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Stabilization of β-Galactosidase (from Peas) by Immobilization onto Amberlite MB-150 Beads and Its Application in Lactose Hydrolysis

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The soluble *Ps*BGAL (from *Pisum sativum*) is extremely unstable with loss of over 80% in enzyme activity within 24 h at 4 °C when the protein concentration was lower than 0.1 mg/mL. Enzyme immobilization onto Amberlite MB-150 beads (diameter = 5 μm) greatly stabilized the enzyme preparation, with almost no loss for 12 months at room temperature (27 °C). Enzyme (21.9 μg) was immobilized by 62.56% onto activated 100 mg of Amberlite MB-150 beads using 4% glutaraldehyde, at pH 6.0 (50 mM, sodium phosphate buffer). Statistical analysis carried out by ANOVA revealed that all parameters used during immobilization were equally important at $P < 0.05$ (level of significance). An approach toward commercial exploitation of Amberlite−*Ps*BGAL especially in lactose hydrolysis was anticipated due to improved physicochemical properties including broad optimum pH and temperature, with a K_m of 4.11 ± 0.21 mM for lactose. Amberlite−*Ps*BGAL hydrolyzed 64.57 and 69.18% of lactose present in milk and milk whey, respectively, within 10 h (at room temperature). Immobilized enzyme has reusability of over 10 batchwise uses, with almost no loss in activity. The easy accessibility of enzyme source, ease of its immobilization on Amberlite, lower cost of Amberlite, enhanced stability of Amberlite−*Ps*BGAL, and comparable lactose hydrolysis in milk and milk whey described here make it a suitable product for future applications at laboratory and industrial scale.

KEYWORDS: β-Galactosidase; *Pisum sativum*; Amberlite MB-150; immobilization; lactose hydrolysis

INTRODUCTION

Immobilized biocatalysts (enzymes and whole cells) are at the heart of innovative biotechnological processes as an alternative to traditional chemical technologies due to their high specificity, ease in product separation, and continuous operation. Irrespective of various uses of immobilized biocatalyst, their kinetic and operational specifications help in screening and designing a bioreactor to be used at an industrial scale. The most important modulating parameters of immobilized biocatalyst that are taken under consideration are temperature stability and reusability and other factors such as broad pH stability and catalytic efficiency. For successful development and application of an immobilized biocatalyst, the enzyme support is generally considered to be the most important component contributing to the performance of the reactor system. Batch reactors are better adapted for treatment of food products than continuous reactors due to higher risk of contamination, requirement of sophisticated expertise, and diffusional constraints in the later ones (*1*). Furthermore, food products produced by continuous culturing are unacceptable by U.S. Food and Drug Administration (FDA) guidelines. Therefore, the food industry always tries to approach batch mode for large-scale process.

Milk is an aqueous mixture consisting mainly of salt, sugar, and protein. The primary sugar in milk is lactose (4.3−4.5%), a disaccharide composed of glucose and galactose. Lactose causes intolerance to a great number of the human population, also being a low soluble sugar, turning the milk into a raw material that is difficult to use in concentrated and frozen products (*2*, *3*). To minimize such problems, an adequate solution would be to carry out lactose hydrolysis by β-galactosidase (BGAL) enzyme. Implementation of enzyme in its immobilized form by the industries based on enzymatic processes led to effective reduction in cost using process operation, which is optimized with a good immobilization technology. BGAL has been successfully immobilized onto various matrices such as metal chelated gels (*4*), epoxy activated acrylic beads (*5*), thiosulfonate agarose (*6*), *p*-amino carbanylated cellulose derivatives (*7*), and silica and agarose (*8*). Some of these methods suffer from low immobilization yields and continuous leakage of enzyme. Industrially used BGAL preparations are from either bacterial or fungal sources. No papers based on lactose hydrolysis by BGAL from plant sources, which could be a better substitute due to its easy availability and cost effectiveness, are available.

Amberlite has been used as a matrix for immobilization for various enzymes such as urease (*9*), lipase (*10*), and α-galactosidase (*11*), and even the immobilization of fructosyltransferase has been patented by the United States (patent 5215905).

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Various pharmaceutical companies such as Advanced Biosciences have commercialized Amberlite for immobilizing various enzymes, including penicillin acylase, lysozyme, β-amylase, and lipase. Apart from immobilization, the Amberlite beads have several advantages over soft gels, especially in processes that require a fast flow rate or high pressure because the flow rates with these beads are linear with pressure. The mechanical strength of these beads could provide reproducible results with constant column parameters. Also, this matrix is resistant to biological degradation and compatible with almost all organic solvents and most concentrated acids. Furthermore, enzyme immobilization using glutaraldehyde has been much desirous due to its ability to react with the lysine amino groups of enzyme and cross-linking it with the matrix. Glutaraldehyde coupling is an enigma that deserves special attention as, it could either stabilize the enzyme, due to the multipoint attachment, presumed to occur with glutaraldehyde, or prevent unfolding of the protein. Furthermore, the polymeric nature of glutaraldehyde provides a long lash, attaching the protein to the matrix and permitting greater flexibility for conformational changes required for activity (*12*).

