Korean J. Chem. Eng., **30**(1), 166-171 (2013)
DOI: 10.1007/s11814-012-0093-1

Bioconversion process for synthesis of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate using liquid-core immobilized *Saccharomyces cerevisiae* CGMCC No 2233

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(*Received 15 March 2012 • accepted 16 June 2012*)

Abstract–*Tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate was synthesized using asymmetric reduction of *tert*-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate with liquid-core immobilized *Saccharomyces cerevisiae* CGMCC No. 2233. The optimum conditions for preparation of the liquid-core immobilized cells were found to be 2% guar gum, 5% $CaCl_2$, 0.8% sodium alginate, capsule diameter 2 mm, 0.3% chitosan (1.0×10^5) solution, and 30 min for formation of the film of liquid-core immobilized cells. The optimum re-cultivation time was 32 h. The optimum reduction conditions were found to be pH 6.8-7.2, 160 r/min, and 30 °C. Conversion was found to reach 100% when initial concentration of substrate was less than 50 g/L. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate exceeded 99%. The liquid-core immobilized cells retained their effectiveness even after 15 uses.

Key words: Asymmetric Reduction, *Tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate, Liquid-core Immobilized Cells

INTRODUCTION

Tert-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate is a key intermediate of hydroxylmethylglutaryl coenzyme A (HGM-CoA) reductase inhibitors. Hydroxymethylglutaryl coenzyme A(HGM-CoA) reductase inhibitors are a class of lipid-lowering drugs; they can cure hypercholesterolemia and prevent the occurrence of atherosclerosis and coronary heart disease [1-3].

It has been reported that an efficient whole-cell biotransformation process using *Lactobacillus kefir* was developed for the asymmetric synthesis of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate using two-step asymmetric reduction of *tert*-butyl 6-chloro-3,5-dioxohexanoate. A diastereomeric excess of >99% was measured for (3R,5S) and (3S,5S)-butyl 6-chloro-dihydroxyhexanoate [4,5]. The production of *Lactobacillus kefir* is expensive, and only small cell densities are achieved [6]. It has been shown to be difficult to obtain large amounts of *Lactobacillus kefir* cells. A polypeptide enzyme capable of asymmetrically reducing *tert*-butyl (S)-6-chloro-5-hydroxy-3-oxohexanoate to *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate was identified in a previous study [7]. Coenzymes (NADH or NADPH) were found to be necessary for synthesis of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate. These coenzymes are expensive, increasing the cost of production. Another paper showed that *tert*-butyl (3R, 5S)-6-chloro-3,5-dihydroxyhexanoate can be synthesized by resolution of racemic compounds with lipase catalyst [8]. Theoretically, the highest possible rate of conversion of resolution by lipase is 50%.

Whole-cell biotransformation is widely used in asymmetric reduction of carbonyl compounds to produce chiral products [9-12]. This is because whole cells contained large amounts of reductase and coenzyme (NADH and NADPH). In theory, conversion rates can reach 100% [13-16]. With the development of immobilized cell technology, large-scale industrial production of chiral products using whole-cell biotransformation has become more viable [17-19]. Reuse of immobilized cells has been shown to improve productivity, facilitate separation of product, and reduce the cost of production. The selection of an appropriate method of immobilization is very important. In this paper, liquid-core immobilized cells were used to convert *tert*-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate to form *tert*-butyl (3R,5S)-6-chloro-3, 5-dihydroxyhexanoate. The reaction is shown in Fig. 1. Components of liquid-core capsules and biotransformation conditions were optimized in detail.

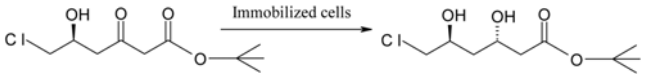

Fig. 1. Synthesis of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate by asymmetric reduction of *tert*-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate with immobilized cells.

MATERIAL AND METHODS

1. Reagents and Instruments

Tert-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate, *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate, and *tert*-butyl (3S,5S)-6-chloro-3,5-dihydroxyhexanoate were obtained from Zhejiang Jiuzhou Pharmaceutica Co. Ltd., China. A gas chromatograph (Agilent 7820) equipped with a chiral column (Cyclodex-B 0.25 mm×30 m×0.25 μm) was used for analysis.

