EXPLORATION OF HALOPHILIC BACTERIAL DIVERSITY FOR THE PRODUCTION OF INDUSTRIALLY IMPORTANT EXTRACELLULAR HYDROLYTIC ENZYMES

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خلاصہ

ھیلوفائلس بنیادی طور پر وہ ماحولیاتی انتہاپسند ہیں جو منفرد موافقت کے ذریعہ نمک حالات میں رہنے کے قابل ہیں. بیکٹیریل ڈومین کے اندر ہالوفیلیزم کی خصوصیت وسیع ہے. بیکٹیریل ھیلوفائلس ماحول میں بہت زیادہ پائے جاتے ہیں جیسے نمک جھیلوں، نمکین مٹیوں، نمک کانوں کی کھدائیوں، نمکینوں اور نمکین کھانے کی مصنوعات. ھیلوفائلس کی تجارتی اہمیت قدیم اوقات سے ریکارڈ کی گئی ہے. یہ مطالعہ منفرد خصوصیات کے ساتھ قابل قدر صنعتی انزائموں کی پیداوار کے لئے بورنگ پانی سے ہیلوفیلک بیکٹیریا کی تناسب، شناخت اور اسکریننگ کی رپورٹ کرتا ہے. ہیلوفیلک انزائمز کی پیداور کو دیکھنے کے لئے کوالٹی پلیٹ طریقہ کار استعمال کیا گیا. آٹھ بیکٹیریل قسموں کو دو بورنگ پانی کے نمونوں سے علیحدہ کیا گیا جو کہ کراچی کے مختلف علاقوں سے حاصل کیے گئے تھے. گرام سٹینینگ کی بنیاد پر زیادہ تر ہیلوفیلک پائے گئے (cocci). بیکٹیریا کو کسائی سب سے زیادہ ہائیڈرولائٹک صلاحیت کو ظاہر کیا ہے SNA1-4 اور SNA1-3 ، SNA1-2علیحدہ کیے گئے بیکٹیریل اقسام. انزائمز پیدا کیے (β-galactocidase) اور بیٹا گلیکٹوسائڈیس (Caseinse) ، کیسیناس، (Gelatinase) جیلیٹنس ،(Cellulase)، سیلولیز ، (Amylase) کیونکہ انہوں نے امائلیز امیلولیٹک انزائمز کو تمام بیکٹیریل اسٹرینس نے سب سے زیادہ پیدا کیا(سو فیصد)۔ تمام اسٹرینس نے تقریباً آٹھ فیصد تک نمک کی کشیدگی کو برداشت کیا نے دیگر بیکٹیریل اقسام کے مقابلے میں اعلی نمک رواداری ظاہر کی ہے SNA2-4 اور SNA2-1 ، SNA1-2، SNA1-1 کیونکہ وہ پندرہ فیصد نمک کی کشیدگی کو برداشت کر رہے تھے

ہیلوفیلک بیکٹیریا کے طور پر تقسیم کیا گیا (moderate) .اور معتبر (slight) اس مطالعے میں کئے گئے نمک کشیدگی پروفائل کے مطابق، علیحدہ کیے گئے ہیلوفیلک بیکٹیریا کو تھوڑا سا اس طرح، ان ہیلوفیلک ہائیڈولائٹک انزائمز کئی صنعتی سیٹس کے لئے بہت دلچسپی رکھتی ہے جہاں پر نمک حراستی کے سخت پیرامیٹر کی ضرورت ہوتی ہے.ا

