Trop J Nat Prod Res, March 2024; 8(3):6693-6699 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Immobilization of *Lactobacillus acidophilus* β-galactosidase on chitosan obtained from the shells of the African giant snail, *Achatina achatina*

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ARTICLE INFO

Article history:
Received 05 December 2023
Revised 13 March 2024
Accepted 18 March 2024
Published online 01 April 2024

ABSTRACT

High enzyme activity and reusability are the major factors that limit enzyme application in the industry. This study explored the properties of *Lactobacillus acidophilus* β-galactosidase immobilized on *Achatina* chitosan, for improved enzyme reusability in industry. β-Galactosidase was produced from environmentally well-adapted *Lactobacillus acidophilus*. The enzyme was purified by ion exchange chromatography using DEAE-cellulose and had a molecular weight of 43kDa. Mg^{2+} was a major positive effector of the β-galactosidase activity. Chitin was extracted from *Achatina* shells by demineralization and deproteination, and deacetylated to chitosan. The chitin and chitosan yields were 74.64% and 58.60%. However, a hypochlorite-decolorized chitin, deacetylated to chitosan, gave a yield of 71%. FTIR spectra of chitin showed major bands at 711 $cm^{-1,}$ 855 cm^{-1}, 1082 cm^{-1}, and 1438 cm^{-1} for chitin and for chitosan at 6778 cm^{-1}, 711 cm^{-1}, 851 cm^{-1}, 1082 cm^{-1} and 1436 cm^{-1}. The β-galactosidase was immobilized on chitosan beads by adsorption and covalent linkage using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS), and glutaraldehyde, separately. The enzyme had optimal temperature and pH of 70°C and 5.5. The Michaelis-Menten constant (K_M) and maximal velocity (V_{max}) of the free and immobilized β-galactosidase were 0.262/0.251mM, and 270.27/290 µmol/min using p-NPG as substrate, and 0.53/10.02 mM and 250/275 µmol/min for lactose as substrate, respectively. Covalent immobilization by glutaraldehyde improved the β-galactosidase activity more than adsorption, in comparison to EDC/NHS. The results show that extracellular β-galactosidase from *Lactobacillus acidophilus*, isolated from dairy wastewater, can be immobilized on chitosan support produced using cheaply available *Achatina* shell chitosan for greater reusability.

***Keywords*:** *Lactobacillus acidophilus*, β-galactosidase, properties, chitosan, *Achitina achatina*, immobilization, adaptation.

Introduction

β-Galactosidase [EC 3.2.1.23] catalyzes the transgalactosylation and hydrolysis of β-D-galactopyranoside substrates.[1] The enzyme is found mainly in plants, animals, and microorganisms.[2] In humans, it is abundant in the gut where it hydrolyzes the main carbohydrate in milk, lactose, into galactose and glucose which are then absorbed across the intestinal epithelium[3]. Lysosomal β-galactosidase is a reliable indicator of the switch mechanism for cells entering senescence and has become useful as a probe for fluorescence-guided diagnosis of some cancers.[4,5]
β-Galactosidase has two crucial applications in the food industry: the hydrolysis of lactose for reducing lactose levels in dairy products, and the transgalactosylation reactions for the synthesis of galactooligosaccharides (GOS).[6,7] Lactose mal-absorption or lactose intolerance is a severe health problem in about 75% of the world population who could be at risk from the consumption of frozen milk products which usually contain large amounts of lactose.[8,9]

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Citation: Chilaka FC, Ezugwu AL, Oparaji EH, Eje OE. Immobilization of *Lactobacillus acidophilus* β-galactosidase on chitosan obtained from the shells of the African giant snail, *Achatina achatina*. Trop J Nat Prod Res. 2024; 8(3):6693-6699. https://doi.org/10.26538/tjnpr/v8i3.32

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Most GOS are classified as prebiotic food (since they are non-digestible) or *Bifidus* growth factor as they can stimulate the establishment of human colonic *Bifidobacteria species.* In addition, GOS, by suppressing the expression of bacterial quorum-sensing (QS) genes, could prevent the growth and production of biofilms by some possible harmful enteric bacteria.[10-13]
Industrial β-galactosidases are usually sourced from microorganisms, especially fungi, bacteria, and yeasts.[8,9,14] Although many bacteria produce β-galactosidases, major β-galactosidase production has majorly focused on the use of some *Lactobacillus species* of the Lactic acid bacteria (LBA) with the "Generally Regarded As Safe (GRAS)" status.[15,16] LBA is naturally found in any environment rich in carbohydrates, such as decomposing plant materials, fermented foods, and in the feces of individuals on high milk, lactose, or dextrin diets.[17] *Lactobacillus acidophilus* ('acid-loving milk-bacillus') is a homo-fermentative species of *Lactobacillus* that grows at pH below 5.0 in the lumen of the gut, especially the lower end of the small intestine where it metabolizes any residual lactose, thus encouraging lactose digestion in individuals with low galactosidase activity.[15]
Good thermostability and cost are major factors to consider in the industrial application of enzymes.[18] At higher temperatures, thermostable enzymes have increased activity, substrates maintain higher solubility and microbial fouling could be considerably reduced.[9] High cost is a critical issue in the industrial use of immobilized enzymes in relation to enzyme reusability and the regenerability of immobilization supports. This issue has been sufficiently addressed by the overwhelming choice of biopolymeric immobilization supports like some proteins and carbohydrates, but more especially the polysaccharides, chitin, and chitosan.[19-21] Chitin is one of the most abundant natural polymers. It can be easily obtained at a relatively low