2. Microorganism and Cultivation

Saccharomyces cerevisiae CGMCC No. 2233 was isolated from

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soil and preserved in the China General Microbiological Culture Collection Center. The solid medium for the preservation of the strain was composed of 10 g/L malt juice, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose and 20 g/L agar. The liquid medium for the cultivation of cells on a large scale was composed of 30 g/L glucose, 3 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4$, 1 g/L $K_2HPO_4 \cdot 3H_2O$ and 1 g/L KH_2PO_4. The strain picked from the solid medium was inoculated into a 100 ml liquid culture medium and cultivated in a 30 °C shaker (160 r/min) for 24 h. A 10 ml cell suspension was then inoculated into 100 ml liquid medium. After cultivation for 24 h, the fermentation broth was centrifuged and the sediment was washed three times with sterile saline. The resting cells were used for cell immobilization.

3. Preparation of Solid-core Immobilized Cells

The cell levitation liquid was prepared by addition of 1 g (dry weight) resting cells into 100 ml sterile water. The cell levitation liquid and 2%% sodium alginate solution were uniformly mixed together at equal volumes. The mixture was dropped into 3% $CaCl_2$ solution to form 2 mm solid-core capsules using a needle.

4. Preparation of Liquid-core Immobilized Cells

The cell levitation liquid was prepared by addition of 1 g (dry weight) resting cells in 100 ml sterile water. Solution A was composed of 1-3% (g/g) guar gum and 3-8% (g/g) $CaCl_2$. The cell levitation liquid and solution A were uniformly mixed together at equal volumes. The mixture was dropped into 0.6-1.0% sodium alginate solution to form 2 mm, 3 mm, 4 mm and 5 mm liquid-core capsules using needles of different sizes. The liquid-core capsules were washed three times with sterile water. The liquid-core capsules were then dispersed in 0.2-0.7% chitosan solution for 10-50 min to form the film. Chitosans of different molecular weights (1.8×10^4, 2.9×10^4, 6.8×10^4, 8.8×10^4, and 1.0×10^5) facilitated the formation of the films of the liquid-core capsules.

5. Re-cultivation of Immobilized Cells

Before being used in reduction, the solid-core immobilized cells and the liquid-core immobilized cells were dispersed in liquid medium for re-cultivation in a 30 °C shaker (160 r/min) for 24-72 h.

6. Asymmetric Reduction of *Tert*-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate for the Preparation of *Tert*-butyl (3R, 5S)-6-chloro-3,5-dihydroxyhexanoate

Substrate was added in sterile potassium phosphate buffer (0.1 mol/L) containing immobilized cells. The reduction was carried out in shaker (160 r/min) at 30 °C for 24-72 h. The substrate and product in the reduction mixture were extracted three times using ethyl acetate at the end of each reduction. Substrate and product content in the ethyl acetate layer were detected by gas chromatography (Agilent 7820).

7. Detection of Cell Dry Weight in Solid-core and Liquid-core Capsule

Broken capsule liquid contained 0.2 mol/L $NaHCO_3$ and 0.06 mol/L $Na_3C_6H_5O_7 \cdot 2H_2O$ (pH 8.0) [20]. Then 1 g of capsule was added to 10 ml of broken capsule liquid. The mixture was shaken for 20 min and then centrifuged at 4,000 r/min for another 20 min. The cell sediment was dried to a constant weight.

8. Analysis of Conversion and Diastereomeric Excess of (3R, 5S)-6-chloro-3,5-dihydroxyhexanoate

The concentrations of substrate and product were determined by GC (Agilent 7820) equipped with a chiral column (Cyclodex-B 0.25 mm×30 m×0.25 μm). The flow rate of nitrogen was 1.0 ml/min. The column temperature, injection temperature, and detector temperature were 250 °C, 80 °C, and 250 °C, respectively. The retention times of *tert*-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate, (3R,5S)-6-chloro-3,5-dihydroxyhexanoate, and *tert*-butyl (3S,5S)-6-chloro-3,5-dihydroxyhexanoate were 11.905 min, 20.496 min, and 22.444 min.

