ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Production and Characterization of Fungal α-Amylase from Marine *Alternaria alternata* Utilizing Lignocellulosic Wastes and Its Application.

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ABSTRACT

A marine *Alternaria alternata* was tested for its ability to produce amylase by solid state fermentation (SSF) and submerged fermentation (SMF).Various lignocellulosic wastes such as corn cobs, wheat bran, potato shells, wheat straw and rice straw were used as solid substrates. The highest titer of amylase activity was obtained on potato shells by SSF (46.47 U/g solid substrate) compared to 41.19 U/g solid substrate by SMF after 14 day of fermentation. The highest extraction yield of α-amylase (183.57 U/g solid substrate) was obtained with acetate buffer (0.05 M, pH 5) as a leaching agent, 75% sea water concentration and pre-treatment of the waste by boiling in water for 60 min. Repeated washes under the optimum conditions showed that most of the enzyme (about 90.31%) was recovered in four repeated extractions. The calculated values of the half life for the crude amylase at 50, 55 and 60^{o}C were 189.86, 99.82 and 93.77 min, respectively. Optimum α-amylase enzyme activity was observed at 40^{o}C and active at wide range of pH. The calculated K_m and V_{max} for α-amylase were 1.90 mg/ml and 0.33 U/mg protein, respectively. Presence of Na^+ enhanced amylase activity by 2-fold. Moreover, the enzyme was able to hydrolyze corn starch under optimized conditions with efficiency 85%.

Keywords: Marine fungi; α-amylase; lignocellulosic wastes; potato shells; Solid state fermentation; Submerged fermentation; *Alternaria alternata;* Application.

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ISSN: 0975-8585

INTRODUCTION

Amylases accounts for about 30% of the world's enzyme production [1]. They randomly catalyze the hydrolysis of the α -(1→4) glucosidic linkages of polysaccharides, such as starch and other polysaccharides of various sizes [2].

They are used in several industries particularly for the hydrolysis of starch as a basic step to generate glucose, maltose, a mixture of maltooligosaccharides, and various α-limit dextrin-containing α-(1–6) bonds [3]. Those products are highly valued in a wide range of nutritional, cosmetic and pharmaceutical processes and applications [4]. To meet the demands of these industries, low cost medium is required for the fermentation of α-amylase [5].

α-Amylases (α -1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are widely present in plants (malt), animal tissues (saliva, pancreas), and microorganisms. Amylase of fungal origin was found to be more stable than the bacterial enzymes on a commercial scale. It is preferred for use in formulation for human or animal consumption involving application under acidic condition and around 37 ^{o}C due to this biocompatibility [6]. Also, due to its biocompatibility, fungal amylases are preferred in baking and food processing. Many attempts have been made to optimize culture conditions and suitable strains of fungi [7].

α-Amylase can be produced by different species of microorganisms using both submerged fermentation (SMF) and solid substrate fermentation (SSF). But the cost of enzyme production in SMF is high which necessitates reduction in production cost by alternative methods such as SSF. However, SSF systems appear promising due to the natural potential and advantages they offer [8]. SSF is generally characterized by the growth of microorganism on and or within particles of a solid substrate in the presence of varying amounts of water.

The solid substrate acts as a source of carbon, nitrogen, minerals and growth factors, and has a capacity to absorb water, necessary for microbial growth. As the microorganisms in SSF are growing under conditions similar to their natural habitats, they may be able to produce certain enzymes and metabolites more efficiently than in submerged fermentation [9,10].

SSF has many advantages over SMF, including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be the most appropriate process for developing countries. A further advantage of SSF is that cheap and easily available substrates, such as agriculture and food industry by-products [11,12,13].

It is well known that the marine microorganisms produce a variety of industrially important metabolites [14]. Studies are mostly focused on the production of biologically active compounds by the fungi while their capability of degrading a wide range of polymeric compounds have not been well exploited.

Over the past few decades, considerable researches have been undertaken with the extracellular α-amylase being produced by a wide variety of microorganisms. The major advantages of using microorganisms for the production of amylases are the economical bulk production capacity of microbes and their easier manipulation to obtain enzymes of desired characteristics. Since many of the commercially available amylases do not withstand industrial reaction conditions and also they do not meet a large industrial demand of this enzyme, therefore, isolation and characterization of novel amylases with desirable properties such as alkaline stability and halophilicity are very important to meet the industrial demands [15].These may be the reasons why researchers all over the globe are now trying to exploit extremophiles which are the valuable source of novel enzymes [16]. Among the extremophiles, halophiles are microorganisms that live, grow, and multiply in highly saline environments. Exoenzymes from these organisms with polymer-degrading ability at low water activity are of interest in many harsh industrial processes where concentrated salt solutions would inhibit enzymatic conversions [17].

