



Effect of different immobilization techniques on α - amylase

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ABSTRACT

Immobilization has emerged since last decade as a very powerful tool to improve almost all enzyme properties like stability, activity, specificity and selectivity, and reduction of inhibition. The immobilization may help to solve some of the problems of enzymes as industrial biocatalysts like enzyme recovery for reuse. In the present investigation *A.niger* was isolated from soil and identified by lactophenol staining and microscope observation. Amylase producing *A.niger* were screened using starch hydrolysis medium. α - amylase enzyme assay was done by DNS method. Immobilization was performed using entrapment method. The Entrapment materials were polyacrylamide, agar-agar, and gelatin and *ca*- alginate. Different matrix was immobilized with amylase and the fermentation period was noted as 36, 72, 108, 144, 180, 216 hrs. Immobilized microbes were subjected to growth in production medium and enzyme activity was noted as every 36 hrs. The 36 hrs sample was subjected to Folin's Lowry procedure to determine the activity of amylase being released by the immobilized cells. The O.D reading was done by periodic sample obtained from the immobilized cell media were noted. Immobilization efficiency was found to be highest for polyacrylamide matrix 76.9% followed *ca*- alginate (57%), agar- agar (33%), and gelatin (5.27%). Polyacrylamide was showed the maximum enzyme activity than three types of immobilized matrixes.

Keywords: Immobilization efficiency, *A.niger*, polyacrylamide, Gelatin, α - amylase, *ca* alginate.

INTRODUCTION

Faithful industrial process depends on process simplicity that ultimately saves energy, labor thereby reducing overhead production cost. In that respect enzyme immobilization technology has gained tremendous popularity and has taken leading edge over free enzyme catalysis process. Several studies indicated that immobilized enzyme has shown increased catalytic activity, increased stability of enzyme (1), easy recovery of enzyme, easy separation from product minimizing or eliminating protein contamination of product, repeated or continuous use of a single batch of enzyme (2).

Several methods of enzyme immobilization technique used (3) covalent binding in gelatin by chemical organic cross linkers like glutaraldehyde or formaldehyde has been very effective in retaining enzymatic activity since availability and accessibility of substrate binding site of enzyme is not compromised(4). The immobilized enzyme can contact easily with substrate and do not detach from inert support matrix maximizing all benefits of immobilization.

Immobilization is defined as confining the molecules or cells to a distinct phase from the one in which the substrates and products are present. It is critical that the substrates and the products move freely in and out of the phase to which the molecules or cells are confined (5).

Enzymes are immobilized by physical adsorption, ionic binding, covalent binding, cross – linking and entrapment methods (6). In the past few decades, many immobilization methods and carrier materials have been investigated (7). There are many reports about immobilization of α -amylase used for the hydrolysis of starch and production of maltose. Immobilization by entrapment differs from covalent binding and Adsorption in that enzyme/ cells are free in solution but restricted in movement by the lattice structure of a gel.

Immobilization of enzymes refers to techniques which represent variety of advantages over free enzyme catalysis including increased stability of enzyme, easy recovery of enzyme, easy separation of reactant and product, repeated or continuous use of a single batch of enzyme (2) which will ultimately save the enzyme, labor and overhead costs (8). Immobilized enzymes have been widely used for many years in different industrial processes. Usually, immobilization of enzymes is carried out by three principle means, matrix assisted entrapment of enzyme, adsorption on a solid support, ionic or covalent binding (9, 10). 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 (11). Physical entrapment of α -amylase in calcium alginate beads has shown to a relatively easy, rapid and safe technique (12) in comparison with other immobilization methods. The method of immobilization should be such that an enzyme faces as little conformational change as possible.

Agar is well known as a solidifying agent in the preparation of microbiological media. It is obtained from red algae. Species of *Gelidium*, *Gracilaria* are used extensively. Agar is polymers of galactose, or galactose-containing compounds, with surface groups. Some of the sulfate groups are involved in the bonds between sugar residues, which are used to make gels or to make solutions viscous. It is also important in the food industry (13).

Alginate is a naturally occurring polymer extracted on industrial scale from various species of brown algae (*Phaeophyceae*), including *Ascophyllum*, *Laminaria*, *Lessonia*. Some species of *Pseudomonas* and *Azotobacter* produce alginate as an exopolysaccharide by means of various extracellular epimerases (14). Alginate is applied for paper manufacturing, paint thickening, dentists for making impression of teeth (13).

