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ORIGINAL ARTICLE

Immobilization of *Rhizopus oryzae* in a modified polyvinyl alcohol gel for L(+)-lactic acid production

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Abstract The production of L(+)-lactic acid (LA) by *Rhizopus oryzae* immobilized in polyvinyl alcohol (PVA) was investigated. To decrease diffusional resistance, we modified the PVA gel through the addition of sodium alginate and phosphate esterification. The production of L(+)-LA improved notably in the immobilized *Rhizopus oryzae*. Maximum L(+)-LA production (106.27 g/L), with a yield of 73.1 % and rate of 2.95 g/L·h, was obtained at a temperature of 38 °C, 6 % PVA, and 0.8 % sodium alginate. The immobilized *R. oryzae* was stable in 14 serial-batch cultures using non-growth medium. The immobilized beads also displayed good tolerance to low temperature and long-term storage at 4 °C with the preservation of biochemical properties.

Keywords Modified PVA · Immobilized · L(+)-lactic acid · *Rhizopus oryzae*

Abbreviations

LA	Lactic acid
PLA	Polylactic acid
PVA	Polyvinyl alcohol

Introduction

L(+)-lactic acid (LA) and its salts are used widely as acidifying agents, flavorings, and preservatives in the food, pharmaceutical, leather, and textile industries (Hofvendahl et al. 1999). Currently, much more attention is being paid to LA in the context of biodegradable and biocompatible polymers, such as polylactic acid (PLA), which is considered to be an environmentally friendly alternative to petroleum-based plastics used in medical, industrial, and consumer products (Gross and Kalra 2002). However, the commercial replacement of plastic with PLA depends on whether L(+)-LA can be produced at a lower cost than petrochemical derivatives.

There has been increasing interest in fungal fermentation with *Rhizopus oryzae* to produce optically pure L(+)-LA as this approach overcomes the disadvantages associated with bacterial fermentation (Tay and Yang 2002). This fungus can grow in a simple medium with glucose, xylose, or starch as the carbon source. Researchers have focused on L(+)-LA production processes that incorporate immobilization techniques because this approach can maintain high cell concentrations and improve the production rate of LA while reducing medium requirements and substrate inhibition. The yields of many fermentation reactions are notably improved when immobilized cell systems are employed.

Cell immobilization can be accomplished by cell entrapment within a polymeric matrix, by cell attachment via adsorption to a surface, or by self-immobilization in cell pellets (Liao et al. 2007; Maneeboon et al. 2010). Several types of immobilization materials have been used in LA production systems, such as sodium alginate, loofa sponge, polyethylene glycol, and κ-carrageenan (Ganguly et al. 2007). However, each of these matrices has its own specific limitations. In the LA production system with alginate used for cell immobilization, the calcium ions that stabilize the alginate gel are displaced by the lactate ions produced during the production process, leading to disruption or dissolution of the beads, while with loofa sponge, mycelia absorbed on the sponge desquamate easily. Thus, there are ongoing attempts to find better matrices for the immobilization of microbial cells. Polyvinyl alcohol (PVA), a synthetic organic polymer, has

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been widely used as an immobilizing material because of its high mechanical strength, durability, and lack of negative effects on both microorganisms and the environment (Pattanapipitpaisal et al. 2001). In reactors, it can improve mass-transfer characteristics, and is often required for immobilized aerobic fungal cultures. An immobilized biocatalyst based on *R. oryzae* fungal cells entrapped in PVA-cryogel using a freeze–thaw procedure has been evaluated for use in L(+)-LA production (Efremenko et al. 2006).

Michal et al. (2005) studied LA production by bacteria immobilized with PVA-boric acid and observed that the immobilized cells showed good stability and performance. Since entrapment in PVA often results in an insufficient oxygen supply due to diffusional resistance, in our study we attempted to improve L(+)-LA productivity by modifying the gel structure with the addition of sodium alginate and immersion in phosphate. The main aims of our study were: (1) to assess the effectiveness of the modified PVA for the immobilization of fungal spores and improved production of L(+)-LA; (2) to determine optimum immobilization and fermentation conditions for L(+)-LA production by immobilized *R. oryzae*, and (3) to evaluate the properties of immobilized beads, i.e., their thermal and operational stability and capacity to preserve L(+)-LA biosynthetic activity at a low temperature.

