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Production of L(+)-Lactic Acid Using Acid-Adapted Precultures of *Rhizopus arrhizus* in a Stirred Tank Reactor

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Abstract Cultivations of filamentous fungi in stirred tank reactors (STRs) to produce metabolites are often limited by insufficient mixing and mass transfer because of the formation of mycelial clumps inside the reactors. This study developed an acid-adapted preculture approach to control the morphology of filamentous *Rhizopus arrhizus* in a STR, consequently to enhance the production yield and productivity of L(+)-lactic acid efficiently using waste potato starch as substrate. Using the acid-adapted precultures as inoculum, the morphology of *R. arrhizus* was maintained as large clumps, coalesced loose small pellets, and freely dispersed small pellets. The highest lactic acid concentration of 85.7 g/L with a yield of 86% was obtained in association with the formation of coalesced loose small pellets. The results indicate that the use of the acid-adapted precultures as inoculum is a promising approach for lactic acid production in STRs.

Keywords L(+)-lactic acid · *Rhizopus arrhizus* · Pellets · Acid-adapted precultures · Morphology · Stirred tank reactor

Introduction

Lactic acid (2-hydroxypropionic, $CH_3CHOHCOOH$) is a valuable multifunctional organic acid, which has been widely used in the food, pharmaceutical, and chemical industries. Lactic acid exists naturally as two optical isomers: D(−)-lactic acid and L(+)-lactic acid. Both can be polymerized to biodegradable and biocompatible poly-lactate polymers, which

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are environmentally friendly alternatives to plastics derived from petrochemicals [1]. The development and commercialization of these biopolymers has led to increased use of lactic acid, and in 2005, 20–30% of the 120,000-ton global production has been estimated to be used in these new applications [2]. As D(−)-lactic acid may be harmful to humans, L(+)-lactic acid is the preferred isomer in the food and pharmaceutical industries [3].

Pure L(+)-lactic acid can be produced by *Rhizopus* species. In the past two decades, L(+)-lactic acid fermentation with *Rhizopus* species has attracted a great interest because of their amylolytic ability and low nutritional requirements [4–6] compared to lactic acid bacteria [7]. As filamentous fungi, *Rhizopus* species can grow in different morphological forms such as filamentous mycelia, clumps, and pellets, depending on the growth conditions. Because of its industrial capacity and reliability, the stirred tank reactor (STR) has been widely utilized in commercial fermentation processes. However, fermentations of filamentous fungi in a STR often lead to the formation of large clumps, which cause operational difficulties, such as anchoring onto the wall of the reactor and probes, wrapping around impellers, and blocking the sampling and feeding ports, and result in lower yield and productivity. To overcome these problems encountered in reactors, immobilized *Rhizopus* cells were employed to improve lactic acid production by a number of researchers [1, 8]. However, in most immobilization studies, lactic acid yields were only 65–78%, with lactic acid concentrations of 40–73 g/L [9–14]. Although there are instances where lactic acid yields reached more than 85% [15–18], the operational processes of immobilization were either complex or uneconomical, which would limit their application in an industrial scale.

Pellets are often the preferable morphological forms in industrial fermentation processes. A less dense pellet form is favorable for fungal cell growth and mass/heat transfer in a reactor because molecular diffusion can take place easily throughout the less dense pellets via turbulent diffusion and convective flow [19]. Pellets of *R. oryzae* were formed easily by the manipulation of the initial spore concentration when *R. oryzae* was cultivated in air-lift reactors (ALRs) and bubble column reactors (BCRs) [20, 21]. However, these methods were not transferable to STRs. In a STR inoculated by precultures prepared in shake flasks with xylose as the carbon source, small pellets were induced, but only 33 g/L lactic acid was produced over 60 h [22]. Pellets of *R.oryzae* were also formed in a STR by controlling preculture conditions, such as the time of $CaCO_3$ addition, inoculum size, and nitrogen concentration in the shake flasks, or agitation speed and aeration rate in the STR [23]. Despite the complexity of the cultivations, only 76.1 g/L lactic acid was obtained from 100 g/L glucose [23]. Therefore, it still remains a challenging task to build up a simple and reliable approach to control the morphology of *R. arrhizus* in pellet forms in STRs and to produce lactic acid efficiently.

This study was aimed at developing an acid-adapted preculture approach to control desirable morphological forms of *R. arrhizus* to enhance lactic acid yield in a STR. A model strain—*R. arrhizus* DAR 36017—which demonstrated a high capacity for lactic acid production in previous studies [4, 24], was employed in this work. Waste potato starch was used as the production substrate.

