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# Immobilization of the Antarctic *Bacillus* sp. LX-1 α-Galactosidase on Eudragit L-100 for the Production of a Functional Feed Additive

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**ABSTRACT:** Partially purified α-galactosidase from *Bacillus* sp. LX-1 was non-covalently immobilized on a reversibly soluble-insoluble polymer, Eudragit L-100, and an immobilization efficiency of 0.93 was obtained. The optimum pH of the free and immobilized enzyme was 6.5 to 7.0 and 7.0, respectively, while there was no change in optimum temperature between the free and immobilized α-galactosidase. The immobilized α-galactosidase was reutilized six times without significant loss in activity. The immobilized enzyme showed good storage stability at 37°C, retaining about 50% of its initial activity even after 18 d at this temperature, while the free enzyme was completely inactivated. The immobilization of α-galactosidase from *Bacillus* sp. LX-1 on Eudragit L-100 may be a promising strategy for removal of α-galacto-oligosaccharides such as raffinose and stachyose from soybean meal and other legume in feed industry. (**Key Words:** α-Galactosidase, *Bacillus*, Eudragit L-100, Immobilization, α-Galacto-oligosaccharides, Feed Industry)

## INTRODUCTION

Soybean is a legume crop and a premier protein source of foods and feeds globally, owing to its well-balanced amino acid pattern (Thippeswamy and Mulimani, 2002; Stein et al., 2008). Nevertheless, the use of soybean-based foods or feeds has been occasionally restricted because they contain α-galacto-oligosaccharides such as raffinose and stachyose, which act as anti-nutritive factors (De Lumen, 1992; Anderson and Wolf, 1995; Prashanth and Mulimani, 2005). Monogastric animals including human, swine, and poultry cannot synthesize sufficient α-galactosidase (EC 3.2.1.22) in their gastrointestinal tracts (Karr-Lilienthal et al., 2005; Prashanth and Mulimani, 2005), which is an exo-glycosidase catalyzing the hydrolysis of terminal non-reducing α-1,6-linked galactosyl residues from a wide range of galacto-oligosaccharides and polysaccharides (Naumoff, 2004). Thus, the α-galacto-oligosaccharides pass into the large intestine, where the resident microbita ferment them into carbon dioxide, hydrogen, and methane, which lead to flatulence and gastrointestinal disorder (Steggerda et al., 1966; Prashanth and Mulimani, 2005). Accordingly, degradation of the oligosaccharides from the soybean-based products by α-galactosidase could be an efficacious tool to solve the nutritional problem (Ghazi et al., 2003; Gote et al., 2004; Falkoski et al., 2009).

Considerable attention has been paid to immobilized enzymes, because of their favorable features such as reusability, stability, and separation of the enzymes from products, compared to free counterparts (Falkoski et al., 2009). Particularly, concerning animal nutrition and feed science, the immobilization technique has been applied in developing a kit for measuring *in vitro* amino acid digestibility of soybean meal, which could be correlated to poultry true amino acid digestibility (Schasteen et al., 2007) and in preparing a forage additive (Monica et al., 2008). Up to now, several matrices including polyacrylamide, alginate, chitin, silica gel, Amberlite IRA-938, and Sepabeads EC have been used as support materials for preparing immobilized α-galactosidases (Onal and Telefoncu, 2003a,b; Prashanth and Mulimani, 2005; Falkoski et al., 2009; Bayraktar et al., 2011). Additionally, there is much interest in Eudragit L-100 (a copolymer of methacrylic acid and methylmethacrylate) which was utilized as a reversibly soluble and insoluble immobilization matrix for immobilization of the widely used technological enzymes

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such as xylanase (Sardar et al., 2000; Gaur et al., 2005), cellulase (Zhang et al., 2010), and amylase (Cong et al., 1995), as well as a pH-dependent enteric coating polymer for the efficacious colon-specific drug delivery system (Venkatesh et al., 2009).

