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标题: An optimized fed-batch culture strategy integrated with a one-step fermentation improves l-lactic acid production by Rhizopus oryzae

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摘要: In previous work, we proposed a novel modified one-step fermentation fed-batch strategy to efficiently generate l-lactic acid (l-LA) using Rhizopus oryzae. In this study, to further enhance efficiency of l-LA production through one-step fermentation in fed-batch cultures, we systematically investigated the initial peptone- and glucose-feeding approaches, including different initial peptone and glucose concentrations and maintained residual glucose levels. Based on the results of this study, culturing R. oryzae with initial peptone and glucose concentrations of 3.0 and 50.0 g/l, respectively, using a fed-batch strategy is an effective approach of producing l-LA through one-step fermentation. Changing the residual glucose had no obvious effect on the generation of l-LA. We determined the maximum LA production and productivity to be 162 g/l and 6.23 g/(l center dot h), respectively, during the acid production stage. Compared to our previous work, there was almost no change in l-LA production or yield; however, the productivity of l-LA increased by 14.3%.

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ORIGINAL PAPER

An optimized fed-batch culture strategy integrated with a one-step fermentation improves L-lactic acid production by *Rhizopus oryzae*

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Abstract
In previous work, we proposed a novel modified one-step fermentation fed-batch strategy to efficiently generate L-lactic acid (L-LA) using *Rhizopus oryzae*. In this study, to further enhance efficiency of L-LA production through one-step fermentation in fed-batch cultures, we systematically investigated the initial peptone- and glucose-feeding approaches, including different initial peptone and glucose concentrations and maintained residual glucose levels. Based on the results of this study, culturing *R. oryzae* with initial peptone and glucose concentrations of 3.0 and 50.0 g/l, respectively, using a fed-batch strategy is an effective approach of producing L-LA through one-step fermentation. Changing the residual glucose had no obvious effect on the generation of L-LA. We determined the maximum LA production and productivity to be 162 g/l and 6.23 g/(l·h), respectively, during the acid production stage. Compared to our previous work, there was almost no change in L-LA production or yield; however, the productivity of L-LA increased by 14.3%.

Keywords Glucose-feeding approaches · L-Lactic acid · Peptone control · Pellet structure · *Rhizopus oryzae*

Introduction

Lactic acid (LA) and its salts are widely used in food and pharmaceutical industries. Use of LA in novel applications has increased and the rising demand for biodegradable plastics has made LA production an attractive investment (Eiteman and Ramalingam 2015; Zhang et al. 2016). *Rhizopus oryzae* is preferred for the production of LA, due to its exclusive formation of the L-isomer with a simple nutritional requirement and its ease of product recovery. Therefore, fermentation with *R. oryzae* to produce pure L-LA has attracted increasing interest in recent years (Yu et al. 2007; Taskin et al. 2012; Zhang et al. 2016).

Production of L-LA through fermentation by *R. oryzae* has been extensively studied in the past decades. For example, solid fermentation (Soccol et al. 1994), several floc morphology control method (Kosakai et al. 1997; Park et al. 1998; Yu et al. 2008), and immobilized cells (Efremenko et al. 2006; Tay and Yang 2002) have all been used for L-LA production by *R. oryzae*. However, the low production efficiency of L-LA prevents industrial mass-scale production (Coban and Demirci 2016; Liao et al. 2007a; Skory 2000). L-LA production by *R. oryzae* pellets would be very efficient and much more economical than currently used methods (Zhou et al. 1999; Liu et al. 2006; Liao et al. 2007b). However, when submerged pellet fermentation was tested, the overall amount and production efficiency of L-LA were low. In our previous work (Fu et al. 2016), we proposed a novel modified one-step fermentation strategy to improve the efficiency of L-LA production by *R. oryzae* pellets. This approach yielded up to158 g/l of L-LA and a peak productivity of 5.54 g/(l·h) in the acid production stage. Overall, we found that a fed-batch culture one-step fermentation process effectively enhance the production efficiency of L-LA.