Here we report immobilization of *Ps*BGAL isolated from seeds of pea (*Pisum sativum*) onto Amberlite MB-150 beads using glutaraldehyde. Comparisons of various physicochemical properties of immobilized enzyme with the soluble enzyme have been carried out. Furthermore, the overall performance of the immobilized enzyme was evaluated for lactose hydrolysis of milk and whey for which the biocatalyst was intended using batch mode.

MATERIALS AND METHODS

Chemicals. All of the chemicals for buffers and other reagents were of analytical grade or electrophoresis grade. Unless stated, all chemicals were purchased from Sigma (St. Louis, MO). Milli-Q water with a resistance of >18 MΩ was used throughout the experiments. Dry seeds of pea (*P. sativum* var. *arvense* AP-3) were generously provided by the Indian Institute of Vegetable Research (IIVR), Varanasi, India.

Enzyme Preparation. *Ps*BGAL was purified from soaked pea seeds (in 25 mM sodium phosphate buffer, pH 6.8, overnight at 4 °C) using the following steps, discussed here briefly [the protocol for *Ps*BGAL isolation from pea is part of a manuscript titled "A β-Galactosidase from Pea (*Pisum sativum* L.) Seeds (*Ps*BGAL): Purification, Stabilization, Catalytic Energetics, Conformational Heterogeneity, and Its Significance" (Dwevedi, A.; Kayastha, A. M., submitted for publication]. Buffers used in various steps of purification contained 1 mM DTT, 1 mM PMSF, and 0.02 mM EDTA unless stated otherwise. Seeds were crushed using a kitchen blender in 25 mM sodium phosphate buffer, pH 6.8 (buffer A). Crude extract prepared by filtering using two layers of muslin cloth and centrifuging at 8420*g* for 20 min at 4 °C. Supernatant was collected and precipitated using 40−55% ammonium sulfate and centrifuged. Pellet was dissolved in a minimum volume of buffer B [25 mM sodium phosphate buffer, pH 6.1, containing 2 M $(NH_4)_2SO_4$] and dialyzed against the same buffer. Enzyme was applied onto Octyl Sepharose-4B equilibrated with buffer B, and later enzyme elution was carried out by 25 mM sodium phosphate buffer, pH 6.1, containing 1 M $(NH_4)_2SO_4$ at 25 °C. High specific activity fractions were pooled, concentrated, and dialyzed against 50 mM Tris-HCl, pH 8 (buffer C). Enzyme was applied to DEAE-Sephacel equilibrated with buffer C and eluted with NaCl gradient from 0 to 0.5 M. High specific activity fractions were pooled, concentrated, and dialyzed against 50 mM sodium acetate buffer, pH 5. Final preparation was 910-fold purified with a specific activity of 77.33 μmol/min/mg and was found to be homogeneous on SDS-PAGE.

Immobilization of *Ps*BGAL onto Amberlite MB-150 Beads. The procedure described by Anita and Sastry et al. (*9*) was adopted with a few modifications for immobilization of *Ps*BGAL. Amberlite MB-150 beads (50−150 mg) of diameter 5 μm [equilibrated at different pH values ranging from 4 to 9 using 0.05 M buffers: sodium acetate (pH 4−5), sodium phosphate (pH 6−7), Tris-HCl (pH 8−9)] were activated using 200 μL of 1−5% (v/v) solution of glutaraldehyde prepared in the equilibration buffer with an overnight incubation at room temperature. The glutaraldehyde-activated Amberlite beads were washed with the equilibration buffer two or three times to remove excess glutaraldehyde. Later, various amounts of enzyme (μg) were added to the activated Amberlite beads, and the coupling was continued for 24 h at 4 °C. The unbound enzyme was washed off with the equilibration buffer (ice cold). The protein and enzyme activity were estimated in washed off fractions. To determine the optimum immobilization conditions, the following parameters during the immobilization process were studied: (a) amount of Amberlite, 50−150 mg; (b) glutaraldehyde concentration, 1−5% (v/v); (c) protein concentration, 7.3−29.2 μg; (d) pH, 4.0−9.0. One parameter was varied and the others kept constant. The percent of immobilization is calculated by using the following relationship:

$$\%\ \text{immobilization} = \frac{\text{specific activity of immobilized } Ps\text{BGAL}}{\text{specific activity of soluble } Ps\text{BGAL}} \times 100$$

Enzyme Assays. *(Soluble) ONPG.*Activity toward ONPG was estimated in 500 μL of 20 mM ONPG prepared in 50 mM glycine−HCl (pH 3.2) and enzyme with a final concentration of 5 μg/mL. Reaction was terminated using 20 mM sodium tetraborate (1.5 mL) (sodium tetraborate is used for both reaction termination and color development) after an incubation of 5 min at 37 °C, and absorbance was recorded at 405 nm. One unit of BGAL activity was defined as the amount of enzyme required to release 1 μmol of *o*-nitrophenol produced per minute per milliliter at 37 °C (extinction coefficient of *o*-nitrophenol = $4.05 \times 10^3\ M^{-1}cm^{-1}$).

Lactose. Activity toward lactose was estimated in 50 μL of reaction mixture containing 50 mM lactose prepared in 50 mM acetate buffer, pH 4.0, and enzyme with a final concentration of 25 μg/mL. Reaction was stopped after 10 min by heating the reaction mixture in a boiling water bath for 5 min. Glucose released was estimated using a commercially available kit (Span Diagnostics Ltd.) based on a glucose oxidase−peroxidase (GOD-POD) method (*13*). Reaction mixture (20 μL) was added to (500 μL) glucose reagent, color was developed for 10 min at 37 °C, and absorbance was recorded at 505 nm. One unit of enzyme activity is defined as 1 μmol of glucose released per minute per milliliter at 37 °C.

