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Research Article

Mining salt pan soil metagenome for functional screening of enzymes

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Metagenomics relates to the uncultivable microbial world, targeting the total microbial genetic material in a specific environment. While a few extreme habitats have been studied for the metagenomic diversity and biotechnological potential, the saline systems of India have not been much explored. This study describes direct DNA extraction methods based on the physical, chemical and enzymatic lysis in various combinations to extract the metagenomic DNA. Metagenomic DNA extracted from salt pan soil has a high molecular weight. Four small insert metagenomic libraries were constructed using two different vectors pUC18 and pET 21a^{+}, and *E. coli* JM109 as the host organism. The libraries were further screened for protease, amylase and lipase. Seven protease and five amylase positive clones were detected, and the relative enzyme activities were assessed. The metagenomic library from the salt pan soil proved to be significant for the exploration of valuable enzymes.

[**Keywords**: Amylase, Lipase, Metagenomic DNA extraction, Metagenomic library, Protease, Salt enriched Soil]

Introduction

Metagenomics describe genomes and biomolecules of the entire microbial communities. It's accepted that only about 1 % microorganisms of a given habitat are cultivated under standard laboratory conditions. This is limiting our knowledge about genomes, biocatalysts and other metabolic potential of the microorganisms[1]. Metagenomics facilitates our understanding of the microbial activity in a given niche using next-generation technology. The survival strategies in a saline environment and interaction with the marine host organisms have recently been described[2-4]. The study of marine metagenomics would help unlock the potential of vast and unique microbes. Gujarat has a 1600 km long coastline harbouring huge and untapped microbial diversity. The quality and quantity of the extracted metagenomic DNA is crucial but often fraught with challenges. However, there's no universal method for extracting metagenomic DNA from various environments, so it's critical to establish a specific method. Over the past two decades, advancements in molecular tools have facilitated the extraction, cloning, screening, and sequencing of these genomes. In this study, a metagenomic DNA extraction protocol based on direct *in situ* lysis of the cells was established.

The most commonly used physical disruption method is "bead beating", which increases DNA yield[5,6]. Chemical lysis of the cells by detergents, such as Sodium Dodecyl Sulfate (SDS) and Cetyltrimethyl-Ammonium Bromide (CTAB), has also been reported for DNA extraction[7-10]. Enzymatic lysis, combined with bead beating, has proven to be the most successful protocol for isolating higher yields of inhibitor-free DNA from soil[11-15]. Polyethylene Glycol (PEG) aids in precipitating the DNA and reduces humic acid contaminant[16]. The extracted metagenomic DNA must be of high quality, suitable for molecular applications, such as diversity, functional genomics, and other areas[11]. Construction of a metagenomic library is a promising approach for identifying novel biocatalysts from prokaryotes[17]. Functional screening of clones helps to understand the potential of yet unknown enzymes. Enzymes from extreme environments, such as those found in saline conditions, are unique and suitable for many industrial processes, as they efficiently function at alkaline pH, high salt concentrations, and high temperatures[18-20].

Materials and Methods

Sampling sites

Two salt-enriched soil samples, designated as 1 and 2, were collected from the salt pan near Mithapur (Latitude 22°24'36" N, Longitude 69°00'0" E) and the saltpan near Dwarka (Latitude 22°13'48" N,

Longitude 68°58'12" E), respectively, from Gujarat, India. The collected saline soil samples were stored at 4 °C for subsequent analysis.

Physicochemical analysis of the salt pan soil

Physicochemical characteristics of the salt pan soil were evaluated for its colour, soil texture, soil consistency and pH. Electrical conductivity was determined with the help of a Systronics digital conductivity meter; chloride by Mohr's method; and total organic carbon by Walkleys and Black rapid titration method. Salinity was determined according to the method reported by Ramoliya *et al*.[21].

Extraction of the metagenomic DNA from the salt pan soil

Metagenomic DNA from the salt-enriched soil was extracted using direct extraction methods, such as soft lysis, bead beating, and their combination[13] with modifications of incorporating Tris-saturated phenol and 1 % PEG. Additionally, for comparison, HiPurA™ soil DNA isolation (HiMedia, India) and NUCLEOSPIN® soil (Macherey–Nagel, Duren, Germany) kits were also used.