Fed-batch culture is a batch culture fed continuously or sequentially with substrate without removing the fermentation broth, is typically superior to batch culture and continuous processing, and is especially beneficial when changing nutrient concentrations affects the productivity and biomass of the desired product (Lee et al. 1999; Roukas and Kotzekidou 1998; Ji et al. 2014). Ding and Tan (2006) developed a process for lactic acid production by *Lactobacillus casei* that used exponential feeding of glucose and yeast extract, where lactic acid production was remarkably improved. Compared to the traditional batch culture, the method used

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in this study improved the final L-LA yield and productivity by 56.5 and 59.7%, respectively. However, to our knowledge, few systematic reports have been published on using fed-batch fermentation for high concentrations and efficient *R. oryzae* production of L-LA. In our previous study (Fu et al. 2016), we did not systematically vary our fed-batch cultures when optimizing one-step fermentation.

In the present study, we optimized the fed-batch culture strategy for use in one-step fermentation to improve L-LA production efficiency. First, batch and fed-batch fermentation were compared to optimize the parameters of fed-batch culture and to identify ideal initial peptone, initial glucose, and sustained residual glucose concentrations. The one-step integrated fed-batch fermentation strategy was then modified to minimize the fermentation period and, thus, enhance L-LA productivity, in order to increase suitability for commercial production. To our knowledge, this is the first report describing the one-step integrated fed-batch fermentation strategy for production of L-LA by *R. oryzae*.

Materials and methods

Microorganisms and medium

R. oryzae LA-UN-1 from our laboratory, which can easily be formed into pellets, was used in this study (Yin et al. 2013). The fungus was grown on a potato dextrose agar (PDA) plate at 30 °C for 7 days. In all experiments, fungal spores were collected by shaving the PDA surface with a sterile loop and extracting spores with sterile water, which were then stored at 4 °C. The one-step fermentation medium recipe is as follows: glucose 20–220 g/l; peptone 1.0–5.0 g/l; KH_2PO_4 0.2 g/l; $MgSO_4{\cdot}7H_2O$ 0.2 g/l; The excess sterile $CaCO_3$ used (about 425 g of $CaCO_3$ total) in the culture medium and the value of pH (pH 6.0) were included in this work. All media were autoclaved at 121 °C for 20 min.

Culture conditions and methods

A culture was inoculated with *R. oryzae* spores at a final concentration of 10^7spores/ml and working volume of 5.0 l, and incubated in a modified 7.5-l fermenter (An interception device (like gauze) was added to the sample connection on the inside of the fermenter) as described previously (Fu et al. 2016). Glucose consumption, cell growth, and LA production displayed no change during the first 24 h. After 24 h, the fermentation volume was reduced by 50% (working volume of 2.5 l) to increase the cell density (The modified fermenter allowed for the sampling on the outside of the fermenter to reduce the volume of the cultivation medium, while retaining the original total biomass within the tank). The aeration rate, agitation speed, and culture temperature were set at 0.5 vvm, 300 rpm, and 30 °C, respectively. Calcium carbonate was used as the neutralizer.

As a control, we batch fermented *R. oryzae* for L-LA production using an initial glucose concentration of 220 g/l. During the course of the entire fermentation, no extra reagents were added, except excess calcium carbonate to maintain the pH at 6.0.

All fed-batch fermentations were initiated by creating batch cultures with different initial peptone (1.0, 3.0 and 5.0 g/l) or glucose concentrations (150, 100, 50, and 20 g/l). During fed-batch fermentation, a glucose solution of 600 g/l was fed into the fermenter when the residual glucose concentration decreased to 0–5.0 g/l. Excess calcium carbonate was added to maintain the pH at 6.0.

Analytical methods

Sugar and L-LA concentrations were analyzed by HPLC as previously described (Fu et al. 2014). The size and interior structure of pellet after culturing for 24 h were analyzed also using a previously described method (Fu et al. 2014). Biomass was determined by washing the mycelia pellets twice with 1M HCl to remove residual calcium carbonate, and the washed biomass was dried at 60 °C for 24 h before weight analysis.

Kinetic parameter calculations

The specific L-LA formation rate (q_p, h^{-1}) was estimated using the method described by Fu et al. (2016). We fitted data by interposing between our experimental L-LA production data at a definite time ($dt = 0.1$ h) and cubic spline interpolation approximations using Origin software (Version 7.5, OriginLab Corp., Northampton, Massachusetts, USA).

