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COMPARATIVE STUDY OF PARAMETERS THAT INFLUENCE THE ACTIVITY OF FREE AND CHITOSAN IMMOBILIZED B-D-GALACTOSIDASE FROM E.COLI.

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ABSTRACT:

An investigation was conducted on the immobilization of β-D-galactosidase onto chitosan and characterized based on its optimal operation pH and temperature, its thermal stability and its kinetic parameters (*Km* and *Vmax*) using o-nitrophenyl β-d-galactopyranoside as substrate. The optimal pH for free β-D-galactosidase activity and immobilized β-D-galactosidase was found to be 7.3 but the immobilized enzyme worked at broader range of pH. Optimal operating temperatures of free β-D-galactosidase and immobilized β-D-galactosidase found to be 37 ◦C. At 55◦C, the immobilized enzyme showed an increased thermal stability, being more stable than the soluble enzyme retaining 60% of its initial activity. The immobilized enzyme was reused for 10 cycles, showing stability since it retained more than 70% of its initial activity. The immobilized enzyme retained approximately 100% of its initial activity when it was stored at 4°C and pH 7.0 for 30 days. The soluble β-gal lost more than 10 % of its initial activity when it was stored at the same conditions.

Key words :- *β-D-galactosidase, chitosan, immobilization, thermal stability, Vmax, km.*

INTRODUCTION:

β-D-galactosidase has been used in the dairy industry for the hydrolysis of lactose (Fig 1). The enzymatic hydrolysis of lactose by β-D-galactosidase plays an important role in the processing of dairy products, such as the production of milk containing low concentrations of lactose, the prevention of crystallization in dairy products, and the use of galactosyltransferase for synthesizing galactooligosaccharides [1,2].

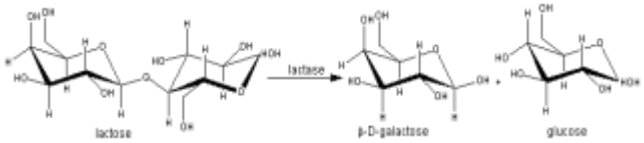

Figure 1: Schematic presentation of lactose hydrolysis by β galactosidase (lactase) enzyme. (Martin Chaplin and Christopher Bucke; Cambridge University Press, 1990)

The main disadvantage of using soluble enzymes as industrial catalysts is their poor stability in aqueous solutions against extreme pH, temperature, high ionic strengths, among others [3]. Various novel approaches have been proposed to solve this problem, including enzyme immobilization on various supports, the use of glycols and other stabilizing additives, reticulation with bifunctional agents, the use of enzymes from thermophilic organisms, or a used of chemicaly modified enzyme that increased its stability[4, 5]. The immobilization of enzymes is usually advantageous in numerous ways: the biocatalyst can be reused, the reaction volume can be reduced because the immobilized enzyme can be used at higher concentrations, there is an ease of control the process parameters when the reactor is operated continuously, it is easier to operational control, and the product can be more easily separated from the immobilized enzyme because the immobilized enzyme is not soluble in the reaction medium [6]. Pessela et al. [7] studied the immobilization of β-D-galactosidase from Escherichia coli on different supports and observed that the recovery activity was dependent on the immobilization support, which may be associated with the different levels of enzyme distortion caused by immobilization. This work aims to study to investigate the immobilization of the commercially available β-D-galactosidase from *E.coli* onto chitosan particles and determine its properties such as the optimal operating pH and temperature, the thermal stability of the enzyme (at 40 ◦C, 50 ◦C and 60 ◦C) and the kinetic parameters *Km* and *Vmax*. Chitosan is the deacetylated form of chitin, the second most abundant polymer in nature after cellulose. It is a low cost, renewable, biodegradable natural product, which has very good biocompatibility, low toxicity, chemically inert and high hydrophilic [9,10]. Different protocols may be

employed for the immobilization of enzymes in chitosan, such as adsorption, encapsulation and covalent bounding. Among the different methods, in this study we used the covalent immobilization onto glutaraldehyde activated chitosan. The method of choice due to the presence of amino groups in the structure of chitosan that react with this agent in mild conditions close to neutrality [9].

