Characterization of immobilized β-galactosidase from Aspergillus niger

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ABSTRACT

β-galactosidase enzyme was isolated from *Aspergillus niger*, and immobilized in sodium alginate gel. The maximum activity of the free enzyme was obtained at 65°C, pH 3.5 and its not affected by immobilization. The free enzyme had pH stability range from 3.5 to 6.5 and it was increased by immobilization process especially at acid pH values. The free enzyme retained 90.28, 85.09, 45.49, and 19.2 % of its initial activity after incubation at 30, 40, 50, and 60°C, for 60 min respectively. Thermal stability was enhanced by immobilization process. The kinetic parameters for soluble and immobilized enzyme were also determined, and immobilization led to decrease in *K*m value (5.12 mM for free form to 1.48 mM for immobilized form), indicating decreased affinity by the enzyme for its substrate. *V*max was also decreased by immobilization process, and it was reached from 86.66 µmol ONP.min^{-1} for free enzyme to .38.02 µM ONP.min^{-1} for immobilized form.

Keywords: β-galactosidase**,** *Aspergillus niger***,** Immobilization

1. INTRODUCTION

β-galactosidase (E.C.3.2.1.23) commonly known as lactase, is a part of a wide group of hydrolyzing enzymes. It is a glycosidase which is catalyze the hydrolysis of lactose to produce a mixture of mono saccharides, D-glucose and D-galactose, which are sweeter, more soluble, and more digestible than lactose (Tanriseven and Doğan, 2002).

β-galactosidase has been found in plants, animals and microorganisms. The latter have been considered as potential commercial sources of this enzyme. The production of this enzyme by Generally Recognize As Safe (GRAS) microorganisms is very important (Saad, 2004). The microorganisms which are used for producing this enzyme are bacteria and many types of filamentous fungi such as Aspergillus niger, Aspergillus oryzae, and Trichoderma sp. (Rasouli and Kulkarni, 1994;

Tanriseven and Doğan, 2002; Seyis and Aksoz, 2004).

In spite of their excellent catalytic properties, enzyme properties have to be usually improved before their implementation at industrialscale. Generally, soluble enzymes have to be immobilized to be reused for long times in industrial reactors and, in addition to that, some other critical enzyme properties have to be improved like stability, activity, inhibition by reaction products, selectivity towards non-natural substrates. In this way, immobilized enzymes may also exhibit much better functional properties than the corresponding soluble enzymes by very simple immobilization protocols. Immobilization of the enzyme on a solid is economically feasible in spite of the cost of the enzyme production and the immobilization processes. This is mainly due to the fact that the enzyme derivatives can be used several times and possibility by developing a continuous hydrolysis process (Szczodark, 2000; Haider and Husain, 2009 b).

The aim of this work is the isolation and immobilization of β-galactosidase from the Aspergillus niger and the study of enzyme properties..

2. Materials and Methods

2.1. Materials

An extracellular β-galactosidase-producing strain of *Aspergillus niger* was obtained from Biology Dept. Faculty of Science, Sulaimani University, which is locally isolated from Onion. *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was from Fluka. All other chemicals used were of analytical grade.

2.2. Growth condition

Aspergillus niger was grown on the medium consist of 30 g l^{-1} potato extract, 5 g l^{-1} peptone and 40 g l^{-1} lactose 100 ml of the medium was transferred to a conical flask and autoclaved at 121°C for 15 min. cooled and then inoculated with a loopful of fungus culture. The inoculated flasks were incubated in a shaker incubator (200 rpm) for 24 h. at 30±2°C.

2.3. Assay of β-galactosidase.

β-galactosidase activity was determined by measuring the release of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at 420 nm (Lederberg, 1950). 200 µl enzyme sample was incubated at 40°C, in a reaction mixture consisting of 1.0 ml of phosphate buffer (0.2 M, pH 7.0) and 1 ml of ONPG solution for 10 min. The reaction was

stopped by adding 0.4 ml of 1M Na_2CO_3 solution. One unit of β-galactosidase activity is defined as the amount of the enzyme which liberates one µmol of *o*-nitrophenol from ONPG per minute under the experiment conditions.

2.4. Immobilization of β-galactosidase

Twenty ml of enzyme solution were mixed with 100 ml of sodium alginate solution 2% (w/v), and added drop wise to a stirred cold solution of calcium chloride (0.15 M). 10ml syringe with attached needle was used for preparation of calcium alginate beads and left in a solution for 60 min to harden (Fraser and Bickerstaff, 1997). The beads were filtered and washed twice with distilled water and suspended in phosphate buffer (0.05 M, pH 7.0) and stored at 4°C until used.

