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Optimum Conditions for the Biological Production of Lactic Acid by a Newly Isolated Lactic Acid Bacterium, *Lactobacillus* sp. RKY2

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Abstract Lactic acid is a green chemical that can be used as a raw material for biodegradable polymer. To produce lactic acid through microbial fermentation, we previously screened a novel lactic acid bacterium. In this work, we optimized lactic acid fermentation using a newly isolated and homofermentative lactic acid bacterium. The optimum medium components were found to be glucose, yeast extract, $(NH_4)_2HPO_4$, and $MnSO_4$. The optimum pH and temperature for a batch culture of *Lactobacillus* sp. RKY2 was found to be 6.0 and 36°C, respectively. Under the optimized culture conditions, the maximum lactic acid concentration (153.9 g/L) was obtained from 200 g/L of glucose and 15 g/L of yeast extract, and maximum lactic acid productivity (6.21 $gL^{-1}h^{-1}$) was obtained from 100 g/L of glucose and 20 g/L of yeast extract. In all cases, the lactic acid yields were found to be above 0.91 g/g. This article provides the optimized conditions for a batch culture of *Lactobacillus* sp. RKY2, which resulted in highest productivity of lactic acid.

Keywords: batch fermentation, culture conditions, homofermentation, lactic acid, *Lactobacillus*

INTRODUCTION

Lactic acid has been used for the fermentation and preservation of human foodstuffs for at least 4~5 millennia [1]. It is a nonvolatile and odorless chemical, and is classified as GRAS (Generally Recognized As Safe) for use as a general-purpose food additive by the FDA. Lactic acid is used in a wide range of applications in the food, cosmetic, pharmaceutical, and chemical industries [2-4]. Lactic acid is also receiving a great deal of attention as a feedstock monomer for the manufacturing of polylactic acid (PLA), which can be used as a biodegradable commodity plastic [2,3,5]. Currently, the scale of lactic acid production is significantly rising to supply raw material for the production of PLA [6,7].

To produce lactic acid through the biological route, there are two types of lactic acid producing microorganisms that can be used: lactic acid bacteria and filamentous fungi [8]. Fungi, such as the *Rhizopus* species, utilize glucose aerobically to produce lactic acid, whereas the lactic acid productivity and yield of a batch culture of filamentous fungi are typically below 2.0 $gL^{-1}h^{-1}$ and 0.8 g/g, respectively [8-10]. Lactic acid bacteria are generally fast-growing organisms, and they consist of the following gram-positive genera: *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Carnobacterium*, *Oenococcus*, *Pediococcus*, and *Streptococcus* [1,11-13]. Recently, the strains used for industrial applications are almost proprietary, but it is believed that most of the organisms belong to the genus *Lactobacillus* [2,14-16]. It is necessary that lactic acid bacteria, for lactic acid production on an industrial scale, convert glucose into lactic acid through the homofermentative pathway [12]. However, as far as we know, to date there have been no reports on homofermentative lactic acid bacteria isolated from soybean paste, which is a kind of traditional, fermented, Korean food, for the production of a high concentration of lactic acid. We pioneered the isolation of a novel lactic acid bacterium from traditional, fermented, Korean soybean paste. In our previous work, this newly isolated strain from soybean paste was established to be homofermentative lactic acid bacteria and was named *Lactobacillus* sp. RKY2 [17,18].

In this article, we investigated the optimum conditions for the biological production of lactic acid by a batch culture of *Lactobacillus* sp. RKY2. For this purpose, at first the optimum compositions of the culture medium for the maximum production of lactic acid were studied, and then the culture conditions, such as culture pH and temperature, were optimized. Finally, we investigated the influence of glucose and yeast extract concentrations on lactic acid fermentation.

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MATERIALS AND METHODS

Microorganism

Lactobacillus sp. RKY2 [17,18], which was screened from soybean paste, was used for all experiments. This species was previously deposited to the Korean Collection for Type Cultures (Daejon, Korea) as KCTC 10353BP. The stock cultures were maintained at -20°C in 5-mL vials containing *Lactobacilli* deMan Rogosa Sharpe (MRS) [19] medium and 50% (v/v) glycerol solution until they were ready to be used.