Results

Effects of initial peptone concentration on L-LA production through one-step fermentation in fed-batch cultures

In our previous work, it was found that L-LA production by *R. oryzae* fed-batch cultures through one-step fermentation was regulated in response to nitrogen sources (Fu et al. 2016). Meanwhile, the peptone concentration had the greatest impact on pellet density and biomass in cell growth stage, which can ultimately affect the accumulation of lactic acid by *R. oryzae* (Fu et al. 2014). Therefore, the effects of different initial peptone concentrations ranging from 1.0 to 5.0 g/l on L-LA production by *R. oryzae* fed-batch cultures through one-step fermentation was investigated and the results are presented in Fig. 1 and Table 1. As displayed in Fig. 1a,

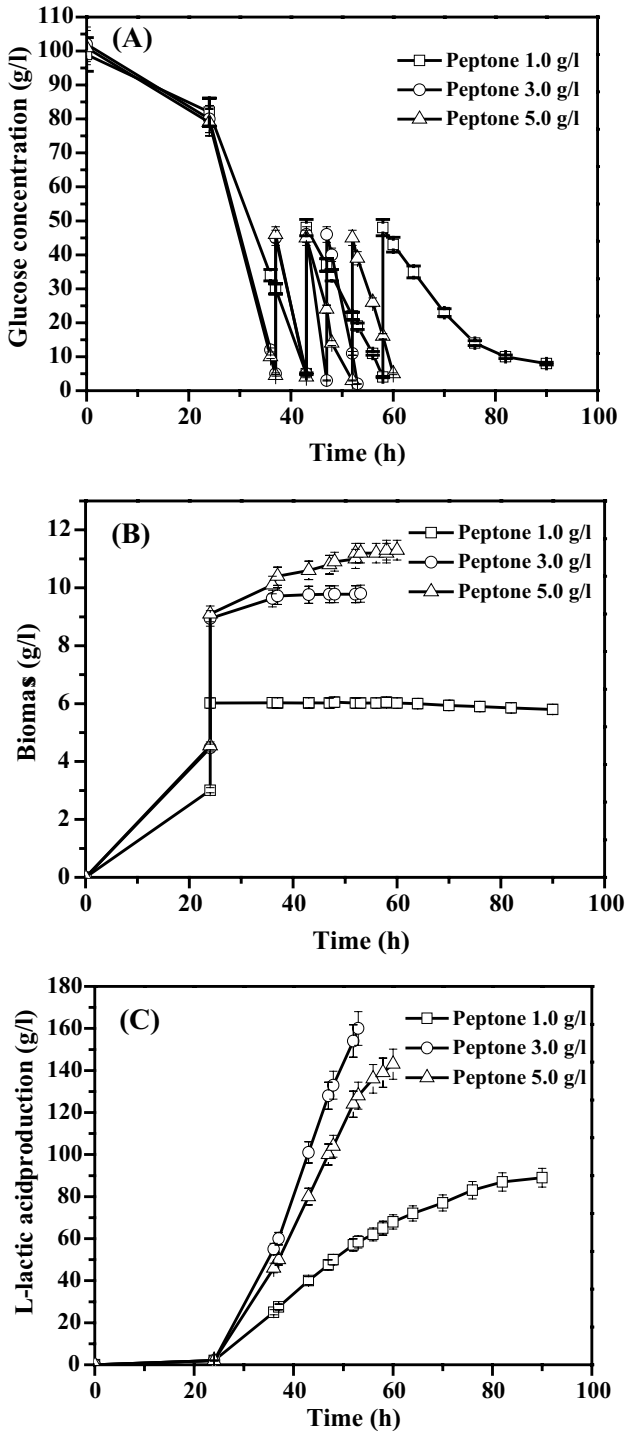

Fig. 1 Effects of initial peptone concentration on L-LA production by *R. oryzae* through one-step fermentation using the fed-batch culture strategy (initial glucose concentration was 100 g/l). **a** Glucose consumption, **b** biomass, and **c** L-LA production

