INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Isolation and Characterization of Thermo-stable Fungal Alpha amylase from Geothermal Sites of Afar, Ethiopia

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ABSTRACT

The hot springs and soils considered in this study lie within the Ethiopian rift system. The temperature of the water were 48.33°C at Afdera ,44.36 °C at Awash National Park, while that of soil was 47.33 °C on the way from Afdera to Semera and 36.46°C at Awash National Park. There is a significant difference in the temperatures of all the soils as well as the waters ($p \leq 0.05$). ThepH in all case werein the alkaline region. Thermophilic fungi, genus Aspergillum (STG3 and STG6)isolated fromAfar hyperthermal spring produced a thermostable α-amylase. Enzyme production from STG3 and STG6 fungi at 55°C after 7 day incubation was 2.73599±0.00818Uand 2.51618±0.00818U, respectively. The optimum temperature for enzyme activity was 45-55°C for STG3 and 55-65°C for STG6.At 65°C and 40 minute incubation STG3 and STG6 retained 15.8% and 16.82% of their activity respectivelyand are stable at pH values of 4.0-10.0. Enzyme activity was strongly inhibited by Ca^{2+},Mg^{2+} and Zn^{2+}.

Key words: Hot springs of Afar, ThermophilicFungi, Thermophilicalpha amylase, enzyme activity, temperature and PH.

1. INTRODUCTION

Thermophilic fungi are a small group in eukaryota that have a unique mechanism of growing at elevated temperature extending up to 60 to 62°C. During the last four decades many species of thermophilic fungi sporulating at 45°C have been reported as cited in Abdullah and Zora, (1993). Thermophilic fungi are those that have a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C.

The occurrence of thermophilic fungi in aquatic sediment of lakes and rivers as first reported by Tubaki*et al.* (1974), is mysterious in view of the low temperature (6-7°C) and low level of oxygen (average 10 ppm, <1.0 ppm at a depth of 31meters) available at the bottom of the lake. A number of thermophilic fungi survive the stresses such as increased water pressure, absence of oxygen and desiccation (Mahajan*et al*., 1986).

Fungi are an important component of the soil micro biota typically constituting more of the soil biomass than bacteria depending on soil depth and nutrient conditions (Ainsworth &Bisby, 1995). Many important plant pathogens (i.e. smuts and rusts) and plant growth promoting microorganisms (i.e., ecto- and endo-mycorrhizae) are fungi. The saprobic fungi represent the largest proportion of fungal species in

soil and they perform a crucial role in the decomposition of plant structural polymers, such as, cellulose, hemicelluloses and lignin, thus contributing to the maintenance of the global carbon cycle. This property, coupled with their ability to produce commercially interesting organic molecules and enzymes explains the significant interest in the biotechnological utilization of filamentous fungi. The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem. They perform ecological services that strongly impact the quality of human life and have enormous potential for providing economic benefits, e.g., the isolation and identification of the soil fungus *Penicillium*led to a large pharmaceutical industry of antibiotics (Diana, 1994). It is estimated that there are 1.5 million fungal species on earth, of which only about 70,000 have been described up to now (Hawksworth&Rossman, 1997).

Amylases are a group of starch degrading enzymes with greatest significance in the biotechnology industries and have huge application in food, fermentation, textile and paper production (Pandey*et al*., 2000). The various sources of amylase are plants, animals and microbes (Kathiresan and Manivannan, 2006). This study is mainly to produce alpha amylase from thermophilic fungi for starch industries.