Modified DNA extraction methods

Soft lysis along with saturated phenol + 1 % PEG

One-gram soil was treated with 10 ml extraction buffer and incubated at 37 °C for 10 – 12 h at 150 rpm. Sample was re-extracted in 1 ml of the extraction buffer, and supernatant was collected by centrifugation (~ 2000 × g for 5 min). A lysis buffer (4 ml) was then added and incubated at 70 °C for 2 h with vigorous intermittent shaking at 15 min intervals. The supernatant was collected by centrifugation at ~ 13000 × g for 10 min and treated with an equal volume of Tris-saturated Phenol (10 mM Tris, 1 mM EDTA; pH-8.0): Chloroform: Isoamyl alcohol (25:24:1). The upper aqueous phase was collected by centrifugation at ~ 13000 × g for 20 min at 4 °C and treated with an equal volume of Chloroform: Isoamyl alcohol (24:1), followed by centrifugation at ~ 13000 × g for 10 min at 4 °C. One tenth volume of potassium acetate (7.5 M) was then added, and DNA was subsequently precipitated with 2 volumes of chilled ethanol and incubated at 4 °C overnight. Afterwards, 2 ml PEG at 1 % final concentration was added and incubated at 4 °C for 1 h. The DNA was pelleted by centrifugation at ~ 13000 × g for 10 min at 4 °C, air dried and dissolved in 100 µl TE buffer for storage.

Bead beating along with saturated phenol + 1 % PEG

The first step of treatment with the extraction buffer was followed as described above. The sample was then blended with glass beads (5 g) for 15 min, followed by incubation at 70 °C for 2 h. Centrifugation was done at ~ 13000 × g for 10 min to collect the supernatant. The remaining steps were the same as those described in the soft lysis method of the Tris-saturated Phenol and 1 % PEG treatment.

Combination of soft lysis and bead beating along with saturated phenol + 1 % PEG

The first step of the method was the same as described in the above section. The sample was blended with 5 g glass beads for 15 min, followed by the addition of lysis buffer (4 ml) to the supernatant and incubation at 70 °C for 2 h under vigorous shaking at 15 min intervals. Supernatant was collected by centrifuging at ~ 13000 × g for 10 min and subjected to further procedures as described in the soft lysis method, along with the Tris-saturated phenol and 1 % PEG method.

Spectrophotometric assessments of the quality and yield of the extracted DNA

The humic acids and proteins are often co-extracted in metagenomic DNA. The humic acids absorb at $A_{230\ nm}$, while DNA and protein absorb at $A_{260\ nm}$ and $A_{280\ nm}$, respectively. To assess the quality of the extracted DNA, the $A_{260\ nm}/A_{230\ nm}$ (DNA/humic acid) and $A_{260\ nm}/A_{280\ nm}$ (DNA/protein) ratios were determined[13].

Agarose gel electrophoresis

The extracted DNA samples (12 µl) were mixed with 3 µl loading buffer and analysed on 0.8 % agarose gels using 1 × TAE as the electrophoresis buffer. Gels were stained with ethidium bromide and evaluated on the Gel Documentation System (BioRad, USA). A DNA marker (1 kb) (Lambda DNA/HindIII Marker, Mol Bio HiMedia, India) was used as a reference.

Construction of metagenomic libraries and screening for protease, amylase and lipase activity

Bacterial strains, plasmids and growth conditions

Escherichia coli JM109 was used as a host and pUC18 plasmid as a vector. *Escherichia coli* transformants were grown at 37 °C in Luria-Bertani (LB) (HiMedia, India) medium supplemented with 100 µg/mL ampicillin.