Abstract

Halophiles are basically extremophiles that are able to live in salty conditions through unique adaptations. The property of halophilism is widespread within the bacterial domain. Bacterial halophiles are found abundant in environments such as salt lakes, saline soils, salt mines, salterns and salted food products. The commercial importance of halophiles has been recorded since ancient times. This study reports the isolation, identification and screening of halophilic bacteria from boring water for the production of valuable industrial enzymes with unique characteristics. Qualitative plate assays were used to screen the productivity of halophilic enzymes. Eight strains were isolated from two boring water samples, obtained from different areas of Karachi. On the basis of Gram staining, most of the isolated halophilic strains were cocci. The isolated strains SNA1-2, SNA1-3and SNA1-4 showed the highest hydrolytic potential as they produce amylase, cellulase, gelatinase, caseinase, and β-galactocidase enzyme. Amylolytic enzymes were recorded to be mostly produced enzymes by all strains (100%).All strains revealed salt tolerance profile up to 8% NaCl concentration. SNA1-1, SNA1-2, SNA2-2, and SNA2-4 showed high salt tolerance as compared to other strains as they tolerated 15% salinity. According salt tolerance profile, carried out in this study, the isolated strains were classified as slightly and moderately halophilic. Thus, these halophilic hydrolytic enzymes may be of great interest for several industrial setups that require harsh parameter of salt concentration.

Introduction

Microbial diversity plays a vital role in many biotechnological processes and product manufacturing. Microorganisms serve as a rich source for the production of novel metabolites, recycling of nutrients and neutralization of harmful toxins from environment (Kiran *et al.*, 2015). These microbes have also tremendous potential to produce industrially valuable enzymes. Production of extracellular hydrolytic enzymes by a variety

of microorganisms can be carried out through environmental friendly processes. These processes can generate bulk quantities of product with improved quality in a limited time under optimum conditions (Delgado-Garcia *et al*., 2014; Sanchez-Porro *et al*., 2003). However, all industrial processes cannot always fulfill the requirements of production under ideal conditions. Several industrial procedures need intense conditions of various physical and chemical parameters (Satyanarayana, 2005).

There is a diverse group of microbes that thrive under extreme environments (temperature, pH, pressure, intensity of light, oxygen concentration, level of nutrients, salinity etc.), called extremophiles. These extremophiles are so unique that exist in unusual habitats, hence their enzymes called extremozymes (Sarmiento *et al*., 2015) which can cope with the harsh industrial processes requirements. The novel properties in these extremophiles are due to genetic or physiological adaptations (Gaur *et al*., 2012). Halophilic microorganisms are salt loving extremophiles that adapt hypersaline environments to grow optimally. Halophiles are widely dispersed in different hypersaline niches of world ranging from natural brines, coastal and deep sea locations, hypersaline lakes and spring to salterns and salt mines. Halophilic population is divided in to major life domains mainly archaea, bacteria and eukarya. Most diverse halophilic bacterial specie is *Halobacterium*, *Halomonas* and *Salinibacter*. Halophiles can be grouped in to three categories according to their NaCl demand: (a) slight halophiles (b) moderate halophiles and (c) extreme halophiles (Waditee-Sirisattha *et al*., 2016; Margesin and Schinner, 2001; Sarwar *et al*., 2015).

Halophilic microbes are involved in significant hydrolytic activities as they are potent source of industrially relevant enzymes. Their biocatalysts possess unique and novel characteristics, quite suitable for biotechnological applications. These halophilic biofactories have been exploited for the production of many extremozymes, which can catalyze specific chemical reaction under extreme chemical or physical surroundings, required in many industrial processes. However, halophilic extremozymes retain high degree of stability with remarkable features as compared to conventional enzymes. In addition, halophilic enzymes not only energetic in extreme salt environments but are also often active at high temperature and alkaline pH. Halophilic enzymes thus seem to be attractive candidates in various commercial applications including bioremediation, waste water management and food industry. Due to immense uses, research interest of scientific community has greatly enhanced towards screening and purification of halophilic enzymes that are enough capable to carry out ideal activities at different levels of extreme conditions (Waditee-Sirisattha *et al*., 2016; Das Sarma and Arora, 2002; Setati, 2010).

Salt tolerant enzymes derived from halophilic bacteria, mainly hydrolases and their potential use has been studied by various groups. Halophilic hydrolases include amylase, lipase, cellulase, protease, xylanase have strong demand in a number of industrial sectors such as textile, food, dairy, bread and baking, paper and pulp, pharmaceutical, leather, detergent industries etc. (Moreno *et al*., 2013; Setati, 2010). The purpose of current study was to explore novel halophilic bacteria from boring water and to examine their salt tolerance profile. The research also aims to investigate the microbial biodiversity in extreme habitats specifically halophilic microflora and their ability to produce extracellular hydrolytic enzymes.