2. MATERIALS AND METHODS

2.1. Sample Collection

Soil and water samples were collected from hot springs and dry areas of Afar; (Afdera water and soil, soil sample from every 60 km of Afdera to Semera and soil and water of Awash national park) after measuring the pH and temperature during December 2012 and the following sterilized materials were used to collect the samples. A steel pipe with a diameter of 50 mm was used to take soil samples. The pipe was pressed 100 mm down into the soil to take the sample. The sample was transferred to sterilized plastic container with a screw care. The pipe was cleaned thoroughly in 70% alcohol after each sampling. At each place five soil samples were taken randomly lie over an area of $1m^2$ to get sample rich inmicroorganism. The samples were mixed by shaking the plastic container vigorously. The distance between Afdera and Semera is 180 km and to get representative sample every 60 km sample was taken three times and mixed. Two meter long plastic pipe was used to take water samples from streams and hot springs by pressing 10 cm into the bottom. Five water samples (500 ml) were taken from each hot springs distributed randomly over an area of $1m^2$. The samples were transported to the laboratory in ice box (Gulelat. and Rakshit 2003).

2.2. Isolation of thermophilic amylase producing Fungi

The water and soil samples were pretreated, diluted, isolated and purified (Figure 1). The different mould cultures were isolated from the samples by serial dilution (Clark *et al*., 1958). One gram of soil sample and 1 ml of the water sample was dissolved in 9 ml of sterilized distilled water in different test tubes. The suspensions were diluted up to 10^{-4} times and 0.1 ml of the diluted suspensions was then transferred to Petri plates containing Potato Dextrose agar medium. The Petri plates were incubated at 30°C for 3-4 days. The young colonies were aseptically picked up and transferred to potato dextrose agar slants. The slants were then incubated at 30 °C for 3-5 days for maximum growth and then stored at 4°C for further study.

2.3. Screening of thermophilic amylase producing Fungi

The isolated fungi were primarily screened for α-amylase synthesis after incubation at 55°C for three days. The plates were flooded with a solution of 0.5% (w/v) I_2 and 5.0% (w/v) KI (Thippeswamy*et al*., 2006). The clear zone surrounding the colony was measured in (mm) from the edge of the colony to the limit of clearing. Colonies exhibiting large halo starch hydrolysis were picked up.

2.4. Enzyme production from fungi

Amylase production by the stored culture of fungi was detected by growing the organism on solid media containing soluble starch and exposing the plates to iodine vapors or flooding them iodine-iodine solution (Modi*et al*., 1985) and those which show clear haloes in the media were selected as amylase producers. All the positive isolates were cultured in yeast broth and incubated for 7 days at 55°C. The whole culture was centrifuged at 4000 rpm in a high speed centrifuge for 1 hr and was filtered through what-man number 1 filter paper. The culture filtrate was used as the enzyme source.

2.5. Characterization of Alpha Amylase

2.5.1. Effect of temperature on activity and stability of alpha amylase

The enzyme activity was measured by incubating the partially purified enzyme at various temperatures (35-95°C) with starch as substrate prepared in 20 mM sodium phosphate buffer at pH 6.0 (Behal*etal*., 2006). In order to determine the temperature stability enzyme solutions in different tubes were incubated at various temperatures at 45, 55 and 65°C for 10 min (Carvalho*et al*., 2008).

2.5.2. Effect of pH on activity and stability of alpha amylase

Activity of enzyme at different pH values from 3-11 with starch as substrate were measured. The different buffers used were 20 mM acetate buffer (pH 3.0-5.0); 20 mMsodium phosphate buffer (pH 6.0-8.0) and 20 mMTris/HCl (pH 9.0-11.0) (Reyed, 2007).To determine the enzyme stability at various pH values (3-11), enzyme solution were incubated in 20 mM of each of the buffer at 55°C for 1 hour and then enzyme assay were performed (Carvalho*et al.*, 2008).

2.5.3. Effect of metal ions on the activity and stability of α-amylase

The activities of the produced α-amylase assay were performed under standard conditions in the presence of 1.0 mM, 5 mM and 10 mM of the following salts: $CaCl_2$, $MgCl_2$ and $ZnCl_2$. The activity assayed without metal ions were considered to be the reference value (100%).

