J. Dairy Sci. 92:491–498
doi:10.3168/jds.2008-1618

Immobilization of recombinant thermostable β-galactosidase from *Bacillus stearothermophilus* for lactose hydrolysis in milk

W. Chen,*[1] H. Chen,* Y. Xia,* J. Yang,* J. Zhao,* F. Tian,* H. P. Zhang,† and H. Zhang*
*State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, China
†Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Huhhot, Inner Mongolia 010018, P. R. China

ABSTRACT

A recombinant thermostable β-galactosidase from *Bacillus stearothermophilus* was immobilized onto chitosan using Tris(hydroxymethyl)phosphine (THP) and glutaraldehyde, and a packed bed reactor was utilized to hydrolyze lactose in milk. The thermostability and enzyme activity of THP-immobilized β-galactosidase during storage was superior to that of free and glutaraldehyde-immobilized enzymes. The THP-immobilized β-galactosidase showed greater relative activity in the presence of Ca^{2+} than the free enzyme and was stable during the storage at 4°C for 6 wk, whereas the free enzyme lost 31% of the initial activity under the same storage conditions. More than 80% of lactose hydrolysis in milk was achieved after 2 h of operation in the reactor. Therefore, THP-immobilized recombinant thermostable β-galactosidase from *Bacillus stearothermophilus* has the potential for application in the production of lactose-hydrolyzed milk.
Key words: β-galactosidase, thermostable, immobilization, lactose hydrolysis

INTRODUCTION

Lactose is the major carbohydrate present in milk and is not easily digested by a significant fraction of the global population (Panesar et al., 2006). Lactose ingested by lactose-intolerant people cannot be hydrolyzed owing to low levels of β-galactosidase in the jejunum; the passage of lactose to the large intestine can lead to tissue dehydration, poor calcium absorption, generation of hydrogen and carbon dioxide gases, diarrhea, bloating, flatulence, blanching, and cramps (Shukla, 1975).

β-Galactosidase (EC 3.2.1.23) is the gene product of the lacZ operon and an important industrial enzyme that is used in the hydrolysis of lactose in milk and whey to avoid the health and environmental problems posed by lactose (Ladero et al., 2005; Panesar et al., 2006). Although β-galactosidase has been found in a wide variety of sources including microorganisms, plants, and animals, the only commercially exploited sources are microorganisms (Agrawal et al., 1989). The maximum activity of β-galactosidase from fungi is generally at pH 3 to 4, whereas the optimum pH of β-galactosidase from yeasts and bacteria is typically in the range of pH 6 to 7. Because the normal pH of milk is around 6.5 to 6.6, β-galactosidase from yeasts and bacteria is more suitable for application in the production of lactose-hydrolyzed milk than that from fungi (Ladero et al., 2005).

In the past decade, thermophilic bacteria have become an object of interest for the commercial production of β-galactosidase; the thermostable β-galactosidases generally have maximum activity at temperatures ranging from 70 to 90°C (Petzelbauer et al., 1999; Pessela et al., 2003; Cheng et al., 2006). When compared with mesophilic enzymes, the application of thermostable enzymes in the production of lactose-hydrolyzed products has significant advantages such as greater reaction velocity, reduced risk of microbial contamination, and longer enzyme half life under operational conditions (Hirata et al., 1985; Petzelbauer et al., 1999; Maciunska et al., 2000).

Because the thermostable β-galactosidase was reported to be only poorly produced in the original host, which was not suitable for large-scale production (Bruins et al., 2001; Kang et al., 2005), recombinant techniques have been applied in the production of thermostable β-galactosidase in a mesophilic host (Kang et al., 2005). In our previous research, a thermostable β-galactosidase from *Bacillus stearothermophilus* was cloned and successfully expressed in *Bacillus subtilis* WB600 (Chen et al., 2008). β-Galactosidase from *B. subtilis* expression system has potential application in dairy industry (Chen et al., 2002) because *B. subtilis* is considered a safe source (Mahoney, 2003).

Because application of immobilized enzyme in lactose hydrolysis could reduce the processing cost, ensure bet-

Received August 11, 2008.
Accepted October 22, 2008.
[1]Corresponding author: weichen@jiangnan.edu.cn

ter control of the process, and eliminate the inhibitory effect of galactose on β-galactosidase, the immobilization technique was applied in the present study to the recombinant thermostable β-galactosidase. The objective of this study was to optimize the immobilization conditions of the thermostable β-galactosidase from the recombinant *B. subtilis* WB600/pMA5-bgaB and explore its application in lactose hydrolysis in milk.

