ISOLATION, SCREENING AND CHARACTERIZATION OF HALOTOLERANT ENZYMES OF EXTREMOPHILES ISOLATED FROM KELAMBAKKAM SALTERN POND

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ABSTRACT

The study of extremophiles provides an understanding of the physicochemical parameters defining life on Earth and may provide insight into how life on Earth originated. The postulations that extreme environmental conditions existed on primitive Earth and that life arose in hot environments have led to the theory that extremophiles are vestiges of primordial organisms and thus are models of ancient life and also are research importance in the field of Biotechnology. However, Halotolerant enzymes have been reported to be active and stable under more than one extreme condition and also extremophiles are excellent sources of enzymes that are not only salt stable but also can withstand and carry out reactions efficiently under extreme conditions (sumit *et al.*, 2012). The objectives of present study was to study extremophiles, and to isolate, screen and characterize the halo tolerant enzymes of extremophiles isolated from kelambakkam saltern pond, Chennai, Tamil Nadu, India. Primary and secondary screening for both protease and amylase production was carried out. Based on the secondary screening of the both amylase and gelatinase producing isolates, the isolate KAA3 showed prominent activity in both of the screening. Hence the isolate KAA3 was taken for further analysis. Qualitative enzymes assay for both amylase and gelatinase enzymes produced by isolates KAA3 and Quantitative amylase and gelatinase enzyme production was carried out. Determination of Primary enzyme production using dual Substrate method surprisingly showed that selected isolate was produced only gelatinase and no amylase have been produced. Both qualitative and quantitative analysis revealed that the presence of gelatinase and no amylase was observed. This is the first of kind

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study have been conducted and confirmed as proteases are the primary enzyme than their counterpart of amylase. The biochemical and physiological characterization of selected enzyme producing isolate KAA3 conclusively proved that isolates KAA3 is rod shape, Gram negative and belong to the taxa ***Proteus mirabilis.*** Zymogram analysis for both amylase and protease enzyme was carried out which revealed that, the selected isolate having two different isoform in amylase with different molecular weight/protein ionic strength and having single isoform in gelatinase enzymes. It was concluded that, the isolate KAA3 can produces both amylase and protease.

Keywords; extremophile, halotolerant, amylase and protease.

INTRODUCTION

Bacteria live in diverse ecological conditions from extreme cold (Antarctica) to hot conditions (hot springs), mesophilic soil to extreme saline conditions of different pH [Gibbons NE 1969.]. Adaptability of the bacteria to such diverse conditions has immense fundamental and applied importance. Besides naturally occurring saline environment, over utilization of ground water is increasing the saline area and about 25% global cultivated area shows excessive salinity [Chowdhury JB et'al., 1993]. Therefore, study and investigation of extremophiles of saline's environment has significant roles biotechnology. Halo tolerant enzymes have enzymatic activities at high salinities and also used in the manufacture of food products, paper products and polysaccharide containing materials, oligosaccharides and they are isolated from ***halophiles or halo tolerant*** organisms.

The **Halophiles** are organisms that live in highly saline environments, and require the salinity to survive, while **Halotolerant** organisms (belonging to different domains of life) can grow under saline conditions, but do not require elevated concentrations of salt for growth. The halotolerant and halophilic organisms are important for maintenance of soil health and nutrition recycling in saline environment too [Vreeland R.H 1996].

Systematic and phylogenetics studies have defined a large number of species to be included within the hyper saline environment, distributed over atlest half of the major phylogenic branches of bacteria. Molecular ecology techniques available nowadays should be used to determine in more detail the ecological distribution of these halophilic microorganisms and the

role they play in hyper saline environments, as well as their contribution to microbial transformation processed. The use of such techniques would enable the elucidation of the biodiversity of extromiphilic bacteria and the identification of species that constitute the predominant populations in these extreme habitats. Hence, it was decided to investigate the systematic morphophysiological, biochemical characters studies on natural saline habitats pond and isolation of halo tolerant enzymes of extremophiles isolated from kelambakkam saltern. However, present study specifically would isolate Amylase and protease from isolated extremophiles. Amlyase are one of the most significant enzymes in biotechnology that had a share of approximately 25% of world enzymes market (Rajagopalan and Krishnan, 2008) and also isolation and identification of soil microorganisms with best amylase activity could contribute a lot for the discovery of novel potential amylases appropriate for industrial and biotechnological applications (Mohapatra *et al*., 2003). The objectives of present study were to screen, isolate and characterize halo tolerant enzymes of extremophiles isolated from kelambakkam saltern pond.