MATERIAL AND METHODS :-

All the chemicals and solvents used in this study were of analytical grade and obtained commercially. Chitosan (GRM9358), Bovine serum albumin (BSA) (MB083), Glutaraldehyde solution Grade II 25%(MB222) and the o-nitrophenyl-β-d-galactopyranoside (99%, ONPG)(RM582) and o-nitrophenol (99%, ONP)(GRM4761), Dinitrosalicylic acid (DNS) (MB232) were purchased from HIMEDIA Laboratories Mumbai. β-D-galactosidase were purchased from Sigma–Aldrich (India). All other analytical grade reagents were purchased from NOVA chemical.

2.1 The immobilization of β-D-galactosidase onto glutaraldehyde-activated chitosan

In this work, chitosan was used as a support for enzyme immobilization [11, 12]. The immobilization of β-D-galactosidase that was carried out in a batch reactor under mild agitation at 25°C by mixing the enzyme in a sodium phosphate buffer at pH 7.0 and the previously activated support given in literature [12], for different contact times between the enzyme and the support (2, 5, 10, 15, 20 and 24 h). In all experiments, the initial quantity of enzyme added to the buffer was 10 ml of enzyme per gram of support. To determine the immobilization yield, samples of supernatant were withdrawn and their hydrolytic activities were measured. A blank assay was also conducted to evaluate the possibility of enzyme deactivation in the buffer while it was undergoing immobilization. Therefore, a solution of β-D-galactosidase in phosphate buffer was prepared in a separate reactor as a control experiment, but there were no chitosan supports placed in that reactor [12].

2.3 Immobilization yield:

After measuring the initial (*Ati)* and final (*Atf*) enzyme activities in the supernatant of the immobilization suspension and in the reference suspension, the remaining enzyme activity in the supernatant (*Ati − Atf*) and the immobilization yield (*IY*) could be calculated according to Eq. (1) [13]. The reference suspension is a control assay that was performed in the absence of support to discard any possibility of loss of enzyme activity during immobilization.

$$IY(immobilization)(\%) = \frac{Ati-Atf}{Ati} \times 100 \quad (1)$$

2.4 The optimal operating pH and temperature of free and immobilized β-gal.

The effects of pH and temperature on the activities of soluble and immobilized β-D-galactosidase were evaluated. Samples were incubated at a pH ranging between 4.0 and 9.0 and a constant temperature of 37°C to evaluate the effect of pH on enzymatic activity. The effect of temperature on enzymatic activity was determined at a constant pH of 7.0 in a temperature range of 25°C to 60 ◦C. In this work, the optimal operating pH and temperature refers to the maximum enzymatic activity.

2.5 An evaluation of the thermal stabilities of soluble and immobilized β-D-galactosidase.

Soluble and immobilized β-D-galactosidase was placed in separate sodium phosphate buffer solutions at pH 7.3 and kept at 3 different temperatures of 40°C, 50°C and 60 ◦C for 1, 2 and 3 h. Samples were periodically withdrawn and their residual activities were evaluated by analyzing their ability to hydrolyze ONPG.

2.6 Determination of the kinetic parameters in the hydrolysis of O-Nitrophenyl-β-d-galactopyranoside(ONPG):

The kinetic parameters of ONPG hydrolysis were determined in the range of 0.5 – 5 mM in sodium phosphate buffer at 37 ◦C. Standard graph of ONP was prepared by taking 0.025, 0.05, 0.075, 0.10, 0.125, 0.15, 0.2, 0.3….0.9 and 1.0 mmol of ONP in borrate buffer at pH 9.8 to determine the concentration of ONP a product release by enzyme at specific time. The *Michaelis–Menten* constant (*Km*) and the maximum reaction velocity (*Vmax*) were calculated by fitting a non-linear Michaelis–Menten model and linear graph of *Linweaver-Burk* plot to the experimental data.

