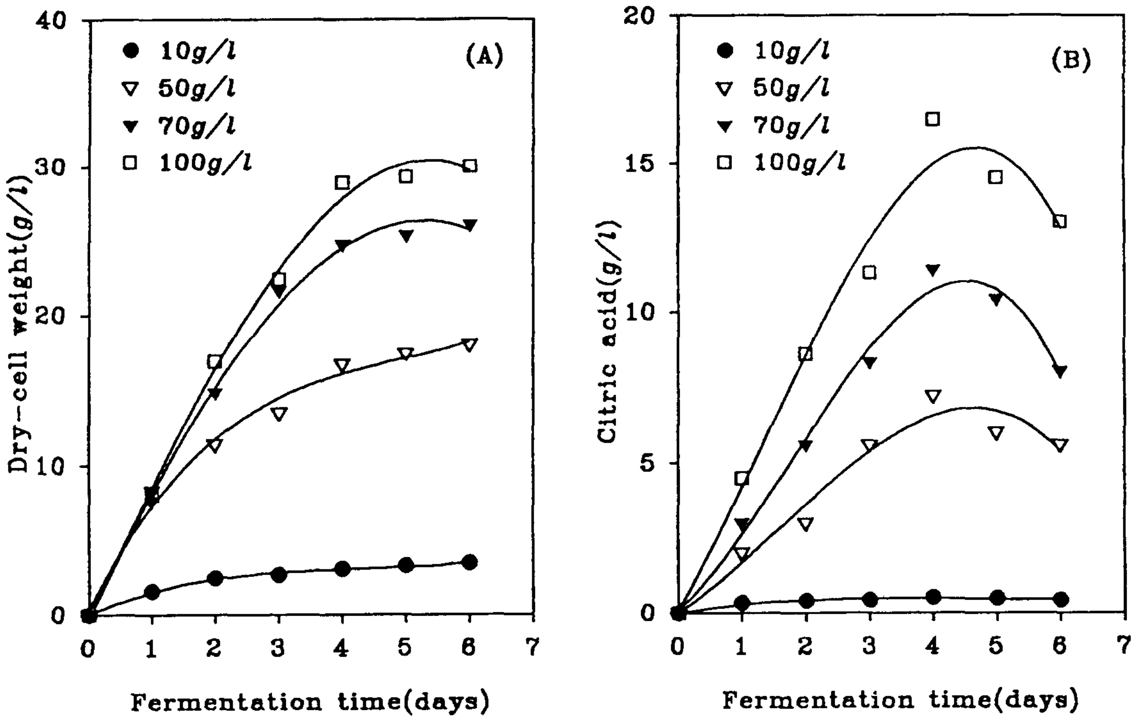

Fig. 3. Time course profiles of citric acid production and mycelial growth for *Aspergillus niger* ATCC 9142 cultures with concentrated milk-wastewater in a batch bioreactor.

used. Increasing the reducing sugar concentrations resulted in the citric acid and cell mass increasing(Fig. 3). The concentrations of citric acid were marked up to 7.2, 11.4 and 16.5g/ℓ in a batch bioreactor with *Aspergillus niger* ATCC 9142 with 50, 70 and 100g/ℓ of reducing sugar concentrations in milk-wastewater, respectively. From this result concentrated medium was increased the production of citric acid about 8.4~33 fold as compared with no concentrated.

Mathematical modelling of batch fermentation

The model employs rate equations for cell(X), product([CIT]), and substrate([GLU]) to describe the fermentation process

1. Cell growth

For cells, the logistic rate equation is used :

$$\frac{dX}{dt} = \mu X \left(1 - \frac{X}{X_{max}}\right) \qquad (1)$$

where μ is the growth constant and X_{max} is the maximum cell-mass

2. Production of citric acid

The production formation rate equation used is that of Luedeking-Piret,

$$\frac{d[CIT]}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad (2)$$

where α is the experimental constant for the growth relation, and β is the experimental constant for the non growth constant. Equation (2) divided by X and substituting equation (1) into this expression gives

$$\frac{1}{X}\frac{d[CIT]}{dt}=(\alpha\mu+\beta)(1-\frac{\alpha\mu}{(\alpha\mu+\beta)X_{max}}X) \quad (3)$$

Applying the Luedeking-Piret equation to an stationary state of batch culture it follows that, since $\beta=0$.

$$\frac{1}{X}\frac{d[CIT]}{dt}=\alpha\mu(1-\frac{X}{X_{max}}) \quad (4)$$

This means that the cell mass increases with fermentation time but the production rate of citric acid decreases.

3. Substrate consumption

Substrate consumption rate is shown below. Substrate consumption is taken to depend on the magnitudes of three factors, the instantaneous cell growth, the product formation rate, and the cell maintenance function.

$$-\frac{d[GLU]}{dt}=\frac{1}{Y_{X/G}}\frac{dX}{dt}+\frac{1}{Y_{A/G}}\frac{d[CIT]}{dt}+K_eX \quad (5)$$

$$-\frac{1}{X}\frac{d[GLU]}{dt}=\frac{1}{Y_{X/G}}\frac{1}{X}\frac{dX}{dt}+\frac{1}{Y_{A/G}}\frac{1}{X}\frac{d[CIT]}{dt}+K_e \quad (6)$$

$$=\frac{1}{Y_{X/G}}\mu(1-\frac{1}{X_{max}})+\frac{1}{Y_{A/G}}\alpha\mu(1-\frac{X}{X_{max}})+K_e \quad (7)$$

$$=(\frac{\mu}{Y_{X/G}}+\frac{\alpha\mu}{Y_{A/G}}+K_e)-(\frac{\mu}{Y_{X/G}X_{max}}X+\frac{\alpha\mu}{Y_{A/G}X_{max}}X) \quad (8)$$

$$=A-BX$$

Since X increases during fermentation, $\frac{1}{X}\frac{d[GLU]}{dt}$ decreases gradually, but the substrate can't increase. Thus, the substrate consumption term can not fall below zero. Substrate consumption parameters were estimated by the Runge-Kutta-Gill method. And the results are shown in Table 2.