Fermentation

Unless stated otherwise, the basal medium for the fermentation experiments consisted of glucose, yeast extract, $(NH_4)_2HPO_4$, and $MnSO_4$. To find the optimum medium compositions for lactic acid production, each component of the fermentation medium was replaced with different sources. Detailed descriptions of the adjustment made to the fermentation medium are stated in the results and discussion section.

To optimize the culture medium and fermentation conditions for the efficient production of lactic acid, two types of fermentation were used: vial-type and fermentor-type fermentations. The vial-type fermentation was performed in a 50-mL vial containing 40 mL of fermentation medium. The inoculated vials were incubated at 38°C and 200 rpm in a shaking incubator (KMC-8480SF, Vision Scientific Co., Daejon, Korea). The fermentor-type fermentation was performed in a 2.5-L jar fermentor (KF-2.5 L, Kobiotech Co., Incheon, Korea) containing 1 L of fermentation medium. During the fermentor-type fermentations, the culture pH was automatically controlled at desired values (5.0~7.0) by the addition of 10 M NaOH. The culture temperature was controlled at 30~42°C, and the agitation speed was set to 200 rpm to insure complete mixing of the fermentation broth.

Analytical Methods

Cell growth, as optical density, was measured by a UV-160A spectrophotometer (Shimadzu Co., Kyoto, Japan) set to 660 nm, and dry cell weight was determined by an appropriate calibration curve. Lactic acid was quantified by a high-performance liquid chromatography (HPLC) system (Waters Co., Milford, MA, USA) equipped with a Waters 486 tunable absorbance detector set to 210 nm. An ion-exclusion column (300 × 7.8 mm, Aminex HPX-87H, Bio-Rad, Hercules, CA, USA) was eluted with 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min, while the column temperature was maintained at 35°C. Glucose was measured enzymatically by a glucose oxidase peroxidase method using a kit from YD Diagnostics (Seoul, Korea). All the experiments were carried out in duplicate and the mean values are reported.

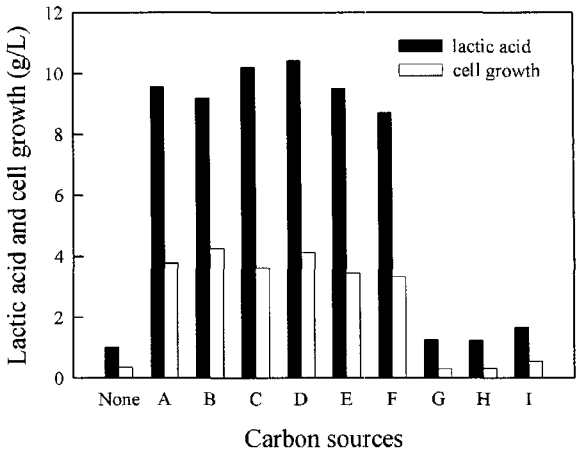

Fig. 1. Effect of carbon sources on lactic acid production and cell growth by a batch culture of *Lactobacillus* sp. RKY2. The fermentations were carried out in a 50-mL vial containing 40 mL of fermentation medium (30 g/L of a carbon source, 15 g/L of yeast extract, 2 g/L of K_2HPO_4, and 0.2 g/L of $MnSO_4$) at 38°C and 200 rpm. A, glucose; B, maltose; C, fructose; D, sucrose; E, lactose; F, galactose; G, glycerol; H, starch; I, xylose

RESULTS AND DISCUSSION

Influence of Carbon Sources

To investigate the effect of carbon sources on lactic acid fermentation by a batch culture of *Lactobacillus* sp. RKY2, the fermentations from various carbohydrates were experimented through vial-type fermentation with the carbohydrates concentration being 30 g/L. The results are shown in Fig. 1. *Lactobacillus* sp. RKY2 showed different fermentation characteristics with respect to carbohydrate source. When the medium containing glucose, fructose, maltose, lactose, galactose, or sucrose was used as a carbon source, *Lactobacillus* sp. RKY2 metabolized all of these carbohydrates into lactic acid, with 3.3~4.3 g/L of dry cell weight and 8.7~10.4 g/L of lactic acid produced. *Lactobacillus* sp. RKY1 rarely utilized xylose, starch, and glycerol, and could not grow on these carbon sources. Although 30 g/L of each carbon source was used as a substrate, the mean amount of lactic acid produced between all carbon sources was around 10 g/L. This might be due to the acidification of culture broth as lactic acid was produced. However, organic acids other than lactic acid were rarely produced. These results suggest that *Lactobacillus* sp. RKY2 metabolize some carbohydrates *via* the homofermentation pathway.