$$\boldsymbol{V0 = \frac{\text{Amount of product produced}}{\text{ml of enzyme} \times \text{Time}}} \quad \ldots\ldots\ldots \quad \textbf{(2)}$$

$$\boldsymbol{Catalytic\ efficiency(\%) = \frac{(Vmax/Km)immobilized}{(Vmax/Km)free} \times \frac{[E0]immobilized}{[E0]free} \times 100} \quad \ldots\ldots\ldots \textbf{(3)}$$

Where E_0 refers to the initial enzyme activity

2.7 Reuse of the immobilized β-D-galactosidase:

The reuse of the immobilized enzyme was assayed by using 0.05 g of the biocatalyst in successive batches of ONPG hydrolysis. The assay conditions

were the same as those described. At the end of each batch, the support was removed from the reaction medium, washed with phosphate buffer to remove any remaining substrate or product, dried under a vacuum and subjected to a fresh batch of ONPG for hydrolysis. The residual activity was calculated as the activity of the immobilized enzyme measured after each cycle relative to the activity of the immobilized enzyme during the first cycle.

2.8 Storage stability of the immobilized β-D-galactosidase:

Biocatalyst samples were also stored at 4 ◦C in a sodium phosphate buffer at pH 7.3, and their residual hydrolytic activities were determined after 1, 2 and 3 month of storage to evaluate the storage stability of the soluble and immobilized enzyme.

2.9 Analytical methods.

Enzyme activity: The hydrolytic activity of soluble and immobilized β-D-galactosidase was determined using o-nitrophenyl- β-d-galactopyranoside as a substrate at pH 7.0 and 37 ◦C, according to the methodology described in literature [12] but with some modifications. A 0.1 mL enzyme sample was added to a test tube containing a 0.5 mL solution of 10 mM of ONPG in deionized water, mixed into a 0.40 mL of sodium phosphate buffer solution and maintained at 37 ◦C for 2 min. The immobilized enzyme activity was measured by adding 1 g of immobilized enzyme to the test tube. The reaction was stopped by adding 3.0 mL of 200 mM Borate buffer with pH 9.8 was added. The o-nitrophenol product concentration was measured at a wavelength of 410 nm. One unit (U) of β-D-galactosidase is defined as the quantity of enzyme that liberates 1 μmol of o-nitrophenol/min under the test conditions.

$$\textbf{Units of enzyme/ml} = \frac{(\Delta A410nmTest - \Delta A410nmBlank) \times V \times DF}{t \times 4.6 \times \text{volume of enzyme used}} \quad \ldots\ldots\ \textbf{(4)}$$

Where: V= total volume of assay
DF= dilution factor of enzyme
t= time
4.6= molar attenuation coefficient of ONP

RESULT & DISCUSSION:

3.1 Immobilization of β-D-galactosidase onto chitosan activated with glutaraldehyde:

The immobilization of β-D-galactosidase onto chitosan activated with glutaraldehyde was investigated in this work, with the aim of obtaining a biocatalyst possessing operational and thermal stabilities.

Table 1 shows the results of the effect of contact time between the enzyme and the support on the immobilization yield (IY).

Table1: Immobilization parameter of β-D-galactosidase on chitosan activated with glutaraldehyde: immobilization yield (IY), enzymatic activities of the free β-D-galactosidase in the supernatant before (At_i) and after (At_f) immobilization.

