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BIOCHEMICAL CHARACTERIZATION OF FREE AND IMMOBILIZED α–AMYLASE FROM *ASPERGILLUS NIGER* AND ITS BIOTECHNOLOGICAL APPLICATIONS

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Keywords:

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ABSTRACT: α-amylase is widely used in various biotechnological applications such as food processing, starch, and paper industries, hydrolyzing starch, glycogen, and other polysaccharides into glucose, maltose, and oligosaccharides. *Aspergillus niger* α-amylase was partially purified by ammonium sulphate fractionation with 1.409 fold purity and 54.88% yield. The partially purified α-amylase was immobilized on sodium alginate with calcium chloride by ionotropic gelation with glutaraldehyde as a cross-linking agent. Immobilized α-amylase exhibited 50% of its original activity. The soluble and immobilized α-amylases exhibited maximum activity at pH values 7.5 and 8.0, respectively. The optimum temperature for both the soluble and immobilized enzymes was 35 ºC. The immobilized enzyme was more pH and thermally stable than the soluble one. The substrate starch was hydrolyzed by soluble (Km 0.6 mg/ml, V_{max} 16.05 mg/ml/min) and immobilized α-amylase (Km 0.65 mg/ml, V_{max} 17.41 mg/ml/min) with high efficiencies. On the basis of the results obtained, immobilized α-amylase could be employed in the saccharification of starch processing.

INTRODUCTION: Alpha-amylases (E.C.3.2.1.1) are enzymes that catalyze the hydrolysis of internal α-1,4-glycosidic linkages in starch into low molecular weight products, such as glucose, maltose, and maltotriose units. Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market [1, 2]. The Aspergillus species produce a large variety of extracellular enzymes, and amylases are the ones with the most significant industrial importance. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry.

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The fungal α-amylases are preferred over other microbial sources due to their more accepted GRAS (Generally Recognized as Safe). This degrading starch enzyme has received a great deal of attention because of its perceived technological significance and economic benefits. The industrial application of enzymes is often hampered by a lack of availability, high price, and limited stability under operational conditions.

Recently, the researchers focused on IE because of its advantages over FE in biotechnological applications. Immobilization facilitates the efficient recovery and reuse of costly enzymes [3]. Additionally, advantages include enhanced stability, great operational control, *e.g.,* choosing an immobilization method that can improve the thermal behavior of an enzyme could potentially extend the operating capabilities of a process and allow a reactor to operate at higher temperatures, hence increasing the reaction rates and product yields [4], the flexibility of reactor design, and easy separation from the catalyst and unreacted substrate

contamination [5]. 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 [6].

Various natural polymers like alginate, carrageenan, cellulose, chitin, chitosan, agar, agarose, gelatin, and synthetic polymers like polyacrylamide have been extensively investigated for the entrapment of cells [6-13]. Entrapment of enzymes in calcium alginate is one of the most widely used methods for enzyme immobilization. It is a fast and simple technique using mild conditions [14, 15-19]. The above features would be important in the development of an economically feasible continuous bioreactor for the starch hydrolysis industry. Thus immobilizing α- amylase would be of great significance.

Thus, in the present study, α-amylase from *Aspergillus niger* was partially purified and immobilized in calcium alginate gel beads with cross-linking agent glutaraldehyde. The conditions of entrapment like a concentration of sodium alginate, calcium chloride, glutaraldehyde and bead size were optimized for highest apparent activity. The immobilized α-amylase was characterized in terms of optimum temperature and pH, thermo stability and pH stability, kinetic parameters and compared with those of free α-amylase.

MATERIALS AND METHODS:

Materials: Sodium Alginate obtained from HiMedia, Pvt. Ltd. (India). DNSA (3, 5-Dinitrosalycyclic acid) were purchased from Himedia (Mumbai). The enzyme-substrate, starch, were obtained from Merck, Glutaraldehyde is from Sd fine, Mumbai, and all other reagents used were of analytical grade.

Microorganism and Fungal Culture: The fungus *Aspergillus niger* capable of producing extracellular α-amylase was obtained from the Micro-biology laboratory (Maharanis Science College for Women). It was maintained on PDA (potato dextrose agar) slant and stored at 4 ºC.