2.5.4. Enzyme activity

Amylase activity was determined by measuring the release of reducing sugar from soluble starch. The reaction mixture contained 0.5 ml of enzyme and 4.5 ml of 0.1 M phosphate buffer (pH 6.0) were added to 2 ml of soluble starch (1%). After incubation at 30°C for 30 min in a shaking water bath, the reaction was stopped by the addition of 2 ml of 3-5-Dinitrosalicylic acid (Bernfeeld, 1955). The tubes were kept in boiling water for 15 min's to develop the color and then cooled to room temperature. The absorbance was read at 540 nm by Spectrophotometer after making up the volume to 10 ml. Maltose was used to construct a standard curve. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol maltose per min under the assay conditions.

3. RESULTS AND DISCUSSIONS

3.1. Screening of Thermo-stable α-amylase Producing Microorganisms

The temperature and pH of the hot spring from which the microorganism was isolated were 47.33°C; 8.67 and 36.8°C; 7.49, respectively. The thermophilic strains designated as STG3 and STG6were later identified as *Aspergillus* according to *Bergey'sManual*of*Systematic Bacteriology* (Sneath et al., 1986).The optimum growth temperature of the organism on starch agar plate was found to be 55°C.

3.2. Thermostable α-amylase producing fungi

Primary screening was carried out by starch hydrolysis method. Among the 680 isolates the 80 fungal isolates exhibited amylolytic activity in starch agar medium and six out of 80 which shows higher amylolytic activity was selected for further studies (Table 1). The three fungi namely STG5, STG6 and STG3 which form large halo diameter were selected for further studies.

3.3. Production of Alpha Amylase

Since temperature on amylase production is related to the growth of the organism, the optimum temperature for enzyme production depends on whether the culture is mesophilicor thermophilic (Ramachandra*et al.*, 2004; Francis *et al.*, 2003). High amount of enzyme was produced by STG3 and STG6 than that of STG5. The enzymes were produced at temperature of 55°C. In contrast to this study most amylase production studies have been done with mesophilic fungi within the temperature range of 25–37°C (Ramachandra*et al.*, 2004; Francis *et al.*, 2003). A raw starch degrading α-amylase was produced by *Aspergillusficuum*at 30 °C.

The enzyme amount was calculated from the maltose produced (Table 3). In other study which is similar to this work amylase production at optimum level has been reported between 50–55 °C for the thermophilic fungal cultures such as *Talaromycesemersonii*, *Thermomonosporafusca*and *Thermomyceslanuginosus*(Jensen and Olsen, 1992).

3.4. Effect of temperature on activity and stability of fungal alpha amylase

The optimum temperature of the enzyme was 45-55°C for STG3E and 55-65°C for STG6E. By taking the activity of the enzymes at the optimum temperature as 100%, the residual activity of the enzymes at 65°C were 50.91% for STG3E and 73.57% for STG6E.

Temperature is the most important factors which markedly influence enzyme activity. Maximum amylase activity was recorded at 55ºC 10 min incubation (1.202U/mg of STG3E and 1.052U/mg of STG6E). Further increase in temperature resulted in decrease in the activity of amylase (Figure 3).

The rates of thermal inactivation of the enzyme at pH 9.0 are shown in Figure 4. STG3 enzyme retained 18.71% of its activity while STG6 enzyme retained 15.68% of its activity for 40 min when heated to 55°C. At 65°C and 40 minute incubation STG3E and STG6E retained 15.8% and 16.82% of their activity respectively. The rate of thermal inactivation was faster at temperatures higher than their optimum and above 10 minutes incubation time which is similar trend with bacterial alpha amylase.

3.5. Effect of pH on activity and stability of fungal alpha amylase

The optimum pH for both of the enzymes (STG3E and STG6E) was 6.0. The amylase retained more

than 67% of its original activity between pH 4 and 10. The enzyme was stable over a wide pH range. The enzyme was not stable below pH 4 or above pH 10.0.

