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Research article

A COMPARITIVE STUDY ON IMMOBILIZATION OF ALPHA AMYLASE ENZYME ON DIFFERENT MATRICES

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ABSTRACT: In present study 7 carriers such as agar –agarose, wood fiber, wood chips, gelatin, wood powder, calcium alginate and butanol sodium alginate were used for immobilization of fungal amylase. Amylase immobilization was carried out by simple adsorption, adsorption followed by cross-linking, combined adsorption and gel entrapment methods. The suitability of the supports and techniques for the immobilization of amylase was evaluated by estimating the enzyme activity, stability at various temperature and pH, immobilization efficiency and treatment of carriers with different chemicals were investigated. Maximum enzyme activity(39.67 U/ml/min) was obtained in sodium alginate method of immobilization, followed by Agar –agarose (38.67U/ml/min), gelatin (37.73U/ml/min), butanol (34.67 U/ml/min), NaOH treated Wood fiber (18.83 U/ml/min), NaOH treated Wood chip (18.67 U/ml/min) ,NaOH treated Wood powder (18 U/ml/min) and lowest activity obtained in case of SDS treated wood powder(14.67U/ml/min) . The optimal pH (6 pH) of the immobilized enzymes was shifted to lower values than for the free enzyme. The optimum reaction temperature was determined to be 40°C for the free amylase, whereas that for the immobilized enzymes was shifted to 50-60°C.

Key words: Amylase, Immobilization, Gelatin and Gluteraldehyde

INTRODUCTION

Amylases constitute a class of industrial enzyme having approximately 25% share of the enzyme market [1,2] Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents [3,4]. The a-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylating enzymes. The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps. An enzyme is immobilized when its mobility has been restricted by chemical means. Immobilization technique is advantageous over free enzyme catalysis as it increase stability, easy recovery, easy separation of reactant and product, repeated or continuous use of a single batch of enzyme [5] which will ultimately save the enzyme, labour and overhead costs [6]. Several efforts have been taken to immobilize alpha -amylase by binding it to solid carriers [7-18]. Immobilization of enzymes is carried out by three principle means matrix assisted entrapment of enzyme adsorption on a solid support, ionic or covalent binding [19,20]. Immobilized enzymes have been widely used for many years in different industrial processes. Entrapment is taken as the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability in microenvironment of matrix, protects enzyme from microbial contamination [21]. Physical entrapment of -amylase in calcium alginate beads has shown to a relatively easy, rapid and safe technique in comparison with other immobilization methods [22]. The method of immobilization should be like that the enzyme structure might have least conformational change as possible. The nature of the solid support or matrix plays an important role in retaining the actual confirmation and activity of enzyme in the processes that utilized immobilized biocatalysts.

MATERIALS AND METHODS

Screening of Amylase producing fungal strain

Fungal colonies were isolated from soil samples by serial dilution method. 50µl of soil samples diluted up to 10^{-5} dilutions were grown on starch agar medium. After seven days of incubation at 28 °C the Grams iodine solution (for amylase) is added to the cultures plates, plates showing highest zone of clearance were selected for amylase production

Preparation of Amylase Production media

The medium used for amylase production was comprised of (g/L)**:** Peptone 5g, NaCl 5g, Beef extract 1.5g, Yeast extract 1.5g, Starch 10g .The pH was adjusted to 5.5. Cultivation was carried in 250 mI Erlenmeyer flasks, each containing 100 ml of sterile medium. The inoculum 3% v/v was transferred to the culture medium and the flasks were incubated at 30°C for 3 days on a rotary shaker. After 3 days, a clear mat of A.niger was seen on top of the production media in the flask. It was then filtered through Whattman filter paper.

Amylase Assay

Amylase was assayed according to the procedure followed by Bernfeld, 1955 [23]. The activity of extracellular amylase was estimated by determining the amount of reducing sugars released from starch. The sugars were quantified by the method of 3,5-dinitrosalicylicacid (DNS), according to Miller [24].The starch solution was prepared from 1% (w/v) soluble starch in distilled water. 0.5 ml of the enzyme extract, 3.5 ml of citrate phosphate (pH 5.5) buffer and 1 ml of 1% starch solution were added in test tube and the mixture was incubated at 40ºC for 30 min. After that 2 ml of DNS was added to terminate the reaction and the reaction mixture was boiled at 100ºC for 5 minutes. The amount of reducing sugars in the final mixture was determined spectro photometrically at 540 nm One unit of enzyme activity (U) was defined as the amount of the enzyme liberating one µmole of reducing sugars as glucose/min.