(Immobilized) ONPG. One hundred milligrams of Amberlite−*Ps*BGAL was incubated in 500 μL of 20 mM ONPG prepared in 50 mM glycine−HCl, pH 3.2, for 5 min at 37 °C. One and a half milliliters of 20 mM sodium tetraborate was added for color development, and absorbance was recorded at 405 nm. One unit of enzyme activity was defined as 1 μmol of *o*-nitrophenol produced per minute per milliliter at 37 °C.

Lactose. One hundred milligrams of Amberlite−*Ps*BGAL was incubated in 50 μL of 50 mM lactose prepared in acetate buffer, pH 4.0, for 10 min at 37 °C. Later, 20 μL was withdrawn from the reaction mixture for glucose estimation as described in section on soluble enzyme assay. One unit of enzyme activity was defined as 1 μmol of glucose released per minute per milliliter at 37 °C.

Protein Estimation. Protein was estimated as described by Bradford (*14*) with the Bradford reagent, calibrated with crystalline bovine serum albumin.

Steady-State Kinetics. For all steady-state kinetics studies, concentrations of soluble enzyme and amounts of Amberlite−*Ps*BGAL (100 mg) were the same as described under Enzyme Assays.

The optimum pH with respect to ONPG for the soluble and Amberlite−*Ps*BGAL were determined by varying the pH of 0.05 M assay buffers from 1 to 5, while in the pH range of 1−7 with lactose. Buffers used were KCl−HCl (pH 1−2), glycine−HCl (pH 2−4), acetic acid−sodium acetate (pH 4−5), and sodium monobasic−sodium dibasic (pH 5−7). The enzyme activity (soluble and immobilized) was determined similarly in each buffer according to the method described under Enzyme Assay. Optimal temperatures for soluble and Amberlite−

*Ps*BGAL were studied by assaying at an increasing temperature range, 20−100 ± 1 °C, using a multitemperature water bath (Pharmacia, Uppsala Sweden). K_m and V_{max} were determined by the Lineweaver−Burk plot with the help of SigmaPlot 8.0 software by varying [ONPG] from 0.5 to 20 mM, pH 3.2, while in the range from 1 to 50 mM for lactose, pH 4.0.

Statistical Analysis. GraphPad StatMat 2.0 software (San Diego, CA) was used for statistical analysis. The effects of various parameters (such as pH, percent glutaraldehyde, and amount of enzyme) on percent immobilization of *Ps*BGAL onto Amberlite MB-150 beads were analyzed using one-way ANOVA with post test (Bonferroni) at $P < 0.05$.

Storage Stability. For storage stability studies, Amberlite−*Ps*BGAL was kept at 4 °C and room temperature under wet (stored in equilibration buffer) and dry conditions (air-dried). The activity of immobilized enzyme was determined using the assay procedure mentioned in the previous section. Freshly immobilized enzyme was taken as control for each assay that was carried out using ONPG.

Thermal Inactivation Studies. The Amberlite−*Ps*BGAL was incubated at 70 °C, in small aliquots at different time intervals. At specified time intervals, immobilized enzyme was withdrawn, chilled, and transferred to assay buffer (0.05 M glycine−HCl, pH 3.2) containing 20 mM ONPG. Residual activity was determined according to the usual assay method at 37 °C. Rate constant was calculated according to the method described earlier (*15*).

Reusability. The immobilized *Ps*BGAL was reused 10 times over a period of 15 days, and the residual activity was measured. After the assay, the immobilized *Ps*BGAL was washed with assay buffer, dried, and stored at room temperature. Furthermore, the immobilized *Ps*BGAL, which showed better stability, was reused for prolonged periods.

Scanning Electron Microscopy (SEM). The microstructural details of immobilized *Ps*BGAL onto Amberlite beads were monitored through SEM (Philips XL 20) under secondary electron imaging.

Preparation and Enzymatic Treatment of Milk and Milk Whey. Twenty-five grams of defatted powdered milk (Nestlé) was dissolved in distilled water in the ratio of 1:10 (w/v). Fractions with lower dilutions were too turbid to be used for analysis by spectrophotometer. Milk whey was produced by lowering the pH of milk (1:10) using 2 N HCl for isoelectric precipitation of proteins and subsequently centrifuged at 8420*g* for 5 min at 4 °C. Milk and milk whey aliquots (50 μL) were incubated with 100 mg of Amberlite−*Ps*BGAL at room temperature for different time intervals. Twenty microliter samples were removed at predetermined time intervals, and then glucose content was estimated in the treated samples using the GOD-POD method as described under Enzyme Assays. Percent of lactose hydrolysis was calculated using following relationship:

$$(\text{glucose present without treatment with Amberlite}-Ps\text{BGAL}/\text{glucose present after treatment with Amberlite}-Ps\text{BGAL}) \times 100$$

$$\%\ \text{lactose unhydrolyzed} = 100 - \%\ \text{lactose hydrolyzed}$$

A plot was generated with log % lactose unhydrolyzed versus time, and rate constant of lactose hydrolysis was determined using the slope of the plot using the equation

$$\text{slope} = -k/2.303$$

where the rate constant $k = (2.303)/(t) \log (100)/(100 - x)$ and x is lactose unhydrolyzed.

