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# Pretreatment of Sugarcane Molasses and Citric Acid Production by *Candida zeylanoides*

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Citric acid is produced via submerged fermentation using yeasts. Among eight different strains of yeast, *Candida zeylanoides* was chosen as the strain for producing citric acid and optimized for various C/N ratios and effects of phosphate or $Fe^{2+}$ ions in a clean carbon source medium (glucose: fructose, 1:1). The yield of citric acid was maximized at a C/N ratio of 40/1, a phosphate addition of 1.0 g/l, and an $Fe^{2+}$ ion concentration of less than 50 mg/l, yielding up to 91 g/L in the broth with 18.5 g/l of iso-citric acid in a six-day fermentation period using a pre-treated molasses medium. The yield of batch culture was 0.51 ($Y_{p/s}$, g/g) in a 5 L-Jar fermenter.

**Keywords:** Citric acid production, submerged fermentation, iso-citric acid, pretreated cane molasses, tricalcium phosphate, *Candida zeylanoides*

Citric acid is a common, yet vital metabolite that is mass-produced by fermentation due to its wide range of uses and applications. The high functionality and versatility of citric acid contributes to its many uses in industrial applications such as an additive for detergents, shampoos, cosmetics and chemical cleaners [16].

Production of citric acid is done generally in two methods: the surface method and the submerged method. The surface method, which utilizes labor, does not require an expensive bioreactor or large amounts of energy and is also less sensitive to trace metal elements than the submerged method. However, because it lacks control and fails to produce steady results along with added labor cost [3, 6], the submerged method is preferred. Currently, the most common commercial production of citric acid is by submerged fermentation using *Aspergillus niger* and a carbohydrate medium.

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Citric acid is commonly derived commercially from alkanes such as n-paraffin from oil refinery processes using various strains of yeast [2] or from sources of sugar such as glucose syrup, fructose, or beet molasses using *Aspergillus niger*. Recently, due to potential health risks that can be caused by benzopyrene which can result as a byproduct in case of incomplete purification, demand for citric acid derived from natural substrates has been receiving increased attention. However, because *Aspergillus niger* is sensitive to contamination and has low yield [11, 12], alternate methods of producing citric acid are required. Although research has been conducted on expanding the range of substrates for *Aspergillus niger*, namely, pumpkin [9] and carrot juice or celery waste [9], the low yield suggests applying the more tolerant and efficient yeast fermentation, to carbon sources of sugar to solve this [4, 23].

Yeast could be a desirable candidate of citric acid producer from its' high yield and efficiency. But one of these strains, *Candida zeylanoides,* requires optimized pre-treatment of molasses with invertase because an inability of sucrose utilization.

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Also, when citric acid is produced by fermentation using yeasts, a considerable amount of iso-citric acid usually occurs in the culture broth as a by-product. Iso-citric acid is an organic acid produced during TCA cycle metabolism which has no significant commercial usage and thus, it is rarely desirable to produce iso-citric acid since it lowers the yield of citric acid and makes the purification process difficult [12].

In this study, we compare the citric acid and iso-citric acid productivity of various yeasts using a solution of mixed monosaccharides or cane sugar molasses as a cheap carbon source. Furthermore, we optimize factors in pre-treatment for the particular yeast strain, *Candida zeylanoides* in order to provide basic applicable data for future studies.

The strains of yeast used in this research, *Candida tropicalis (Castellani)* ATCC 20115, *Candida oleophila* ATCC 20373, *Candida oleophila* ATCC 20177, *Candida zeylanoides* ATCC 20367, *Yarrowia lipolytica* ATCC 20237, *Yarrowia lipolytica* ATCC 20346, *Yarrowia lipolytica* ATCC 46330, *Aspergillus niger* ATCC 10577, and *Aspergillus niger* ATCC 13794 were purchased from ATCC (Maryland, USA). *Saccharomycopsis lipolytica* IFO1658 was obtained from IFO (Osaka, Japan). Lyophilized yeast cells were revived, streaked and cultured at 30°C on YEPD plates (Yeast Extract 10 g, Peptone 20 g, Glucose 10 g, Agar 1.5%, DW 1 L). Cane sugar molasses obtained from CJ Co. (Incheon, Korea), was the waste of sugar refinery process from Indonesian raw sugar. Invertase and other reagents were purchased from Sigma-Aldrich.