Polyacrylamide is a polymer formed from acrylamide subunits. It can be synthesized as a simple linear-chain structure or cross-linked. Polyacrylamide is not toxic. However, unpolymerized acrylamide, which is a neurotoxin, can be present in very small amounts in the polymerized acrylamide (15) therefore it is recommended to handle it with caution. One of the largest uses for polyacrylamide is to flocculate solids in a liquid. This process applies to water treatment, and processes like paper making. Polyacrylamide can be supplied in a powder or liquid form, with the liquid form being subcategorized as solution and emulsion polymer.

Gelatin is a mixture of protein and peptides produced by the partial hydrolysis of collagen extracted from the skin, bones, and connective tissues of animals such as domesticated cattle, chicken, pigs, and fish. Photographic and pharma grades of gelatin are generally made from beef bones, although some beef bone gelatin is used by the food industry. Gelatin is an animal protein unlike many other gelling agents used by the food industry. The natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily. Gelatin melts to a liquid when heated and solidifies when cooled again. Together with water, it forms a semi-solid gel. Gelatin forms a solution of high viscosity in water, which sets to a gel on cooling, and its chemical composition is, in many respects, closely similar to that of its parent collagen (16).

Amylase is an enzyme that catalyzes the hydrolysis of starch into sugar that is water soluble and has low molecular weight, this enzyme is used extensively in drink industry for example the production of high fructose syrup (HFS) or textile industry. Amylase can be made from various microorganisms especially from *Bacillus*, *Pseudomonas* and *Clostridium*, *Aspergillus*, *Rhizopus*, *Penicillium* etc. The α -amylase is a starch hydrolyzing enzyme which has many applications in various fields. It is used in breweries, chocolate, pharmaceuticals industries etc (17). α -Amylase is an endo-enzyme that catalyzes hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharide of various chain lengths (18).

A.niger is a fungus and one of the most common species of the genus *Aspergillus*. It cause a disease called black mold on certain fruits and vegetables such as grapes, onion and peanuts, and is a common contaminant of food. It is ubiquitous in soil. In the classic book, the genus *Aspergillus* by listed close to 200 species and great many varieties.

EXPERIMENTAL SECTION

Sample collection

The soil sample was collected from Mannargudi, Thiruvavur district, Tamil Nadu, India. The collected sample was transferred to the sterile plastic bags and sealed.

Isolation of fungi

After sample collection, serial dilution was performed for isolating microbial growth from the collected samples.. Potato dextrose agar was prepared and poured into sterile separate petridishes of allowed to solidify. After solidification the selected dilution factor of fungi such as 10^{-3} to 10^{-5} were spreaded on the medium then the plates were incubated at 27°C for 42 to 72 hours respectively. Lactophenol cotton blue technique is used to identify fungal morphology (19, 20)

Determination of amylase activity (21)

The *Aspergillus niger* were tested for amylase production by starch hydrolysis. When starch agar medium (Peptone-0.5g, Beef extract-0.15g, Yeast extract- 0.15g, NaCl-0.5g, Starch-1g, Agar-2g, Distilled water-100ml) was inoculated with *Aspergillus niger* and the plates were incubated at 37°C at 24 hours. Subsequently the plates were flooded with iodine solution (Iodine-0.2%, potassium iodide-0.4%, and distilled water- 100ml) and incubated for 15 min, the zone of clearance around the microbial growth indicated the production of amylase.

Quantitative assay (22)

Quantitative assay was done by centrifugating the fermented broth of each strain at 1000 rpm. The supernatant was used as crude enzyme. The fermented liquid culture test strains were taken and centrifuged at 5000 rpm for 10 min and the supernatant was discarded. The cell pellets were washed thoroughly with sterile 20.0g/l potassium chloride solution followed with sterile distilled water subsequently. Finally the cell mass suspended in sterile sodium chloride solution (9.0 g/l), and the cell suspension was used for immobilization as well as for free -cells fermentations. The whole cell was immobilized using entrapment method.

