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Enzymatic synthesis of cyclohexyl-α and β-D-glucosides catalyzed by α- and β-glucosidase in a biphase system

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ABSTRACT

Two secondary alcohol glucosides, cyclohexyl-α-D-glucoside and cyclohexyl-β-D-glucoside, were synthesized via the condensation reaction of cyclohexanol with D-glucose in a biphase system catalyzed by α-glucosidase and β-glucosidase, respectively. The effects of pH, water content, glucose concentration and metal ions on the yield of glucosides were studied. The optimum catalytic conditions established for α-glucosidase was 25% (v/v) water content, 2.5 mol/L glucose concentration and pH 2.0, and for β-glucosidase was 30% (v/v) water content, 2.0 mol/L glucose and pH 5.0. The maximum yield of glucoside was 13.3 mg/mL for cyclohexyl-α-D-glucoside and 8.9 mg/mL for cyclohexyl-β-D-glucoside. Synthesis progress was monitored by TLC and quantitatively analyzed by pre-derived capillary gas chromatography (GC). The retention time was 12.34 min for the α isomer and 12.96 min for the β isomer, respectively. With an anomeric purity of more than 99.5%, the two glucosides display excellent site-specific catalysis by α- and β-glucosidase. Herein, we present a general method to produce anomerically pure glucosides via a one-step bio-reaction in a biphase system. This method could potentially be applied in glucosylation of primary and secondary alcohols or other reactions requiring glucosylation.

1. Introduction

Glucosides are ubiquitous in nature and possess diversity of biological properties. Most bioactive glucosides are anomerically pure glucosides, such as gastrodine (*p*-hydroxymethylphenyl-β-D-glucopyranoside) and arbutin (*p*-hydroxyphenyl -β-D-glucopyranoside). According to the spatial orientation of the C_1 hydroxyl in glucose, there are two types of glucosides: α-glucosides and β-glucosides, which are anomers. Alkyl glucosides, a group of non-ionic surfactants, have been used widely in detergents, cosmetics, foods and pharmaceuticals [1,2]. Due to limited natural resources, production of glucosides is traditionally carried out by a chemical process in which by-products, environmental pollution and expensive consumption are inevitable. Also, it is difficult to separate one anomerically pure glucoside from another [1]. An alternative approach is the enzymatic synthesis of glucosides. The enzymatic route offers certain advantages such as having mild reaction conditions, being environmental friendly, and moreover, it enables anomerically pure products to be obtained in a one-step reaction [3–7].

Both α-glucosidase (EC 3.2.1.20) and β-glucosidase (EC 3.2.1.21) are hydrolases for glucosides, but they reverse hydrolysis in favor of synthesis in low-water-activity media, high concentration of glucosyl donor media, or in organic media [8,9]. Corresponding glucosides were obtained through intermolecular condensation between glucose and alcohols in the reverse hydrolysis reactions (Fig. 1.). Methyl-, ethyl-, propyl- [6], butyl- [10], hexyl- [11,12], octyl- [13,14], benzyl- [7], arbutin [15], myrtenyl and neryl β-D-glucosides [16], have been synthesized via condensation reactions, but few of α-D-glucosides have been reported to date [17].

In this paper, we explore a general approach to prepare anomerically pure glucosides through an enzymatic method. In our study, both isomers, cyclohexyl-α and β-D-glucosides, were synthesized through the condensation of glucose and cyclohexanol by α-glucosidase and β-glucosidase catalysis in a biphase system. Moreover, factors affecting the yield, such as water content, glucose concentration, pH, and metal ions were studied. We also investigated the detection method of α-D-glucoside and β-D-glucoside by capillary gas chromatography.