glucose consumption by *R. oryzae* was relatively low when the initial peptone concentration was 1.0 g/l. The glucose consumption rate increased as the initial peptone concentration increased until it peaked at 3.0 g/l of peptone. When the peptone concentration was increased to levels higher than 3.0 g/l, the rates of glucose consumption decreased. Meanwhile, as shown in Fig. 1b, the biomass after 24 h was small, only 6.02 g/l, when the initial peptone concentration was 1.0 g/l. The biomass increased as the initial peptone concentration increased, where it reached 8.94 and 9.1 g/l for initial peptone concentrations of 3.0 and 5.0 g/l, respectively, after culturing for 24 h, similar to results seen in our previous work (Fu et al. 2014). Additionally, there was a slight decrease in biomass during the acid production stage when the initial peptone concentration was low (1.0 g/l) and a slight increase as the peptone concentration increased (9.8 and 11.3 g/l biomass for initial peptone concentrations of 3.0 and 5.0 g/l, respectively). The production (Fig. 1c), productivity, and yield (Table 1) during the lactic acid production stage increased as the peptone concentration increased and peaked at an initial peptone concentration of 3.0 g/l at 158 g/l, 5.45 g/(l·h), and 0.79 g/g, respectively. These parameters then decreased with values of 143 g/l, 3.92 g/(l·h), and 0.71 g/g, respectively, at an initial peptone concentration of 5.0 g/l.

Meanwhile, the initial nitrogen concentrations effected the pellet structure (Fig. 2). As displayed in Fig. 2a, when the initial peptone concentration was lower (1.0 g/l), a hollow structure was formed with a thin condensed layer (red and green) around the outside of the pellet, while the center was either composed of extraordinarily loose biomass or was hollow. As expected, there was a significant decrease in production when a hollow structure was generated, mainly due to the heterogeneous distribution of biomass and the overabundance of inactive biomass inside the pellet. As the initial peptone concentration increased to 3.0 g/l, a core shell structure was obtained. As shown in Fig. 2b, almost homogeneous compact material (blue and green) was distributed throughout the thick outer layer of the pellet and the center contained loosely packed material. The highest production of L-LA was observed when cultures contained this structure. When the initial peptone concentration reached 5.0 g/l, a very dense aggregated structure (Fig. 2c) was formed with dense blue and green layer in the center and a thin loose red and pink layer around the edge. It was probably because that the mass transfer (data not shown) was limited in homogeneously structured, dense pellets, thus causing a slower overall rate of turnover.

Comparison of batch and fed-batch culture L-LA production through one-step fermentation

Batch cultures have been commonly used for industrial lactic acid production, where initial glucose concentrations have been as high as 200 g/l (Kadam et al. 2006; Gao et al. 2012). However, osmotic pressure caused by high initial glucose concentrations can negatively affect lactic acid production.

Table 1 Comparison of the parameters of L-LA produced during one-step fermentation by *R. oryzae* using different culture strategies

Culture strategy	Biomass after 24 h (g/l)	Acid production stage (after 24 h)				
		Acid production time (h)	Final biomass concentration (g/l)	L-LA production (g/l)	Productivity (g/(l h))	Yield (g L-LA/g glucose)
Batch	6.85	40	7.54	101	2.52	0.51
Fed-batch with different initial peptone concentrations (g/l)						
1.0	6.02	66	5.8	87	1.32	0.56
3.0	8.94	29	9.8	158	5.45	0.79
5.0	9.1	36	11.3	141	3.92	0.71
Fed-batch with different initial glucose concentrations (g/l)						
150	8.24	36	8.98	139	3.86	0.69
100	8.94	29	9.80	158	5.45	0.79
50	9.01	26	9.80	160	6.15	0.82
20	9.02	40	9.90	147	3.68	0.73
Fed-batch with 50 g/l initial glucose concentration with residual glucose maintained at different concentrations (g/l)						
0–60	9.01	26	9.80	160	6.15	0.82
0–30	9.03	26	9.90	162	6.23	0.83
30–60	9.0	26	9.93	159	6.12	0.81

In our previous study (Fu et al. 2016), the total glucose concentration reached 220 g/l when using a novel modified one-step fermentation strategy. Meanwhile, the biomass was high. Therefore, in this present study, batch cultures with an initial glucose concentration of 220 g/l were compared to fed-batch cultures with different initial glucose concentrations of 150, 100, 50, and 20 g/l (The initial peptone concentration was 3.0 g/l). Similar to the results of our previous study (Fu et al. 2016), L-LA production by the batch and fed-batch cultures occurred in two distinct stages (Fig. 3a–e; Table 1). When comparing batch to fed-batch cultures, L-LA production, productivity, and *R. oryzae* biomass were all higher in the fed-batch cultures than the batch cultures, where the batch cultures reached values of only 101 g/l, 2.52 g/(l·h), and 6.85 g/l, respectively, during the acid production stage. This could be a result of the high osmotic pressure on the cells in the batch culture. Above a critical substrate concentration, the reduced water activity combined with plasmolysis causes a decrease in the rate of fermentation and sugar utilization (Ding and Tan 2006).

