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Research Report

L-lactic Acid Fermentation under Non-neutralizing Conditions and Oligomerization

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ABSTRACT L-lactic acid is a raw material for biodegradable and renewable polylactic acid (PLA). To simplify the purification process after lactic acid fermentation, we have developed extractive fermentation and adsorption fermentation methods performed under non-neutralizing conditions with metabolically engineered yeast to produce optically pure L-(+)-lactic acid. In the extractive fermentation method, 80 g/L lactic acid in the solvent was obtained by means of a hollow fiber module when tri-*n*-decylamine/oleyl alcohol was used as the extractant. In the adsorption fermentation method, we also developed an integrated process involving fermentation using activated carbon (AC) as an adsorbent for lactic acid fermentation, separation, and oligomerization. It was confirmed that pH has a significant effect on the adsorption of lactic acid on AC. The use of AC for in situ removal of lactic acid successfully decreased the inhibitory effect of lactic acid, resulting in significant increases in both productivity and yield. Acetone was successfully used to desorb lactic acid without decreasing the optical purity of lactic acid, i.e., the optical purity was as high as 99.5% after desorption. Oligomers with a high optical purity of above 96% and a mean molecular weight of 2,400 were obtained from the desorbed lactic acid in the oligomerization process. It is expected that this process will be adapted for practical usage of PLA production in the future.

KEYWORDS L-lactic Acid, Fermentation, Adsorption, Desorption, Oligomerization, Optical Purity, Polylactic Acid, Non-neutralizing Condition, Extractive Fermentation

1. Introduction

Lactic acid is a naturally occurring hydroxy-carboxylic acid, which is widely used in the food, pharmaceutical, cosmetic, and chemical industries. Recently, there has been growing interest in lactic acid production as a raw material for biodegradable and renewable plastics such as polylactic acid (PLA). Lactic acid is commonly produced by lactic acid bacteria which need specific minerals, B-vitamins, and several amino acids to ensure their optimum growth.[1,2] Since most lactic acid bacteria are not resistant to low pH, neutralizing agents, such as NaOH, NH_4OH, and $CaCO_3$, have to be added to prevent a decrease in pH. Using neutralizing agents may increase the production cost of lactic acid because the purification process to convert lactic acid salt to undissociated lactic acid is complicated. To simplify the purification process after lactic acid fermentation, we studied lactic acid fermentation under non-neutralizing conditions.

Yeasts such as *Saccharomyces cerevisiae* that have been used in the production of ethanol can only produce a small amount of lactic acid. In our previous study, lactic acid-producing yeast was developed by introducing six copies of the bovine *L*-lactate dehydrogenase (*L-ldh*) gene into the genome under the control of the pyruvate decarboxylase 1 promotor.[3,4] The engineered yeast produced 122 g/L of lactic acid with a high optical purity of over 99.9% in a pH-controlled fermentation.[3] The transgenic yeast has been evaluated using tri-*n*-octylamine (TOA), tri-*n*-decylamine (TDA), Alanine 336, and tri-*n*-butylphosphate (TBP) as extractants.[5] The results showed the high tolerance of the transgenic yeast to the solvent. At the end of extractive fermentation, 20 g/L lactic acid in the broth and 35 g/L lactic acid in the solvent were obtained.[6] However, in extractive lactic acid fermentation, separation of oligomers in the solvent was difficult due to low lactic acid concentration in the solvent. To increase the lactic acid concentration in the solvent, we examined the possibility of extractive fermentation with a hollow fiber module (EF-HFM).[7]

We also attempted to develop an adsorption fermentation method using activated carbon (AC), which is an extremely porous material with a high surface area ratio and exhibits specific affinity to organic materials.[8] Currently, there have been limited investigations on the use of AC for fermentative production of organic acids. Koide et al. assumed that AC cannot adsorb lactic acid, and thus they used AC as a cell immobilization carrier in the fermentation with *Rhizopus oryzae* at a controlled pH of 6.0 for efficient production of lactic acid.[9] Chen et al. investigated the characteristics of lactic acid adsorption onto AC.[10] They used AC as an adsorbent for lactic acid as well as for cell immobilization in the fermentation controlled at pH 5.5, achieving a lactic acid production of above 500 h.[11] However, accumulation of lactic acid (60 g/L) in the fermentation broth occurred, indicating the low adsorption of lactic acid onto AC at this pH value. Since adsorption of polar alcohols and acids onto AC is considered to be physical adsorption and depends mainly on electronic polarizability,[12] the pH of the solution used would influence the adsorption of lactic acid onto AC.

