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PURIFICATION & CHARACTERIZATION OF β-AMYLASE PRODUCED BY ASPERGILLUS FLAVUS USING RICE HUSK

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ABSTRACT: *Solid state fermentation was carried out by using rice husk in order to produce enzyme from Aspergillus flavus (KIBGE IB-34). The highest enzyme activity was obtained at pH 6.5 and 30°C, after 72 hours. The extracted enzyme was then subjected to concentration and partial purification by ammonium sulphate fractionation. After purification maximum activity was obtained with 50-60% fractionation and 3.5 times increase in activity was seen in comparison with crude enzyme. The enzyme was optimally active at pH 7.5 and 50°C when 1 % starch was provided as substrate. The purified enzyme was stable at 40°C and it loses its activity as the temperature rises, and deactivates completely at 105°C. Moreover, the presence of EDTA on enzyme shows inhibitory effects. The enzyme activity is reduced to half in the presence of EDTA. Furthermore, the half life of enzyme was also studied at 40-80°C.*

Keywords: β-amylase, *Aspergillus flavus,* rice husk, purification, characterization.

1. INTRODUCTION:

Amylase is an enzyme that is found in many forms. It is a digestive enzyme that aids in the breakdown of carbohydrates by breaking the bonds between sugar molecules in polysaccharides through a hydrolysis reaction in animals, plants, and bacteria etc. Amylase can be classified into three types: Alpha-amylase, Beta-amylase and Gamma-amylase. All the types of amylase differ on the basis of their nature of hydrolyzing the bonds to create short-chain sugars. (EC 3.2.1.1) α-amylase is a chemical found in different structures in the nature. Two isoforms are found in the human body; salivary alpha amylase and pancreatic alpha amylase. Both variations join in the assimilation of starch. α-amylase is likewise present in growths, microscopic organisms and diverse plants. The final items obtained after hydrolysis by α-amylas are maltose and glucose from amylopectin and maltose and maltotriose from amylose. (EC 3.2.1.2) β-amylase is a catalyst found in organisms, microscopic organisms and plants however not in people. Not at all like α-amylase. β-amylase can just debase starch from the non-decreasing end of the polymer chain by hydrolysis of the second α-1,4 glycosidic bond. The final item is therefore maltose, i.e. two glucose units. (EC 3.2.1.3) γ-amylase is a catalyst that corrupts starch from the non-hydrolysing so as to diminish end last α(1-4) glycosidic linkages, along these lines yielding one glucose unit. Additionally, it hydrolyzes α(1-6) glycosidic linkages. The catalyst has a lower pH ideal contrasted with different amylases.[1]

Utilization of agro-waste as a substrate for SSF by fungal sources has proved to be a cost effective approach in the field of biotechnology. Rice husk is a good source of amylase production used in many industries such as fuel, textile, paper, food and pharmaceutical industry [2]. According to a study, 649.7 million tons of rice is grown worldwide annually whose 20% is comprised of rice husk [3]. Dry mass of husk contain 59.2% (w/w) carbohydrate in which 8.7% starch is present. This starch acts as specific substrate for fungi and results in production of amylase [4]. The filamentous fungus, *Aspergillus flavus* is a potential producer of amylase for industrial use [5, 6]. The present study deals with the purification and characterization of amylase using rice husk and *Aspergillus flavus* (KIBGE IB-34).

2. MATERIALS AND METHODS:

2.1 Microorganism and Maintenance:

The fungal strain of *Aspergillus flavus* (KIBGE IB-34) was used for this study. The culture was grown on petridish containing Sabouraud dextrose agar (SDA), incubated at 30ºC for 7 days and stored at 4ºC.

2.2 Inoculum Preparation:

Spores were dislodged from 7 days old SDA culture and added in 10 ml sterilized saline solution (0.9% NaCl) under aseptic conditions. It was then vortex gently in order to obtain a homogenized spore suspension. Hemocytometer was used for cell counting in inoculum.

2.3 SSF:

Agro waste substrate (10 g) containing (g/100ml) 0.1g K_2HPO_4 and 0.1g $MgSO_4$ was taken in 250 ml flask and autoclaved at 121ºC for 20 minutes. Thereafter, the flask material was inoculated with 5 ml spore suspension of fungal strain and incubated at 30ºC for 7 days.