Therefore, the time required for 50% lactose hydrolysis is given by

$$t_{1/2} = \frac{0.693}{k}$$

RESULTS AND DISCUSSION

Immobilization. Figure 1 shows SEM micrographs of *Ps*BGAL immobilized onto Amberlite beads. Analysis of the SEM micrograph of Amberlite−*Ps*BGAL at a resolution of 3699× showed immobilized *Ps*BGAL resembled the rod-shaped particles present on Amberlite beads (**Figure 1B**). Furthermore, a variation in size of the rod-shaped enzyme particles were seen, suggesting that varied amounts of enzyme were immobilized at different sites of the matrix. Especially, peripheral portions had large rod-shaped enzyme particles due to the fact that the periphery has the highest probability of glutaraldehyde activation and enzyme immobilization. Various conditions have been tested to obtain optimum immobilization, and the best conditions were found to be as follows: 100 mg of Amberlite MB-150 beads when activated with 4% (v/v) glutaraldehyde for 12 h at room temperature coupled with 21.9 μg of *Ps*BGAL for 24 h at 4 °C resulted in 62.56% immobilization at pH 6.0. According to statistical analysis using one-way ANOVA, all factors including pH, amount of enzyme, percent glutaraldehyde, and amount of Amberlite beads were equally important for *Ps*BGAL immobilization at $P < 0.05$ (level of significance).

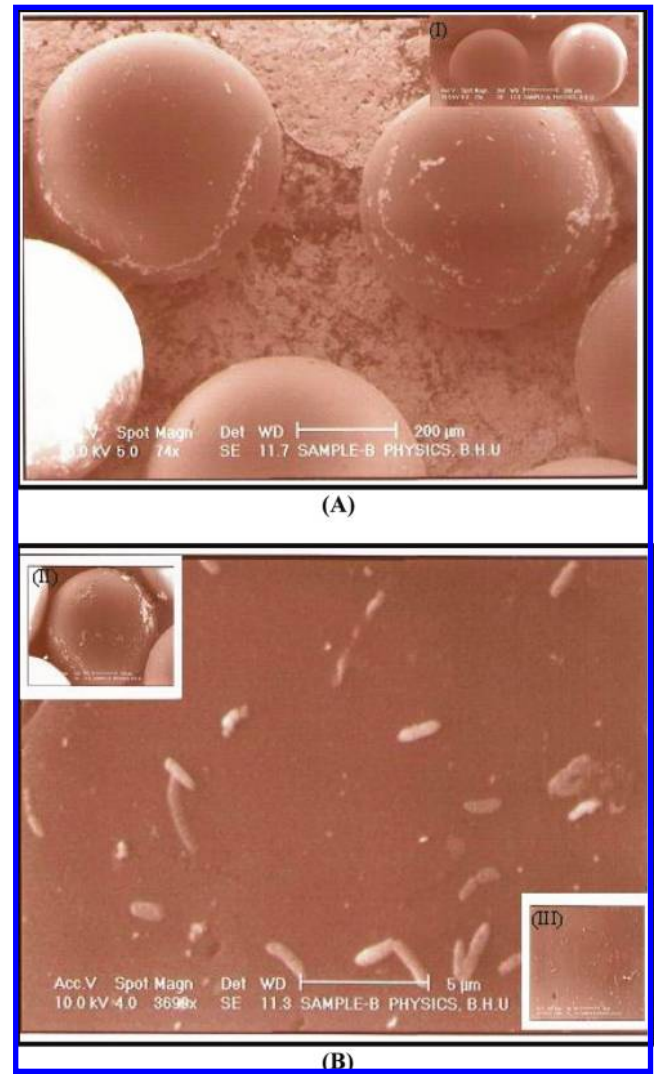

Figure 1. (**A**) SEM micrograph of *Ps*BGAL immobilized on Amberlite MB-150 beads and its control (inset I) at resolution of 74×; (**B**) SEM micrograph of Amberlite−*Ps*BGAL at highest resolution (3699×). Insets (II, III) are shown at resolutions of 148× and 1480×, respectively.

pH around neutrality was found to be best suited for enzyme immobilization using glutaraldehyde due to its tendency to form alcohols at low pH and acids at higher pH. As summarized in **Table 1**, it was found that there was a decrease in percent immobilization as the glutaraldehyde concentration was increased above 4% (v/v). Higher concentration of glutaraldehyde led to aggregation, precipitation, loss of enzyme activity, and

Table 1. Various Conditions Used for Optimizing Immobilization of *Ps*BGAL onto Amberlite Beads

condition	vol (μL)	incubation time (h)	incubation temperature (°C)	immobilization[a] (%)
glutaraldehyde				
1%	200	12	RT	26.84
2%	200	12	RT	28.41
3%	200	12	RT	32.67
4%	200	12	RT	**44.05**
5%	200	12	RT	38.31
pH				
4.0			RT	27.78
5.0			RT	46.11
6.0			RT	56.07
7.0			RT	**47.95**
8.0			RT	35.90
9.0			RT	23.06
amount of Amberlite (mg)				
50				37.79
100				**55.13**
150				50.76
amount of enzyme (μg)				
7.3				44.09
14.6				54.82
21.9				**62.56**
29.2				38.13

