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World Journal of Microbiology and Biotechnology (2018) 34:74
https://doi.org/10.1007/s11274-018-2455-2

Published online: 21 May 2018

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. zae* 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. zae*.

Materials and methods

Microorganisms and medium

R. zae 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. zae* 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. zae* 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.

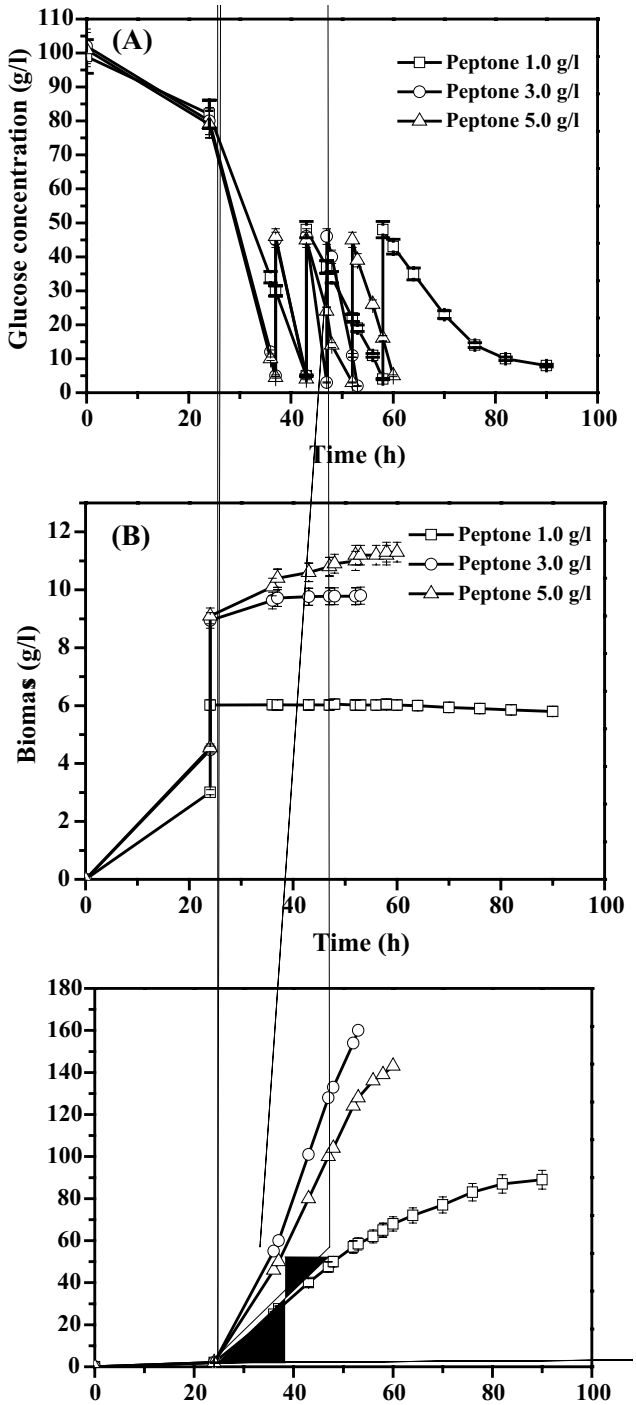
Kinetic parameter calculations

The specific L-LA formation rate (, 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 ($d_{-}=0.1$ 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. zae* 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. zae* (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. zae* 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,



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.

