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PERFORMANCE OF β-GLUCOSIDASE IMMOBILIZED ON CALCIUM ALGINATE BEADS

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Abstract

Performance of immobilized β-glucosidase obtained from almonds (EC 3.2.1.21) on calcium alginate beads was studied by measuring the activity of the enzyme in terms of the generation of *p*-nitrophenol from the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (PNPG).The immobilized enzyme activity was compared with soluble enzyme and was found to decrease by 36.6% albeit with an increased operational stability in terms of easy recovery from the finished product, recurrent use and scale-up in various reactor configurations. The hydrolysis rate data exhibit Michaelis-Menten kinetics. The Michaelis constant were determined using Langmuir linearized plot which obtained v_{max} and K_m as 20.88 µmol/mL.min and 0.0125 mol/L respectively. However, the immobilized *β-Glucosidase* did not show a sufficiently good operational stability on reuse.

Keywords: Immobilized enzyme, enzyme activity, Michaelis-Menten kinetics, β-glucosidase, *p*-nitrophenyl-β-D-glucopyranoside (PNPG), alginate gel

1. Introduction

Immobilization of enzymes is one of the method to impart operational stability and longevity for multiple usage of the enzyme. It also facilitates easy separation from the reaction mixture and reuse of the enzyme. Different immobilization methods are currently in practice (Sheldon, 2007). In the present study, a β-glucosidase obtained from almonds (EC 3.2.1.21) was immobilized by entrapment in alginate gel (Ortega et al., 1998; Roy et al., 1989). A wide variety of carriers have been used for the immobilization of β-glucosidase such as alginate, polyacrylamide gel and CNBr-activated sepharos entrapped in polyacrylamide gel (Ortega, 1998; Roy et al., 1989). Then the hydrolysis of *p*-nitrophenyl-β-D- glucopyranoside (PNPG) to *p*-nitrophenol by β-glucosidase was studied at different substrate (PNPG) concentration. The performance of the enzyme was studied by measuring its activity in terms of generation of *p*-nitrophenol which was calculated using absorbance measured by spectrophotometer. The rate of substrate consumption has 1:1 stoichiometric relation with *p*-nitrophenol generation. The rate vs. substrate consumption show Michaelis-Menten type of dependence. Linearized plots are used to evaluate Michaelis constants. The efficiency of immobilized enzyme is compared with free soluble enzyme. Enzyme activity is generally expected to decrease with immobilization, however with enhanced operational performance in terms of stability, reuseability and easy separation of the enzyme [Dey, 2003; Sheldon, 2007].

2. Materials and Methods

β-Glucosidase from almonds (EC 3.2.1.21), and *p*-nitrophenyl-β-D-glucopyranoside (PNPG) were obtained from Sigma-Aldrich, sodium alginate was obtained from Fisher Chemical, and calcium chloride was purchased from Merck. All other chemicals used are commercially available reagent grade. The lyophilized enzyme was dissolved in 2 mL of 1M Potassium Phosphate buffer at pH 6. The unit of enzyme per bottle was 100 U and for each run of enzymatic reaction, only 2U equal to 40 μL of enzyme was used.

2U enzyme (in 40 μL) was mixed with 960 μL sodium alginate solution to finally give a 4% (w/v) sodium alginate (Dey et al., 2003; Ortega, 1998; Workman & Day, 1982). This mixture was dropped into 0.2 M calcium chloride using 1 mL pipette from a height of approximately 20cm which finally solidified into spherical beads. The beads are cured for 2 hours in the calcium chloride solution to give bead size of 3mm. The calcium alginate beads containing the enzyme were thoroughly washed with distilled water two times for further studies. Beads of sizes 4 mm and 5 mm were made by cutting 5 mm and 10 mm diameter of the 1 mL pipette tip respectively.