[a] Percent immobilization presented here was expressed in terms of specific activity Amberlite−*Ps*BGAL/specific activity of soluble *Ps*BGAL. The specific activity of soluble of *Ps*BGAL was 77.33 μmol/min/mg, whereas the apparent specific activity of immobilized enzyme was calculated by subtracting specific activity of washed fractions. RT stands for room temperature (27 °C).

distortion of the enzyme structure (*16*). Furthermore, aggregation of enzyme at higher concentration of glutaraldehyde led to fewer sites available for attachment to the matrix; the same was true when the protein concentration used was >21.9 μg. A substantial decrease in percent immobilization was observed when the amount of Amberlite was increased from 100 to 150 mg (**Table 1**). This was due to a decrease in the surface area of the matrix (directly proportional to percent immobilization) available for enzyme immobilization. This was further supported by the SEM micrograph of Amberlite−*Ps*BGAL (**Figure 1**) showing that larger rod-shaped enzyme particles were seen at the periphery of the matrix (portion that is unattached to neighboring beads).

Steady-State Kinetics. *Optimum Temperature.* Amberlite−*Ps*BGAL has broad optimum temperature in the range of 65−80 °C (**Figure 2**), whereas soluble enzyme has a sharp temperature optimum at 60 °C (inset, **Figure 2**) showing that the former is more active at higher temperatures. Immobilization of enzyme to the matrix led to a barrier toward free movement of enzyme. Therefore, gain in kinetic energy by the immobilized enzyme due to an increase in temperature is limited to a great extent, leading to an increase in optimum temperature. Similar displacement of optimum temperature for immobilized enzymes was observed in many cases, but the extent of displacement differed with type of matrix and interactions between the enzyme and the matrix. BGAL immobilized onto various matrices such as gel fiber made of cellulose acetate, titanium isopropoxide, Sephadex beads, and alginate/gelatin showed optimum temperatures around 55 °C (*17*−*19*).

Kinetic Parameters. These parameters for immobilized enzyme depend on three major factors: (i) Enzyme attached to the matrix must be attached in precisely the correct conformation. Also, the enzyme attached should be able to change its conformation during catalysis. (ii) Diffusion barriers, both *internal* and *external*, must be present. An internal diffusion barrier is present in the case of immobilized enzyme system,

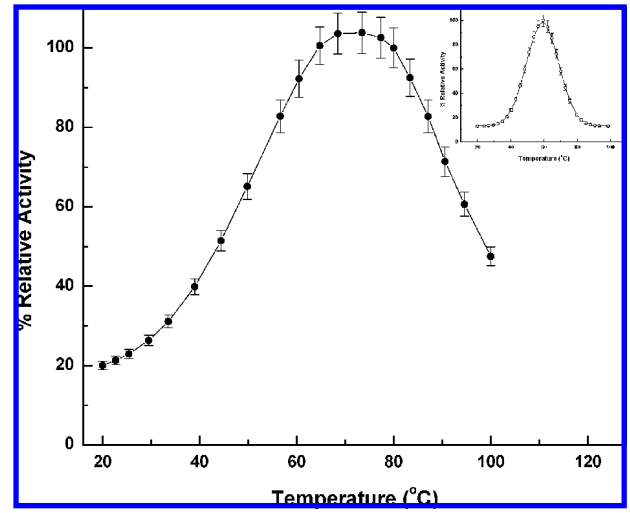

Figure 2. Temperature optima of Amberlite−*Ps*BGAL and soluble *Ps*BGAL (inset). Vertical bars represent standard error during experiment. Percent relative activity was determined by using the following relationship: (enzyme activity at given temperature/maximum enzyme activity corresponding to a particular temperature) × 100.

Table 2. Kinetic Parameters Determined Using a Lineweaver−Burk Plot at 37 °C

dnzyme	kinetic parameter	ONPG	lactose
soluble *Ps*BGAL	K_m (mM)	0.97 ± 0.05	3.03 ± 0.12
	V_{max} (μmol/mL/min)	15.38 ± 0.59	0.58 ± 0.04
	V_{max}/K_m (min^{-1})	15.86	0.19
Amberlite−*Ps*BGAL	K_m (mM)	1.00 ± 0.06	4.11 ± 0.21
	V_{max} (μmol/mL/min)	2.85 ± 0.17	0.10 ± 0.006
	V_{max}/K_m (min^{-1})	2.85	0.02

where enzyme is present inside the matrix. Here, substrate has to diffuse inside the matrix to be hydrolyzed to its products. On the other hand, an external diffusion barrier (important for an immobilized enzyme system where enzyme is present on the surface of the matrix, as in the present case) is a result of the thin, unstirred layer of solvent that surrounds the polymer particle, called the "Nernst layer". Solutes diffuse in this layer by a combination of passive molecular diffusion and convection. The higher the concentration of solute, the greater would be the external diffusion barrier. The thickness of this layer is affected (within limits) by the speed at which the solvent around the immobilized enzyme particle is stirred. Increasing the stirring rate will reduce this external diffusion layer (eq 1; ref *20*)

$$\alpha = V_{max}/K_m \quad (1)$$

where α is the diffusion constant.