Partial purification of α -amylase was done (23)

Immobilization (Entrapment) (24, 25)

Polyacrylamide

A cell suspension was prepared by adding cell suspension to 10 ml chilled sterile distilled water. In another 10 ml of 0.2 M sterile phosphate buffer (pH 7.0) was taken and the following chemicals were added: 2.85 g acrylamide, 0.15 g bisacrylamide, 10 mg ammonium persulphate, and 1 ml TEMED (NNN1N1 tetra methyl ethylene diamine). The cell suspension and phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm – diameter petriplates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4mm³), transferred to 0.2 M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 hour. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4°C until use

Agar-agar

A definite quantity of agar-agar (Hi media, Mumbai, India) was dissolved in 18ml of 0.9% sodium chloride to get final concentration of 2% and sterilized by autoclaving. The cell suspension (2ml) was added to the molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4 inch-diameter petriplates and allowed to solidify. The solidified agar block was cut into equal cubes (4mm³), added t sterile 0.1M phosphate buffer (pH 7.0) and kept in the refrigerator (1 hour).phosphate buffer was then decanted and the cubes were washed with sterile distilled water 3 to 4 times.

Ca alginate

The alginate entrapment of cells was performed aseptically under laminar flow unit. Sodium alginate solution was prepared by dissolving sodium alginate in 100 ml boiling water and autoclaved at 121°C for 15 minutes. Both alginate slurry and cell suspension were mixed and stirred for 10 min to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into 0.2M CaCl₂ solution from 5-cm height and kept for curing at 4°C for

1 hour. The beads were washed with sterile distilled water 3 to 4 times. When the beads were not being used, they were preserved in 0.9% sodium chloride in a refrigerator.

Gelatin

Five milliliters of cell suspension was added to 15 ml of 20% sterile gelatin (hi-media), maintained at 45°C. The mixture was then poured into a sterile Petridish. The gel was then left for hardening at 30°C. The resulting block was cut into small- size cubes (4mm³) and the cubes were washed thoroughly with sterile distilled water.

Immobilized Enzyme Assay

The activity of immobilized enzyme was assayed by incubating 1 ml of 3 % (w/v) starch solution (prepared in 50 mM TrisHCl buffer of pH 7.5) with 1 g of beads of different immobilization, at 70°C for 5 minutes. The α -amylase level was determined by measuring the reducing sugar released from soluble starch (26). An enzyme unit is defined as the amount of α -amylase that liberates 1 mol of reducing sugar from the substrate per gram of beads under the assay conditions.

Immobilized enzyme activity for starch hydrolysis (22)

Immobilized enzyme 0.1 g (1 ml enzyme solution) was mixed with buffered 5% starch solution and shaken in a water bath shaker. One milliliter of the product was mixed with 5 ml iodine solution and the absorbance was read at 610 nm. One unit of enzyme activity is defined as the amount required to hydrolysing 1 mg starch per minute under the assay conditions. All results are presented in a normalized form with the activity under optimum conditions being assigned a value of 100%. Influence of pH on activity was determined by carrying out the reaction at varying pH in the range and keep in all other reaction conditions constant. The pH stability was determined by pre-incubating the catalyst and buffer for different time intervals between 15 min and 20 hours at 30°C followed by the reaction.

Enzyme assay

Assay of amylase done by measuring the reducing sugars (RS) dinitrosalicylic acid (DNS) method (27). The protein contented was determined (28).

Preparation of starch substrate (0.1%)

100 mg of soluble starch was dissolved in 80-90 ml of distilled water and the solution was boiled in a micro oven for complete dissolution. Then the volume was made up to 100 ml with distilled water for each time this solution prepared freshly.

Estimation of reducing sugar by DNS method and Estimation of protein was done by Lowry's method

Immobilization Efficiency (29)

Immobilization Efficiency (IE) (%) = $(I/A-B) \times 100$

Where, A = Added enzyme (U/g of beads or cubes), B= Unbound enzyme (U/g of beads or cubes), I = Immobilized enzyme (U/g of beads or cubes)

