Application of Chitosan-Entrapped β-Galactosidase in a Packed-Bed Reactor System

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Received 11 October 2002; accepted 23 May 2003

ABSTRACT: The model enzyme β-galactosidase was entrapped in chitosan gel beads and tested for hydrolytic activity and its potential for application in a packed-bed reactor. The chitosan beads had an enzyme entrapment efficiency of 59% and retained 56% of the enzyme activity of the free enzyme. The Michaelis constant (K_m) was 0.0086 and 0.011 μmol/mL for the free and immobilized enzymes, respectively. The maximum velocity of the reaction (V_{max}) was 285.7 and 55.25 μmol mL^{-1} min^{-1} for the free and immobilized enzymes, respectively. In pH stability tests, the immobilized enzyme exhibited a greater range of pH stability and shifted to include a more acidic pH optimum, compared to that of the free enzyme. A 2.54 × 16.51-cm tubular reactor was constructed to hold 300 mL of chitosan-immobilized enzyme. A full-factorial test design was implemented to test the effect of substrate flow (20 and 100 mL/min), concentration (0.0015 and 0.003*M*), and repeated use of the test bed on efficiency of the system. Parameters were analyzed using repeated-measures analysis of variance. Flow ($p < 0.05$) and concentration ($p < 0.05$) significantly affected substrate conversion, as did the interaction progressing from Run 1 to Run 2 on a bed ($p < 0.05$). Reactor stability tests indicated that the packed-bed reactor continued to convert substrate for more than 12 h with a minimal reduction in conversion efficiency. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 1294–1299, 2004

Key words: enzymes; biopolymers; crosslinking; hydrolytic activity; packed-bed reactor

INTRODUCTION

Chitosan is an abundant, natural polymer with promising applications in the immobilization and controlled release of biological compounds. Various enzymes have been immobilized with chitosan using absorption,[1,2] covalent binding,[3–9] or matrix entrapment.[10–14] A primary benefit of matrix entrapment is that is can be used to immobilize enzymes and other proteins in a chitosan gel under mild processing conditions. Other, more harsh methods can also be used to immobilize enzymes onto a chitosan matrix.[1–9,12] However, these methods of covalent binding often rely on the use of glutaraldehyde, a toxic chemical often unsuited for applications in the food and biomedical industries. Another benefit of enzyme entrapment is that the immobilized enzyme can eventually be recovered because covalent bonds are not involved.[15]

A number of studies have investigated the use of chitosan-immobilized enzymes in packed-bed reactors. Packed-bed reactors are practical and efficient reactors, showing higher conversion efficiency than that of continuously stirred tank reactors, provided that ideal plug flow conditions are met and that equal residence time is considered. Typically, enzymes have been attached to premanufactured chitosan bead supports. Penicillin G acylase attached to chitosan beads by adsorption and covalent linking successfully converted substrate in a packed-bed reactor for more than 350 h.[16] Similarly, purchased chitosan beads were used as a support for the covalent attachment of urease and the enzyme-immobilized beads showed 93% of their original hydrolytic activity after 8 cycles in a column reactor.[17] β-Galactosidase and a broad-spectrum β-glycosidase were also successfully immobilized onto chitosan beads and used to hydrolyze lactose in column reactor systems.[6,9,18] In each case, the enzyme was covalently attached to the bead support with glutaraldehyde, and the immobilized systems retained activity for numerous cycles.

β-Galactosidase is an important enzyme in the food industry, primarily used to hydrolyze lactose found in cheese whey, a byproduct of cheese making. This process permits the recovery and use of the sugars from lactose (glucose and galactose) as well as rendering the whey suitable for transport because of the removal of the relatively insoluble lactose. Another potential use for β-galactosidase is the reduction of lactose in milk, given that lactose is a natural component of milk

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Journal of Applied Polymer Science, Vol. 91, 1294–1299 (2004)

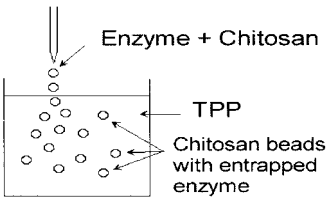

Figure 1 Preparation of chitosan beads.

that the majority of the world's adult population cannot digest.

The objective of this study was to investigate the use of chitosan-entrapped β-galactosidase in a packed-bed reactor. Although entrapment is a mild immobilization method with significant potential for enzyme reuse, diffusional limitations, enzyme leakage, and undesirable changes in kinetic parameters could limit its use. In this project we evaluated the capture efficiency and activity of β-galactosidase entrapped in chitosan gel beads. Optimal pH and kinetic parameters of the entrapped enzyme were determined, and the chitosan beads were applied in a continuous flow packed-bed reactor system.

