Research Article

Purification and Characterization of Extracellular, Polyextremophilic α-amylase Obtained from Halophilic *Engyodontium album*

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Background: α-Amylases (EC 3.2.1.1) are covering approximately 25% of total enzyme market and are frequently used in food, pharmaceutical and detergent industries.
Objectives: The first ever detailed characterization of amylase from any halophilic *Engyodontium album* is presented.
Materials and Methods: An extracellular α-amylase was studied from halophilic *E. album* TISTR 3645. The enzyme was extracted and purified by column chromatography. SDS-PAGE was performed to find the molecular weight of the enzyme. The effects of pH, temperature and salinity on the isolated enzyme were determined. The effects of various additives on enzyme were studied.
Results: The molecular weight of the amylase was 50 kDa. The enzyme specific activity was 132.17 $U.mg^{-1}$ with V_{max} and K_m values of 15.36 $U.mg^{-1}$ and 6.28 $mg.ml^{-1}$, respectively. The optimum enzyme activities were found at pH 9.0, 60ºC and 30% (w/v) NaCl. $BaCl_2$, $CaCl_2$, $HgCl_2$ and $MgCl_2$ improved amylase activity. β-mercaptoethanol, EDTA, $FeCl_2$ and $ZnCl_2$ decreased enzyme activity.
Conclusions: Polyextremophilic characteristics of α-amylase from halophilic *E. album* TISTR 3645 were revealed during the characterization studies, demonstrating promising features, making it a useful candidate for various industries.

Keywords: α-amylase; *Engyodontium album*; Extremozyme; Halophilic fungi

1. Background

Fungi capable of growing in salinities above 1.7 M *in vivo* and 3 M of salt *in vitro* are classified as halophilic fungi (1, 2).

Halophiles and their metabolites have tremendous applications such as bioactive compounds, biosurfactants, biocompatible solutes and food additives (3-5). Moreover, halophiles produce potential hydrolytic enzymes that are capable of working in many industrial processes (6). Currently, halophilic bacteria and their metabolites monopolized the related industries (7). On the contrary, little data has been augmented for halophilic fungi (2), despite the fact that more often fungal species are better sources of metabolites and halophilic enzymes (8). α-Amylases are enzymes used in varieties of industries such as food, feed, pharmaceuticals and detergents, taking up 25% of the enzyme market (9-11).

2. Objectives

Here, a native halophilic fungus, *E. album* TISTR was isolated and characterized both morphologically and via molecular analysis. The fungal α-amylase properties were analyzed following enzyme purification. To the best of our knowledge this is the first report of amylase from *E. album*.

3. Materials and Methods

3.1. Fungal Isolation

E. album TISTR 3645 was isolated from extreme hypersaline conditions at man-made solar saltern,

located in Ban Laem district of Phetchaburi province, Thailand (12). In growth characterization studies the fungus was found to be a halophile (13). The strain was later deposited to Thailand Institute of Scientific and Technological Research (TISTR) culture collection.

3.2. Enzyme Screening

The presence of α-amylase from *E. album* TISTR 3645 was performed by plate screening method according to Ali *et al*. (2). *E. album* was grown on PDA plates, supplemented with 1% (w/v) starch.

3.3. Growth Conditions for Enzyme PSroduction

The media for amylase production was made according to Hernandez *et al*. (14), containing 8.0 $g.1^{l-}$ $CaCO_3$, 0.15 $g.1^{l-}$ $FeSO_4.7H_2O$, 3.5 $g.1^{l-}$ KH_2PO_4, 0.10 $g.1^{l-}$ $MgSO_4.7H_2O$, 3.0 $g.1^{l-}$ mycological peptone, 6.6 $g.1^{l-}$ $(NH_4)_2SO_4$ and 10 $g.1^{l-}$ starch .

3.4. Amylase Purification

Amylase was isolated according to Chakraborty *et al*. and Hmidet *et al*. (15, 16). The culture broth was centrifuged for 10 min at 13,000 ×*g* in 4ºC. Enzyme was precipitated by 90% saturated $(NH_4)_2SO_4$. The mixture was left overnight at 4ºC. The precipitate was centrifuged for 30 min at 12,000 ×*g* and dissolved in 100 mM Tris-HCl buffer (pH 8.0). The enzyme was dialyzed against same buffer for 48 h. For further purification Sephadex G-100 column (2.6 cm-150 cm) pre-equilibrated with 25 mM Tris-HCl buffer (pH 8.0, containing 0.5% v/v Triton X-100). Enzyme fractions of 5 ml were eluted with the same buffer at a flow rate of 30 $ml.h^{-1}$.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Hmidet *et al*. (2008) to determine molecular weight and enzyme purity in a 15% resolving gel. For the determination of molecular weight the molecular marker kit (Bio-Rad) was used as reference.