2.4 Recovery of Enzyme:

After fermentation, 50 ml of distilled water was added in each flask and placed on shaker for 60 minutes, so that crude enzyme can be separated from the fermented mash. Afterwards, the mixture was filtered through filter paper and centrifuged at 8000 rpm for 15 min at 4ºC to remove fungal spores and unwanted particles. The supernatant, thus, acquired was used as a source of crude enzyme.

2.5 Enzyme Assay:

The activity of amylase was assayed by incubating 50µl enzyme with 50µl soluble starch (1% w/v) prepared in 0.1M phosphate buffer pH 7.0 at 50ºC for 15 minutes. Reaction was stopped by adding 150µl 3, 5-dinitrosalicylic acid reagent. The blank was made in the same way except that 50µl enzyme was added after DNS. Deionized water (750 µl) was added in the reaction mixture and blank and read at 546 nm. All the experiments were conducted in triplicates [7].

One enzyme activity unit was defined as "the amount of enzyme releasing 1 µmol of reducing sugar from the substrate in 1 minute at 50ºC".

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2.6 Concentration and Partial Purification:

In present study, attempts were made to partially purify enzyme by ammonium sulphate fractional precipitation. The crude enzyme was precipitated by subjecting the cell-free culture supernatant to 50%, 60% and 70% saturation. The precipitated enzyme was separated by centrifugation at 4ºC, 10,000 rpm for 20 minutes. The pellet was dissolved in small volume of phosphate buffer (pH 7.5). Both the pellet and supernatant were dialyzed overnight against same buffer. The purified fractions were then analyzed for enzyme activity.

2.7 Characterization of Enzyme:

a- Determination of Temperature Optima:

For determination of optimum temperature of enzyme activity, the assay was carried out at different temperature (40, 45, 50, 55, 60, 70, 75 and 80ºC).

b- Determination of pH Optima:

For pH optima, starch solution was prepared in buffers of different pH (4.5-9). For; pH (4.5-5.5) Na-Acetate buffer, pH (6.5-7.5) Potassium phosphate buffer and pH (8-9) Tris-HCl buffer was used. The enzyme activity was assayed for each starch solution.

c- Determination of Volumetric Productivity (g/ltr.h):

Volumetric productivity is defined as the amount of enzyme units produced per kilogram per hour of substrate under SSF conditions. For the determination of volumetric productivity rates plates containing 2g of rice-husk supplemented with 4 ml of media were prepared and autoclaved at 121°C for 30 min. All plates were inoculated with 1 ml of inoculum (containing 5.465 E6 spores/ml) while un-inoculated plate was used as control and were incubated at 30ºC for specified time period. The fermented mash was subjected to extraction of enzyme and enzyme activity was determined.

d- Determination of Melting Temperature (T_m):

In order to evaluate thermal stability of enzyme, one of the parameter tested was melting temperature($\mathbf{T_m}$). For the determination of $\mathbf{T_m}$, the aliquots containing crude enzyme were heated at temperatures ranging from 40 to 105ºC, followed by cooling at 4ºC for 1 hour. The unheated aliquot served as control. Enzyme activity was measured by DNS method.

e- Determination of Half-life of Enzyme ($T_{1/2}$):

For the determination of half life($\mathbf{T_{1/2}}$), the crude enzyme was incubated in different aliquots at 40, 50, 60, 70 and 80ºC for 60 minutes. First aliquot was withdrawn immediately followed by subsequent withdrawal after every 15 minutes. Before the determination of enzyme activity, the aliquots were cooled at 4ºC for 1 hour and enzyme activity was measured by DNS activity.

f- Effect of EDTA:

To check the effect of EDTA on enzyme, the substrate (1% starch) was prepared in phosphate buffer pH 7.5 supplemented with 10mM EDTA. Substrate prepared in buffer without EDTA served as control.

3. RESULTS:

3.1 Concentration and Partial Purification of Enzyme:

Before purification, the crude enzyme activity was found to be 1.628±0 IU/ml. The results after purification are shown in the Table 1.