Digestion of the metagenomic DNA and ligation into the vector

Two micrograms of the extracted DNA were digested in 100 µl reaction mixtures with BamHI and SalI (New England BioLab, Inc.) for 1 h under the conditions as specified by the manufacturer. The digested samples were analysed on 1.2 % agarose gel with a broad range DNA marker (HiMedia, Mol Bio, India). The ligation reaction mixture contained, vector and insert DNA in 1:3 ratio, T4 DNA ligase, and a 2 × ligation buffer. The final reaction mixture volume was brought to 20 µl with sterile Milli-Q water and incubated at 16 °C for 20 h. The ligated mixture was subsequently used to transform *E. coli* JM109.

Preparation of the competence cells

Escherichia coli JM109 was grown in LB medium and incubated at 37 °C for 3 – 4 h at 180 rpm. The bacterial cells were transferred to sterile (50 ml) tubes when the culture reached an A_{600} of 0.6, and it was brought to 0 °C by incubating on ice for 10 min. The cells were collected by centrifugation at ~ 3200 × g for 10 min at 4 °C and the tubes were kept in an inverted position for 1 min to remove traces of the media. The pallets were suspended by gentle vortexing in 30 ml of the ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$). The competent cells were collected by centrifugation at 5000 rpm for 10 min at 4 °C. The pellets were then resuspended by gentle vortexing in 2 ml of ice-cold 0.1 M $CaCl_2$ solution for each 50 ml of the original culture. The competent cells were directly utilised in the transformation process.

Transformation

For transformation, 200 µl of the competent cells were added into a sterile, chilled microfuge tube with a pre-chilled and sterilised tip. Plasmid DNA or ligated vector was then added, and the tubes were gently swirled to mix. The tubes were stored on ice for 30 min followed by heat shock treatment for 90 seconds in a preheated 42 °C water bath without shaking. Tubes were then quickly transferred an ice bath for 1 – 2 min. To this, 800 µl of the LB media was added and the culture was incubated for 1 h at 37 °C. Subsequently, aliquots of 50 µl, 100 µl and 200 µl of the transformed cells were spread on the LB agar plates with ampicillin (100 µg/ml), IPTG (0.1 mM), X-Gal (40 µg/ml) and were incubated overnight at 37 °C[ref. 22].

Blue white screening and master plate of white clones

The recombinant clones of the metagenomic library were detected by Blue-white screening. The clones were inoculated on the LB ampicillin agar plates containing X-gal and IPTG and incubated overnight at 37 °C. After transformation, the blue (non-recombinants) and white (recombinants) colonies were screened.

Screening of the clones for Protease, Amylase and Lipase activity

The metagenomic library was screened for protease, amylase and lipase activities using gelatin, starch and *tributyrin* agar plates, respectively. The zone of clearance around the colony was measured. The recombinant clones were inoculated as spots on the gelatin and starch agar medium containing 100 µg/ml ampicillin and 0.1 mM IPTG. After 24 h incubation at 37 °C, the protease activity was detected by flooding the plates with Frazier's reagent and the amylase activity by flooding Gram's iodine. The ratio of the hydrolysis zone to the colony diameter was calculated for the relative enzyme secretion as a function of growth.

Protease production in liquid culture

A loopful of pure culture was added into 250 mL sterile gelatin broth, followed by incubation at 37 °C on a rotary shaker for 24 h. A 10 % inoculum from the culture (O.D. 1.0 at $A_{600\ nm}$) was inoculated into the gelatin broth. After incubation for 24 h at 37 °C under shaking conditions (150 rpm), the growth was measured at 600 nm. For 10 consecutive days, 2 ml of the culture was harvested and centrifuged at ~ 7200 × g for 10 min at 4 °C to prepare cell-free extracts, which were then used as crude enzyme preparation. Alkaline protease was estimated using the Anson Hagihara method[23] as described previously[24,25].

Result and Discussion

Soil characteristics

Table 1 describes the physicochemical properties of the salt-enriched soil samples. Soil sample-2, as compared to sample-1, contained high electrical conductivity, chloride, organic carbon and sulfate.