Here, we investigated two fermentation methods to separate lactic acid under non-neutralizing conditions. The purpose of this study was to develop an integrated process involving fermentation, separation, and direct oligomerization of lactic acid.

2. Materials and Methods

2. 1 Microorganism and Culture Media

S. cerevisiae OC-2T T165R[3,4] was used as the L-lactic acid producing microorganism in this study. The strain was precultured at 30°C and 80 rpm in YPD medium containing a yeast extract (10 g/L), peptone (20 g/L), and glucose (20 g/L). The medium for fermentations contained molasses (40 g/L) and sucrose (134 g/L).

2. 2 Extractive Fermentation

A schematic diagram of in situ extractive fermentation with an organic solvent is shown in **Fig. 1**(A). The broth for fermentation contained molasses (40 g/L) and sucrose (134 g/L). 30% TDA/oleyl alcohol was added to the fermenter prior to fermentation, which was carried out in a 1-L jar fermenter (working volume: 0.5 L) at 32°C with an initial OD_{660} of 5. The volume ratio of the solvent and the fermentation broth was set at 1:2. In the case of extractive fermentation with a hollow fiber module (EF-HFM), shown in Fig. 1(B), a hollow fiber module and a solvent column were used to prevent direct contact between the yeast cells and the solvent. A tube-type hollow fiber module (TS-1.0S, TORAY Co., Japan) with an effective filtration area of 0.2 m^2 and a nominal pore size of 0.1 μm was used to separate the yeast cells from the fermentation broth. When extractive fermentation was inititated, the fermentation broth was pumped into the hollow fiber module. The filtrate flowed into the column for lactic acid extraction. After extraction, the filtrate flowed into the fermenter, while yeast cells concentrated in the hollow fiber module were recycled to the fermenter.

2. 3 Adsorption Fermentation

2. 3. 1 Adsorption of Lactic Acid

The adsorption and desorption of lactic acid were investigated prior to adsorption fermentation. The adsorption of lactic acid was performed with activated carbon (AC) as an adsorbent, which was a guaranteed-grade product from Wako, charcoal-activated and granulated. The adsorption of lactic acid onto AC was carried out with an initial lactic acid concentration of 50 g/L on a rotary shaker at 80 rpm. To determine the effect of pH on lactic acid adsorption, the pH of the lactic acid solution was adjusted in the range of 2-11 by the addition of 10 M sodium hydroxide. Next, 10 g of AC was added to 100 mL of the lactic acid solutions with different initial pHs. To determine the effect of the AC concentration on lactic acid adsorption, 10-50% (wt./vol.) of AC was added to the lactic acid

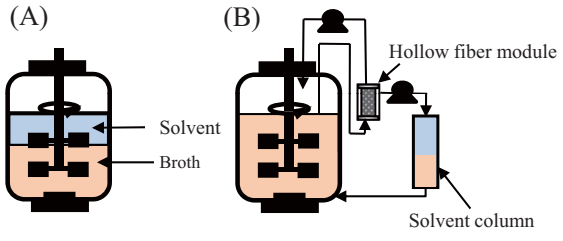

Fig. 1 Schematic diagrams of extractive fermentation.
(A) In situ extractive fermentation;
(B) extractive fermentation with a hollow fiber module.

solution without pH adjustment; the initial pH of the lactic acid solution was 2.0. Shaking was carried out overnight to reach equilibrium. After equilibrium had been reached, samples were withdrawn from the aqueous lactic acid solutions and the lactic acid concentrations in the aqueous phase were determined with a bio-sensor BF-4S (Oji Keisoku Kiki, Japan). Lactic acid concentrations in the solvent were calculated according to mass balance.