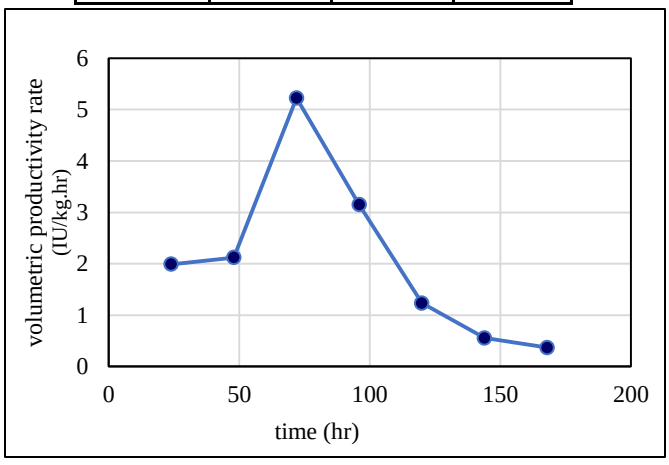
Table 1: Enzyme Activity in Pellet and Supernatant

Enzyme activity recovered in pallet		Residual activity in supernatant	
Ammonium sulphate Saturation. (%)	**E.A IU**	**Ammonium sulphate Saturation (%)**	**E.A IU**
50	60.778 (±0.034)	50	212.135 (±0.030)
60	39.442 (±0.017)	60	282.6 (±0.007)
70	8.0125 (±0.002)	70	86.58 (±0.027)

The results show that maximum specific enzyme activity was present in supernatant with 60% ammonium sulphate saturation and was found to be 5.624±0.007553 IU/ml i.e; the activity was increased by 3.5 folds (Table 2).

Table 2: Partial Purification of Amylase by Ammonium Sulphate

Sample	Volume (ml)	Enzyme Activity (IU)	Folds
Crude Enzyme	50	81.4	-
50-60% saturation	50	282.6	3.5



3.2 Determination of Volumetric Productivity Rate:

Figure 1: Volumetric productivity rate

The results of enzymatic activity are summarized in Figure 1. Figure shows that maximum volumetric productivity rate was observed after 72 hours and if the time period either decreases or increases by 72 hours volumetric productivity rate also decreases.

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3.3 pH Optima for Enzyme Activity:

The result of pH optima for enzyme activity is summarized in Figure 2. Figure shows that the pH optima for enzyme activity was 7.5 as highest enzyme activity (3.996±0.272 IU/ml) was noted at this pH. Furthermore, a slight improvement in enzyme activity was also observed at pH 9. Appearance of two peaks in enzyme activity may indicate the presence of at least two isozymes. Our findings are almost similar to Alva et al., in which enzyme has optimum pH value 5.8, 7.5 and 9. [8]

3.4 Temperature Optima for Enzyme Activity:

The results for enzyme activity for temperature optima are

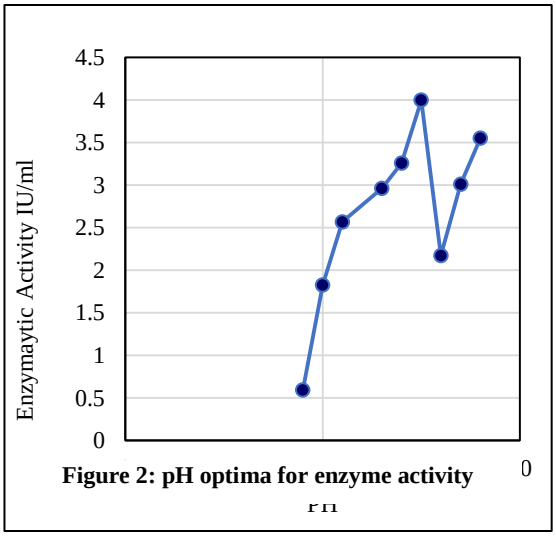

Figure 2: pH optima for enzyme activity

summarized in Figure 3. For temperature optima, the enzyme activity was measured after incubation at different temperatures ranged between 40 to 80ºC. The data generated suggests that a temperature of 50 ºC was optimum for the activity.

3.5 Effect of EDTA:

The results for effect of EDTA can be seen from Figure 4.