RESULTS AND DISCUSSION

Aspergillus niger was isolated from soil samples, collected from Mannargudi, Thiruvavur District, Tamil Nadu. Basal media was used for *Aspergillus niger* isolation, amylase produced under submerged fermentation. Enzyme assay was done by DNS method. Immobilization was done by sodium alginate, Agar agar, gelatin, polyacrylamide gel with α -amylase and immobilized enzyme efficiency was also calculated. The 36 hrs sample was subjected to Folin's Lowry procedure to determine the activity of amylase being released by the immobilized cells. The O.D reading was done by periodic sample obtained from the immobilized cell media were noted. In polyacrylamide matrix maximum immobilization activity was noticed in 36 hrs, OD 0.095 at pH 9.2, minimum immobilization activity was noticed at the pH 7.5 and the duration 216 hrs. Gelatin maximum activity was occurred at 36 hrs, pH 9.2 and the Optical Density value 0.082. In agar- agar, fermentation period was 36 hrs Optical Density noted as 0.079, at pH 9.1. In pH 7 enzyme activity was declined. Ca alginate, Optical Density was noticed as 0.089, at pH 9.0

and the duration 36 hrs. Overall immobilization activity was noticed in polyacrylamide matrix (15 IU/ml), ca-alginate (14 IU/ml) followed by agar-agar(12 IU/ml),gelatin(8 IU/ml).

Immobilization efficiency was found to be highest for polyacrylamide matrix 76.9% followed ca- alginate (57%), agar- agar (33%), and gelatin (5.27%).Maximum immobilization efficiency was occurred in the polyacrylamide.

In present study, amylase enzyme was produced from *A.niger* from that immobilization done by various matrixes, such as ca alginate, polyacrylamide, agar- agar, and gelatin. Polyacrylamide was showed the maximum enzyme activity than three types of immobilization, the fermentation were performed at 36 hrs.

TABLE-1 Polyacrylamide Immobilization of *A.niger* with Different Matrix

Entrapment matrix	Final pH	Fermentation Period	Immobilization activity (OD)	Amylase volumetric productivity (IU/ml/hour)
Polyacrylamide	9.2	36	0.095	15
	8.5	72	0.093	16
	8.2	108	0.090	18
	8.0	144	0.087	22
	7.8	180	0.080	18.5
	7.5	216	0.077	17

TABLE-2 Agar – agar Immobilization of *A.niger* with Different Matrix

Entrapment matrix	Final pH	Fermentation Period	Immobilization activity (OD)	Amylase volumetric productivity (IU/ml/hour)
Agar-agar	9.2	36	0.082	12
	8.8	72	0.079	13.5
	8.4	108	0.075	15
	7.0	144	0.073	17.5
	7.0	180	0.050	15.5
	7.0	216	0.045	14.5

TABLE-3 Gelatin Immobilization of *A.niger* with Different Matrix

Entrapment matrix	Final pH	Fermentation Period	Immobilization activity (OD)	Amylase volumetric productivity (IU/ml/hour)
Gelatin	9.1	36	0.075	8
	8.3	72	0.071	10.5
	8.0	108	0.069	13
	7.0	144	0.067	15.5
	7.0	180	0.040	13
	7.0	216	0.032	11.5

TABLE-4 Caalginate Immobilization of *A.niger* with Different Matrix

Entrapment matrix	Final Ph	Fermentation Period	Immobilization activity (OD)	Amylase volumetric productivity (IU/ml/hour)
Ca alginate	9.0	36	0.089	14
	8.9	72	0.086	12.5
	8.8	108	0.082	14
	8.5	144	0.079	16.5
	8.3	180	0.075	16
	8.0	216	0.069	15.5

TABLE-5 Immobilization Efficiency

S.No	Entrapment Matrix	Immobilization Efficiency (%)
1	Polyacrylamide	76.92
2	Ca alginate	57.14
3	Agar agar	33.3
4	Gelatin	5.27

FIGURE -1 Immobilization Activity (OD)