Materials and methods

Microorganisms and medium

Rhizopus oryzae RQ4015, which is maintained in the Key Laboratory of Ion Beam Bioengineering, Chinese Academy of Sciences, was used throughout this research (Wang et al. 2009). Spores suspensions at a concentration of 1×10^7 spores/mL were obtained by washing the slant with sterile water.

The seed culture medium contained (per liter): glucose, 100 g; NH_4NO_3, 3.0 g; KH_2PO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.75 g; $CaCO_3$, 10 g (sterilized alone). The proliferation culture medium contained (per liter): glucose, 130 g; urea, 2.0 g; KH_2PO_4, 0.2 g; $MgSO_4$, 0.25 g; $ZnSO_4{\cdot}7H_2O$, 0.05 g; $CaCO_3$, 20 g (sterilized alone). The fermentation medium contained (per liter): glucose, 150 g; $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 0.3 g; $MgSO_4{\cdot}7H_2O$, 0.75 g; $ZnSO_4{\cdot}7H_2O$, 0.2 g; $CaCO_3$, 50 g (sterilized alone). The non-growth medium contained (per liter): glucose, 150 g; KH_2PO_4, 0.3 g; $MgSO_4{\cdot}7H_2O$, 0.75 g; $ZnSO_4{\cdot}7H_2O$, 0.2 g; $CaCO_3$, 50 g (sterilized alone).

Immobilization of *R. oryzae* spores in alginate-modified and phosphate-modified PVA

Polyvinyl alcohol (degree of polymerization 1,800±100; Sinopharm Chemical Reagent Co., Shanghai, People's Republic China) and sodium alginate (low viscosity: approx. 200 cps, average MW 3.0×10^5; Sinopharm Chrmical Reagent Co.) were used as the immobilization agent for *R. oryzae* RQ4015 according to the methods described by Chen and Lin (1994). Sodium alginate was mixed with PVA to produce final sodium alginate concentrations ranging from 0.4 to 2.0 % (w/v) and final PVA concentrations ranging from 4 to12 % (w/v) as described in the Effects of immobilized matrix concentrations on L(+)-LA production section. The mixture was heated to dissolve to sodium alginate and PVA and then sterilized. After cooling to room temperature, 50-mL samples of the mixture were mixed with 15 mL of spore suspension and then injected through a syringe into 250 mL of sterile solution containing 5 % boric acid and 2 % $CaCl_2$, forming spherical beads. The obtained alginate-modified PVA beads, with a diameter of 2.5–3 mm, were allowed to harden for 24 h at 4 °C and then washed with sterile saline solution to remove excess calcium ions and untrapped spores.

To obtain phosphate-modified PVA beads, the above alginate-modified PVA beads were placed in 0.3 M sterile monosodium phosphate solution for 0.5–1 h for simultaneous phosphorylation of the gel and disintegration of the calcium–alginate polymer. The phosphate-modified PVA beads were rinsed with sterile water and collected for culture.

L(+)-LA fermentation by free fungal mycelia

The spore suspension was cultivated in seed medium (50 mL in a 250-mL flask) in a rotary shaker at 200 rpm, 38 °C for 12 h, then 4 % (v/v) of the culture from seed medium was transferred to fermentation medium (50 mL in a 250-ml flask) and cultivated at 200 rpm, 38 °C; $CaCO_3$ was added to bring the pH to 6.0. The broth in flask was periodically sampled for assay.

L(+)-LA fermentation by immobilized *R. oryzae*

The alginate-modified and phosphate-modified PVA beads containing entrapped *R. oryzae* spores were inoculated into the proliferation medium [bead inoculum amount of 20 % (v/v)] and cultured in a rotary shaker (150 rpm) at 38 °C for 24 h. The immobilized mycelia beads were then removed from the proliferation medium, washed, and then used for L(+)-LA fermentation, which was conducted in 250-mL Erlenmeyer flasks containing 50 mL of the fermentation medium with 12 g of beads per flask. All flasks were cultured for 30–36 h at 38 °C and 170 rpm.

Repeated-batch fermentations by mycelia of *R. oryzae* immobilized in phosphate-modified PVA were also conducted in 250-mL Erlenmeyer flasks using non-growth medium.

Immobilization conditions

The optimal immobilization conditions for L(+)-LA production by *R. oryzae* immobilized in phosphate-modified PVA were determined in 250-mL Erlenmeyer flasks containing 50 mL of the fermentation medium under aerobic culture conditions (170 rpm).