Contact time (h)	At_i (U/ml)	At_f (U/ml)	IY (%)
2	1.383	0.526	61.96
5	1.340	0.436	67.46
10	1.389	0.314	77.39
15	1.386	0.120	91.34
24	1.391	0.021	98.49

No expressive loss of enzyme activity was observed in the reference suspension during immobilization. Table 1 shows that higher immobilization yields were achieved with an increased contact time. These results indicate that the first bonds between enzyme molecules and activated support are built up quickly. Nevertheless, the next bonds take more time to be formed since the protein itself becomes a barrier to enzyme molecules diffusion [15]. Thus, longer contact times are needed to overcome the intra-particle diffusion effects, caused by the presence of the enzyme inside the pores, and promote more enzyme molecules to link to the support. The immobilization yields observed to increase for 24 h (see Table 1). But the maximum immobilization observed After 10 h, thereafter maximum immobilization required 24 h. Therefore, a contact time of 24 h was selected as the optimal contact time for further immobilization assays.

3.2 Storage stability:

Soluble and immobilized β-D-galactosidase samples were stored in sodium phosphate buffer at a pH 7.0 and a temperature 4 ◦C for 1, 2 and 3 months. The immobilized enzyme retained 100% of its initial activity after being stored for 30 days, lost 2.3 % of its initial activity after being stored for 60 days and 3.6% of its initial activity after being stored for 90 days. In contrast, the soluble β-D-galactosidase

experienced a 9% to 10% reduction in its activity when stored under the same conditions for the same period of time. This is study compare with the results that have been published in the literature [3, 10, 16, 17].

In comparison to our study, Makkar *et al.* (1981) [27] reported storage stability of free and immobilized β-D-galactosidase enzyme from *Lactobacillus bulgaricus* for a period of 60 days at 4 °C. They observed no loss in activity for 30 days by the immobilized β-D-galactosidase enzyme and 30 % loss in activity was observed after 60 days of storage whereas the free enzyme showed a 60 % loss in activity after 60 days of storage. Similarly, Zhou *et al.* (2013) [28] reported immobilization of β-D-galactosidase by chitosan beads. They observed that the free β-D-galactosidase lost more than 80 % of its initial activity after storage for 50 days whereas the immobilized β-D-galactosidase was found to lose less than 20 % of its initial activity. The results showed that the immobilized β-D-galactosidase exhibited higher stability than the free β-D-galactosidase. Thus, immobilized enzyme showed better storage stability. Ansari and Husain [16] immobilized β-D-galactosidase obtained from Aspergillus oryzae onto a support constructed out of concanavalin A-Celite 545 and observed that the enzyme retained 78% of its initial activity after 2 months of storage while its free β-D-galactosidase counterpart retained only 40% of its initial activity. Bayramoglu et al. [17] reported that β-D-galactosidase immobilized on magnetic poly-(glycidyl methacrylate ethylene dimethyl methacrylate) beads retained 59% activity after 2 months of storage, while the free enzyme experienced a complete loss of activity after 5 weeks of storage.

3.3 The effects of temperature and pH on the activity of free and immobilized β-D-galactosidase:

The hydrolysis of ONPG was used as a model reaction to study the effects of temperature and pH on the properties of free and immobilized β-D-galactosidase. The effect of temperature was investigated at temperatures ranging from 25°C to 60 ◦C as shown in Fig. 2, at a constant pH of 7.0

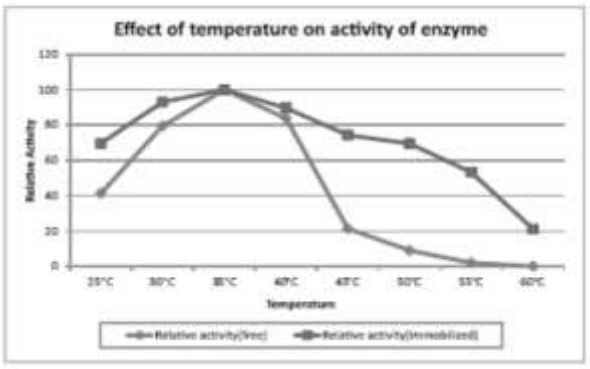

Fig2: Temperature effects on the hydrolytic activity of fre and immobilized β-D-galactosidase at pH 7.0.