(iii) Partitioning effects arise due to polyionic matrices, due to interaction between polyionic matrix and ionic solute. The partition coefficient $p = S_i/S_o$, where S_i is the concentration of ions around enzyme and S_o is the concentration of ions in bulk phase. Thus, the Michaelis−Menten equation becomes

$$v = V_{max}S_o p/K_{max} + S_o p \quad (2)$$

and thus

$$K_{m(app)} = K_m/p \quad (3)$$

Table 2 shows that there was almost no change in K_m with ONPG and lactose of Amberlite−*Ps*BGAL, but there was significant decrease in V_{max} of Amberlite−*Ps*BGAL as compared

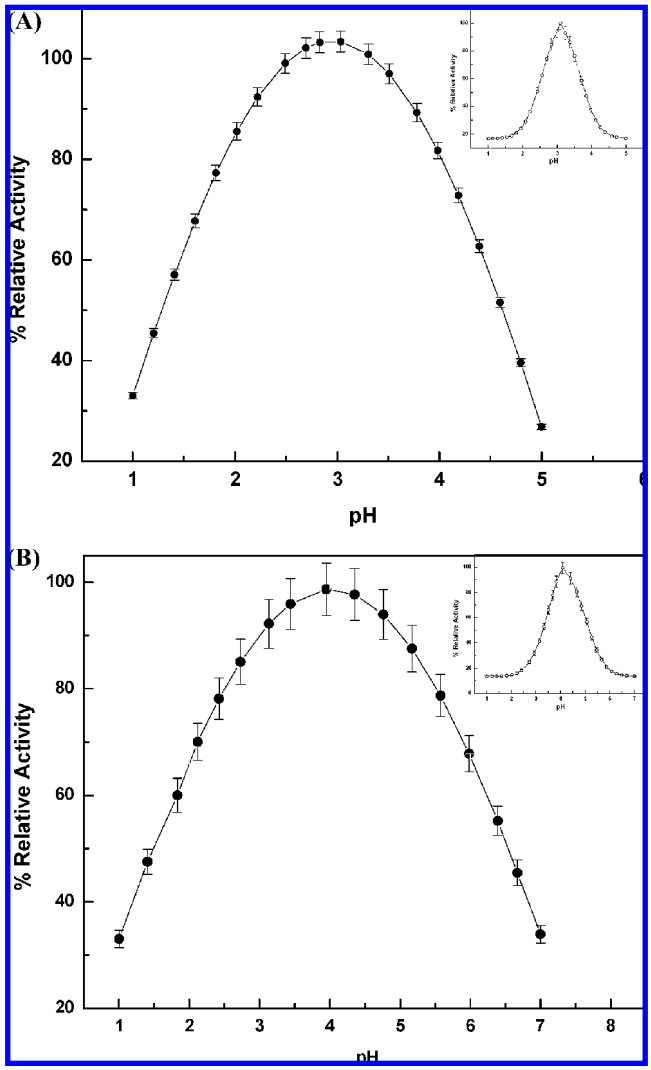

Figure 3. (**A**) Optima pH of Amberlite−*Ps*BGAL and soluble *Ps*BGAL (inset) with ONPG. Vertical bars represent standard error during experiment. Percent relative activity was determined by using the following relationship: (enzyme activity at given pH/maximum enzyme activity corresponding to a particular pH) × 100. (**B**) Optima pH of Amberlite−*Ps*BGAL and soluble *Ps*BGAL (inset) with lactose. Vertical bars represent standard error during experiment. Percent relative activity was determined similarly to the method stated for panel **A**.

to soluble enzyme. Unchanged K_m indicates that the partition effect is almost negligible (eq 3). This is due to the fact that Amberlite MB-150 beads bear negative charges on their surface, and attachment of enzyme using glutaraldehyde lowers the surface charge density of beads. Due to unchanged K_m, rate of reaction V_{max} becomes directly proportional to diffusion of the substrates (eq 1). It is concluded that slower diffusion of substrate is one of the causes of lower V_{max} in the case of Amberlite−*Ps*BGAL. Moreover, lower V_{max} also showed the enzyme's inability to undergo conformational changes while catalyzing a reaction due to attachment at various points onto Amberlite by glutaraldehyde.

Optimum pH. Amberlite−*Ps*BGAL has a broad pH optima in the range of 2.7−3.3 with ONPG (**Figure 3A**) but in the range of 3.9−4.4 with lactose (**Figure 3B**). Soluble enzyme has sharp pH optima at pH 3.2 and 4.0 with ONPG and lactose as substrates, respectively (inset, **Figure 3A**,**B**). According to Trevan and Grover (*21*), pH profiles where both limbs are broadened showed that the effective rate of the enzyme becomes