IMMOBILIZATION METHODS

Immobilization of amylase on hot water treated wood chips, wood fiber and wood powder

Wood fibres, Wood chips and Wood powder were delignified by boiling in hot water several times until the resulting water was clear then the fibres, chips and powder were air dried and 1 gram of each carrier was incubated with 2 ml of enzyme solution (α-amylase from fungal source) for at least 2-3 hrs while maintaining the cold condition. The unbound enzyme was removed from the carriers by washing with distilled water until no activity or soluble protein was detected.

Immobilization of amylase on SDS (Sodium Dodecyl Sulphate) treated wood chips, wood fiber and wood powder

Wood fibres, Wood chips, Wood powder were separated and boiled in water containing 0.01% (w/v) sodium dodecylsulphate for 1 hr. Then fibres, chips and powder were dried completely at room temperature and soaked in 2-3 ml of enzyme solution (α-amylase) for 2-3 hrs at 30°C .After adsorption, the enzyme solution was decanted and the unbound α -amylase was washed off with distilled water until no activity was detected in the washing.

Immobilization of amylase on NaOH treated wood chips, wood fiber and wood powder

The Wood fibres, Wood chips and Wood powder were delignified by placing them in 1% NaOH solution for atleast 1-2 hrs then the fibres, chips and powder were air dried at room temperature and soaked in about 2-3ml of enzyme (α-amylase from fungal source) at 30°C for 2 hrs . The excess crude enzyme was drained and the fibres, chips, powder were washed with distilled water to remove the unbound enzyme and dried for atleast for 30 minutes.

Immobilisation of alpha amylase using gelatin and gluteraldehyde

Gelatin solution of different concentrations (10%-35%) was prepared by dissolving gelatin in distilled water by heating at 50°C and thereafter cooling to room temperature 2 ml of crude enzyme was added to varied concentration of 9 mL gelatin solution with thorough mixing. The suspension was casted on preassembled glass plates and when the suspension jellified, 10 mL of 10% glutaraldehyde solution was added to each glass plate of different concentration of gelatin and left for complete hardening. The gelatin was then cut into small beads (5x5 mm) and washed thoroughly with distilled water to remove any enzyme attached to the surface of beads.

Enzyme **loading capacity of gelatin solution**

Small gelatin beads were prepared according to above said method except varying the enzyme concentration from 1 ml to 2.5 ml.

Stability of gelatin immobilized enzyme at various temperature

Incubation temperature was varied from 20°C-110°C and enzyme activity was measured by Dinitrosalicylic acid method.

Immobilization of alpha amylase on agarose and agar gel

Agarose concentration was varied from 0.5% to 2% and Agar from 1%to 6% to check the stability. Beads were found stable when when 1% agarose solution and 4% agar solution was prepared in 25 mM sodium acetate buffer (pH 5.5) by warming them at 50°C. After cooling down to room temperature, 1 ml crude enzyme was mixed with 9 ml agarose and agar solution (the total volume of matrix and enzyme mixture being 10 ml) and immediately casted on preassembled glass plates .After solidification at room temperature, gel was cut into small beads of 5 x 5 mm size and washed several times before use to remove any unbound enzyme attached to the gel surface.

Enzyme loading capacity of agarose and agar gel

Small beads were prepared according to above said method except varying the enzyme concentration from 1 ml to 2.5 ml.

Stability of agar-agarose immobilized enzyme at various temperature

Incubation temperature was varied from 20°C-110°C and enzyme activity was measured by Dinitrosalicylic acid method.

Immobilisation of alpha amylase in calcium alginate beads

1mlcrude enzyme was mixed with 5 ml of 3% sodium alginate solution .This enzyme and sodium alginate mixture was taken in the syringe and was dropped in the beaker containing 0.2 M $CaCl_2$ solution. Enzyme entrapped in Calcium Alginate beads were formed.

Enzyme loading capacity of Calcium Alginate beads

Small beads were prepared according to above said method except varying the enzyme concentration from 1 ml to 2.5 ml.