EXPERIMENTAL

Materials

High molecular weight chitosan was supplied by Aldrich Chemical Co. (Milwaukee, WI). β-Galactosidase (from *Aspergillus orysae*, activity of 11.7 units/mg), Folin and Coicalteu phenol reagent, sodium phosphate, *o*-nitrophenyl β-D-galactopyranoside (ONPG), magnesium chloride, Trizma hydrochloric acid, cupric sulfate, sodium potassium tartrate, and mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO). Food-grade tripolyphosphate (TPP) was donated by FMC BioProducts (Lawrence, KS). All other chemicals were of reagent grade or higher and were purchased from EM Science (Gibbstown, NJ) or Fisher Scientific (Fair Lawn, NJ).

Entrapment of β-galactosidase

Enzyme was entrapped in a chitosan matrix according to the following procedure. A 1.5% (w/v) chitosan solution in 3% (v/v) acetic acid was prepared and stirred for 2 h. β-Galactosidase was added to the chitosan gel at a concentration of 200 U/mL and mixed thoroughly with an automatic stirrer. The resulting gel was extruded through a 1-mL plastic syringe (VWR Scientific Products, catalog number BD309602, West Chester, PA) into a solution of 15% (w/v) tripolyphosphate (pH 5.0) at a vertical distance of approximately 15 cm (Fig. 1). After extrusion and curing for 10 min, the beads were removed from the TPP solution and rinsed with distilled water.

Determination of β-galactosidase activity

After curing, the amount of β-galactosidase remaining in the beads was measured as total protein. The beads were homogenized (Kinematica, Cincinnati, OH) in 5-mL of 2.0*N* hydrochloric acid and a 200-μL sample of the resulting solution was tested for protein spectrophotometrically (Model DU-64; Beckman Instruments, Fullerton, CA) using the Lowry assay.[19]

The β-galactosidase activity of the gel beads was determined using the method described by Brena et al.[20] Beads produced from 1 mL of the chitosan/enzyme solution (hereafter referred to as a 1-mL sample of beads) were added to 30 mL of the assay buffer with ONPG substrate and the hydrolytic formation of free nitrophenol was measured spectrophotometrically at 405 nm. Protein concentration and enzyme activity were analyzed in three 1-mL sets of beads for each of the three independent experiments.

pH optimization

Batch studies were conducted on the enzyme-entrapped beads to determine the effects of pH on β-galactosidase activity. The pH of the ONPG assay buffer was varied to determine the optimum pH for production of free nitrophenol. The pH values tested were 5.0, 6.5, 7.3, 8.5, and 9.0. The same pH values were used to evaluate the free enzyme activity for comparison. The pH optima were determined in three 1-mL sets of beads for each of the three independent experiments.

Determination of K_m and V_{max}

The β-galactosidase–entrapped chitosan beads (1-mL sample) were immersed in 30 mL of five different concentrations of ONPG assay buffer: 0.0015, 0.0075, 0.015, 0.075, and 0.15*M*, at 25°C, subjected to gentle stirring (Fisher Scientific stirring hot plate, catalog number 11-502-49SH), and the hydrolytic formation of free nitrophenol was measured. The same concentrations of ONPG were also used to determine the V_{max} and K_m for the free enzyme. Kinetic parameters were analyzed in three 1-mL sets of beads for each of the three independent experiments.

Reactor design and experimental evaluation

A reactor was designed (Fig. 2) using the design equation for a packed-bed reactor,[21] and assuming plug flow and Michaelis–Menten kinetics. The design called for 300 mL (as measured by the original chitosan/enzyme solution) of β-galactosidase–entrapped

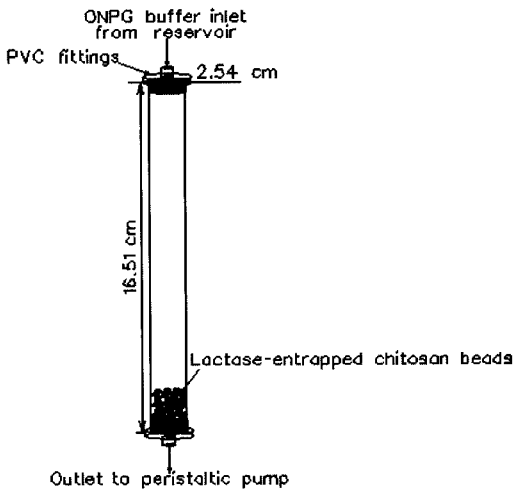

Figure 2 Schematic of packed-bed reactor.

