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Extractive Fermentation of Lactic Acid with Hiochi Bacteria in a Two-Liquid Phase System

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Abstract

Lactic acid production by fermentation has attracted interest because optically pure lactic acid is the raw material of biodegradable polymer, polylactic acid, which has been mainly used in food packing. *In situ* extractive fermentation of lactic acid with reactive extraction has two critical problems: the toxicity of diluents and extractant and the difference in the optimum pH between fermentation and reactive extraction. In this study, the extractive fermentation was conducted with alcohol-tolerant Hiochi bacteria, *Lactobacillus homohiochii* and *Lactobacillus fructivorans*, using the co-encapsulation of the bacteria and calcium carbonate to depress the decrease in pH in the capsules.

L. fructivorans NRIC0224 and 1-decanol were selected as the Hiochi bacterium and the diluent, respectively, based on the tolerance of aliphatic alcohol and extractability. We found that the presence of powdered $CaCO_3$ in the medium considerably alleviated the toxicity of tri-n-octylamine (TOA) and that the components in the medium, yeast extract and peptone, affected the extraction of lactic acid with TOA in 1-decanol. Then, we co-immobilized both *L. fructivorans* NRIC0224 cells and $CaCO_3$ into Ca-alginate capsules and constructed an *in situ* extractive fermentation system using TOA and the Ca-alginate capsules. This system operated successfully, and the yield and productivity were improved over those of control fermentation.

Keywords: Extractive fermentation; Lactic acid; Hiochi bacterium; Ca alleviation effect; Toxicity

Introduction

Lactic acid is produced by chemical synthesis and fermentation. This product and its derivatives are important industrial chemicals in the fields of food processing, pharmaceuticals and cosmetics [1], and they have attracted great interest as the raw materials of biodegradable polymer, polylactic acid [2]. In the fermentative production of lactic acid, traditionally, the fermentation broth was neutralized with lime and recovered as calcium lactate [1-5]. After adding sulfuric acid, lactic acid is concentrated by evaporation and purified by methods involving short-path distillation [3]. This recovery and purification of lactic acid from the fermentation broth is the most costly process [4]. Therefore, many recovery techniques such as diffusion dialysis, solvent extraction, adsorption, chromatographic methods, membrane use, drying and electrodialysis have been proposed [5]. Additionally, the *in situ* recovery technique during fermentation offers the potential to not only recover lactic acid but also relieve end-product (lactic acid) inhibition [5]. Among the proposed separation techniques, reactive extraction is a promising separation method that can be applied *in situ* and has the potential to provide a high recovery ratio [6]. The reactive extraction of organic acids with amines and ammonium salts has already been heavily investigated. Tri-n-octylamine (TOA) in 1-octanol is reported to be the extractant-diluent combination with the highest distribution ratio [6]. To apply a TOA/1-octanol system to *in situ* extractive fermentation, two critical problems remain. One is the toxicity of the diluents and extractant. Marinova and Yankov [7] studied the toxicity of solvents and extractants for *Lactobacillus casei*, and all of the alcohols investigated involving 1-octanol were found to show high phase toxicity. Therefore, we focus on Hiochi bacteria, *Lactobacillus homohiochii* and *Lactobacillus fructivorans*, which are known for their ability to spoil Japanese rice wine (sake) and for being the most alcohol-tolerant organisms [8]. On the other hand, extractant TOA has been reported to be highly toxic for *Lactobacillus* strains [7,9,10]. Studies on the alleviation of the toxic effect have been attempted, such as co-immobilization of soybean oil [11] or sunflower oil [12] with the cells and passing extracted broth through a column filled with organic solvent [13]. The other problem is the difference in optimum pH between fermentation and extraction. It is well known that the polar diluents and low pH (<pKa=3.86 for lactic acid) increase the extractability because they can stabilize the ion-pair (lactic acid-amine complex) formed by hydrogen bonds or by solvation [14]. Generally, fermentation pH is in the range of 6–8, while acid production acidifies the medium. Therefore, one method to overcome a difference in optimum pH is to develop an acid-tolerant organism1. The other method is to remove the acid *in situ* by a chemical or physical method without pH control [3,15].