3.5. Amylase Assay

Dinitrosalicylic acid (DNS) method for the measurement of reducing sugar was used for the determination of amylase activity (17). Amylase (0.1 ml) was added to 0.5 ml of 0.1 M phosphate buffer supplemented with 1% (w/v) soluble starch and incubated for 10 min at 40ºC. The reaction was stopped by the addition of 3 ml of 3, 5-dinitrosalicylic acid and heated for 5 min in boiling water. The reaction mixture brought up to 10.6 ml using dH_2O and the absorbance was recorded at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that produced 1 µmol of glucose in 1 min.

3.6. Protein Estimation

The amount of protein was estimated by the Lowry method (18). Bovine serum albumin (BSA) was used as the standard.

3.7. Characterization of Amylase

The enzyme was characterized by studying the effects of pH, temperature and salinity. The results are explained by percentage relative activity. The effect of pH was determined by incubating the sample mixture in pH 5-12 at constant temperature. For acidic range of pH, 0.1 M acetate buffer was used, while for alkaline and neutral ranges of pH, 0.1 M phosphate buffer was used. The effect of temperature was determined by incubating the sample mixtures in 30-90ºC at constant pH. The effect of salinity was estimated by supplementing the sample mixture with 0-40% NaCl concentration (w/v), at constant pH and temperature.

The effect of various additives on enzyme activity was determined by using 2 mM $HgCl_2$, $BaCl_2$, $ZnCl_2$, $FeCl_2$, $MgCl_2$, $CaCl_2$, ethylene diamine tetra acetic acid (EDTA) and β-mercaptoethanol.

3.8. Determination of Kinetic Parameters

The Kinetic studies were performed by incubating the amylase with different concentrations of soluble starch. Optimum conditions were used in kinetic studies. The values of V_{max} and K_m were obtained from the Lineweaver-Burk plot.

4. Results

4.1. Purification of Amylase

The *E. album* was found to have amylase by plate screening method. The crude enzyme was extracted from the media and applied to column chromatography. The fractions showing the highest amylase activity was selected for the determination of molecular weight and purity of enzyme. The enzyme was found to have a single band with relative molecular mass of 50 kDa (Figure 1). The specific activity of amylase was 132.17 $U.mg^{-1}$.

4.2. Characterization of Amylase

The percentage relative activity of enzyme in different pH showed that enzyme was able to work better in neutral and alkaline pH ranges (Figure 2). A steady

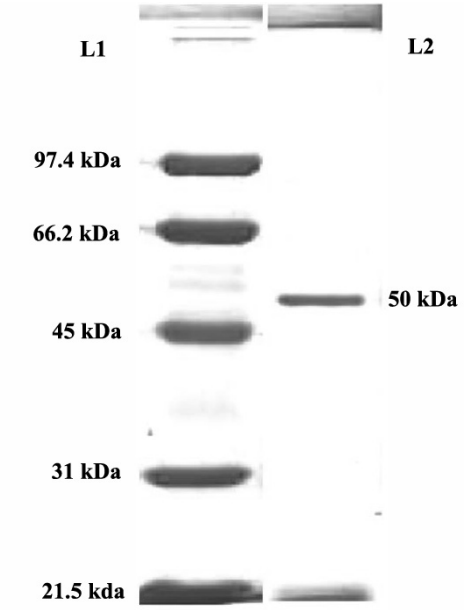

Figure 1. SDS-PAGE analysis of the purified α-amylase from *E. album* TISTR 3645. L1: represents lane 1, which is the molecular mass ladder. L2: represents lane 2, which is the purified α-amylase lane, showing single band at approximately 50 kDa

increase in enzyme activity was observed from pH 5-9. The highest enzyme activity was observed at pH 9.0. A sharp decline in enzyme activity was noted at pH values greater than 9.0.