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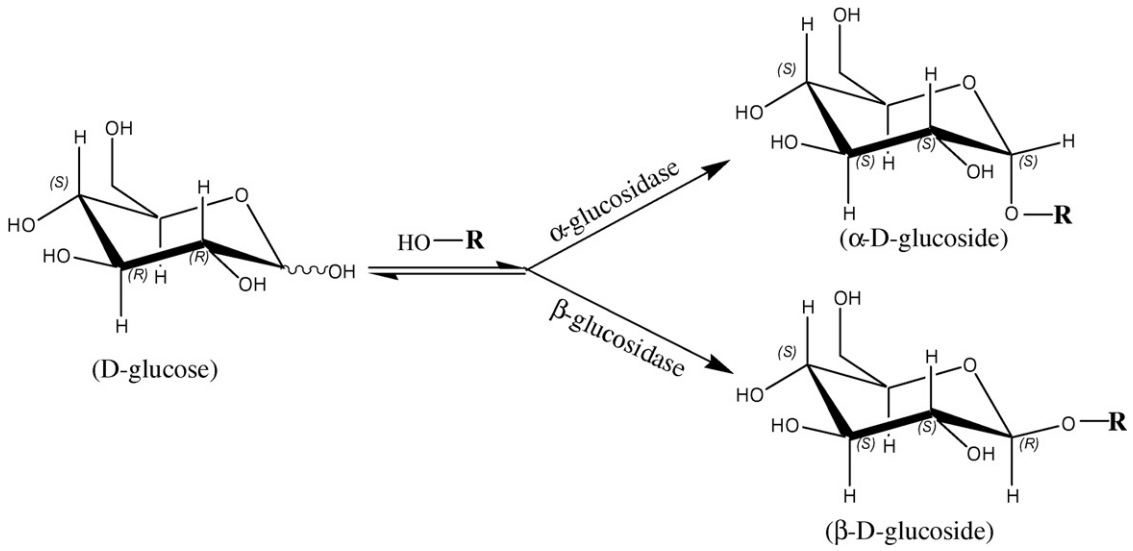

Fig. 1. Enzymatic synthesis of cyclohexyl-α and β-D-glucoside.

2. Materials and methods

2.1. Materials

α-Glucosidase (EC 3.2.1.20) from *Aspergillus niger* (300,000 U/mL) was purchased from Amano Enzyme Inc., Nagoya, Japan, and used without purification in a liquid state. β-Glucosidase (EC 3.2.1.21) from almonds (27 U/mg), *p*-nitrophenyl α- and β-glucoside (α-*p*-NPG, β-*p*-NPG) were purchased from Sigma, St. Louis, MO, USA. Hexamethyl-disilazane (HMDS) and chlorotrimethylsilane (TMS) were purchased from Fluka Chemic (GmbH, Buchis, Germany). Silica gel G and silica gel G plate are from Qingdao Haiyang Chemical Co., Ltd., Shan Dong, China. D-glucose, cyclohexanol and other reagents are all analytical reagents.

2.2. Synthesis of cyclohexyl glucosides in a biphase system

In our biphase system, the aqueous phase consists of 2.0–2.5 mol/L glucose, 0.1 mol/L sodium acetate and enzymes, the organic phase is cyclohexanol presaturated with 0.1 mol/L sodium acetate buffer. In a typical reaction, glucose, sodium acetate buffer and cyclohexanol were pre-equilibrated in a vial tightly screw-capped at 50 °C for 10 min. By adding corresponding glucosidase in above equilibrated reaction system to initialize the reaction, the condensation between cyclohexanol and glucose was carried out under vigorous shaking at 50 °C for 24 h. The reaction process was monitored by thin-layer chromatography (TLC) with acetone:ethyl acetate:cyclohexane (4:1:1) as the developing solvent and with 0.2% 1-naphthoresorcinol (w/v, ethanol) as the visualization reagent at 105 °C for 2 min. By the end of the reaction, the organic phase and the aqueous phase were separated from each other, the cyclohexyl glucoside in the organic phase and the aqueous phase were analyzed by gas chromatography (GC) with β-*p*-NPG as the internal standard. The yield of cyclohexyl glucosides was defined as the amount of products per milliliter reaction mixture (mg/mL). Various concentrations of purified cyclohexyl-α-D-glucoside were used as standard in quantitative analysis.