Materials and Methods

Microorganism

Rhizopus arrhizus DAR 36017 was obtained from the Orange Agricultural Institute, Sydney, Australia. This strain was maintained and grown for spore production on potato dextrose agar slants at 30 °C for 7 days and stored at 4 °C.

Preculture Medium

The preculture medium contained (g/L): soluble starch, 10; peptone, 5.0; yeast extract, 5.0; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2 [4]. The initial pH of the preculture medium was adjusted by the addition of 2 M HCl solution before sterilization. The pH of the control preculture medium was pH 6.8. The preculture medium was autoclaved at 121 °C for 20 min.

Production Medium

The concentration of the waste potato starch was calculated and presented as concentration of glucose in this paper, unless otherwise stated. The production medium used in the STR consisted of 100 g/L waste potato starch, 3.0 g/L $(NH_4)_2SO_4$, 0.25 g/L KH_2PO_4, 0.15 g/L $MgSO_4 \cdot 7H_2O$, and 0.04 g/L $ZnSO_4 \cdot 7H_2O$. The waste potato starch used in this study was provided by Smiths Chips Ltd. (Australia). About 600–700 g/L waste potato starch was liquefied at 95–100 °C for 4 h by addition of 0.05% α-amylase (Termamyl® Classic, Novoenzymes, Denmark). The concentration of the liquefied starch solution was adjusted to 100 g/L. 2.5 mL antifoam (Dow Corning® 1510, BDH Chemicals, UK) was added to the reactor before sterilization. The production medium was autoclaved at 121°C for 20 min.

Preparation of Acid-Adapted Precultures

The acid-adapted preculture was prepared in a 250-mL shake flask containing 100 mL preculture medium. Spores were harvested from slants using a platinum loop and suspended in sterilized water. The pH of the acid-adapted preculture medium was adjusted to 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 by adding 2 M HCl. The acid-adapted precultures and control precultures inoculated with spores (a final concentration of 10^5 spores/mL) were grown in an orbital shaker at 150 rpm and 30 °C for 18 h. These precultures were named as the 1st precultures. The 2nd preculture was prepared using 5 mL of the 1st preculture as inoculum and grown for 12 h. This process was serially repeated for preparing the 3rd and 4th precultures. The cultivation time for the 3rd and 4th precultures was 12 h. Unless otherwise stated, the pH was the initial pH for each preculture.

Lactic Acid Production in the STR

The production of lactic acid was carried out in a 3.3-L STR with a 2.5-L working volume (New Brunswick Scientific, USA), which was inoculated with 125 mL of the 1st, 2nd, and 3rd precultures. The cultivation temperature in the STR was maintained at 30 °C throughout the experiments. The aeration rate and agitation speed were set at 1.0 vvm and 300 rpm, respectively. Growth pH was controlled at pH 6.0 by the addition of 10 M NaOH solution. The cultivation time in the experiments ranged from 60 to 78 h as indicated. Approximately 2 mL of fermentation broth was taken from the reactor at 6- to 12-h intervals. The sample was centrifuged at 10,000 rpm for 5 min and the supernatant was frozen at −20°C for further analysis.

Analytical Methods

The biomass was collected from one shake flask and the STR, and washed three times with tap water. The clumps and pellets of biomass were collected separately from the STR. The clumps were the biomass attached on the impellers, probes, and the sparger, and the pellets

were the spherical or oval aggregates dispersed in the reactor. Biomass weight was determined after drying at 60 °C for 72 h. The percentage of pellet biomass was calculated as pellet biomass to total fungal biomass, as PB/TB % (*w*/*w*). To record the morphology, biomass was transferred to a 9-cm Petri-dish and photographed using a digital camera (Power Shot A95, Canon, Japan). A Rezex ROA-Organic Acid analysis column (300× 7.8 mm, Phenomenex, Australia) and a refractive index detector (Model 350, Varian, Australia) were used to analyze the organic compounds, including glucose, lactic acid, fumaric acid, and ethanol. The mobile phase was 4 mM H_2SO_4 solution at a flow rate of 0.6 mL/min. The column temperature was maintained at 70 °C. The results were the means of triplicate experiments in shake flasks and at least duplicate experiments in the STR. Liquefied starch was diluted 50 times; 0.5 mL diluted liquefied starch, or supernatant from the centrifuged sample, was diluted 10 times and mixed with 37% HCl at a ratio of 10:1 [18]. The mixture was autoclaved at 121 °C for 1 h and filtered for glucose analysis by HPLC. Another 0.5 mL supernatant was diluted 25 times and filtered for analysis of lactic acid, fumaric acid, and ethanol by HPLC.