Preparation of Crude Enzyme Extract: The active culture was produced through submerged cultivation of the *A. niger* using substrates of zero cost, namely Wheat Bran, Rice Husk. For 0.5 gm of powdered wheat bran and rice husk were taken in 250 ml flasks and moistened with nearly 20 ml of MSM containing the following in gm/l (0.016g NaCl, 0.016 g KCl, 0.002 g $CaCl_2$, 0.04g Na_2HPO_4, 0.004g $MgSO_4$, 0.002g $FeSO_4$, 0.16 g Glucose, 0.04 g NH_4Cl volume was made up to 100 ml pH 6.2. After autoclaving, the flasks were inoculated with spores (2×10^6) of *A. niger*. Submerged fermentation was carried out in 250 ml of Erlenmeyer flasks, each containing 100 ml of culture medium. The flasks were incubated at 37 ºC for 5 days on an orbital shaker at 120 rpm. On the 5th day, mycelia were separated from culture broth by filtration through Whatmanno.1 filter paper, and the filtrate was used as crude α-amylase (2.511 U ml^{-1}).

Ammonium Sulfate Precipitation: The cell-free crude extracts of the enzyme were subjected to sequential ammonium sulfate saturation. Different concentrations of ammonium sulfate in the range of 0-80% were evaluated to attain saturation point for *A. niger* amylase. The preparations were kept overnight at 4 °C and then centrifuged, which resulted in the separation of precipitates and supernatants. Precipitates from each preparation so obtained were dissolved in the minimum volume of Tris-HCl buffer (20 mM, pH 8.0) separately and were refrigerated until further use.

Dialysis: Ammonium sulpahte salted out protein precipitates of *A. niger* amylase was dialyzed against 20mM Tris-HCl (pH-8.0) buffer with five changes overnight at 4 ºC using 14 kDa cut-off dialysis membrane.

Immobilization of α-amylase in Alginate: Ammonium sulphate precipitated and dialyzed α-amylase was entrapped in calcium alginate with glutaraldehyde as the cross linking reagent. The enzyme was immobilized in calcium alginate beads according to the method of Naganagouda and Mulimani (2006) [20]. 4 ml of sodium alginate solution (3.75%) was mixed with 1 ml enzyme solution (7.5 mg/ml) to a homogenous final alginate concentration of 3%. The mixture was extruded drop by drop using a sterile hypodermic syringe needle into 0.2M $CaCl_2$ solution maintained at 4 °C to form beads. The beads were allowed to harden in the $CaCl_2$ solution for 2 h. The resulting spherical beads were washed with sterile distilled water.

The beads were stored in a 0.2M acetate buffer (pH 4.8) at 4 °C. Glutaraldehyde was used as a cross linking agent according to the method of Naganagouda and Mulimani (2006) [20]. Calcium alginate beads were treated for 3 min in 5 ml of 1% glutaraldehyde. The beads were filtered off, washed with sterile water, and stored at 4 °C until used.

Assay of Free and Immobilized Enzyme: The ammonium sulphate precipitated free amylase (500 µl) and immobilized enzyme (0.4 grams of alginate beads equivalent to 100µl of free enzyme) activity is measured using a colorimetric method with DNS reagent (3,5-dinitrosalicylic acid), and starch is used as substrate. The starch is hydrolyzed on the catalytic action of amylase to fragments, which can be determined with 3,5-dinitrosalicylic acid, due to their semi-acetalic reducer groups. The formed nitro-aminosalycilic acid concentration corresponds to the enzymatic activity of amylase.

Activity Yield: The activity yield of α-amylase immobilized in calcium alginate, was calculated. The activity yield (%) was defined by

AY = activity of immobilized enzyme × 100 / Activity of the soluble enzyme

Characterization of Free and Immobilized Enzyme:

Determination of Optimum pH of Free and Immobilized α- amylase: Incubate 0.5 ml of suitably diluted enzyme extract and immobilized enzyme (0.4 grams of alginate beads equivalent to 100µl of free enzyme) with 0.4 ml of buffers of different pH for 5 min. At the end of 5 min, start the reaction with 0.5 ml of 1% starch, incubate at room temperature for 15 min and arrest the reaction with 0.5 ml of DNS reagent. Heat the tubes in boiling water bath for 15 min, cool and dilute with water to 10 ml. Read the absorbance at 540 nm against the blanks made with the respective buffers. Plot the activity as a function of pH. From the plot, the optimum pH for the enzyme, and predict the reactive group involved in the catalysis.