cost from waste from the shells of crabs, shellfish, shrimps, krills and lobsters, snails, insect cuticles, and fungal cell walls, especially from mushrooms.[22] Chitin, when deacetylated, is termed chitosan.[23] Chitosan disrupts microbial quorum sensing (QS) by preventing microbial contamination and fouling of industrial processes.[24-27]

On the consideration that the best and safest sources for commercial microbial *β*-galactosidase are some strains of lactose-fermenting bacteria, we explored the potential of a novel lactic acid bacteria isolated from traditional fermented milk product industrial wastes in Port Harcourt in Rivers State, Nigeria to produce *β*-galactosidase by an optimized submerged fermentation to improve its yield.[28] An adequate industrial application of this *β*-galactosidase would require satisfactory immobilization on cheap reusable supports in relation to reusability and regenerability. There is a high consumption of giant snails (*Achatina*) in Nigeria with the low biodegradable shells while constituting an enormous waste, could provide a cheap source of chitin and chitosan. *Achatina* shell chitosan beads were prepared and used to immobilize a purified extracellular *Lactobacillus acidophilus β*-galactosidase by adsorption and cross-linking, and the immobilized enzyme was characterized.

Methods

Chemicals

Chemicals of analytical grade were used in this research and were products of May, and Baker Limited (England), BDH Chemical Limited (England), Sigma Aldrich, and Merck (Germany).

Isolation, screening, identification, and production of Lactobacillus acidophilus β-galactosidase

The wastewater (50 mL) was diluted 10^{-7} folds and the solution (1 mL) was added to DeMan Ragoshie sharpie (MRS) agar medium, followed by incubation at 38˚C for 3 days. Different colonies were isolated by sub-culturing them on different plates and the step was repeated to obtain pure isolates, which were characterized using the method described by Ezeonu et al.[29] Screening, and 16S rDNA identification of β-Galactosidase producing microbial strain from dairy effluent, production, and some properties of *Lactobacillus acidophilus β*-galactosidase, have already been reported.[28,30]

Assay for β-galactosidase activity

The activity of *β*-galactosidase was assayed by using the p-NPG as a substrate.[31] After 30 min of incubation, 4 mL of 0.1M NaOH was used to stop the reaction and develop the color. Absorbance was read at 400 nm using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas). One unit (U) of enzyme activity is given as µmole p-nitrophenol liberated per minute. Protein concentration was determined by the method of Lowry et al.[32] using bovine serum albumin (BSA) as the standard protein.

The molecular weight of Lactobacillus acidophilus β–galactosidase

A dialyzed 70% ammonium sulfate precipitated *β*-galactosidase was loaded onto a pre-swollen DEAE 52 column (2.0 x 14) cm equilibrated with 0.01M sodium phosphate buffer (pH 7.5). After washing off unbound proteins, the enzyme was recovered by step elution using 0.1 to 1M of sodium chloride in 0.01 phosphate buffer (pH 7.5). The 5 mL combined active fractions from the ion exchange chromatography were loaded and eluted on the Sephadex- G-100 gel column (65 x 1.6cm) initially calibrated with appropriate molecular weight (Mr) markers.[33] The Mr of the *Lactobacillus acidophilus* β-galactosidase was extrapolated from a Mr ladder constituted by the Mr markers.

Effects of metal ions on β-galactosidase activity

The effect of the different concentrations of Mg^{2+} and Mn^{2+} was determined by a 30 minute incubation of *β*-galactosidase in varied concentrations (20-50 mM) of the metal ions using pNPG as substrate.