To the best of our knowledge, the present study is the first report on the immobilization of α-galactosidase on Eudragit L-100. The α-galactosidase was derived from an Antarctic bacterial isolate, *Bacillus* LX-1 as previously described (Lee et al., 2012).

## MATERIALS AND METHODS

### Reagents

Eudragit L-100, which is a copolymer of methacrylic acid and methylmethacrylate at a ratio of 1:1, was a product of Rohm Pharma, Weiterstadt, Germany. The substrate, *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

### Preparation of free α-galactosidase

*Bacillus* sp. LX-1 was cultivated in 25 ml of Luria-Bertani (LB) medium supplemented by containing 1% galactose in an Erlenmeyer flask of 250 ml capacity for 24 h at 28°C. Then, 1 L of the same medium in two Erlenmeyer flasks of 2 L capacity was aseptically inoculated with 1% seed culture broth and aerobically grown with vigorous shaking (220 rpm) for 48 h at 28°C. The culture medium containing secreted α-galactosidase was centrifuged (10,000×g; 20 min; 4°C) to remove cell, and then protein in the supernatant was precipitated with ammonium sulfate (75% saturation). The pellet was dissolved in 25 mM Tris-HCl (pH 8.0) and dialyzed overnight against 25 mM Tris-HCl (pH 7.4) at 4°C. The dialyzed solution was used as the free enzyme throughout this work.

### Immobilization of α-galactosidase on Eudragit L-100

2% Eudragit L-100 solution was prepared as previously described (Roy et al., 2003). The free enzyme (0.1 to 2 ml) was added to 0.75 ml of the Eudragit L-100 solution and the final volume was made up to 5 ml with 50 mM sodium phosphate (pH 7.0). After 1 h incubation at room temperature, polymer was precipitated by lowering pH to 4.0 with 3 M acetic acid. After 20 min, the suspension was spun down by centrifugation (12,000×g, 20 min). The precipitate was washed with 4 ml of 10 mM sodium acetate (pH 4.0) until no enzyme activity was detected in the washings. Finally, the pellet so formed was suspended in 5 ml of 50 mM sodium phosphate (pH 7.0) and used as the immobilized enzyme preparation.

### Enzyme activity assay

The α-galactosidase activity was determined by the amount of *p*-nitrophenol released from the *p*NPG. The reaction mixture (1 ml) containing 0.1 ml of diluted soluble enzyme (free or immobilized), 0.8 ml of 50 mM sodium phosphate (pH 7.0 ) and 0.1 ml of 10 mM pNPG in 50 mM sodium phosphate (pH 7.0) was incubated at 40°C for 15 min. The reaction was terminated by adding 1 ml of 1 M $Na_2CO_3$. The absorbance was measured at 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per minute under the assay conditions.

### Reusability of the immobilized enzyme

The immobilized α-galactosidase was used for repeated cycles to check its reusability. The preparation was incubated at 40°C with 1 mM *p*NPG in 50 mM sodium phosphate (pH 7.0) for 15 min. After each cycle of hydrolysis, the pH of the supernatant was lowered to 4.0 by adding 0.2 ml of 3 M acetic acid when the immobilized enzyme was precipitated. This was collected by centrifugation (12,000×g, 30 min) and the amount of released *p*-nitrophenol in supernatant estimated according to the analytical method described above. For running the second cycle, the immobilized enzyme was redissolved in 5 ml of 50 mM sodium phosphate (pH 7.0) and added to the *p*NPG and processed the same way as before.

### Determination of pH optimum

The effects of optimum pH on the free and immobilized LX-1 α-galactosidase were investigated by assaying both preparations in the pH range of 3 to 8.5 (50 mM glycine-HCl (pH 3); 50 mM sodium acetate (pH 4 to 5.5); 50 mM sodium phosphate (pH 6 to 7) and 50 mM Tris-HCl (pH 7.4 to 8.5)) at 30°C.

### Determination of optimum temperature

The effects of optimum temperature on the free and immobilized enzyme were determined by assaying their activities at different temperatures (0 to 80°C) in 50 mM sodium phosphate (pH 7.0).