In this study, the aim of this work is to establish the *in situ* extractive fermentation of lactic acid with alcohol-tolerant Hiochi bacteria using the co-encapsulation of the bacteria and calcium carbonate to depress the decrease in the pH in the capsules. First, the tolerance of Hiochi bacteria to aliphatic long-chain alcohols as diluents and TOA as an extractant was examined. Then the effects of pH and the components in the medium on the extraction of lactic acid with TOA in the selected diluent, 1-decanol, were investigated. Finally, an *in situ* extractive fermentation process of lactic acid was developed by co-immobilizing

decanol-tolerant Lactobacillus fructivorans NRIC0224 cells and calcium carbonate into Ca-alginate capsules without pH control [6-9].

Experimental

Organisms and cultivation

We used the four lactic acid-producing Hiochi bacteria listed in Table 1. All bacteria were provided by NODAI Culture Collection Center (NRIC, Japan). The GYP-$CaCO_3$ medium was used for the organisms NRIC 0119 and 0224. SI medium (commercial medium for Hiochi bacteria [16]) was used for NRIC 1814 and 1815. In the cases of organic solvents containing tri-n-octylamine, both the GYP-$CaCO_3$ and GYP media were used for the organisms NRIC 0224. Cultivations were performed at 30°C.

Long-chain aliphatic alcohols	Log Po/w	Lb. homohiochi NRIC 0119	Lb. homohiochi NRIC 1815	Lb. fructivorans NRIC 0224	Lb. fructivorans NRIC 1814
1-Butanol	0.8	+	+	+	+
1-Pentanol	1.3	–	–	–	+
1-Hexanol	1.8	–	–	–	–
1-Heptanol	2.3	–	–	–	–
1-Octanol	2.9	–	–	–	–
1-Decanol	3.8	–	–	+	–
1-Dodecanol	5	+	+	–	+

Table 1: Toxicity of long-chain aliphatic alcohols to Hiochi bacteria. +, Relative activity >0.5; relative activity <0.5. Relative activity is defined as the ratio of lactic acid concentration of the sample to that of the control.

Growth in presence of organic solvents

Growth in the presence of a second phase was determined based on a method described previously [17]. An inoculum of 2.5% (v/v) from an overnight culture was transferred to a fresh medium. When the culture reached the exponential growth phase (OD560 ≈ 0.3), 2 mL were transferred to new culture bottles (20 mL), and 1% (v/v) of one of the organic solvents listed in Tables 1 and 2 was added. Added organic solvents formed the second phase. The culture bottles were closed with Teflon valves to prevent evaporation and then incubated for 48 h at 30°C. Then, the optical density (OD600 for NRIC 0199 and 0224, and OD550 for NRIC 1814 and 1815) and the concentrations of glucose and lactic acid were measured [10,11].

Extraction of lactic acid from fermentation broth

Tri-n-octylamine (TOA) was used as the extractant without further purification. An organic solution was prepared by dissolving 0.5 mol/L of TOA in 1-decanol. In the aqueous media, aqueous solutions were prepared by dissolving lactic acid in deionized water. Prior to the experiment, commercial lactic acid was diluted with distilled water and heated for at least 12 h to hydrolyze the lactic acid polymers. In the fermentative media, lactic acid fermentation was carried out using *L. fructivorans* NRIC0224 cells and lactic acid was produced from culture grown in 100 mL of GYP-$CaCO_3$ medium at 30°C for 48 h. The fermentation broth was centrifuged at 40,000 g for 30 min, and then the supernatant was used as an aqueous solution for the extraction experiment. The pH values of the aqueous solutions were adjusted by adding a 1 mol/L H_2SO_4 or NaOH aqueous solution. The aqueous and organic solutions of equal volume (10 mL) were mixed in a vial and shaken at 30°C to attain extraction equilibrium. After the two phases were separated, the concentration of lactic acid in the aqueous solution was analyzed. The concentration of lactic acid in the organic solution was calculated from the mass balance. The pH of the aqueous solution before and after equilibration was determined by a Horiba F-12 pH meter.