gel beads. To house this volume of beads, a 2.54 × 16.51-cm packed-bed reactor was constructed. The reactor consisted of clear polyvinyl chloride pipe (Portland Plastic, Portland, ME), column adapters and end fittings (Bangor Pipe, Bangor, ME), 180-μm pore-size nylon filters (type NY8H, lot R6JM13905; Millipore Corp., Bedford, MA), and 0.3175-cm tubing (Cole Parmer Instrument Co., Vernon Hills, IL).

A full-factorial design was implemented to test the effects of flow and concentration of the ONPG assay buffer on the efficiency of the packed-bed reactor. Two levels of each factor (flow and concentration) were used. The flow rates were 20 and 100 mL/min and the concentration levels were 0.0015 and 0.003*M*. Flow rates were achieved using a peristaltic pump (Masterflex L/S peristaltic pump, model 7518-00, Cole-Parmer Instrument Co.). The full factorial was tested in triplicate for a total number of 12 test beds. To initiate the experiment, 300 of β-galactosidase–entrapped gel beads were loaded into the column. ONPG buffer (pH 7.3 and 21°C) was immediately pumped through the column and the hydrolytic formation of free nitrophenol was measured in the exiting liquid at 2, 5, 10, 15, 25, 45, and 60 min. After 60 min, 500 mL of distilled water was pumped through the bed at a flow rate of 100 mL/min. Immediately after the wash step, the same flow and concentration conditions were implemented, for a total of three times per bed on each of the 12 test beds. Each of the three identical tests performed on each individual bed are hereafter referred to as Run 1, Run 2, and Run 3. Percentage substrate conversion was calculated by a mass balance on the reactor, given that the product (nitrophenol) was in a 1 : 1*M* reaction ratio with the substrate (ONPG).

Reactor stability

The flow rate and concentration that resulted in (1) the highest percentage substrate conversion and (2) the greatest repeatability as measured by the lowest standard deviation between the replicate treatments were chosen to perform stability tests on the packed-bed reactor. For the stability experiment, the bed was packed with 300 mL of β-galactosidase–entrapped gel beads and 0.003*M* ONPG buffer was pumped through the column at a flow rate of 20 mL/min. Hydrolytic formation of free nitrophenol was monitored for 12 h. Reactor stability tests were performed in triplicate.

RESULTS AND DISCUSSION

Entrapment of β-galactosidase

β-Galactosidase was successfully entrapped within a chitosan gel matrix (Table I). Average capture efficiency, as defined by the amount of protein entrapped in the bead divided by the amount of protein in the original solution, was 59%. The resulting activity was 111.9 U/mL. The specific activity of the immobilized enzyme was 46.6% of the original specific activity of the free enzyme. This decrease in specific activity may have been attributable to inaccessibility of a portion of the enzyme or to diffusional limitation such that the substrate was unable to readily diffuse through the chitosan matrix and be converted to product. However, the resulting activity was still very good compared to that of the free enzyme (56% of free enzyme). Capture efficiency data were comparable to that of Ghanem and Skonberg,[14] who reported an average of 55.7%.

pH optimization

The pH range for the immobilized β-galactosidase optimal enzyme activity was broadened, compared to

TABLE I
Protein Capture Efficiency and Resulting Activity for Immobilized β-Galactosidase in Chitosan Gel Beads

Capture efficiency[a] (%)	Enzyme activity in beads (U/mL original chitosan)	Specific enzyme activity in beads (U/mg protein entrapped)	Protein entrapped in bead (mg)
58.5 ± 7.8	111.9 ± 11.4	62.2 ± 14.6	1.8 ± 0.8

[a] Results of eight analyses of three separate experiments. Each value is the average of three replications ± SD. Original amount of protein in β-galactosidase was 18% of total mass.

