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Kinetics for Citric Acid Production from the Concentrated Milk Factory Waste Water by *Aspergillus niger* ATCC 9142

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The waste water from a milk factory was investigated for possibility of use to the production of citric acid by cells of *Aspegillus niger* ATCC 9142. The addition of Mn^{2+}, Fe^{2+} and Cu^{2+} ions to waste increased citric acid production steadily, but addition of metal ion Mg^{2+} decreased the citric acid production. The amount of produced citric acid by *Aspegillus niger* ATCC 9142 with addition 50 g/l and 100 g/l of reducing sugar in milk factory waste water were 7.2 g/l and 16.5 g/l respectively. Mathematical model was simulated for their predictability of cell growth, citric acid production and substrate consumption rate and coincided with experimental data.

Key words – Milk factory waste water, *Aspegillus niger* ATCC 9142, citric acid production, reducing sugar concentration, mathematical model

Recently the consumption of alkaline instant food processed using natural food such as egg, milk, potato, etc has increased rapidly. Mass production of these food in factories causes pollution of the water[6]. Citric acid is widely used in the food and beverage industries as an acidulant, preservative and precursor for soluble aspirins and stabilizes of ascorbic acid. Commercial citric acid is produced by submerged fungal fermentation of a glucose, sucrose, cane or molasses medium etc[5]. Several investigators have reported the citric acid production using *Aspergillus niger* and diverse sources of carbohydrates[7,15,16]. Most of researches till now have been described to methods for reusing of waste water. Milk factory waste water is important source of high-strength waste streams in the dairy industry. For a fermentation process based on milk factory waste water, if a microorganism is capable of utilizing all of the sugars present in the substrate, it is advantageous both on the basis of product yield and to minimize any waste disposal problem resulting from residual sugar. Much information were not reported about use of milk waste water as a substrate for secondary process such as citric acid fermentation up to now. The purpose of this investigation is to find the possibility of use for milk factory waste water as a medium source for production of citric acid instead of sugars[10]. Also, mathematical model was simulated for predictability of cell growth, citric acid production and substrate consumption rate.

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Materials and Methods

Organisms and medium

A citric acid producing strain of *Aspergillus niger* ATCC 9142 was obtained from American Type Culture Collection (ATCC). It was grown on a potato dextrose agar (PDA, Difco Lab., Detroit, USA) slant at 30℃ for 7 days, and stored at 4℃. The agar slant was subcultured by every two month. Milk factory wate water used in this study was obtained from a local dairy industry. It contained 1.2~3.5% reducing sugars and its moisture constent was 97%. A inoculation was performed by 0.2%, suspended by 10^5~10^6 spores in distilled water, to culture medium.

Fermentation studies

A preculture of the strain were performed in a 250 ml erlenmeyer flask containing 100 ml milk waste water with shaking. All media and equipment were sterilized at 121℃ for 20 min. The flasks were incubated at 30℃ on a rotary shaking incubator (Vision scientific Co., Korea) and operating speed of 180 rpm. Fermentation experiments were performed in a batch reactor (working volum 1 l). Agitation speed was 400 rpm. The fermentation temperature was maintained at 30℃ and the initial pH was adjusted to 2, 3, 4, and 7.2.

Analytical method

Mycelial dry weight was determined by filtering, washing with distilled water and drying at 105℃ overnight. Amount of reducing sugars were measured by the method of dinitrosalicylic acid (DNS)[3], and citric acid was measured as reported Mareier et al. before[12].