2.3. Purification of cyclohexyl glucosides

Organic phase was separated form aqueous phase by centrifugation when the reaction finished. Cyclohexanol in the organic phase was recovered by vacuum concentration with the remaining residues containing glucosides. The aqueous phase was extracted with ethyl acetate and the separated ethyl acetate layer was dried by evaporation. The residues of organic and aqueous phases were redissolved in acetone and mixed together. The cyclohexyl glucosides in the mixture were separated from other components by silica gel chromatography with acetone as the mobile phase. The process monitored by TLC was described as above. After collecting the fractions and evaporating acetone, the glucosides and cyclohexyl-α- or cyclohexyl-β-D-glucoside were obtained at last, respectively.

2.4. GC analysis of cyclohexyl glucoside

30 μL of the organic phase mixed with 3 μL β-*p*-NPG (100 mg/mL, pyridine) was put in an uncapped vial and evaporated to dryness at 180–190 °C for 3 min. The residua was redissolved in 30 μL pyridine. Then it was further mixed with 30 μl HMDS and 3 μL TMS. The mixture was incubated at 80 °C for 10 min. Reactant was centrifuged at 12,000 rpm for 1 min, and 1 μL of supernatant was taken out for GC analysis. 30 μL of the aqueous phase mixed with 3 μL β-*p*-NPG (100 mg/mL, pyridine) was extracted twice by acetone. The extracts were mixed and evaporated to dryness at 50 °C. Then the residua was derivatized and analyzed by GC, as above.

The capillary gas chromatography system (GC-2014, Shimadzu Corporation, KYOTO, Japan) was equipped with a silica capillary column (O: 0.25 mm × 30 m, film thickness: 0.25 μm) and FID detector. N_2 was the carrier gas with a pressure of 110 kPa. A typical GC program was performed as follows: 160 °C for 2 min, 10 °C/min from 160 °C to 260 °C, and 260 °C for 5 min was performed. The temperature of the injection chamber and detector were 270 and 300 °C, respectively.

3. Results and discussion

3.1. Water content in the reaction system

The aqueous phase content in the reaction system was titrated from 5% to 35% (v/v), the condensation reaction was carried out in the presence of 9000 U/mL of α-glucosidase or 6 U/mL of β-glucosidase. The results showed that the yield of glucoside was significantly dependent on the water content. For α-glucosidase, the yield of glucosides increases with the increase of water content when it is lower than 30%. For β-glucosidase, the glucoside yield is

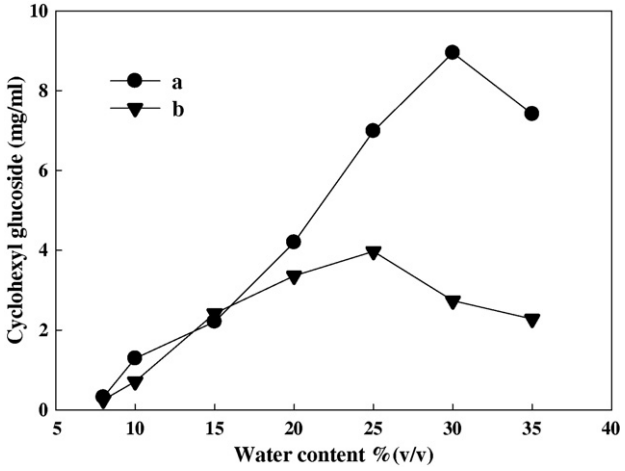

Fig. 2. Effects of water content on the yield of glucosides at 50 °C, 2.0 mol/L glucose, 0.1 mol/L sodium acetate and 9000 U/mL of α-glucosidase (●) or 6 U/mL of β-glucosidase (▼). (a) cyclohexyl-α-D-glucoside; and (b) cyclohexyl-β-D-glucoside.

maximal when the water content is 25% (see Fig. 2). The optimum water content is 30% for α-glucosidase and 25% for β-glucosidase.

