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High-efficiency L-lactic acid production by *Rhizopus oryzae* using a novel modified one-step fermentation strategy

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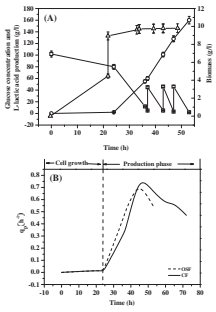
HIGHLIGHTS

- Modified one-step fermentation strategy for L-lactic acid production was developed.
- The higher cell density greatly increased L-lactic acid production efficiency.
- The L-lactic acid production and productivity reached 158 g/l and 5.45 g/(l h).
- This strategy is a convenient and economical method for L-lactic acid fermentation.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, lactic acid fermentation by *Rhizopus oryzae* was investigated using the two different fermentation strategies of one-step fermentation (OSF) and conventional fermentation (CF). Compared to CF, OSF reduced the demurrage of the production process and increased the production of lactic acid. However, the *qp* was significantly lower than during CF. Based on analysis of μ, q_s and q_p, a novel modified OSF strategy was proposed. This strategy aimed to achieve a high final concentration of lactic acid, and a high *qp* by *R. oryzae*. In this strategy, the maximum lactic acid concentration and productivity of the lactic acid production stage reached 158 g/l and 5.45 g/(l h), which were 177% and 366% higher, respectively, than the best results from CF. Importantly, the q_p and yield did not decrease. This strategy is a convenient and economical method for L-lactic acid fermentation by *R. oryzae*.

1. Introduction

Lactic acid and its derivatives are widely used in food, pharmaceutical, leather, and textile industries (Eiteman and Ramalingam, 2015). In addition, there has been an increased use of lactic acid in novel applications and biodegradable plastics have made lactic acid production an attractive investment (Zhang et al., 2016). Currently, industrial lactic acid fermentation is primarily carried out using lactic acid bacteria (Ding and Tan, 2006). However, unlike lactic acid bacteria, production of L-lactic acid by the fungus *Rhizopus oryzae* exclusively generates the L-isomer, has simple nutritional requirements and allows for easy product recovery, and, thus, is a preferential method over the use of bacteria (Zhang et al., 2007a). Therefore, fermentation using this fungus to produce pure L-lactic acid has been attracting increased interest in recent years (Bai et al., 2008; Coban and Demirci, 2016; Liao et al., 2007a; Zhou et al., 1999).

The generation and secretion of lactic acid by *R. oryzae* occurs under aerobic conditions in a high-glucose medium containing a limiting amount of nitrogen (Fu et al., 2014; Papagianni, 2004). In order to develop a cost-effective process for lactic acid production by *R. oryzae*, research has mainly focused on improvement of the fungal strain, control of morphology, and the utilization of

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cheap feedstock and novel bioreactors (Maas et al., 2008; Zhang et al., 2007a; Kosakai et al., 1997; Miura et al., 2003). However, in most studies on lactic acid fermentation, only 0.7–2.5 g/l/h of lactic acid with a total production of 60–120 g/l are produced by *R. oryzae*, which is a lower amount than generated by lactic acid bacteria (Yu et al., 2007; Fu et al., 2014; Park et al., 1998). A lot of effort has been devoted to using mycelial pellets or immobilized cells for lactic acid production because of their satisfactory levels of productivity (Efremenko et al., 2006; Bai et al., 2003). Efremenko et al. (2006) reported that PVA-cryogel-immobilized cells were the most productive lactic acid generating form of *R. oryzae*. However, this shake-flask technique is not suitable for large-scale production of lactic acid (Yu et al., 2007; Liao et al., 2007a).

Therefore, building a simple, reliable and efficient approach to produce lactic acid by *R. oryzae* still remains a challenge. High cell-density fermentation can greatly enhance the production efficiency of metabolites (Riesenberg and Guthke, 1999). However, since high cell-density in organic acid fermentation can greatly reduce the production and yield of lactic acid, *R. oryzae* is rarely cultivated in this manner in batch/fed-batch fermentation processes (Fu et al., 2009a; Liao et al., 2007b; Yu et al., 2007). Büyükkileci et al. (2006) introduced a method of lactic acid production using *R. oryzae*, where the lactate production medium was inoculated directly with a spore suspension. The one-step fermentation (OSF) involved using preculture and L-lactic acid production before proceeding into this method. However, this method was not discussed in depth in their work. Meanwhile, an interesting and unexpected phenomenon noted in our study was that *R. oryzae* cell growth and L-lactic acid production can occur in a OSF, where the higher cell density in this process greatly increased L-lactic acid production efficiency. To the authors' knowledge, there have been few systematic reports on OSF using a high cell density for generation and secretion of lactic acid by *R. oryzae*. The aim of the present study was to develop a novel OSF strategy for the highly efficient generation of L-lactic acid by *R. oryzae*. Furthermore, L-lactic acid OSF was characterized, and the factors influencing L-lactic acid production, and the resulting productivity and biomass of the OSF strategy, including different nitrogen source, peptone concentration, cell density and the inclusion of a fed-batch fermentation step, were studied in this paper.