Results and Discussion

Effect of initial pH on the production of citric acid, mycelial growth and total reducing sugars

The effect of initial pH on the production of citric acid[11] was shown in Fig. 1. A citric acid concentration increased steadily until 1 day, but after then stoped increasing for all pH regions except for pH 3.0. The medium adjusted initial pH 3.0 constinuosly increased production of citric acid until 4 days, achieving a maximum concentration of 3.6 g/l. At the initial pH rather than pH 3, the maximum yield of citric acid for other pH regions showed 1.12 g/l at pH 2, 2.8 g/l at pH 4 and 2.5 g/l at pH 7.2. These findings were similiar to the previous report that *Aspergillus foetidus* produced a maximum concentration of 6 g/l citric acid at pH 3.4 [9]. The results of residual sugar concentrations as a time function were shown in Fig. 1c. The cell mass increased to a maximum of 13.4 g/l at pH 2.0 in Fig. 1b. As shown in Fig. 1b, the cell concentration at pH 2.0, adjusted the initial pH 2.0, was 65% higher than at pH 7.2. As expected, the concentration of residual sugars were decreased in assayed all pH regions in contrast on an increase in cell mass and citric acid production.

Effect of ammonium nitrate addition as a nitrogen source for citric acid production

Ammonium nitrate as a nitrogen source was used for citric acid production. pH is known to play very important role in citric acid fermentation and can be maintained at a low level by using ammonium nitrate as a nitrogen source[14]. A series of flask cultures was conducted with different initial ammonium nitrate concentrations from 0 to 3 g/l. The effect of ammonium nitrate on citric acid conversion from the milk factory waste water was shown in Fig. 2. It revealed that none of the added ammonium nitrate increased the formation of citric acid. This results

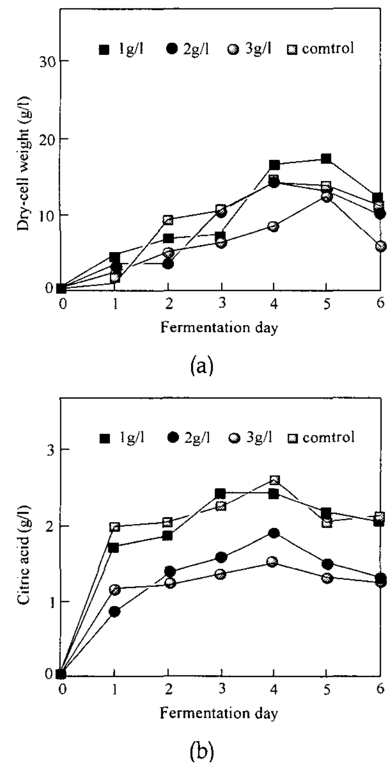

Fig. 2. Effect of NH_4NO_3 as nitrogen source on citric acid production (a) and effect of NH_4NO_3 as itrogen source on mycelial growth (b).

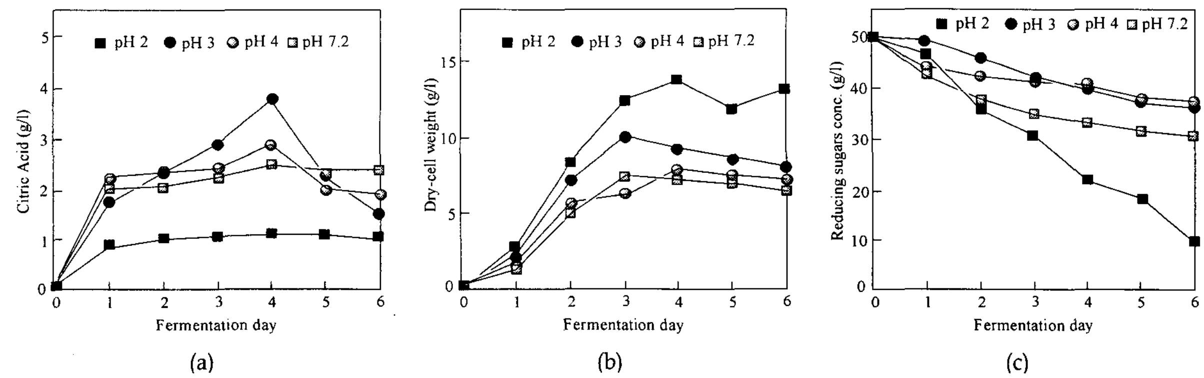

Fig. 1. Effect of initial pH to production of citric acid (a), effect of initial pH to mycelial growth (b), and effect of initial pH to production of total reducing sugars (c).