Immobilization of cells in Ca-alginate capsules

Cells of *L. fructivorans* NRIC0224 for encapsulation were obtained from culture grown in 20 mL of GYP-$CaCO_3$ medium at 30°C for 18 h. The cells were harvested from the fermentation broth by centrifugation at 40,000 g for 30 min and then washed with sterile saline solution. The cells were resuspended in 10 mL of a 2.0% (w/v) steric $CaCl_2$ solution containing 20 g/L $CaCO_3$. The suspended solution was mixed with 10 mL of a 2.0% (w/v) sterile sodium alginate solution. The mixture was added dropwise into a sterile 1.0 mol/L $CaCl_2$ solution at room temperature. The beads were hardened in this solution for 1 h. The beads were washed with sterile saline prior to use [12-16].

Fermentations

Batch fermentations were performed in 250-mL culture bottles containing 100 mL of GYP medium and 20 mL of alginate beads with cells. Samples were withdrawn periodically during fermentation and analyzed for glucose and lactic acid. We also carried out lactic acid production using immobilized cells in a packed bed reactor (dia. 15 mm, length 100 mm) with broth recycled to a stirring tank in a batch mode as illustrated in Figure 1. The stirring tank initially contained 175 mL of GYP medium including 25 g/L glucose. The broth in the tank was held at 30°C, 500 rpm agitation rate and an initial pH of 6.75. From this, the medium was pumped to the top of the packed bed. The medium emerging from the bed was returned to the tank. The flow rate of the medium in the bed was kept to 1.5 mL/min by the two pumps. In an extractive fermentation, 175 mL of 1-decanol solution containing 0.5 mol/dm^3 TOA was placed on top of the medium solution. Samples were withdrawn periodically from the medium and the organic phase during fermentation and analyzed for glucose and lactic acid. Ca-alginate capsules were broken down by the homogenizer, and the number of viable cells (CFU/mL) in the cell suspension was counted before and after fermentation.

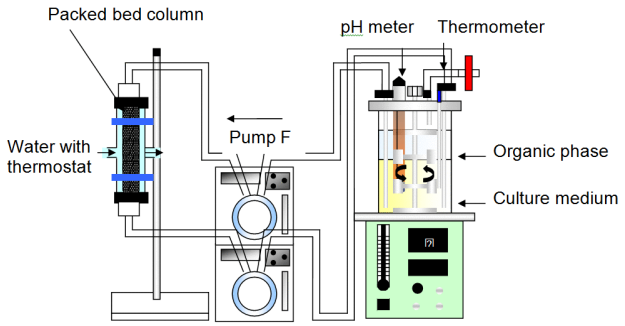

Figure 1: Schematic diagram of extractive fermentation with packed bed column.

Analysis

Optical density was determined using a spectrophotometer (Shimadzu UV-2500PC). Lactic acid in the organic phase was stripped with 1 mol/dm^3 NaOH solution. The concentrations of lactic acid and glucose in the resultant aqueous solutions were determined by HPLC with a Shodex SH-1011 column and a 5 mM H_2SO_4 solution as the mobile phase. Lactic acid and glucose were detected with an RI detector (Shimadzu RID10A). All experiments were duplicated [17-19].