Results

Effect of pH on Precultures

Table 1 shows the effect of the adaptation pH on biomass production and final pH in shake flasks with preculture medium. As the pH changed from 6.8 (control) to 2.5, the biomass of the 1st precultures decreased from 2.6 g/L to 0.7 g/L. Large mycelial clumps were formed in the 1st precultures at pH 6.0 and control preculture (pH 6.8) after 18 h cultivation (data not shown). Because of the formation of mycelial clumps in the 1st precultures at pH 6.0 and pH 6.8 (control), further transfer of these precultures was not carried out. These clump cultures were not suitable as inoculum for reactor use. Little mycelial growth was observed in the 1st preculture at pH 2.0. This result indicated that low pH inhibited cell growth. The final pH dropped sharply in the precultures, which were acidified at a pH equal to or higher than 4.5 (Table 1) as a result of the accumulation of lactic acid (data not shown). The final

Table 1 Final biomass and pH of precultures adapted at different pH and batches in shake flasks*.

Initial pH	Final Biomass (g/L)				Final pH			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
6.8 (control)	2.6	–	–	–	4.8	–	–	–
6.0	2.5	–	–	–	4.5	–	–	–
5.5	2.2	1.8	1.6	1.5	4.0	3.5	3.4	3.3
5.0	2.2	1.8	1.7	1.5	3.6	3.4	3.3	3.3
4.5	2.1	1.7	1.6	1.5	3.5	3.4	3.2	3.2
4.0	2.1	1.6	1.5	1.4	3.4	3.2	3.1	3.1
3.5	1.9	1.6	1.4	1.2	3.2	3.1	3.0	3.0
3.0	1.1	1.4	1.2	1.2	2.9	2.9	2.8	2.7
2.5	0.7	0.7	0.9	0.8	2.5	2.5	2.5	2.5
2.0	0.0	–	–	–	2.0	–	–	–

*Final biomass and pH were measured after 18 h cultivation for 1st precultures, and 12 h cultivation for 2nd, 3rd, and 4th precultures. Standard deviations were 0.0–0.3 g/L for biomass and 0.0–0.2 for pH.

pH remained constant in the precultures adapted at pH 2.5, and dropped slightly in the preculture acidified at pH 3.0.

Figure 1 shows representative morphological forms generated in the acid-adapted precultures. Short freely dispersed mycelia were formed in the 1st preculture at pH 2.5 (Fig. 1a1), whereas long and entangled mycelia were observed in the 1st precultures adapted at pH 5.5 (Fig. 1b1). Small radial and fluffy pellets were observed in the 2nd preculture at pH 2.5 (Fig. 1a2), whereas short dispersed mycelia were formed in the 2nd preculture at pH 5.5 (Fig. 1b2). Big fluffy and radial pellets were found in the 3rd preculture adapted at pH 2.5 (1a3). Fluffy pellets mixed with mycelia were observed in the 3rd preculture at pH 5.5 (Fig. 1b3). It was interesting to note that large, radial, and fluffy pellets were formed in all the 4th precultures at pH 2.5–5.5 (Fig. 1a4 and b4). The observation results showed that repeated cultivations of the precultures of *R. arrhizus* DAR 36017 at low pH could induce the formation of pellets in shake flasks.

Effect of Acid-Adapted Precultures on the Morphology of *R. arrhizus* in the STR

Precultures adapted at pH from 2.5 to 5.5 were inoculated into the STR with production medium. Figure 2 shows the representative morphology of *R. arrhizus* in the STR at 60 h inoculated with acid-adapted precultures at pH 2.5 (Fig. 2a1–a3) and 5.5 (Fig. 2b1–b3). A few clumps but a large amount of pellets were observed in the STR using the 1st preculture at pH 2.5 (Fig. 2a1). However, as the pH of the preculture increased from 2.5 to 5.5, more clumps but fewer pellets were formed (Fig. 2a1 and b1). The PB/TB, as a measurable indicator of pellet production in the STR using different precultures, was summarized in Fig. 3. The PB/TB was 70% in the STR using the 1st preculture at pH 2.5, whereas only about 7% pellets was obtained by inoculation of the 1st preculture at pH 5.5. Surprisingly, more 65% pellets were formed in the reactors, which were inoculated with the 2nd and 3rd precultures adapted at pH 2.5–5.5. The highest PB/TB was around 90% in the STR inoculated with the 2nd precultures at pH 4.5–5.5 (Figs. 2b2 and 3). Obviously, the use of the repeated acid-adapted precultures significantly enhanced the formation of pellets

