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SCREENING OF HALOTHERMOPHILIC BACTERIAL STRAIN FOR STARCH SACCHARIFICATION ENZYME

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ABSTRACT

Extremophiles can thrive in extreme environmental conditions due to their unique and surprising adaptations towards harsh surroundings. In nature, these extremophiles may be capable to cope with two or more ecological stresses, hence called polyextremophiles. Halothermophiles is one of the important class of polyextremophiles, whose physiology is adapted to tolerate high temperature and salt concentration, therefore halothermophilic enzymes can be the classical tool in various biotechnology based industrial setups. Amylases (starch saccharifying enzyme) from halothermophiles have numerous applications in many industries. The purpose of current work is to isolate a novel halothermophilic bacterial strain from different extreme ecological regions i.e. salt mine, sea, hot spring etc. and to explore its ability to survive and produce amylolytic enzyme under raised temperatures and NaCl concentrations. For this, qualitative plate screening was done and enzyme index (EI) was calculated. According to results, the strain grew well and produced more amylase at 50˚C with 7 and 10% NaCl concentration and at 55˚C with 5% NaCl conc. That's why it is suggested that this strain has great potential in industrial biotechnology.

Keywords: Extremophiles, halothermophiles, amylolytic enzyme, enzyme starch saccharification.

INTRODUCTION

For the continuity of life on earth, moderate environmental parameters are mandatory. Moderate habitats are ideal environments comprised of favorable temperature ranges from 20˚C-40˚C, neutral pH, 1 atm pressure with enough supply of nutrients and water. In nature, extreme environments also exist where it is difficult for normal life to acclimatize. Extreme conditions may extent from extraordinary elevated or low levels of temperature, pressure, pH and to the proportion of O_2 and CO_2 in air. Presence of high intensity of radiations, toxic compounds and NaCl concentration and absence of water availability is also marked as extreme state.

A large number of microbes have been found to thrive in such unusual extreme conditions, known as extremophiles. Extremophiles have remarkable ability to withstand and remain active under particular severe conditions (Satyanarayana *et al*., 2005) due to their countless adaptations. These adaptations allow their cellular proteins to be intact under harsh environments. One of the adaptative strategy elaborates the influence of chemical composition of extremophilic protein on the protein folding pathway in specific circumstances (Reed *et al*., 2013). Extremophiles are grouped in to various classes based on their survival and defense in a certain extreme environment. These include (i) thermophiles (ii) psychrophiles (iii) halophiles (iv) acidophiles (v) alakaliphiles (vi) barophiles and (vii) geophiles (Babu *et al*., 2015). Additionally, most of these microorganisms are polyextremophiles i.e. adapted to accommodate under two or more physiochemical stresses (Rampelotto, 2013).

Halothermophiles constitute an important category of polyextremophiles that propagate optimally under high salt (NaCl) concentration and high temperature. A technical definition of halothermophile is "a microbe that is needed at least 1.5M NaCl concentration and ≥ 50˚C temperature for maximum growth" (Mesbah and Wiegel, 2005).

Halothermophiles follow special collaborative adaptation phenomenon in nature. They hold unique mechanisms in order to cope with increased environmental temperature and salt concentration. Halophilic microorganisms halt the rapid deprivation of water by regulating their cytoplasmic content at least isoosmotically with the external surroundings. Moreover, hyper osmotic cytoplasm generates turgor pressure inside the cell. Almost all halophilic microbes control turgor pressure (Oren, 1999). Maintenance of plasma membrane permeability at high temperature is a big challenge for thermophiles. At raised temperature, the movement of lipid molecules fastens, which enhances permeability of positively charged ions. This movement also allows water molecules to entrap with in lipid portion of plasma membrane, hence permitting positive ions to jump from molecule to molecule. Ions other than protons can diffuse across the biomembrane as temperature also affects the diffusion, so permeability of membrane to ions is also increased (Konings *et al*., 2002).

Halothermophiles are ideal research tools in the discipline of industrial biotechnology as they retain vast industrial potential and the extremozymes obtained from halothermophiles can be recommended for those industrial setups that are stable at high salt concentration and temperature ((Mesbah and Wiegel,2005). These enzymes can be employed in the various processes of food, pharmaceutical, biomedical, textile and detergent industries (Kumar *et al*., 2012).