MATERIALS AND METHODS

Materials

Chitosan was obtained from Golden-Shell Biochemical (Zhejiang, China). Tetrakis(hydroxymethyl) phosphonium chloride (80%, wt/vol) was obtained from Sunrise Science and Trade (Tianjin, China). Glutaraldehyde (25%, wt/vol) was obtained from Wulian Chemicals Co. Ltd. (Shanghai, China). *O*-nitrophenyl-β-D-galactopyranoside (**ONPG**) was purchased from Sigma Chemicals (Sigma-Aldrich Corp., St. Louis, MO). All other reagents were of analytical grade.

Escherichia coli TG1 was used as host strain for cloning and for the preparation of template plasmids. *Bacillus stearothermophilus* ATCC8005 was the donor of thermostable β-galactosidase coding sequence, and *B. subtilis* WB600 was used for expression of β-galactosidase. The plasmid pBSKII KS+ was the clone vector, and pMA5 was used for construction of the thermostable β-galactosidase expression plasmid in *B. subtilis*.

Expression and Extraction of the Recombinant Thermostable β-Galactosidase

The construction of expression vector and expression of the recombinant thermostable β-galactosidase was conducted according to the method described by Chen et al. (2008). The cells of *B. subtilis* WB600/pMA5-bgaB were then suspended in 20 m*M* sodium phosphate buffer (pH 7.0) and treated with sonication. The supernatant was collected after centrifugation at 4°C at 9,000 × *g* for 10 min (J2–21M centrifuge, Beckman Coulter Inc., Fullerton, CA). Subsequently, the solution was heated at 60°C for 50 min and centrifuged at 30,000 × *g* for 30 min, and 30% ammonium sulfate was added to the supernatant at 4°C. After the centrifugation at 30,000 × *g* for 30 min, the pellet was discarded, and the supernatant was brought to 65% ammonium sulfate saturation and centrifuged at 30,000 × *g* for 30 min again. The pellet was dissolved in 20 m*M* sodium phosphate (pH 7.5), dialyzed at 4°C for 24 h, and lyophilized.

Immobilization of β-Galactosidase and Optimization of Immobilization Conditions

The enzyme immobilization was prepared by reaction of soluble β-galactosidase with chitosan activated by the coupling agent Tris(hydroxymethyl)phosphine (**THP**) and glutaraldehyde according to the method described by Oswald et al. (1998). According to preliminary results, β-galactosidase was dissolved in 0.1 *M* sodium phosphate buffer (pH 7.0) and mixed with 0.63 mg/mL THP-activated and 0.25% glutaraldehyde-activated chitosan solution to reach 100 U/mL, respectively. The suspensions of the enzyme with THP and glutaraldehyde were shaken at room temperature for 10 min and 1 h, respectively. Then, they were washed free of excess of coupling agents with distilled water and a 1 *M* NaCl solution, respectively. Experiments were performed under the following conditions to optimize the immobilization procedure: at 5 immobilization durations of 15, 30, 60, 120, and 180 min; at 3 immobilization temperatures of 30, 40, and 50°C; and at 4 pH values of 6.0, 6.5, 7.0, and 7.5.

Effect of Temperature, pH, and Metal Ions on Enzyme Activity

The effect of temperature on the activity of free and immobilized β-galactosidase was determined with a 5-h incubation at 60, 65, and 70°C (pH 7.0) before analysis. The effect of pH (pH 6.0 to 8.0) on the relative enzyme activity of free and immobilized β-galactosidase was determined with a 5-h incubation at 70°C before analysis. The effects of univalent cations K^+ and Na^+, and divalent cations Mn^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, and Ca^{2+} (10 m*M*) on the enzyme activity of free and immobilized β-galactosidase was determined after the incubation at 25°C for 20 min in potassium phosphate buffer (200 m*M*, pH 6.5) with various reagents.

Reusability and Storage Stability of Immobilized Enzyme

The reusability of the immobilized enzyme was evaluated by incubation with ONPG 9 times at 70°C for 10 min (between each successive use, the immobilized enzyme was washed with distilled water). The storage stabilities of the free and immobilized enzymes were evaluated during storage at 4°C for 6 wk (in 0.1 *M* potassium phosphate buffer, pH 7.0). The samples were analyzed for the residual activity every week. The thermostability of β-galactosidase was measured after 5 h of incubation at 60, 65, and 70°C.