In fed-batch culture, L-LA production, productivity, and yield increased as the initial glucose concentration decreased (Table 1). The maximum L-LA production, productivity, and yield of 160 g/l, 6.15 g/(l·h), and 0.82 g/g, respectively, occurred when fed-batch cultures were initiated with 50 g/l of glucose. This is a 1.57-fold increase in L-LA production, a 2.44-fold increase in productivity, and a 1.68-fold increase in yield compared to the batch culture. The productivity was also higher compared to the productivity of 5.45 g/(l·h) observed when using the fermentation approach described in our previous study (Fu et al. 2016). Meanwhile, we noted an interesting and unexpected phenomenon when 20 g/l of glucose was used as the starting glucose concentration. Initially, this culture had higher production and productivity of L-LA compared to all the other culture conditions. However, after 37 h of fermentation (started from the inoculation moment), the glucose consumption and L-LA production decreased and became lower than in fed-batch cultures in all other conditions.

The biomass and pellet characteristics could also affect L-LA production efficiency. The pellets with the smallest biomass (6.85 g/l) and a highly dense aggregated structure (Fig. 4) formed in batch cultures after 24 h. These structures caused a decline in L-LA production. After 24 h, as the initial glucose concentration decreased, the biomass increased. Meanwhile, the interior pellet structure gradually shifted from highly dense and aggregated to fully dispersed, where the highly dense aggregated structure occurred at a glucose concentration of 150 g/l, the core shell structure at 100 g/l, and the fully dispersed structure at 50 g/l. Presumably, these pellets were more capable of withstanding secretion of L-LA. At the initial glucose concentration of 20 g/l, the pellets were very light and fluffy with a degree of dispersion that was higher than at 50 g/l and tended to form filaments. This morphology could probably be well-suited for oxygen and mass transfer before 37 h of culturing. However, as fermentation progresses, this would increase the viscosity of the culture broth, leading to an increase in resistance to mass transfer in the fermenter, which would further lower glucose consumption and L-LA production.

To further characterize the kinetics of our different one-step fermentation strategies, the specific rate of L-LA