It is well known that sufficient water is necessary for enzyme activity. But in the synthesis of glucosides it is necessary to maintain a low water content [8,9]. In the biphase system, the synthesis of glucosides is an interface reaction and the products, cyclohexyl glucosides, will be extracted from reaction interface into organic phase, which is beneficial to the synthesis of glucoside [18]. Our data indicated that it is important to maintain a proper water content to obtain high yields of glucosides, and the optimum water content could be various according to the type or source of enzymes themselves.

3.2. Glucose concentration in the reaction system

The concentration of glucose in aqueous phase of the reaction system was titrated from 0.2 mol/L to 3 mol/L. The condensation reaction was carried out in the presence of α-glucosidase (12,000 U/mL) or β-glucosidase (8 U/mL). Fig. 3 shows the effect of glucose concentration on the yields of cyclohexyl glucosides. The yield increased with the concentration of glucose from 0.5 mol/L to 2.0 mol/L, but there was no visible increase above 2.5 mol/L. The optimum glucose concentration is 2.5 mol/L for α-glucosidase and 2.0 mol/L for β-glucosidase.

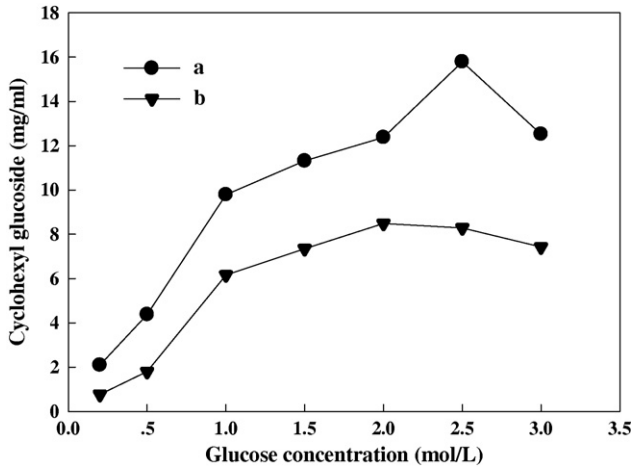

Fig. 3. Effects of glucose concentration on the yield of glucosides at 50 °C, 0.1 mol/L sodium acetate, 25% (v/v) water content and 12,000 U/mL of α-glucosidase (●) or 8 U/mL of β-glucosidase (▼). (a) cyclohexyl-α-D-glucoside; and (b) cyclohexyl-β-D-glucoside.

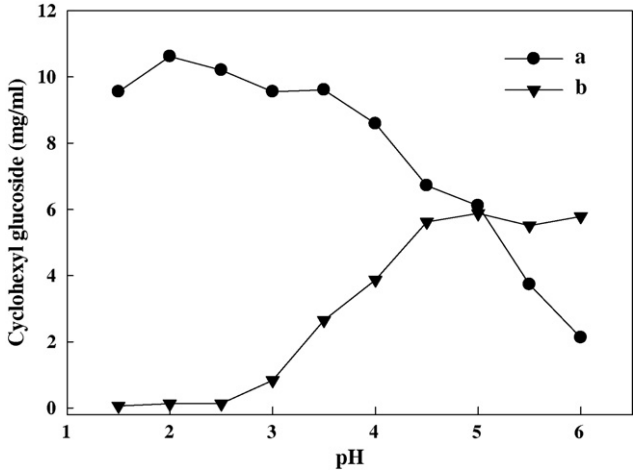

Fig. 4. Effects of pH on the yield of glucosides at 50 °C, 2.5 mol/L glucose, 0.1 mol/L sodium acetate, 25% (v/v) water content and 9000 U/mL of α-glucosidase (●) or 6 U/mL of β-glucosidase (▼). (a) cyclohexyl-α-D-glucoside; and (b) cyclohexyl-β-D-glucoside.