Material And Methods

Samples collection

The boring water samples were collected from two different locations of Karachi in sterile glass jars aseptically, properly labelled, transported to laboratory and stored at 4˚C.

Isolation of halophilic bacteria

Isolation of halophilic bacteria was achieved by serially diluting (up to 10^{-4}) water samples and spreading on nutrient agar plates with appropriate salt concentration using pour plate method. The plates were incubated at 37˚C for 48 hours. In order to obtain pure culture, the bacterial isolates were further subcultured by using streak plate method.

Morphological studies and biochemical analysis for the characterization of bacterial isolates

For the identification and characterization of isolated bacterial cultures, colonial morphology was observed. The bacterial isolates were also subjected to Gram's staining and various biochemical assays for further identification by using Bergey's manual of determinative bacteriology. The obtained purified cultures were subcultured routinely and preserved as glycerol stocks.

Screening of bacterial isolates for extracellular enzymatic activities

The bacterial isolates were screened qualitatively for different extracellular hydrolase enzymes including amylase, protease, lipase, esterase, cellulase, pectinase, beta galactosidase and DNase. Screening assays for these enzymes are highlighted below,

Extracellular amylolytic activity
The bacterial isolates were monitored for amylase production by inoculating all isolates on medium containing glucose 0.1%, yeast extract 0.25%, starch 1%, NaCl 5% and agar 2%. Plates were incubated at 37 ˚C for 48 hours. After growth, the plates were flooded with 2% iodine solution. The presence of halos around the colony indicated the starch hydrolysis (Mazzucotelli *et al*., 2013).

Extracellular lipase activity
The potential of lipolytic activity of isolated strains was determined by growing colonies on medium containing $CaCl_2$ 0.01%, peptone 1%, NaCl 5%, tween 80 1% and agar 2%. Plates were incubated at 37 ˚C for 48 hours. The isolates that produced zones of precipitation around the colonies were identified as lipase producers (Kumar *et al*., 2012).

Extracellular cellulase production
For the qualitative evaluation of cellulase producing capability of bacterial isolates, the strains were spot inoculated on nutrient agar medium incorporated with 1% cellulose as substrate and 5% NaCl. The plates were incubated at 37 ˚C for 48 hours. To observe cellulose hydrolysis, plates were exposed to 2% potassium iodide solution, presence of clear halos around colonies elaborated enzymatic activity (Kasana *et al.*, 2008).

Extracellular gelatinase production
Gelatin hydrolysis test was assayed by using nutrient agar medium, which was provided with substrate- gelatin and 5% NaCl. All isolates were allowed to grow on gelatin agar plates at 37 ˚C for 48 hours. After visible growth, the plates were flooded with saturated solution of ammonium sulphate, in order to visualize clear zones around the colonies, which showed gelatinase activity.

Extracellular casein hydrolyzing activity
Potential for caseinase production of the isolated strains were carried out by spot inoculation on nutrient agar plates containing NaCl 5% and casein 1%. Plates were incubated at 37 ˚C for 48 hours. Then the protease producing strains were identified on the basis of zone of clearance (Perez *et al*., 2009).

Extracellular pectin degrading activity
In order to screen the pectin hydrolyzing capability of bacterial isolates, all strains were spotted on the medium containing KH_2PO_4 0.06%, $(NH_4)_2$ SO_4 0.14%, $MgSO_4,7H_2$ 0 0.02%, pectin 1%, yeast extract 0.1% , NaCl 5% and agar 2%. Plates were incubated at 37 ˚C for 48 hours. After incubation, the plates were flooded potassium iodide solution for 5 min. The clear zones around colonies were indicated the qualitative pectinase activity (Rhoban *et al*., 2008).

Extracellular esterase enzyme activity
All cultures were inoculated on the medium having $CaCl_2.2H_2O$ 0.01%, peptone 1%, NaCl 5%, peptone 1%, tween 20 1% and agar 2%. Plates were incubated at 37 ˚C for 48 hours. After incubation, the zones of precipitates around the growth were considered as positive reaction (Kumar *et al*., 2012).