Results and discussion

Tolerance of Hiochi bacteria to long-chain aliphatic alcohols

In a previous study [17], we investigated the tolerance of lactic acid producing bacteria, including *L. homohiochii* NRIC0119, 1815, *L. fructivorans* NRIC0224, 1814, to organic solvents. The long-chain aliphatic alcohols, such as octanol and decanol, were reported to be active diluents for lactic acid extraction [18]. Consequently, we examined the tolerance of Hiochi bacteria to long-chain aliphatic alcohols. Table 1 shows the relationship between the bacterial activity retained by cells exposed to a long-chain aliphatic alcohol and their log $P_{O/W}$ values for four Hiochi bacteria. Microbial activity in the presence of organic solvents was determined on the basis of the concentration of lactic acid produced after adding alcohol, with relative activity being defined as the ratio of the lactic acid concentration of the sample to that of a control. As pointed out by Laane et al. [19], solvents having a log $P_{O/W}$ lower than 2 are not suitable for biocatalytic systems due to the high solubility of the solvent in the water phase. Unfortunately, 1-octanol was toxic to all Hiochi bacteria investigated. *L. fructivorans* NRIC0224 had a tolerance to 1-decanol, which is an active diluent for lactic acid extraction. In the following experiment, we selected *L. fructivorans* NRIC0224 as the Hiochi bacterium and 1-decanol as the diluent.

Tolerance of Hiochi bacteria to tri-n-octylamine extractant

As described above, TOA has been reported to be highly toxic to Lactobacillus strains [7,9,10]. In this study, we examined the toxicity of extractants to Hiochi bacteria and their relative activities in the presence and absence of $CaCO_3$ (10 g/L) are summarized in Table 2. In the absence of $CaCO_3$, TOA depressed the microbial activities of Hiochi bacteria. It was found that the presence of $CaCO_3$ in the medium alleviated the effect of TOA on the Hiochi bacteria, but $CaCO_3$ had no effect on the other extractants, tri-n-butylphosphate and tri-n-octylmethylammonium chloride, for lactic acid extraction. And then it was found that other carbonates and calcium salts had also no effect, suggesting that the effect of calcium carbonate was specific to extractants. There have been reports on the mechanism of calcium alleviation for mineral toxicities, where a specific effect is considered to be the collective ameliorative effect of Ca^{2+} based on the Ca^{2+}-toxicant interactions at the cell surface [20]. A small amount of dissolved Ca^{2+} from slightly soluble calcium carbonate may interact with TOA. Figure 2 shows the effect of $CaCO_3$ concentration in the medium on the microbial activity. The presence of $CaCO_3$ at more than 2.5 g/L caused an alleviation effect for TOA.

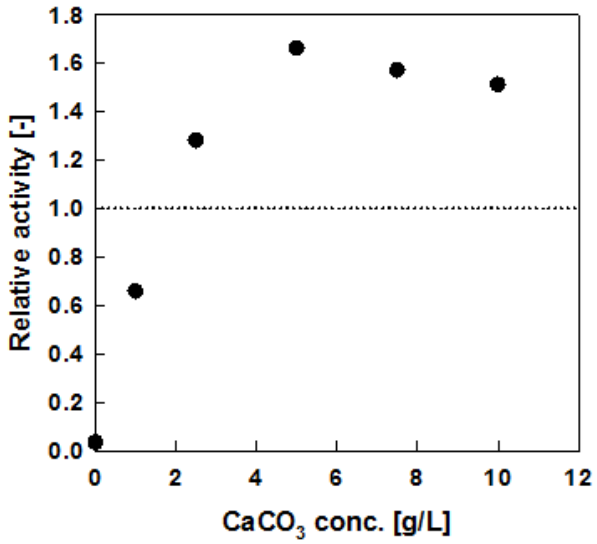

Figure 2: Effect of $CaCO_3$ in the medium on relative activity in the presence of TOA as a second phase

Generally, a viscous extractant such as TOA is diluted by the appropriate diluents, and the TOA solution is used as an extract. As described above, 1-decanol was an active diluent for lactic acid extraction. Therefore, the toxicity of a TOA solution diluted by 1-decanol was also examined as shown in Table 1. From these experimental results, a 1-decanol solution containing TOA is promising for the extractive fermentation of lactic acid with Hiochi bacteria in the presence of $CaCO_3$.