2. Materials and methods

2.1. Microorganisms and medium

R. oryzae LA-UN-1, which is easy to form the pellet morphology, from our laboratory 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 d. For the experiments, fungal spores were collected by shaving the PDA surface with a sterile loop and extracting spores with sterile water and then were stored at 4 °C. The conventional fermentation medium included preculture medium (g/l): glucose 20; peptone 2.0; KH_2PO_4 0.2; $MgSO_4{\cdot}7H_2O$ 0.2 and L-lactic acid fermentation medium (g/l): glucose 80; peptone 2.0; KH_2PO_4 0.2; $MgSO_4{\cdot}7H_2O$ 0.25; $ZnSO_4{\cdot}7H_2O$ 0.04; $CaCO_3$ 50. The one-step fermentation medium (g/l): glucose 100; nitrogen sources (peptone 2.0–4.0, urea 2.0, $(NH)_2SO_4$ 2.0, yeast extract 2.0); KH_2PO_4 0.2; $MgSO_4{\cdot}7H_2O$ 0.2; $CaCO_3$ 50.

2.2. Fermentation conditions and methods

Conventional fermentation (CF): A preculture was inoculated with *R. oryzae* spores at a final concentration of 10^7 spores/ml and volume of 50 ml, and then incubated in a 250-ml shake flask at 150 rpm at 30 °C for 24 h. The L-lactic acid fermentation was carried out in a 7.5-L fermenter (New Brunswick Scientific, USA) in a working volume of 5.0 L, where a 10% (v/v) of the preculture was used to inoculate the fermenter. 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.

One-step fermentations (OSF): A culture was inoculated with *R. oryzae* spores at a final concentration of 10^7 spores/ml and working volume of 5.0 L, and incubated in a modified 7.5-L fermenter. The modified fermenter with liquid filtration and collecting components added. The fermentation medium was filtered and collected in bottles and *R. oryzae* cells were retained in the tank through the component. The collected filtered fermentation medium can be reused. The aeration rate, agitation speed, culture temperature and neutralizer were the same as for the CF.

For certain strategies, a modified OSF with a fed-batch culture was applied in order to increase the concentration of lactic acid. The fermentations were set up at an initial glucose concentration of 100 g/l, and the feeding substrate was pumped into the fermenter using a computer coupled peristaltic pump. During the fed-batch fermentation, a glucose solution of 400 g/l was fed into the fermenter with different pulse feeding times to maintain a residual glucose concentration in the range of 0–40 g/l.

2.3. Analytical methods

Sugar consumption and L-lactic acid concentrations were analyzed by HPLC as reported previously (Fu et al., 2014). Biomass was determined by weighing the mycelial mass after drying at 60 °C overnight.

2.4. Kinetic parameters calculation

The specific cell growth rate (μ, h^{-1}), specific glucose consumption rate (q_s, h^{-1}) and specific L-lactic acid formation rate (q_p, h^{-1}) were estimated from experimental or fitted data of cell growth (x, g/l), residual glucose concentration (s, g/l), and L-lactic acid production (p, g/l) by Eqs. (1)–(3), respectively (Fu et al., 2009b). The fitted data were obtained by interposing between experimental data of cell growth, residual glucose concentration or L-lactic acid production at definite time (dt = 0.1 h) with the approximation method of cubic spline interpolation in Origin software (Version 7.5, OriginLab Corp., Northampton, Massachusetts, USA).

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{1}{x}\lim_{\Delta t\to 0}\frac{\Delta x}{\Delta t} \tag{1}$$

$$q_s = -\frac{1}{x}\frac{ds}{dt} = -\frac{1}{x}\lim_{\Delta t\to 0}\frac{\Delta s}{\Delta t} \tag{2}$$

$$q_p = \frac{1}{x}\frac{dp}{dt} = \frac{1}{x}\lim_{\Delta t\to 0}\frac{\Delta p}{\Delta t} \tag{3}$$