Isolated amylase worked better in moderate to high temperature values (Figure 3). A sharp incline in enzyme activity was observed from 40-60ºC. The enzyme optimum temperature was 60ºC and in temperatures above the activity declined. The enzyme was able to retain almost 60-85% of its relative activity at higher temperatures of 80 and 90ºC, where most of enzymes become denatured.

Increase in salinity by 0-30% (w/v) of NaCl increased the activity of the enzyme (Figure 4). The highest enzyme activity was found at 30% NaCl. Although the enzyme activity was decreased in greater salt concentrations, it still kept about 85% of its relative activity at 40% of salt concentration, which is over the saturation point of salt solution.

$BaCl_2$, $CaCl_2$, $HgCl_2$ and $MgCl_2$ increased the amylase activity, but not greater than 110% (Table 1). In contrast, β-mercaptoethanol, EDTA, $FeCl_2$ and $ZnCl_2$ decreased the enzyme activity. The greatest inhibition occurred in the presence of $ZnCl_2$. The decrease in enzyme activity was never less than 60% by the addition of any inhibitor.

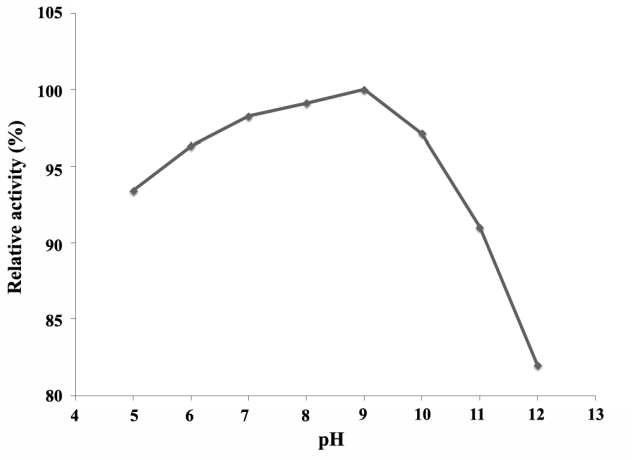

Figure 2. Effect of pH on the purified α-amylase activity from *E. album* TISTR 3645 at constant temperature. The results are presented in percentage relative activity

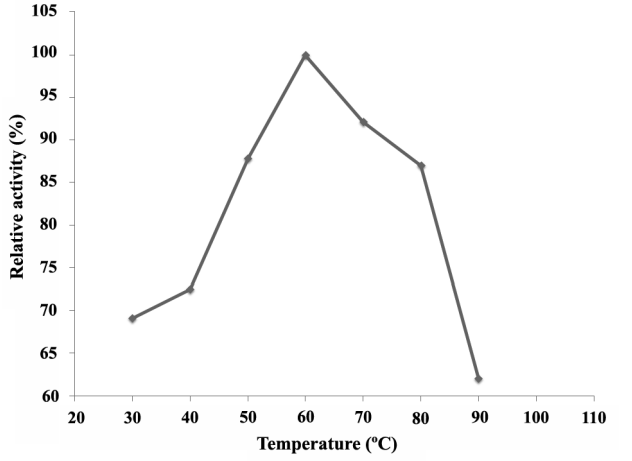

Figure 3. Effect of temperature on the purified α-amylase activity from *E. album* TISTR 3645 at constant pH. The results are presented in percentage relative activity

5. Discussion

Researchers are constantly working on finding suitable fungal strains for amylase production (19). Amylases from fungi have mostly been reported from mesophilic fungi (20), especially from *Aspergillus* and *Penicillium* genus (21). Even though, the enzymes from fungi are preferred to be used in industries (8), the enzymes from halophilic fungi have been neglected as compared to the bacterial counterparts (2). Except few preliminary screenings no reports of purification and detailed characterization of α-amy-

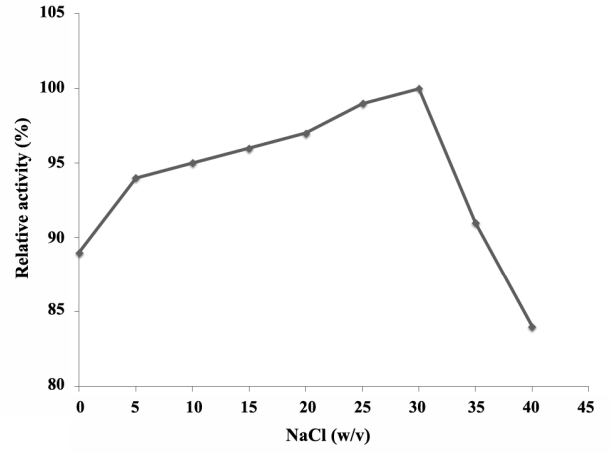

Figure 4. Effect of NaCl concentrations (% w/v) on the purified α-amylase activity from *E. album* TISTR 3645 at constant pH and temperature. The results are presented in percentage relative activity