Extraction of chitin and production of chitosan

Chitin was extracted from the shells of the snail by a three-step process of demineralization, deproteinization, and decolorization. Chitosan was then produced by deacetylation of the chitin. The shells of the African giant snail, *Achitina maginata*, were obtained from Itam Market in Uyo, Akwa Ibom State, Nigeria. Pulverized shells were demineralized in 2M HCl. After 24 h, the demineralized product was washed with distilled water until the pH became neutral. This was followed by deproteination in 1N NaOH for 24 h at 80 ℃. The chitin product was washed to neutrality. The chitin was treated with different concentrations of decolorization agents, such as sodium hypochlorite, permanganate, ethanol, acetone, ethanol + acetone, and acetone + ethanol + permanganate. The chitin was eventually decolorized with 0.1% hypochlorite solution for 48 h and washed to neutrality. The decolorized chitin was deacetylated to chitosan in 40 % sodium hydroxide (10 M NaOH) at 100 ℃ for six (6) hours. After washing it thoroughly, the chitosan was oven-dried at 60℃ for 48 h in an oven box (Gellemp haump, Germany). The obtained chitosan powder was stored in airtight containers. The percentage yields for chitin and chitosan were calculated using equation 1 and 2.[34]

$$\% \text{ Yield of chitosan} = \frac{\text{weight of dried chitin}}{\text{weight of ground shell}} \text{x } 100 \quad -----1$$

$$\% \text{ Yield of chitosan} = \frac{\text{weight of dried chitosan}}{\text{weight of chitin}} \text{x } 100 \quad ----------2$$

Analyses of chitin and chitosan by FTIR Spectrophotometry

Samples of chitin and chitosan were prepared in KBr for analysis of unique functional groups using FTIR spectrophotometry (Model: Agilent Carry 630, Germany). Absorbance Spectra were recorded in the region (4000 to 400 cm^{-1}) with a resolution of 4 cm^{-1}. [35] The degree of deacetylation (DD) was evaluated from the spectra.

Preparation of the chitosan beads

A quantity (2.5 g) of chitosan was dissolved in 1% acetic (100 mL) and concentrated at 75 ℃ until a chitosan hydrogel was formed as described by Kamburov and Lalov.[36] To form the macrobeads, the chitosan hydrogel was introduced as droplets into 30% sodium hydroxide (100 mL). The chitosan beads produced were then washed to neutrality and oven-dried overnight at 60 ℃.

β-galactosidase immobilization on chitosan beads.

Crude and purified *β*-galactosidase were immobilized on chitosan beads by methods of adsorption and covalent linkage.[36,37] Immobilization by adsorption involved the incubation of dried chitosan beads (0.5 g) in 10 mL of enzyme solution for 2 h and the unbound enzyme was washed off. The activities and protein concentrations of the adsorbed and unbound *β*-galactosidase respectively were determined, and the specific activity (SA) of the adsorbed enzyme was calculated using equation 3:

$$\text{SA} = \frac{\text{Enzyme activity (U)}}{\text{Protein concentration}} \quad ----------3$$

Percentage immobilization efficiency (IE) by adsorption of the enzyme on chitosan beads was calculated from equation 4:

$$\text{IE} = \frac{[E_1]-[E_2]}{[E_1]} \text{ X } 100\% \quad ----------4$$

Where $[E_1]$ = Concentration of enzyme (mg/mL) being added during the immobilization process. $[E_2]$ = Concentration of unbound enzyme (mg/mL).[38]

For covalent immobilization, the chitosan beads (0.5 g) were stirred in a 4 mL solution of 2 mM EDC containing 5 mM NHS solutions in 0.01M phosphate buffer pH 6.0. After standing at 37 ℃ for 2 h, the beads were recovered after decantation of the crosslinking mixture. After washing off any remaining solution, 1 mL of *β*-galactosidase was poured into the carbodiimide-activated chitosan gel beads and allowed to stand for 3-48 h at 37 ℃.[37] The unbound enzyme was washed off. The activity and protein concentration of the bound and unbound *β*-galactosidase were determined and the IE of the bound enzyme was evaluated as described above. Samples containing no EDC served as controls for physical protein adsorption. Equally, fresh chitosan beads (0.5 g) were immersed in a glutaraldehyde `solution containing 200 µL of 25% glutaraldehyde (w/v) in 100 mL of distilled water.[36] After 3 h,

the excess glutaraldehyde was decanted and the beads were thoroughly washed with distilled water. The beads were introduced to 1 mL of *β*-galactosidase solution. After a 5-48 h interval, the unbound enzyme was washed off. The activity and protein concentration of bound and unbound *β*-galactosidase were determined. The percentage immobilization efficiency of the bound *β*-galactosidase was calculated using Equation 3.