Extracellular β- galactosidase production
β galactosidase production was checked by inoculating all the isolates on nutrient agar medium supplemented with 5% NaCl and 1% lactose. Plates were incubated at 37˚C for 48 hours. Clear zones were identified around the colonies (Carrim *et al*., 2006).

Extracellular DNase enzyme production
To detect DNase activity, the strains were cultured on DNase test agar medium provided with 5% NaCl. Plates were incubated at 37 ˚C for 48 hours. Colonies showed clear halos were considered as DNase producers.

Analysis of salt tolerance potential of bacterial isolates
All strains were analyzed to test their ability to survive at different salt (sodium chloride) concentrations. For this purpose, nutrient agar with varying concentrations of NaCl (1%, 2%, 5%, 8%, 10% and 15%) inoculated with 0.1ml respective bacterial cultures. Tubes were incubated at 37 ˚C. Afterwards, growth at different salt concentrations for different time intervals (24 and 48 hours) was measured by spectrophotometer analysis at 600nm.

Results

Isolation and characterization of halophilic bacteria

In present work, total eight halophilic bacterial strains were isolated from two local boring water samples (SNA1 & SNA2), named as SNA1-1, SNA1-2, SNA1-3, SNA1-4, SNA2-1, SNA-2, SNA2-3, SNA2-4. Pure cultures of these isolated strains were obtained by streak plate method. Afterwards, the purified colonies were stored in glycerol stocks.

Among 8 strains, seven were Gram positive with spherical shape, named cocci while only one strain was Gram's negative, identified as *Bacillus* (rod shaped). Morphological studies and biochemical analysis was also done for further characterization.