3. Results and discussion

3.1. Characteristics of CF and OSF

As shown in Fig. 1A, CF consisted of the two processes of proculture and lactic acid production. The preculture was used for *R. oryzae* spore germination and cell growth, and, at the end of this process, L-lactic acid became apparent. Then, a 10% (v/v) of the preculture was inoculated into the fermenter, which was used for L-lactic acid production. A lag occurred at the beginning of lactic acid production, which affected the overall levels generated. For example, the L-lactic acid production at 8 h was 0.69 g/(l h), which was only 58% of the average total levels produced by fermentation (1.19 g/(l h)). The productivity increased as the fermentation time

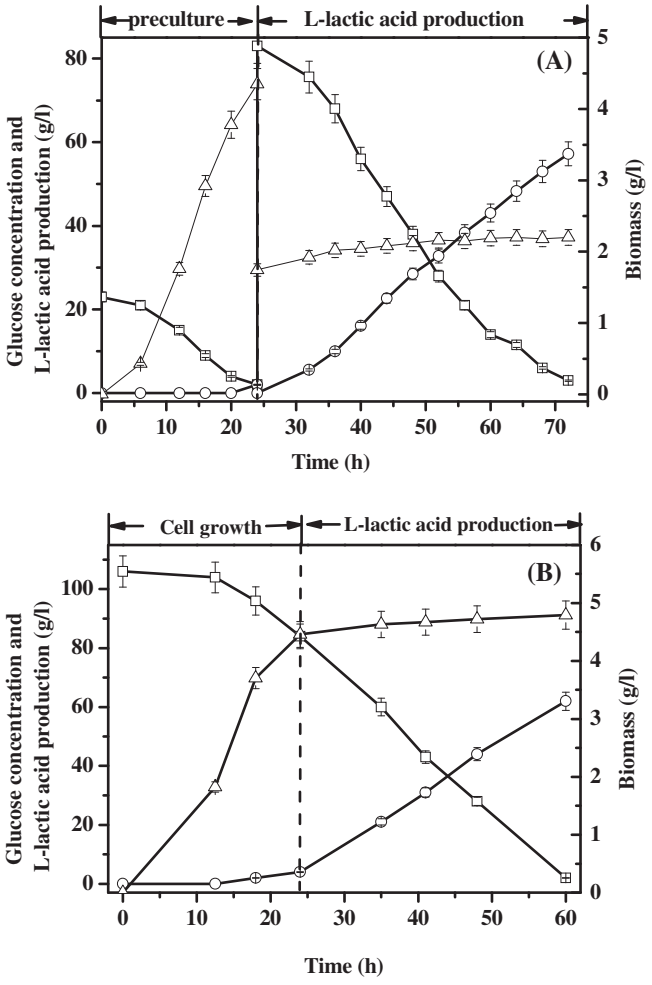

Fig. 1. Time course of L-lactic acid production using CF (A) and OSF (B) based of *R. oryzae*. □ Glucose consumption, ○ L-lactic acid production and △ Biomass.

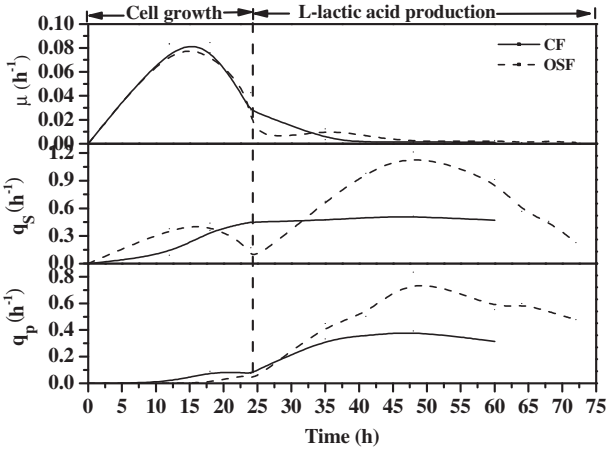

Fig. 2. Comparison of kinetic parameters of L-lactic acid fermentation by *R. oryzae* using CF (solid line) and OSF (dashed line): Specific cell growth rate (μ), specific glucose consumption rate (q_s), and specific L-lactic acid formation rate (q_p).