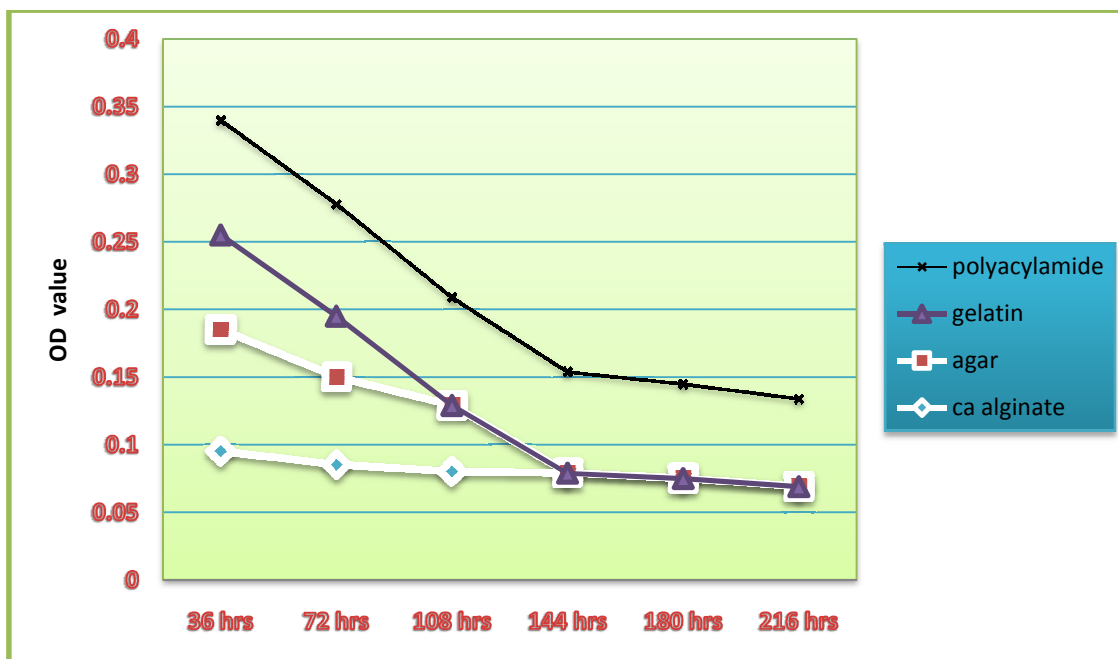
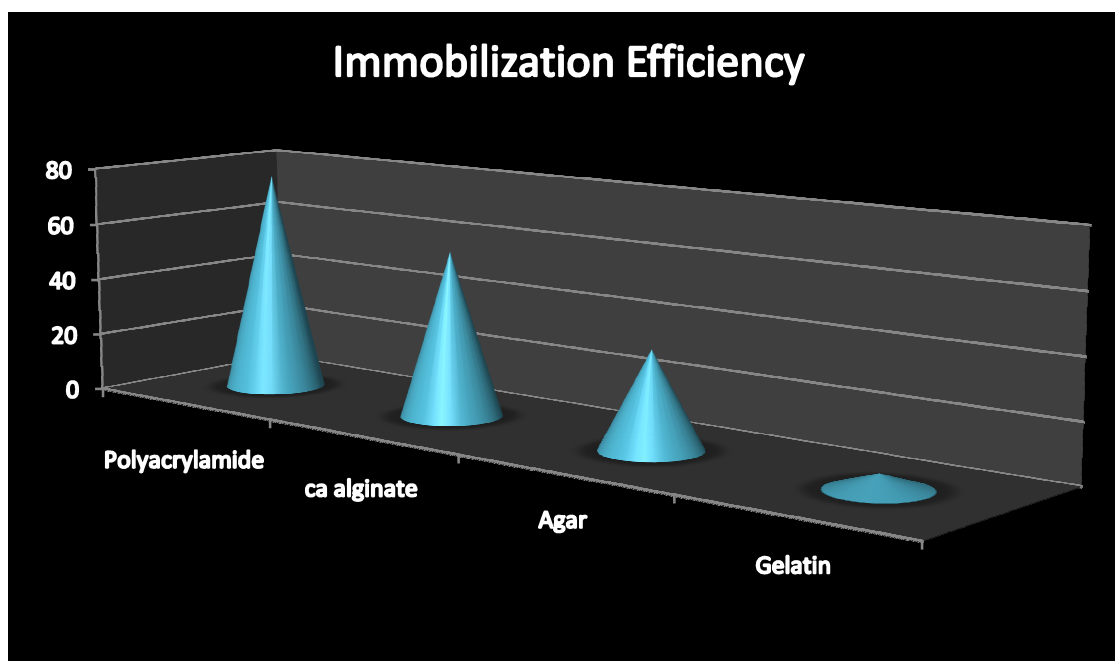


FIGURE -2 Immobilization Efficiency on Different Matrix



CONCLUSION

Entrapment is a promising method of immobilization for easy production. Immobilised cells are superior to that of free cells because it leads to higher volumetric activities within the same line of fermentation. Specific advantages of immobilization technique such as long life term stability, reusability, and possibility of regeneration to be adoptable for the production.

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