Influence of Nitrogen Sources

To investigate the effect of nitrogen sources on lactic acid fermentation by a batch culture of *Lactobacillis* sp. RKY2, several nitrogen sources were tested. Fig. 2 shows

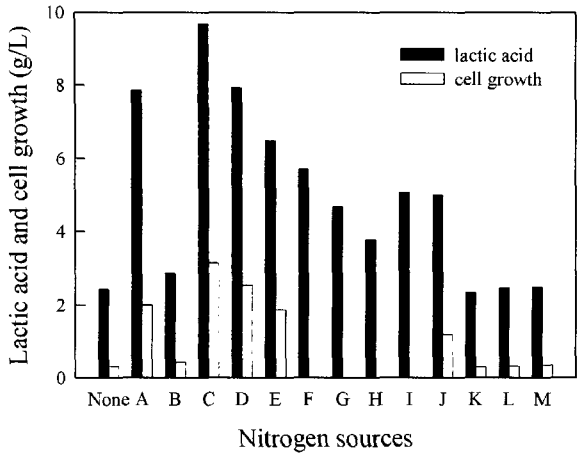

Fig. 2. Effect of nitrogen sources on lactic acid production and cell growth by a batch culture of *Lactobacillus* sp. RKY2. The fermentations were carried out in a 50-mL vial containing 40 mL of fermentation medium (30 g/L of glucose, 15 g/L of a nitrogen source, 2 g/L of K_2HPO_4, and 0.2 g/L of $MnSO_4$) at 38°C and 200 rpm. A, beef extract; B, malt extract; C, yeast extract; D, peptone; E, polypeptone; F, soybean flour; G, cotton seed flour; H, whey; I, pharmamedia; J, corn steep liquor; K, urea; L, ammonium sulfate; M, ammonium chloride.

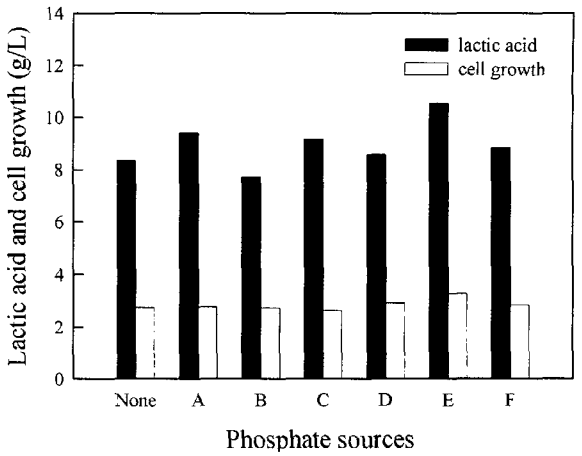

Fig. 3. Effect of phosphate sources on lactic acid production and cell growth by a batch culture of *Lactobacillus* sp. RKY2. The fermentations were carried out in a 50-mL vial containing 40 mL of fermentation medium (30 g/L of glucose, 15 g/L of yeast extract, 2 g/L of a phosphate source, and 0.2 g/L of $MnSO_4$) at 38°C and 200 rpm. A, K_2HPO_4; B, KH_2PO_4; C, Na_2HPO_4; D, NaH_2PO_4; E, $(NH_4)_2HPO_4$; F, $NH_4H_2PO_4$.

