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Research Article

Detection of Thermostable Amylases Produced by Thermophilic Fungi Isolated from some Ethiopian Hyper-Thermal Springs

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ABSTRACT

One thousand five hundred and nine thermophilic strains of water and soil fungi from six hyperthermal springs of Ethiopia (*Arbaminch*, *Awassa*, *Nazret*, *Shalla*/*Abijata*, *Wendo Genet* and *Yirgalem)* were isolated on Potato Dextrose Agar at 45°C for 3 to 5 days. Amylase active fungi were detected by growing the purified organisms on starch agar plates and flooding by Gram's iodine solution. During their metabolism 61 strains (about 4.04%) formed a clear halo around the colony while flooded with Gram's iodine solution indicating starch degradation. While nearly 37.5% of the crude alpha amylase extracts obtained from the strains isolated from *Shalla/Abijata* showed activity at 60°C only about 8% of those obtained from *Arbaminch* effectively hydrolyzed starch at this temperature. Further purification and characterization studies of enzyme will lead to a better knowledge of promising candidates of thermophilic microorganisms and processing conditions for enhanced production of thermostable amylolytic enzymes.

Key words: Ethiopian hot springs, thermostable alpha amylase, thermophillic fungi, starch

INTRODUCTION

The recent discovery of extremophiles from exotic environments perhaps has extended the notion of the limits of life. While they provided a broader understanding of biological structures and processes, they have also contributed towards the development of a number of enzymes capable of action under extreme conditions. The research areas on the subject include screening and application of new thermophiles from plants, animals and microbes (Kathiresan and Manivannan, 2006) and thermostable proteins, factors contributing to thermostability of their macromolecules, phylogeny, metabolic pathways and regulatory mechanisms.

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 (Hawksworth, D.L. et al., 1995). Many important plant pathogens (e.g. smuts and rusts) and plant growth promoting microorganisms (e.g., 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. In addition, these catabolic activities enable fungi to grow on inexpensive substrates. This property, coupled with their ability to produce commercially interesting organic molecules and enzymes explains the significant interest in the biotechnological utilization of fungi.

Alpha-amylases are ubiquitous enzymes and amylases from plant and microbial sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry. Fungal amylases have been widely used for the preparation of oriental foods. Microbial sources, namely fungal and bacterial amylases are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Burhan et al., 2003, Monnet et al., 2010; Kumari and Kayastha, 2011).

Because of wide range of physiological, analytical, industrial application and their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation, enzymes from microorganisms have attracted attention of researchers all over the world (Chakrabortya *et al.,* 2009). Thermozymes (thermostable enzymes) are one type of extremozymes isolated from thermophiles and are valuable tools for studying both protein stability and for producing special industrial and biotechnological products. Structural comparisons between mesophilic enzymes and thermozymes suggested that protein stabilization mechanisms include

hydrophobic interactions, salt-bridges, packing efficiency, hydrogen bonds, reduction of conformational strain, and reduction of entropy of unfolding, resistance to covalent destruction and loop and alpha helix stabilization (Vieille and Zeikus, 1996). Thermozymes are being used in molecular biology, detergent and starch processing industries and are candidates for applications requiring high protein stability.

Enzyme production generally relies on the screening of a large number of organisms for a specific enzyme activity with a specific set of biochemical and physical characteristics that suit the targeted application.

Microbial screening processes at present involve the evaluation of purified enzymes isolated from pure cultures obtained from culture collections, microorganisms isolated from environments rich in the substrate of interest and microorganisms that have a history of being good enzyme producers. An extensive research has been conducted to isolate thermostable amylases, which can act on starch gels at the pasting temperature. The search however, has remained challenging, as alpha amylases with improved characteristics are required for commercial applications (Pandey et al., 2000, Monnet *et al*., 2010; Kumari and Kayastha, 2011). In this work, survey has been made to asses the existence of thermostable alpha amylase producing thermophilic *fungi* and *eubacteria* in six Ethiopian hyperthermal springs (*Arbaminch, Awassa, Nazret, Shalla and Abijata, Wendo Genet* and *Yirgalem*). Further tests on the activity of the amylases extracted from selected organisms at elevated temperatures were conducted to detect the best thermostable enzyme from these sources.