Table 2
Effect of nitrogen source on lactic acid production phase of OSF.*

Nitrogen source	Time (h)	L-Lactic acid production (g/l)	Productivity (g/(l h))	Yield (g/g)	Biomass (g/l)
Peptone	36	62	1.72	0.76	4.79
Urea	–	–	–	–	0.23
$(NH)_2SO_4$	72	31	0.43	0.44	3.21
Yeast extract	30	43	1.43	0.41	6.43

* Two distinct phases occurred in OSF with different nitrogen source: the cell growth (0–24 h) and L-lactic acid production (>24 h) phases.

increased. In order to decrease the lag time, the preculture and L-lactic acid production processes were used before proceeding into OSF. As shown in Fig. 1B, two distinct phases occurred in OSF: the cell growth (0–24 h) and L-lactic acid production (>24 h) phases. Depletion of glucose before 24 h was primarily due to cell growth, similar to the preculture before CF. There was almost no lag time after 24 h (For example, the L-lactic acid productivity following 8 h of fermentation reached 1.75 g/(l h), which was similar to the average productivity of the entire fermentation (1.72 g/(l h)), and the production of L-lactic acid increased rapidly, reaching the highest concentration of 62.0 g/l after 60 h. CF took 72 h to reach the same concentration. A comparison of the experimental results from the two types of lactic acid fermentation is displayed in Table 1. It was found that L-lactic acid accumulation occurred at a significantly faster rate in OSF than CF, where the productivity reached 1.72 g/(l h) in the OSF production phase, which was 45% higher than CF (1.19 g/(l h)). This is likely due to a lack of lag time before the acid production phase and a higher biomass in OSF compared to CF (4.79 and 2.18 g/l, respectively). At the same time, the L-lactic acid production and yield from OSF, which were 62.0 g/l and 0.76 g/g, respectively, were both higher than CF at 57 g/l and 0.71 g/g, respectively. Therefore, it can be concluded that using OSF at a high cell-density has the potential for efficient production of L-lactic acid.

3.2. Kinetic analysis of CF and OSF

In order to further characterize the kinetics of CF and OSF, three parameters were analyzed: specific rate of glucose consumption (qs), specific rate of cell growth (μ) and specific rate of L-lactic acid formation (qp). These parameters were calculated based on the data in Fig. 1A and B using an interpolation method and the results are shown in Fig. 2. As seen in Fig. 2, the qp and μ of both processes displayed similar changes during the first 24 h. However, the qs of the two were different. The qs during OSF increased and then reached its maximum value. By contrast, the qs during CF first increased and then decreased. After 24 h, although the μ differed between OSF and CF, this difference was not significant. By contrast, the qs and qp were significantly different between OSF and

Table 1
Comparison of the parameters of L-lactic acid production by *R. oryzae* using different fermentation processes.

Culture strategy	Stage	Time (h)	L-Lactic acid production (g/l)	Productivity (g/(l h))	Yield (g/g)	Biomass (g/l)
CF	Proculture	24	2.0	–	–	4.35
	Production	48	57.0	1.19	0.71	2.18
OSF	Cell growth	24	2.0	–	–	4.46
	Production	36	62.0	1.72	0.76	4.79