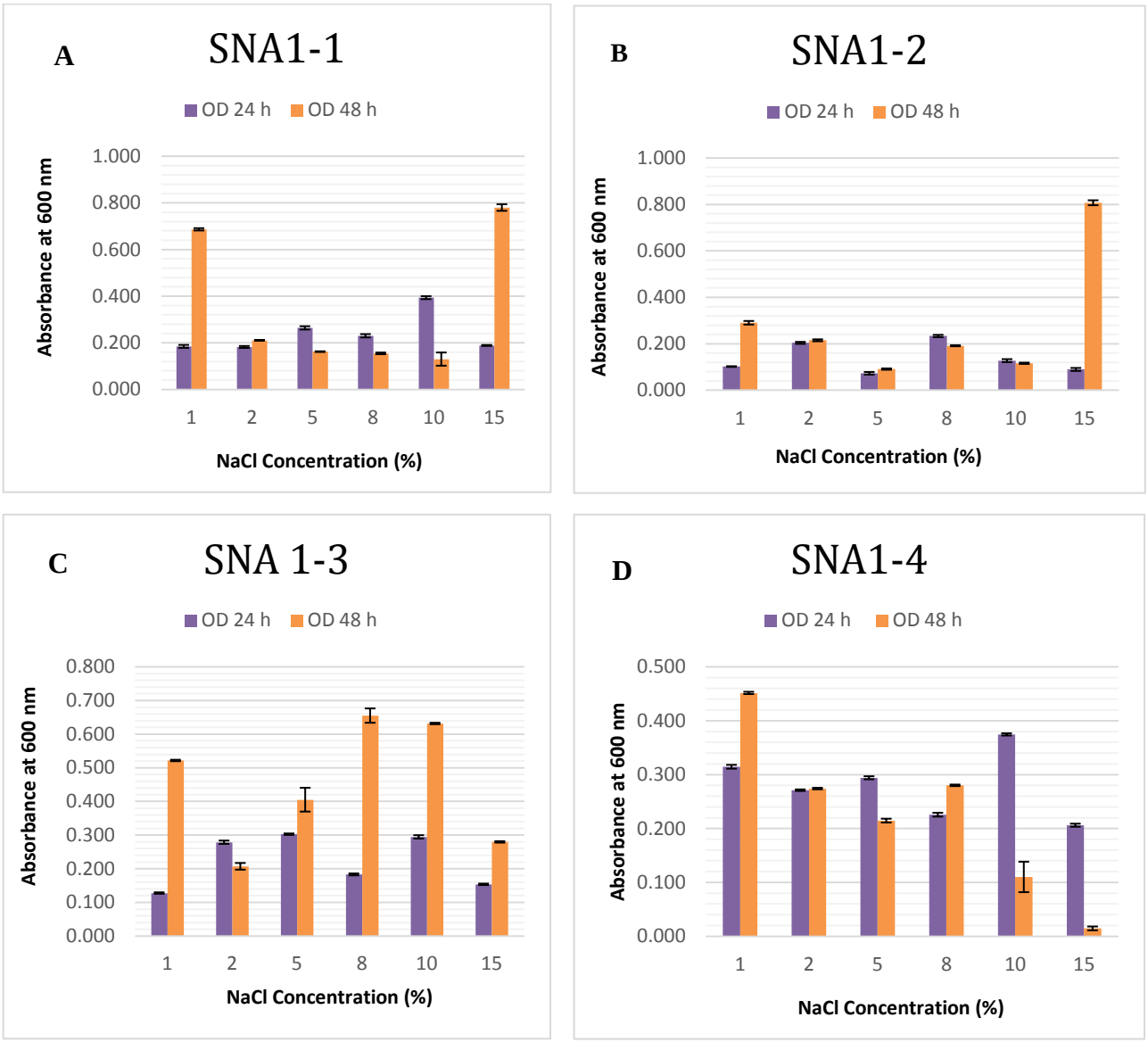

Fig 1: (A) Growth of halophilic strain SNA1-1 at different NaCl Concentrations for 24 & 48 hours; (B)Growth of halophilic strain SNA1-2 at different NaCl Concentrations for 24 & 48 hours; (C) Growth of halophilic strain SNA1-3 at different NaCl Concentrations for 24 & 48 hours; (D) Growth of halophilic strain SNA1-4 at different NaCl Concentrations for 24 & 48