### Determination of shelf storage stability

For testing the shelf storage stability of enzymes, both free and immobilized LX-1 α-galactosidase were stored at 37°C for 18 d. Then the remaining activity of the enzymes was measured under the standard assay conditions.

## RESULTS AND DISCUSSION

### Immobilization of LX-1 α-galactosidase

LX-1 α-galactosidase was immobilized on the smart polymer, Eudragit L-100, which is a relatively cheap, easily

available, non-toxic, and water soluble support, and which binds non-covalently to proteins (Gaur et al., 2005). The optimal immobilization efficiency of 0.93 was achieved at 0.77 U of enzyme load and, beyond the amount of enzyme, the immobilization efficiency tended to decrease (Figure 1). Figure 2 depicted the activity staining of the immobilized enzyme. The decrease of immobilization efficiency with larger enzyme load is generally caused by overcrowding of the enzyme on the Eudragit surface (Sardar et al., 2000; Roy et al., 2003). Meanwhile, only 0.55 of the optimal immobilization efficiency was observed when α-galactosidase from *Penicillium griseoroseum* was immobilized onto modified silica gel using glutaldehyde linkages (Falkoski et al., 2009). Thus, the immobilization yield of LX-1 α-galactosidase seemed to be acceptable.

**Reusability of immobilized enzyme**

As an immobilization matrix, the merit of Eudragit L-100 is its solubility during catalysis with its insolubility below pH 4.0, enabling catalyst recovery and separation of the soluble reaction products by lowering the pH (Roy et al., 2003; Gaur et al., 2005). Reusability of an immobilized enzyme is of great importance for economical use of the enzyme in repeated batch or continuous processes (Bayraktar et al., 2011). As shown in Figure 3, the immobilized LX-1 α-galactosidase could be recycled for six times without significant loss in activity, despite the fact that the enzyme was recovered by lowering the pH to 4.0 after each cycle. Such acidic process for enzyme recovery, which has routinely used in other enzymes immobilized on Eudragit matrices (Gaur et al., 2005; Silva et al., 2006; Zhang et al., 2010) did not have any negative effect on the LX-1 α-galactosidase activity. This may be somewhat associated with previous report that immobilization of

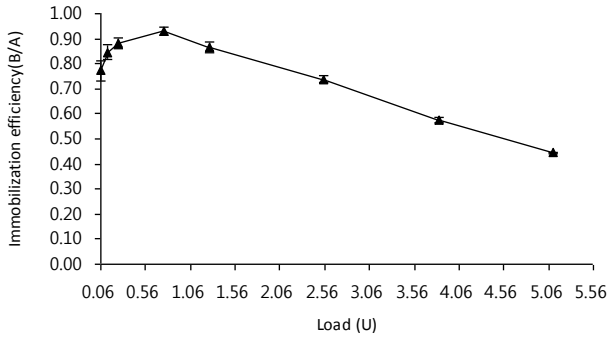


**Figure 1.** Immobilization of LX-1 α-galactosidase on Eudragit L-100. "A" represents the amount of enzyme theoretically bound to the matrix. This is calculated by subtracting the unbound activity in the supernatant from initially added enzyme. "B" represents the expressed activity of the particular immobilized preparation, measured after incubating the immobilized enzyme with the substrate. Data were expressed as mean±standard error from three experiments.

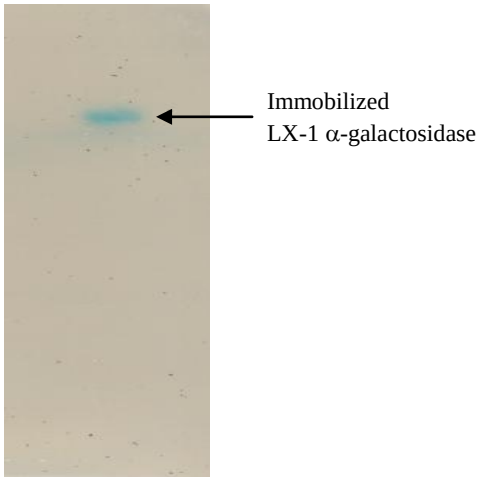


**Figure 2.** Zymogram analysis of α-galactosidase activity in the immobilized enzyme.

*Aspergillus niger* xylanase on Eudragit L-100 has led to stabilization of the enzyme toward alternate exposure to pH 4.0 and pH 5.5 (Sardar et al., 2000).