Table 1. Effect of various additives on the purified α-amylase from *E. album* TISTR 3645. The results are presented in percentage relative activity

Additives (2 mM)	Relative activity (%)
None	100
$BaCl_2$	100.23 ± 1.5
$CaCl_2$	110.48 ± 0.4
$FeCl_2$	63.11 ± 0.01
$HgCl_2$	103.04 ± 1.5
$MgCl_2$	101.02 ± 1.5
$ZnCl_2$	71.34 ± 1.5
β-mercaptoethanol	76.48 ± 0.2
EDTA	76.15 ± 0.5

lases from halophilic fungi exists. *E. album* has not been much reported for its potential of extracellular enzymes except few reports of proteases (22, 23).

Presence of single band of approximately 50 kDa was found during the purification of amylase (Figure 1), which shows that there is no need for any further purification of enzyme. The specific activity of the enzyme is found in a good range (15). The value of K_m has been found to fall in the most reported ranges of 0.35-11.66 mg.ml^{-1} for amylases (24).

The pH characterization study shows the typical behavior of halophilic fungi adaptation to its habitat. The hypersaline habitats are mostly found to have neutral to alkaline range of pH (4). In our earlier report (12), we found that most of the fungi in growth characterization were able to grow best from neutral to alkaline pH as well. The pH values of 9 and 10 are mostly considered enough to designate the enzyme as alkalophilic (25, 26). Alkalophilic amylases are mostly applied in the detergent industries (9).

Most of the enzymes are unable to perform the catalytic activity at high temperature values of 50-60ºC (27). The α-amylase in this study has been found to have optimum activity at 60ºC and retain more than 85% of its activity at high temperatures of 70-80ºC, which are considered as thermophilic range for enzymes (28, 29). This character shows that, it has the capability to withstand high temperature processes that are mostly carried out in starch industries (27).

The *E. album* in the previous study has been found to withstand the salinity ranges of 0-20% of NaCl (w/v) (13). This extreme characteristic requires modification in the working capabilities of the metabolites in the halophiles (1). The catalytic ability of α-amylase in this study at high salt concentrations makes this enzyme novel. This capability is much greater than the previously reported bacterial and Archeal amylases (6, 9, 30). Ability of this amylase to work at extreme salt concentrations makes them the best available candidate to work for bioremediation processes, particularly, saline waste management (31, 32). Similarly the limiting water resources and availability of hard water for domestic uses in current climate change, requires the additive amylases for detergents to work better in low water activity for minimizing the consumption of water, as well as to make more cleaning effects in the presence of high salt concentrations (33). Halophilic enzymes are also reported to work better than normal enzymes in biofuel productions (34).

The increase of activity due to the addition of almost half of the additives makes this amylase to work better in the presence of several additives. The inhibition of amylase from few additives has not been found more than 40% which shows the resistance of inhibition from the studied amylase.

Halophilic microbes are frequently been reported to provide polyextremophilic amylases (6, 8). Mostly they have found to be haloalkalophilic having thermotolerant characteristic (35). Polyextremophilic enzymes are better candidates than monoextremophilic enzymes since most industrial processes require enzymes to withstand multiple harsh conditions (36), such as: pH, temperature and salinity.

6. Conclusions

α-amylase from *E. album* TISTR 3645 found to be more extremophilic in properties than the fungus itself. This finding may suggest that other primary and secondary metabolites from halophilic fungi demonstrate polyextremophilic behavior. Accordingly, it would be fair to state that looking into halophilic fungi may promise greater advantages over using bacterial counterparts in related industries. The polyextremophilic behavior shown by α-amylase obtained from *E. album*, makes this enzyme a suitable choice to be used in extreme conditions of industries; particularly as additive in detergents as well as for the treatment of extreme saline waste water.

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Authors' contributions

IA conducted the research, AA helped in enzyme purification, MA provided consultation, BY helped in characterization studies, SP and PL provided consultations and lab facilities and HP supervised the research.

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