The current work is based on the isolation and screening of halothermophilic strain for starch saccharification. This research also determines the effect of temperature and NaCl concentration not only on the growth of strain but also on the production of amylase. The overall study mainly narrates the commercial importance of halothermotolerant amylase (EC:3.2.1.1), a class of hydrolases that is widely applied in different industrial sectors including food, detergent, textile, brewing, distilling, pharmaceutical (Moreno *et al*., 2013), paper and pulp (Setati, 2010; Gurung *et al*., 2013), bread and baking industry (Ahmed *et al*., 2016; DeSouza *et al*., 2010).

MATERIALS AND METHODS

Collection of samples:

In order to isolate halothermophiles, different soil and water samples were collected from extreme ecological niches i.e. extreme salt and extreme temperature conditions. Soil and water samples were aseptically collected in sterile containers from Khewra salt mine, Punjab Mubarak Village, Karachi and Manghopir hot spring, Karachi. Then the samples were stored at 4˚C.

Isolation of halothermophilic strain:

For the isolation of thermophiles, the water and soil samples of hot spring were serially diluted for up to 10^{-6} in normal saline and spread on Luria agar. All plates were incubated at 37˚C for 24 hours. For the isolation of halophiles, the soil and water samples of Khewra salt mine were inoculated in halophilic broth containing yeast extract 10g/lit, KCl 5g/lit, $MgSO_4$10g/lit, $CaCl_2$0.2 g/lit and Glycine 2g/lit (Jadhav and Musaddiq, 2011) with varying concentration of NaCl (10%, 15%, 20% and 25%). All flasks were incubated at 37˚C for 3-7 days. After visible turbidity in broth medium, these flasks were used as original inocula and used to make dilutions. The dilutions were plated on halophilic agar with 5% NaCl and incubated at 37˚C for 3-7 days. Marine extremophilic microbes were isolated by plating least dilutions on Luria agar incorporated with 5% NaCl, followed by incubation at 37˚C for 3-5 days. Afterwards, individual bacterial colonies were further purified by quadrant streak plate method.

Screening of starch saccharifying ability of isolates:

For the evaluation of amylase production by isolated pure cultures, qualitative plate assay was done. Luria agar supplemented with 1% starch, inoculated with purified colonies and incubated at 37˚C for 24 hours. After incubation, plates were flooded with 2% iodine solution to examine the zone of hydrolysis (Mazzucotelli *et al*., 2013).

Assessment of temperature influence on the growth and enzyme production:

Strains with amylolytic activity were selected for the determination of thermo-tolerance and its impact on amylase production. Qualitative screening was carried out. Luria agar with 1% starch inoculated with isolated colonies and incubated at different temperatures i.e. 37˚C, 40˚C, 45˚C, 50˚C and 55˚C. Plates were exposed to iodine solution to observe the zone of starch degradation. The culture with best properties was selected for identification and further studies.

Qualitative analysis of thermal and saline stresses on amylase production:

The strain was checked for its ability to flourish and to produce amylase under varied thermal and saline tensions. The growth and amylolytic enzyme production by strain was analyzed by inoculating Luria agar incorporated with 1% starch with multiple NaCl concentrations 3, 5, 7 and 10% and incubating at various temperatures 45˚C, 50˚C and 55˚C for 24 h. After 24 h, the plates were flooded with iodine solution to interpret the clear halos around the colony, which was measured to calculate the Enzyme Index by using formula (Islam *et al.*, 2016; Florencio *et al*., 2012).

Enzyme Index= Diameter of zone of hydrolysis (mm)/ Diameter of colony (mm)

Culture preservation:

The purified culture was preserved as glycerol stock (50%) by taking sterilized glycerol and 18 hours old culture (1:1) in vials, vortexed and stored at -4˚C.

RESULTS AND DISCUSSION

The study was started with the aim to exploit polyextremophiles with considerable industrial applications. The samples were collected from unusual extreme ecological regions. Total 73 strains were isolated and purified. Further, they were investigated for their capacity to hydrolyze a polymer, starch in to smaller subunits of dextrins and glucose (Rehman and Saeed, 2015) by qualitative plate assay (Mazzucotelli *et al*., 2013).