**Effect of pH on activity of free and immobilized enzyme**

As shown in Figure 4, the optimum pH values for the free and immobilized LX-1 α-galactosidase were 6.5 to 7.0 and 7.0, respectively. Although the pH profile change in immobilized enzymes was very common (Thippeswamy and Mulimani, 2002; Bora et al., 2005; Sanjay and Sugunan, 2005), immobilization of enzymes on the reversibly soluble-insoluble polymers such as Eudragit L-100 and Eudragit S-100 had frequently marginal effect on the optimal pH activities (Ai et al., 2005). For instance, there were no and slight changes in the optimum pH values for *Aspergillus niger* and *Scytalidium thermophilum* xylanase immobilized on Eudragit L-100, respectively (Sardar et al., 2000; Gaur et al., 2005). In addition, the optimum pH value of tomato α-galactosidase immobilized on galactose-containing polymeric beads and the free enzyme was identically pH 4.0 (Okutucu et al., 2010).

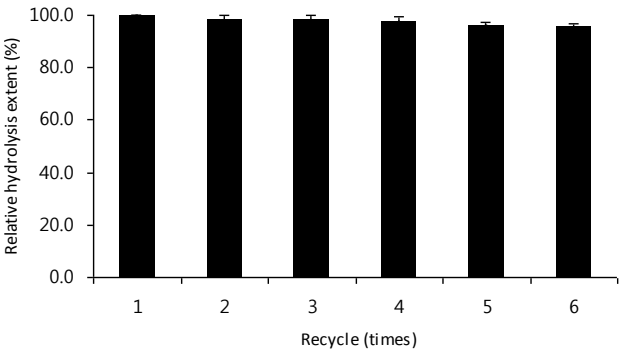


**Figure 3.** Reusability of the immobilized LX-1 α-galactosidase. Data were expressed as mean±standard error from three experiments.

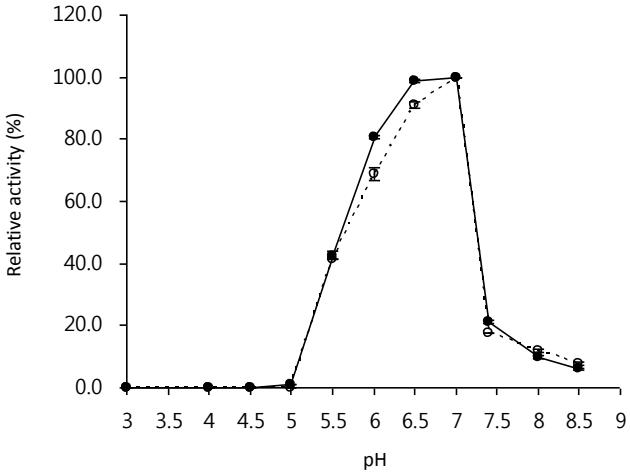


**Figure 4.** Effect of pH on LX-1 α-galactosidase activity. Data were expressed as mean±standard error from three experiments. Immobilized enzyme (open circle with dotted line); free enzyme (closed circle with solid line).

### Effect of temperature on activity of free and immobilized enzyme

The optimum temperature for the free and immobilized LX-1 α-galactosidase was 40°C (Figure 5). Immobilization had no influence on the optimum temperature of the enzyme, which was also observed in a commercial protease, Esperase, immobilized on Eudragit S-100 (Silva et al., 2006), the *Penicillium griseoroseum* α-galactosidase immobilized on silica gel (Falkoski et al., 2009) and the tomato α-galactosidase immobilized on galactose-containing polymeric beads (Okutucu et al., 2010). However, the optimum temperature of the *Streptomyces olivaceoviridis* E-86 xylanase immobilized on Eudragit S-100 moved from 60°C (the optimum temperature of free enzyme) to 65°C (Ai et al., 2005).