2.5. Characterization of β-galactosidase

2.5.1 Effect of Temperature on β-galactosidase Activity.

The effect of temperature on the activity of free and immobilized β-galactosidase was determined by assaying the activity at various temperature degrees ranging from 30 to 90°C. The activity was measured at the pH 7.0.

2.5.2 Effect of pH on β-galactosidase Activity.

The effect of pH on the activity of free and immobilized β-galactosidase was studied by assaying the enzyme activity at various pH values ranging from 2.5 to 7.0 using phosphate buffer.

2.5.3 Effect of Temperature on β-galactosidase Stability.

Thermal stability of the free and immobilized β-galactosidase was determined by incubation of 1 ml of the free and 2.5 gm alginate beads for 1 hour at various temperature degrees ranging from 30 to 80°C. The residual activity was measured every 15min.

2.5.4 Effect of pH on β-galactosidase stability.

Stability of the free and immobilized enzyme at various pH values was determined by measuring the residual activity after holding the enzyme solution in phosphate buffer (0.2 M) at various pH values at room temperature (25±2°C) for 30 min. prior to the addition of substrate.

2.5.5 Determination of Kinetic Parameters.

The free and immobilized β-galactosidase was assayed using ONPG concentrations ranging from 0.4 to 4.0 mM. Michaels constant (Km) and Maximum velocity (Vmax) were calculated using Lineweaver-Burk plot.

3. RESULTS And Discussion

3.1. Effect of temperature on β-galactosidase.

The maximum activity of the free enzyme was achieved at 65°C, above which a decrease in the enzyme activity was observed, and the activity was dropped to less than 27% at 75°C. There was no alteration in optimum temperature of immobilized enzyme but there was a broadening in temperature activity for immobilized form (figure 1).

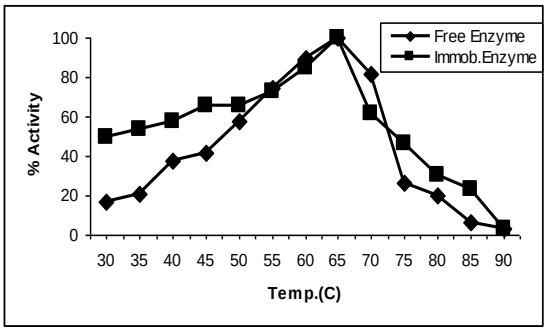

Figure1. Temperature activity profiles for free and immobilized β-galactosidase.

This result for free enzyme is similar to that obtained by Agrawal *el al.* (1980) for *Aspergillus fumigatus*. In the study of β-galactosidase form *Aspergillus carbonarius* ATCC6276 O'Connell and Walsh (2008) showed that the crude preparation contained two β-galactosidase activities and they are differ from optimum tem. 55°C and 65°C for β-gal 1 and β-gal 2, respectively. Tanriseven and Doğan (2002) showed that the optimum temperature (50°C) for *Aspergillus oryzae*, β-galactosidase was not affected by immobilization in fiber composed of alginate and gelatin hardened with glutaraldehyde. Similar results were obtained by Gaur *et al.* (2006) and Haider and Husain (2007) when they are used three different techniques and calcium alginate entrapped preparations for immobilizing β-galactosidase from *Aspergillus oryzae* respectively, while Haider and Husain (2009 a) showed that the immobilized enzyme has higher temperature activity approximately 10 degree than the free enzyme. The enzymatic reaction dependence on the temperature resembles other chemical reactions except that there is an optimum temperature in the enzymatic reaction above which the activity decreases due to the denaturation of the enzyme protein. (Mann and Thompkinson, 1988; Zhou and Chen, 2001).

3.2 Effect of pH on β-galactosidase.

Figure 2 demonstrates the pH-activity profiles of the free and immobilized β-galactosidase. Maximum activity for the immobilized form was obtained at pH 3.5 compared with 4.0 for the free form of the enzyme. This enzyme shows the activity in acid pH side and less activity near the neutral values. Tanriseven and Doğan (2002) previously showed that β-galactosidase from *Aspergillus oryzae* was not affected by immobilization and the optimum pH for free and immobilized enzyme were 4.5, while Gaur *et al.* (2006) discussed that the immobilization of *Aspergillus oryzae* β-galactosidase by covalent coupling to chitosan shifts the optima pH toward the acid value from 4.5 to 4.5.

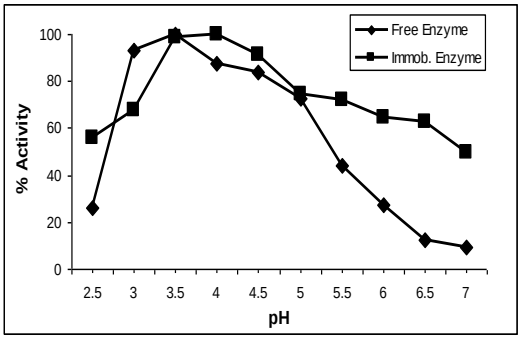

Figure2. pH activity profiles for free and immobilized β-galactosidase.