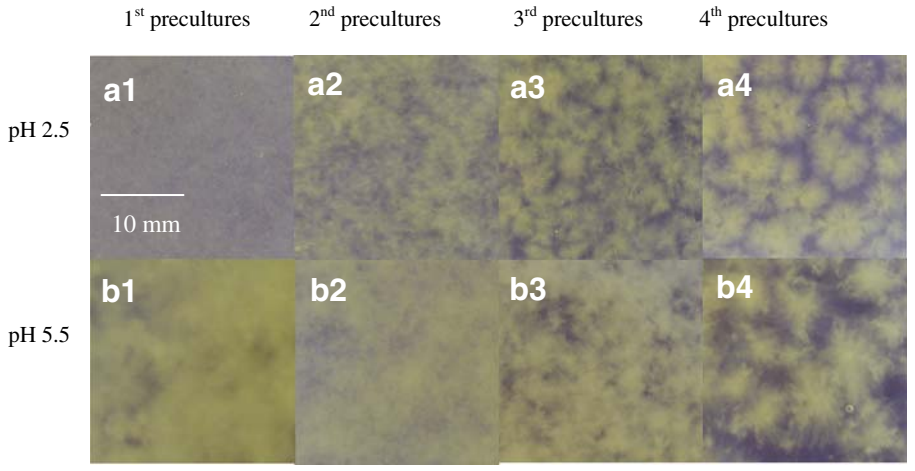

Fig. 1 Morphology of *R. arrhizus* in the precultures at pH 2.5 (**a**) and pH 5.5 (**b**) in shake flasks. The photos were taken from a 9-cm Petri-dish at the end of the cultivations

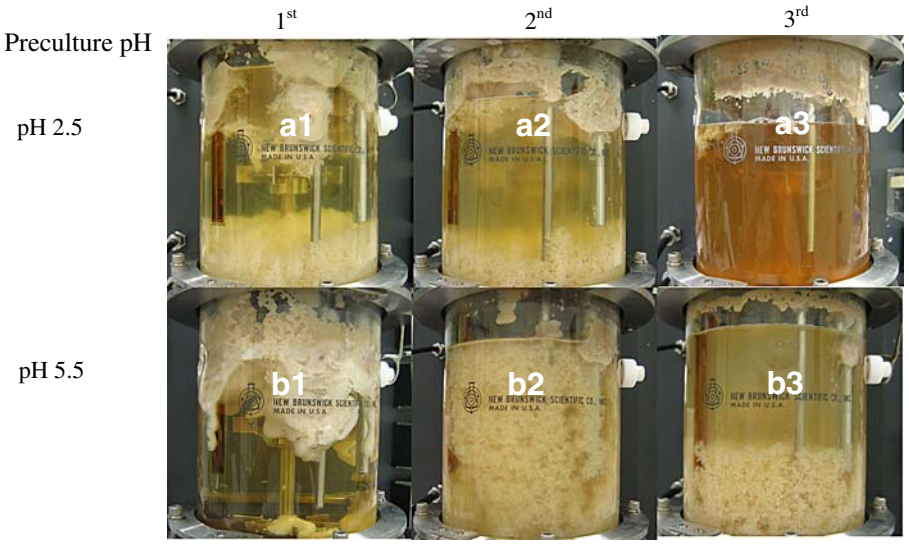

Fig. 2 Morphology of *R. arrhizus* at 60 h in the STR inoculated with the 1st, 2nd, and 3rd precultures adapted at pH 2.5 (**a**) and pH 5.5 (**b**). The photos were taken after agitation and aeration were stopped. Note: The pH of the reactor was controlled at pH 6.0 for all the reactor experiments

compared to those using the 1st preculture, especially, when the adaptation pH of the preculture was at pH 4.5–5.5.

Interestingly, suspension and sedimentation of the biomass occurred in the STR when aeration and agitation stopped (Fig. 2). This was caused by the density difference in the fungal biomass produced with different morphological forms. Coalesced loose small pellets were observed in the STR with the 2nd preculture at pH 4.5–5.5, whereas freely dispersed small pellets were formed in the STR using other precultures (data not shown). Coalesced