2. 3. 2 Procedure for Adsorption Fermentation and Recovery of Lactic Acid

A schematic diagram of adsorption fermentation is shown in **Fig. 2**. AC used as the adsorbent was sterilized at 200°C for 1-2 h prior to fermentation. The AC was then directly added to the fermentation broth on both flask and fermentor scales. On the fermentor scale, there was no agitation because mass transfer of oxygen could be improved by flowing air only, due to the extremely porous nature of AC. After fermentation, the fermentation broth with AC was passed through a plastic perforation sheet (pore size: about 1.0 mm) to separate the AC. Next, the AC was mixed with acetone directly at a ratio of 1:2 (w/v). When the AC was mixed with acetone, the cells adsorbed onto the AC during fermentation were released quickly from the AC so that the cells became suspended in the solution. The AC was again passed through the plastic perforation sheet, then the cells in the solution were removed by filtration with a PTFE membrane filter (Advantec, H050A, pore size: 0.5 μm). Lastly, the recovered solution was mixed with the washed AC again for further lactic acid desorption. The procedure for AC washing was the same as described above except for cell removal. After removal of acetone, a concentrated lactic acid solution was obtained, i.e., concentrations of 150-200 g/L. The lactic acid concentrations of the solutions were determined to calculate the rate of recovery of lactic acid.

In the case of fermentations with repeated use of AC, the AC washed twice with acetone was sterilized at 200°C for 1-2 h for use in the next fermentation.

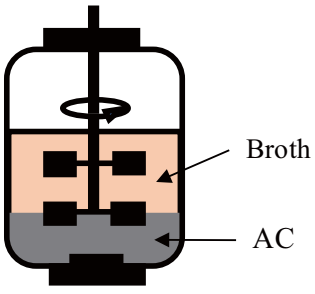

Fig. 2 Schematic diagrams of adsorption fermentation.

2. 4 Oligomerization of Lactic Acid

When fermentation with 50% AC was complete, desorption of lactic acid was performed, as described above. The acetone-free solution contained 150-200 g/L lactic acid with an optical purity of above 99.5%. The oligomerization procedure described by Moon et al.[7] was modified by varying the temperature. The reaction for lactic acid oligomerization was performed at 100°C, 10,000 Pa for 2 h, followed by 130°C, 1,300 Pa for 2 h, 150°C, 1,300 Pa for 2 h, and 160°C, 1,300 Pa for 2 h. After the reaction, the oligomers were cooled to room temperature and then 2 mL of NaOH (2 M) per 20 mg of oligomer was added to prepare samples for HPLC measurement.[8]

For reference, lactic acid oligomerization in a pH-controlled fermentation broth (pH 6.0) was carried out. The fermentation broth was not treated with AC or acetone. The oligomerization was performed after the pH of the fermentation broth had decreased to 2.0 upon addition of H_2SO_4.

3. Results

3. 1 Extractive Fermentation

Since yeasts are more tolerant to extractant toxicity than lactic acid bacteria, in situ extractive fermentation was first considered without pH control. The results of fermentation are shown in **Fig. 3**. In in situ extractive fermentation, sucrose was quickly hydrolyzed to glucose and fructose and was completely depleted within 20 h, resulting in increases in the concentrations of glucose and fructose. However, the consumption rates of glucose and fructose did not increase after exhaustion of the sucrose. Although fermentation was performed for more than 100 h, lactic acid production was stopped within 30 h, and 30 g/L of glucose and 50 g/L of fructose remained in the fermentation broth.