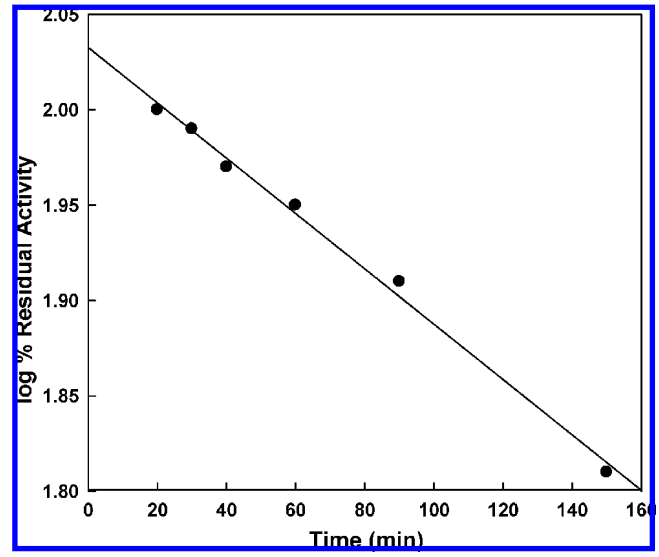

Figure 4. Thermal inactivation studies of Amberlite−*Ps*BGAL at 70 °C. Obtained values for log % residual activity corresponding to incubation time were fitted through a linear regression program available at SigmaPlot 8.0 with $r^2 = 0.9987$. Please refer to Results and Discussion for further details.

less sensitive to pH changes, as found during immobilization. An immobilized enzyme preparation having high loading (that is, a large quantity of enzyme activity per unit of polymer) is subjected to substrate diffusion limitation. As the enzyme has a high intrinsic specific activity (occurs during immobilization), the substrate concentration gradient through the particle will be steep, and consequently the substrate may not penetrate to the center of the immobilized enzyme particle. With the constraint of pH, the substrate concentration gradient will become less steep, thus allowing the substrate to penetrate further into the immobilized enzyme particle having high intrinsic specific activity (due to increased enzyme concentration during immobilization, Zulu effect; ref *22*). Two factors therefore work antagonistically on the reaction rate, the change in pH reducing the rate and the rise in effective enzyme concentration tending to increase the rate, thereby moderating the effect of the pH change. In the present case, high local enzyme concentration and substrate limitation have led to broadening of the optimum pH as found with substrates ONPG and lactose. Furthermore, it was found that there was an upward shift of optimum pH curve with lactose. It was due to the fact that Amberlite−*Ps*BGAL has a higher catalytic rate (V_{max}/K_m) for ONPG than for lactose, because the release of glucose was slower than that of *o*-nitrophenol. Moreover, *o*-nitrophenol has a lower pK_a than glucose, creating greater perturbation in the apparent pH of immobilized enzyme. Tosa et al. (*23*) have reported similar results with papain immobilized on polyanionic membrane.

Thermal Inactivation Studies. The soluble enzyme retained 86.26% of its activity when kept for 10 min at 40 °C but lost 90.85% of its activity at 50 °C and 94.89% at 60 °C, respectively. According to thermal stability studies at 70 °C (**Figure 4**), there was an almost negligible loss even after 30 min. The complete loss in activity with free enzyme was due to irreversible denaturation induced by covalent changes such as deamination of asparagine residues or non-covalent changes such as rearrangement of the protein chain. The elevated temperatures cause changes in the tertiary structure of a protein (mainly by breaking H-bonds).

Thermal inactivation of enzymes followed first-order kinetics:

$$\ln A/A_0 = -kt \quad (4)$$

where A_0 is the initial activity (at $t = 0$) and A is the residual activity at time t, after the treatment, where k is the rate constant. Equation 4 can also be written in the form of $y = mx + c$, as follows:

$$t = \frac{-2.303}{k} \log A/A_0$$

Therefore, the rate constant k can be determined from the slope of the plot ($\log A/A_0$ versus time) and $t_{1/2}$ using the relationship $t_{1/2} = 0.693/k$.

According to **Figure 4**, the rate constant (k) for thermal inactivation of Amberlite−*Ps*BGAL was 0.0015 min^{-1} and $t_{1/2}$ was 462 min, respectively. Therefore, it can be stipulated that immobilization has enhanced the thermal stability significantly by limiting its free movement at higher temperatures and protecting from disruption. According to Tosa et al., enhanced thermal stability during enzyme immobilization was due to molecular rigidity and the creation of a protected microenvironment (*23*). Reddy and Kayastha have reported that 50% enzymatic activity of urease immobilized onto arylamine and alkylamine glass beads was retained after 90 min, at 77 °C (*24*).

Stability. Amberlite−*Ps*BGAL was stable for over 12 months under dried condition at room temperature with no loss in its activity (data not shown). On the other hand, soluble *Ps*BGAL was extremely unstable with loss of >80% within 24 h at 4 °C when the protein concentration was <0.1 mg/mL. During *Ps*BGAL immobilization onto Amberlite beads, a substantial increment in local enzyme concentration [Zulu effect (*22*) due to high surface area of Amberlite MB-150 bead having a diameter of 5 μm]. Therefore, at normal substrate concentrations only fractions of enzyme molecules are involved in substrate conversion to its products. Thus, despite enzyme inactivation, immobilized enzyme appears to be stable with no loss in its activity. Furthermore, increase in protein concentration is a crucial factor responsible for its stabilization due to increase in protein−protein interactions (*25*). Enhanced stabilization during enzyme immobilization has been reported by various authors (*26, 27*).