The effect of PVA concentration on fermentation was assessed at concentrations of 4, 6, 8, 10, and 12 % (w/v) PVA when 0.9 % (w/v) of sodium alginate was used. The effect of sodium alginate concentration was assessed at concentrations of 0.4, 0.8, 1.2, 1.6, and 2 % sodium alginate when 6.0 % (w/v) PVA was used (Pattanapipitpaisal et al. 2001). Each flask was incubated with 12 g of immobilized *R. oryzae* beads and cultured at 38 °C for 36 h.

Thermal stability of immobilized *R. oryzae*

After 24 h of proliferation, the phosphate-modified PVA beads were incubated at various temperatures (0–80 °C) for 1 h and then transferred into fermentation medium. The residual activity after fermentation was determined by the levels of L(+)-LA and glucose in fermentation broth.

Preservation of immobilized *R. oryzae*

The alginate-modified immobilized beads entrapped with spores were immersed in sterile water and stored at 4 °C for 0–70 days. They were then modified by sterile monosodium phosphate solution, proliferated, and used for flask fermentation. All experiments were performed in triplicate.

Analytical methods

The residual glucose and L(+)-LA were quantified using a biosensor (model SBA-40C; Shandong Academy of Sciences, Shanghai, People's Republic China). This instrument measures the content of glucose (which is oxidized to gluconic acid by immobilized glucose oxidase) and is specifically sensitive to L(+)-LA produced by immobilized lactate oxidase (Wang et al. 2009).

Scanning electron microscopy (SEM) micrographs of fungus mycelia and PVA granules were obtained using samples fixed in 2.5 % glutaraldehyde and dried by vacuum freeze-drying equipment. The photographs were taken at 20 kV (model 3000 series; Amray, Bedford, MA).

Statistical analysis

Data are presented as the mean ± standard deviation (SD) determined from three separate experiments.

Results and discussion

Modification of the PVA immobilization method

Polyvinyl alcohol shows a high elasticity, but the density of the crosslinked gel structure results in poor permeability. In our study, we modified the gel structure to increase porosity as an approach to improve mass transfer. Alginate-modified beads were produced by adding sodium alginate to the PVA gel solution to eliminate bead agglomeration. The beads were then modified by phosphate solution, which hardens the PVA gel structure through esterification and simultaneous disintegration of calcium–alginate polymers inside the PVA beads. The resultant PVA gel beads were more porous, with improved gel strength, gas permeability, diffusion, and bioactivity as documented below.

The appearance of PVA granules by SEM showed that the gel void space was increased after the phosphorylation treatment (Fig. 1a, b). The inside layers of the phosphate-modified PVA gel beads contained numerous pores for occupancy by fungal spores (Fig. 1c). The structure also provided a suitable environment for substrate diffusion and cell growth. Spores insider and outside the phosphate-modified PVA beads germinated and grew during the cultivation period (Fig. 1d), forming a thick shell of mycelia outside the bead after proliferation (Fig. 1e).

Fermentation kinetics of free mycelia and immobilized mycelia of *R. oryzae*

Batch fermentations with glucose (150 g/L) were conducted using free mycelia of *R. oryzae*, mycelia immobilized in alginate-modified PVA, and mycelia in phosphate-modified PVA (Fig. 2). Free mycelia produced the highest concentration of L(+)-LA at 110.55 g/L, while mycelia immobilized in phosphate-modified PVA had the highest production rate of 2.81 g/L·h during the fermentation.

Three phases of activity (lag, exponential growth, and stationary phase) occurred during fermentation with free mycelia and with mycelia immobilized in alginate-modified PVA. In the free-mycelia fermentation, the density of mycelia was low at the beginning and, consequently, a noticeable change in the glucose concentration did not occur immediately, resulting in the observed lag phase. Increases in the mycelial density was accompanied by increased in the rate of glucose consumption, ultimately achieving a rate similar to that of *R. oryzae* immobilized in phosphate-modified PVA. The fermentation profile of mycelia immobilized in alginate-modified PVA (without phosphorylation) was characterized by a long lag-phase of 24 h. This long lag phase might be attributable to two factors. First, the mycelia grew poorly due to the resistance of the gel matrix, and second, it takes time to achieve diffusional equilibrium