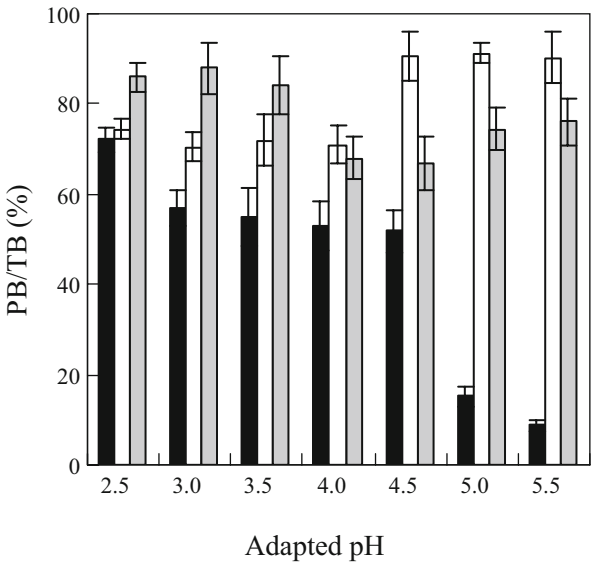

Fig. 3 Percentage of pellet biomass to total biomass (PB/TB) in the STR inoculated with the 1st (dark bar), 2nd (blank bar), and 3rd (gray bar) precultures

loose small pellets could suspend in the fermentation broth, whereas dispersed small pellets sediment subsided (Fig. 2).

Kinetics of Lactic Acid Production in the STR

Figure 4 shows the representative kinetic profiles of lactic acid production and starch consumption in the STR, with the acid-adapted precultures at pH 2.5–5.5. Obviously, lactic acid production rate and starch consumption with the 1st preculture at pH 5.5 were the lowest because of the formation of a large amount of mycelia clumps in the reactor (Fig. 3). Only 62.5 g/L lactic acid was produced after 72 h cultivation with the 1st preculture at pH 5.5 (Fig. 4c). In contrast, with the formation of more pellets in the reactor inoculated by the 1st preculture at pH 4.0 and 2.5 (Fig. 3), the lactic acid concentration reached 72.2 g/L and 81.5 g/L at 54 h, respectively. The use of the 2nd and 3rd precultures at pH 2.5, 4.0, and 5.5 resulted in a PB/TB more than 65% and lactic acid concentrations up to 81 g/L at 54 h (Figs. 3 and 4). However, the fermentation time with the 3rd precultures at pH 2.5 and 4.0 was prolonged to 60 h (Fig. 4a, b). This may be caused by the formation of more or less compact pellets in reactors (data not shown).

Figure 5 shows profiles of dissolved oxygen (DO) level measured in the fermentation broth in the STR, with the precultures adapted at pH 2.5, 4.0, and 5.5. The DO level in the fermentations dominated by pellets, especially by coalesced loose small pellets was low (Fig. 5). The lowest DO level of 18% was found in the cultivation with the 2nd preculture at pH 5.5 (Fig. 5c), corresponding to 90% pellets formed (Fig. 3). In contrast, the DO level was above 90% in the cultivations inoculated with the 1st preculture at pH 5.5 (Fig. 5c). It was observed that a high DO level was associated with the formation of large mycelial clumps (Fig. 2b1). The DO level in the STR using the 1st preculture at pH 2.5 was only 30–40% after 12 h cultivation (Fig. 5a), corresponding to 72% pellets of total biomass (Fig. 3). The lowest DO level in the fermentations using the 2nd or 3rd precultures at other pH varied between 60% and 70% (Fig. 5), lower than that with the 1st preculture at pH 5.5.

Summary of Production of Lactic Acid and By-Products in the STR

Figure 6 shows the maximum concentrations and productivities of lactic acid in the STR with different precultures. It can be seen that the production of lactic acid was affected significantly by the precultures and their adaptation pH. More than 80 g/L lactic acid was produced in the fermentations inoculated with the 1st precultures at pH 2.5, the 2nd and 3rd precultures at pH 2.5–5.5. With the 1st precultures, lactic acid concentration increased from 62.5 to 81.5 g/L as the adapted pH of the precultures decreased from 5.5 to 2.5 (Fig. 6a). The corresponding productivity increased from 0.9 to 1.5 g/L/h (Fig. 6b); 84.9–85.7 g/L lactic acid was produced in the STR using the 2nd precultures at pH 4.5–5.5, with the highest lactic acid concentration of 85.7 g/L obtained in the fermentation using the 2nd precultures at pH 5.0. There was only a slight decrease in lactic acid concentration with the 3rd precultures compared to that with the 2nd precultures (Fig. 6a). However, using the 3rd preculture, lactic acid productivity decreased from 1.6 to 1.3 g/L/h, with the increase of adaptation pH from 2.5 to 4.0 (Fig. 6b).