Marine microorganisms are capable of catalyzing various biochemical reactions with novel enzymes such as amylase, lipase, deoxyribonuclease and protease [18].

In view of the potential uses of amylases and advantages of marine microorganisms, the study of this enzyme from the marine-derived fungus *Alternaria alternata* is desirable. The main objective of this study was to investigate optimization α-amylase production by *A. alternata* under SSF and SMF conditions with potato shells (PS) as a relatively cheap substrate, The characterization of α-amylase and determination of its kinetics. Moreover, the application of the produced enzyme.

MATERIALS AND METHODS

Microorganism and Maintenance

The filamentous marine-derived fungus *Alternaria alternata* used in this study was isolated from decayed wood pieces of old fishing boats from Ismalia, Egypt. Identified in the National Research Centre, Chemistry of Natural and Microbial Products Dept. [Microbial Culture Collection Unit (MCCU)] according to Kohlmeyer and Kohlmeyer [19]. Stock cultures of the fungus was maintained on malt extract agar slants (biomalt 20g/l, agar15g/l, 800 ml sterile sea water and 200 ml distilled water) at 4°C and periodically subcultured.

Enzyme Production

The enzyme production was studied by using five lignocellulosic wastes (corn cobs, wheat bran, potato shells, wheat straw, rice straw) as a fermentation substrate in two different methods.

Solid State Fermentation (SSF):

Three gram of the solid substrate was moistened with 15 ml of sea water and 1.0 ml inoculum containing (5×10^6 spores/ml) of 7days old culture. Incubation was carried out at 28-30ºC in 250 ml Erlenmeyer flasks for up to 21 days.

Submerged Fermentation (SMF)

The same weight of the solid substrate with 50 ml of sea water were inoculated with 1.0 ml inoculum containing (5×10^6 spores/ml) of 7days old culture and incubated under shaking conditions at 150 rpm and 28-30 ºC, for different time intervals (7, 10, 14 and 21 days).

Enzyme Extraction

The solid cultures were suspended in 50 ml of distilled water and placed on a shaker for 1h. The suspension was filtered through a nylon cloth, and then centrifuged at 5,000 rpm for 15 min. [20].

In submerged cultivation, solids were separated by filtration and the culture broth was centrifuged at 5,000 rpm for 15 min. the clear supernatant was used for enzyme assay.

Estimation of α-amylase Activity

Amylase activity was estimated by analysis of reducing sugar released during hydrolysis. Incubation of the reaction mixture (0.5 ml of diluted enzyme solution was added to 0.5 ml of 1% soluble starch in 0.05 M acetate buffer (pH 5.00)) was performed for 20 min at 40˚C. The released reducing sugars were determined by the method of Somogyi [21]. One unit (U/g) of amylase activity was defined as the amount of enzyme that releases 1µmol of reducing sugar as glucose per gram of dry substrate per minute, under standard assay conditions.

Factors Affecting α-amylase Production

Method of Extraction

This was done according to the method of Ahmed and Mostafa [22]. After the fermentation period, the enzyme was extracted by suspending the fermented material in 50 ml of different solvents; distilled water,

acetate buffer (0.05 M; pH 5) and non-ionic surfactant (Tween-80, 1%). Flasks were shacked at (150 rpm) for 1h. Following extraction, the suspended materials and fungal biomass were separated by centrifugation at 5000 rev/min for 20 min at 4^{o}C. The clarified supernatant was used as the source of the crude enzyme.

Salinity

The influence of salinity on α-amylase production by *A. alternata* was determined by using different seawater (SW) proportions, as follows: 100%(DW), 25%(SW), 50%(SW), 75%(SW) and 100%(SW) (v/v).

Pre-treatment of Potato shells

Pre-treatment of (PS) is an essential factor for efficient α-amylase production. 20 g of (PS) treated by boiling in water, autoclaving, 1% H_2SO_4, NaOH, PEG, T-80 and triton for 60 min. The remaining waste after treatment was dried, and used as fermentation media compared to the control (the shells without any treatment).

Number of Extraction Cycles

Number of extraction cycles was studied by adding 50 ml of acetate buffer (0.05 M) to the solid state fermentation media after the extraction for each wash, the enzyme activity was determined in each extract.