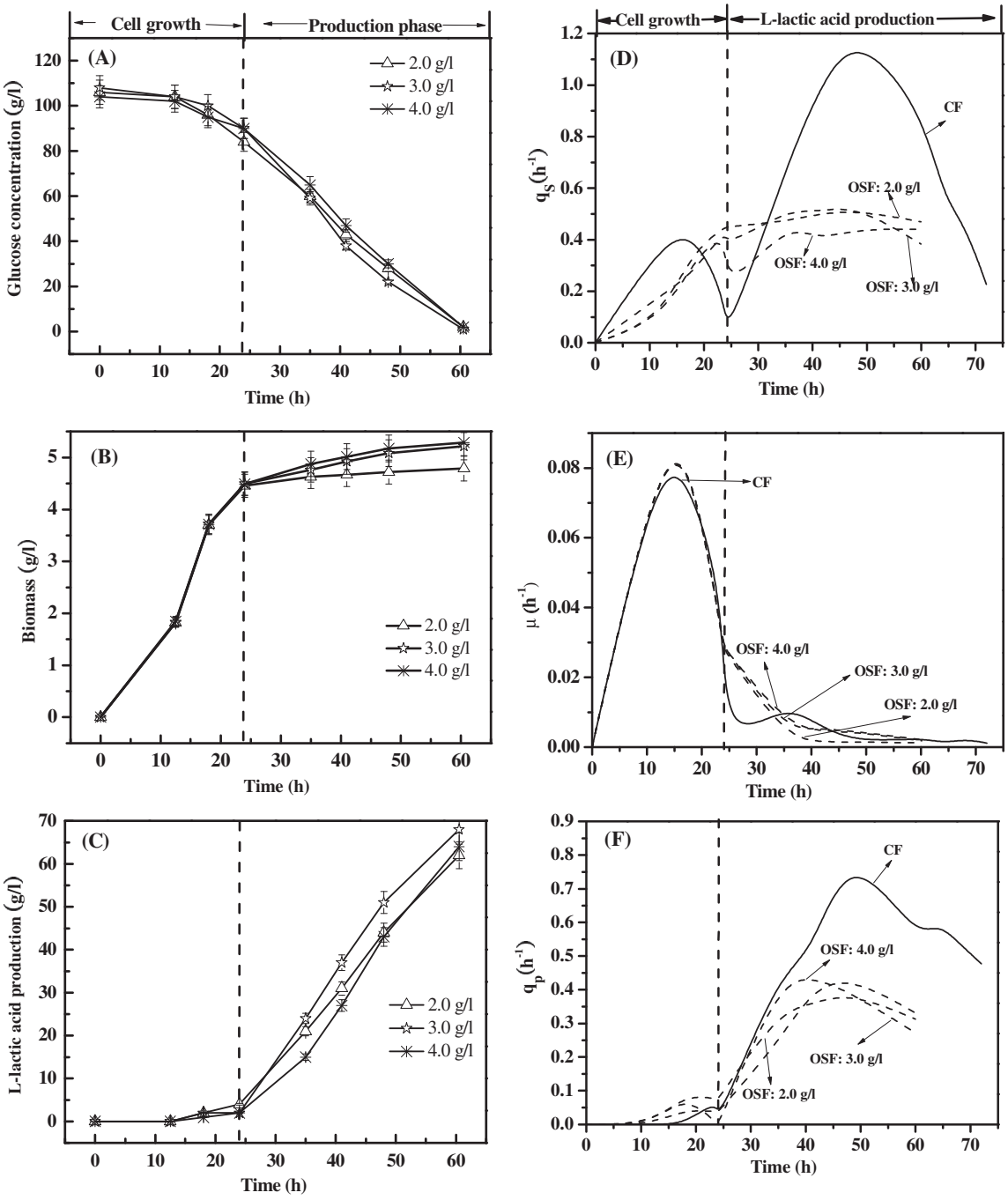

Fig. 3. Time course examining the effect of different initial peptone concentrations on L-lactic acid production by OSF by *R. oryzae* (A–C) and a comparison of the kinetic parameters of CF (solid line) and OSF (dashed line) (D–F). (A, D) Glucose consumption, (B, E) Biomass and (C, F) L-lactic acid production.

Table 3
Effect of peptone concentration on lactic acid production phase of OSF.

Peptone concentration (g/l)	Time (h)	L-Lactic acid production (g/l)	Productivity (g/(l h))	Yield (g/g)	Biomass (g/l)
2.0	36	62	1.72	0.76	4.79
3.0	36	68	1.88	0.76	5.22
4.0	36	64	1.78	0.73	5.28

CF. At the majority of the time points evaluated, the *qs* and *qp* of OSF were lower than CF. Meanwhile, the maximum *qs* and *qp* from OSF, 0.51 and 0.39 h^{-1},were only half that of CF, 1.2 and 0.84 h^{-1}, respectively.

Comprehensive analysis determined OSF not only had a reduced lag time, but also an increase in cell density, both of which further improved lactic acid production. Paradoxically, increased biomass can decrease the *qp*. Therefore, improving *qp* during OSF would effectively resolve the bottleneck problem in lactic acid fermentation by *R. oryzae*.

3.3. Different OSF strategy

In order to increase the *qp* during OSF, different OSF strategies were designed. To optimize the parameters for this, three experiments were performed using different nitrogen source, initial peptone concentrations and cell densities during OSF.