It was noted that the variation in fumaric acid concentration followed a similar profile to lactic acid concentration. A high concentration of fumaric acid was associated with a high lactic acid production (Figs. 6a and 7a). This may be because the synthesis of both lactic acid and fumaric acid takes place in an aerobic process and their production activities can be affected significantly by the oxygen transfer conditions. The highest concentration of

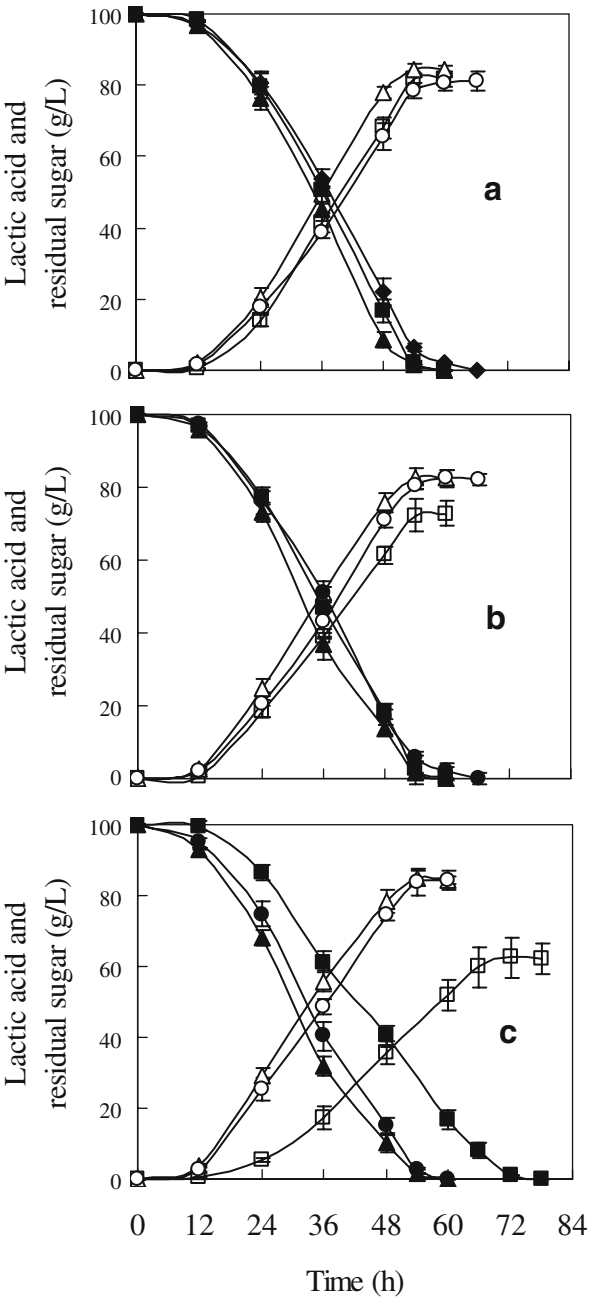

Fig. 4 Kinetic profiles of lactic acid production (open symbol) and starch consumption (close symbol) in the STR with the 1st (square), 2nd (triangle), and 3rd (circle) precultures at pH 2.5 (**a**), pH 4.0 (**b**), and pH 5.5 (**c**)

fumaric acid of 2.1 g/L to 2.3 g/L was obtained in the STR using the 2nd precultures at pH 4.5–5.5 (Fig. 7a).

The ethanol concentration varied from 16.4 to 2.6 g/L as the adaptation pH for the 1st precultures decreased from 5.5 to 2.5 (Fig. 7b). The ethanol concentration corresponded well to the variation in the morphology of *R. arrhizus* in the STR. A high concentration of