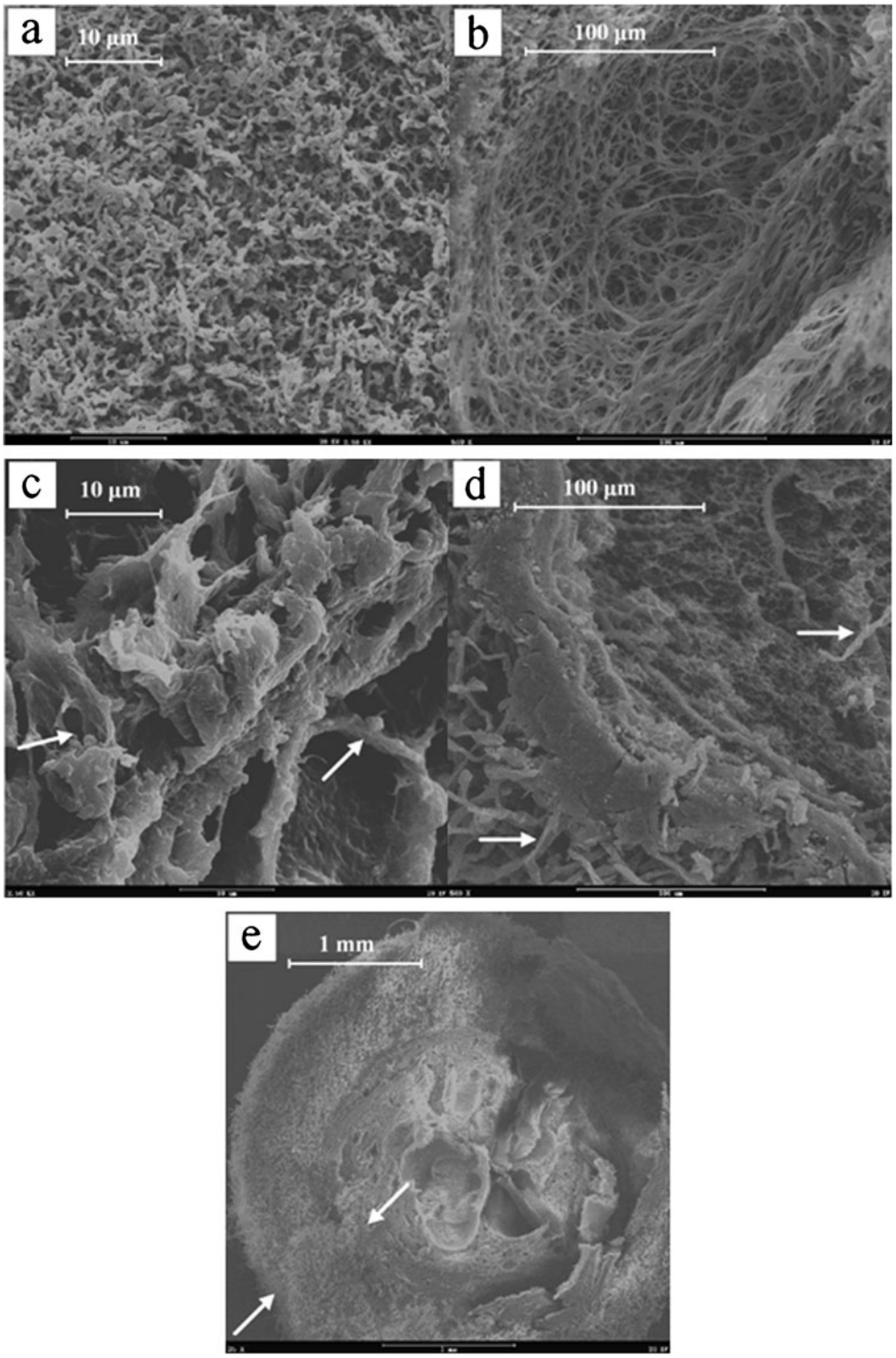

Fig. 1 Scanning electron micrographs of polyvinyl alcohol (PVA) beads and immobilized *Rhizopus oryzae*. **a** Cross-linking and internal structure of an alginate-modified bead (2,500×), **b** internal structure of a phosphate-modified bead (500×), **c** internal structure of a phosphate-modified bead (2,500×), **d** sectional profile of a phosphate-modified bead after fungal proliferation (500×), **e** sectional profile of a phosphate-modified bead after proliferation (35×). Fungal spores (Fig. 1c) and mycelia (Fig. 1d, e) are marked with *arrows*

between the PVA beads and the solution. The fungus immobilized in alginate-modified PVA may have used more glucose for mycelial growth instead of L(+)-LA production, resulting in a lower L(+)-LA concentration (90.45 g/L) and a yield of 60.1 %.