Factors Affecting Enzyme Activity

The thermal stability of the crude amylase was determined by pretreatment of the crude enzyme at different temperatures (40, 45, 50, 55, 60^{o}C) for different periods (15, 30, 45, 60min) prior reaction incubation time with substrate. Optimum temperature of enzyme activity was determined by incubating the enzyme substrate reaction mixture at different temperatures (30, 35, 40, 45, 50 and 55^{o}C) for 20min. The effect of pH on amylase activity was determined by incubating the reaction mixture with different buffers of 0.05 molarity (pH 4-7 phosphate buffer, pH 7.5 and 8 phosphate buffer, pH 8.5 and 9 Tris-HCl buffer). The optimum substrate (soluble starch) concentration for maximum amylase activity was determined by using different concentrations ranging from 2.5-20 mg/ml. Effect of various metal ions like Ca^{2+}, Ba^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, NH^{+}, K^{+}, Fe^{2+} and Cu^{2+} on enzyme activity was determined in presence of each of the metal ions at final concentration of 0.05M. The effect of different Na^{+} concentration (0.025- 0.2 M) on enzyme activity was determined.

Application of Crude Amylase Preparation from *Alternaria alternata* in Hydrolysis of Corn Starch

The produced amylase from *A.alternata* was evaluated for its capability to hydrolyze corn starch according to Farid [23] with some modifications. Different concentrations of corn starch (0.25-1.5 g) were dispersed with stirring into 10 ml tap water. This was followed by the addition of 5 IU of fungal amylase preparation per mg solids and the temperature was maintained at 40^{o}C for precooking, in a water bath, for 30 min. The mash was then cooked under pressure in an autoclave at 120^{o}C and 15psi for 30min. After gelatinization, The content of the flask was allowed to cool and another dose of amylase (5 IU/mg solids) was added while the temperature was maintained at 40^{o}C for another 30min for starch liquefication. The liberated reducing sugar was determined by Somogyi [21] after 30, 60, 120min and 24h.

Conversion efficiency was calculated as follows:

Reducing sugars produced by hydrolysis/Reducing sugars obtainable from starch in corn x 100.

RESULTS AND DISCUSSION

Substrates for α-amylase Production by SSF and SMF

In recent years, researches on the selection of suitable substrate for SSF have mainly centered around more efficient utilization of different agro-industrial residues, including spent brewing grain, sugarcane

bagasse, wheat bran, wheat straw, sunflower meal, rice husk, cottoned seed meal, soy bean meal, rye straw and corncob leaf and oil cakes, for amylases production [8,24].

The selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation [25].

All the substrates used in the study as shown in Figure 1 supported the growth and enzyme production by the isolate, The order of substrate suitability was potato shells>wheat bran> rice straw> wheat straw> corn cobs. Potato shells proved to be the best solid substrate with the highest titer of amylase activity by both SSF and SMF techniques 39.05 and 33.34 U/g solid substrate, respectively. Nothing that the production by SSF was higher than that by SMF and this coincides with that reported by Dharani and Kumaran [26] for amylase production using SSF and SMF techniques by *Aspergillus niger*. The amylase production in our study by SSF by *Alternaria alternata* on potato shells after 10 days (39.05 U/g solid substrate) was higher than that produced by *Aspergillus flavus* (3.128 U/g solid substrate) on sugarcane bagasse and by *A. niger* (0.933 U/g solid substrate) isolated from Municipal compost soil on rice bran [27,28].