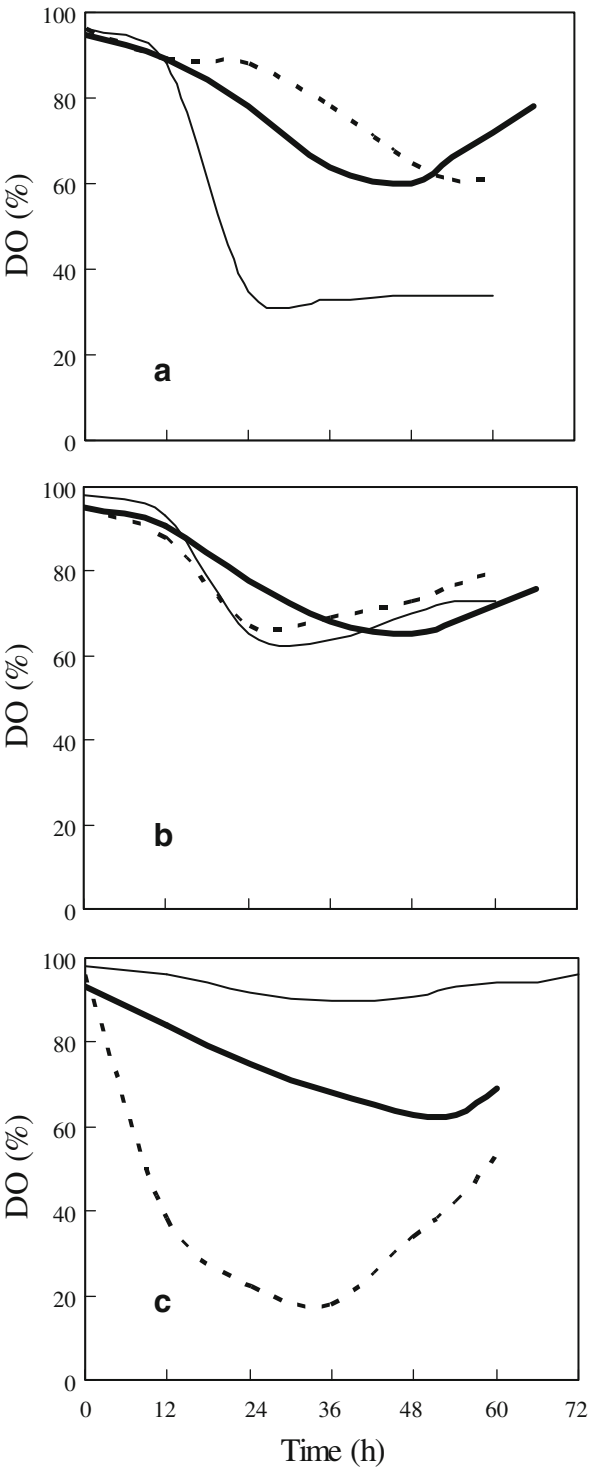

Fig. 5 DO profiles during the cultivations in the STR with the 1st (thin line), 2nd (dashed line), and 3rd (thick line) precultures adapted at pH 2.5 (**a**), pH 4.0 (**b**) and pH 5.5 (**c**)

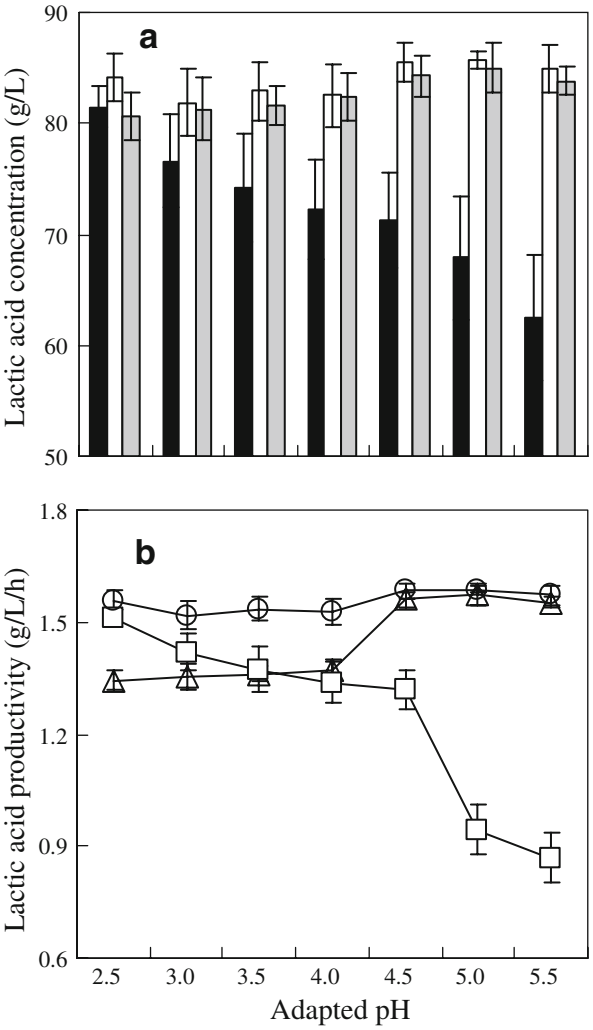

Fig. 6 Maximum lactic acid concentrations with the 1st (dark bar), 2nd (blank bar), and 3rd (gray bar) precultures (**a**), and productivities in the STR inoculated with the 1st (□), 2nd (○), and 3rd (Δ) acid-adapted precultures (**b**)

ethanol was produced in the fermentations in which less pellets but more clumps were produced. The ethanol concentrations were maintained at a low level between 1.7 to 2.6 g/L in the STR using the 2nd and 3rd precultures because of the high percentage of pellets formed (Fig. 3). Only 1.7–1.8 g/L ethanol was accumulated in the fermentations with the 2nd and 3rd precultures at pH 4.5–5.5.