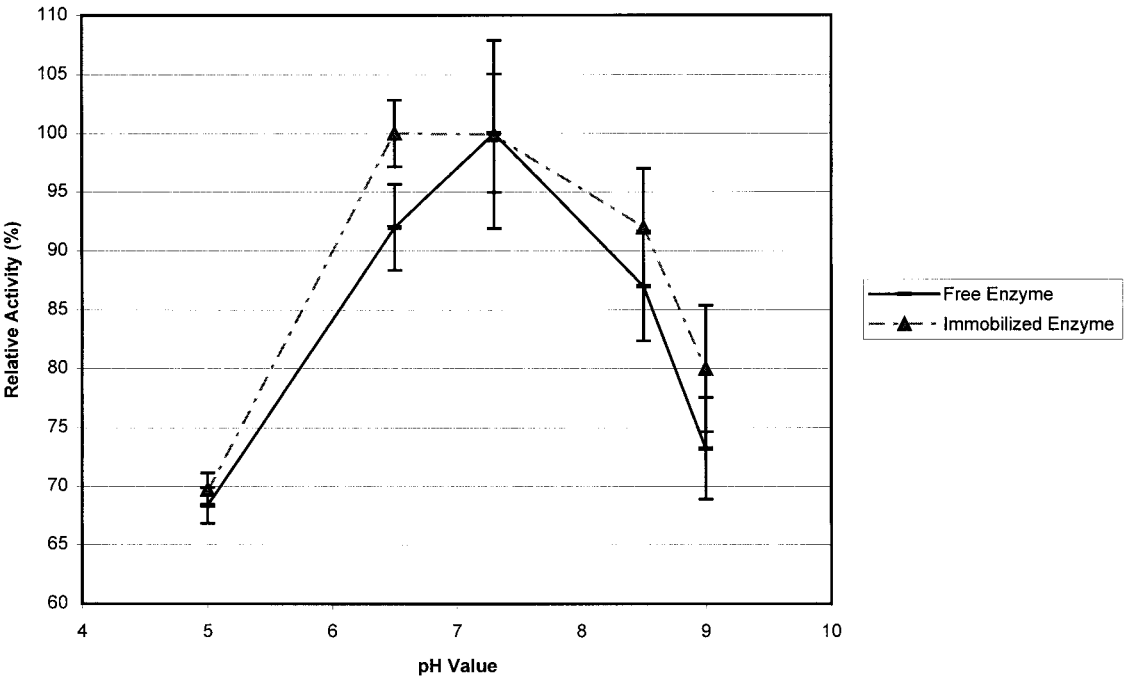

Figure 3 Effect of pH on the relative activity of free and chitosan-immobilized β-galactosidase. Relative activity = [activity (U/mL)/highest activity at all pH ranges tested (U/mL)] × 100. Each value is the average of three replications with sample size of three per replicate ± SD.

that of the free enzyme (Fig. 3). The pH optimum of the free enzyme peaked at 7.3, whereas the immobilized enzyme exhibited a plateau from pH 6.5 to 7.3, suggesting the optimal working pH range of the immobilized enzyme was higher than that of the free enzyme. In broadening the working range of the enzyme, its flexibility for potential applications increases. The ability of the entrapped enzyme to maintain 100% relative activity at a lower pH was similar to results observed with the enzyme tannase.[12] Broadening the optimal pH range to include more acidic conditions may have been caused by a shift in pH in the microenvironment resulting from the chitosan matrix. The amino groups of chitosan are protonated at high H^+ concentrations. The protonated group would be more likely to attract hydroxyl ions that would maintain a higher local pH than when the enzyme is in bulk solution. In effect, this control over the microenvironmental pH may allow for greater pH flexibility and stability of the immobilized enzyme.[22]

It was determined through V versus [S] plots that β-galactosidase followed Michaelis–Menten kinetics for both the immobilized and free enzyme (data not shown). Therefore, using Lineweaver–Burk plots of the free and immobilized β-galactosidase, the kinetic parameters of apparent V_{max} and apparent K_m were determined and are shown in Table II. Immobilization of the enzyme resulted in an increase in the apparent K_m and a decrease in apparent V_{max}. Mass transfer resistance, electrostatic, or steric effects may have caused the increase in the K_m value after immobilization. Mass transfer resistance appears to be significant for macromolecular substrates such as ONPG because they must diffuse into the bead to the entrapped enzyme.[22] The higher K_m also may have been attributable to enzyme active sites being less accessible to the substrate than in free solution. Encapsulating the enzyme also may have reduced its ability to undergo conformational changes that are intrinsic to enzyme–substrate interaction. The decrease in V_{max} may have been attributable to the conformational changes of the enzyme caused by encapsulation. The encapsulated chitosan matrix may result in a longer residence time of the converted substrate or solely a longer enzymatic reaction time because of the inability of the enzyme to undergo conformational changes. The increase in K_m values and decrease in V_{max} values for the immobilized enzyme seen here are in agreement with the observations of other researchers who conducted work on immobilized enzymes.[3,4,12,22,23]