Metagenomic DNA extraction, quantification and assessment of the purity

A direct *in-situ* method was used for DNA extraction, which included soft and harsh lysis, as well as their combination, employing physical,

Table 1 — Physicochemical analysis of the soil samples

Characters	Sample 1	Sample 2
Sampling site	Mithapur	Dwarka
Soil appearance	Wet, Sandy, Reddish	Dry, Blackish
Electrical conductivity (dm/m)	67	75
Salinity (gm/L)	26.8	30.0
Chloride (gm/L)	42.60	74.65
Organic carbon (gm/Kg)	0.48	0.63
Sulfate (gm/L)	0.007	0.009
pH	7.9	7.7

chemical and enzymatic microbial cell disruption. Significant modifications were made to the methods of soft lysis, bead beating and their combinations[13]. Two commercial metagenomic DNA extraction kits: HiPurA^{TM} and NUCLEOSPIN® were also used for comparison. The extracted metagenomic DNA was high in purity and had a high molecular weight. In soft lysis, a lower quantity of DNA was obtained as compared to the combination of soft lysis and bead beating methods. While the bead beating method yielded a higher amount of DNA, the quality of which was relatively lower as compared to the soft lysis and the combination methods. It is essential that the extracted DNA is free of inhibitors and minimally sheared for its suitability in PCR, cloning and sequencing applications. The study suggests that physical methods alone were not as efficient for extracting DNA from the salt-enriched soil, due to the co-extraction of humic acid. Therefore, chemical, physical, and enzymatic methods in combination were found most suitable. Bead beating, a mechanical method, yielded homogenous cell disruption leading to high DNA yield. However, it's not a preferred method due to high DNA shearing. Nevertheless, in combination with chemical lysis, it's quite effective.

In the soft lysis method, proteinase K and lysozyme break down proteins and the bacterial cell wall to release the DNA. While the treatment with surfactants and chelating agents, such as CTAB, SDS, and N-Lauryl sarcosine, was useful in removing lipids. Combining bead beating and the enzymatic lysis method was most effective for higher yields of inhibitor-free DNA. As reported earlier, a combination of bead beating and lysis buffer yields a high quantity and high-quality environmental DNA from saline soils[12]. The inclusion of tris-saturated phenol and 1 % polyethene glycol 8000 (PEG 8000) enhanced the quality and quantity of the metagenomic DNA. HiPurA^{TM} Soil DNA and NUCLEOSPIN® Soil

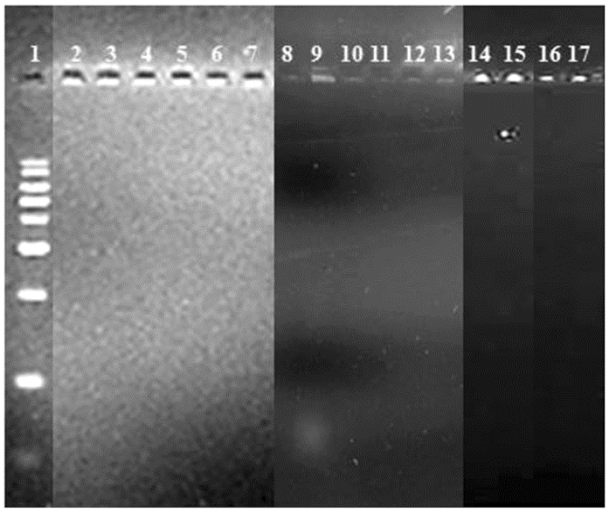

Fig. 1 — Agarose gel electrophoresis of the total metagenomic DNA extracted from samples 1 and 2 by various methods. Lane 1: 1 Kb ladder, Lane 2: Sample 1 soft lysis (MM), Lane 3: Sample 1 bead beating (MM), Lane 4: Sample 1 soft lysis + bead beating (MM), Lane 5: Sample 2 soft lysis (MM), Lane 6: Sample 2 bead beating (MM), Lane 7: Sample 2 soft lysis + bead beating (MM), Lane 8: Sample 1 soft lysis, Lane 9: Sample 1 bead beating, Lane 10: Sample 1 soft lysis + bead beating, Lane 11: Sample 2 soft lysis, Lane 12: Sample 2 bead beating, Lane 13: Sample 2 soft lysis + bead beating, Lane 14: Sample 1 HiPurA^{TM} Soil DNA isolation kit, Lane 15: Sample 2 HiPurA^{TM} Soil DNA isolation kit, Lane 16: Sample 1 NucleoSpin® soil kit, Lane 17: Sample 2 NucleoSpin® soil kit. MM - Modified Method