Extractant	Lb. homohiochi NRIC 0119	Lb. homohiochi NRIC 1815	Lb. fructivorans NRIC 0224	Lb. fructivorans NRIC 1814
TOA	–	–	–	–
TOA [a]	+	+	+	+
1.0 mol/dm^3 TOA in 1-decanol[a]	+	+	+	+
Tri-n-butylphosphate[a]	b	b	–	b
Tri-n-octylmethyl ammonium chloride[a]	b	b	–	b

Table 2: Toxicity of extractants to Hiochi bacteria. a: Cultivation in the presence of 10 gL-1 $CaCO_3$. b: not determined.

Extraction of lactic acid from fermentation broth

The distribution ratio, D, of lactic acid, HA, is defined by Eq. (1).

$$D = \frac{[\mathrm{HA}]org}{[\mathrm{HA}]aq} \quad (1)$$

Figure 3 shows the distribution ratios of lactic acid in an aqueous medium and the fermentative medium at pH 3.5. Evidently, the distribution ratio dramatically decreased from 15 in the aqueous medium to 2 in the fermentative medium. In order to specify the causative component in the medium, the effects of glucose, yeast extract and peptone on the distribution ratio are also shown in Figure 3. The causative components were found to be yeast extract and peptone, although it was reported that peptone, yeast extract and lactose did not affect the lactic acid extraction with TOA in a mixture of dodecane and 1-decanol [18]. However, the concentration ranges investigated were unclear in the previous paper. It is because the relative high concentrations of yeast extract and peptone were used in this fermentation. Probably, the organic acids involved in yeast extract and peptone, and lactic acid completed for TOA and the organic acids interfered with the extraction of lactic acid using TOA. In the future, the optimum medium composition must be considered for both fermentation and extraction.

As described above, in the extractive fermentation of lactic acid with a chemical reaction using tertiary amines, one of the critical problems to be solved was the difference in the optimum pH between the fermentation and extraction. Therefore, the pH effect on the distribution ratio was examined, and the results are shown in Figure 4. As described in previous papers [13,18,21] the distribution ratio decreased with increasing pH, suggesting that undissociated lactic acid was the extracted species. In this study, even under the non-optimized medium composition, the distribution ratio of lactic acid from the fermentation broth was larger than unity at pH 5.

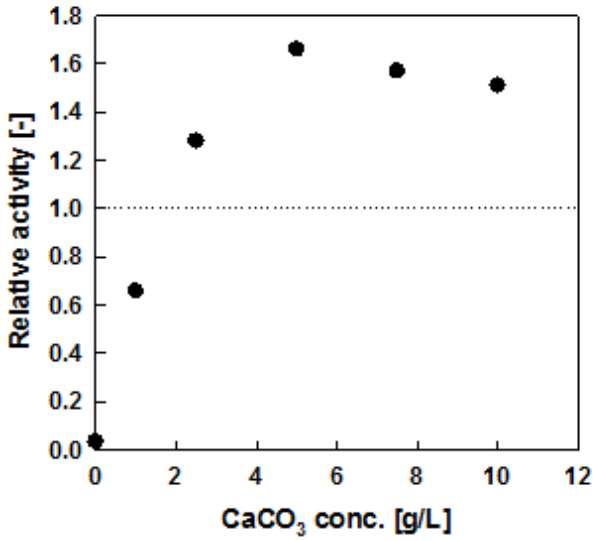

Figure 3: Effects of components in the aqueous medium on distribution ratio of lactic acid at pH 3.5; Fermentation medium contains 10 g/L glucose, 5 g/L yeast extract and 5 g/L peptone.