Materials and method

Site description and sample collection

Kelambakkam is a rapidly growing suburb of Chennai in Tamil Nadu state in India, Asia. It is situated on the Old Mamallapuram Road. Its geographical coordinates are $12^0$48' 0" North, 80^0 13" 47" East and its population in this area is around 20,000.

For present study, samples were collected from three locations of Kelambakkam saltern pond, Tamil Nadu, India. Top layer of soil sample (about 1cm) was removed, sample were mixed thoroughly and put in sterilized polythene packets with proper levels named as sample (1), sample (2) and sample (3).

Isolation of Bacteria

One gram of soil samples were serially diluted in sterilized physiological saline water containing Nacl to get a concentration range from 10^{-1} to $10^{-6}$and a volume of 0.1 ml of each dilution was plated aseptically to nutrient agar amended with 5% NaCl plates. The sample was spread uniformly and also the plates were incubated at 37°C for 3 days.

Primary Screening for amylase Production

The microbial isolates were streaked on the starch agar plates to obtain pure culture and incubated at 37°C for 48 hours. The cultures were flooded with iodine solution dropper for 30 seconds on the starch agar plate to indicate clear zone of hydrolysis (Marc J.E.C *et al*., 2002).

Secondary Screening of selected Isolates for Amylase Production

Based on the primary screening for amylase production, four isolates were taken for the secondary screening. The same starch nutrient agar was used. The Gram's iodine was used to detect the amylase activity by the isolates.

Primary Screening for Protease Production

The microbial isolates were streaked on the gelatin agar plates to obtain pure culture and incubated at 37°C for 48 hours. The cultures were flooded with Ammonium sulphate solution dropper for 30 seconds on the starch agar plate to indicate clear zone of hydrolysis (Marc J.E.C *et al*., 2002).

Secondary Screening of Selected Isolates for Gelatinase Production

Based on the primary screening for gelatinase production, four isolates were taken for the secondary screening. The same gelatin agar was used. Saturated ammonium sulphate was used to detect the gelatinase activity by the isolates.

Enzyme assay for amylase enzyme

A suitable volume of bacterial culture broth was centrifuged at 5000 rpm for 20 min at 4°C. Cell free supernatant recovered by centrifugation was used for amylase activity and considered equivalent to crude enzyme. 1.0ml of culture extract 'enzyme' into a test tube and 1ml of 1% soluble starch in citrate-phosphate buffer (pH 6.5) was added in test tube. The test tubes were covered and incubated at 30^0c for 45 minutes in water bath. Then 2.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 5 minutes. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer.

Enzyme assay for protease enzyme

The enzyme activity was assayed by the modified method of kunitz using casein as a substrate. A suitable volume of bacterial culture broth was centrifuged at 5000 rpm for 20 min at 4°C. Cell free supernatant recovered by centrifugation was used for amylase activity and considered equivalent to crude enzyme. 1.0ml of culture extract 'enzyme' into a test tube and

1ml of 1% soluble starch in citrate-phosphate buffer (pH 6.5) was added in test tube. The test tubes were covered and incubated at 30^0c for 45 minutes in water bath. Then 2.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 5 minutes. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer (Hadj-Ali *et al*., 2007).

Selection of best isolate for enzyme production

Based on the primary and secondary screening of the tested enzymes, amylase and gelatinase production, the best isolates which produces both amylase and gelatinase was selected for further analysis.

Determination of Primary enzyme production using dual Substrate method

Dual substrate media (starch and gelatine) was used to determine primary enzyme production. The sterilised broth was inoculated with selected isolates and kept in a rotary shaker for 48 h. After incubation, the culture free supernatant was collected after get centrifuged. The culture filtrate was used for the enzyme detection using qualitative method of starch and gelatine agar hydrolysis methods.