TABLE II
Kinetic Parameters of Free and Immobilized β-Galactosidase

	Immobilized enzyme	Free enzyme
V_{max}	55.25 μmol/mL^{-1} min^{-1}	285.7 μmol/mL^{-1} min^{-1}
K_m	0.011 μmol/mL	0.0086 μmol/mL

TABLE III
Effect and *p*-Values of Tested Factors on Packed Bed Performance[a]

Effect	*p*-value
Concentration	<0.0001
Flow rate	<0.0001
Run 1 versus run 2	0.0060
Run 2 versus run 3	0.0500

[a] Repeated measures statistics were performed on absorbance data.

Column experiments

The full-factorial tests using flow rate and concentration of ONPG substrate as factors were analyzed using repeated-measures analysis of variance (Systat version 9.0). There was a significant ($p < 0.05$) effect of flow rate and concentration on bed performance, as measured by percentage substrate conversion (Table III). As the flow rate was increased from 20 to 100 mL/min, substrate conversion decreased by an average of 86% for both ONPG concentrations (Figs. 4 and 5). This was expected because a shorter substrate residence time within the bed means the substrate was less likely to be converted to product by the enzyme. A significantly ($p < 0.05$) higher substrate conversion efficiency was also observed within the 0.0015*M* ONPG treatment compared to that within the 0.003*M* treatment. Doubling the ONPG concentration from 0.0015 to 0.003*M* (while keeping flow rate constant) resulted in an average of 16% loss in conversion efficiency. However, the total conversion of substrate was still 48.9% higher in the 0.003*M* ONPG treatment.

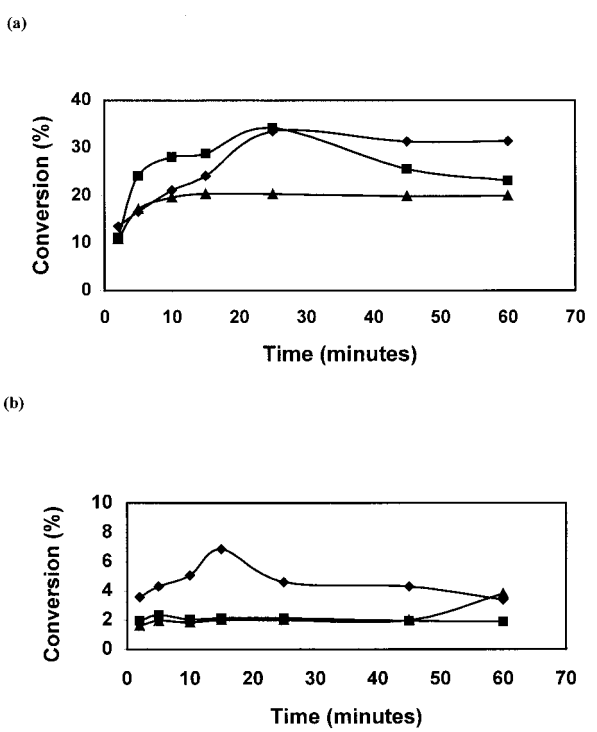

Figure 4 Conversion of 0.0015*M* ONPG as a function of time and run number: (a) flow rate of 20 mL/min; (b) flow rate of 100 mL/min. ◆ = Run 1, ■ = Run 2, ▲ = Run 3. Each value is the average of three replications.