The mathematical model was tested for predictability of cell growth, citric acid production and substrate consumption rate and results showed good agreement with experimental data(Fig. 4).

Acknowledgement

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Table 2. Estimated model parameters

Initial concentration of reducing sugar milk-wastewater(g/ℓ)	Parameters				
	X_{max}(g/ℓ)	μ(hr^{-1})	α	A	B
50	18.0	0.048	3.281	8.677	0.320
70	26.1	0.053	4.774	7.851	0.362

X_{max}(g/ℓ) : maximum cell-mass

μ(hr^{-1}) : growth constant

α : experimental constant for the growth relation

A, B : reference to equation(8)

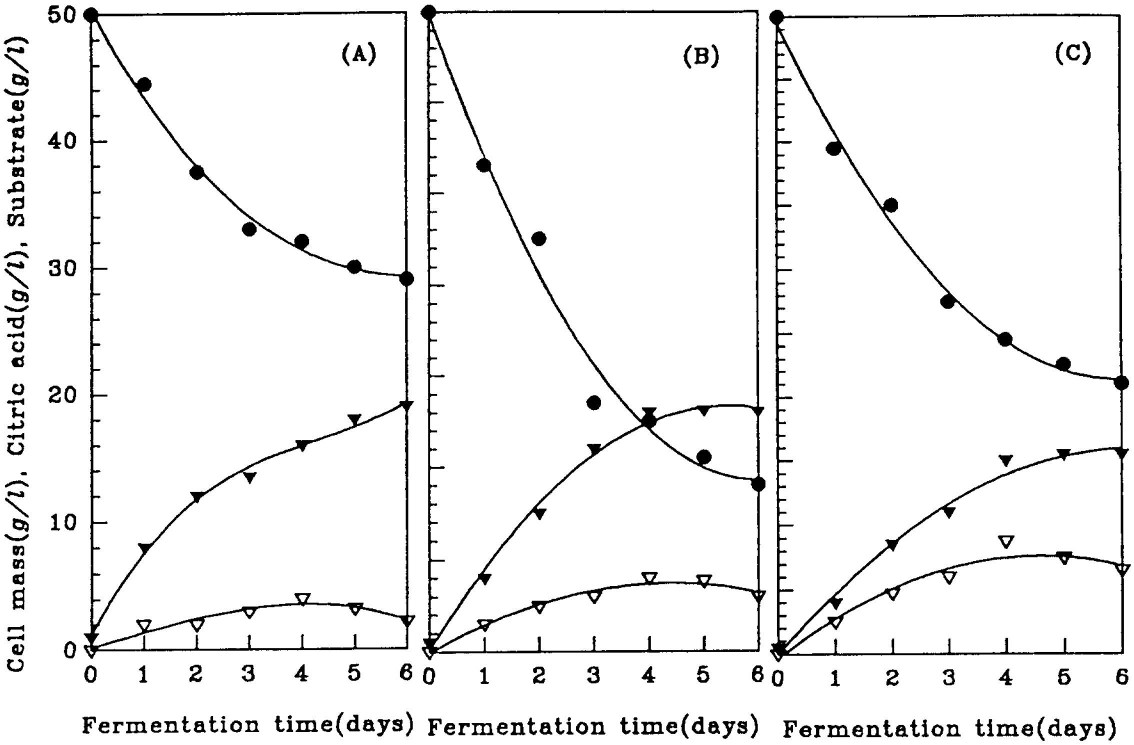

Fig. 4. Comparision of the experimental data with simulation results of milk-wastewater at (A) 50g/ℓ, (B) 70g/ℓl, (C) 100g/ℓ reducing sugar concentration

▽ citric acid ; ▼ cell mass ; ● substrate.

▽, ▼, ● : Experimental, ______ : Simulation

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초록 : *Aspergillus niger*를 이용한 우유폐수로 부터의 구연산 생산

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우유공장에서 배출되는 폐수를 *Aspergillus niger* ATCC 9142의 발효기질로 하여 회분식발효기에서 구연산의 생산에 관하여 연구하였다. 구연산의 생산성을 높이기 위하여 각종 금속이온, Mn^{2-}, Fe^{2+}, Cu^{2+} 및 Mg^{2+} 등을 첨가하여 발효시킨 결과, Mn^{2-}, Fe^{2-} 및 Cu^{2+}의 첨가시 구연산의 생산이 증가하였으나, Mg^{2-}를 첨가한 경우는 오히려 감소하였다. 우유폐수의 환원당 농도를 각각 50g/ℓ 및 100g/ℓ로 농축하여 *Aspergillus niger* ATCC 9142를 이용하여 생산된 구연산은 7.2g/ℓ 및 16.5g/ℓ 이상이였다. 실험적인 결과에 의한 세포의 성장, 구연산의 생산 및 기질의 소모속도는 수학적인 모델링에 의해 예측한 경우와 잘 일치하고 있음을 알 수 있었다.