The β-glucosidase activity was routinely determined with *p*-nitrophenyl-β-D-glucopyranoside (PNPG) as the substrate (Ortega et al., 1998). The reaction mixture, containing 1 mL of a 10 mM PNPG solution in 0.05 M sodium citrate buffer, pH 4.8 and the calcium alginate beads was incubated at 50°C in a water-bath. After 15 min, 1.5 mL of 1.0 M sodium carbonate was added to stop the reaction and to develop the colour of any *p*-nitrophenol (generating *p*-nitrophenolate) that has been released from the substrate. The absorbance was read at 400 nm, and the amount of *p*-nitrophenol

liberated was determined. *A unit of activity was defined as the amount of enzyme required to liberate 1 µmol of p-nitrophenol per min from the substrate.* The standard graph of absorbance vs. *p*-nitrophenol concentration was prepared. Similarly, the activity of the free enzyme was determined and compared with the immobilized enzyme for the optimum bead size.

Determination of kinetic constants

Enzymatic reaction was carried out at 50°C with different substrate concentrations. The stock solution for the substrate was prepared for 0.1 M PNPG and was diluted till 0.01 M. The kinetic constants K_m and v_{max} were calculated using Langmuir and Lineweaver-Burk plots.

Operational stability of the immobilized β-Glucosidase

After each β-glucosidase activity assay, the beads were removed, washed thoroughly with distilled water and stored at 4°C chiller. Then the beads were reassayed for the second cycle of enzyme activity which is after 4 days (96 hours) and the third cycle was at the fifth day (120 hours).

3. Results and Discussion

Immobilized enzyme activity for different bead sizes (3, 4 and 5 mm diameter) were studied and the highest activity was observed for 4 mm beads which were used for subsequent experiments and analysis.

The enzyme activities for immobilized and free enzymes are compared in Figure 1. Immobilized enzyme shows a decreased activity by 36.6% as compared to free enzyme which is obvious due to mass transfer resistance to substrate diffusion through alginate beads.

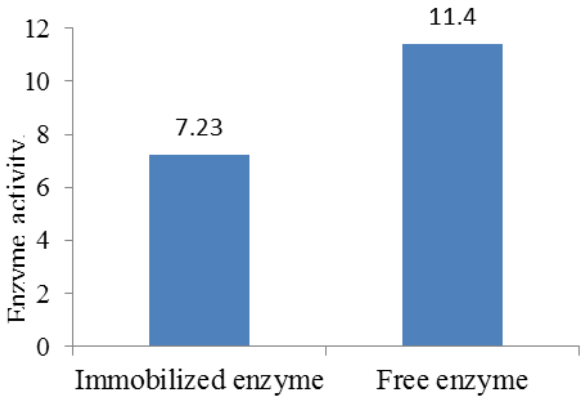

Figure 1: Enzyme activity of immobilized enzyme (4mm beads) and free enzyme.

Determination of Kinetic Parameters

Plot of enzyme activity as a function of substrate concentration shows saturation of reaction rate at high substrate concentration as depicted in Figure 2. This behavior is typical of Michaelis- Menten kinetics. Thus it can be inferred that the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (PNPG) by β-glucosidase follows Michaelis-Menten kinetics. The Michaelis constants were evaluated using linearized plots of Langmuir and Lineweaver Burk as shown in Figures 3 and 4 respectively. Although most of the works reported in literature use Lineweaver- Burk plot for determining Michaelis parameters (Dey et al., 2003; Ortega et al., 1998; Roy et al., 1989), our data are better represented by Langmuir plot as is evident from comparision of Figures 3 and 4 and corresponding R^2 values. Langmuir plot shows a better fit of our experimental data due to the fact that there is minimum distortion of experimental errors in the values of *s* and *v*, especially at low substrate concentrations in Langmuir plot compared to Lineweaver-Burk plot (Doran, 1995).

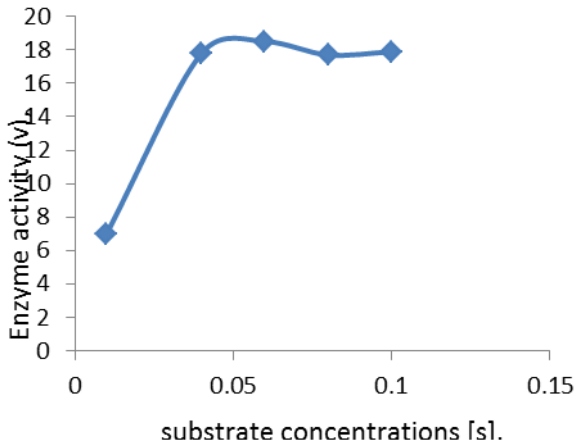

Figure 2: The enzyme activity as afunction of substrate concentrations.