Determination of the pH Stability of Free and Immobilized α-amylase: Incubate 0.5 ml of suitably diluted enzyme extract and immobilized enzyme (0.4 grams of alginate beads equivalent to 100 µl of free enzyme) with 0.1 ml of buffers of different pH for 30 min. At the end of 30 min, start the reaction, with 0.8 ml of 1% starch in 0.2 M acetate buffer, incubate at room temperature for 15 min, and arrest the reaction with 0.5 ml of DNS reagent heat the tubes in boiling water bath for 15 min, cool and dilute with water to 10 ml. Read the absorbance at 540 nm against blanks made with respective buffers. Plot the activity as a function of pH. From the plot, determine the pH stability of the enzyme.

Effect of Optimum Temperature of Free and Immobilized α- amylase: Pipette out 0.9 ml of 1% starch in 0.1M acetate buffer, pH 5.5 into different test tubes, pre incubate at 5, 10, 15, 20, room temperature 35, 40, 45, 60, 70 and 97 ºC for 10 min. At the end of pre-incubation, start the reaction by adding 0.1 ml of suitably diluted enzyme extract and immobilized enzyme (0.4 grams of alginate beads equivalent to 100 µl of free enzyme). Incubate at the respective temperatures for 15 min. Arrest the reaction with 0.5 ml of DNS reagent and heat on a boiling water bath for 15 min. Cool, and make up the volume in each case to 10 ml with distilled water. Read the absorbance at 540 nm against a suitable blank prepared at the above temperature. Plot activity in µmoles as a function of temperature in ºC. Construct the Arrhenius Plot, and using the slope of the plot, determine the energy of activation of the given enzyme.

Temperature Stability of Free and Immobilized α- amylase: Pipette out 0.1 ml of suitably diluted enzyme extract and immobilized enzyme (0.4 grams of alginate beads equivalent to 100µl of free enzyme) into different test tubes, incubate at 5, 10, 15, 20, room temperature 35, 40, 45, 60, 70 and 97 ºC for 15 min. At the end of the incubation period, rapidly cool the tubes in ice. Assay the tubes at room temperature by adding 0.9 ml of 1% starch in 0.1M acetate buffer, pH 5.5. Arrest the reaction for 15 min with 0.5 ml of DNS and heat on a boiling water bath for 15 min. Cool, and make up the volume in each case to 10 ml with distilled water. Read the absorbance at 540 nm against suitable blanks prepared at the above temperatures. Plot activity in µmoles as a function of temperature in ºC.

Determination of Km and V_{max}: Pipette out different aliquots of 2.5% substrate in 0.1 M Acetate buffer, pH 5.5 to obtain 50 to 1000 mg %

of substrate concentrations. Make up the volume to 0.9 ml with buffer and start the reaction by adding 0.1 ml of suitably diluted enzyme extract and immobilized enzyme (0.4 grams of alginate beads equivalent to 100µl of free enzyme). Incubate for 15 min, and stop the reaction by adding 0.5 ml of DNS reagent to all the tubes. Boil the contents in the tubes in a water bath for 15 min, cool, and read the absorbance at 540 nm after making up the volume in each case to 10 ml with distilled water and plot (i) substrate concentration v/s activity (ii) Lineweaver - Burk plot. Determine the Km and V_{max}.

RESULTS AND DISCUSSION: Recently, interest and demand for enzymes with novel properties are very high in various industries, and it leads to the discovery of various types of amylases with unique properties. Each application of amylases requires properties with respect to specificity [21-23]. Amylases from microbial sources, especially fungi (Aspergillus spp.), have gained much attention because of the availability and high productivity of fungi, which are also amenable to genetic manipulation [24].

Since Aspergillus spp, proved to be the most potent alpha-amylase producer; hence it was selected for the purpose of partial production purification and investigating properties of the enzyme.

Incubation Time for Alpha-Amylase Production: The production of amylase was highest on the 5th day under submerged fermentation, and the activity is found to be (2.511 U ml^{-1}).

Ammonium sulphate Fractionation: The enzyme extracts of *A. niger* was subjected to sequential ammonium sulphate saturation from 0-80%. α-amylase got precipitated from 30-80% saturation of ammonium sulphate, with 31.61 mg protein and specific activity of 2.845 IU/mg **Table 1**. 1.409 fold purification of the enzyme was achieved with 54.88% amylase yield after 80% ammonium sulphate fractionation.