Next, extractive fermentation with a hollow fiber module (EF-HFM) was carried out without pH control for comparison with in situ extractive fermentation as shown in **Fig. 4**. The rate of hydrolysis of sucrose in EF-HFM appeared to be a little slow, and residual fructose and glucose were observed in the fermentation

broth. It should, however, be noted that the residual sugar concentrations were much lower than those in in situ extractive fermentation. 14 g/L of glucose and 32 g/L of fructose remained in the fermentation broth as compared to 30 g/L of glucose and 50 g/L of fructose in in situ extractive fermentation. Thus, the yield also increased from 33% in in situ extractive fermentation to 42% in EF-HFM. Moreover, the extraction degree was much better because the problem of yeast cells accumulating at the interface between the fermentation broth and the solvent for lactic acid extraction was solved through the use of the hollow fiber module. In EF-HFM, the lactic acid concentration in the solvent was over 70 g/L, i.e., it was much higher than that in in situ extractive fermentation (39 g/L).

Moreover, EF-HFM was carried out at pH 3.5 in attempt to obtain higher extractive fermentation performance as shown in **Fig. 5**. The concentrations of residual sugars decreased during fermentation and fell rapidly during the first 50 h of fermentation relative to those in pH-uncontrolled extractive fermentation. On the other hand, the lactic acid concentration in the fermentation broth increased, and a lactic acid concentration of 80 g/L in the solvent was achieved.

3. 2 Adsorption Fermentation

3. 2. 1 Adsorption of Lactic Acid

When sodium hydroxide was not added to the model lactic acid solution, the initial pH was 2.0. About 35% lactic acid was absorbed onto AC as shown in **Fig. 6**.

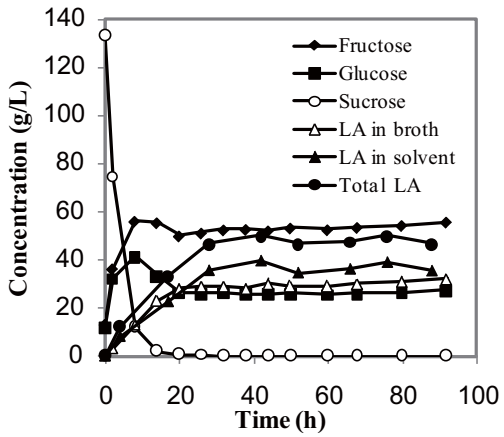

Fig. 3 Time course of pH uncontrolled extractive fermentation.

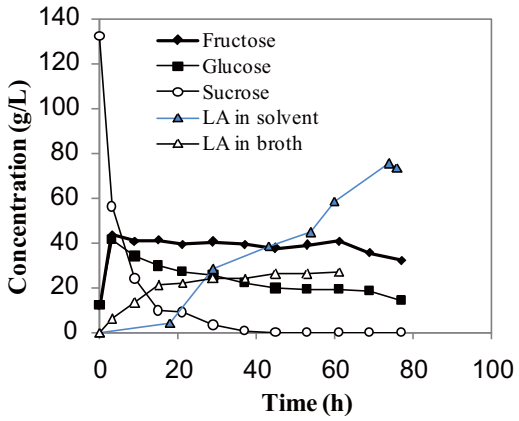

Fig. 4 Extractive fermentation with a follow fiber module under pH-uncontrolled condition.

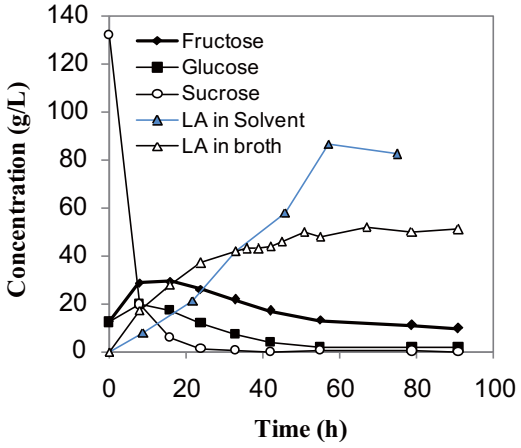

Fig. 5 Extractive fermentation with a follow fiber module under pH 3.5 controlled condition.