Effect of pH, temperature, and substrate concentration on free and immobilized β-galactosidase

The effect of pH and temperature on purified free *β*-galactosidase has already been described.[30] The method was adopted for the effect of pH on the immobilized β-galactosidase by using 2 mM p-NPG in 0.1M CH_3COONa (pH 3.5-5.5), 0.1M Na_2HPO_4 (pH 6.0-7.0) and 0.1M Tris-HCl (7.5-9.0) buffers. The optimum pH was determined by plotting the enzyme activities against pH. The optimal temperature of the immobilized *β*-galactosidase, as well, was monitored by incubating the immobilized enzyme and 2 mM p-NPG in (0.1 M) phosphate buffer solution (pH 6.5) at different temperatures (30-70 °C) for 30 minutes.[30] The enzyme activities were plotted against temperature. The effect of different p-NPG concentrations on *β*-galactosidase activity was determined as described by Chilaka *et al*.[39] with different concentrations (0.05 - 0.9 mM) of the substrate (p-NPG) at pH 5.0 and temperature of 70 °C.

The effect of varied concentrations of lactose on the free and immobilized *β*-galactosidase activity was also determined. In this case, the concentration of released reducing sugar was calculated by reaction with dinitrosalycilic acid (DNSA). The Michaelis constant (K_M) was obtained from the Lineweaver-Burk plot of the initial velocity of enzyme activity at varied concentrations of p-NPG and lactose.

Statistical Analysis

All assays were carried out in triplicates and the values were analyzed using Statistical Product and Service Solution (SPSS) to obtain their mean and standard deviation.

Results and Discussion

Lactobacillus β-galactosidase-producing species was confirmed as *Lactobacillus acidophilus* using the 16S rDNA sequencing technique[30]. The results of agarose gel electrophoresis showed a characteristic band at approximately 750 bp (Figure 1).[30] As a single amino acid is coded by three bps, and the average molecular weight of one amino acid is 110, a Mr of 27,000 daltons (27KDa) for *Lactobacilli acidophilus β*-galactosidase was deduced from 750bp.

Lactobacillus acidophilus submerged culture produced an extracellular *β*-galactosidase with the highest activity on the 12th day of incubation. It has also been shown that in cultures of *Bacillus coagulans* RCS3, the extracellular β-galactosidase activity increased rapidly and continued to increase after 10 days which suggested that the synthesis and secretion of the extracellular β-galactosidase in bacteria occurs largely after the cells have matured.[40]

Two types of *β*-galactosidases are widely present in LAB: a GH2 LacLM *β*-galactosidase and a GH42 LacA *β*-galactosidase.[9,41] GH2 LacLM *β*-galactosidases focus on extracellular and intracellular lactose hydrolysis, while GH2 LacZ *β*-galactosidases promote galactoligosacchide (GOS) synthesis. Most *Lactobacillus* GH2 LacLM *β*-galactosidases are thermophilic *β*-galactosidases mostly active at acid to neutral conditions with pH optima between 4.0 to 7.5.[9,42,43] As extracellular enzyme from *Lactobacillus acidophilus* had a pH optimum of 5.0 with an optimum temperature of 70 °C, it would appear that extracellular *L. acidophilus β*-galactosidase is a thermophilic *β*-galactosidases resembling the GH2 LacLM type *β*-galactosidases. The molecular weight of the purified extracellular *β*-galactosidase from *L acidophilus* obtained from gel filtration was 43kDa. This is similar to the purified monomeric *L. salivarius* and *Bacillus subtilis* SK09 *β*-galactosidases that showed molecular weights of 30 and 43 kDa, respectively.[44] GH2 LacLM type *β*-galactosidases are multimeric enzymes with the predominant quaternary structure of a heterodimeric protein of native molecular weight of ~115 kDa comprising an active larger subunit of Mr 60-90 kDa,[9,42] and inactive 35 kDa subunit. The active extracellular *Lactobacillus LacLM β*-galactosidase with A Mr of 43 kDa from gel-filtration compares favorably with GH2 LacLM type *β*-galactosidases active larger subunit of Mr 60-90 kDa,[9] and is close to the computed molecular weight of about 27kDa based on a 750 bp in the 16S rDNA sequencing. However, the difference could arise from one or more spontaneous deletion events within an ancestral *β*-galactosidase gene in a dispensable domain or post-transcriptional processing and modifications of the ancestral *β*-galactosidase required to translocate and export extracellularly, an active and stable enzyme requiring extensive proteolysis by cleavage of signal peptides and chaperone propeptides might be important in the production of tailor-made fully folded active conformations of extracellular enzymes with sequences likely shorter than their intracellular counterparts and possibly made in a protease-resistant conformation for longer shelf life.[45]

Studies on the effect of metal ions on enzyme activity showed that Mg^{2+} and Mn^{2+} enhanced enzyme activity when compared with the control (Figure 2). *L. acidophilus β*-galactosidase activity was enhanced by Mn^{2+} and Mg^{2+}. The LacLM heterodimeric *Lactobacillus acidophilus* R22 *β*-galactosidase was activated by Mg^{2+} which improved both activity and stability suggesting the possible presence of binding sites for Mg^{2+} within the active site which might interact with Glu residues and, thus, could contribute to both subunit interactions and global enzyme stabilization.[9,46]

Chitin is insoluble in water and organic solvents but could be processed by extensive alkaline deacetylation to obtain a more soluble product called chitosan. The chitin yield from the *Achatina* shells was 74.64%, while the chitosan yield was 58.60%. Decolorizing the chitin with 0.1% hypochlorite increased the yield of chitosan to 71%. This result shows that the chitin and chitosan, extracted from *Achatina* shell waste, are available at a low cost and in high abundance.