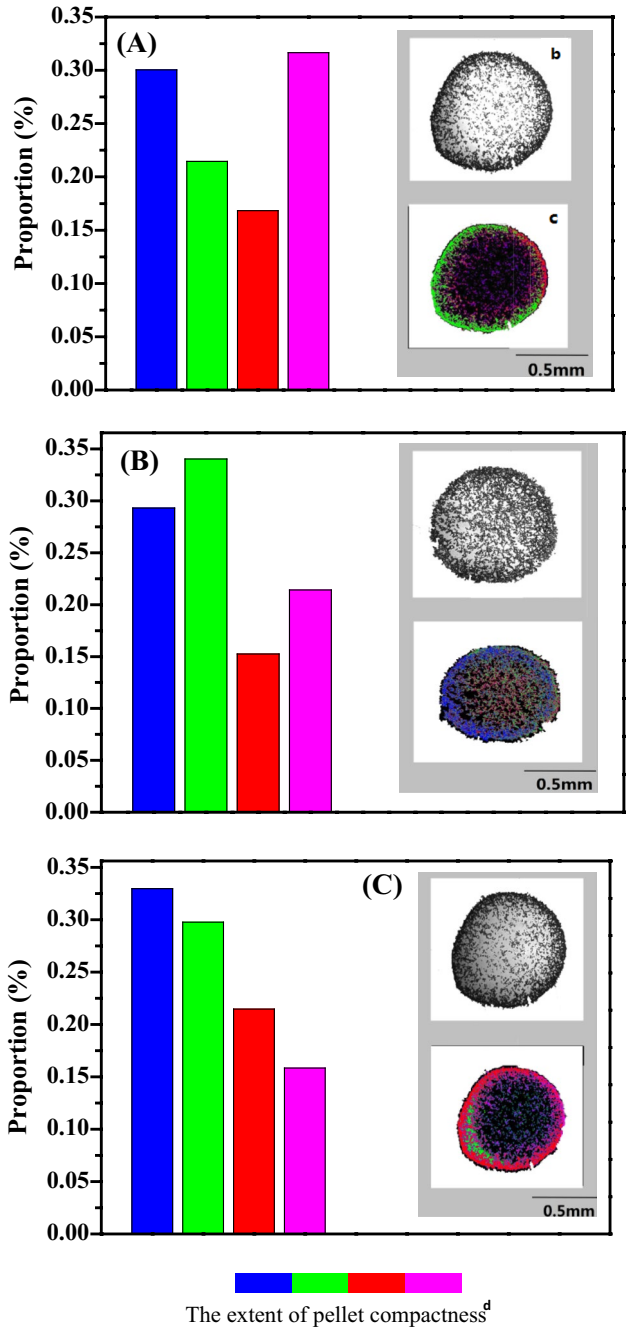

Fig. 2 The interior structure of *R. oryzae* pellets after culturing with different initial peptone concentration (Since the software could not calculate the ratio of hollow pellets, the ratio of hollow pellets is not given in the figure, and the black part of the internal structure of the pellets represent hollow portions) of **a** 1.0, **b** 3.0, **c** 5.0 g/l. *b* Suspended pellets were imaged using a CCD camera with an exposure time of 40 ms. Using digital image analysis (Image J 3.0), the pellet size was determined by measuring the cross-sectional area. *c* Using image analysis software from the Institute of Plasma Physics Chinese Academy of Sciences, an adaptive binary mask was computed, and the binary image was automatically segmented into four zones based on gray-level segmentation. *d* Compactness degree: blue was the most compact and pink was the loosest

formation (*qp*) was calculated with the data presented in Fig. 3a–e using an interpolation method. The results are shown in Fig. 3f. As seen in Fig. 3f, the *qp* values of the fed-batch cultures were higher than the batch culture after 24 h. The *qp* increased as the initial glucose concentration decreased. After 37 h of fermentation (started from the inoculation moment), the *qp* of the cultures with 20 g/l initial glucose peaked and began to decrease. Meanwhile, the *qp* of the other cultures continued to increase. After 43 h (started from the inoculation moment), the *qp* of the other cultures peaked and exceeded the *qp* of the culture with 20 g/l glucose initially. The *qp* of the fed-batch culture with a 50 g/l initial glucose concentration was typically higher than all other conditions.

Effects of residual glucose control on L-LA production

The fed-batch cultures were fermented with an initial glucose concentration of 50 g/l and the residual glucose concentration were maintained at 0–60, 0–30, and 30–60 g/l. Table 1 shows that varying the controlled residual glucose levels had a negligible effect on L-LA production when the initial glucose concentration was kept constant.

Discussion

A substantial number of studies had systematically investigated the use of fed-batch fermentation for high concentration and efficient production of L-LA by lactic acid bacteria. In these studies, different nutrient contents, such as nitrogen and carbon sources, were explored for the optimal feeding strategies (Bai et al. 2012; Ding and Tan 2006; Gao et al. 2012). For example, Bai et al. (2012) developed a process for producing ammonium lactate with *Lactobacillus lactis* using pH-control fed-batch fermentation. The ammonium lactate production was remarkably improved by the continuous supply of glucose. Ding and Tan (2006) developed a process for lactic acid production by *Lactobacillus casei* that employed the exponential feeding of glucose and yeast extract. Meanwhile, various studies on the necessary nutrients for LA production have reported that higher concentration of LA could be obtained with increased supplement of nitrogen sources (Jin et al. 2003; Yin et al. 1997). However, L-LA production by *R. oryzae* in fed-batch culture with one-step fermentation is subjected to its own nitrogen source control mode. In this work, too low (1.0 g/l) or too high (5.0 g/l) initial peptone concentration in culture did not benefit L-LA production by *R. oryzae* pellets. In fact, these concentrations of initial peptone resulted in more hollow or denser pellet structures that were not conducive to the transfer of oxygen and mass, which ultimately led to a decline in L-LA production.