Phosphate modification of the PVA gel enlarged the interspaces, improving substrate and gas diffusion and resulting in greater mycelial growth. Therefore, fermentation started without any lag period, and glucose was virtually exhausted within 35 h. The rate of L(+)-LA production increased to 2.81 g/L·h, which was twofold higher than that of *R. oryzae* immobilized in alginate-modified PVA. Compared to the fermentation process with free mycelia, the rate of L(+)-LA production by *R. oryzae* immobilized in

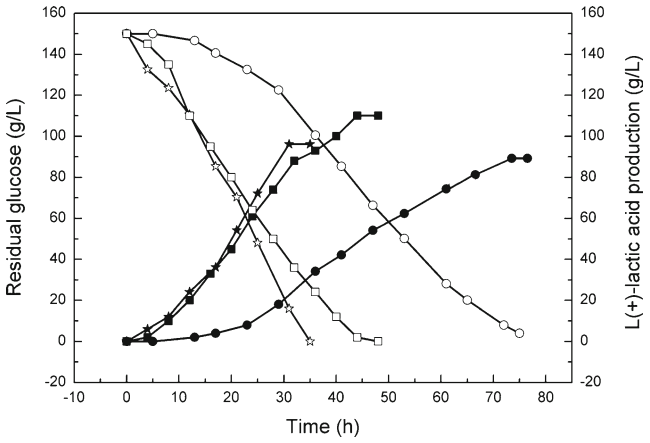

Fig. 2 Time profiles during L(+)-lactic acid [L(+)-LA] fermentation. *Filled* and *open square*s Free mycelia, *filled* and *open circles R. oryzae* immobilized in alginate-modified PVA, *filled* and *open stars R. oryzae* immobilized in phosphate-modified PVA. *Filled symbols* L(+)-LA, *open symbols* Residual glucose. The fermentation was conducted in 250-mL Erlenmeyer flasks containing 50 mL of the fermentation medium at 38 °C; the concentrations of PVA and sodium alginate were 8.0 % and 0.9 % (w/v), respectively

phosphate-modified PVA was slightly higher, but the production of L(+)-LA (98.49 g/L) was lower, presumably due to the fact that the proliferated mycelia had formed a shell around the bead and only the mycelia in the outer layer of the shell were in a good contact with the substrate. Fermentation by mycelia in the inner layer was limited by oxygen diffusion, resulting in more ethanol and less L(+)-LA production under oxygen-limited conditions (Skory et al. 1998).

The highest LA production (127 g/L), with a production rate of 2.5 g/L·h, was obtained with immobilized *R. oryzae* (Tay and Yang 2002). However, this result was obtained with a lower initial glucose concentration (7 %) as the substrate in a fed-batch fermentation system using immobilized cells of *R. oryzae* in a rotating fibrous bed bioreactor. In our immobilization system, substrate inhibition and the accumulation of high concentrations of product under batch conditions could limit the yield. This problem can be reduced by running the fermentation in a continuous mode. Moreover, controlling the growth and thickness of the mycelial layer on the surface of beads is critical to L(+)-LA production. Therefore, it is necessary to optimize immobilization conditions to improve the rate and yield of L(+)-LA production. The results of our study suggest that the use of alginate-PVA modified by phosphate for the immobilization of *R. oryzae* is a promising alternative method for L(+)-LA production because it significantly improves the rate of production and makes the broth free of mycelia, which allows the L(+)-LA to be easily recovered from the medium. Therefore, the immobilized beads used in subsequent studies were all modified with phosphate.

Effects of immobilized matrix concentration on L(+)-LA production

Varying the polymer concentration changes the porosity and, correspondingly, the mass-transfer characteristics of polymeric matrices (Lozinsky and Plieva 1998). Therefore, PVA and sodium alginate concentrations should be optimized according to the requirements for gel strength, bioactivity, and mass transfer resistance. In our study, L(+)-LA production was negatively correlated with PVA and sodium alginate concentrations. We determined that the optimum condition should be 6 % PVA in the presence of 0.8 % sodium alginate, which provided an L(+)-LA production of 106.27 g/L (73.5 % yield) and production rate of 2.95 g/L·h.