Pretreatment of molasses includes 50 mg of invertase (300-500 Units/mg) added to 1 L of molasses (sugar content 52-56%) at 55°C, for 2 hours. For the main carbon sources, a clean carbon source (glucose: fructose, 1:1 mixture 180 g/l) or invertase treated molasses (sugar content is diluted to 18%) was used with varying amounts of nitrogen and phosphate. 0.5 to 4.0 g/l of potassium phosphate (monobasic) was added to the clean sugar medium. Fermentation in a shaken flask was carried out with 18% (W/V) of total carbon concentration (glucose: fructose, 1:1) at pH 6.5, 30°C, in a reciprocal shaker (200 rpm) for 6 days.

When fermentation finished, cell growth was measured via spectrophotometry (at 600 nm) after being diluted 100 times. For analysis, samples were centrifuged or filtered through Whatman filters. Citric acid was measured by acetic anhydride pyridine method [10] and sugar by DNS method or HPLC. Also, citric acid and iso-citric acid in filtrates were determined by Shimadzu LC-10AD HPLC with ODS-10 reverse phase column, and the mobile phase was 2% of $KH_2PO_4$ adjusted to pH 2.4 using $H_3PO_4$. Citric acid and iso-citric acid were detected by the differential refractometer (Shimadzu RID-10A) [2].

A large number of micro-organisms including fungi and yeasts have been used to produce citric acid. However, most of them are not able to produce commercially acceptable yields. Among these, only *A. niger* and certain yeasts such as *Yarrowia (formerly Saccharomycopsis)* sp. and

**Table 1. The summary of productions of citric acid by submerged fermentation.**

| Strain | Raw material | Productivity (g/l) | $Y_{p/s}$ (g/g) | Reference No. |
|---|---|---|---|---|
| Fungi | | | | |
| *A. niger* | Glucose | ~88.1 | -0.6 | [6, 16, 20] |
| *A. niger* | Molasses (Beet, Cane) | ~103.3 | ~0.75 | [5, 9, 13, 21] |
| *A. niger* | Starch hydrolysate | 64.7 | ~0.49 | [12] |
| Yeasts | | | | |
| *Y. lipolytica* | Glucose (hydrol) | 78.5 | 0.50~0.79 | [15] |
| *Y. lipolytica* | Carrot juice | 62.6 | 0.57 | [22] |
| *Y. lipolytica* | Cellulose hydrolysate | 42.4 | - | [8] |
| *Y. lipolytica* | n-paraffin | 9.8 | NA | [2, 18] |
| *C. oleophila* | Sucrose | 57.8 | - | [18, 23] |
| *C. tropicalis* | Glucose, Sucrose | - | 0.53 | [14] |
| *C. zeylanoides* | n-paraffin | ~84.0 | ~1.02 | [7] |
| *C. zeylanoides* | Cane molasses (invertase-treated) | | | this study |

*Candida* sp. are employed for commercial production of citric acid. A list of some microorganisms used to produce citric acid is shown in Table 1. These examples were studied under different conditions. Generally, yeast fermentation is advantageous over *A. niger* in terms of the speed of production, yield and aeration or mixing control in a tank reactor due to its unicellular shapes. All of the yeast strains were purchased from ATCC or IFO then tested for productivity of citric acid, the results of which are shown in Table 2. *C. zeylanoides* was chosen based on its high yield to the producing strain of citric acid for further experiments.

Since molasses contains a sufficient amount of nitrogen, a clean carbon source (glucose: fructose, 1:1) was used to determine the effect of C/N ratio on citric acid production. Optimal C/N ratio was found to be 40/1 (C/N) in maximizing yield (Table 3). Although composition of the molasses varies depending on the season, location, period of import, etc., the average of C/N ratio of used molasses was approximately 55/1. Accordingly, further experiments were attempted at 40/1 by adding 1.5 g of nitrogen source (Ammonium chloride) into 1 L of invertase-treated molasses medium. Excessive amounts of nitrogen causes cell mass growth but decrease in yield [17]. Fig. 1(A) shows the effects of phosphate ion on the citric acid production by