Biochemical test

The selected bacterial isolate was subjected to various biochemical and staining techniques as described and the key provided in the Bergy's Manual of Determinative Bacteriology (Holt *et al.*, 2004)

Biochemical test were performed as suggested by Garrityet et al., 2004, which include following tests Citrate utilization test, Methyl red test, Nitrite test, Oxidase test, Voges proskauer (VP) test, Urease test, Indole production test, Catalase test, Starch hydrolysis test, Gelatin hydrolysis, Gram staining and Triple sugar iron (TSI) test.

Estimation of Protein Using Bradford Reagent

The protein content in the tissue culture samples was estimated according to the dye binding method of Bradford (1976). One ml of the culture filtrate was added with 5 ml of the Bradford's reagent (CBB) and the intensity of the blue colour that developed was read at 595 nm in a spectrophotometer. The amount of protein was determined using bovine serum albumin fraction V (Sigma, USA) as the standard.

RESULTS AND DISCUSSION

Sample collection

All the three samples, sample (1), sample (2) and sample (3) were collected from three different locations of Kelambakkam saltern pond, Kancheepuram Distric, Tamil Nadu, India. This result of sample collection was in agreement with Dr.F. Emerson (2013).

Isolation of Enzymes producing Bacteria

Microorganisms present in Estuary soil are in high numbers. The exact number could not be found out when their discrete colonies are not growing in the medium. The isolation of enzymes producing bacteria was done by using serial dilution agar plate method. The principle of serial dilution agar plate method is that when microorganisms are grown through this method possibly all the live propagules will grow and develop individual colony. A total of 24 colonies were isolated from three different samples collected, Kelambakkam saltern and all of them were named as KAA1-KAA24 (Table 1).

Sample Code	Isolate Code
I	KAA1 – KAA8
II	KAA9-KAA20
III	KAA21 – KAA24

This result was corresponded with work done by N. Amaresa (2013) and Dr.F. Emerson (2013) who determined the heterophilic Bacterial population on Nutrient Agar medium.

Primary Screening for amylase Production

All the 24 selected isolates were subjected to primary screening on the starch nutrient agar media in which Gram's iodine was used to detect the amylase activity by the isolates and zone of clearance was observed. Among the 24 isolates, only four isolates were showed high clear zone and they were selected for further secondary screening of amylase production (Table 2).

Isolates Code	Zone of clearance (cm)
KAA1	2.5
KAA2	2
KAA3	2.1

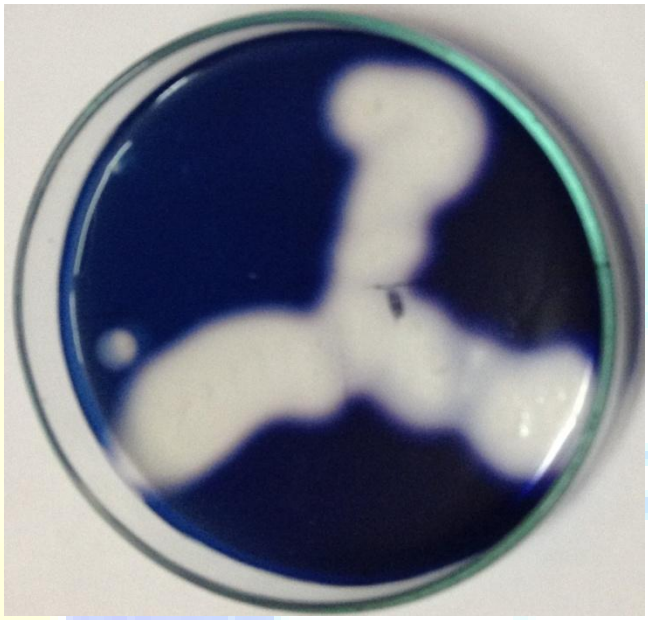
KAA4	0.1
KAA5	1.5
KAA6	1
KAA7	-
KAA8	-
KAA9	1.5
KAA10	0.4
KAA11	0.8
KAA12	0.6
KAA13	1
KAA14	0.6
KAA15	0.8
KAA16	2
KAA17	-
KAA18	0.2
KAA19	0.3
KAA20	-
KAA21	2.5
KAA22	-
KAA23	-
KAA24	-