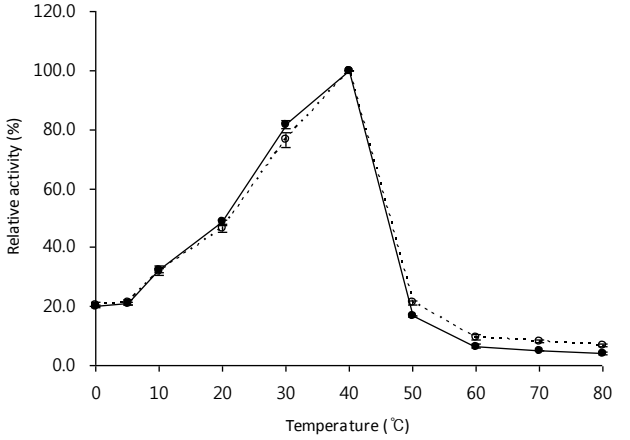


**Figure 5.** Effect of temperature on LX-1 α-galactosidase activity. Data were expressed as mean±standard error from three experiments. Immobilized enzyme (open circle with dotted line); free enzyme (closed circle with solid line).

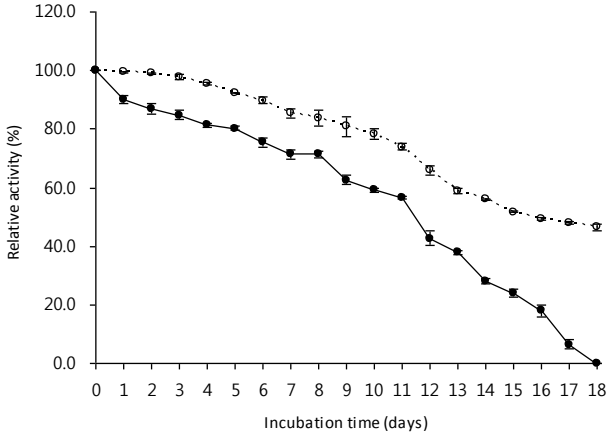


**Figure 6.** Storage stability of free and immobilized LX-1 α-galactosidase at 37°C. Data were expressed as mean±standard error from three experiments. Immobilized enzyme (open circle with dotted line); free enzyme (closed circle with solid line).

### Storage shelf stability of free and immobilized enzymes

In general, an enzyme in solution is not stable during storage and the activity is gradually decreased over time (Bayraktar et al., 2011). From a practical point of view, it is an important criterion that an enzyme designed for use in animal feed should survive storage at ambient temperature (Francesch and Perez-Vendrell, 1997; Sulabo et al., 2011). As shown in Figure 6, the storage stability at 37°C was greatly improved by immobilization. The immobilized LX-1 α-galactosidase retained about 50% of its initial activity even after 18 d at this temperature, while the free enzyme was completely inactivated. The improved stability of immobilized enzymes may be associated with the prevention of autolysis (Sharma et al., 2003). In addition, the storage stability of immobilized enzymes varies depending on the applied immobilization method and storage conditions (Onal and Telefoncu, 2003b; Celem and Onal, 2009; Okutucu et al., 2010).

## CONCLUSIONS

Nowadays, advanced feed production can rely on biotechnological approaches. The immobilization of α-galactosidase from *Bacillus* sp. LX-1 on Eudragit L-100 may be a promising strategy for removal of α-galacto-oligosaccharides such as raffinose and stachyose from soybean meal and other legume in feed industry, which enables to increase the available energy and feed efficiency (Suarez et al., 1999; Vila and Mascarell, 1999), due to its relatively durable enzyme activity and good storage stability.

## ACKNOWLEDGEMENTS

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