**Table 2. Comparison of the citric acid productivity of the Yeasts by submerged fermentation.**

| Strains | Cell growth ($OD_{600}$) | Citric acid (g/l) | Yield ($Y_{p/s}$) |
|---|---|---|---|
| *C. tropicalis* ATCC 20115 | 26 | 45.0 | 0.25 |
| *C. oleophila* ATCC 20177 | 23 | 20.7 | 0.12 |
| *C. oleophila* ATCC 20373 | 31 | 60.1 | 0.33 |
| *C. zeylanoides* ATCC 20367 | 42 | 91.4 | 0.51 |
| *S. lipolytica* IFO1658 | 53 | 56.2 | 0.31 |
| *Y. lipolytica* ATCC 20237 | 36 | 50.8 | 0.28 |
| *Y. lipolytica* ATCC 20346 | 32 | 62.3 | 0.35 |
| *Y. lipolytica* ATCC 46330 | 29 | 46.6 | 0.26 |

Initial concentration was 18% (w/v) of mixed carbon source (glucose: fructose = 1:1). Citric acid produced at 30°C, 200 rpm, 6 days' fermentation in shaken flask.

**Table 3. Effect of C/N ratio on the citric acid production using 18% of carbon source by *Candida zeylanoides*.**

| Carbon/Nitrogen | Cell growth ($OD_{600}$) | Citric acid (g/l) | iso-Citric acid (g/l) |
|---|---|---|---|
| 100/0 | 8 | 2.0 | - |
| 100/1 | 27 | 65.8 | 5.5 |
| 40/1 | 40 | 91.9 | 19.8 |
| 20/1 | 75 | 80.3 | 14.5 |

Total carbon concentration (glucose: fructose, 1:1) was 18% (w/v) in the media broth. Citric acid produced at 30°C, 200 rpm, 6 days' fermentation in shaken flask.

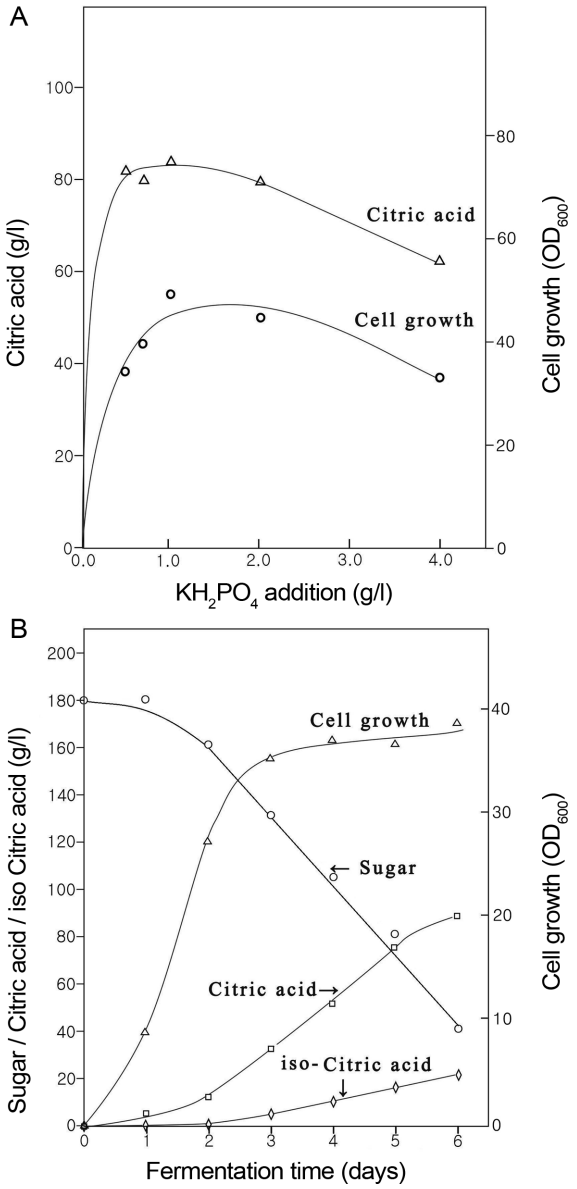


**Fig. 1. The citric acid production of *Candida zeylanoides*.** (A) Effect of phosphate concentration on the citric acid production. (B) Typical time course of the citric acid production of *Candida zeylanoides* in the 5 L-Jar fermenter. Main carbon source is an invertase-treated molasses (18% of carbohydrates, W/V) and metal ions were precipitated by TCPH. pH was controlled to be 6.5 with 0.2 N NaOH at 700 rpm agitation. Di/Dt = 0.56 of disc turbine type impellers were used.