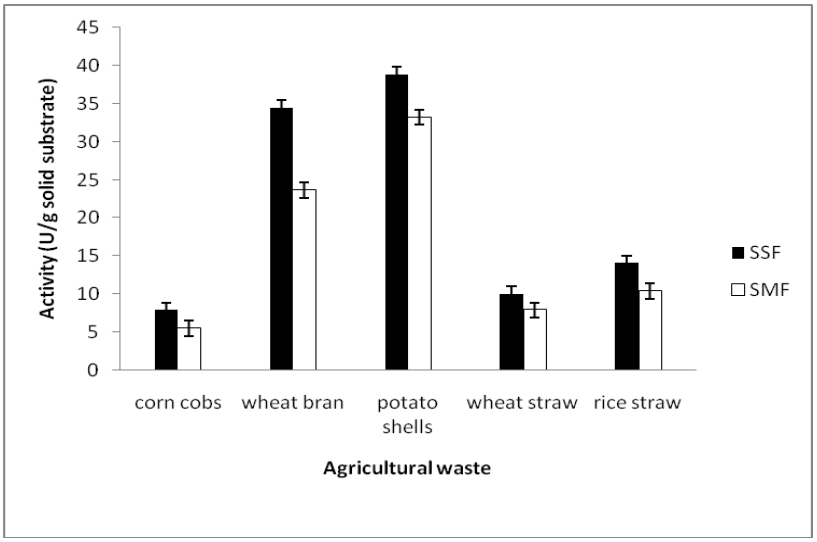


Figure 1: α-amylase production by *A. alternata* under solid state fermentation (SSF) and submerged fermentation (SMF) with different agricultural wastes.

Also, wheat bran was found to be suitable for amylase production (35.16 U/ g solid substrate) and for necessary manipulation [25, 29, 30, 1] following potato shells. Widespread suitability of wheat bran may be due to the presence of sufficient nutrients and its ability to remain loose even in moist conditions thus providing a large surface area [31].

Effect of Incubation Time

In SSF and SMF, The change of α-amylase production with incubation time, in which medium contained (PS) and sea water only, is shown in Figure 2. The enzyme production increased gradually by increasing incubation period until reached its maximum (46.47 U/g solid substrate) with SSF, and (41.19 U/ g solid substrate) with SMF on day 14. Further increase in the incubation period led to a reduction in amylase production. This might be due to the depletion of nutrients in the medium.

ISSN: 0975-8585

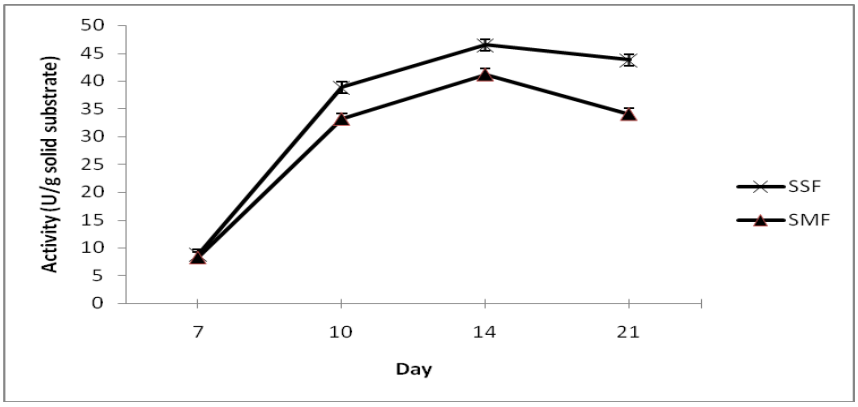


Figure 2: Time course of α-amylase production by *A. alternata* under solid stat fermentation (SSF) and submerged fermentation (SMF) with potato shellss (PS).

The results showed that the fungus *A. alternata* utilized the (PS) effectively with SSF, producing α-amylase in amounts higher than that of SMF. These results are in agreement to that reported by Lekha and Lonsane [32], where they found that SSF can give higher yields of certain enzymes, such as amylases compared to submerged fermentation (SMF).

Most raw starch digesting enzymes reported to date hardly digest potato starch because of the larger size of these granules. On the other hand, next to corn, potato is the most important source of starch. Therefore, enzymes that are capable of digesting raw potato starch are economically attractive for they can increase the range of starch sources for direct hydrolysis [33].

Effect of Extraction Leaching Agent

Extraction with different leaching agents considered an important factor affecting the enzyme activity, therefore, distilled water, acetate buffer(0.05 M) and non-ionic surfactant (Tween-80 1%) were used for separation and recovery of enzyme from SSF of potato shellss. The results in Figure 3 showed that, acetate buffer (0.05 M; pH 5) gave the best result and was found to be efficient in recovering α-amylase (56.75 U/g) from fermented PS (about 1.22 -fold as compared to the extraction with water and about 1.69 -fold as compared to Tween-80).