MATERIALS AND METHODS

Chemicals: All of the microbiological media were obtained from Hi Media laboratories Limited, Mumbai-400 086, India. Magnesium sulphate hydrated, soluble starch, $FeSO_4.7H_2O$ were obtained from the Division of Asia Pacific Specialty Chemicals Limited (APS Finechem). Dipotasium hydrogen phosphate, potassium dihydrogen phosphate, potassium chloride, potassium iodide, iodine, hydrochloric acid and sodium hydro-oxide were obtained from Sigma-Aldrich Laborchemikalian GMBH. Magnesium sulphate, calcium chloride, sodium chloride and sodium nitrate were from MERCK Euro lab.

Equipment: In all the analysis, an orion pH meter of Model 420A for laboratory and a portable pH meter from HANA instruments for field pH and temperature measurement, a Gallenkamp incubator, a sensitive analytical balance (METTLER TOLEDO, Bergman As), Keimzahlgerat BZG 28 colony counter, a KT-30LD model autoclave and a Hettich centrifuge were utilized.

Site selection: Soil and water samples were collected from *Arbaminch, Awassa, Nazreth, Shalla* and *Abijata, Wendo Genet* and *Yirgalem* in Ethiopia. Temperature and pH of the water were measured directly. Samples were immediately transported to the laboratory.

Sample collection: A steel pipe with a diameter of 50mm was used to take soil samples. The pipe was pressed 50-100mm down into the soil to take the sample. The samples were then deposited in a sterile container with a screw lid. After each sampling the pipe was cleaned thoroughly in 70% alcohol. At each position, five soil samples were taken which are randomly distributed over an area of $1m^2$. Water samples from the hot springs were taken directly.

Isolation of amylase producing thermophilic fungi: Samples were kept at 55^oC overnight after which 10gm of sample was diluted in 90ml of sterile water. 0.1ml of sample was spread over Potato Dextrose Agar (PDA) and incubated at 45^oC for 3 to 5 days. After the growth, colonies were counted and purified isolates were maintained on PDA slants at 4^oC. Amylase active fungi were detected by growing the organisms on starch agar plates and flooding by Gram's iodine solution.

Basal medium: The basal medium for fungi for enzyme production consisted (g/l distilled water): $NaNO_3$, 3.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1.0; $FeSO_4.7H_2O$, 0.1 and starch 10.0. The pH was adjusted to 6.5 before sterilization using either HCl or NaOH.

Preparation of the enzyme extract: One hundred milliliters of the basal medium was inoculated using 4ml of culture, which was previously prepared using normal saline solution and incubated at 45^oC for 5 to 7 days. The incubation was made at 45^oC with rotary shaking for 48 hrs. Cell free culture supernatant was used as the crude enzyme source.

Plate assay method: Starch agar plates were prepared by using 2% (w/v) soluble starch and 1.5% agar powder. Five millimeter diameter holes were prepared on the starch agar plates by the help of a gel puncher. Fifty microliter of the crude enzyme extract previously heated at 60^oC for 10 minutes was then added to the holes and the plates were incubated at 37, 45 and 60^oC. After overnight incubation, plates were flooded with Gram's iodine solution.

RESULTS AND DISCUSSION

The Great East African Rift (GEAR) is famous world wide for its active rifting and volcanism in sub-aerial conditions from developed to early rifting stages. The hot springs considered in this study lie within the Ethiopian rift system which involves the northern part of GEAR. The altitude of the hot springs ranged from 1200 to 1690 meters above sea level. The temperature of the water varied considerably from 36.6°C at *Arbaminch* and 93.4°C at *Shalla* and *Abijata*. However, there was no significant difference in the temperature values between *Awassa* and *Wendo Genet* hot springs during the sampling period (Table 1).

Table 1. Altitude, temperature, pH and location of the hot springs studied in Ethiopia

Study area	Altitude	Temperature	pH	Latitude*	Longitude*
Arbaminch	1380	36.6	6.39	6°.02'N	37°.36'E
Awassa	1680	79.3	6.69	7°.04'N	38°.31'E
Nazreth	1200	59.4	6.25	8°.33'N	39°.15'E
Shalla & Abijata	1560	93.4	8.38	8°.00'N	38°.43'E
Wendo Genet	1520	78.4	6.58	7°.19'N	38°.38'E
Yirgalem	1690	44.8	7.07	6°.40'N	38°.23'E

*National Atlas of Ethiopia, 1988. Temperature and pH are mean values

There is a significant difference in the temperatures of all the other combinations ($p < 0.05$). The pH of the springs was close to neutral except that of *Shalla* and *Abijata* which is alkaline (Table 1), and the difference in the pH values of the spring at *Shalla* and *Abijata* vs all and *Yirgalem* vs *Arbaminch* and *Nazret* is significant ($p < 0.05$). According to Kumar and Swati (2001) the water temperature and pH of hot springs cover a wide range (30-100°C; pH 1-11) and such a natural geothermal environment is usually diverse as a habitat.