This result was corresponded with Anbazhagan Mageswari *et al.*, (2012), Berhanu Andualem (2014) and Sumit kumar *et al.*, (2012) who reported the isolates producing enzymes were selected based on ratio of zone of clearance and The production of amylase and rate of bacterial isolates growth were analyzed in the medium containing 1% starch as source of carbon and 0.5% peptone as a source of nitrogen.

Secondary Screening for amylase Production

Among the 24 colonies screened, 4 isolates (KAA1, KAA3, KAA9 and KAA21) were showed prominent amylase production in primary screening and they were subjected to

secondary screening on the same starch nutrient agar media for the consistence of the isolate amylase producing ability. Based on the secondary screening of the amylase producing isolates, the isolate KAA3 showed prominent activity in both of the screening. Hence the isolate KAA3 was taken for further analysis It was agreed with Sumit Kumar *et al*., (2012) result who reported isolate was selected based on hydrolytic activity and zone of clearance (Figure 1).



Primary Screening for Protease Production

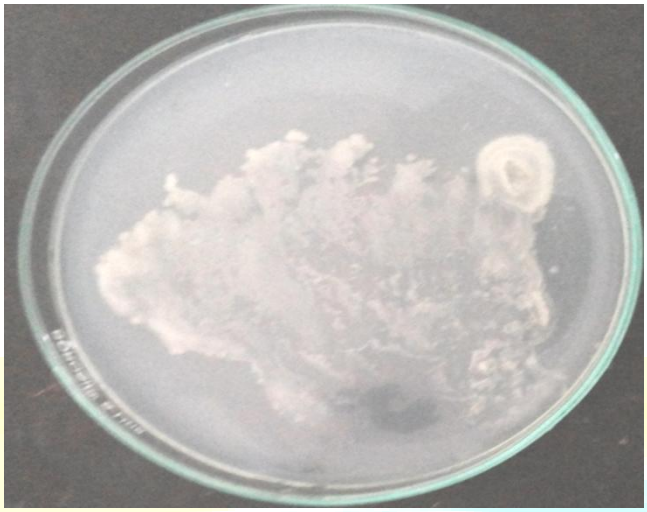
The 24 isolates from Kelambakkam Saltern were subjected to primary screening on the gelatin agar media in which saturated ammonium sulphate was used to detect the gelatinase activity by the isolates and zone of clearance indicated the positive production of gelatinase. Four isolates were showed high clear zone and they were selected for secondary screening.

Isolates Code	Zone of clearance (cm)
KAA1	1.8
KAA2	-
KAA3	3
KAA4	2.5
KAA5	-
KAA6	-

KAA7	-
KAA8	-
KAA9	4
KAA10	-
KAA11	3
KAA12	3.2
KAA13	-
KAA14	-
KAA15	2.5
KAA16	-
KAA17	-
KAA18	-
KAA19	-
KAA20	-
KAA21	2
KAA22	-
KAA23	-
KAA24	-

Secondary Screening for Protease Production

Among the 24 colonies screened, 4 isolates (KAA3, KAA9, KAA11 and KAA12) were showed prominent protease production in primary screening. They were subjected to secondary screening on the same gelatin agar media for the consistence of the isolate protease producing ability. The isolate KAA3 produces surplus gelatinase enzyme and hence it was selected for further analysis. This result was in agreement with C. Suganthi (2013) who reported the total of 15 halotolerant bacteria from saltern pond have been screened for the presence of protease production and six strains were identified as protease producers by zone of hydrolysis around the colonies(figure 3).



Based on the secondary screening of the both amylase and gelatinase producing isolates, the isolate KAA3 showed prominent activity in both of the screening. Hence the isolate KAA3 was taken for further analysis (Figures 4).