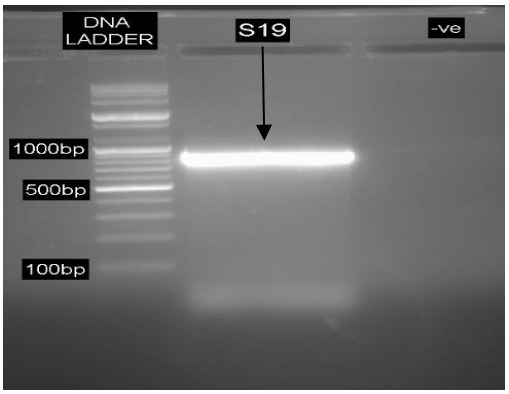

Figure 1: Agarose gel electrophoretogram of the amplicons. The lane S19 represents the amplified bacterial genome.

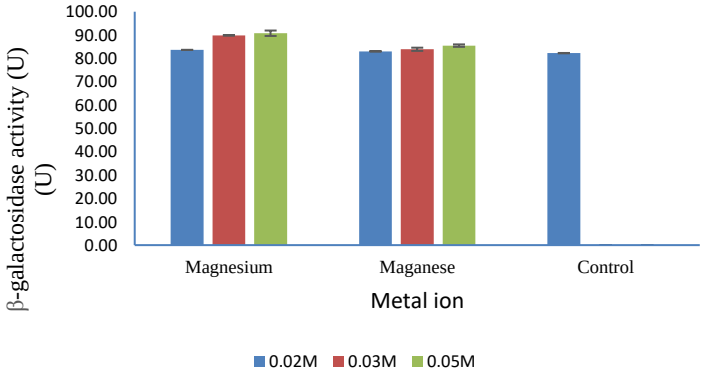

Figure 2: Effect of Mg and Mn ions on *Lactobacilli acidophilus β-galactosidase* activity

Chitin occurs in three polymorphic forms (α, β, γ) in nature arising from specific hydrogen bonding interactions reflected by different but explicit FTIR signatures. FTIR spectra of chitin and chitosan showed major bands at 711 $cm^{-1,}$ 855 cm^{-1}, 1082 cm^{-1}, and 1438 cm^{-1} for chitin; and at 6778 cm^{-1},711 cm^{-1}, 851 cm^{-1}, 1082 cm^{-1} and 1436 cm^{-1} for chitosan (Figure 3 and 4). As the chitosan spectra did not show any sharp absorptions at about 3500 cm^{-1} - 3000 cm^{-1} attributable to the –OH groups in positions C2 and C6 of the chitosan usually involved in intra- and intermolecular hydrogen bonds.[47] The absence could indicate that the snail chitin and chitosan exhibit no marked absence of free OH groups. In this region, α-chitin usually expresses a more detailed structure than β-chitin. As the NH of the amide group is also involved in intermolecular hydrogen bonding CO· · ·HN, bands at 3261 and 3110 cm^{-1} are normally seen in α-chitin spectra, but are weak and not easily observed in β-chitin.[48] The observed peak at 1438 cm^{-1} suggests a strong amide 1 (C=O) band, a characteristic feature of chitin, and usually assigned to β-chitin due to a different environment of the primary hydroxyl groups in β-chitin.[49] Thus, the snail's chitosan is a β-chitin with a 71% degree of deacetylation. Acetylated chitins of 70–90%, and low protein content, are considered good final products.[50] α-Chitin extracted from shrimp and crab shells showed the highest acetylation degree with up to 88.5% and 78.6%, respectively. β-chitin has a lower acetylation rate, about 70.1%, compared with α-chitin obtained under the same conditions.[35]

The content of polysaccharide was represented by bands between 850 and 1156 cm^{-1} with the peak at 1082 cm^{-1} representing anti-symmetric stretching of the C-O-C bridge and the peaks between 600-895 cm^{-1} due to the C–H bonds of the anomeric carbon assigned to β-linkage of the polysaccharide. The OH-out-of-plane bending peaks were between 6778-851 $cm^{-1,}$ which is suggestive of β-chitin.

Analyses of FTIR spectra of chitin treated with different decolorization agents showed that chitosan production with sodium hypochlorite, ethanol, acetone + ethanol + permanganate, acetone, and ethanol + acetone showed 58.60, 37.30, 31.20, 29.90 and 26.34% chitosan yield, respectively, and 71, 54, 58, 24 and 28% deacetylation, respectively. The results indicate that sodium hypochlorite was the best decolorization agent.