Extractive fermentation by immobilized *L. fructivorans* in Ca-alginate capsules

As described above, an *in situ* extractive fermentation process with a chemical reaction using TOA has two critical problems: solvent toxicity and pH. In the previous sections, we found that the presence of $CaCO_3$ in the medium considerably alleviated the toxicity of TOA in 1-decanol for the lactic acid fermentation with *L. fructivorans* NRIC0224, and lower pH values caused the high extractability of lactic acid with TOA. Based on these results, we proposed the co-immobilization of cells and $CaCO_3$ powders in Ca-alginate capsules. In such a scenario, the cells do not receive the phase toxicity, that is, there is no direct contact of the cells with organic solvent, and the presence of $CaCO_3$ in the vicinity of the cells alleviated the molecular toxicity of TOA dissolved in the aqueous solution. Although initial fermentation pH is 6.8, the medium pH gradually decreases as the fermented product, lactic acid, is produced if the pH is not kept at the initial value. However, the medium pH within the Ca-alginate capsules can

be maintained due to the neutralizing effect of $CaCO_3$ [22]. On the other hand, the pH of bulk medium may be lowered due to the release of lactic acid from the capsules. Furthermore, a lower pH of the medium is preferable for extraction with TOA. Based on this notion, we constructed an *in situ* extractive fermentation system with a chemical reaction using TOA as shown in Figure 1.

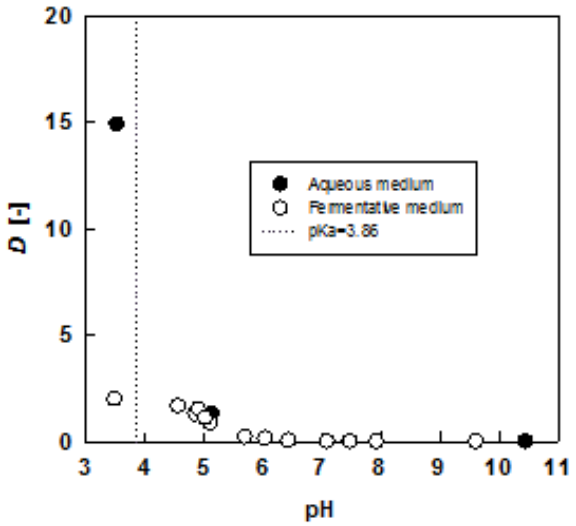

Figure 4: pH effects on distribution ratio of lactic acid in fermentative medium and aqueous medium.

Prior to the extractive fermentation with the packed bed, we examined the effect of co-immobilization of *L. fructivorans* NRIC0224 cells and $CaCO_3$ in Ca-alginate capsules on the lactic acid fermentation in a batch operation. Table 3 gives a summary of the lactic acid produced in the medium under various conditions. While TOA was toxic in the GYP medium, co-immobilization of $CaCO_3$ in Ca-alginate capsules had an alleviation effect on the toxicity of TOA. In all cases, the presence of $CaCO_3$ caused higher lactic acid production, suggesting that $CaCO_3$ depresses the decrease in pH because $CaCO_3$ neutralizes lactic acid fermentation.

$CaCO_3$	–	+	–	+	–	+
TOA	–	–	+	+	+	+
1-Decanol	–	–	–	–	+	+
Lactic acid [g/L]*	5.95	8.03	1.05	4.17	3.08	5.29

Table 3: Summary of batch fermentation of lactic acid using Ca-alginate capsules including L. fructivorans NRIC 0224. Initial glucose concentration was 9.44 g L^{-1}. Values are concentrations of lactic acid in the medium after 48 h.

	Yield*1	Productivity*2	108Vial cells*3	
			Before	After
Control	0.694	0.34(30 h)	7.35	5.63
Extractive	0.846	0.43(30 h)	11.9	0.758
		0.31(56 h)		

Table 4: Summary of extractive fermentation with packed bed and viability of *L. fructivorans* NRIC 0224 during the fermentation *1) g-lactate produced per g, glucose consumed; *2) g-lactate produced $L^{-1}h^{-1}$; *3) CFU mL^{-1}.