RESULTS AND DISCUSSION

1. Growth Curves of Free Cells, Immobilized Cells Embedded in Solid-core Capsules, and Immobilized Cells Embedded in Liquid-core Capsules

Single-gram (dry weight) quantities of resting cells and of both solid-core and liquid-core immobilized were inoculated separately into 500 ml liquid medium and cultivated to produce the growth curve. The formation conditions of liquid-core capsule were 2% guar gum, 5% $CaCl_2$, 0.8% sodium alginate and 0.3% chitosan (M_w 1.0×10^5). The diameter of immobilized capsules was 2 mm. The biomasses were 21.5 g/L, 39.5 g/L, and 56.0 g/L for free cells, solid-core immobilized cells, and liquid-core immobilized cells cultivated for 24, 32, and 32 h. The optimal cultivation times for free cells, solid-core immobilized cells, and liquid-core immobilized cells were 24, 32, and 32 h. Greater biomasses were produced by re-cultivation of liquid-core immobilized cells than through the cultivation of resting cells or solid-core immobilized cells. Bioprocess for synthesis of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate involving liquid-core immobilized cells as catalysts was further investigated.

2. Effects of Substrate Concentration and Reaction Time on Reduction with Liquid-core Immobilized Cells as Catalysts

Different initial concentrations of substrate were added to the reduction mixture containing liquid-core immobilized cells. Conversion increased with the extension of reduction time. The optimum reduction time varies with initial concentration of substrate. The optimum reduction times were 36, 36, 48, 60, and 60 h when initial substrate concentration was 10, 20, 30, 40, and 50 g/L. Conversion decreased with the increase of initial substrate concentration during the same reduction time. Conversion was found to reach 100% when initial substrate concentration was less than 50 g/L. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate

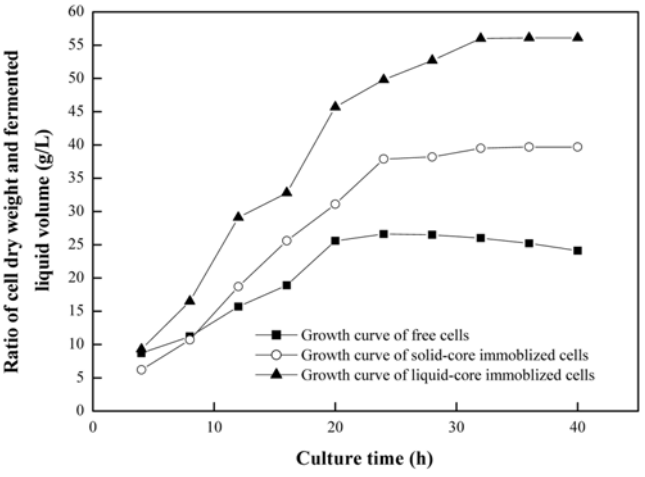

Fig. 2. Growth curves of free cells and immobilized cells.

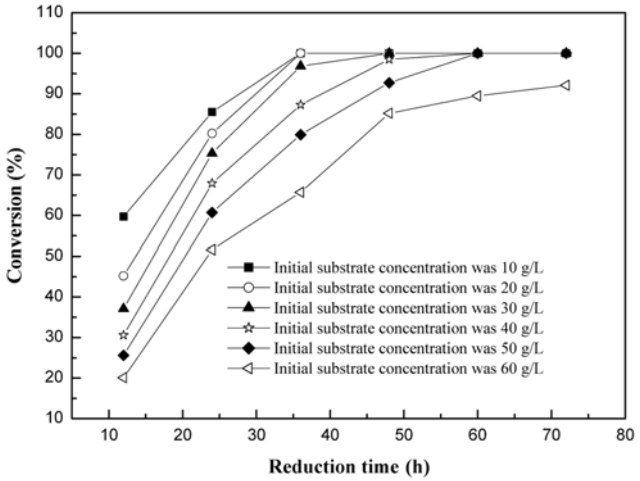

Fig. 3. Effects of substrate concentration and reaction time on reduction with liquid-core immobilized cells as catalyst. Reduction conditions: 2% guar gum, 5% $CaCl_2$, 0.8% sodium alginate, 0.3% chitosan (M_w 1.0×10^5). Diameter of liquid-core capsules, 2 mm. Cell dry weight in reduction mixture, 56.0 g/L. pH 7.0, 30 °C, 160 r/min.

exceeded 99%.