For applications in the food industry, chitosan has key properties of great interest, including high colloidal stability in suspensions, hydrophilicity, good biocompatibility, nontoxicity, antimicrobial activity, a remarkable affinity for proteins, high mechanical strength, and good chemical stability.[51-54] Chitosan displays excellent gel-forming properties with enough malleability and high adaptability for reshaping into various geometrical configurations for the production of different 3D forms as in chitosan gels of various forms, such as beads, membranes, coatings, capsules, powder, gels and films, sponges, spherical micro/nanoparticles and electrospun fibers which can be remodeled to serve as appropriate support for different immobilization techniques.[55]

There are three major common enzyme immobilization techniques: physical adsorption, entrapment/encapsulation, and cross-linking/covalent binding.[55,56] The -NH_2 and -OH groups in chitosan molecules are attractive sites that can bind or allow the introduction of additional chemical groups that can bind or react, with the amino acid residues on enzyme surfaces for enzyme immobilization. Chitosan is a cationic amino polysaccharide with its primary amino (NH^{3+}) groups.[55] In solutions of lower pH (pK of 6.3 to 6.8), most of the amino groups are protonated and chitosan is soluble in aqueous acid media, while at higher pH the amino groups are deprotonated and chitosan becomes insoluble forming viscous solutions. The cationic chitosan forms water-insoluble complexes with anionic polyelectrolytes as the high positive charge on $–NH^{3+}$ groups enables chitosan to adhere to negatively charged surfaces and, bind with, and aggregate polyanionic compounds. Many enzymes with polyanionic groups can be immobilized on chitosan by simple physical adsorption.[19,51-53] Initial experiments showed that the pH optimum for adsorption of *β*-galactosidase onto chitosan beads was 6.0 for both crude extracts and purified *β*-galactosidase. It required 120 min and 60 min for optimum adsorption of crude and purified *β*-galactosidase, respectively. After adsorption, the immobilization efficiency (IE) of the crude was 69.4 % with a specific activity of 274.12 U/mg whereas the purified enzyme had an immobilization efficiency of 70.09%, with a specific activity of 317 U/mg, indicating that there was greater immobilization by adsorption of the purified enzyme than the crude.

Chitosan beads are relatively faint, with weak mechanical stability, and suffer from dissolution under acidic conditions. To produce mechanically resistant support for enzyme immobilization applications, crosslinking of the chitosan chains is required and takes advantage of abundant and available highly reactive functional amine and hydroxyl groups in the chitosan molecules often using the bifunctional reagents such as carbodiimide hydroxysuccinimide (EDC/NHS) and glutaraldehyde to form stable covalent complexes between the support matrix and functional groups of the enzyme. At a pH of 6.0, an immobilization period of 2 h was optimal for the cross-linking of β-galactosidase using either EDC/NHS or glutaraldehyde. The IE and SA of crude *β*-galactosidase cross-linked with EDC/NHS were 69.18% and 258.23 U/mg, whereas the purified enzyme had an immobilization efficiency of 76.14%, with a specific activity of 356.54 U/mg.