TABLE 1: PURIFICATION PROFILE OF α-AMYLASE FROM *A. NIGER*

Purification step	Total activity	Total protein	Specific activity	Purification fold	%Recovery / Yield
Crude enzyme	823.39	40.12	2.025	1	100
Ammonium sulphate fractionation (30-80%)	451.93	31.61	2.845	1.409	54.88
Dialysis	405.23	29.99	3.371	1.66	49.20

*Total activity: Enzyme activity in given volume (IU); **Total protein: mg/ml; ***Specific activity: Enzyme activity per unit protein concentration (IU/mg); ****Purification fold: is increase in specific activity; *****Percent recovery is remaining protein concentration as % of the initial protein concentration.

In literature also, advanced purification steps followed by ammonium sulphate precipitation has been reported by several workers. In agreement with our results, the majority of them reported 80% saturation as optimum for efficient precipitation of α-amylase. 80% saturation of ammonium sulphate has been adjudged as best for precipitation of *B. subtilis* α-amylase by Hamza (2004) [25]. Gangadharan *et al.,* (2008) [26] reported 30-90% saturation as optimum for salting out maximum amylase isolated from *B. amyloliquefaciens.* Shafiei *et al.* (2010) [27], observed 80% ammonium sulphate saturation as best for precipitation of α-amylase from a moderate halophile (*B. methylotrophicus* P11-2).

Characterization of Free and Immobilized Alpha-Amylase Produced from *Aspergillus niger*: Although the stability of enzyme is its intrinsic property dictated by its primary structure, many external factors such as physical factors and chemical reagents influence the overall stability and thus the activity of an enzyme. Keeping in view these facts, the effect of various physical and chemical factors *viz*. pH, pH stability, temperature, thermostability, substrate concentration, *etc*. were evaluated on the behavior of purified α-amylase from *A. niger*.

Effect of pH on Activity and Stability of Free and Immobilized α-amylase: Enzymes being proteins are sensitive to changes in the environment in which they work. Any change in hydrogen ion concentration (pH) can profoundly affect the activity of an enzyme. This is very important in the industrial use of enzymes to be active at varying pH levels. The pH must be controlled, and industrial processes usually try to get the maximum

rates of activity by choosing the appropriate pH at which the enzyme is active but is not denatured.

The pH activity profiles for the free and immobilized enzyme are shown in **Fig. 1**. From the figure, it is observed that the maximum enzyme activity was obtained at pH 7.5 and 8.0, respectively. The enzyme activity was gradually increased from pH 3-7.5 in case of free enzyme, whereas in an immobilized enzyme, it is found that shift of pH 0.5 units towards alkaline pH was observed that is pH from 3-8.0. There was a gradual decrease in enzyme activity after a pH of 7.5. Varalakshmi *et al.,* 2009 [28], have reported optimum pH 9.5 for the strain *Aspergillus niger* JGI 24. In accordance with this statement, acidic to neutral α-amylase finding great applications in food and baking industries from fungi and bacteria have been reported in the vast majority in the literature [29-30]. However, there are very few reports on alkaline amylase giving activity at pH 9.0 and 10.0. Hmidet *et al.,* (2008) [31] reported α-amylase from *B. licheniformis* NH1, which was highly active in the wide pH range of 5.0-10.0, with maximum activity at pH 9.0. Similarly, Roy *et al.,* (2014) [32] reported a *B. megaterium* Strain KAN1 showing elevated amylase activity at pH 11.0. In this regard, alkaline α-amylase from *A. niger* is a potent candidate in the industry.

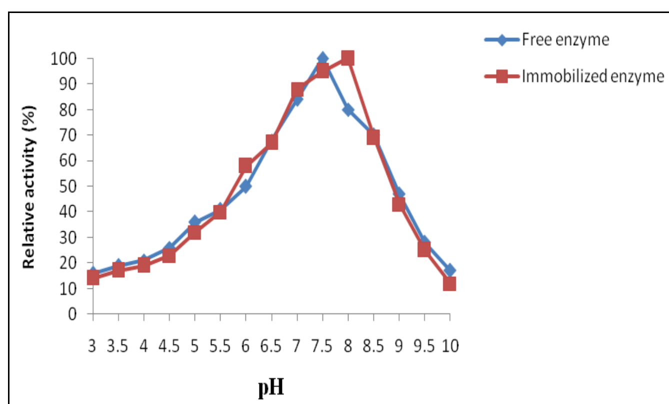

FIG. 1: EFFECT OF pH ON FREE AND IMMOBILIZED α-AMYLASE ACTIVITY

Many industrial processes involving enzymatic processes do not have adequate pH control, and thus the pH usually fluctuates around the required value. Thus, it is important to determine the pH activity curve in quantitative detail for a particular substrate enzyme system under consideration. In such conditions, an enzyme with a wider pH stability range is sought after. The wide pH stability range for *A. niger* α-amylase spanning 7.0-11.0) makes them a promising candidate in the paper, detergent, textile, and biofuel industries requiring near neutral to alkaline pH **Fig. 2**. It has been documented that 90% of all liquid detergents contain α-amylases [33].