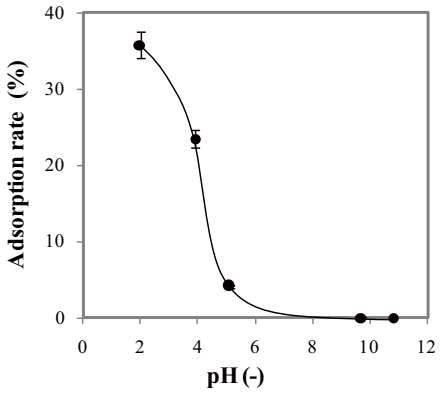

Fig. 6 Effect of pH on lactic acid adsorption onto AC. *Reaction conditions*: initial lactic acid concentration of 50 g/L, AC concentration of 10% (w/v), 30°C, 80 rpm.

Along with an increase in pH value, the absorption rate decreased, becoming 0% at pHs over 9.7.

Figure 7 shows the effect of the AC concentration on the adsorption of lactic acid. The results followed the expected pattern, in that the absorption rate increased with increasing AC concentration. The absorption rate increased very quickly with low concentrations up to 30%, after which the increase in AC concentration had less effect on the absorption rate. When the AC concentration was over 30%, over 80% of the lactic acid in the solutions was adsorbed.

3. 2. 2 Effect of AC Concentration on pH-uncontrolled Lactic Acid Fermentation

To determine the effect of the AC concentration on pH-uncontrolled lactic acid fermentation, flask level fermentations were carried out with various AC concentrations, from 10% to 50% (w/v). The total lactic acid concentration was determined by desorbing lactic acid from AC with NaOH after fermentations were over. As shown in **Fig. 8**, when AC was not added, the lactic acid concentration in the fermentation broth was the highest among the fermentations, but over 20 g/L glucose remained after 47 h, corresponding to the lowest total lactic acid concentration, 35 g/L. In contrast, the lactic acid concentrations in the fermentation broth could be controlled by adding AC to the fermentation broth. Along with the increase in the AC concentration, both the concentrations of lactic acid and glucose in the fermentation broth decreased while the total lactic acid concentration increased. When 50% AC was used, the productivity and yield in fermentations increased by 2 times and 1.5 times, respectively, as compared to those in fermentation without AC.

3. 2. 3 AC Recycling for Adsorption Fermentation

As shown in **Fig. 9**, the effect of the adsorption ability of lactic acid with repeated use of AC was not significant. The residual glucose in the fermentation broth was lower than 0.2 g/L in all cases. In the four runs, the amounts of lactic acid recovered were in the range of 42-48% of the initial glucose concentration. In the fifth run (data not shown), NaOH was used instead of acetone for the desorption of lactic acid to obtain an accurate yield of lactic acid. As a result, 60 g/L lactic acid was produced from 85 g/L glucose.

3. 3 Oligomerization of Lactic Acid Desorbed from AC with Acetone

After adsorption fermentations, lactic acid was desorbed from the AC with acetone. The acetone was

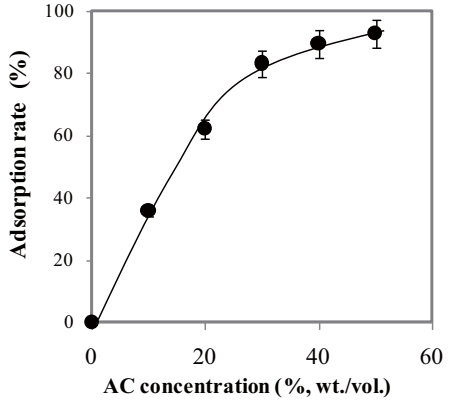

Fig. 7 Effect of AC concentration on lactic acid adsorption onto AC. *Reaction conditions*: initial lactic acid concentration of 50 g/L, initial pH of 2.0, 30°C, 80 rpm.