On the other hand, all calcium alginate entrapped preparations of β-galactosidase from *Aspergillus oryzae* studied by Haider and Husain (2007) showed no change in pH-activity profiles but had a remarkable broadening in the pH activity as compared to the free enzyme.

3.3 Thermo-stability of β-galactosidase.

The effect of temperature on the stability of the free and immobilized enzyme was determined at temperature range of 30-70°C. Thermal stability of the free enzyme was shown in the figure 3. The β-galactosidase was retained more than 90% of its original activity after 60 min at 30°C while, at 50°C, the enzyme retained about 45% of its initial activity after the same incubation time. Increasing the temperature to 70°C resulted in complete inactivation of the enzyme after 45 min. of incubation. The result of the present study was close to several previous studies. In the study of *Alternaria palmi* β-galactosidase Agrawal *el al.* (1980) found that this enzyme lost only 10.5% of its activity after 2 h at 65°C and retained 50% of its activity after 40 min. at 70°C, while Saad (2004) reported that β-galactosidase from *Aspergillus japonicus* was about 35% of its initial activity at 60 °C for 3h. For the β-galactosidase from thermophilic fungus *Talaromyces Thermophilus* CBS-236.58 Nakkharat and Haltrich (2006) found that this enzyme was stable at 40°C and the half life of it was 196 hour at 40°C.

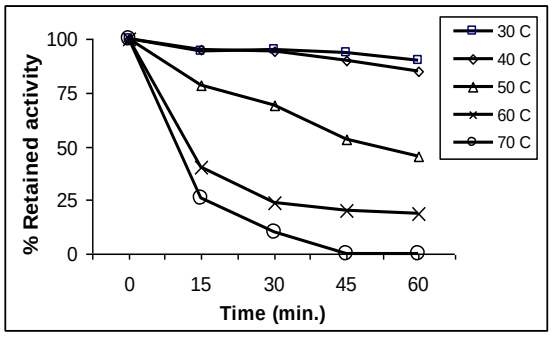

Figure 3: Effect of temperature on free β-galactosidase stability.

The immobilized β-galactosidase showed remarkable higher thermal stability as compared to the free form, and it was retained more than 24% of its initial activity after 60 min. at 60°C. (Fig.4) this result was agreed with that obtained by Tanriseven and Doğan (2002) for β-galactosidase from *Aspergillus oryzae*, who observed that the immobilization process led to the increasing of the thermal stability. Gaur *et al.* (2006) showed that the different techniques of immobilization led to enhancement in the half-lives of *Aspergillus oryzae* β-galactosidase. Haider and Husain (2009 a) have also shown that the immobilization of β-galactosidase from *Aspergillus oryzae* by bioaffinity adsorption on concanavalin-A layered calcium alginate-starch hybrid beads led to retained significant higher activity than the free enzyme at various temperatures.

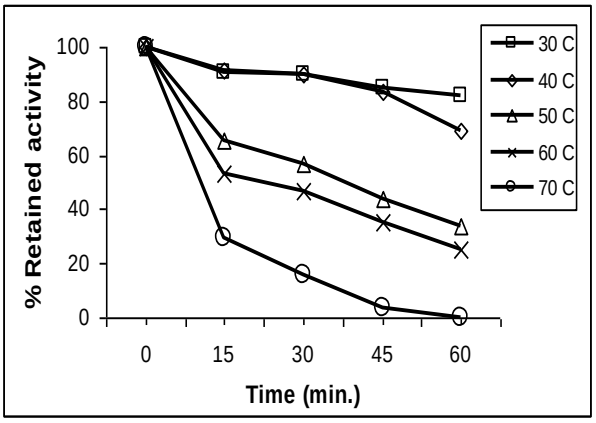

Figure 4: Effect of temperature on immobilized β-galactosidase stability

The increased stability observed in the immobilized enzyme may be attributed to the reduction in the protein mobility, due to anchorage to the support, which shield and protect the enzyme from damaging effects of the environment (Neri et al. 2008).