the results of 50 mL vial-type fermentations using the vial containing 15 g/L of a different nitrogen source. The growth at soybean flour, cotton seed flour, whey, and pharmamedia, could not be measured due to the insoluble property of these nitrogen sources. Complex nitrogen sources, such as beef extract, yeast extract, and peptone, showed comparable results. Polypeptone, pharmamedia, and corn steep liquor each showed some possibilities as an alternative nitrogen source. When yeast extract was used as a nitrogen source, the highest cell growth (3.1 g/L) and lactic acid production (9.7 g/L) were obtained. Although malt extract is a kind of complex nitrogen source, *Lactobacillus* sp. RKY2 could not grow on it. The addition of inorganic nitrogen sources, such as ammonium sulfate, ammonium chloride, and urea, minimally stimulated both cell growth and lactic acid production. Therefore, the most effective nitrogen source for the batch culture of *Lactobacillus* sp. RKY2 seemed to be yeast extract.

Influence of Phosphate and Mineral Sources

We investigated the effect of phosphate sources on lactic acid fermentation by a batch culture of *Lactobacillis* sp. RKY2. Fig. 3 shows the results of 50 mL vial-type experiments using the vial containing 2 g/L of a different phosphate source. Dibasic phosphate sources, such as K_2HPO_4, Na_2HPO_4, and $(NH_4)_2HPO_4$, showed better results in terms of lactic acid production than monobasic phosphate sources, such as KH_2PO_4, NaH_2PO_4, and $NH_4H_2PO_4$. In particular, maximum cell growth (3.3 g/L) was obtained with $(NH_4)_2HPO_4$. Therefore, the optimum phosphate source for lactic acid production by *Lactobacillus* sp. RKY2 was thought to be $(NH_4)_2HPO_4$.

Lactic acid bacteria generally require several minerals as growth factors, and it is well known that Mg^{2+} and Mn^{2+} significantly affect the cell growth of lactic acid bacteria. Since typical lactic acid bacteria cannot degrade H_2O_2 due to the absence of catalase activity, Mn^{2+} may play an important role in protecting many lactic acid bacteria against the toxic effects of H_2O_2 or oxygen [20]. To investigate the effect of Mg^{2+} and Mn^{2+} on lactic acid fermentation by a batch culture of *Lactobacillus* sp. RKY2, the fermentations supplemented with 0~1.0 g/L of $MgSO_4$ or 0~1.0 g/L of $MnSO_4$ were performed in a 50-mL vial containing 40 mL of fermentation medium. As shown in Fig. 4, Mn^{2+} significantly stimulated both lactic acid production and cell growth. However, Mg^{2+} rarely affected cell growth and lactic acid production (data not shown). The addition of 0.1 g/L of $MnSO_4$ enhanced cell growth by 1.7-fold compared to the case where $MnSO_4$ was absent. This result is in agreement with a previous report by Angelis and Gobetti [21], who reported that, in lactic acid fermentation by *L. sanfranciscensis*, both the cell growth and lactic acid production rate are promoted by the addition of 0.225 mM Mn^{2+}.

Influence of Culture pH and Temperature

The fermentations were conducted in a 2.5-L jar fermentor at 30~42°C and pH 5.0~7.0 using 100 g/L of glucose to investigate the effect of culture pH and temperature on lactic acid fermentation by a batch culture of *Lactobacillus* sp. RKY2. As shown in Table 1, while maximum cell growth at pH 6.0 (13.5 g/L) was similar to that at pH 5.0 (13.8 g/L), lactic acid fermentation at pH 6.0 was completed faster than that at pH 5.0. *Lactobacillus* sp. RKY2 seemed to prefer the acidic conditions