show that the citric acid yield is inversely related to the nitrogen content of the culture medium. As the nitrogen content increased, so did the amount of sugar consumption, but this appears to be converted to storage carbohydrate, and possibly carbon dioxide, rather than to citric acid. In general, the data support the observation of Kristansen et al.[8] that the level of excess nitrogen affects citric acid production.

Effect of metal ions additionon citric acid production

Metal ions are the micronutrients which modulate biochemical conversions[2], and intend the sequence of metabolic conversions to result in overproduction of desired secondary metabolites. *Aspergillus niger* ATCC 9142 produces large amount of citric acid extra cellularly when grown on a minimal salts medium, but overabundance of the metal ions leads to excessive vegetative growth at the expense of citrate accumulation[13]. Enhancing for citric acid production optimal levels of various trace metals were investigated. The various levels of Mn^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} were added and analyzed in milk factory waste water. Our results showed that yield of citric acid was enhanced at high concentrations of Mn^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} (Table 1). In contrast, high concentrations of Mg^{2+} ion, showed an inhibitory effect on citric acid production.

Effect of substrate concentration on citric acid production

Concentration of total reducing sugars included in milk waste water varies by 12 g/l~35 g/l according to each milk production process. To heighten concentration of citric acid production, reducing sugars were used at different levels in batch bioreactor. For this experiment, the reducing sugar concentrations in milk factory waste water were concentrated by 50 g/l, 70 g/l and 100 g/l. When concentration of reducing sugars increased, production of citric acid and cell mass increased (Fig. 3). The concentrations of citric acid were marked up to 7.2 g/l, 11.4 g/l and 16.5 g/l in a batch bioreactor by *Aspergillus niger* ATCC 9142 with the reducing sugar concentrations used to concentrate milk factory waste water by 50 g/l, 70 g/l and 100 g/l respectively. From this results concentrated medium increased the production of citric acid about 8.4~33

Table 1. Data of addition metal ions.

Metal ions	Added Concentration (g/l)	Mycelial dry wt. (g/l)	Reducing sugar consumption (g/l)	Reducing sugar consumption (%)	Yield of citric acid (g/l)	Yield of citric acid (%)
Mn^{2+}	0	8.1	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	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	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	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	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

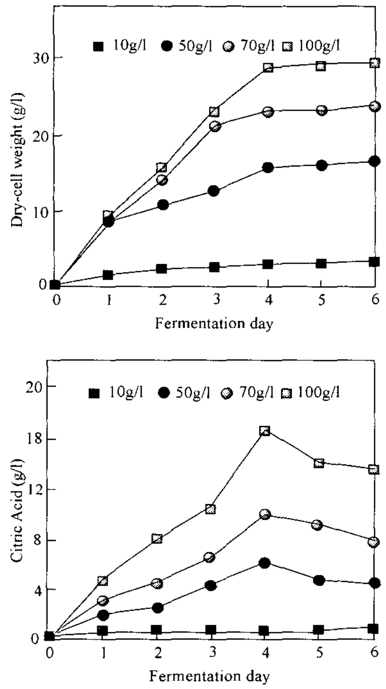

Fig. 3. Time course profiles of citric acid production and mycelial growth for *Aspergillus niger* ATCC 9142 cultures with concentrated milk factory waste water in a batch reactor.

fold as compared with no concentrated.

Mathematical modelling of batch fermentation

The model employs rate equations for cell mass (X), product ([CIT]), and substrate ([GLU]) to describe the fermentation process[4].

Cell growth

For cell growth, the logisitic rate equation is used:

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

where μ is the growth constant and X_{max} is the attainable maximum cell concentration[1].