Only 26 isolates showed amylase production. These isolates were further screened for extracellular amylolytic enzyme production at different temperatures (37˚C, 40˚C, 45˚C, 50˚C and 55˚C). Strain C5W showed desired characteristics (Fig.1), it produced more enzyme as the temperature increased. Maximum enzyme production was found at 55˚C. It was also observed that bacterial growth was maximum at 45˚C with moderate halo but as the temperature increased, the growth of bacterial cells was reduced but enzyme production was remarkably enhanced. No growth and enzyme production noticed at 60˚C. Colonial morphology (Fig.2) and Gram's Reaction (Fig.3) of isolate C5W elucidated that it was *Bacillus* species.

Fig.1. Qualitative starch hydrolysis assay of isolated halothermophilic culture strain.

Fig.2. Isolated halothermophilic strain C5W.

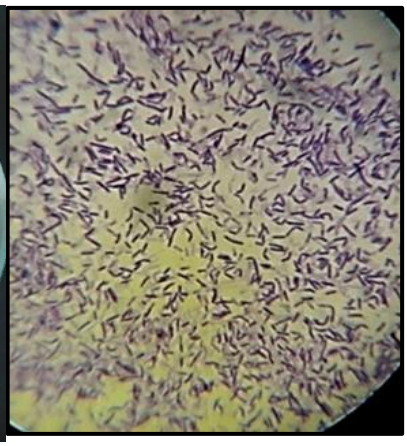

Fig.3. Cellular morphology.

The combined effect of temperature and NaCl concentration on the growth and amylase production by C5W was also assessed qualitatively and enzyme index was calculated. Enzyme index (EI) is estimated by using simple plate screening technique. In addition, the use of EI is convenient and rapid approach. For the selection of those microbes that can utilize miscellaneous complex polysaccharides. Ten *et al.* (2004) reported that the diameter of clear zone of degradation of various polysaccharides such as starch, cellulose and xylan by microorganisms on agar plates is beneficial for choosing the potent strain with zymogenic capability. Enzyme index greater than 1.5 considered to be significant outcomes (Florencio *et al*., 2012).

According to EI profile (Table 1), the strain showed worthy consequences at 50°C with 7 and 10% NaCl concentrations and at 55˚C with 5% NaCl concentration. Based on these findings, it may be inferred that the isolated strain could be moderate thermophile (45˚C-70˚C) (Canganella and Weigel, 2011) and moderate halophile (5-20% NaCl) (DasSarma and Arora, 2002).

Fahimeh *et al.* (2013) presumed that the optimum temperature and NaCl concentration for amylase production by halothermophilic strain were 40˚C and 15% (2.5M). Mathabatha (2010) and Carvalho *et al.* (2008) demonstrated the optimum amylase production by halophilic *Bacillus* species at 40˚C-70˚C. Fukushima *et al.* (2005) and Hutcheon *et al.* (2005) also reported amylase from *Haloarcula* species that works effectively at 50˚C under 4.3 M salt concentration with stability in benzene, toluene and choloroform. These highlighted previous researches are similar to present work which has been qualitatively done however for more accuracy and authenticity, quantitative analysis of growth of isolated strain in the presence of NaCl concentrations at various temperatures is required.

Table 1. Assessment of thermo-tolerance in salinities of 3, 5, 7 and 10% NaCl on enzyme production.

S.NO.	NaCl Conc. %	Temperature ˚C	Enzyme Index (Mean ± SE)
1.	**3**	**45**	1.25 ± 0.021
		50	2.11 ± 0.018
		55	2.0 ± 0.100
2.	**5**	**45**	1.81 ± 0.083
		50	1.60 ± 0.079
		55	1.5 ± 0.096
3.	**7**	**45**	1.77 ± 0.046
		50	2.0 ± 0.067
		55	NA
4.	**10**	**45**	1.69 ± 0.038
		50	1.81 ± 0.017
		55	NA

NA= No Activity; Data represents as mean ± standard error (SE) for triplicates

Therefore, the stability and adaptation of halothermophilic strain towards high temperature and NaCl concentration with ability of halo-thermotolerant amylase production proposed that this strain could be an ideal option for biotechnology based industrial processes. In conclusion, above mentioned findings lead to future investigations involving optimization and purification studies of amylase by isolated halothermophilic bacteria.

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