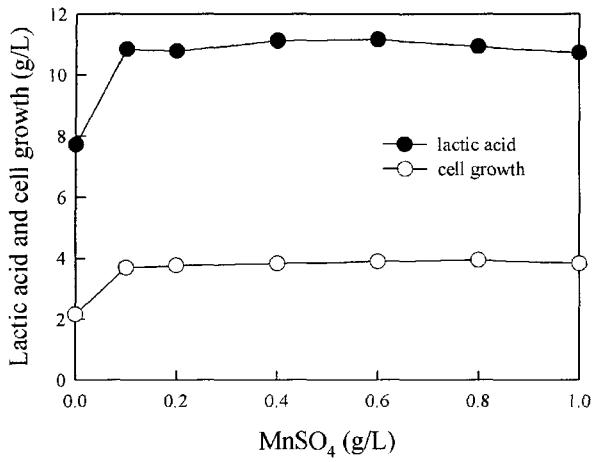

Fig. 4. Effect of manganese ion on lactic acid production and cell growth by a batch culture of *Lactobacillus* sp. RKY2. The fermentations were carried out in a 50-mL vial containing 40 mL of fermentation medium (30 g/L of glucose, 15 g/L of yeast extract, 2 g/L of $(NH_4)_2HPO_4$, and 0~1.0 g/L of $MnSO_4$) at 38°C and 200 rpm.

Table 1. Effect of culture pH and temperature on lactic acid production, yield, cell growth, and productivity by a batch culture of *Lactobacillus* sp. RKY2[a]

	pH			Temperature (°C)				
	5.0	6.0	7.0	30	34	36	38	42
Lactic acid (g/L)	93.7	93.8	91.8	95.2	92.0	94.5	93.8	44.5
Yield (g/g)[b]	0.92	0.93	0.96	0.93	0.92	0.92	0.93	0.90
Maximum cell growth (g/L)	13.8	13.5	3.9	13.2	13.7	13.8	13.5	5.8
Productivity ($gL^{-1}h^{-1}$)	3.12	5.21	2.78	4.53	5.11	5.25	5.21	1.35

[a] Fermentation experiments under each condition were carried out in a 2.5-L jar fermentor containing 1 L of fermentation medium composed of 100 g/L of glucose, 15 g/L of yeast extract, 2 g/L of $(NH_4)_2HPO_4$, and 0.1 g/L of $MnSO_4$.
[b] The yield of lactic acid was calculated based on the amount of consumed glucose.

to alkaline conditions in lactic acid fermentation. Lactic acid productivity was maximized at pH 6.0 (5.21 $gL^{-1}h^{-1}$), at which the lactic acid yield and maximum cell growth were obtained as 0.93 g/g and 13.5 g/L, respectively. In addition, even though optimum cell growth was obtained at 30~38°C, the lactic acid fermentations at 34~38°C were completed faster than those beyond these temperatures. In the case of lactic acid fermentation at 38°C, maximum cell growth was obtained as 13.5 g/L, and lactic acid productivity was slightly lower than that at 36°C. Bruno-Bárcena *et al.* [22] previously reported that, when *L. casei* is used for lactic acid fermentation, cell growth decreases with the increase of culture temperature, while lactic acid productivity increases up to 42°C but further decreases beyond this value. However, in the case of *Lactobacillus* sp. RKY2, the cell growth and lactic acid productivity increased up to 36°C but further decreased beyond this value. Therefore, when considering lactic acid productivity and cell growth, the optimum temperature for lactic acid fermentation by a batch culture of *Lactobacillus* sp. RKY2 seemed to be 36°C.