kits yielded a low quantity but high-purity DNA. Applications of commercial kits for the extraction of DNA from soil have been reported in several earlier studies[26-29]. The modified combination of soft lysis and bead beating, along with saturated phenol + 1 % PEG is the best DNA extraction method compared to other optimised methods. The molecular size of the extracted metagenomic DNA was above 10 kb, as judged on the agarose gel electrophoresis, suggesting its intactness devoid of shearing (Fig. 1). The DNA band patterns on the agarose gel were similar. Still, the band intensity varied from method to method. Figure 2 illustrates the spectrophotometric analysis of the extracted metagenomic DNA. Recently, the extraction of metagenomic DNA from a thermophilic water body using various approaches, followed by analysis of diversity and polymorphism, has been reported[30].

Restriction analysis and metagenomic libraries

The metagenomic DNA was double-digested by two restriction enzymes, SalI and BamHI. The digested DNA (3.5 kb) was analysed on the agarose gel. The Sau3AI digested metagenomic DNA from forest soil generated fragments ranging from 4 – 20 kb

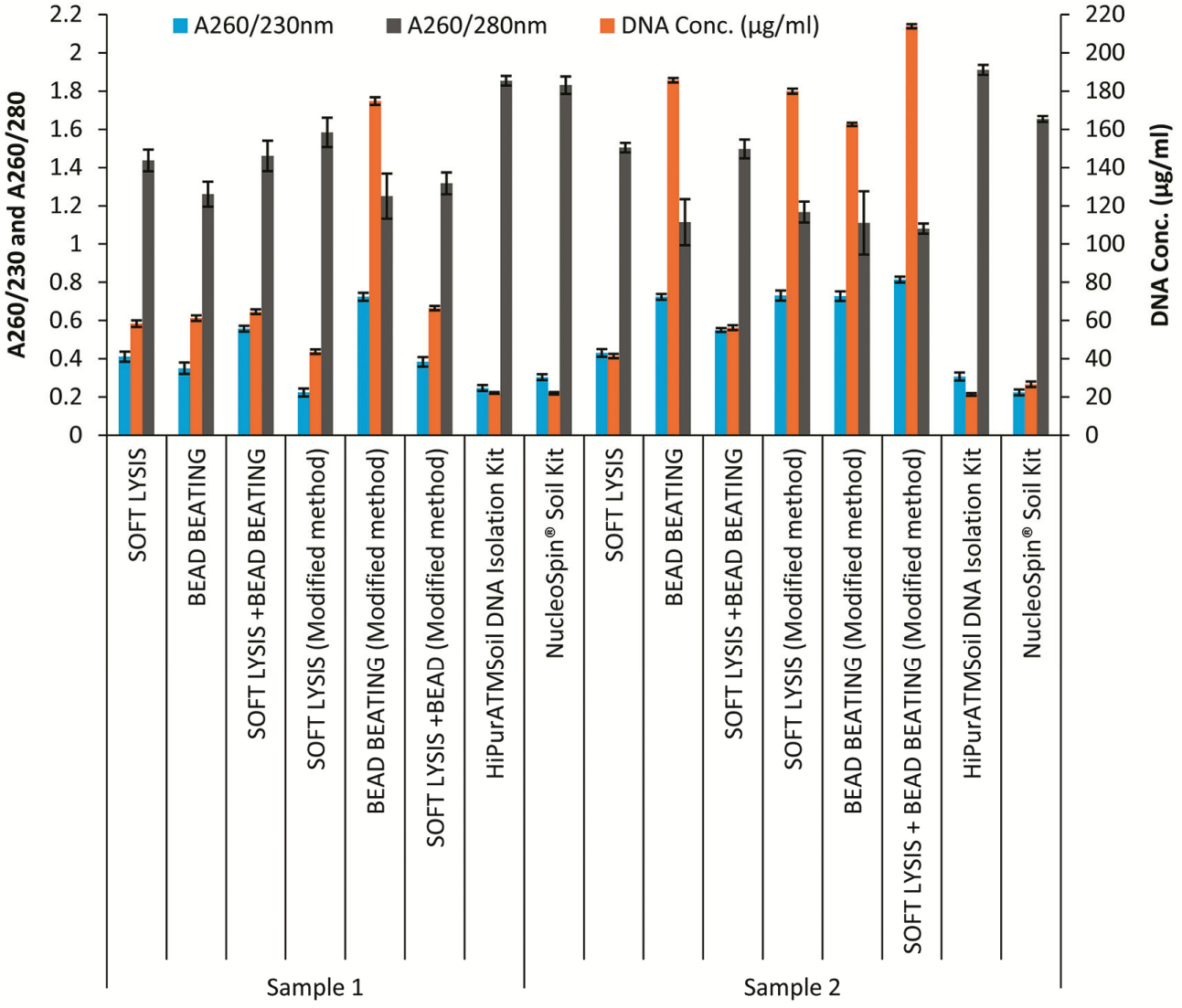

Fig. 2 — Purity and yield of metagenomic DNA

inserted into BamHI digested pHT01 vector[31], while EcoRI digested metagenomic DNA from various soils and compost yielded fragments of 3 – 8 kb[32]. The double-digested DNA was inserted into two different vectors, pUC 18 and pET 21a^{+}, followed by transformation into *E. coli* JM109. A