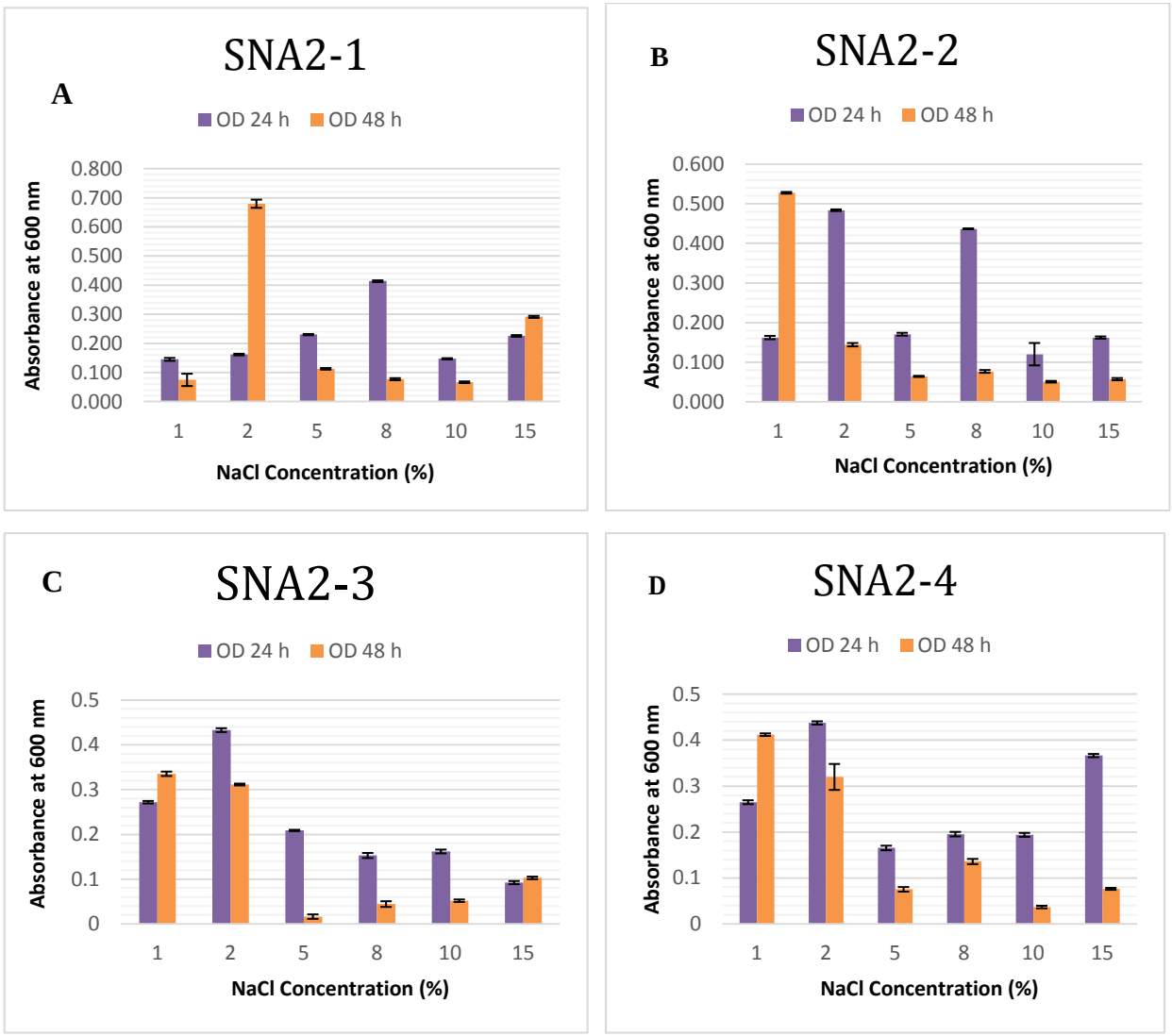

Fig 2: (A) Growth of halophilic strain SNA2-1 at different NaCl Concentrations for 24 & 48 hours; (B) Growth of halophilic strain SNA2-2 at different NaCl Concentrations for 24 & 48 hours; (C) Growth of halophilic strain SNA2-3 at different NaCl Concentrations for 24 & 48 hours; (D) Growth of halophilic strain SNA2-4 at different NaCl Concentrations for 24 & 48 hours.

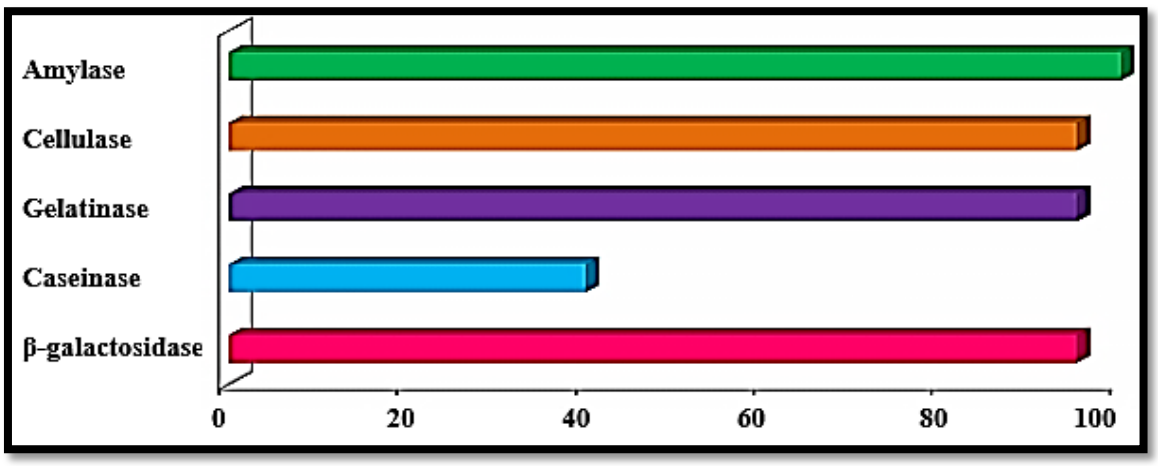

Fig 3: Hydrolytic enzyme production in % of isolated strains

Table 1: Result of enzyme screening of isolated strains.