Discussion

Morphology of filamentous fungi in submerged cultures is a determining factor for the rheology of the fermentation broth, and thereby for the bioreactor performance. Growth of filamentous fungi in pellet forms decreases the viscosity of the fermentation broth, and therefore enhances the mixing and mass transfer properties of the broth considerably. The morphology of *Rhizopus* fungi in the submerged fermentations varies from mycelial clumps

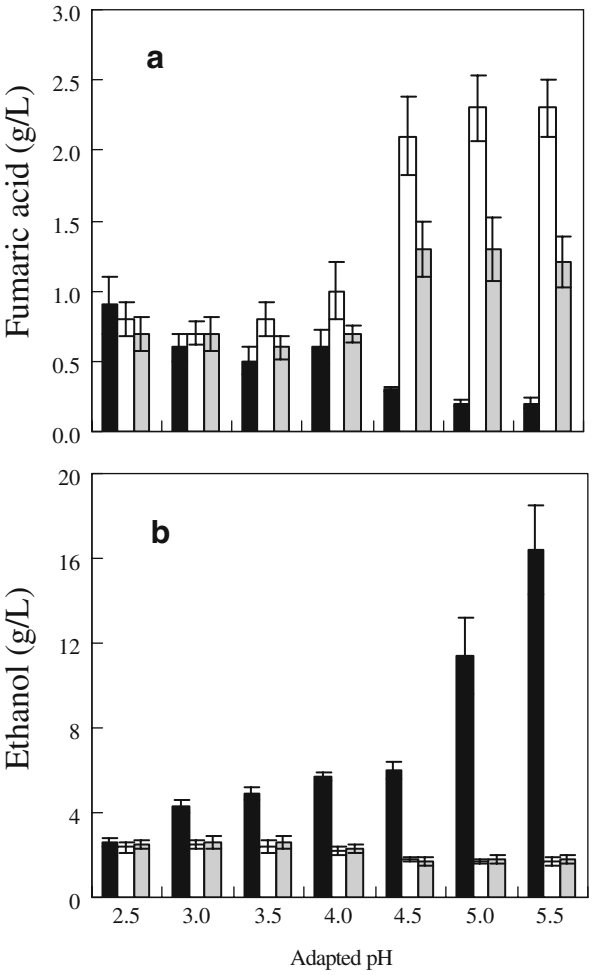

Fig. 7 Maximum concentrations of fumaric acid (**a**) and ethanol (**b**) in the STR inoculated with the 1st (dark bar), 2nd (blank bar), and 3rd (gray bar) precultures

to pellets, depending on the growth conditions. A desirable morphology of the fungal biomass can be maintained using the acid-adapted precultures to overcome the challenging engineering barriers in the submerged fermentation systems. A high lactic acid yield up to 86% was achieved in our study in a STR with the formation of small pellets in our study, which were induced by the inoculation of acid-adapted precultures.

It was stated that a high DO level was essential to achieve high productivity and yield of lactic acid using *Rhizopus* species [18]. However, in our study, the clumps-dominated fermentation demonstrated a very high DO level (over 90%) under given aeration rate, but produced only 62.5 g/L only lactic acid with a very high ethanol concentration (16.4 g/L), indicating that limitation of oxygen supply occurred inside the clumps. A low DO level (18%) was observed in the fermentations dominated by small pellets; however, more lactic acid (84.9 g/L) was produced with a small amount of ethanol (1.7 g/L). Therefore, it is important to note the DO level is a measure of DO in the fermentation broth, and this is likely not to be a reliable measure of oxygen availability inside the clumps. These results further prove that small pellets, especially the less dense small pellets, are favorable for mass/oxygen transfer.

The lactic acid yield of 86% in our study is comparable to those achieved in reactors with the assistance of immobilized *Rhizopus* cells in batch fermentations [16, 17]. This yield is also 10–45% higher than those reported, with suspended cells of *Rhizopus* in stirred tank reactors [22, 23, 25]. The lactic acid concentration is expected to be further improved by increasing waste potato starch concentration. The low productivity in our study may be caused by mechanical shear force and low DO level in the STR. The shear stress in STRs is generally recognized to be higher than ALRs and BCRs. High shear stress may inhibit mycelial growth at early cell growth phase and prolong the lactic acid production period and, therefore, result in a low productivity. Although the DO level in the fermentation broth may not be a very crucial factor for lactic acid production if the fungal biomass can be maintained in small pellet forms, a low DO level may hamper the cell growth rate, causing a low lactic acid productivity. Nevertheless, our results reveal that the use of the acid-adapted precultures is a very promising operation method to control the morphology of filamentous *R. arrhizus* DAR 36017 in small pellet forms in the STR. It is expected that both lactic acid concentration and productivity can be further enhanced via optimizing operational conditions in the STR.

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