The relative activity of the fungal enzymes in different pH was above 50% and both of the enzymes (i.e. STG3E and STG6E) were more active at pH 9. The enzymes were active in basic conditions. Both of the enzymes retain more than 60% of their activity from pH 4 to 10 with the optimal at pH 9. Acidic conditions for activity of amylase have been reported by others (Mayzaud and Conover, 1976; Mayzaud and Mayzaud, 1981) pH 6.0 as optimal for mixed total zooplankton and copepods which is similar with this study. Boucher and Samain (1974) found pH 6.8 to be optimal for copepods and Mayzaud (1985) found pH 6.4 for Acartiaclausi as optimal. Van Wormhoudt et al. (1983), however, reported an alkaline condition, 7.2 pH, as the optimum for α-amylase of the Antarctic krill, Euphausiasuperba.

3.6. Effect of metal ions on the activity and stability of fungal α-amylase

In this study the metal ions (i.e. Ca^{2+}, Mg^{2+} and Zn^{2+}) were potential to reduce the activity of fungal enzymes (i.e. STG3E and STG6E) at all the indicated concentrations (1, 5 and 10Mm) above 27% as compared to the activity assayed without metal ions. As indicated in other studies after analyzing of fungi enzyme activity to be carried in the presence of different metal ions, it was found that enzyme activities were enhanced by calcium on the contrary to the results of Reyed (2007), where calcium slightly inhibited the amylase activity. Asgher*et al.* (2007) reported that Ca^{+} had no effect on enzyme activity.

4. CONCLUSIONS

The study areas (Afdera, Every sixty kilometer from Afdera to Semera and Awash national park) were potential source of thermo-stable alpha amylase producing microorganisms. The thermophilic microorganism STG3E and STG6E were appeared to have good potential for thermo stable amylase production and was screened, characterized. The optimum temperature of the enzymes was 45-55°C for STG3E and 55-65°C for STG6E. The enzyme also has good activity in the pH range of 4.0-10.0. The enzyme produced was active at wide temperature and PH range which makes it suitable to use it in starch processing industries.

5. ACKNOWLEDGEMENTS

Special thanks go to my father Mr.WeldayAtsbha, my mother Mrs. TlbetsBerhe for nursing, shaping and educating me properly, my sisters EdltiWelday, SelemawitWelday, Genet Welday, Sosna, Semhar, Betelihum and my brothers Dr. TesfahunWelday, Samson, Araya and Natnael for the financial help, over all support and care. Above all is the Almighty Lord God who provided me this opportunity with all the strength and makes my entire path straight.

Table 1 Average halo diameter formed by the selected fungi

Code	Halo diameter(mm)	Remark
STG1	0.45 ± 0.014^{d}	Fungi from AFW
STG2	0.325 ± 0.007^{e}	Fungi from ANS
STG3	0.715 ± 0.021^{a}	Fungi from AFS
STG4	0.465 ± 0.021^{d}	Fungi from ANW
STG5	0.615 ± 0.021^{b}	Fungi from AF60
STG6	0.545 ± 0.007^{c}	Fungi from AF60

Values are mean ±standard deviation. Values followed by different letters with in a column indicate significant difference ($P < 0.05$).
STG1: Solomon TeklebrhanGulelat fungi1; STG2: Solomon TeklebrhanGulelat fungi2; STG3: Solomon TeklebrhanGulelat fungi3; STG4: Solomon TeklebrhanGulelat fungi4; STG5: Solomon TeklebrhanGulelat fungi5; STG6: Solomon TeklebrhanGulelat fungi6.

Table 2 Enzyme amount produced from the selected fungi at 55°C

Code	U
STG3	2.73599 ± 0.00818^{a}
STG5	1.81339 ± 0.00409^{c}
STG6	2.51618 ± 0.00818^{b}

Values are mean ±standard deviation. Values followed by different letters indicate significant difference ($P < 0.05$).
STG3: Solomon TeklebrhanGulelat fungi3; STG5: Solomon TeklebrhanGulelat fungi5; STG6: Solomon TeklebrhanGulelat fungi6.