3. Effects of Guar Gum Content and the Diameter of Liquid-core Immobilized Cells on Cell Growth and Reduction

Liquid-core immobilized cells prepared using different amount of guar gum were used in reduction. The guar gum helps the liquid core to form. When guar gum content was less than 2.0%, the breakage rate of liquid-core capsules was high enough to reduce both the number of cells acquired and the conversion of reduction. The fluid viscosity in the liquid-core capsule was so high that that the resistance of the transfer of medium components into the interior of the capsule was very high. Few cells were obtained when guar gum content was over 2.0%. The optimal guar gum content was found to be 2.0%. Liquid-core immobilized cells of different diameters were used in reduction. The number of cells and conversion both decreased with the increase of capsule diameter, reaching 56.0 g/L and 100% when the diameter of capsule was 2 mm. The optimal diameter of liquid-core immobilized cells was 2 mm. This was probably because surface area was bigger and material spread more fully. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate exceeded 99% for different diameters of liquid-core immobilized cells and different levels of guar gum content.

4. Effects of the Concentration of $CaCl_2$ and Sodium Alginate on Cell Growth and Reduction

Different concentrations of $CaCl_2$ and sodium alginate were used to assess the effects of the sodium alginate content on cell growth and reduction. The function of sodium alginate was to form the alginate network with $CaCl_2$. The amount of $CaCl_2$ and sodium alginate can affect the molding and mechanical strength of capsule. When the concentration of $CaCl_2$ and sodium alginate was less than 5% and 0.8%, the breakage rate of liquid-core capsule was higher and there were fewer cells, decreasing the conversion of reduction. When the concentration of $CaCl_2$ and sodium alginate was more than 5% and 0.8%, respectively, the alginate network was dense and affected the mass transfer of medium components into the interior of the capsule. The optimum concentrations of $CaCl_2$ and sodium alginate were found to be 5% and 0.8%. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate exceeded 99% at different concentrations of $CaCl_2$ and sodium alginate.

5. Effects of Chitosan of Different Molecular Weights and of Chitosan Concentration on Cell Growth and Reduction

Liquid-core immobilized cells prepared by chitosans of different molecular weight were used in reduction. The role of chitosan was to form the film of the liquid-core capsule. The number of cells and conversion both increased as the molecular weight of chitosan increased. This was because the chitosan molecules diffused into the alginate network more easily when chitosans of smaller molecular weight were used, allowing a thicker film to form. This made it more