total of 4 small insert metagenomic libraries were thus created. Till date, different plasmid vectors and host organisms have been used to construct the metagenomic library from different environments. pUC19 plasmid vector and *E. coli.* DH10B host was used for the cloning of the goat skin surface metagenome[33]. Similarly, pWEB-TNC plasmid vector and *E. coli* host were used to create a metagenomic library from marine soil sediments[34]. In another study, the Death Valley desert sand library was constructed using the pBSKII plasmid vector and *E. coli* DH10B as host[35].

One hundred ninety-one clones with an average insert size of 3.5 kb were obtained from the salt pan soil metagenomic libraries (Table 2). Among the 191 clones, 7 were positive for the proteolytic activity; 6 from sample-2 and 1 from sample-1 (Fig. 3). Five amylase-positive clones were detected from the metagenomic library generated from sample-1 using pUC18 vector and *E. coli* JM109 as host (Fig. 4). None of the clones in all libraries displayed lipase activity. The zone of hydrolysis was measured and plotted as a ratio of its size to the colony diameter (Figs. 5 & 6).

Table 2 — Transformants derived from salt pan soil metagenomic libraries displaying protease and amylase activities

Sr. No.	Samples	Vector	Host organism	Blue clones	White clones	Protease positive clones	Amylase positive clones
1	1	pUC18	*E .coli* JM109	79	23	1	5
2	1	pET 21a+	*E. coli* JM109	2	20	-	-
3	2	pUC18	*E. coli* JM109	7	23	6	-
4	2	pET 21a+	*E. coli* JM109	4	33	-	-