Based partly on their absorptive metabolism, the fungi have been placed in a super-kingdom (Whittaker and Margulis, 1978). Simple cultivation procedures of organisms and desirable physiochemical properties of the enzymes have enabled certain species of *Bacillus* and *Aspergillus* to be almost exclusively employed for the commercial production of amylases (Barfoed, 1976). Among other fungal sources, however, reports have been limited to a small number of species.

In this study, fungi that are capable of producing an α-amylase were screened. Of 1509 fungal isolates (246, 221, 203, 211, 234 and 394 from Arbaminch, Awassa, Nazret, Shalla and Abijata, Wendo Genet and Yirgalem, respectively), 12 (4.88%), 11 (4.98%), 6 (2.95%), 8 (3.79%), 10 (4.27%) and 14 (3.55%) produced amylase (Table 2).

Table 2. Total fungal isolates and amylase positive colonies

Hyperthermal spring	Total isolates ($\overline{X}$ ± S.D)*	Amylase positive colonies ($\overline{X}$ ± S.D)*	Percentage
Arbaminch (AM)	246 (27.33 ± 9.42)	12 (1.33 ± 0.50)	4.88
Awassa (AW)	221 (24.56 ± 4.12)	11 (1.22 ± 0.44)	4.98
Nazret (NA)	203 (22.56 ± 7.20)	6 (0.67 ± 0.50)	2.97
Shalla and Abijata (SA)	211 (23.44 ± 6.57)	8 (0.89 ± 0.33)	3.79
Wendo Genet (WG)	234 (26.00 ± 3.50)	10 (1.11 ± 0.33)	4.27
Yirgalem (YA)	394 (43.78 ± 9.20)	14 (1.56 ± 0.52)	3.55
TOTAL	1509	61	4.04

* Mean values of replicate petridishes from three sampling dates.

The diameter of the halo formed by some of the fungal colonies grown on starch agar is presented in Figure 1.

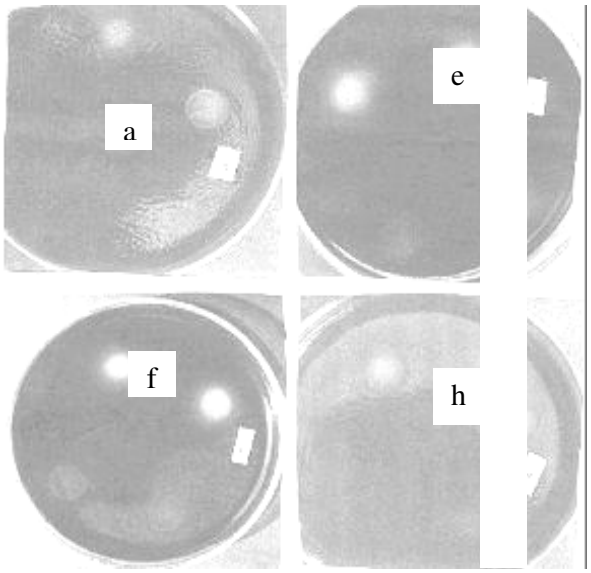

Figure 1. Starch agar plates after detection of amylase activity of selected fungi from Arbaminch (a), Awassa (e,f) and Shalla and Abijata (h).

Clear zones on a blue background indicate the extent of starch hydrolysis.
Most of the enzymes from the fungal origin, performed well at 37 and 45°C (Table 3).

Table 3. Percentage of amylases isolated from fungi active at 37, 45 and 60°C

Site	Total amylase active bacteria	% α-amylases active at:		
		37°C	45°C	60°C
Arbaminch	12	100	83.3	8.3
Awassa	11	100	63.6	27.2
Nazret	6	100	50.0	33.3
Shalla & Abijata	8	100	75.0	37.5
Wendo Genet	10	90	70.0	30.0
Yirgalem	14	100	64.3	28.6

Utilization of stable amylases at the saccharification stage of starch is economical for it reduces the amount of acids to be added to lower the pH from liquefying to saccharifying range. In addition, the formation of unwanted materials like maltulose at lower pH values will be significantly minimized. The present work has recorded a variety of fungi capable of producing thermostable amylolytic enzymes. The enzymes are active and stable at a higher temperature. To ensure a broad spectrum of application, the enzymes need to be further purified and characterized. This is being done in our laboratories with the most promising organisms that have been screened.

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