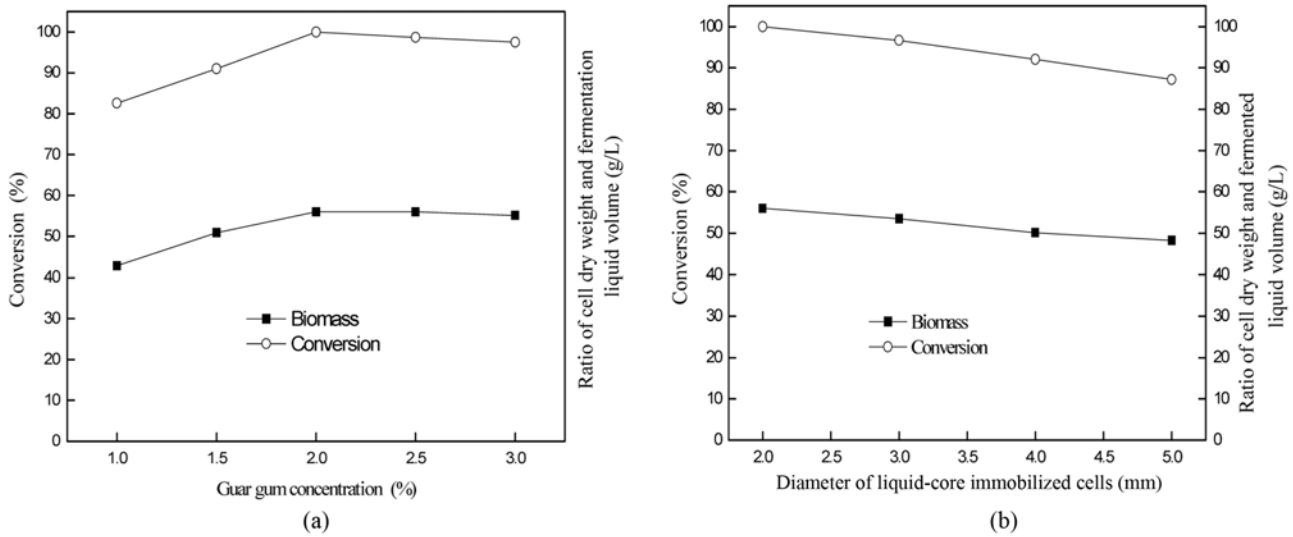

Fig. 4. Effects of the guar gum content (a) and the diameter of liquid-core immobilized cells (b) on cell growth and reduction. Reduction conditions: 5% $CaCl_2$, 0.8% sodium alginate, and 0.3% chitosan (M_w 1.0×10^5). Cell dry weight in reduction mixture, 56.0 g/L. Initial substrate concentration, 50 g/L. pH 7.0, 30 °C, 160 r/min, 60 h.

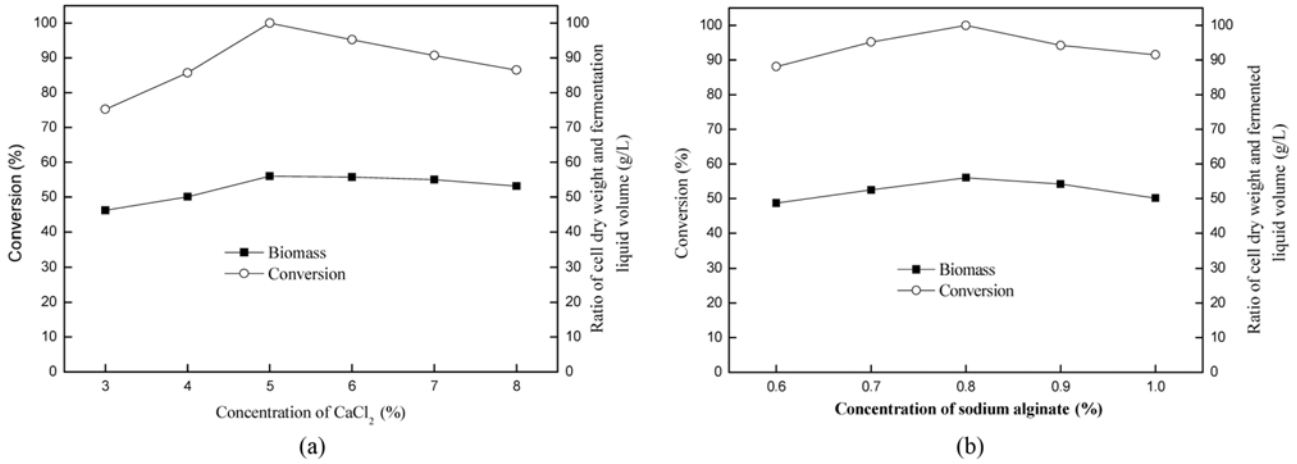

Fig. 5. Effects of the concentration of $CaCl_2$ (a) and sodium alginate (b) on cell growth and reduction. Reduction conditions: 2% guar gum and 0.3% chitosan (M_w 1.0×10^5). Diameter of liquid-core capsules, 2 mm. Cell dry weight in reduction mixture, 56.0 g/L. Initial substrate concentration, 50 g/L. pH 7.0, 30 °C, 160 r/min, 60 h.

difficult for medium components to enter the immobilized cells. In this way, thicker membranes reduced cell growth. The optimum chitosan molecules were found to be 1.0×10^5. The liquid-core immobilized cells made with different concentration of chitosan (1.0×10^5) were used in reduction. The rate of liquid-core capsule breakage was high when 0.2% chitosan was used as the film-forming agent. At chitosan concentrations above 0.3%, the number of cells and the conversion both decreased as the concentration of chitosan increased. When keeping the same film formation time, the higher the chitosan concentration, the thicker the film of liquid-core capusle. It can increase the difficulty of transfer of medium components and substrate into immobilized cells. The optimum chitosan concentration was found to be 0.3%. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate exceeded 99% with chitosans of different molecular weights and different chitosan concentrations.