Table 3 Activity of fungal enzymes at different pH and their optimum temperature and time of incubation

pH	Activity (U/mg)		Relative activity (%)	
	STG3E at 55°C	STG6E at55°C	STG3E at 55°C	STG6E at55°C
4	0.56	0.60	67.46	68.96
7	0.64	0.73	77.10	83..90
9	0.66	0.74	79.51	85.05
10	0.62	0.64	74.69	73.56
Control	0.83	0.87	100	100

Table 4 Residual alpha amylase activity of fungal after characterization with metal ions incubated for 10 minutes

Metal ion	Concentration(Mm)	Activity(U/mg)		Residual activity (%)	
		STG3E	STG6E	STG3E	STG6E
Ca	1	0.61	0.63	73.49	72.41
Ca	5	0.59	0.60	71	68.96
Ca	10	0.48	0.57	57.8	65.51
Mg	1	0.52	0.53	62.65	60.91
Mg	5	0.47	0.52	56.62	59.77
Mg	10	0.471	0.50	56.74	57.47
Zn	1	0.53	0.58	63.85	66.66
Zn	5	0.50	0.56	60.24	64.36
Zn	10	0.49	0.46	59.03	52.87
Standard without metal ion		0.83	0.87	100	100

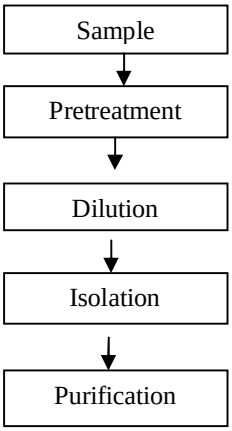

Figure 1 Stages in the isolation of thermo stable Fungi

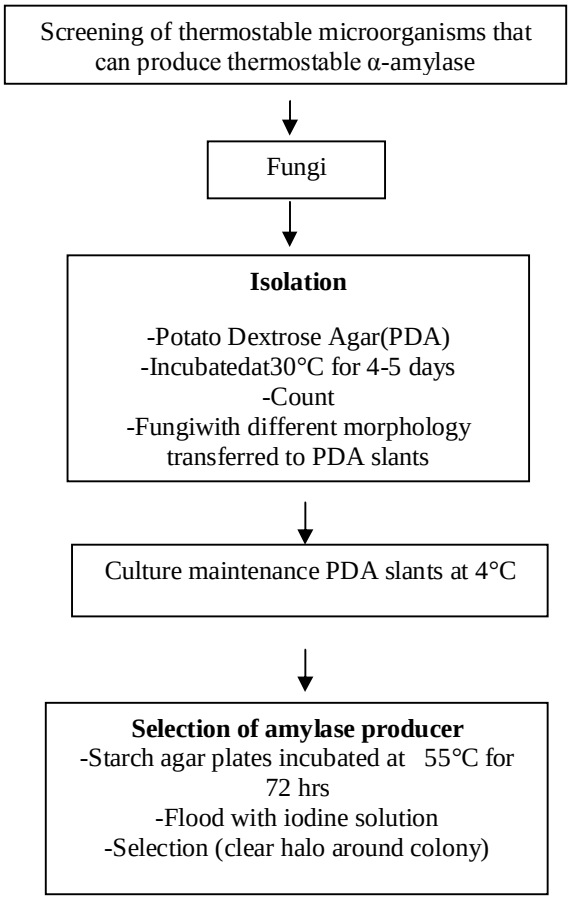

Figure 2 Flow chart that shows the process of screening of amylase positive fungi