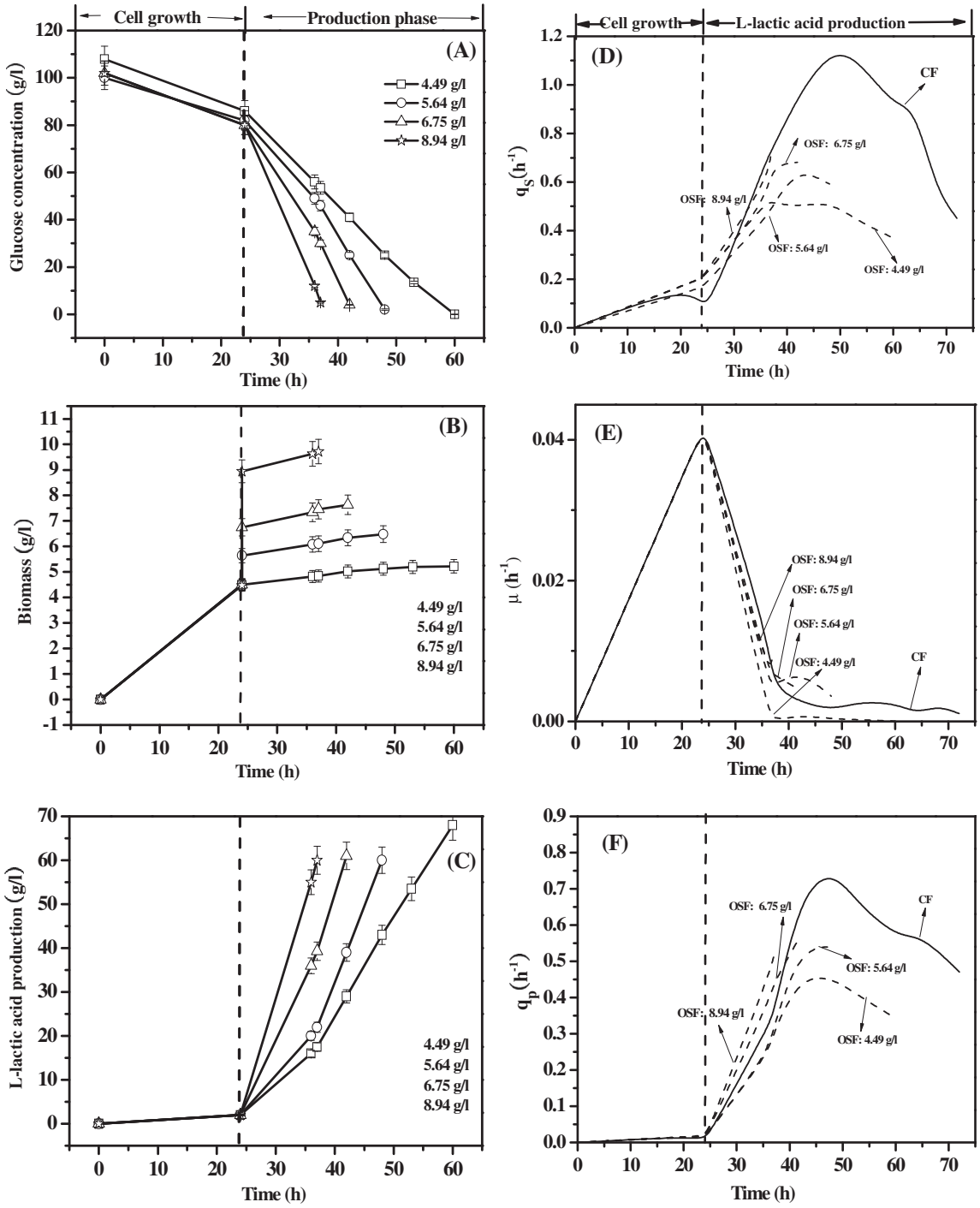

Fig. 4. Time course examining the effect of different cell densities after 24 h on L-lactic acid production by *R. oryzae* by OSF (A–C) and a comparison of the kinetic parameters of CF (solid line) and OSF (dashed line) (D–F). (A, D) Glucose consumption, (B, E) Biomass and (C, F) L-lactic acid production.

Table 4
Effect of cell density after 24 h of lactic acid production phase by OSF.

Cell density after 24 h (g/l)	Acid production Time (h)	Final biomass concentration (g/l)	L-Lactic acid production (g/l)	Productivity (g/(l h))	Yield (g/g)
4.49	36	5.22	68.0	1.88	0.76
5.64	24	6.48	60.0	2.5	0.75
6.75	18	7.63	61.0	3.39	0.8
8.94	13	9.72	60.0	4.62	0.8