3.4 Effect of pH on β-galactosidase Stability

The free β-galactosidase was found to be stable at the pH range of 3.5-6.0 with a little wider range for the immobilized enzyme at the acid side. There was a remarkable drop in stability on holding the both forms at pH values above 8 (Fig. 5). This loss in activity is attributed to the irreversible denaturation for the enzyme and to change in conformation of the active site (Whitaker, 1994). Wide pH stability (5.5-7.5) was observed for β-galactosidase produced by Talaromyces thermophilus CBS-236.58 (Nakkharat and Haltrich, 2006). β-galactosidase derived from filamentous fungi seem to have a wide range of pH stability. The β-galactosidase from Aspergillus oryzae was found stable in the pH range of 4.0-9.0 (Tanaka et al. 1975), also Seyis and Aksoz (2004) reported thermophilic fungus Trichoderma viride ATCC 32098 β-galactosidase retained more than 90% of its initial activity in the pH range of 3.0-7.5. Immobilization process caused increasing in β-galactosidase stability. The immobilized form was slightly more stable at lower pH. At pH 3.0 immobilized enzyme retained more than 93% of its activity compared with 84% for free enzyme.

The same result was found for Aspergillus oryzae β-galactosidase (Tanriseven and Doğan, 2002). In another study carried out with Kluyveromyces fragilis, immobilized β-galactosidase on silanized porous glass was found to be stable at pH less than 6.5 (Szczodark, 2000).

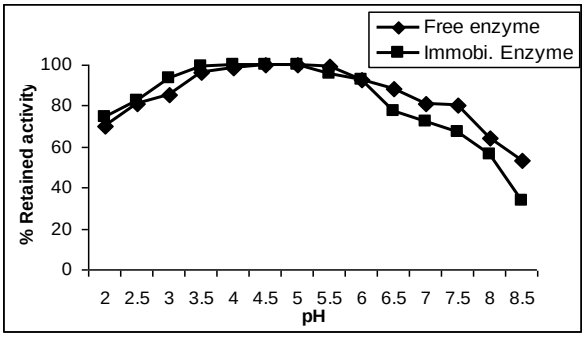

Figure 5: Effect of pH on β-galactosidase stability

3.5 β-galactosidase Kinetics.

The kinetic parameters Km and Vmax of free and immobilized β-galactosidase were determined from the effect of substrate concentration (ONPG) on the reaction velocity. The relationship was plotted by Lineweaver-Burk method (Fig. 6). Immobilization process led to decrease in Km value (5.12 mM for free form to 1.48 mM for immobilized form), Vmax was also affected by immobilization and it was reached from 86.66 µmol ONP.min-1 for free enzyme to 38.02 µM ONP.min-1 for immobilized form.

The Km value for free form was lower than obtained by Tanriseven and Doğan (2002) for Aspergillus oryzae β-galactosidase (42mM), while it was higher than was showed by Gaur et al. (2006) and Chen et al. (2008) for β-galactosidase from Aspergillus oryzae and for Bacillus stearothermophilus (2.63 and 2.96 mM, respectively).

The Vmax (86.66 µmol ONP min-1) value for free enzyme found in this study was higher than estimated by (Gaur et al. 2006) from Aspergillus oryzae in which the Vmax was determined to be 0.36 µmol ONP min-1 for free enzyme by using ONPG as a substrate, and for Bacillus stearothermophilus which is claimed by Chen et al. (2008) in which Vmax value was 6.62 µmol min-1.

The differences in Km and Vmax values among the present and other results could be attributed to many factors such as enzyme source, substrate type, reaction pH, temperature and ionic strength of the buffer.

The immobilization of β-galactosidase affected kinetic parameters (Km and Vmax), they were decreased to 1.48 mM and 38.02 µmol min-1, respectively. The same observation about Km value was observed for Kluyveromuces lactis β-galactosidase by Numanoğlu and Sungur (2004) they were recorded a decrease in Km value after immobilization.

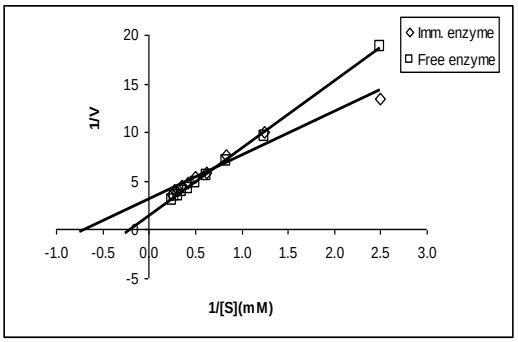

Figure 6: Lineweaver-Burk plot for free and immobilized β-galactosidase

A different result was reported by Tanriseven and Doğan (2002) and Gaur et al. (2006) for Aspergillus oryzae β-galactosidase after immobilization.

The decrease in Vmax value in this study agreed with that observed for Aspergillus oryzae β-galactosidase when immobilized in fiber composed of alginate and gelatin hardened with glutaraldyhyde (Tanriseven and Doğan, 2002). The same result was demonstrated by Gaur et al. (2006). However, Haider and Husain (2009 b) found that Vmax was less affected than Km upon immobilization

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