Reusability. With repeated reuse, the strength of binding between the matrix and enzyme is weakened, leading to loss in activity. Moreover, frequent encountering of substrate in the active site causes its distortion, thus reducing catalytic efficiency. However, as shown in **Figure 5**, immobilized *Ps*BGAL can be reused over 10 times with almost no loss in its activity, which implies that the enzyme is strongly bound to Amberlite beads. Furthermore, as discussed in the section on stability-enhanced local enzyme concentration enzyme immobilization led to the appearance of no loss in enzymatic activity at normal substrate concentrations. Obtained batchwise uses of Amberlite−*Ps*BGAL were found to be comparable as reported previously (*17*−*19, 28*).

Milk and Whey Treatment with Immobilized PsBGAL in Batch Mode. As shown in Figure. **6**, after 10 h of the proposed treatment at room temperature, 64.57 and 69.18% of lactose hydrolysis were observed in milk and whey, respectively. First-order rate constants were determined to be 0.151 and 0.138 h^{-1} for milk and whey, respectively. Thus, the efficiency of lactose hydrolysis present in milk and milk whey by Amberlite−*Ps*BGAL was found to be severalfold higher than that given in previous papers (*29, 30*). *Ps*BGAL immobilization onto Amberlite has led to a severalfold increase in intrinsic specific enzyme activity (Zulu effect) due to which higher lactose hydrolysis efficiency was observed.

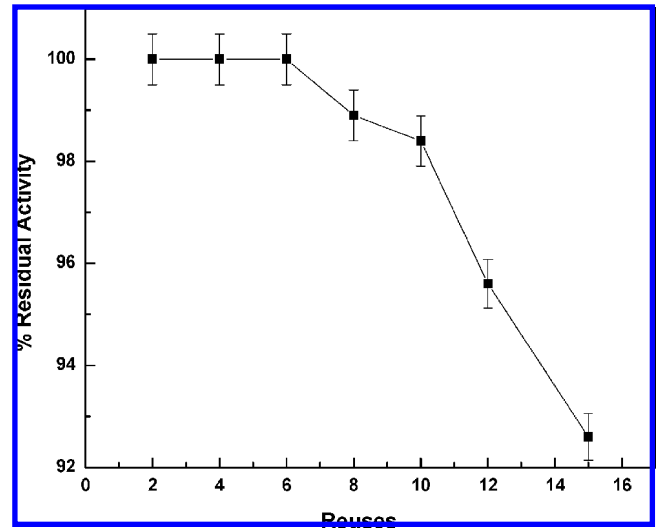

Figure 5. Reusability of Amberlite−*Ps*BGAL. Vertical bars represent standard error during experiment.

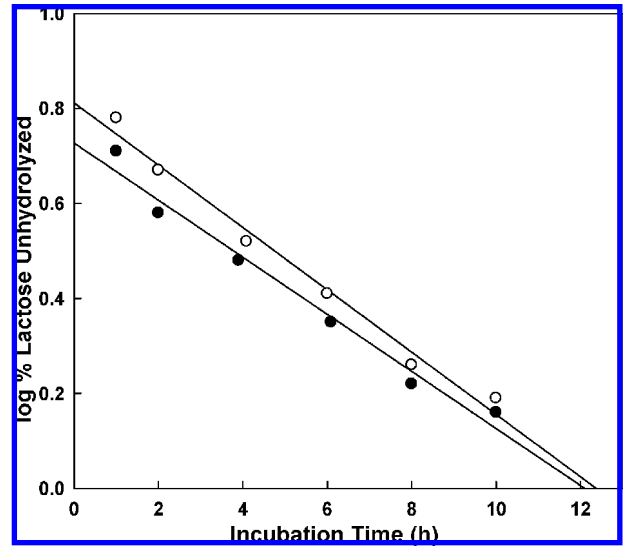

Figure 6. Lactose hydrolysis by Amberlite−*Ps*BGAL at room temperature: (●) log % lactose remaining in milk whey; (○) log % lactose remaining in milk. Values corresponding to log % lactose remaining were fitted through a linear regression program available at SigmaPlot 8.0 with $r^2 = 0.9983$ and 0.9986 in the case of milk and milk whey, respectively. Rate constants of lactose hydrolysis were determined using a first-order equation. Please refer to Materials and Methods for further details.

These results show that the rate of hydrolysis of lactose present in whey was faster than that of lactose present in milk. The higher efficiency of Amberlite−*Ps*BGAL in hydrolyzing lactose of milk whey compared to milk lactose is explained by the differences in pH between the tests, that is, pH 4.5 for whey and pH 6.34 for milk lactose. Amberlite−*Ps*BGAL has pH optima with lactose lying in the range of 3.9−4.4 (see section on optimum pH). Despite the reduced hydrolytic activity shown by Amberlite−*Ps*BGAL above pH 6.0, the rate of hydrolysis of milk lactose (64.57%) can be considered to be quite satisfactory. According to a review by Gikas and Lopez-Leiva (*31*), some traditional enzymatic processes for milk lactose removal available in the market present a final hydrolytic value of 50−70% at reaction times longer than 18 h. Therefore, Amberlite−*Ps*BGAL can be exploited commercially for carrying out lactose hydrolysis in milk and whey.

ABBREVIATIONS USED

ANOVA, analysis of variance; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GOD-POD, glucose oxidase−peroxidase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; *Ps*BGAL,*Pisum sativum* β-galactosidase.

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