Stability of calcium alginate immobilized enzyme at various temperature

Incubation temperature was varied from 20°C-110°C and enzyme activity was measured by Dinitrosalicylic acid method.

Stability of immobilized enzyme with varying pH

Amylase assay was performed by varying the pH of buffer from 3pH to 9 pH.

Immobilisation of alpha amylase in butanol-sodium alginate beads

1 ml crude enzyme was taken and mixed with 5 ml of 1 % Butanol and 3% of Sodium alginate (1: 2 ratio) solution. The enzyme and sodium alginate mixture was taken in the syringe and was dropped in the beaker containing 4% $CaCl_2$ solution.

Enzyme loading capacity of Butanol-Sodium Alginate beads

Enzyme concentration was varied from 1 ml to 2.5 ml and small beads were prepared according to above said method.

Stability of butanol sodium immobilized enzyme at various temperature

Incubation temperature was varied from 20°C-110°C and enzyme activity was measured by Dinitrisalicylic acid method

RESULTS

Effect of different chemical treatment on enzyme activity

Immobilisation of α-amylase on hot water treated and chemically treated (NaOH and SDS) Wood fiber, wood chip and wood powder were studied. On comparing these three methods ,by calculating the enzyme activity we came to know that α-amylase enzyme was immobilised maximum on NaOH treated wood fiber (18.83U/ml/min) then wood chip (18.67U/ml/min), wood powder (18U/ml/min),SDS treated wood fiber (17.83U/ml/min) and minimum on SDS treated wood powder(14.67U/ml/min) (Table 1).

Immobilisation of α-Amylase on different concentration of Gelatin solution

The maximum enzyme activity of 37.73U/ml/min was observed with 25% gelatin and 10% glutaraldehyde concentration was used .Enzyme loading capacity was found maximum when 1 ml of enzyme was used as shown in Table 2.Optimum activity of Gelatin entrapped enzyme was seen at 50-60°C.(Table 3).

Table 1: Effect of different chemical treatment on enzyme activity

Different carriers	SDS treatment Enzyme activity (U/ml /min)	Hot water treatment Enzyme activity (U/ml /min)	NaOH treatment Enzyme activity (U/ml /min)
Wood fibers	17.83	17.00	18.83
Wood chips	16.33	16.83	18.67
Wood powder	14.67	15.67	18.00

Table 2: Enzyme loading capacity of various matrices used for immobilization

Matrices for immobilization	Enzyme activity (U/ml/min) when 1 ml enzyme used	Enzyme activity (U/ml/min) when 1.5 ml enzyme used	Enzyme activity (U/ml/min) when 2 ml enzyme used	Enzyme activity (U/ml/min) when 2.5 ml enzyme used
Gelatin - gluteraldehyde	37.73	25.16	18.50	12.92
Agar agarose	38.67	23.73	19.5	10.4
Sodium alginate	39.67	23.78	17.33	13.07
Butanol sodium	34.67	22.22	16.17	12.27

Table 3: Effect of temperature on immobilized enzyme

Temp °C	20 °C	35°C	50-60°C	110 °C
Gelatin entrapped enzyme (U/ml)	26.67	32.33	37.33	20.34
Agar agarose entrapped enzyme (U/ml)	27.80	34.30	38.67	23.37
Sodium alginate entrapped enzyme (U/ml)	24.55	34.50	39.67	20
Butanol sodium entrapped enzyme (U/ml)	26	32.66	34.67	22

Alpha amylase immobilization on agarose and agar gel

Enzyme activity found 38.67U /ml/min when 4% agar and 1%agarose was used for enzyme immobilization. Enzyme loading capacity was maximum when 1 ml of enzyme were used. (Table 2).Immobilized enzymes showed maximum activity 37.14U/ml /min at 50-60°C (Table 3).

Enzyme loading capacity of calcium alginate beads

Enzyme loading capacity was maximum when 1 ml of enzyme was used. (Table 2)
Optimum activity of immobilized enzyme was seen at 50-60°C (Table 3).
Enzyme showed maximum activity at pH 6 when entrapped in calcium alginate beads (Table 4).

Enzyme loading capacity of Butanol-Sodium Alginate beads

Enzyme loading capacity was maximum when 1 ml of enzyme was used**.** (Table 2) Optimum activity of immobilized enzyme was seen at 50-60°C (Table 3).