The reverse hydrolysis of glucosidases is thermodynamically controlled [7,11], and a higher concentration of glucose leads a shift of the reaction equilibrium toward the synthesis of glucosides. Here, our experiments show that high glucose concentration is essential for high yield of cyclohexyl glucoside [8,9], while the yield tends to decrease at the higher glucose concentration (above 2.5 mol/L). In the melicera microenvironment of the high glucose concentration, the enzymes were limited to contact with the organic phase, which may have in turn limited the synthesis of glucoside and resulted in lower yield.

3.3. Effects of pH on the yields of glycosides

With the other conditions fixed, sodium acetate buffer (0.1 mol/L) at various pH values ranging from 1.5 to 6.0 were tested in the reaction system. Fig. 4 shows the effects of pH on the

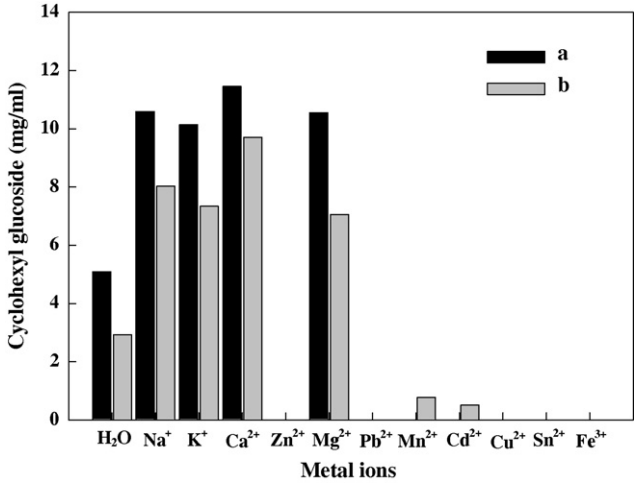

Fig. 5. Effects of metal ions on the yield of glucosides at 50 °C, 2.5 mol/L glucose, 0.1 mol/L sodium acetate, 25% (v/v) water content and 9000 U/mL of α-glucosidase (black) or 6 U/mL of β-glucosidase (gray). (a) cyclohexyl-α-D-glucoside; and (b) cyclohexyl-β-D-glucoside.

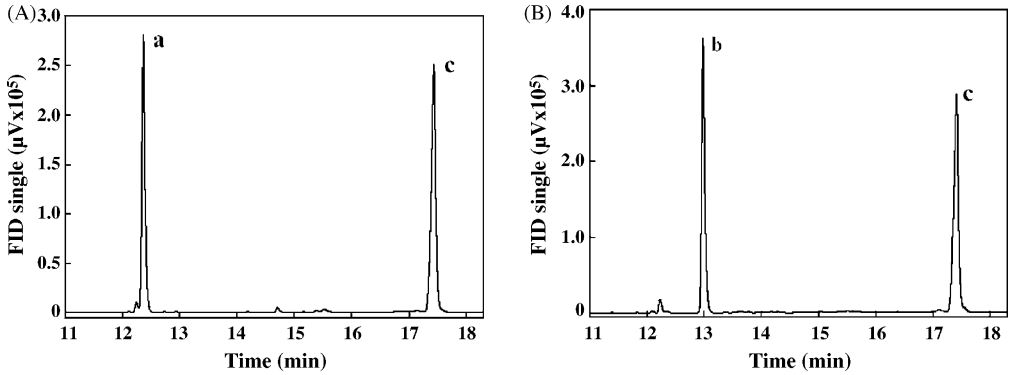

Fig. 6. (A) GC detect of cyclohexyl-α-D-glucoside. (B) GC detect of cyclohexyl-β-D-glucoside. (a) cyclohexyl-α-D-glucoside; (b) cyclohexyl-β-D-glucoside; and (c) β-*p*-NPG.