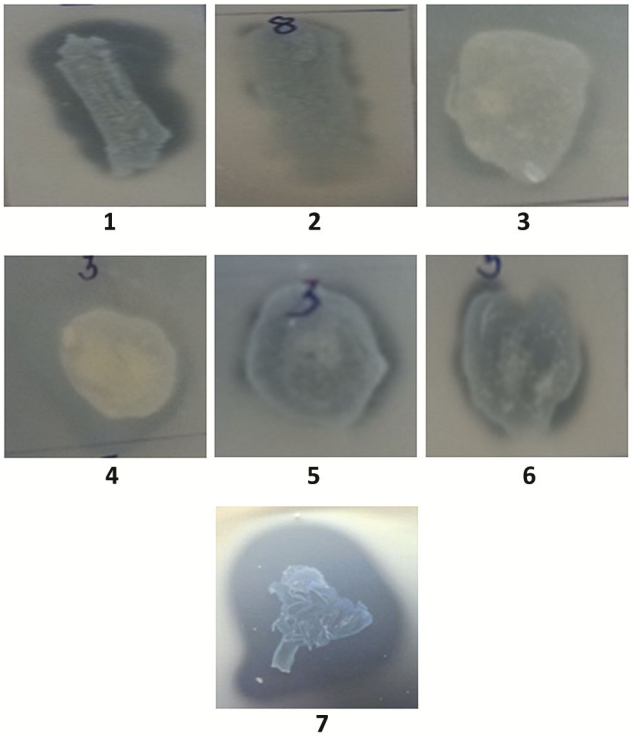

Fig. 3 — Protease positive clones 1-6 and 7 were derived from the metagenomic libraries constructed from the sample 2 (Dwarka) and sample 1 (Mithapur), respectively

There are only a few studies on the exploration of the proteases by the metagenomic approaches. A new oxidant-stable serine protease from a forest-soil metagenomic library[31], an alkaline serine protease from goat skin surface metagenome[33], a protease from the marine soil sediments[34], two serine proteases from the metagenomic libraries of the Gobi and Death Valley deserts[35], proteases from the Antarctic soil metagenome[36], an alkaline protease from the salt enriched soil[37], thermotolerant protease from hot spring sediment[38], and serine metalloprotease from estuarine sediment samples[39] have been reported in recent years. A novel α-amylase was detected in a soil metagenomic library constructed from the soil of the Western Ghats of Kerala[40]. A halotolerant α-amylase has also been reported from the Arabian Sea sediments metagenomic library[41]. Amylase and protease have been recently reported from mangrove soils using a fosmid metagenomic library[42].

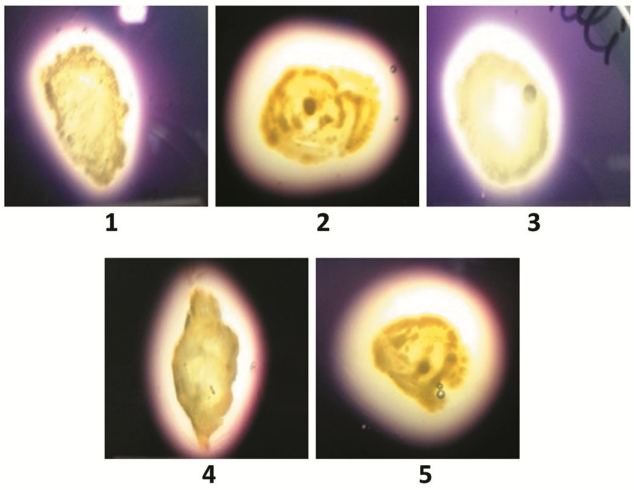

Fig. 4 — Amylase positive clones 1 – 5 derived from the metagenomic libraries constructed from the sample 1 (Mithapur)

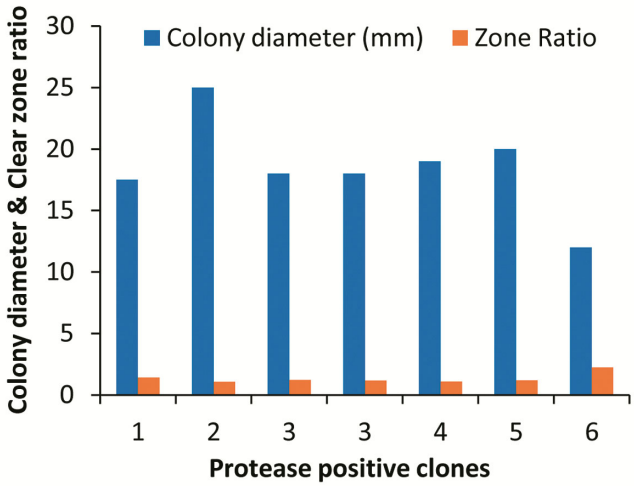

Fig. 5 — Protease secretion by the positive clones as reflected by the ratio of the zone of clearance and colony diameter