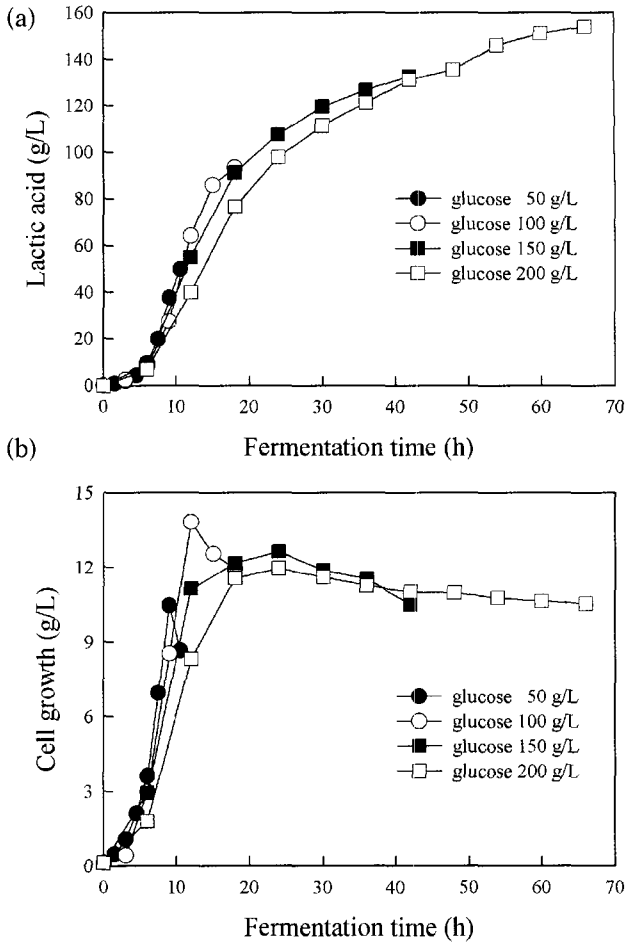

Fig. 5. Lactic acid production (a) and cell growth (b) during lactic acid fermentation from different concentrations of initial glucose by a batch culture of *Lactobacillus* sp. RKY2. The fermentations were carried out in a 2.5-L jar fermentor containing 1 L of fermentation medium (50~200 g/L of glucose, 15 g/L of yeast extract, 2 g/L of $(NH_4)_2HPO_4$, and 0.1 g/L of $MnSO_4$) at 36°C, pH 6.0, and 200 rpm.

Influence of Glucose Concentrations

To investigate the effect of initial glucose concentrations on lactic acid fermentation by a batch culture of *Lactobacillus* sp. RKY2, medium containing 50, 100, 150 or 200 g/L of glucose was tested. The fermentations were performed in a 2.5-L jar fermentor containing 1 L of fermentation medium at 36°C and pH 6.0. As shown in Fig. 5(a), the final lactic acid concentration increased with the increase of initial glucose concentration. The maximum lactic acid concentration (153.9 g/L) was obtained after 66 h of fermentation at 200 g/L of initial

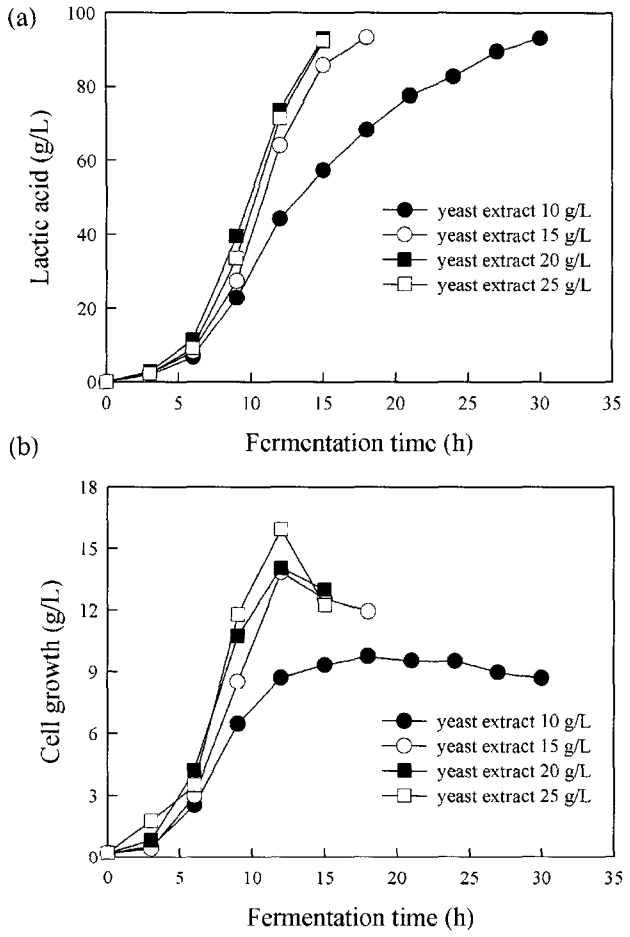

Fig. 6. Lactic acid production (a) and cell growth (b) during lactic acid fermentation from different concentrations of yeast extract by a batch culture of *Lactobacillus* sp. RKY2. The fermentations were carried out in a 2.5-L jar fermentor containing 1 L of fermentation medium (100 g/L of glucose, 10~25 g/L of yeast extract, 2 g/L of $(NH_4)_2HPO_4$, and 0.1 g/L of $MnSO_4$) at 36°C, pH 6.0, and 200 rpm.