Too much PVA (>10 %) resulted in the formation of dense beads that were resistant to substrate diffusion; thus, spores inside and outside the beads did not obtain sufficient nutrients to germinate. However, the beads were too soft when the PVA concentration was lower than 6 %, resulting in the loss of spores from the beads. In terms of PVA concentration, L(+)-LA production was maximum at 4 % PVA, owing to the joint effects of fermentation by free mycelia and immobilized mycelia (Fig. 3a). The addition of sodium alginate had an impact on both bead structure and L(+)-LA production (Fig. 3b). The beads agglomerated with a sodium alginate concentration of less than 0.8 %, which is in contrast to the results of Wu and Wisecarver (1992) who reported that the lowest concentration of sodium alginate that would prevent bead agglomeration was 0.02 %. This discrepancy presumably depends on the characteristics of the PVA and sodium alginate used. Increases in the sodium alginate concentration are accompanied by increases in the number of biopolymer molecules per unit solution and of the binding sites for Ca^{2+} (Clementi et al. 1998; Idris and Suzana 2006). As a result, a more densely cross-linked gel structure was probably formed. The presence of a dense gel encapsulating the fungal spores or mycelia is expected to create resistance to diffusion through the beads, resulting in insufficient cell growth and lower product formation.

Effect of repeated batch fermentations on L(+)-LA production by immobilized *R. oryzae*

One of the major advantages of immobilized *R. oryzae* is that the fungal mycelia can be separated easily from the fermentation broth and used repeatedly for long-term fermentation. Moreover, the use of immobilized cells that are not growing could markedly improve the efficiency of L(+)-LA production because *R. oryzae* can continuously produce LA in non-growth medium.

The re-use efficiency of immobilized *R. oryzae* for L(+)-LA production from glucose in repeated batch fermentations is shown in Fig. 4. The immobilized *R. oryzae* was stable for 14 batches in non-growth medium, with an average production