Table 4: Effect of pH on stability of calcium alginate entrapped enzyme

Citrate phosphate Buffer pH	Enzyme activity (U/ml/min)
3	21
5	35
6	39.67
7	30
9	18

DISCUSSIONS

Immobilization of α-amylase enzyme on various carriers were presented together with the emphasis on loading capacity and stability of enzyme at various temperatures on different carriers. Various chemical treatments were given to matrices (Wood fiber, Wood chip, and Wood powder) to remove lignin. Maximum activity were seen in case of NaOH treated wood fibre

(18.83U/ml/min) and least in case of SDS treated wood chips (14.67U/ml/min).

Maximum immobilization was achieved at 25% gelatin concentration. The results revealed that at low gelatin concentration, unstable and fragile gelatin beads were obtained which led to poor immobilization which may be due to the larger pore size in the gel which probably caused leaching of the enzyme. At concentrations above 25%, the percent immobilization was low which may be the result of steric hindrance due to high concentration of gelatin. the glutaraldehyde concentration above 10% led to a decline in percent immobilization which again may be the result of the steric hindrance caused by the presence of the glutaraldehyde which may have affected the accessibility of the substrate to amylase. Thus, the maximum enzyme activity of 37.73U/ml/min was observed with 25% gelatin and 10% glutaraldehyde concentration. At concentration of 1%, stable Agarose beads were obtained Further increase above 1% resulted into decrease in percent immobilization and at a concentration of 2% the solidification of the gel before the addition of the enzyme resulted. The optimum immobilization was obtained at 4% concentration of agar (w/v). At lower concentrations, the beads were very soft and fragile giving very low percent immobilization. The porosity of the calcium alginate beads depend upon the alginate type and the gelling agent concentration [25] Various concentrations of sodium alginate were used for preparation of calcium alginate beads in order to vary the relative degree of cross linking which would create different pore size. The immobilization yield was found to be highest for a final sodium alginate concentration of 3% (w/v). Decreasing immobilization yield with increase in sodium alginate concentration has been due to the decrease in the porosity of the gel beads, which caused diffusion limitation of the substrate. In practice reducing the porosity can reduce leakage but some initial leakage of the enzyme molecule is certain to occur [26]. Amylase entrapped in Ca-alginate beads showed a gradual decrease in the immobilization yield with the increase of sodium alginate concentration above 3%. A decrease in the relative protease activity with increase in calcium chloride concentration has been reported [27, 28]. It might be due to change in pH of calcium chloride solution with change in calcium chloride concentration. Stable beads were formed when 3% sodium alginate and 0.2 M $CaCl_2$ were used. Alpha-Amylase enzyme was immobilized by the Butanol-Sodium alginate method. Three different ratio percentage of Butanol: alginate were used 1:2, 2:2 and 2:1. Results showed that using 1% Butanol and 2% sodium alginate provides greater stability as compare to other concentration. To determine the temperature stability of α-amylase experiments were conducted and the extent of activity loss under various temperature regimes was determined. Optimum temperature of the immobilized enzymes was found to be 50-60°C for all the methods we used. For temperatures lower than the optimum, the relative activity of the immobilized enzyme is greater than in the case of the free one. At temperatures higher than the optimum, the decrease in activity of physically bonded alpha-amylase is more emphasized than in the case of the other immobilized enzymes. This is mainly due to the denaturation of the enzyme structure at high temperature. The pH is one of the major parameters capable of shifting enzyme activities in reaction mixture. Immobilization usually results in shift of optimum pH due to conformational changes in enzymes. The optimum pH for the activities of immobilized alpha-amylase were shifted to acidic range (optimum pH 6 in comparison to the free enzyme (optimum pH 7). This shift in optimum pH could be resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site [29].

During alpha -amylase immobilization, similar shift in the optimum pH towards acidic direction had been observed [30]. All the methods were found to be good but maximum enzyme activity (39.67U/ml/min) was obtained in Sodium alginate beads method of immobilization, followed by Agar Agarose (38.67 U/ml/min), Gelatin beads (37.73 U/ml/min), Butanol Sodium alginate (34.67U/ml/min).Among different chemical treated Wood chips, Wood fiber and Wood powder; NaOH treated Wood fiber showed maximum activity (18.83U/ml/min) as compare to others.

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