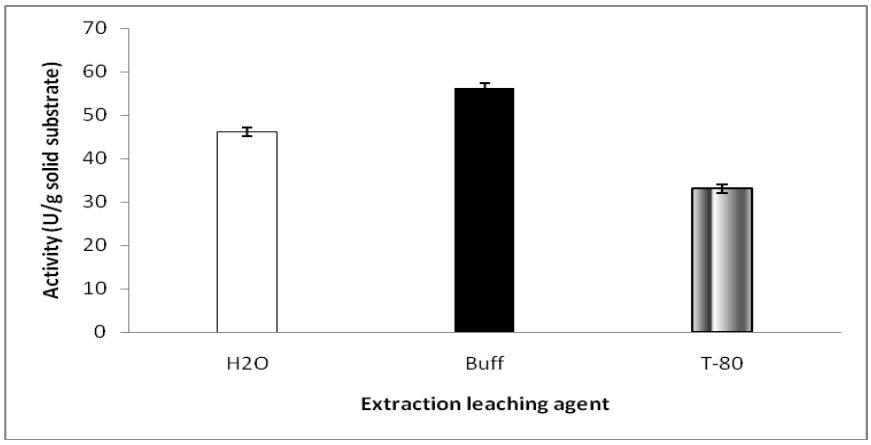


Figure 3: Effect of different leaching agents on α-amylase activity.

Our results are in agreement with the extraction of some enzymes produced under SSF [34,22]. Extraction increased with buffer as compared to distilled water probably due to the salting-in effect of the salt.

Effect of Salinity on Amylase Production

Salinity was a significant parameter influencing the growth and enzyme production by this marine-derived fungal isolate. The effect of salinity concentrations on α-amylase production were determined in SSF

of (PS) prepared with different sea water proportions. After incubation period of 14 days at 28-30ºC, the results showed that maximum enzyme production (87.12 U/g) was determined at 75% sea water. Whereas, the medium prepared with distilled water without any addition of sea water inhibited the enzyme production (27.16 U/g) Figure 4.

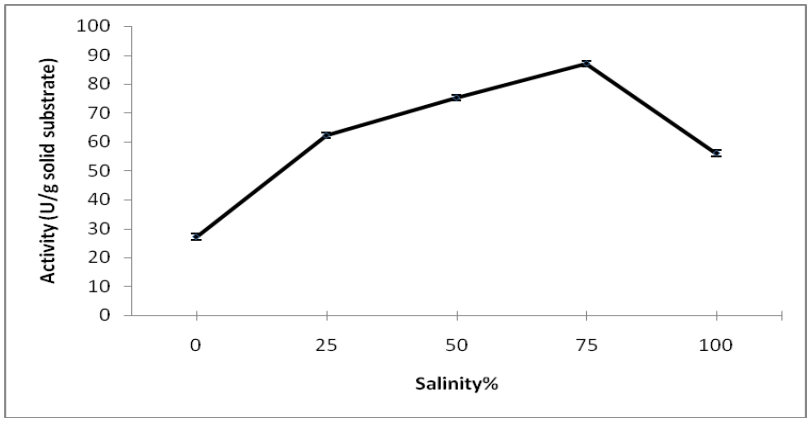


Figure 4: Influence of salinity on α-amylase production.

It appears that the source of isolation plays an important role in obtaining isolates with selected traits. Geofungi growing in marine environments appear to be conditioned to their environment [35]. Therefore, *A. alternata* isolated from decayed wood pieces of old fishing boats from Ismalia, appears to be a good candidate for many applications in saline conditions.

Effect of Substrate Pretreatment

The barrier to the production and recovery of valuable materials from lignocellulosic wastes is the structure of lignocelluloses which has evolved to resist degradation due to crosslinking between the polysaccharides (cellulose and hemicelluloses) and the lignin via ester and ether linkages [36]. The main goal of any pretreatment, therefore, is to alter or remove structural and compositional impediments to hydrolysis and subsequent degradation processes in order to enhance digestibility, improve the rate of enzyme hydrolysis and increase yields of intended products [36].

The pre-treatment of the solid substrate (PS) before the fungal fermentation considered an important factor affecting the enzyme production. The pretreatments which are good for some materials are not good for others [37]. The results illustrated in Figure 5 indicated that the fungus produced the enzyme on all pre-treated (PS) tested but with different degrees. As shown in Figure 5 The amylase production was decreased on H_2SO_4 pretreated potato shells (66.63 U/g solid substrate) and this may be due to that this pretreatment may be not effective in dissolving lignin [38]. While the NaOH pre-treatment caused 1.37 -fold increase in amylase production in comparison to untreated PS due to removal of lignin and part of the hemicelluloses, and efficiently increase the accessibility of enzyme to starch and this concides with that reported by Silverstein [39] and Zhao [40].