The immobilized β-D-galactosidase possessed a higher activity at a temperature range of 25◦ – 55◦C but higher activity at 37◦c in comparison with the free β-D-galactosidase ranging from 30◦- 40◦c but higher activity at 37◦c. However, the immobilized enzyme exhibited a lower activity at temperatures above 55 ◦C. Furthermore, the optimal operating temperature for the free β-D-galactosidase and the immobilized β-D-galactosidase was found to be same; 37 ◦C. This result is comparable to what has been published in a different study [12, 3] where the immobilized enzyme is better at maintaining its activity at lower temperatures but loses it faster when subjected to temperatures above 37 ◦C in comparison with the soluble enzyme. The change in the optimal temperature toward a lower value is unexpected, but this change has also been observed by other authors [3, 12, 18, 19]. One possible explanation is that the impurities contained in the crude extract (soluble enzyme) may stabilize the enzyme molecule, protecting it against thermal inactivation. Those impurities may have been removed from the nearby of the enzyme after immobilization, probably reducing enzyme stability.

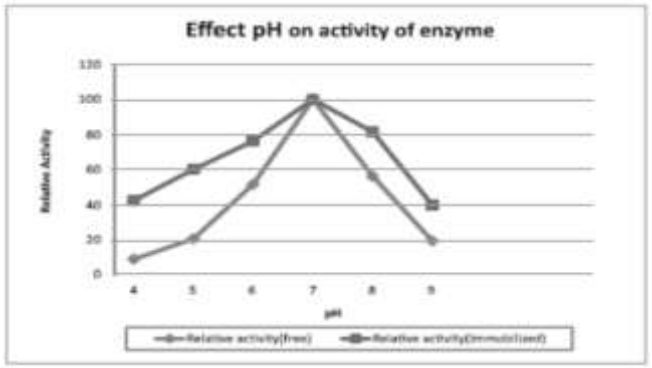

Fig3: pH effects on the hydrolytic activity of free and immobilized β-D-galactosidase at temperature 37°C.

Fig.3 shows the pH dependence of the immobilized enzyme activity at a constant

temperature of 37 ◦C in comparison with the activity of the soluble enzyme. The free β-D-galactosidase and immobilized enzyme exhibited a high activity at the pH of approximately 7.3. The free β-D-galactosidase exhibited a low activity at the pH ≤ 5.0. The immobilized enzyme, on the other hand, retained 60% of its activity at the same pH, indicating that the immobilization helped to increase the stability of the enzyme. .The higher stability of the immobilized β-gal may be explained by the new microenvironment provided by the chitosan matrix. At high H+ concentrations, the amino groups of chitosan are protonated, attracting OH− ions, resulting in a higher local pH in comparison with the bulk solution [20].pH range of immobilized enzyme found to be 4.0 to 9.0 with retaining 40% of activity and soluble enzyme found to be 5.0 to 8.0 with retaining 20% of activity. Furthermore, Immobilized enzyme has found to be a broader optimum pH range when compared to the soluble enzyme [12]. The results from the temperature and pH changes on the enzymatic activity, suggests that the immobilized enzyme possesses a maximum catalytic activity at a temperature same as a soluble enzyme and it is believed that this result of optimum temperature was neither promoted any conformational changes of the enzyme molecules that involve in changing pH but it increase the stability at low and high temperature. However, it is highly possible that the micro-environment in the vicinity of the support may be very different from the bulk solution environment, which may explain the pH stabilization observed for the immobilized enzyme. Immobilization may promote the stability of enzymes against extreme reaction conditions that are deleterious to soluble enzymes [21].