In the current study, the extracellular protease production was detected in gelatin broth. A protease activity of 16.36 U/mL was detected for clone 1 after 96 h of growth (2.86×10^9 cells/ml), while other active clones produced proteases at different times of growth. The optimum enzyme secretion was detected after 144 h, with a sharp decline at the end of the exponential phase of the host. Growth was measured in terms of an increase in the number of cells. Figure 7 shows the highest protease enzyme activity

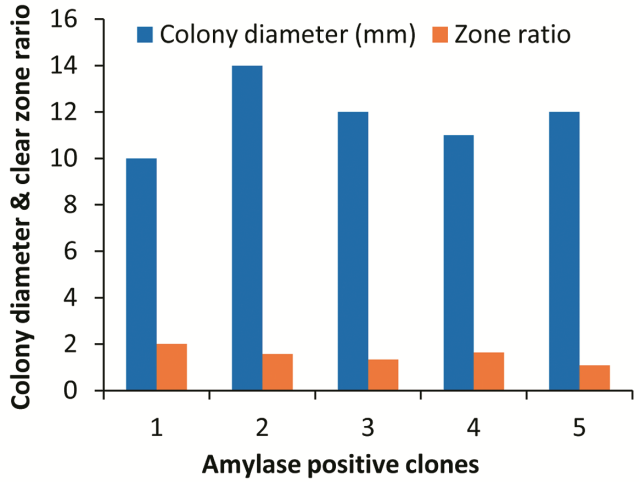

Fig. 6 — Amylase secretion by the positive clones as reflected by the ratio of the zone of clearance and colony diameter

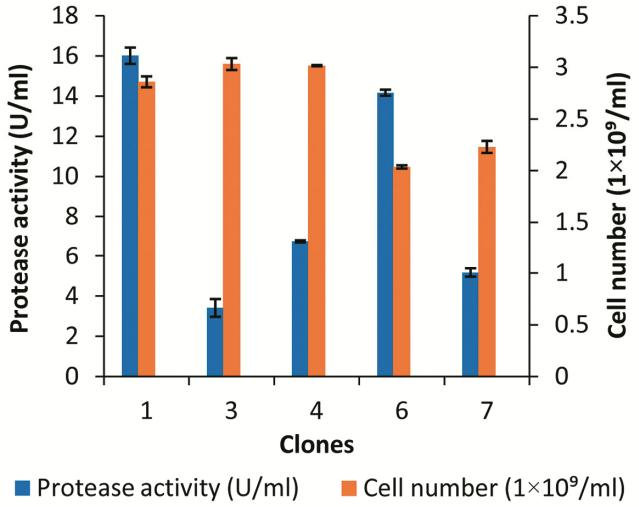

Fig. 7 — Protease enzyme production and growth study of 5 protease clones

detected at that particular cell number per ml. Protease production has recently been studied in some cultivable haloalkaliphilic bacteria and actinomycetes from saline habitats[24,25,43]. However, the exploration of the metagenomically derived proteases needs to intensify.

Conclusion

Metagenomics of extreme habitats offers new perspectives into the microbial diversity and the vast world of uncultivable microorganisms, their metabolic potential and biomolecules. In this study, DNA extraction methods to obtain the total environmental DNA of high quality in significant yield were established. Metagenomic libraries created from the extracted DNA were screened for the hydrolytic enzymes. The study is noteworthy for the exploration of the microbial diversity and enzymatic potential of the largely unexplored saline habitats.

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Conflict of Interest

All authors declare that there are no conflicts of interest.

Ethical Statement

This study did not involve using humans, animals, or procedures that could harm living organisms.

Author Contributions

Conceptualization: SPS & NMR; Samples and physicochemical analysis: KGD & SPS; Experiments: NMR; Data analysis: NMR, KGD & SPS; and Manuscript writing, reviewing & editing: NMR & SPS.

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