Enzymes	SNA1-1	SNA1-2	SNA1-3	SNA1-4	SNA2-1	SNA2-2	SNA2-3	SNA2-4	SNA1-1
Amylase	+	++	+++	+	+++	+++	+	++	+
Lipase	-	-	-	-	-	-	-	-	-
Cellulase	-	+	+	+++	+	+++	+	+	-
Gelatinase	-	+	+++	+++	+	+++	+	+	-
Caseinase	-	+++	++	+	-	-	-	-	-
Pectinase	-	-	-	-	-	-	-	-	-
Esterase	-	-	-	-	-	-	-	-	-
β-galactociase	+	+	+	+	-	+	+	++	+
DNase	-	-	-	-	-	-	-	-	-

+++ (High production), ++ (Moderate production), + (Slight production), - (No production)

Salt tolerance profile at various NaCl concentrations

After identification, bacterial cultures were screened for their ability to tolerate various salt concentrations (1%, 2%, 5%, 8%, 10% and 15%) for 24 and 48 hours. The salt tolerance profile of isolated microorganiams exhibited that they were classified in to two groups i.e. slight halophile (1-5%) and moderate halophiles (5-15%) (Fig 1& 2). Each strain showed different growth patterns at various salt concentrations. Highest growth at 15% NaCl was showed by SNA1-1 and SNA1-2 for the incubation period of 48 hours, while SNA2-1 and SNA2-4 exhibited highest growth at 15% NaCl for 24 hours.

Qualitative screening for the production of extracellular enzymes

All isolates were tested for nine different hydrolytic enzymes (amylase, lipase, cellulase, gelatinase, caseinase, pectinase, esterase, β- galactosidase and DNase) in the presence of 5% NaCl. Results of hydrolytic activities are highlighted in Table 1.

Among all strains, 3 strains SNA1-2, SNA1-3 & SNA1-4 showed highest hydrolytic potential as they were degrading maximum numbers of substrates (starch, lactose, cellulose, casein and gelatin). However, no production of lipase, pectinase, esterase and DNase was observed when all the strains were subcultured in to their respective medium.

All isolates were found to be amylase producers (100%), where as 90% of total isolates were found capable to produce protease (gelatinase),cellulase and β-galactosidase (Fig 3).

Discussion

Halophilic bacteria have considered a true asset for a variety of industrial setups. Halophilic bacteria are the factories of useful biosubstances such as enzymes that show maximum stability and activity in hypersaline conditions. Their enzymes mainly extracellular hydrolases offer immense uses in various sub disciplines of biotechnology (Margesin and Schinner, 2001). The research on halophiles has opened new aspects of information including geographical distribution, culture independent studies on the basis of DNA sequencing which demonstrates the strategies to isolate novel halophilic diversity, widely distributed in different surroundings (Mendparal *et al*., 2013). In 1970, the importance of halophiles was firstly recognized in salt making process (Reema *et al*., 2015; Aljohny, 2015). So in this manner, the present study was done to reveal the significance of halophiles, isolated from local boring water and their ability to produce extracellular hydrolytic enzymes

Microorganisms are cultivated in lab by providing growth conditions, similar to their natural settings (Muthulakshmi *et al*., 2011). Niches with high salinity are the preferred areas for the isolation of halophiles. Keeping this in concern, natural boring water samples were selected for the isolation halophilic bacteria. Previously, some reports showed their substantial concern on the ecology, phylogeny and taxonomy of halophilic bacteria, along with the focus on biotechnological applications. Halophilic bacteria can be divided in to three categories: (a) slight (b) moderate (c) extreme halophiles on the basis of their NaCl tolerance. The outcomes of our screening study clearly indicated the abundancy of slight and moderate halophiles than extreme halophiles. Previously Quesada *et al* (1982) and Rodriguea-Valera (1998) demonstrated that the moderate halophilic bacteria exist with high frequencies in natural hypersaline habitats as compared to extreme halophiles. Extreme halophiles flourish at high NaCl concentrations with the essential requirements of Mg^+ ions, whereas slightly and moderately halophilic bacteria eliminate the need of Mg^+ ions for their optimum growth (Roohi *et al.,* 2012). Our halophilic isolates cultivated well at temperature 37˚C on the medium provided with 1-15% NaCl concentration for 24-48 hours incubation period. Jadhav and Musaddiq (2011) isolated the halophilic bacteria from saline soil in India, which could grow at >12.5% NaCl concentration on elevated incubation time.