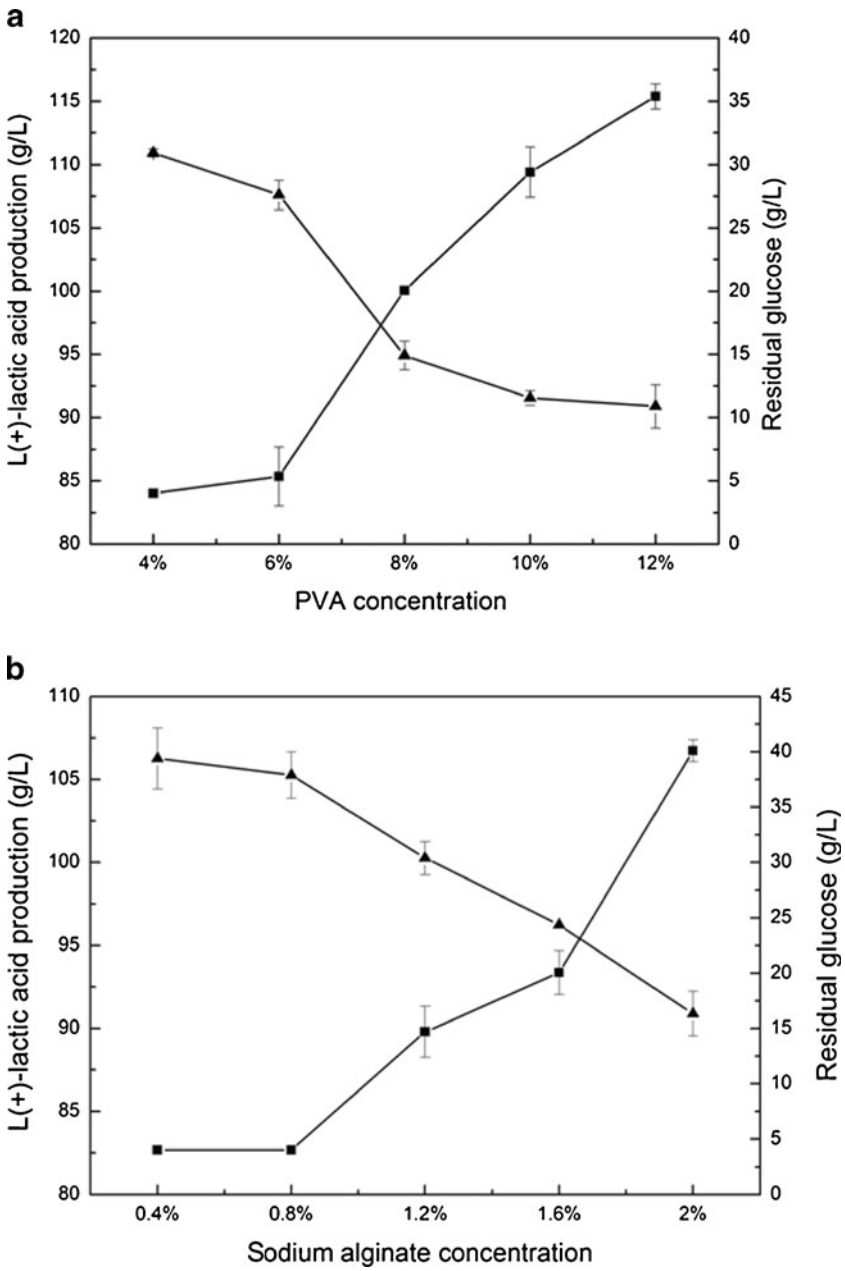

Fig. 3 Effect of the immobilization matrix concentration on L(+)-LA production. **a** Effect of PVA concentration on L(+)-LA production (*filled triangle*) and residual glucose (*filled square*) when 0.9 % sodium alginate was added to the medium. **b** Effect of sodium alginate concentration on L(+)-LA production (*filled triangle*) and residual glucose (*filled square*) when 6 % PVA was used. After 24 h of fungal proliferation, the fermentation by immobilized *R. oryzae* was carried out in 250-mL flasks containing 50 mL of the fermentation medium, at 170 rpm and 38 °C for 36 h

rate of 2.5 g/L·h and yield of 70.4 %. Roukas and Kotzekidou (1998) proposed that the stability of immobilized *R. oryzae* is due to the protection provided by the immobilization matrix to the cells. Immobilized cells retain their enzyme activities for a long time owing to the different composition of their cellular contents (proteins, lipids, RNA, DNA and inorganic substances) compared with free cells (Alexandre et al. 2010).

Effects of thermostability on L(+)-LA production by immobilized *R. oryzae*

The thermostability of immobilized cells is a very important parameter for potential industrial applications. To test the tolerance of immobilized *R. oryzae* to temperature, beads containing mycelia were incubated at various temperatures for 1 h, and then the residual metabolic activity of the encapsulated fungal cells was measured in fermentation reactions. There was no activity loss in immobilized *R. oryzae* up to 40 °C and the production of L(+)-LA (106.58 g/L) remained constant (Fig. 5). At 60 and 80 °C, the beads retained a spherical shape but become soft due to melting of the PVA and calcium-alginate. In addition, the encapsulated mycelia were killed at these temperatures, and the enzymes related to L(+)-LA production were completely inactivated, resulting in no L(+)-LA production and no glucose consumption. The results also suggest the

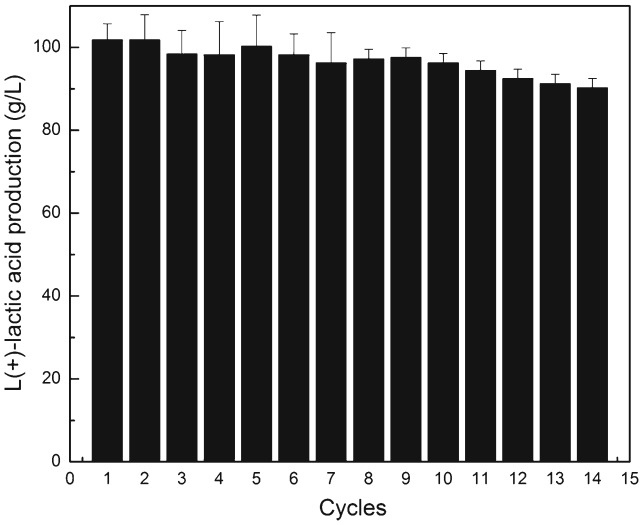

Fig. 4 Effects of repeated batch fermentation on L(+)-LA production. The fermentation was carried out in 250-mL flasks containing 50 mL of non-growth medium, at 170 rpm, 38 °C for 36 h