Production of citric acid

The product formation rate equation is taken as that of Luedeking-Piret[1],

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

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

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

Appling Luedeking-Piret equation to an stationary state of batch culture it follows that, since β=0,

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

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

Substrate consumption

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

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

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

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

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

$$= A - BX$$

Since X increases during the fermentation, $-\frac{1}{X}\frac{d[GLU]}{dt}$ decreases gradually, but the substrate can't increase. Thus, the substrate consumption term can't fall down below zero. In substrate consumption parameters were estimated by Runge-Kutta-Gill method. And the re-

Table 2. Estimated model parameters.

Initial concentration of reducing sugar milk factory waste water(g/l)	Parameter				
	X_{max}(g/l)	μ(hr^{-1})	*a*	A	B
50	18	0.048	3.281	8.677	0.320
70	26.1	0.053	4.774	7.851	0.362
100	30.4	0.056	8.963	11.616	0.39

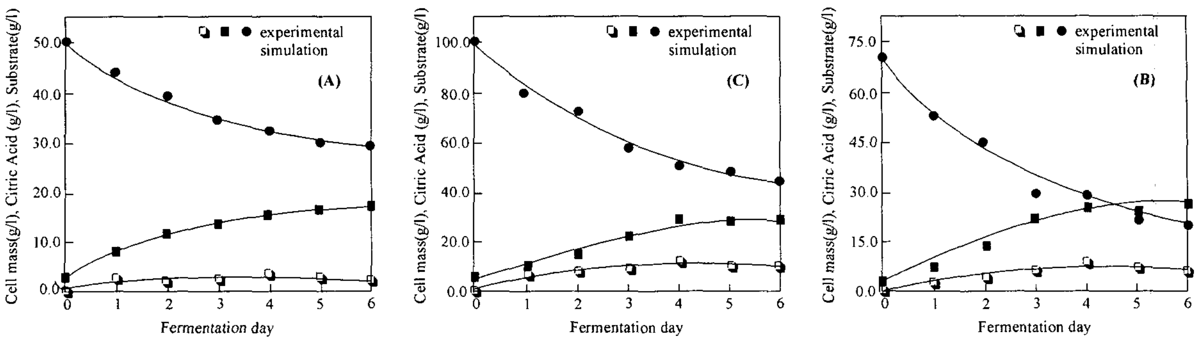

Fig. 4. Comparison of the experimental data and simulation results of milk factory waste water at 50 g/l (A), 70 g/l (B), 100 g/l (C) reducing sugar concentration. □: citric acid, ■: cell mass, ●: substrate.

sults were represented at Table 2. Methematical model was simulated for predictability of cell growth, citric acid production and substrate consumption and exhibited good agreement to result with experimental data (Fig. 4).

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초록 : *Aspergillus niger* ATCC 9142 세포에 의해 농축된 우유공장폐수로부터 구연산생산에 대한 동력학 연구

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Aspegillus niger 세포에 의한 구연산 생산에 대한 발효 매체로서 우유공장폐수가 연구되었다. 발효매체에 Mn^{2+}, Fe^{2+}, Cu^{2+}의 첨가가 구연산 생산을 일정하게 증가시켰다. 그러나 다른 금속이온인 Mg^{2+}의 첨가는 구연산 생산을 감소시켰다. 구연산의 농도는 우유공장폐수에서 환원당 농도의 농도가 50 g/l와 100 g/l로 *Aspegillus niger* ATCC 9142에 의한 batch bioreactor에서 각각 7.2 g/l과 16.5 g/l로 높게 표시되었다. 본 실험에서 적용한 수리적 모델식들이 세포성장의 예상, 구연산 생산, 기질소모 속도에 대해 잘 적용되는지를 알아보기 위해서 발효반응 모델식에 적용시켜 본 결과 실험치와 잘 부합되었다.