3.4 The influence of immobilization on ONPG hydrolysis:

Kinetics *Km* and *Vmax* were estimated by using a non-linear regression of the *Michaelis–Menten* equation and linear regression of *Lineweaver-Burk* plot to fit the experimental data obtained for the initial rates of ONPG hydrolysis. The results obtained for free and immobilized β-D-galactosidase are shown in Fig. 4, 5 and 6. For the free enzyme, *Km* was approximately 3.856 mM and *Vmax* was approximately 0.728 mM/min. For the immobilized enzyme, For the immobilized enzyme, *Km* was approximately 5.319 mM and *Vmax* was approximately 1.209 mM/min. For instance, Goto et al. [23] observed that the immobilization of β-D-galactosidase caused a decrease in the Km value, but the *Vmax* value was increased. In A.F. Lima et al. [12] it can be observed that the Km values for the immobilized enzyme and the free β-D-galactosidase are similar when taking into account experimental errors, but The maximum velocity of the immobilized β-D-galactosidase was double that of the soluble enzyme. The maximum velocity of the immobilized β-D-galactosidase was increased from 0.728 mM/min in free state to 1.209 mM/min after immobilization, although enzyme loads in the assays were similar, where there was 0.139 U of soluble enzyme and 0.137 U of immobilized enzyme. The catalytic efficiency of the immobilized enzyme in this work was approximately 189% that of the soluble enzyme.

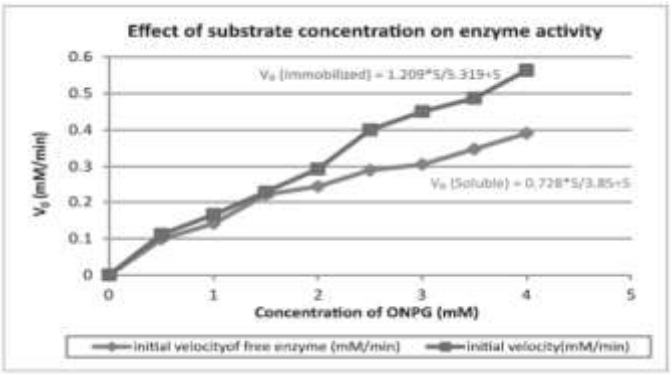

Fig 4: Influence of substrate concentration on the initial rates of ONPG hydrolysis by free and immobilized β-D-galactosidase from *E.coli* at 37° C. The lines represent the fitting of the non-linear *Michaelis–Menten* model to the experimental data. V_0 is the rate of ONPG hydrolysis catalyzed by free and immobilized β-D-galactosidase enzyme.

Hsu CA et al. [3], Mazzei R et al. [22], Goto et al.[23] have discussed the effect of immobilization on enzymes and have concluded that the impact is not always negative. For instance, Goto et al. [23] observed that the immobilization of β-D-galactosidase caused a decrease in the Km value, but the Vmax value was increased. In A.F. Lima et al. [12] it can be observed that the Km values for the immobilized enzyme and the free β-D-galactosidase are similar when taking into account experimental errors, but The maximum velocity of the immobilized β-D-galactosidase was double that of the soluble enzyme. The kinetic parameters estimated in this work are similar to those that have been reported by other researchers. Zhou and Chen [14] determined the Km and Vmax for β-D-galactosidase from the K. lactis Maxilact LX 5000 strain in soluble form and when it was immobilized on graphite. For the free β-D-galactosidase, Km was 1.74 mM and Vmax was 77.45 mmol ONP/min mg, while they were Km was 9.34 mM and Vmax was

8.75 × 10−3 mmol ONP/min mg for the immobilized enzyme. The authors noted that an increase in Km is related to the difficulty experienced by the substrate when coming into contact with the enzyme, which was observed in this work. The kinetic parameters estimated in this work are similar to those that have been reported by other researchers.

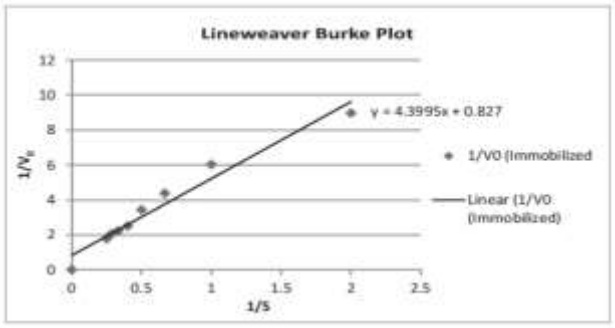

Fig 6: Influence of substrate concentration (1/s) on the initial rates of ONPG hydrolysis by free β-D-galactosidase from *E.coli* at 37° C. The lines represent the linear *Lineweaver-Burk* model to the experimental data. $1/V_0$ is the rate of ONPG hydrolysis catalyzed by immobilized β-D-galactosidase enzyme.