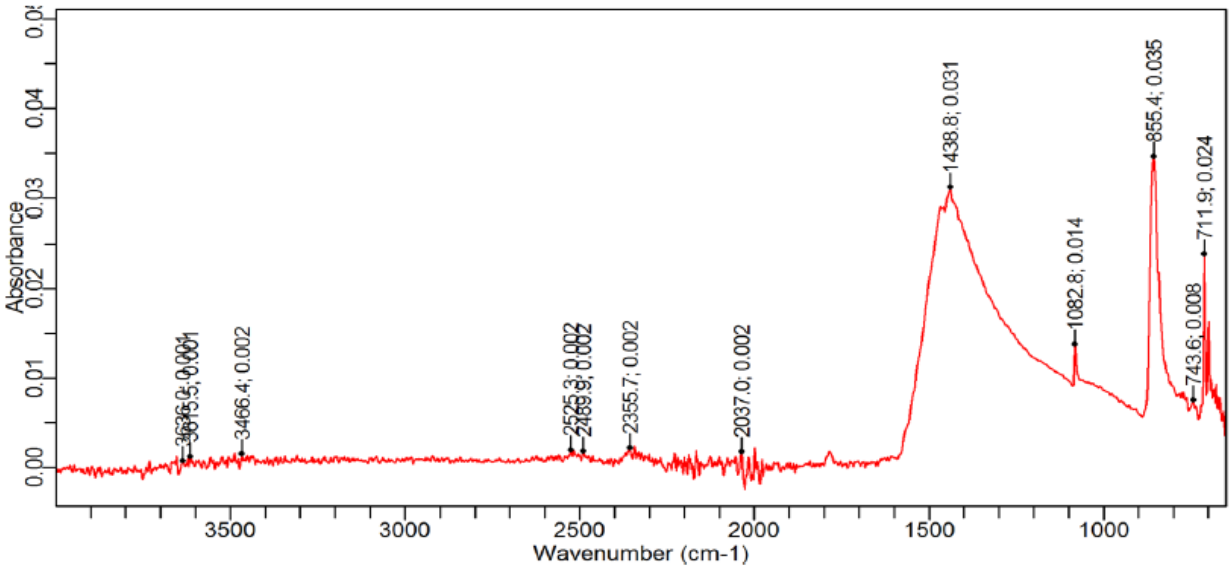

Figure 3: FTIR spectrum of the extracted chitin from shells of water snail.

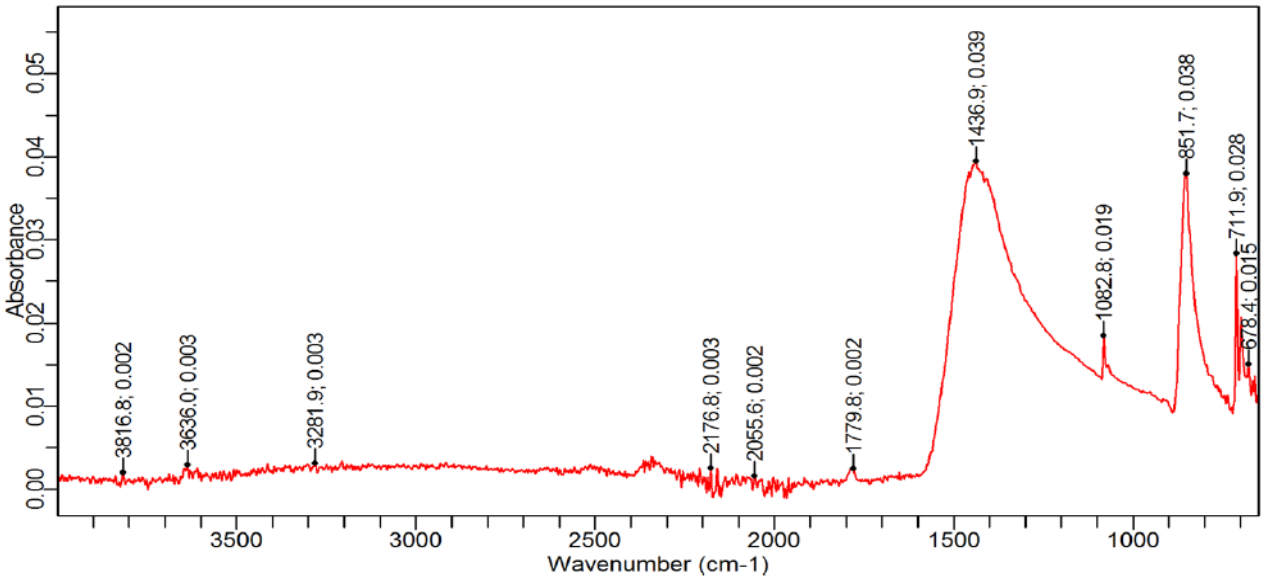

Figure 4: FTIR spectroscopy analysis of chitosan obtained when chitin was decolourized with 1% sodium hypochlorite.

The crude *β*-galactosidase cross-linked with glutaraldehyde had IE and specific activity of 68.71% and 264 U/mg, whereas that for the purified enzyme were 77.76% and 349 U/mg. These results indicate that glutaraldehyde was a slightly better cross-linking agent compared to EDC/NHS. Although adsorption involving ionic interactions is a simpler method for immobilization of the *β*-D-galactosidase, covalent immobilization on chitosan beads improved the β-galactosidase activity over adsorption, probably due to poor adsorption of the enzyme on the chitosan beads. As we achieved an immobilization efficiency (IE) of 69.4% to 77.76% for the *β*-galactosidase, it has been suggested that immobilization efficiency above 50 % during enzyme immobilization using enzyme cross-linkers could be suggestive of a strong interaction between the reactive groups on the immobilization matrix and the reactive groups on the enzyme.[57]