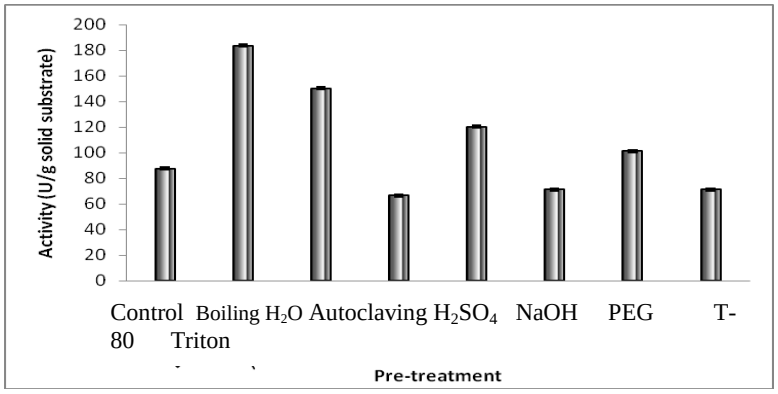


Figure 5: Effect of pre-treatment of (PS) on α-amylase production.

The optimum enzyme production (183.57 U/g) was achieved when using the (PS) pre-treated by boiling in water for 60 min, which increased about 2.1 -fold compared to the control (without any treatment). This may be due to that water under high pressure can penetrate into the biomass, hydrate cellulose, and remove hemicelluloses and part of lignin. The major advantages are no addition of chemicals and produces lower amounts of neutralization residues compared to many processes [38].

It was also found that the pre-treatment of (PS) by autoclaving and and 1% Tween-80 were favorable for α-amylase production (120.12 and 101.62 U/g solid substrate, respectively). On the other hand the pre-treatment of (PS) by 1% PEG and triton have the same effect on enzyme production where the enzyme levels reduced to 71.23 and 71.14 U/g solid substrate, respectively.

Repeated Extraction Cycles

Repeated extraction cycles for the enzyme from the optimum solid state fermentation of (PS) was studied to determine whether most of the α-amylase could be recovered in one extraction. Six consecutive extractions were performed and results are presented in Table 1. About 34.72% (183.57 U/g) of total activity was achieved from the first extraction. Considering that 100% of the enzyme could be extracted with only six extractions, 79.20% (418.82 U/g) of α-amylase was recovered from fermented PS during the first three extractions. The demand for the repeated extraction may be due to the adsorption of the α-amylase to cells or solid waste by ionic bond, hydrogen bond and Van der Waal's forces [41].

Table 1: Repeated extraction of α-amylase enzyme from PS

Number of recovery stage	α-amylase activity (U/g)	α-amylase activity (U/g)%	Cumulative enzyme activity (U/g)
First washing	183.57	34.71	183.57
Second washing	140.25	26.52	323.82
Third washing	95	17.96	418.82
Fourth washing	58.75	11.11	477.57
Fifth washing	35.89	6.79	513.46
Sixth washing	15.33	2.90	528.79

Characterization of Amylase Preparations

The stability of the crude amylase depended on the temperature and the duration of the pretreatment (data not shown). The enzyme began to lose part of its activity (14.89%) after pretreatment at 40^{o}C for 60 min. The loss in activity increased as the pretreatment duration and temperature increased. i.e. amylase lost more than 50% of its activity after pretreatment at 50^{o}C for 30 min (51.02%). This coincides with α-amylase from *Aspergillus sp.* JGI 12 which showed the least stability at 60^{o}C [42]. The high temperature inactivation may be due to hydrolysis of peptide chain, destruction of amino acid, or aggregation [43]. The calculated values of the half-life for the crude amylase at 50, 55 and 60^{o}C were 189.86, 99.82 and 93.77 min, respectively.