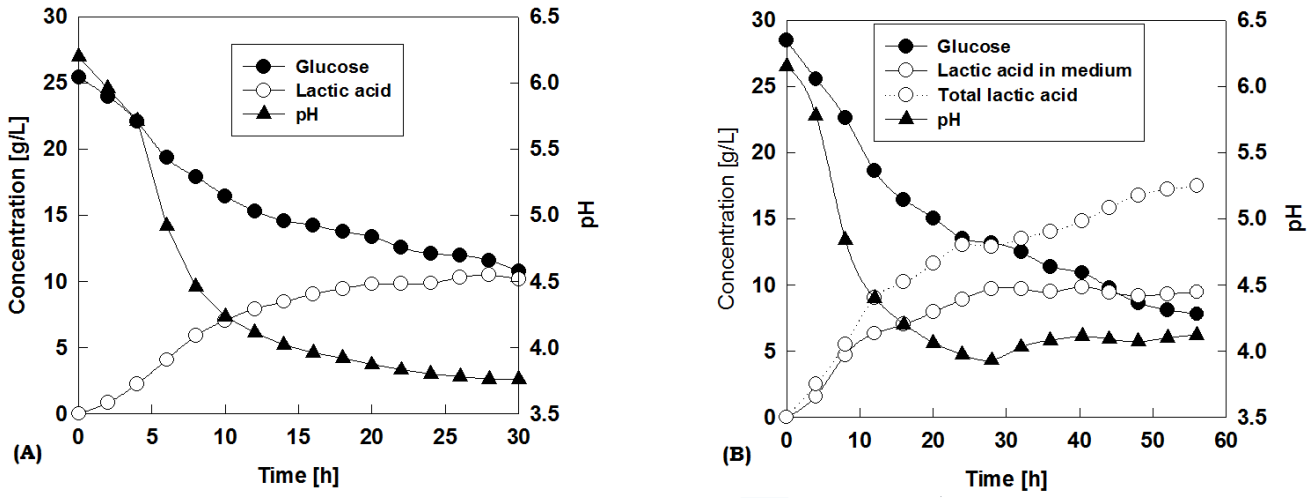

Figure 5: Time courses of glucose and lactic acid concentrations, and pH in control (A) and extractive (B) fermentations using packed bed column.

Figure 5 shows time courses of glucose and lactic acid concentrations in the extractive fermentation of lactic acid using a packed bed column without pH control. In the control fermentation in the absence of an organic phase (Figure 5A), the rates of glucose consumption and lactate production gradually decreased due to the decrease in the medium pH. When pH was kept at the initial value, glucose was completely consumed. The extractive fermentation (Figure 5B) proceeded successfully. Table 4 shows a summary of the fermentation, along with the viability of the cells. Productivity of the extractive fermentation was larger than that of the control at 30 h, and the yield of the extractive fermentation was improved. In the presence of an organic solution, encapsulated cells survived, but the vial cells slightly decreased due to the toxicity of the organic solution. Therefore, improvement of productivity was found to be depressed by the molecular toxicity of TOA. More hydrophobic amines with less water solubility should be developed. Recovery of lactic acid may be enhanced by continuous stripping of lactate from the loaded organic phase [20-22].

Conclusion

The extractive fermentation of lactic acid with Hiochi bacteria was carried out. We selected *L. fructivorans* NRIC0224 as the Hiochi bacterium and 1-decanol as a diluent from the viewpoints of the tolerance of organic solvents to microbes and the extraction capacity. Although TOA has been reported to be highly toxic to Lactobacillus strains, we found that the presence of powdered $CaCO_3$ in the medium considerably alleviated the toxicity of TOA. We also found that yeast extract and peptone affected the extraction of lactic acid with TOA in 1-decanol. Then, we immobilized both *L. fructivorans* NRIC0224 cells and $CaCO_3$ in Ca-alginate capsules and constructed an *in situ* extractive fermentation system with a chemical reaction using TOA and a packed bed of Ca-alginate capsules without pH control. This system worked successfully, and the yield and productivity were improved over those of the control fermentation.

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