3.5 Reuse of the immobilized β-gal

Fig.7 shows a reuse study of the immobilized β-D-galactosidase, using the subsequent hydrolysis of ONPG as a model reaction. In this work, the immobilized enzyme was reused for 10 cycles, showing stability since it retained more than 70% of its initial activity. . In this work, one cycle is defined as a batch of ONPG hydrolysis at 37 ◦C and pH 7.3, lasting 5 min. the immobilized enzyme was reused for 10 cycles with a good stability as more than 70% of the initial activity was retained after the tenth cycle. In this work, one cycle is defined as a batch of ONPG hydrolysis at 37 ◦C and pH 7.3, lasting 5 min. The result achieved may be considered satisfactory in comparison with the results obtained by other researchers [24–26].

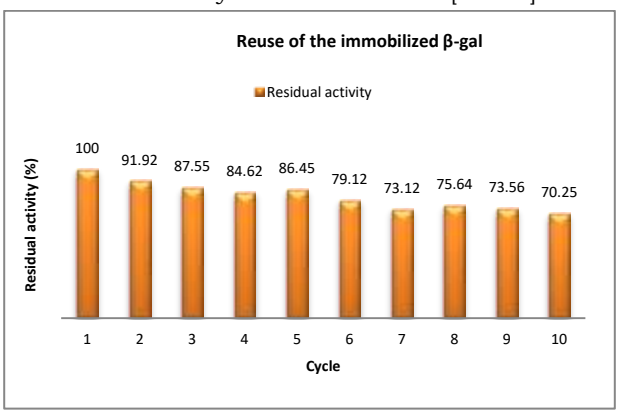

Fig.7 Reuse and stability of immobilized β-D-galactosidase when hydrolyzing ONPG at 37°C.

Verma et al. [24] immobilized β-D-galactosidase from K. lactis on functionalized silicon dioxide nanoparticles and used the same model reaction to evaluate the immobilized enzyme at 40 ◦C over 11 cycles, where the biocatalyst retained approximately half of its initial activity at the end of the last cycle. Tardioli et al. [25] observed that the immobilized enzyme had a higher stability than the soluble enzyme. After four cycles of lactose hydrolysis lasting 20 min each at 40 ◦C and pH 7, the immobilized enzyme retained 83% of its initial activity. Other authors [26] used composite microspheres of tamarind gum and chitosan as a support but only retained 53% of its initial activity after 9 cycles of ONPG hydrolysis at 37 ◦C and pH 7.3. This study compare with A.F lima et al. [12] these found that the immobilized enzyme was retained more than 70% of the initial activity after the tenth cycle. Fig.7. Reuse and stability of immobilized β-D-galactosidase when hydrolyzing ONPG at 37 ◦C.

CONCLUSION:

The higher stability of immobilized β-D-galactosidase was most likely a result of the new microenvironment provided by the chitosan matrix that result into increase in catalytic efficiency of enzyme because of positive impact on thermal, operational and storage stabilities in comparison with the free β-D-galactosidase. The kinetics of ONPG hydrolysis catalyzed by free and immobilized β-D-galactosidase suggests that there is increase in *Km* is related to the difficulty experienced by the substrate when coming into contact with the enzyme but the *Vmax* of immobilized enzyme increases. From the present study, it is concluded that chitosan immobilized β-D-galactosidase used for the hydrolysis of milk and milk products because immobilization improved the performance of the biocatalyst in the hydrolysis of lactose, probably by altering the kinetic parameters and stability of the enzyme at different conditions

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