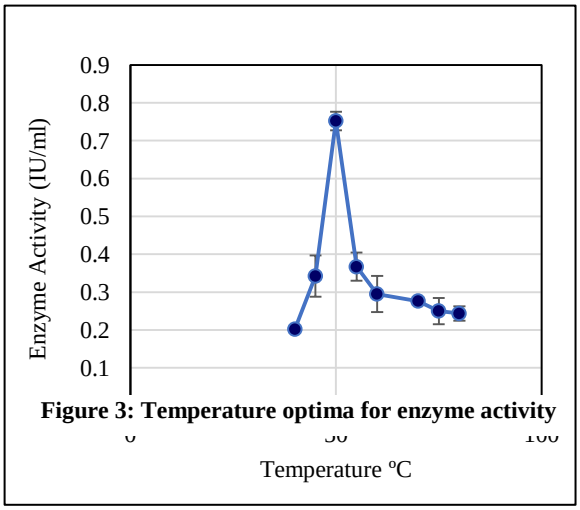

Figure 3: Temperature optima for enzyme activity

Results show decrease in enzyme activity, when the enzyme reaction was carried out in the presence of EDTA.

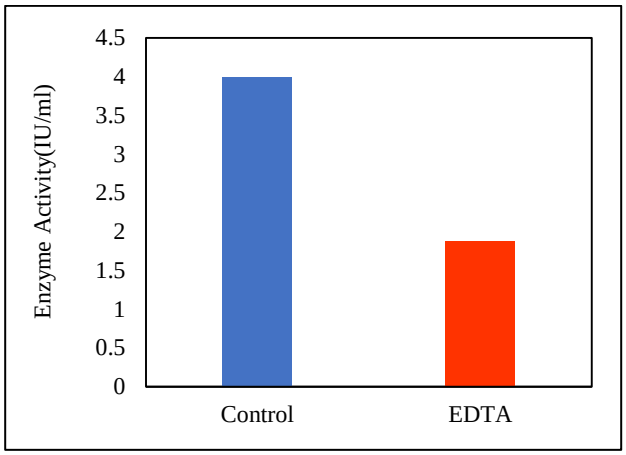

Figure 4: Effect of EDTA

3.6 Melting Temperature:

Every enzyme has optimum temperature for its activity and is thermo sensitive in nature. Its activity gradually decreases with increase in temperature and is lost beyond certain limit. Figure 5 shows that after storing enzyme at 40ºC or at higher temperatures for 60 minutes enzyme activity decreases. In addition, the temperature at which enzyme loses its activity and enzyme denaturation occurs is 105 ºC.

3.7 Half-life of Enzyme:

Results for half life for enzyme at 40, 50, 60, 70 and 80 ºC

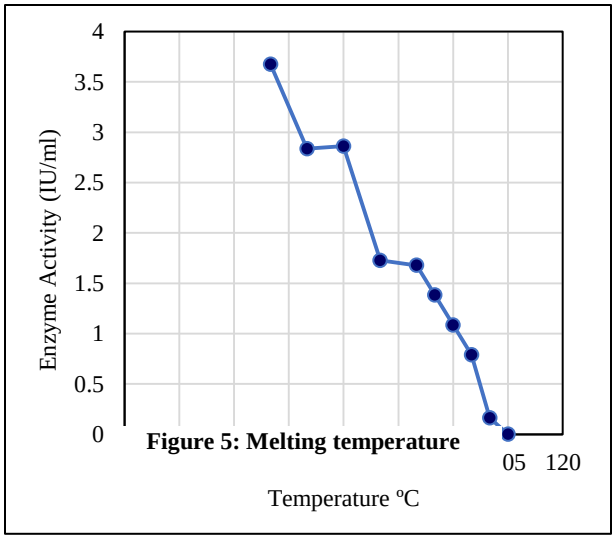

Figure 5: Melting temperature

are shown in Figures 6. The results show that enzyme maintains its 50% activity at 40, 50, 60, 70 and 80 ºC within 46, 38, 40, 45 and 15 minutes respectively.