The purified *β*-galactosidase had a pH optimum of 5.0 and an optimum temperature of 70 °C. The glutaraldehyde covalently immobilized enzyme had an optimum pH of 5.5 (Figure 5) and an optimal temperature of 70 °C (Figure 6). Lineweaver-Burk plots of the initial velocity of the free and immobilized β-galactosidase determined at pH 5.0 and temperature of 70 ˚C at varied concentrations of p-NPG, gave the Michaelis-Menten constant (K_M) of 0.262 and 0.251 mM, and maximal velocity (V_{max}) of 270.27 and 290 µmol/min, respectively. The K_M of the free and immobilized *β*-galactosidase for lactose was evaluated to be 10.53 and 10.02 mM and V_{max} was 250 and 275 µmol/min, respectively at pH 5.0 and temperature of 70 ˚C. This would indicate that the immobilization of *Lactobacillus acidophilus β*-galactosidase on chitosan did not greatly affect the kinetic properties of *β*-galactosidase. The fact that there was little or no change in the pH and temperature optima, as well as the K_M, of the immobilized *β*-galactosidase in comparison to the non-immobilized, would indicate the enzyme retained most of its unique properties on immobilization. The covalent immobilization of β-galactosidase on chitosan supports has been extensively studied which revealed dramatically improved enzyme properties such as stability of the enzyme at low, medium, or high pH and temperatures as well as the kinetic parameters, K_M, V_{max}, and *Ki*.[58] In relation to stability and efficiency in synthesis, a comparison of chitosan-immobilized *β*-galactosidase with free enzyme and cross-linked aggregates revealed that chitosan-immobilized *β*-galactosidase gave maximum yield but was more suitable for cost-effective industrial production due to enzyme reusability and matrix regenerability.[55,56]

The major problem that limits the use of immobilized *β*-galactosidase in lactose hydrolysis and other immobilized enzyme systems is microbial fouling. For long-term operations, extra costs might be incurred as periodic washing and pasteurization could be required.[59] A very unique property of chitosan is its low tendency for microbial contamination due to its ability to disrupt microbial quorum sensing, QS. Chitosan has been shown to interact with bacterial cells via electrostatic adsorption of the polycationic groups of chitosan to the anionic bacterial cell envelope leading to disruption of cellular membranes, and by a process of ionic interaction between bacteria cells and charged polymer surfaces, effectively delivering the quorum-quenching compounds to the microbes causing cell damage and inhibiting biofilm formation.[24-27]

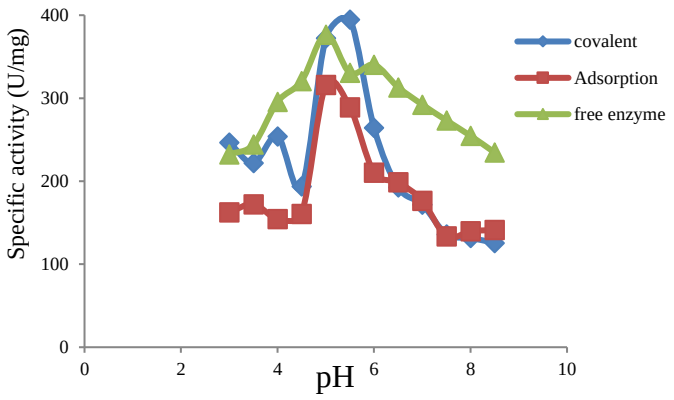

Figure 5: pH activity profile of free and immobilized *Lactobacillus acidophilus*β-galactosidase.

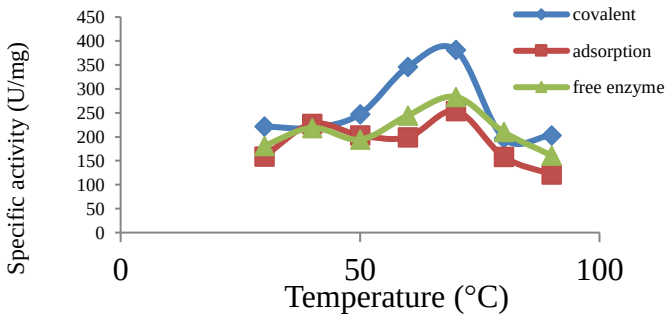

Figure 6: Temperature activity profile of free and immobilized *Lactobacillus acidophilus*β-galactosidase.

Conclusion

This study has shown for the first time that chitosan from *Achatina* shells can be good and cheap immobilization support for *L. acidophilus* *β*-galactosidase. The immobilization of *Lactobacillus acidophilus* β-galactosidase on chitosan did not greatly affect the kinetic properties of *β*-galactosidase. Also, the results suggest that glutaraldehyde was a slightly better cross-linking agent compared to EDC/NHS. Covalent immobilization improved *β*-galactosidase activity more than adsorption, probably because of a stronger immobilization of the enzyme. The optimal pH, temperature, Mg^{2+} requirement, and high immobilization efficiency (70.09%) of the *L. acidophilus β*-galactosidase on chitosan beads have shown its suitability for industrial applications such as in the pretreatment of dairy wastewater. The use of chitosan tripolyphosphate (TPP) nanoparticles and electrospun nanofibers for further immobilization and application of the immobilized *β*-galactosidase on chitosan beads are being explored.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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