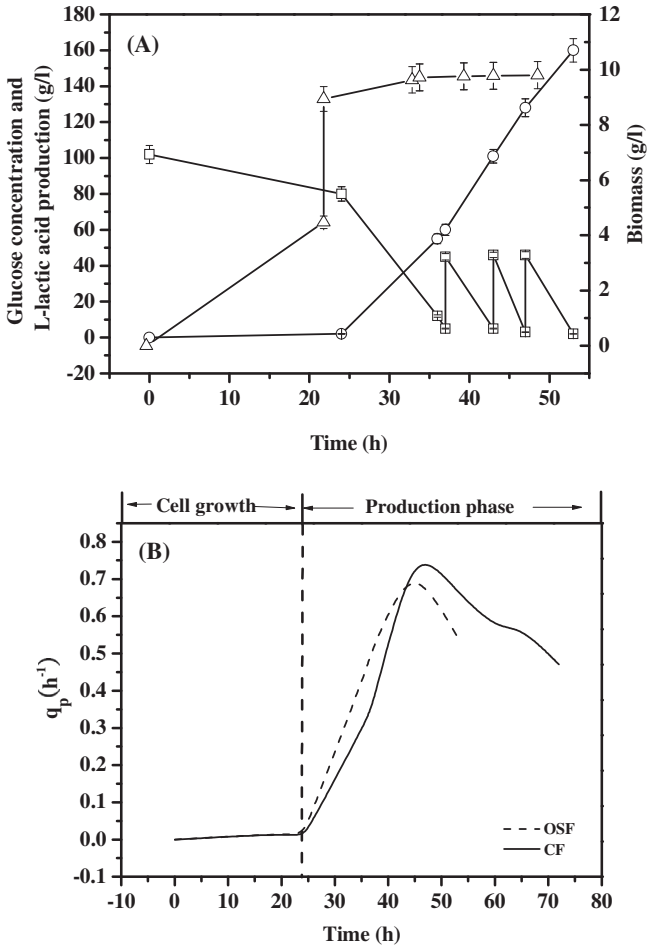

Fig. 5. Time course of modified one-step with fed-batch fermentation strategy (A) and comparison of specific L-lactic acid formation rate (q_p) during CF (solid line) and OSF (dashed line) (B). □ Glucose consumption, ○ L-lactic acid production and △ Biomass.

3.3.1. OSF using different nitrogen source

Nitrogen source affects the biomass and synthesis of a variety of enzymes, which will ultimately influence the amount of metabolites synthesized (Taskin et al., 2012; Zhang et al., 2007b). Therefore, the effect of different nitrogen source, including organic nitrogen: peptone, urea and yeast extract and inorganic nitrogen: $(NH)_2SO_4$ on OSF was investigated and the results are shown in Table 2. The initial concentration of all nitrogen sources was 2.0 g/l. As seen from Table 2, urea and $(NH)_2SO_4$ appeared to be less favorable nitrogen sources for increasing biomass and L-lactic acid production. These results are similar to those found in a study by Taskin et al. (2012). Yeast extract was found to be beneficial for cell growth, but it was not conducive for the production of lactic acid. Compared to other nitrogen sources, peptone is a relatively good nitrogen source for cell growth and lactic acid production by *R. oryzae* when using OSF.

3.3.2. OSF using different initial peptone concentrations

The effect of different initial peptone concentrations ranging from 2.0 g/l to 4.0 g/l on OSF was investigated and the results are shown in Fig. 3 and Table 3. As displayed in Fig. 3A–C, the glucose was primarily used for cell growth before 24 h as there was almost no lactic acid formation during this time. The L-lactic acid accumulated rapidly after 24 h and at 36 h reached the highest concentrations of 62, 68 and 64 g/l for the initial peptone concentrations of 2.0, 3.0 and 4.0 g/l after 60 h, respectively (Table 3). Additionally, there was almost no cell growth during the acid production phase when the peptone concentration was low, 2.0 g/l, with little increase as the peptone concentration was increased. However, the production, efficiency, and yield during the lactic acid production phase displayed no significant changes when the initial peptone concentration increased from 2.0 g/l to 4.0 g/l (Table 3). Furthermore, analysis of the kinetic parameters μ, *qs* and *qp* determined the utilization rate per unit biomass did not significantly change as the initial peptone concentrations were changed (Fig. 3D–F). Overall, while changing the peptone concentration may change the biomass, it failed to improve the *qp*.

3.3.3. OSF using different cell densities during the acid production phase

In this fermentation strategy, the initial peptone concentration was 3.0 g/l. The glucose consumption, cell growth and lactic acid production displayed no change during the first 24 h. Then the fermentation medium was reduced to 0%, 25%, 33% and 50% of the original amount, while retaining the original total biomass within the tank, to create the different cell densities. The reduced fermentation medium could then be reused (date not shown). The changes in cell density for OSF are presented in Fig. 4B. The highest cell-density achieved was 8.94 g/l during the acid production phase, which was almost 5 times higher that attained in our earlier work (Fu et al., 2014) and 10 times higher than that achieved by Liao et al. (2007a,b). From Fig. 4A and C, it can be seen that the sugar consumption and lactic acid productivity notably increased with increases in cell-density after 24 h. During the lactic acid production phase, the fermentation time decreased from 36 to 13 h and L-lactic acid productivity increased from 1.88 to 4.62 g/(l h) when the cell density was increased from 4.49 g/l to 8.94 g/l, respectively. Overall, this is a 64% decrease in fermentation time and 148% increase in productivity, respectively (Table 4). This further illustrates that cell density can dramatically change the lactic acid productivity. However, the yield did not notably change with different cell densities. When further analyzing the μ, *qs* and *qp* data, we unexpectedly found that the *qs* and *qp* increased with increases in cell density. When the cell density reached 8.94 g/l, the *qs* and *qp* of OSF was higher than CF (Fig. 4D–F). This demonstrates that within a certain range, increasing the cell density can decrease the *qp*. When the cell density continues to increase, the *qp* may also increase.