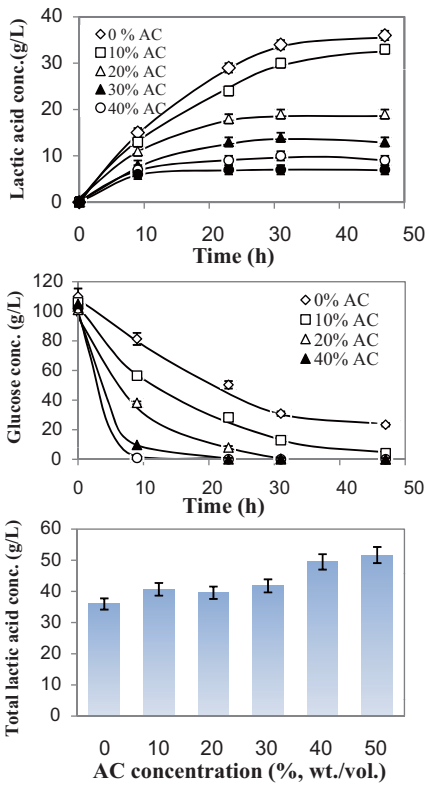

Fig. 8 Effect of AC concentration on lactic acid production in flask level fermentations.
Reaction conditions: initial OD of 5, 30°C and 80 rpm, 0.1 L production medium.

then removed and the resulting acetone-free solutions contained about 150 g/L lactic acid, which was used directly for the oligomerization step. As a result, only very little residual glucose was detected in the four acetone-free solutions, i.e., from 0.1 g/L to 0.2 g/L. When the acetone-free solutions were used for the oligomerization of lactic acid, a small decrease in optical purity was observed and oligomers exhibiting optical purities ranging from 95.2% to 96.2% were obtained. In the fourth run, an oligomer exhibiting a MW as high as 2,400 was obtained, although an oligomer exhibiting a MW of 1,200 was obtained for the direct oligomerization in the pH-controlled fermentation broth.

4. Discussion

Lactic acid fermentation under non-neutralizing conditions is important in order to simplify the processes for PLA synthesis. In this study, we proposed an extractive fermentation method using an extracting agent and an adsorption fermentation method using an adsorbent. In extractive fermentation, the lactic acid productivity of the extractive fermentation method with a hollow fiber module (EF- HFM) was higher than that of in situ extractive fermentation. We proposed that it is important to avoid direct contact between yeast cells and solvent by a hollow fiber module and a solvent column.

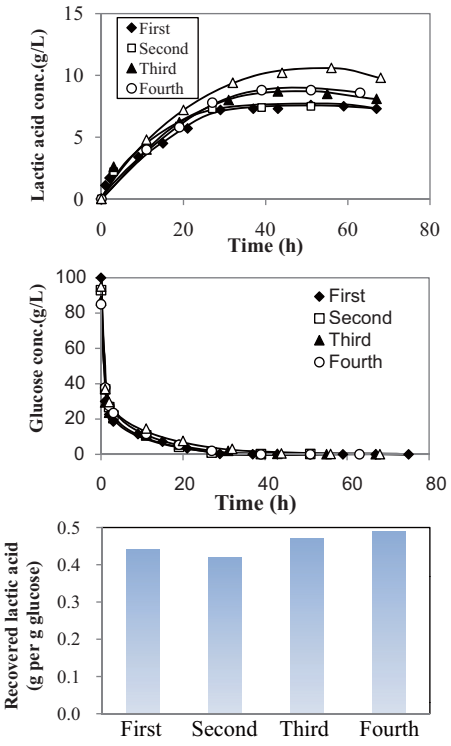

Fig. 9 Repeated use of AC in fermenter level fermentations.
Reaction conditions: initial OD of 5, 32°C, 0.5 L production medium, AC concentration of 50%.

First, EF-HFM was carried out without pH control for comparison with in situ extractive fermentation described in Section 3.1. As shown in Fig. 4, the rate of hydrolysis of sucrose in EF-HFM appeared to be a little slow compared to that in in situ extractive fermentation. However, the residual sugar concentrations in EF-HFM were lower than those in in situ extractive fermentation, and the yield increased from 33% in in situ extractive fermentation to 42% in EF-HFM. Therefore, keeping the yeast cells out of the solvent is a good method for improving the efficiency of extractive fermentation. Moreover, the productivity of lactic acid was higher in the EF- HFM controlled at acid conditions. EF- HFM is a method for lactic acid separation under non-neutralizing conditions.