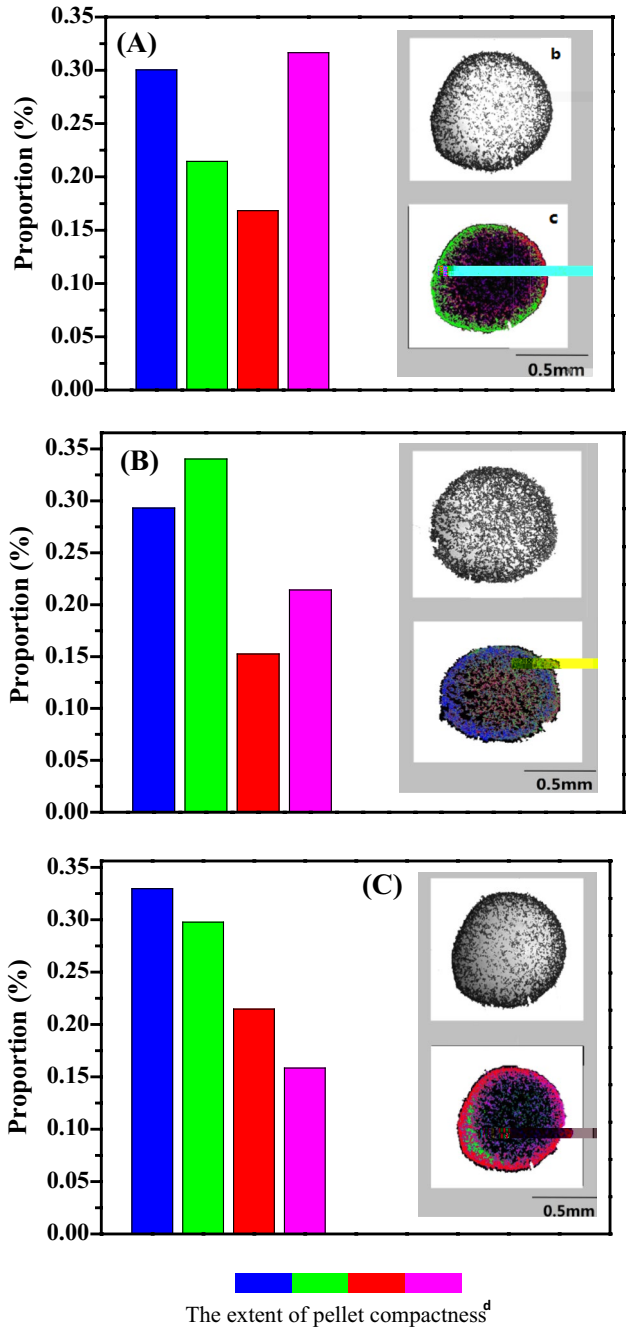
Meanwhile, the initial nitrogen concentrations effected the pellet structure (Fig. 2). As displayed in Fig. 2a, when the initial peptone concentration was lower (1.0 g/l), a hollow structure was formed with a thin condensed layer (red and green) around the outside of the pellet, while the center was 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.

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.* *zae* 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



The interior structure of *R.* *zae* 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

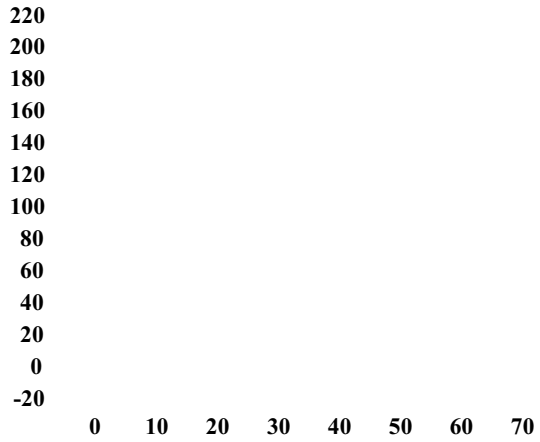
formation ( ) was calculated with the data presented in Fig. 3a–e using an interpolation method. The results are shown in Fig. 3f. As seen in Fig. 3f, the values of the fed-batch cultures were higher than the batch culture after 24 h. The increased as the initial glucose concentration decreased. After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

E□ects 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 *Lacto bacillus 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 *Lacto bacillus 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.* *zae* 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.* *zae* 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.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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.* *zae* 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.* *zae*.

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.

This work was financially supported by the National Natural Science Foundation of China (Grant No. 21106091).

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