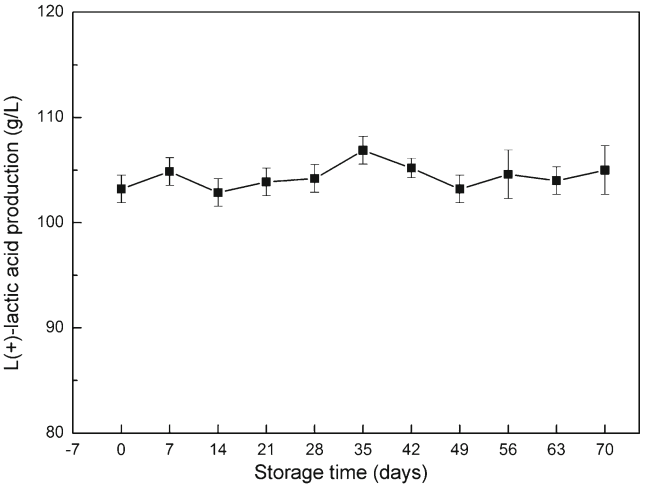

Fig. 6 Effect of storage duration of immobilized *R. oryzae* spores on L(+)-LA production. The fermentation was performed in 250-mL flasks containing 50 mL of the fermentation medium, at 170 rpm and 38 °C for 36 h

possibility of storing immobilized *R. oryzae* cells at 4 °C to preserve their biochemical and physiological properties.

Effects of storing immobilized cells at 4 °C on L(+)-LA production

To analyze the influence of cold storage on the metabolic potential of *R. oryzae* spores immobilized in modified PVA gel, L(+)-LA production by spores immobilized in beads was examined after storage at 4 °C, from 0 to 70 days. The storage time had no obvious influence on L(+)-LA production (Fig. 6). The immobilized spores grew well and L(+)-LA production remained at 102.24–106.50 g/L throughout the storage period, demonstrating that the PVA gel is an effective matrix for preserving the physiological and biochemical properties of the cells. This fact may be immensely useful for the industrial application of immobilized cells in the production of L(+)-LA.

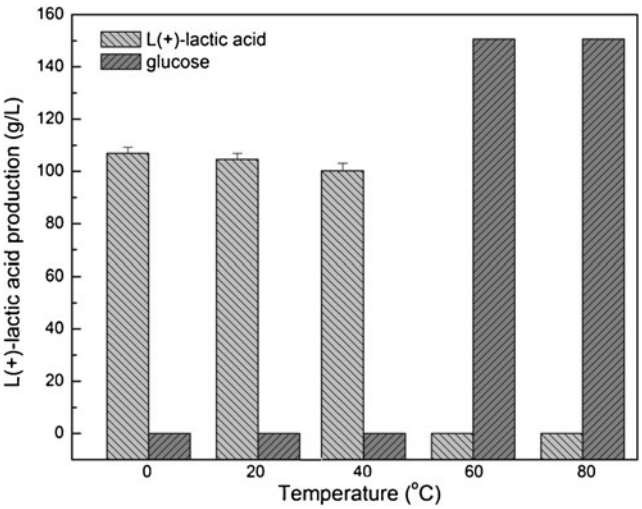

Fig. 5 Thermal stability of immobilized *R. oryzae* in terms of metabolic activity. The proliferated immobilized *R. oryzae* were incubated at different temperatures for 1 h and then used for L(+)-LA fermentation for 36 h at 38 °C in 250-mL flasks containing 50 mL of fermentation medium

Conclusions

The results of our study show that it is feasible to use modified PVA gel to immobilize *R. oryzae* spores for L(+)-LA production. PVA modified by alginate and phosphate has a more porous structure that allows more active growth of the immobilized spores and more efficient production of LA efficiently at superior L(+)-LA production rates compared to free cells. The PVA and alginate concentrations have important influences on gel structure and L(+)-LA production. After optimizing the immobilization condition, L(+)-LA production of immobilized *R. oryzae* in modified PVA was improved to 106.27 g/L, which is nearly comparable to the production by free cells. Fermentation using immobilized *R. oryzae* results in a virtually cell-free fermentation broth that provides many advantages over conventional fermentation processes. The most obvious advantage is the sturdy nature of the immobilized biocatalyst, which provides rapid substrate utilization and stable L(+)-LA production in repeated fermentation cycles in non-growth medium. The ability to re-use the biocatalyst in non-growth medium reduces nitrogen consumption, which is extremely important in reducing fermentation costs. Biocatalyst recycling will enable future large-scale production of L(+)-LA with immobilized *R. oryzae* through a continuous fermentation process with

simultaneous product removal. Therefore, we suggest that *R. oryzae* immobilized in a modified PVA gel is a promising approach for the industrial production of L(+)-LA.

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