glucose concentration. The lactic acid yields, based on glucose consumed, were obtained as 0.99, 0.92, 0.93, and 0.91 g/g at 50, 100, 150, and 200 g/L of initial glucose concentrations, respectively (*i.e.*, a decrease in lactic acid yield was observed at higher glucose concentrations). When the medium was supplemented with 200 g/L of glucose, around 20 g/L of glucose remained even after 66 h of fermentation without utilization (data not shown), which might be due to substrate inhibition. This was expected because there was also a decrease in maximum cell growth at this level (Fig. 5(b)). The profiles of cell growth during the lactic acid fermentations are shown in Fig. 5(b). Cell growth increased with the increase of initial glucose concentration up to 100 g/L but then decreased beyond this value. When the initial glucose concentration exceeded 100 g/L, maximum cell growth decreased, possibly due to inhibition by the high substrate concentration. Maximum cell growth was obtained as 13.8 g/L at 100 g/L of the initial glucose concentration. These results may suggest that a high glucose concentration contributes to the substrate inhibition in lactic acid fermentation. Åkerberg *et al.* [23] previously reported similar substrate inhibition in lactic acid fermentations for *L. lactis* ssp. *lactis*, Ohara *et al.* [24] for *S. faecalis*, and Hujanen *et al.* [25] for *L. casei*, grown on glucose.

Influence of Yeast Extract Concentrations

To investigate the effect of yeast extract concentrations on lactic acid fermentation by a batch culture of *Lactobacillus* sp. RKY2, the fermentations were performed in a 2.5-L fermentor containing 1 L of fermentation medium, because yeast extract was proven to be the most effective nitrogen source. Fig. 6 shows the profiles of lactic acid production and cell growth in the medium supplemented with 100 g/L of glucose and 10~25 g/L of yeast extract. Although volumetric productivity and maximum cell growth were obtained as 3.11 $gL^{-1}h^{-1}$ and 9.8 g/L, respectively, at 10 g/L of yeast extract, these values increased linearly as yeast extract concentrations supplemented to the culture medium increased. The volumetric productivity, however, increased linearly up to 20 g/L of yeast extract, but then remained constant beyond this value. Fermentation efficiency was generally enhanced by the addition of higher amounts of yeast extract. These results are quite similar to those of a previous report by Hujanen and Linko [26], who reported that lactic acid production and cell growth increase linearly with the increase of initial yeast extract as an optimum nitrogen source, because it generally contains some amino acids and vitamins essential for the cell growth of lactic acid bacteria. It has been well-known that yeast extract is the most effective nitrogen source for the growth of lactic acid bacteria, but yeast extract is highly expensive because lactic acid is a large-volume and low-price chemical. Therefore, a cheaper nitrogen source, as an alternative to yeast extract, must be further developed to bring about an economical process of lactic acid production.

CONCLUSION

The optimum conditions for the biological production of lactic acid were studied by using *Lactobacillus* sp. RKY2, which is a homofermentative lactic acid bacterium, and it was isolated from traditional, fermented, Korean soybean paste. The optimum medium for a batch culture of *Lactobacillus* sp. RKY2 was found to be as follows: glucose (variable), yeast extract (20 g/L), $(NH_4)_2HPO_4$ (2 g/L), and $MnSO_4$ (0.1 g/L). The optimum pH and temperature for the batch culture of *Lactobacillus* sp. RKY2 was found to be 6.0 and 36°C, respectively. *Lactobacillus* sp. RKY2 produced up to 153 g/L of lactic acid from 200 g/L of glucose and 15 g/L of yeast extract. Maximum lactic acid productivity was obtained as 6.21 $gL^{-1}h^{-1}$ at 100 g/L of glucose and 20 g/L of yeast extract. However, we are currently searching for low-cost nitrogen sources available for industrial lactic acid production

by *Lactobacillus* sp. RKY2, because yeast extract is considered to be an expensive nitrogen source.

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