6. Effects of Temperature, Shaking Speed, and pH of the Potassium Phosphate Buffer on Reduction

Conversion was affected by shaking speed because the shaking speed can affect the spread rate of the outside material to the surface of liquid-core immobilized cells. Speeds of 100, 120, 140, 160, 180, and 200 r/min were selected for study of the effect of shaking speed on reduction. When the shaking speed was less than 160 r/min, conversion increased with the increase of shaking speed. Conversion reached 100% and the restriction of the spread of exterior material was found to have been eliminated when shaking speed exceeded 160 r/min. The optimum shaking speed and temperature were found to be 160 r/min and 30 °C. The liquid-core immobilized cells were dispersed in reduction mixture containing potassium phosphate buffer at different pH levels and 50 g/L substrate. The liquid-core immobilized cells were found to have good reduction ability from pH 6.8 to pH 7.2. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate was not affected by shaking speed, temperature, or the pH of the potassium phosphate buffer and remained >99%.

7. Reuse of Liquid-core Immobilized Cells in Reduction

One important advantage of immobilized cells is that they can be reused many times. Reuse of immobilized cells in biotransfor-

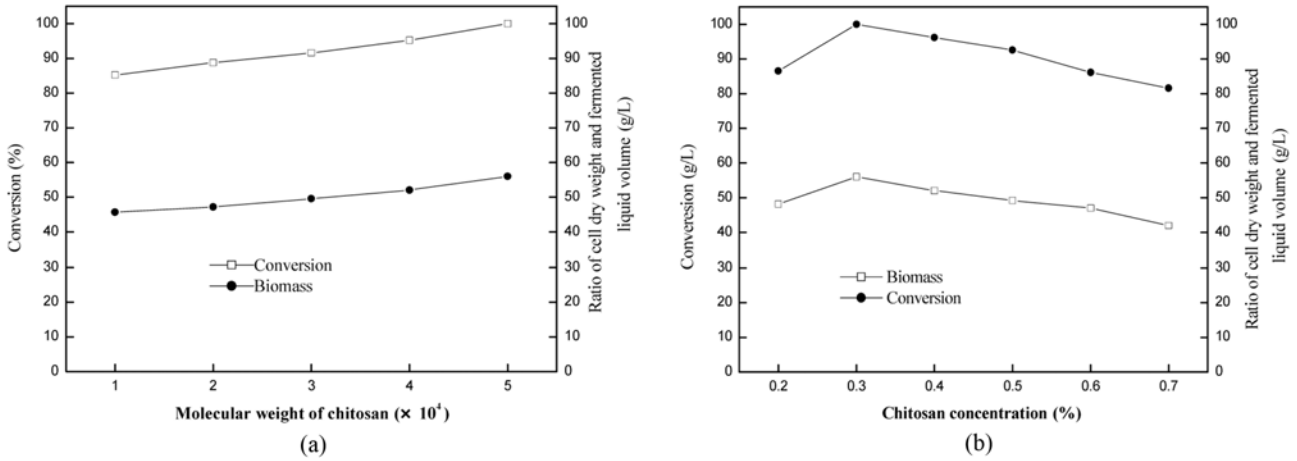

Fig. 6. Effects of different molecular weight chitosan (a) and chitosan concentration (b) on cell growth and reduction. Reduction conditions: 2% guar gum, 5% $CaCl_2$, 0.8% sodium alginate. Diameter of liquid-core capsules, 2 mm. Cell dry weights in reduction mixture, 56.0 g/L. Initial substrate concentration, 50 g/L. pH 7.0, 30 °C, 160 r/min, 60 h.