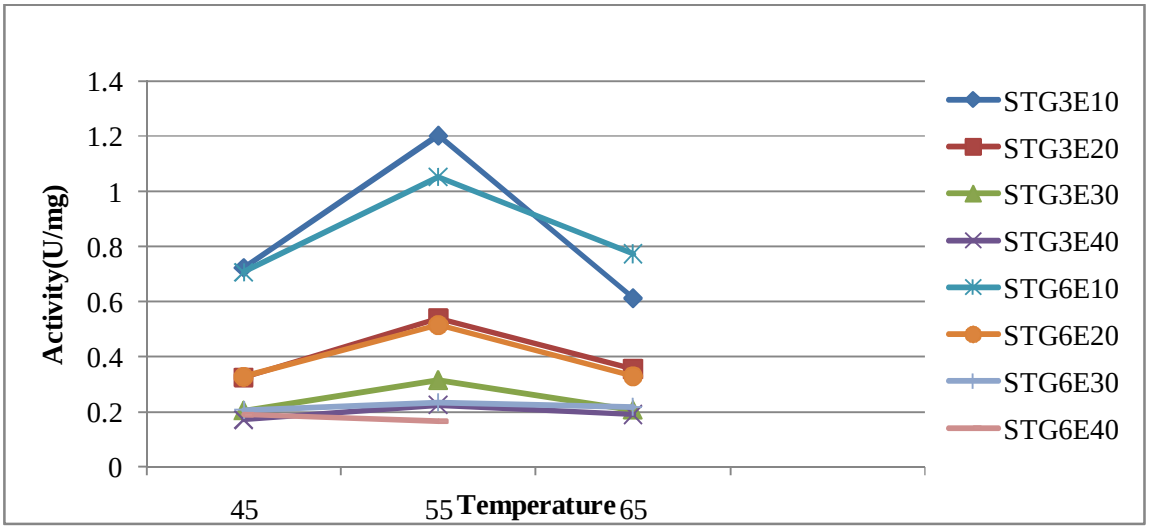

Figure 3 Effect of incubation time and temperature on the activity of partially purified fungal alpha amylase

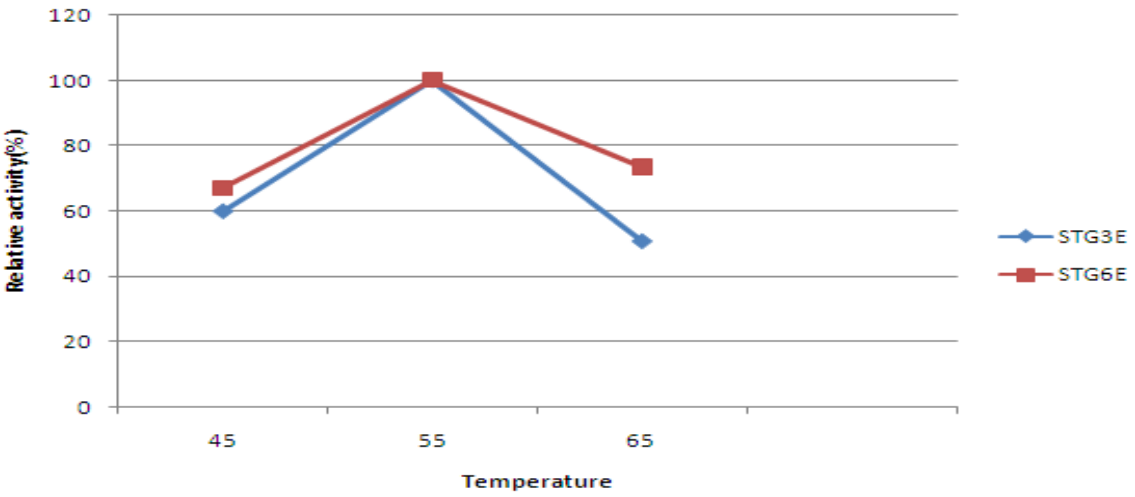

Figure 4 Relative enzyme activity at different temperatures incubated for 10 minute

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