glycoside yields in the presence of 2.5 mol/L glucose, 25% water content, 9000 U/mL of α-glucosidase or 6 U/mL β-glucosidase. The β-glucosidase from almonds can work well between pH 4.5 and 6.0, with an optimum pH of 5.0 (see Fig. 4b). In contrast to β-glucosidase, α-glucosidase from *A. niger* is an acidophilic enzyme in the synthesis of cyclohexyl-α-D-glucoside. It performed well between pH 1.5 and 3.5 and with an optimum pH 2.0. The yield of cyclohexyl-α-D-glucoside decreased sharply from pH 3.5 to 6.0 (see Fig. 4a). At pH 6.0, it showed only about 20% of the yield compared with that at pH 3.5. This is different from the optimum pH 5.0–5.5 for hydrolase activity in the manufacturer's protocol. Based on these results, our data suggest an acid-activating mechanism in the glucosidase-catalyzed synthesis of cyclohexyl-α-D-glucoside.

3.4. Effects of metal Ions on the yields of glycosides

To inspect whether metal ions are essential to glucosidases during synthesis of glucosides, some univalent, bivalent and trivalent metal ions were selected to prepare the reaction buffer. All metal salt solutions (0.1 mol/L) were adjusted to pH 4.0 with acetic acid, except for Fe^{3+} and Sn^{2+}, which were pH 1.8 and 2.0, respectively. Fig. 5 shows the effects of metal ions on the yields of glucosides under the condition of 2.5 mol/L glucose, 25% water content, 9000 U/mL of α-glucosidase or 6 U/mL β-glucosidase. The two glucosidases can catalyze the synthesis of glucosides in lower yields without metal ion. Na^+, K^+, Ca^{2+} and Mg^{2+} can obviously increase the yields of glucosides, while significantly lower yields were observed in the presence of Zn^{2+}, Pb^{2+}, Mn^{2+}, Cd^{2+}, Cu^{2+}, Sn^{2+} or Fe^{3+}. These results indicate that metal ions are not essential for enzymatic synthesis of glucosides. However, the presence of specific ions can cause a marked increase in the yields. The mechanisms of the effect of these metal ions on the enzyme activity are not known.

3.5. Purification and GC analysis of glucosides

Synthesized glucosides with high anomeric purity (>99.5% by GC assay) were separated easily from other components by silica gel chromatography. The product of cyclohexyl-α-D-glucoside is a yellow oleaginous substance and cyclohexyl-β-D-glucoside is orange oleaginous substance. Cyclohexyl-α-D-glucoside can be crystallized at high concentration to obtain a yellowish crystal, while cyclohexyl-β-D-glucoside cannot be crystallized.

One of the obstacles to studying the saccharide is a lack of an appropriate detection method. GC, HPLC and GC–MS are usually used in saccharide analysis [12,19,20]. Unfortunately, these glucosides could not be separated and detected well in HPLC. Here, a highly sensitive approach to detect α- and β-D-glucosides by capillary GC is described. Derived cyclohexyl-α- and β-D-glucoside can be separated thoroughly by GC with retention times of 12.34 and 12.96 min, respectively (Fig. 6). When slightly modified, this method can be used in the analysis of other glucosides (data not shown).

3.6. The condensation between glucose and cyclohexanol

In the synthesis reactions, high concentrations of the glucosyl donor (glucose) were present in the aqueous phase, while water-immiscible alcohols used as glucosyl acceptors were in the organic phase. The high efficiency of the method was attributed to the shift of the thermodynamic equilibrium due to the extraction of the product from the aqueous phase, where the reaction occurs, into the organic phase [21]. As a reaction model, α- and β-glucosides have been synthesized from some primary and secondary alcohol including *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-octanol, *n*-decyl alcohol, benzenemethanol, 2-phenethanol, 2-pentanol, and 2-octanol. These products can be analyzed by TLC and GC under similar conditions (data not shown).