3.4. A modified OSF using a fed-batch strategy

To further enhance the total amount and efficiency of L-lactic acid production, the OSF was modified with a fed-batch strategy. The initial glucose concentration was 100 g/l, the residual glucose was maintained at 0–40 g/l, the initial peptone concentration was 3.0 g/l and the fermentation volume was reduced by 50% after 24 h to increase the cell density. The results are displayed in Fig. 5. During the entirety of fermentation, the acid production stage lasted only 29 h and the L-lactic acid production and productivity peaked at158 g/l and 5.45 g/(l h), respectively. During the acid production stage, this strategy resulted in a 366% increase over CF. At the same time, the *qp* was typically higher than CF, although it was lower than in CF during late fermentation. This is likely due to decreased oxygen and mass transfer in the reactor from the excess calcium lactate generated.

3.5. Comparison of fermentation systems

The kinetics of solid and submerged fermentations for lactic acid production from glucose by *R. oryzae* has been intensively studied and are compared in Table 5. A disadvantage of solid fermentation is low mass transfer, which causes low lactic acid pro-

Table 5
Summary of lactic acid production from glucose by cultures of *R. oryzae*.

Culture method		Final concentration (g/l)	Productivity (g/(l h))	Yield (g/g)	Reference
Solid fermentation		137	1.4	0.76	Soccol et al. (1994)
Flocs on support					
In jar-fermentor		103.6	1.7	0.86	Kosakai et al. (1997)
In air-lift bioreactor		104.6	1.8	0.87	Park et al. (1998)
In stirred tank bioreactor		113	4.03	0.9	Yu et al. (2008)
Immobilized on					
PVA-cryogel		112.7	4.5	0.94	Efremenko et al. (2006)
Cotton cloth(in rotating fibrous bed bioreactor)		126	2.5	0.9	Tay and Yang (2002)
Small pellets					
In bubble column		83	2.58	0.88	Zhou et al. (1999)
In jar-fermentor		92	0.7	0.77	Liu et al. (2006)
Modified one-step fermentation strategy					This work
Stirred tank bioreactor with pellets	Total process	160	3.01	0.72	
	Production stage	158	5.45	0.79	

ductivity (Soccol et al., 1994). Several floc morphology control methods have been developed to achieve a higher fungal biomass and eliminate mass transfer limitations inside the fungal mycelia in an effort to increase the total amount of the final product and efficiency of its production (Kosakai et al., 1997; Park et al., 1998; Yu et al., 2008). In these studies, the floc morphology was successfully controlled, and thus the performance significantly improved, by adding mineral supports and PEO or replenishing the nitrogen source to the culture. The best results obtained in these studies were a 113 g/l final lactate concentration in broth, 4.03 g/(l h) productivity, and a 90% lactic acid yield from the floc fungal biomass. However, fermentations of *Rhizopus* sp. with floc morphology require different methods of control and often lead to operational difficulties, such as the microorganisms wrapping around impellers, fouling agitation blades, and blocking the sampling and feeding ports, and, thus, are not suitable for large-scale production. Therefore, notable effort has been devoted to using immobilized cells for lactic acid production (Efremenko et al., 2006; Tay and Yang, 2002). In these studies, fungal mycelia were either entrapped in a polymeric matrix or attached to a support surface. In this system, Efremenko et al. (2006) reported that PVA-cryogel-immobilized cells had the highest productivity. However, this shake-flask technique is not suitable for large-scale lactic acid production and the immobilization of the cells incurs extra costs in these systems of lactic acid production. Thus, if the cell can directly form pellets, operation would still be very efficient and much more economical. However, the overall amount produced and efficiency of production of L-lactic acid was low using the submerged pellet fermentation (Zhou et al., 1999; Liu et al., 2006; Liao et al., 2007b). In the present work, the fermentation strategy used in this study resulted in the highest lactic acid productivity.

4. Conclusions

Conventional lactic acid fermentation by *Rhizopus oryzae* results in low production efficiency of L-lactic acid, which hinders its use in industrial mass scale production. In this paper, a novel modified one-step L-lactic acid fermentation strategy by *R. oryzae* was developed and presented. In this strategy, the maximum lactic acid concentration and productivity during the lactic acid production stage reached 158 g/l and 5.45 g/(l·h), respectively. Meanwhile, compared to CF, the *qp* and yield of OSF did not decrease. This strategy proved to be a convenient and economical method of L-lactic acid fermentation by *R. oryzae*.

Acknowledgements

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