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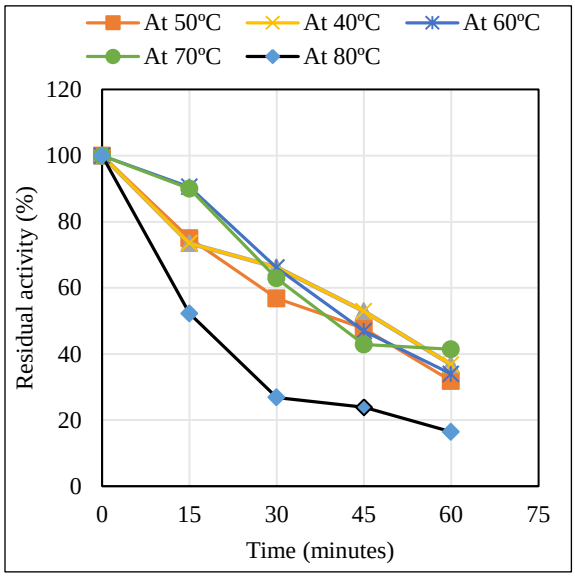

Figure 6: Half Life

4. DISCUSSION:

Ammonium Sulphate precipitation is the most efficient method to purify enzymes due to its high solubility in solution resulting in removal of contaminants proteins from the solution. Thereby, enzyme activity is improved to 2-5 folds. In the present study, ammonium sulphate precipitation technique was adopted because it gives positive and good results [9]. Our results are in accordance with Smitha et al. who reported 3.5 fold increase in activity after purification with 40-60% ammonium sulphate fractionation[10].

Like most of the enzymes, amylases do need specific conditions for their activity. In present study, maximum enzyme activity was seen at pH 7.5. Adekunle used *A. niger* and citrus fruit for amylase production and mentioned that enzyme is active in pH range 4 to 8 and reported 6.5 as optimum pH [11]. Other researchers also worked on pH maxima of amylase and reported maximum enzyme activity at pH 6.2 [12], some reported 7.0 [13] while some reported 7.5 as the most suitable pH for alpha amylase activity [14]. Chakraborty et al., found that at pH 7 amylase showed maximum activity [15]. While, one mentioned 8.0 and another mentioned 9.0 as optimum pH value for amylase [16,17]. These differences are maybe due to source of isolation of microorganism, its strain and culture medium utilized [18].

Figure 3 shows that enzyme is active over a range of 40-80ºC. The highest enzyme activity was, however, noted at 50ºC; suggesting that the enzyme was thermophilic in nature. The ability of amylase to optimally work at higher temperatures makes it a potential candidate to be employed in industry. Thus, it may be used in a variety of industrial applications. Other researchers mentioned 55ºC as temperature maxima for enzyme activity using *Baccilus subtilis* [19]. It is worth to mention that pancreatic amylase also has optimum temperature of 40ºC and the present enzyme also shows good activity at 40ºC. Therefore, as far as the temperature activation is concerned, it could be used in pharmaceutical applications after complete pharmacological testing [20]. Adekunle mentioned that *A. niger* shows enzyme activity in range of 20 to 45ºC [11]

To check whether the enzyme under study requires a divalent cation as co-factor, EDTA was supplemented in the buffer used for the enzyme activity; EDTA acts as a chelating agent hence can chelate out the divalent cations from the enzyme molecule and to retard its activity. The results show that activity was reduced to half in the presence of EDTA; suggesting that the enzyme was a metallo-amylase and requires a divalent cation as a co-factor for its activity (Figure 4). Other investigators also indicated that in the presence of EDTA enzyme activity of amylase decreases [21].

For thermal stability of amylase, it is imperative to find out melting temperature and half life of an enzyme. It is worth to mention that enzyme does not lose its complete activity even at 100ºC (Figure 5). Thus, it is useful for textile and other industries where high temperature is required. Mamo and others also worked with *Bacillus sp*. and reported that amylase is stable upto 100 ºC [22]. Furthermore, Adekunle reported that at 80ºC amylase loses its complete activity within 30 minutes [11].

5. CONCLUSION:

It is concluded that *A. flavus* IB-34 is capable of producing significant amount of amylase just after partial purification. So, its complete purification can be done to enhance its activity and to make it useful for industrial processes.

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