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*Candida zeylanoides.* 0.5 to 4.0 g/l of $KH_2PO_4$ was added to the clean sugar medium and tested for yield. The optimum concentration of phosphates was found to be between 0.5 g/l to 1.5 g/l. Presence of excess of phosphate led to a decrease in $CO_2$ fixation, which caused an adverse effect on the citric acid production [7, 14]. Concentration of phosphate in cane sugar molasses was measured to be within optimum range (data not shown) in its natural state. Therefore, addition of phosphates was determined to be unnecessary for citric acid production in the cane sugar molasses medium.

Metal ions also have a significant impact on citric acid production. In case of *Aspergillus niger*, divalent metal ions ($Fe^{2+}$, $Mn^{2+)}$ in the medium, exceeding concentrations over 200 μg/l, can cause restricted growth, altered morphology, and decreased acid production. This may occur if the raw substrate is not sufficiently pre-treated [16]. For the pretreatment of raw materials, HCF (potassium hexacyanoferrate) was used to precipitate the metal ions as a chelating agent. In the molasses medium used in this study, 120 mg/l of ferrous ion concentration was detected and thus, HCF and TCPH (tricalcium phosphate) were used as chelating agents. Using TCPH produced higher yield than that when HCF (data not shown) was used. This is attributed to the cell growth inhibition of HCF.

Fig. 1(B) shows a typical time course of citric acid fermentation of *Candida zeylanoides* in the 5 L-Jar fermenter using pretreated molasses medium. The two Di/Dt = 0.56 of disc turbine type impellers were used for agitation of the 5 L-Jar fermenter at 700 rpm.

Cells displayed rapid growth patterns in the first three days, producing citric acid from the second day onwards. In the course of six days, citric acid concentrations had reached up to 91 g/l in the broth, with 18.5 g/l of iso-citric acid. For *A. niger* to produce the same concentration of citric acid, up to seven days of cultivation is required [5]. For the batch fermentation of *Candida zeylanoides*, pH was controlled at 6.5 with 0.2 N NaOH. The yield of this batch culture was 0.51 ($Y_{p/s}$, g/g), which followed a mixed growth-associated production pattern. In order to make the citric acid production by yeast commercially more practical, further research should be dedicated to developing a higher yield strain (more citric acid, less iso-citric acid), and optimization of operating conditions and pre-treatment substrates.

Akiyama suggested the sensitive strains of *C. lipolytica* against Monofluoroacetic acid( MFA sensitive) could produce less iso-citric acid than mother strain [1], but Tani reports the opposite result that a MFA resistant mutant of *Candida* sp. shows less iso-citric acid. Two reports don't match [19]. These are to be clarified by further studies.

In summary, *C. zeylanoides* was selected and optimized for to be the producing strain of citric acid, producing 91 g/l of citric acid with 18.5 g/l of iso-citric acid when fermented in a 5 L-Jar fermenter using invertase-treated molasses (18% of carbohydrates, W/V) in 6 days. Precipitation of metal ions was carried out with tricalcium phosphate as a pretreatment of molasses. This report provides basic applicable data to produce citric acid by yeast using cheap molasses as an economic raw material.

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## 국문초록

**사탕수수당밀의 전처리법과 *Candida zeylnoides*에 의한 시트르산의 생산**

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효모의 액침배양법에 의한 구연산을 발효생산하였다. 여덟 가지 구연산 발효균주의 생산성을 비교하여 가장 생산성이 높은 *Candida zeylnoides*를 선발하였다. 발효조건의 최적화를 위하여 glucose와 fructose 혼합당 배지에서 C/N 비, 인산염의 농도, $Fe^{2+}$ 이온의 최적 농도를 결정하였다. 삼각 플라스크 실험결과 C/N 비는 40/1이 가장 좋았으며, 최적화된 인삼염과 $Fe^{2+}$ 농도에서 구연산 91.4 g/l와 이소-구연산 19.8 g/l를 생성하였다. 이 결과를 토대로 TCPH 전처리 당밀 배지를 사용한 실험에서는 인삼염 농도 1.0 g/l, $Fe^{2+}$ 이온 50 mg/l 이하의 농도에서 구연산 91 g/l와 이소-구연산 18.5 g/l를 생성하였다. 5 L-Jar 발효조를 사용한 실험에서의 구연산 수율($Y_{p/s}$, g/g)은 0.51이었다.

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