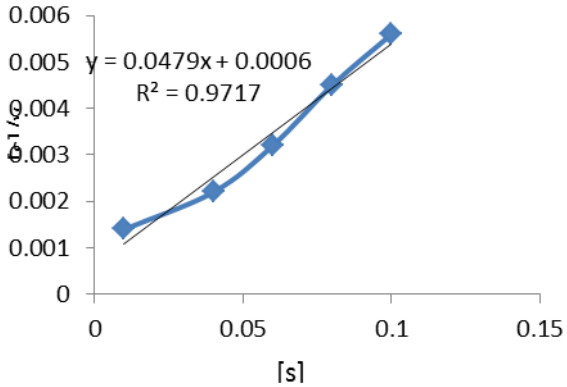

Figure 3: Langmuir plot

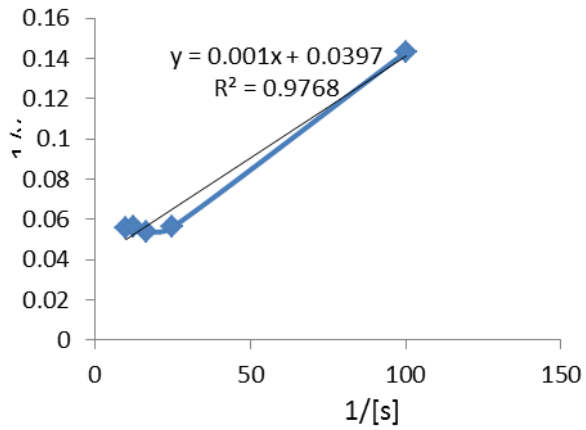

Figure 4: Lineweaver-Burk plot

The linearized Michaelis-Menten equation due to Langmuir is given by (Doran, 1995),

$$s/v = K_m/v_{max} + s/v_{max}$$

where s is the substrate concentration (mol/L), and v is the reaction velocity or the rate of reaction expressed as µmol/mL.min. v_{max} is the maximum rate of reaction at infinite reactant concentration. K_m is the Michaelis constant for the substrate. The best fit straight line to data of s/v *vs.* s is represented by the equation $y = 0.0479x + 0.0006$. Thus v_{max} and K_m are calculated as 20.88 µmol/mL.min and 0.0125 mol/L respectively.

Operational stability of the immobilized β-

Glucosidase

The operational stability of immobilized enzymes is one of the most important factors affecting the utilization of an immobilized enzyme system. The operational stability of the β-Glucosidase was evaluated by three cycles of enzyme assay with PNPG as the substrate. The enzyme showed 28.91% activity during the second reuse and 15.21% activity on its third use. This decrease in activity was due to the enzyme denaturation and leakage of enzyme from the beads due to the washing of beads at the end of each cycle.

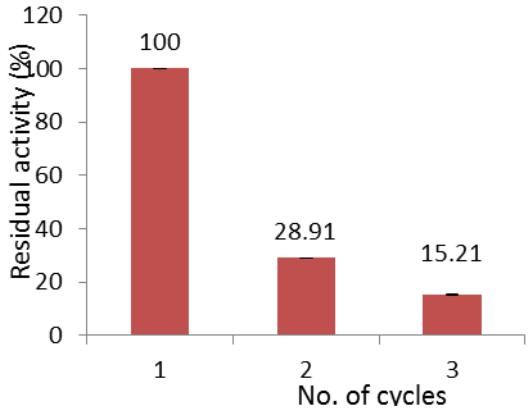

Figure 5 Repeated use of immobilized β-Glucosidase

4. Conclusions

Efficiency of β-glucosidase enzyme immobilized on calcium aliginate bead was compared to the native enzyme by measuring enzyme activity. Immobilized enzyme show significant operational stability albeit at the cost of decrease in the activity by 36.5% after immobilization. Hydrolysis of PNPG by β-Glucosidase follow Michaelis-Menten kinetics. The kinetic parameters were evaluated using linearized Langmuir plot which showed a better fit compared to Linewaever-Burk plot.

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