Qualitative enzyme assay for amylase produced by selected isolate KAA3

The qualitative amylase enzyme assay was performed using starch agar plate assay. The starch nutrient broth grown culture filtrate was used in this study. Different concentration of culture filtrate was taken for the enzyme analysis. The increased concentrations of enzyme also increase the zone of clearance. This result was contestant with work of (Dhara Desai, Trupti K (2014 and Hansa *singh et al., 2014*) who reported that quqlitative determination of amylase

performed using well cut and zone of clearance indicate presence of zone of hydrolysis (Table 4).

Table 4: Zone of clearance of amylase activity of KAA3

Culture filtrate concentration	Zone of clearance (mm)
25 µl	11
50 µl	13
75 µl	17
100 µl	19

Qualitative enzyme assay for gelatinase produced by selected isolate KAA3

The qualitative gelatinase enzyme assay was performed using gelatin agar plate assay. The gelatin nutrient broth grown culture filtrate was used in this study. Different concentration of culture filtrate was taken for the enzyme analysis. The increased concentrations of enzyme also increase the zone of clearance (Table 5).

Table 5: Zone of clearance of gelatinase activity of KAA3.

Culture filtrate concentration	Zone of clearance (mm)
25 µl	16
50 µl	19
75 µl	22
100 µl	24

Quantitative amylase enzyme production

The DNS method was used to determine the amylase activity using the selected isolate KAA3 grown on starch nutrient broth. The amylase was produced by selected isolate KAA3 as 12.5 units/ml of culture filtrate and the protein was produced as 56µg/ml and the specific activity was 223 units/mg proteins. This result was in agreement with work of (Hansa Singh*et al*., (2014).

Quantitative gelatinase enzyme assay

The protease enzyme was determined using the method of Kunitz (1982) spectrophotometrically and casein was used as substrate. The selected isolate KAA3 was grown on gelatin nutrient broth and used for the analysis. The selected isolate KAA3 was produced 7.5 units/ml and the specific activity was 230 units/mg proteins. (Alka S. Petkar (2013).

Determination of Primary enzyme production using dual Substrate method

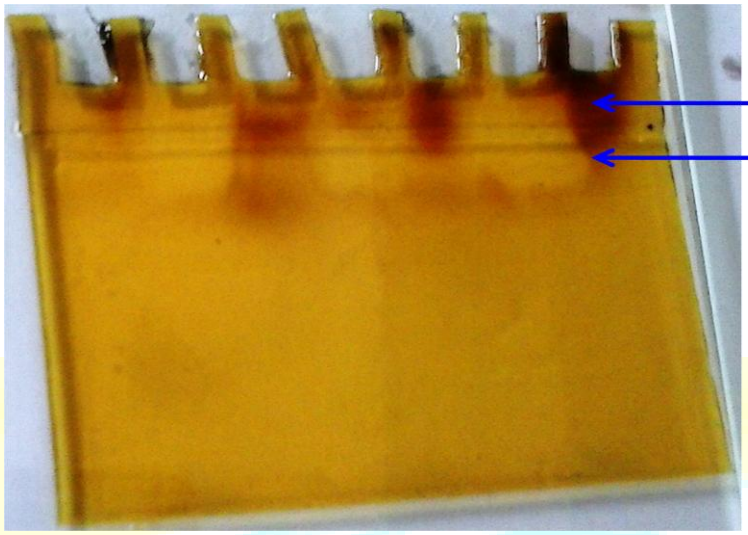
The selected isolate was cultured with dual substrate culture media having blended with starch and gelatin for enzyme production. Surprisingly the selected isolate was produced only gelatinase and no amylase have been produced. Both qualitative and quantitative analysis revealed that the presence of gelatinase and no amylase was observed. This is the first of kind study have been conducted and confirmed as proteases are the primary enzyme than their counterpart of amylase.