4. Conclusion

A one-step bio-reaction model has been established to perform the condensation between glucose and alcohols to produce anomeric glucosides. Anomerically pure (above 99.5%) glucosides, cyclohexyl-α- and β-D-glucosides, with yields of 13.3 and 8.9 mg/mL, have been synthesized for the first time by this method. This reaction model can be used in the glucosylation of primary and secondary alcohols or other reactions required glucosylation. At the end of the reactions, glucose and cyclohexanol can be recovered conveniently, which is also cost beneficial. We also developed a highly sensitive approach to detect α- and β-D-glucosides by GC.

As a whole, enzymatic synthesis in biphase system may be a general approach to produce anomerically pure glucosides, which are widely used in detergents, cosmetics, foods and pharmaceuticals. In addition, GC can be more efficiently used in the analysis of anomeric glucosides.

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References

[1] P. Busch, H. Hense, H. Tesmann, et al., Tenside Surfact. Det. 2 (1993) 116–121.
[2] D.B. Marl, Tenside Surfact. Det. 6 (1991) 419–425.

[3] M. Weuthen, R. Kawa, K. Hill, et al., Fat. Sci. Technol. 97 (1995) 209–211.
[4] C. Panintrarux, S. Adachi, Y. Araki, et al., Enzyme Microb. Technol. 17 (1995) 32–40.
[5] P. Monsan, F. Paul, V. Pelenc, et al., Ann. N.Y. Acad. Sci. 799 (1996) 633–641.
[6] N.J. Parry, D.E. Beever, E. Owen, et al., Biochem. J. 353 (2001) 117–127.
[7] E. Kawahara, M. Fujii, K. Kato, et al., Chem. Pharm. Bull. (Tokyo) 53 (2005) 1058–1061.
[8] E.N. Vulfson, R. Patel, J.E. Beecher, et al., Enzyme Microb. Technol. 12 (1990) 950–954.
[9] G. Vic, D. Thomas, Tetrahedron Lett. 33 (1992) 4567–4570.
[10] A. Ismail, S. Soultani, M. Ghoul, Biotechnol. Prog. 14 (1998) 874–878.
[11] Q. Yi, D.B. Sarney, J.A. Khan, et al., Biotechnol. Bioeng. 60 (1998) 385–390.
[12] P. Turner, D. Svensson, P. Adlercreutz, et al., J. Biotechnol. 130 (2007) 67–74.
[13] O.S. Kuptsova, N.L. Kliachko, A.V. levashov, Bioorg. Khim. 27 (2001) 429–433.
[14] A. Ducret, M. Trani, R. Lortie, Biotechnol. Bioeng. 77 (2002) 752–757.
[15] Y.H. Moon, S.H. Nam, J. Kang, et al., Appl. Microbiol. Biotechnol. 77 (2007) 559–567.
[16] E. Kawahara, M. Fujii, Y. Ida, et al., Chem. Pharm. Bull. (Tokyo) 54 (2006) 387–390.
[17] J. Kosaĭry, Eĭ. Stefanovits-Baĭnyai, L. Boross, J. Biotechnol. 66 (1998) 83–86.
[18] C. Panintrarux, S. Adachi, Y. Araki, Enzyme Microb. Technol. 17 (1995) 32–40.
[19] S. He, S.G. Withers, J. Biol. Chem. 272 (1997) 24864–24867.
[20] H.L. Yu, J.H. Xu, W.Y. Lu, G.Q. Lin, J. Biotechnol. 133 (2008) 469–477.
[21] M.-P. Bousquet, R.-M. Willemot, P. Monsan, et al., Appl. Microbiol. Biotechnol. 50 (1998) 167–173.