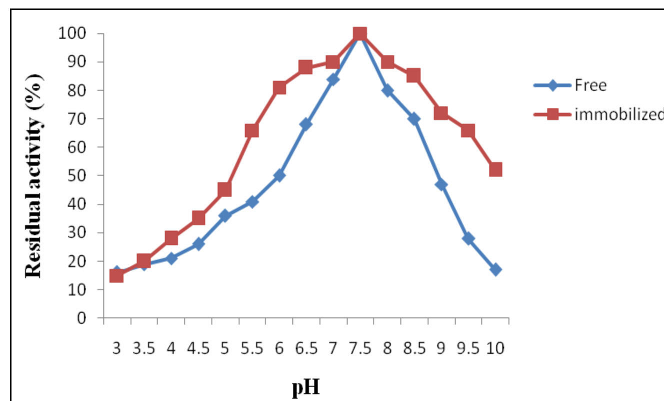

FIG. 2: DETERMINATION OF pH STABILITY ON FREE AND IMMOBILIZED α-AMYLASE ACTIVITY

Effect of Temperature and Thermo-Stability on Activity of Free and Immobilized α-amylase: The effects of temperature on the activity of an enzyme are complex and can be considered as two forces acting simultaneously, but in opposite directions. As the temperature is raised, the rate increases, but at the same time, there is a progressive inactivation (denaturation) of the enzyme protein. This becomes more pronounced as the temperature increases so that an apparent temperature optimum is observed.

The temperature-dependent amylase was determined, and the effect of temperature on enzyme activity profile is shown in **Fig. 3**. From the figure, it is evident that the optimum temperature was 35 ºC for both the free and immobilized enzymes, respectively. Varalakshmi *et al.,* 2009 [17] have reported an optimum temperature of 30 ºC for the strain *Aspergillus niger* JGI 24.

Thermostability is one of the crucial properties of enzymes for industrial applications. Thermostable α-amylases which are stable and active at high temperature and can withstand organic solvent, detergent, low and high pH, and other denaturing agents are highly valued in the industries for their commercial exploitation. According to the data which have been published in the last 20 years, amylases can act in the temperature range of 40-100 °C [34].

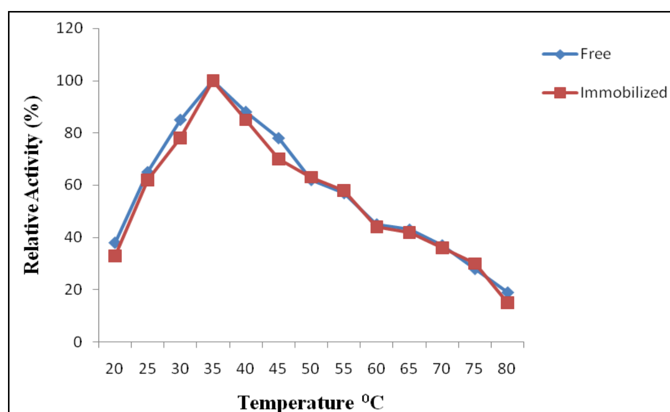

FIG. 3: EFFECT OF TEMPERATURE ON FREE AND IMMOBILIZED α-AMYLASE ACTIVITY

The thermostability of immobilized α- amylase from *A. niger* is thus a striking feature that makes this enzyme a potential candidate to be used in the industry. The enzyme was even active at a higher temperature of 80 °C with a relative activity of approximately 20% **Fig. 3**. Thermostable *α*-amylases are generally preferred in the industry as their application minimizes contamination risk and reduces reaction time, thus providing considerable energy-saving [22]. The major utility of α-amylases is in the starch industry for the liquefaction process that converts starch into fructose and glucose syrups. This process requires the use of a highly thermostable α-amylase, which can act at temperatures around 70-100 ºC.