Influence of Temperature and pH on Enzyme Activity

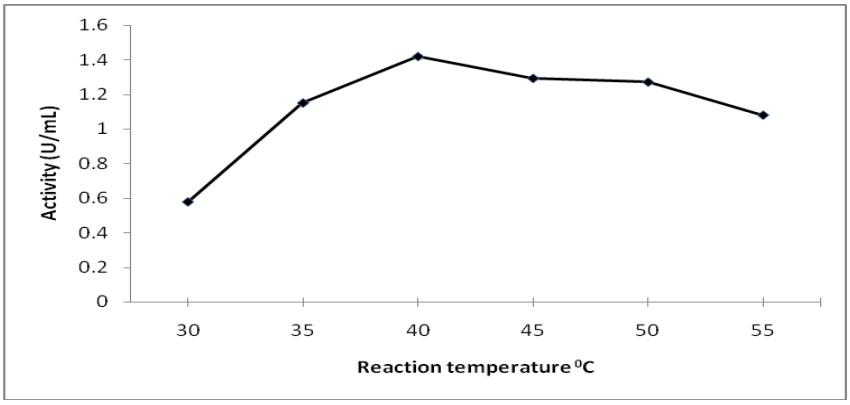


Figure 6: Effect of reaction temperature on α-amylase activity

ISSN: 0975-8585

Temperature and pH are the most important factors, which markedly influence the enzyme activity. Maximum amylase activity (1.419 U/ml) was recorded at 40°C. Further increase in temperature resulted in decrease in the activity of amylase (Figure 6). While amylases from *Aspergillus sp*. JGI 21 and *A. niger* recorded the maximum activity at 30°C and 70°C, respectively [42,28]. The calculated value of the activation energy was 10.40 K cal/mol.

The effect of pH on the enzyme activity indicated that the amylase was active in the pH range 4-9 with two peaks, one acidic pH 5.5 (2.767 U/ml) and one basic pH 8 (2.445) (Figure 7). This coincides with that found by Alva et al [42] and Kumari et al [28]. This suggests that the enzyme would be useful in processes that require wide range of pH change from slightly acidic to alkaline range. The multiple pH optima observed suggests the presence of at least two amylolytic activities in the crude amylase preparation; an α-amylase and a glucoamylase [7].

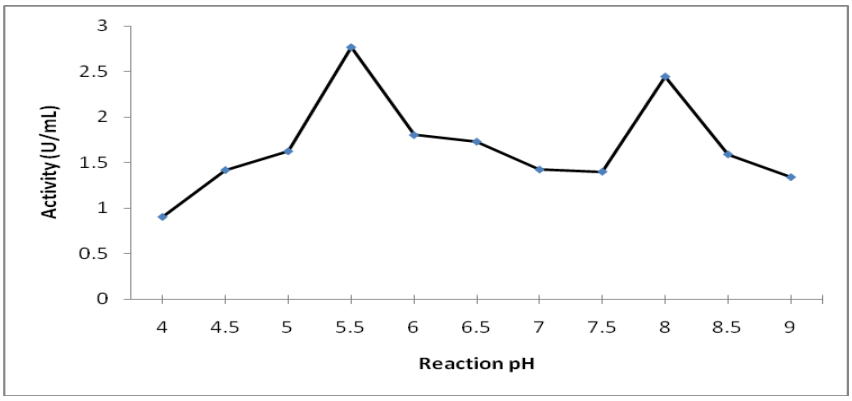


Figure 7: Effect of reaction pH on α-amylase activity

Influence of Substrate Concentration

Evaluation of enzyme activity with different concentrations of soluble starch revealed that a maximum activity 2.767 U/ml was obtained with 7.5 mg/ml starch (0.75%) as the substrate (Figure 8). Increasing the substrate concentration above the aforementioned starch concentration exhibited no further increase in enzyme activity. While amylases from *Aspergillus sp*. JGI 21 and *A. flavus* recorded the maximum activity with 1% starch as substrate [42, 27].

The calculated K_m and V_{max} for α-amylase were 1.90 mg/ml and 0.33 U/mg protein, respectively.

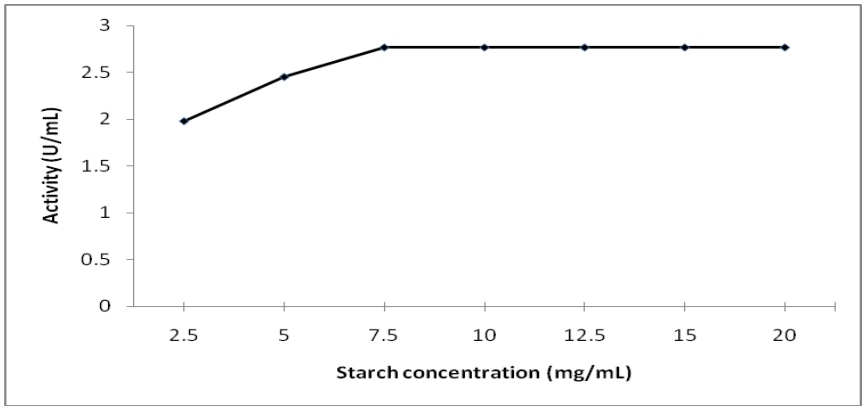


Figure 8: Effect of substrate (starch) concentration in reaction mixture on α-amylase activity

Influence of Some Metal Ions

Most of amylases are known to be metal ion-dependent enzymes. Study of the different metal ions that influence amylase activity as shown in Table 2, indicated that the enzyme was activated by Na^+ ion and

this may be due to that the enzyme preparation was from marine-derived fungus. While the use of other metals caused amylase inhibition with different degree. i.e. the use of Ca^{2+} inhibited the amylase activity by 35.80% and this coincides with that found by Reyed [43]. The inhibitory effect of Hg^{2+} has also been reported on amyalse produced by *A.awamori* and *A.flavus* [44, 27].