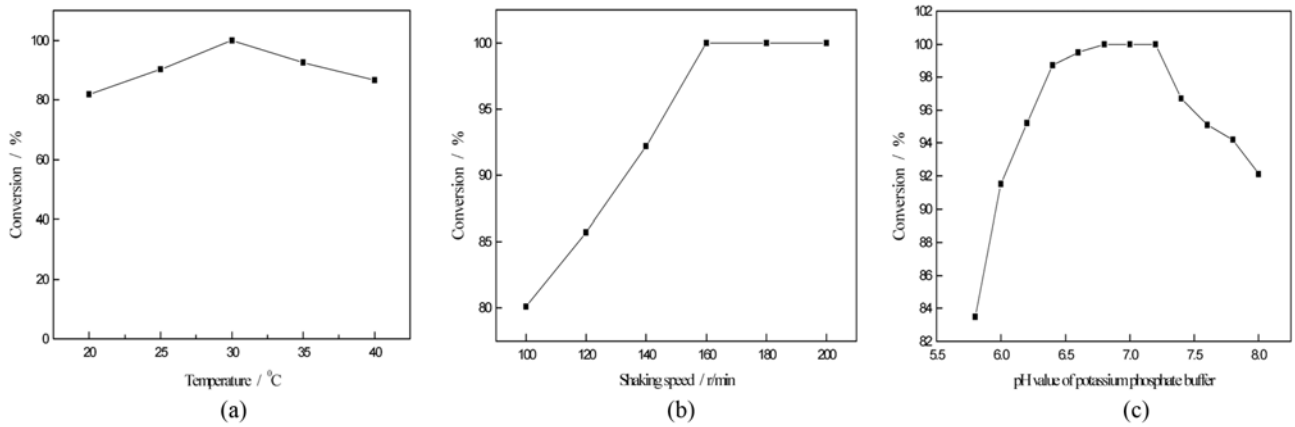

Fig. 7. Effects of temperature (a), shaking speed (b) and pH value of potassium phosphate buffer (c) on reduction. Reduction conditions: Cell dry weights in reduction mixture, 56.0 g/L. Initial substrate concentration, 50 g/L. 60 h.

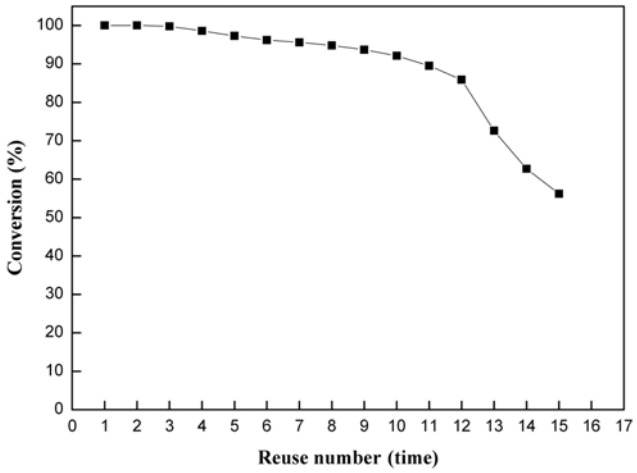

Fig. 8. Recycled uses of liquid-core immobilized cells.

mation can improve productive efficiency. Here, 56.0 g/L liquid-core immobilized cells were used in reduction of 50 g/L substrate for 60 h. Liquid-core immobilized cells were collected for next use in reduction of 50 g/L substrate. The reduction mixture was extracted by ethyl acetate for analysis of conversion and diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate. The results showed that the liquid-core immobilized cells could be reused in reduction 15 times. The reduction activity of liquid-core immobilized cells dropped to 56.2% of the initial reduction activity after 15 uses. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate was >99% during every reduction that used recycled liquid-core immobilized cells as the catalyst.

CONCLUSION

Tert-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate was synthesized by asymmetric reduction of *tert*-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate with liquid-core immobilized cells. Liquid-core immobilized cells showed better reduction ability than free cells or solid-core immobilized cells. The optimized conditions for preparation of liquid-core immobilized cells were found to be as follows: The optimal concentrations of guar gum and $CaCl_2$ in solution A were 2% and 5%. The cell levitation liquid (1 g/ml, cell dry weight/cell levitation liquid volume) and solution A were uniformly mixed together at equal volumes. The mixture was dropped into 0.8% sodium alginate solution to form 2 mm liquid-core capsules. The liquid-core capsules were then dispersed in 0.3% chitosan (1.0×10^5) solution for 30 min to form the film. The asymmetric reduction conditions of *tert*-butyl (S)-6-chloro-5-hydroxy-3-oxo-hexanoate were optimized. The optimal reduction time was found to vary with the initial concentration of substrate. The optimal reduction time was found to be 36 h when the initial substrate concentration was 50 g/L. Conversion increased with longer reduction times and decreased with higher initial concentrations of substrate (when reduction time was constant). The optimum reduction conditions were pH 6.8-7.2, 160 r/min, and 30 °C. Conversion reached 100% when initial substrate concentration was less than 50 g/L. The diastereomeric excess of *tert*-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate was >99%. The liquid-core immobilized cells remained effective after 15 uses.

ACKNOWLEDGEMENTS

We thank the Natural Science Foundation of Zhejiang province (Y4110130) for its financial support of this project.

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