We also developed another method to separate lactic acid by adsorption of lactic acid onto AC. AC is not considered to be an absorbent for lactic acid because all lactic acid fermentations have been performed under neutralizing conditions. It was clear that the adsorption of lactic acid was sensitive to the pH of the used solution, and the adsorption decreased at a high pH (Fig. 6). Since the adsorption of polar alcohols and acids onto AC is considered to be physical adsorption and depends mainly on electronic polarizability,[13] the decreased adsorption of lactic acid at a high pH is probably due to decreased hydrophobic interactions. The adsorption of lactic acid onto AC increased with an increase in AC concentration (Fig. 7), which was attributed to the increased carbon surface area. The use of AC reduced the inhibitory effect of lactic acid, leading to higher productivity and yields of lactic acid (Fig. 8). AC can also adsorb glucose when a large amount of AC is used.[14,15] However, we obtained high yields (70%) of lactic acid in the fermenter level fermentations, e.g., 60 g of lactic acid per 85 g of glucose. The reasons why glucose can be consumed completely are considered as follows: (1) competitive adsorption between glucose and lactic acid occurred and the affinity of glucose was less than that of lactic acid. Consequently, the glucose adsorbed onto AC in the initial period of fermentation was substituted gradually by the lactic acid produced, (2) glucose was

eluted from the AC gradually along with the decrease in glucose concentration due to consumption by the yeast in the fermentation broth.

The desorption of lactic acid from AC was performed with acetone. The desorption by acetone can be attributed to the higher hydrophobicity of acetone than that of lactic acid, which makes acetone more competitive than lactic acid to adsorb onto AC. The adsorbed acetone can be easily removed from the AC by heating under vacuum, which is also the process used to sterilize AC. The repeated use of AC in lactic acid fermentations was also examined in this study. The effect of AC on lactic acid production decreased slightly with the repeated use of the AC. Assuming that all the fermentations gave the same amount of lactic acid, 60 g/L, the rate of recovery of lactic acid would range from 70% to 81%, according to the data for the recovered lactic acid in Fig. 9.

In the direct oligomerization of lactic acid after adsorption fermentation, we obtained lactic acid oligomers of MW 2,400 with an optical purity of above 96%. On the other hand, the oligomers obtained by direct oligomerization after pH-controlled fermentation (pH 4.8) had a MW of 1,200. The lower MW oligomers would be due to the presence of a large amount of the salts in the broth for pH-controlled fermentations. Moon et al. reported that Sn(II) catalysts activated by various proton acids produced high molecular weight PLA (MW $\geq$ 100,000).[16] We propose that the higher MW oligomers are advantageous over the lower MW oligomers to obtain higher polymers of PLA.

Finally, a flow diagram for PLA synthesis is shown in **Fig. 10**. We developed this method to produce oligomers with high optical purities by an integrated process involving fermentation using AC, desorption with acetone, and direct oligomerization of lactic acid.

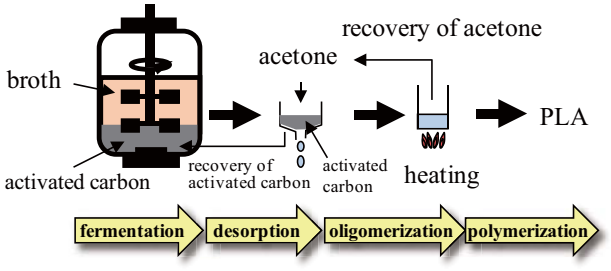

Fig. 10 Flow diagrams for Polylactic acid (PLA) synthesis: from fermentation to polymerization.

It is expected that this process will be adapted for practical usage of PLA production in the future.

Acknowledgments

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