Thermal Inactivation Parameters: Thermo stability studies were carried out by pre incubating purified α-amylase for 120 min at various temperatures ranging from 35 °C to 80 °C.

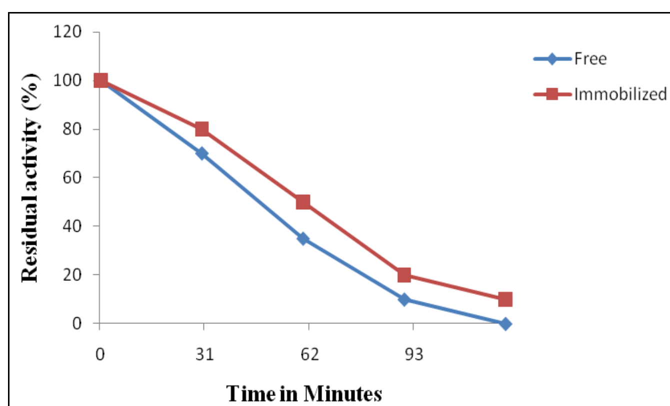

FIG. 4: THERMOSTABILITY OF FREE AND CA-ALGINATE IMMOBILIZED α-AMYLASE

The thermal stability of the enzyme was determined by studying the time-dependent inactivation of an enzyme at temperatures it was subjected to. As shown in **Fig. 4**, α-amylase was stable at a range spanning 30-50 ºC with retention of approximately 50% of the maximum activity for 60 min; however, at 80 °C, 20% relative activity for 30 min was observed. Activity retention of up to 50% was observed at higher temperatures of 60 and 70 °C for 15 min.

Effect Substrate Concentration on Free and Immobilized Alpha-Amylase Activity: Km is a useful and fundamental characteristic for an enzyme and a particular substrate. It can be viewed as an index of how easily the enzyme can be saturated by the substrate (*i.e.,* the affinity) under defined conditions of temperature and pH. The smaller the value of Km, the more readily the enzyme may be saturated with the substrate.

The kinetic parameters for hydrolysis of soluble starch were determined by plotting the initial velocities towards different concentrations of soluble starch as substrate, incubated with the fixed amount of enzyme.

From the experiments, the Km and V_{max} values for the free enzyme are found to be 0.6 mg/ml and 16.05 mg/ml/min at 35 ºC and pH 7.5 with 50 mM phosphate buffer, respectively. Whereas in immobilized alpha-amylase Km is 0.65 mg/ml, and V_{max} is 17.41 mg/ml/min at 35 ºC and pH 8.0 with 50 mM phosphate buffer, respectively **Table 2**. Higher V_{max} and lower Km had confirmed the efficiency of this enzyme for diverse applications. This Km value is nearer to Km value (0.055mM) and V_{max} (22.075 mg/ml/min) of alpha-amylase from *Monascus sanguineus* [35].

TABLE 2: ACTIVITY YIELD AND KINETIC PARAMETERS FOR THE FREE AND IMMOBILIZED α- AMYLASE

	Activity yield	Km (mg/ml)	V_{max} (mg/ml/min)
Free enzyme	-	0.6	16.05
Immobilized enzyme: Ca-alginate	50	0.65	17.41

Low values of Km indicate a high affinity of the enzyme for the substrate. The V_{max} and Km values are enzyme specific for different enzymes and are difficult to compare due to the difference in origin, conditions, and the substrate used. Gangadharan *et al.,* (2008) [15] reported V_{max} and Km values of 3.04 mg/min and 2.34 mg respectively, for purified α-amylase of *B. amyloliquefaciens.* In another study,

Shafiei *et al.,* (2010) [16] reported V_{max} and Km values of 1.18 mg/ml/min and 4.5 mg/ml, respectively. Rasiah and Rehm (2009) [36] studied the kinetics of purified α-amylase from *B. licheniformis,* and V_{max} and Km values were found to be 506 U/mg and 5 μmol respectively. A perusal of these reports revealed that purified immobilized α-amylase from *A. niger* had shown very good kinetic characteristics, thereby making them efficient enzymes in terms of quality and quantity.

CONCLUSION: Though some preliminary enzymatic parameters like pH optimum, pH stability, temperature optimum, thermostability, substrate specificity have been determined for free and immobilized α-amylase from *A. niger,* and the immobilization method was used for its application in the food industry. Further work for its complete application of α-amylase and its application in the food industry would be conducted with the aid of other biochemical techniques.

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