Table 2: Effect of some metal ions on α-amylase activity

Metal ion	Relative activity%
None	100
Ca^{2+}	64.2
Ba^{2+}	49.84
Na^{+}	151.49
Mg^{2+}	2.57
Mn^{2+}	27
Zn^{2+}	0
Hg^{2+}	0
NH^{+}	19.75
K^{+}	95.16
Fe^{2+}	0
Cu^{2+}	0

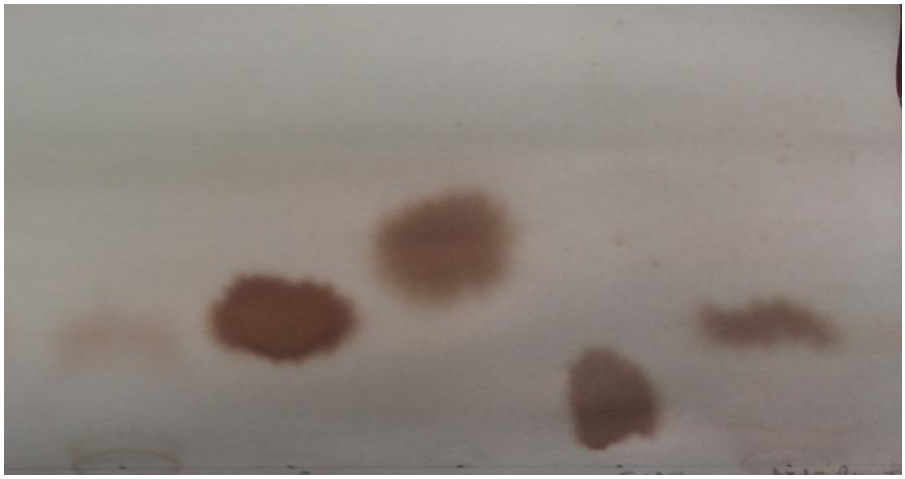
As shown in Table 3 the highest stimulatory effect of Na^{+} was obtained with 0.1 M concentration causing 2 -fold increase in amylase activity.

Table 3: Effect of different concentrations of Na^{+} on α-amylase activity

Metal ion concentration (M)	Relative activity%
None	100
0.025	151.49
0.05	185.75
0.1	200
0.15	187
0.2	175.55

Application of amylase preparation from *Alternaria alternata*

In the hydrolysis of corn starch



Product of fungal hydrolysis **glucose** **fructose** **sucrose** **product of acidic hydrolysis**

Figure 9: Product of fungal and acidic hydrolysis of corn starch

The obtained amylase from *Alternaria alternata* was evaluated for its ability to hydrolyze corn starch. The highest conversion efficiency of about 85% was obtained after 24 h (data not shown). The main product of hydrolysis by fungal preparation was glucose as shown in Figure 9 similar to the chemical hydrolysis by sulphuric acid. The ability of amylase preparation from *Alternaria alternata* to bring about significant hydrolysis of corn starch with an overall conversion efficiency of about 85% after 24 h suggests that this enzyme can be employed for the hydrolysis of corn starch.

CONCLUSIONS

Results of this study indicated that the marine *Alternaria alternata* isolated from decayed wood pieces of old fishing boats from Ismalia was a good producer for amylase using potato shell as solid substrate. Optimization of the fermentation parameters resulted in 5.5 -folds increase in the enzyme yield. The enzyme was found to be active over a wide range of pH.The ability of the marine-derived *A. alternata* to produce α-amylase with high activity in a wide range of salt concentrations suggest that the enzyme may be useful in industrial process containing high salt concentrations.The ability of amylase preparation from *Alternaria alternata* to hydrolyze corn starch with an overall conversion efficiency of about 85% after 24h suggests that this enzyme can be used in combination with *Saccharomyces cerevisiae* for production of ethanol.

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