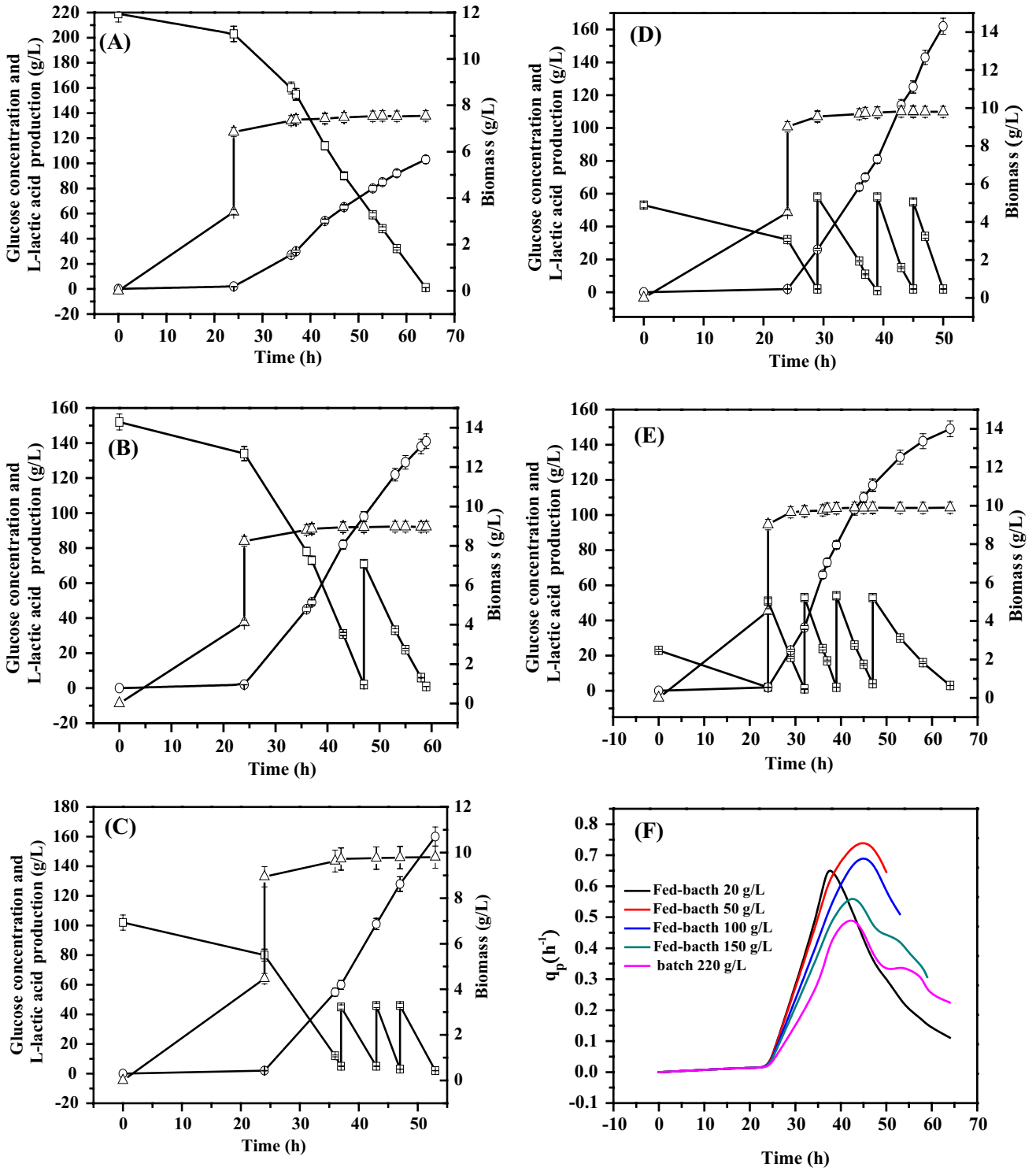

Fig. 3 Time course of L-LA production through one-step fermentation by *R. oryzae* in batch and fed-batch cultures. **a** Batch culture with 220 g/l initial glucose; **b** Fed-batch culture: Initial glucose concentration of 150 g/l, with 70 g/l of supplemental glucose fed in to the fermenter after 47 h of fermentation, resulting in a total glucose concentration of 219 g/l; **c** Fed-batch culture [used in our previous work (Fu et al. 2016)]: Initial glucose concentration of 100 g/l, with solutions of 45 g/l glucose fed in to the fermenter at 37, 43, and 47 h, resulting in a total glucose concentration of 224 g/l; **d** Fed-batch culture: Initial glucose concentration of 50 g/l, with two solutions of 60 g/l glucose added into the fermenter at 29 and 39 h, and a solution of 55 g/l glucose added at 45 h, resulting in a total glucose concentration of 217 g/l; **e** Fed-batch culture: Initial glucose concentration 20 g/l, with 50 g/l glucose added to the fermenter at 24, 32, 39 and 47 h, resulting in a total glucose concentration of 218 g/l; **f** Comparison of specific L-LA formation rate (q_p). (□) Residual glucose concentration; (○) Biomass; (△) L-LA production

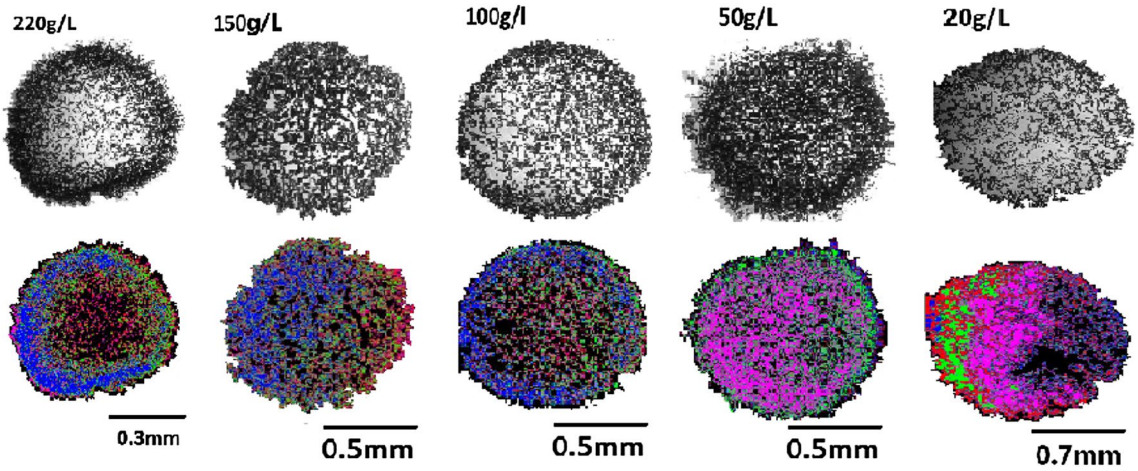

Fig. 4 The interior structure of the *R. oryzae* pellet after culturing with different initial glucose concentrations (initial peptone concentration was 3.0 g/l)

Meanwhile, at the optimal initial peptone concentration (3.0 g/l), the fermentation time was relatively short (only 29 h for the acid production stage), which suggested that the nitrogen source feeding culture was not suitable for this work (data not shown).