Utilization of single strain on industrial level produces multipurpose enzymes simultaneously which can reduce the total cost and time course for manufacturing setups with qualitative yields (Kiran *et al*., 2015). Several screening studies related to the exploration of halophilic hydrolytic enzymes showed that 70% of moderate halophiles possess remarkable hydrolytic capability (Moreno *et al*., 2009). Conventionally, plate screening method is employed worldwide for the identification and selection of potent bacterial isolates with enormous enzymatic ability (Ten *et al*., 2005). The plate screening method elucidated that all the isolates were incapable to hydrolyze pectin. Pectin is complex heteropolysaccharide and due to this chemical nature, it is a tough substrate for single unit of microbial species to degrade, however a complex set of pectin hydrolyzing enzyme is needed so that pectin can be broken down sequentially (Kiran *et al*., 2015).

Halophilic amylolytic enzymes are extensively utilized in starch saccharification, textile, brewing, food and distilling industries. Halophilic amylases showed their intense versatility and stability in the various processes of these industries (Moreno *et al.*, 2013). In this study, 100% halophilic bacterial isolates were able to produce amylase (Fig 5). Sanchez Porro *et al* (2003) reported that among various hydrolases, amylolytic enzymes are largely produced enzymes by different halophilic genera such as *Salinococcus* sp.

Although halophilic lipolytic enzymes (lipases and estrases) have huge commercial applications in detergent, food and paper industries (Moreno *et al*., 2013) but screening of lipolytic enzymes from isolated halophiles showed no activity. It was also found that lipase and esterase growth media showed inhibitory effects against culture growth. According to Michael *et al* (1996), free amount of oleic acid is present in tween 80 as contaminant, which exert the inhibition against the growth of some bacteria due to changes in physical state of fatty acid chain. Same phenomenon is applicable for tween 20 (esterase activity) that is comprised of lauric acid. Another important class of industrial enzymes are proteolytic enzymes that constitute a great commercial importance. Halophilic proteases are appealing hydrolases for biotechnology based industrial procedures. Proteases have broad uses in laundary detergents, baking, brewing, cheese and leather industries (Moreno *et al*., 2013). This study screened for two different proteases i.e. gelatinase and caseinase. The results of plate screening method for protease activity by isolated halophilic strains revealed that 90% of total isolates were found to be gelatinase active, while casein was produced by 40% isolates (Fig 5).

Lignocellulose is the most plentiful bioresource present on earth. Appropriate microbes are involved in the degradation of lignocellulose in to simple sugars for the synthesis of many fermented biproducts such as bioethanol, biobutanol, enzymes and organic acids. Cellulases are one of the important class of hydrolytic enzymes that hydrolyze cellulosic material. Activity of cellulase is compatible with pretreatment process that uses ionic liquids (ILs), which are basically salts. Thus, ILs might not be reliable with conventional cellulases for enzymatic saccharification process. There, there is necessity to produce and purify salt tolerant cellulases and for this purpose, halophiles are best choice (Gunny *et al*., 2015). Current research analyzed bacterial isolates for cellulase production and among all 90% strains were able to hydrolyze cellulose as a carbon source.

On the other hand β-galactosidase has potential applications in the production lactose free whey and milk, manufacturing of oligosaccharides for the enhancement of intestinal microflora and clarification of plant saccharides from fruit juices (Sheridan *et al*., 2000). Hence halophilic β-galactosidase can be used in the industrial process where operational conditions are extreme in terms of salinity. Our study showed that 90% of isolates were found to be β- galactosidase positive.

Conclusion

This study provides knowledge about proteases, amylases, cellulases and β-galactosiadases from halophilic bacteria which may be useful in many industrial processes that require unusual conditions. It is suggested that the isolated halophilic strains may be the active resource for optimization studies

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