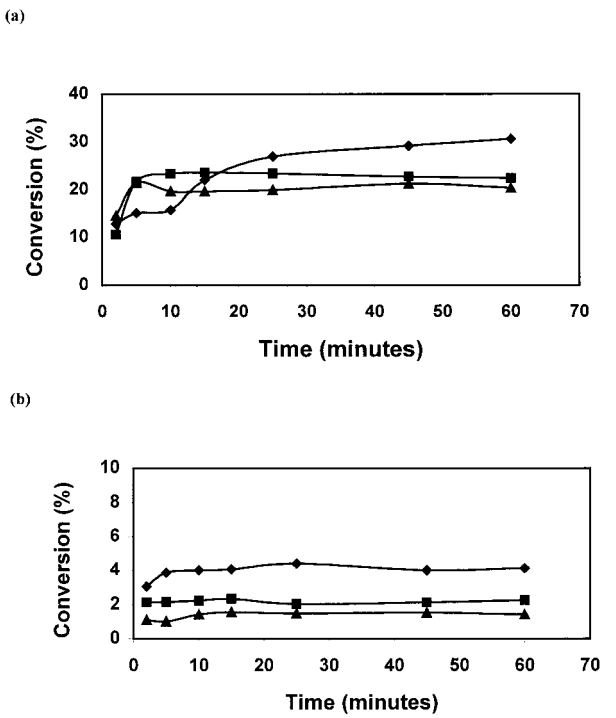

Figure 5 Conversion of 0.003*M* ONPG as a function of time and run number: (a) flow rate of 20 mL/min; (b) flow rate of 100 mL/min. ◆ = Run 1, ■ = Run 2, ▲ = Run 3. Each value is the average of three replications.

Higher substrate conversion efficiencies with lower flow rates and higher substrate concentrations were also reported by Carrara and Rubiolo.[6] However, the overall substrate conversion percentages were much higher (34.4–93.1%) than values reported here (1.02–34.15%; Figs. 4 and 5). This was likely attributable to the much lower flow rates tested (1.9–8 mL/min compared to 20 and 100 mL/min in this study) as well as higher reactor temperatures (43°C compared to 21°C in this study). Differences in substrate conversion may also be attributed to immobilization method, given that Carrara and Rubiolo[6] covalently linked β-galactosidase to the surface of chitosan beads using glutaraldehyde. Rejikumar and Devi[9] and Petzelbauer et al.[18] also reported higher substrate conversions in a packed-bed reactor containing β-galactosidase or β-glycosidases covalently bound to chitosan bead surfaces using glutaraldehyde. Again, the different immobilization methods and operating conditions (higher temperatures, lower flow rates) contributed to differences in conversion efficiencies.

There was a statistically significant effect of repeated bed use on substrate conversion. Significant

differences were observed between Run 1 and Run 2 but not between Run 2 and Run 3 (Table III). During Run 1, less substrate was converted to product during the first 10 min than in subsequent runs. However, substrate conversion continued to increase and by 60 min total conversion was an average of 35% higher than that at the same time period for the two subsequent runs. In contrast, Runs 2 and 3 were characterized by higher initial conversion rates, followed by a plateau after 10 min. The higher substrate conversion in Run 1 of each experimental bed may have been the result of enzyme leakage from the beads during subsequent runs. Previous results by this research group[14] showed that immobilization of β-galactosidase using the same method used in this study, resulted in some initial leakage of protein/enzyme that was entrapped in the matrix, under batch conditions. It was for this reason that the column tests conducted in this study were undertaken: to determine whether the immobilized enzyme could be used successively. These results show that the enzyme can be used continuously and repeatedly in column form with a small initial decrease in activity (Run 1) leading to steady activity seen in Runs 2 and 3. Although there was no statistical difference in substrate conversion between Runs 2 and 3, there was a consistent trend for a lower substrate conversion in Run 3. The difference in initial conversion during the first 10 min between Runs 1 and 2 versus Runs 2 and 3 may have been attributed to the beads adjusting to new localized microenvironmental conditions.

Reactor stability

Reactor stability tests were performed in triplicate with a flow rate and concentration level of 20 mL/min and 0.003*M*, respectively. The test bed continued to convert ONPG to free nitrophenol for more than 12 h. Substrate conversion was reduced by an average of 16.8% from the 1st hour to the 12th hour. The reduction in substrate conversion was likely attributable to the enzyme leaking from the chitosan matrix and washing out of the reactor with the nitrophenol product.

CONCLUSIONS

The overall performance of the chitosan-entrapped β-galactosidase was evaluated in terms of enzymatic activity, pH stability, and multiple reuse in a packed-bed system. There was a negative effect of repeated bed usage on substrate conversion between Runs 1 and 2, and a continued trend for a reduction in substrate conversion on the following run. However, within each run, substrate conversion reached a plateau before 60 min. Additional research is needed to observe the effects of long-term reactor use on substrate conversion. The use of lower flow rates, to more closely mimic commercial practices, should also positively impact longer-term use, resulting in the constant and dependable conversion of substrate that is desired in industrial reactors. When used in a packed-bed reactor, chitosan-entrapped β-galactosidase has promising applications; however, further work is required to further optimize the use of nontoxic chitosan gel beads in enzyme-entrapment systems for the food and biomedical industries.

This manuscript is No. 2665 of the University of Maine Agricultural and Forest Experiment Station.

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