The effect of initial glucose concentration on fed-batch culture for L-LA production was similar to those observed in most studies (Ding and Tan 2006; Kotzamanidis et al. 2002). The high osmotic pressure caused by the initial carbohydrate concentration led to lower productivity of the fed-batch and batch culture. Feeding low level of initial glucose during fermentation greatly improved the efficiency of the process by preventing the inhibitory effects of glucose on L-LA production. Interestingly, we found that initial glucose concentration below < 20 g/l was not efficient for the final production of L-LA. Additionally, initial glucose concentration dictated the morphology of *R. oryzae* pellets, which ultimately influenced the production of L-LA. On the other hand, residual glucose concentration exhibited negligible effect on the final production of L-LA. The insights gained from this work is useful for optimizing large-scale fermentation by *R. oryzae*.

Conclusions

In the present study, to enhance L-LA production efficiency, the effects of different initial peptone concentrations and glucose-feeding approaches (including different initial glucose concentrations and maintained residual glucose levels) on fed-batch cultures undergoing one-step fermentation were systematically investigated. It was demonstrated that using an one-step fermentation integrated fed-batch culture strategy with initial peptone and glucose concentrations of 3.0 and 50 g/l, respectively, significantly shortened the fermentation period and enhanced productivity. These findings have the potential to greatly impact large-scale L-LA production.

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