Taxonomical characterization of producer isolate KAA3

Biochemical test were performed as suggested by Garrityet et al., 2004, which include following tests Citrate utilization test, Nitrite Test, Oxidase test, Voges proskauer (VP) test, Urease test, Indole production test, Catalase test, Starch hydrolysis test, Gelatin hydrolysis, Gram staining and Triple sugar iron (TSI) test. The selected isolate KAA3 was positive to citrate, Voges Proskover, Triple Suger Test, Catalase Test, Starch Hydrolysis and Gelatin Hydrolysis but negative to Indole Production Test, Nitrate Test, Urease Test, Oxidase Test, Gram Stain and Methyl red test. This result was showed that isolate KAA3 is an extremophile live in kelambakkam salern pond and also the method used was based on work of Holt *et al.*, (2004). Based on the biochemical and physiological characterization the selected enzyme producing isolate KAA3 is belong to the taxa ***Proteus mirabilis.***

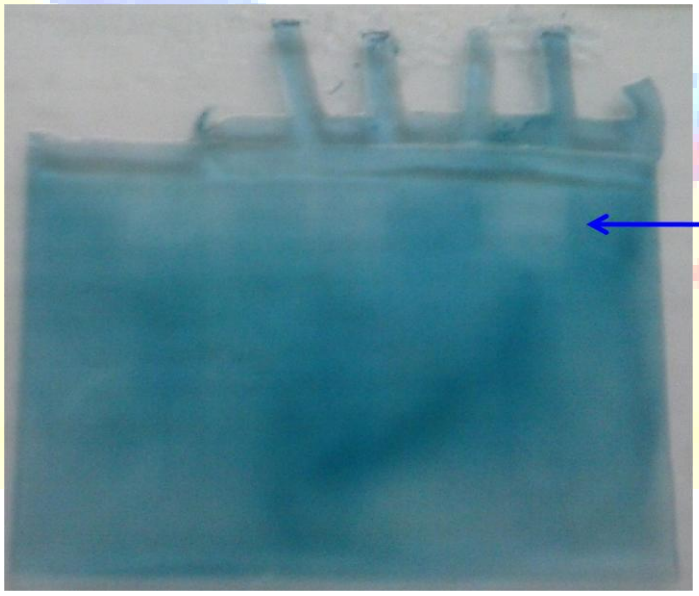
Zymogram analysis of amylase

The native poly acrylamide gel electrophoresis was performed to analyse the amylase isoform in the culture filtrate. The amylase enzyme used in the zymogram effectively digested the substrate and found with zone of clearance. The zymogram analysis revealed that, the selected isolate having two different isoform with different molecular weight/protein ionic strength. This result was contrary with Murakami et al (2007), who reported two -amylase from *Bacillus halodurans* 38C-2-1 with molecular weights of 100 kDa and 75 kDa; similarly Caf et al (2013) have reported two -amylase from *Bacillus* sp. AC-7 with molecular weights of 174 kDa and 137 kDa and also with Yasemin CAF et'al.,(2014) who also reported zymogram analysis showed the presence of single activity band with molecular weights of 114kDa.



Zymogram analysis of protease (gelatinase)

The non denatured poly acrylamide gel electrophoresis was performed to analyse the gelatinase isoform in the culture filtrate. The zone of clearance around the band indicated the presence of the galatinase enzyme. The zymogram analysis revealed that, the selected isolate having only one type gelatinase (Figure 7). This result was contrary with the work of Toshikazu Nishiwaki (2009) who confirmed the proteolytic activity of the ProGf by zymography and reported four clear bands on the native PAGE gel and was in agreement with Dhara Desai (2014) who reported activity stain by zymogram showed clear band indicates protease enzyme.



CONCLUSION

In the present study, we have isolated ***Proteus mirabilis*** from Kellambakam saltern pond.It is a rod in shape and gram negative. We also find out that the selected isolate (KAA3) when was cultured with dual substrate culture media having blended with starch and gelatin for

enzyme production. Surprisingly the selected isolate was produced only gelatinase and no amylase have been produced. Both qualitative and quantitative analysis revealed that the presence of gelatinase and no amylase was observed. This is the first of kind study have been conducted and confirmed as proteases are the primary enzyme than their counterpart of amylase.

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