International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 5, 2016

Original Article

EXTRACELLULAR ALKALINE PROTEASE PRODUCING HALO-ALKALITOLERANT BACTERIA ISOLATED FROM MARINE COASTS OF ODISHA

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Received: 27 Feb 2016 Revised and Accepted: 08 Apr 2016

ABSTRACT

Objective: The objective of the present study was on the isolation of alkali-tolerant bacteria from sediment samples of different coasts of Odisha, having potentiality to produce alkaline protease.

Methods: About 25 sediment samples were collected and analyzed for pH and moisture contents. Then isolation of alkali-tolerants was done using Horikoshi media at 10.3 pH. Isolates were analyzed for producing alkaline protease by plate assay method both at pH 6 and 10. Effects of temperature on protease production were also determined. Besides a new method of quantification of enzymes were adapted. Along this the isolates were partially characterized and identification was done using PIBWin software.

Results: About 80 isolates were initially isolated, and 11 isolates were considered based on maximal zones of clearances at alkaline pH. Maximum solubilisation index (SI) was found to be 30 mm by 3 isolates viz. AP2, AP8 and AP13 while maximum hydrolytic run percentage (HR%) was found to be 65.39% by AP3. About 45.46% isolates had capability for protease production at 37 °C and 18.18% at 57 °C while 81.82% isolates showed production at 17 °C. AP8 was the good producers of alkaline protease having SI 39 mm at pH 10 while incubating at 47 °C. Isolates were characterized partially by cultural, morphological, biochemical and physiological tests, which were belonged to the genera of *Bacillus, Virgibacillus* and *Micrococcus*. The isolated bacteria showed growth at pH ranges from 4-12 and can tolerate 12% NaCl concentrations for their growth.

Conclusion: Due to the above unique features and capability to produce alkaline proteases by the marine isolates, can be used significantly in various industries.

Keywords: Alkali-tolerant, Alkaline protease, Halo-tolerant, Hydrolytic run, Odisha-coast

INTRODUCTION

'Extreme' or 'normal', are those two words that differentiate the extremophiles from other microorganisms. Alkaliphiles are those microorganisms, which can thrive optimally at pH above 9 and showing little or no growth at near neutral pH. Nevertheless, alkalitolerants are capable of growing over a wider range of pH, with pH optima at approaching neutral pH [1]. Alkaliphilic microorganisms are widely distributed in nature and can be found in almost all environments without the restriction of alkalinity. Even so, a few of the naturally-occurring alkaline environments, namely soda soils, lakes, and deserts, harbor a wide range of these types [2]. The dilute alkaline springs, desert soils and oils containing decaying proteins or forest soil also act as the habitat for them, where the pH values of these environments are commonly around 10 and above [3-6]. There are many physiological differences in the adaptive nature of alkaliphiles than the neutrophiles. The major difference is in the cell wall; alkaliphiles have cell wall containing acidic polymers such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, and phosphoric acid [7]. However, they also have an excess of hexoamines and amino-acids in peptidoglycan layer. Alkaliphiles usually require at least sodium ions for growth as it is essential for effective solute transport [8].

Proteases or exopeptidases (EC 3.4.11-18) are a complex group of hydrolytic enzymes capable of hydrolyzing the peptide bonds in a protein molecule to amino acids and smaller peptides [9]. Proteases are classified into 4 major groups, i.e. serine-, thiol-, metallo- and acid proteases [10]. Further, it was sub-grouped assuming the side chain specificity of the enzyme and properties of their active centers [11]. The protease types depend on the composition of the production medium. Therefore, one microorganism can produce various types of proteases.

Microbial proteases have immense applications as compared to other hydrolytic enzymes in various spheres of science and industries and there is a high demand of alkaline protease in detergent industries, which make it as a potent enzyme. Besides, utilizing in detergent industries alkaline proteases can be used in bioremediation, food and dairy industries, leather processing and bio-film degradation [12].

Proteases can be available from plants, animals and microorganisms. However, proteases from microbial source are preferred due to diversified uses and compatibility. There are many reports of getting alkaline proteases from bacteria and fungi. The dominant genera of bacteria producing alkaline proteases are *Bacillus* and *Pseudomonas* due to their survival at extreme alkalinity. About 15 alkaliphilic aerobic bacteria from soil and water samples of Egyptian Soda Lake reported, producing alkaline protease (61 U/ml) at 48 h of incubation [13]. About 20 alkaliphilic were isolated bacteria from industrial effluents, Kott Lakh-Patt, Lahore, Pakistan and belonged to the genera of *Escherichia, Alcaligenes, Aeromonas, Pseudomonas, Marinococcus, Neisseria, Natronobacterium, Micrococcus, Sporosarcina, Pleisomonas and Cupriavidus* [14]. Dodia and co-workers isolated 8 moderately halophilic and alkaliphilic bacteria from saline habitat of coastal Gujarat, India [15].

There are very few reports on alkaline protease producing bacteria from the coastal region of Odisha. Therefore, the present study focuses on the isolation of alkali-tolerant bacteria having the potentiality to produce alkaline protease and quantifying the enzyme activity by a new method i.e. determining hydrolytic run percentage.

MATERIALS AND METHODS

Sample collection and analysis

A total of 25 sediment samples were collected from 5 sites of coastal regions of Odisha i.e. Chandipur (21.47 ° N, 87.02 ° E), Chilika

(19.7167 ° N, 85.318 ° E), Gopalpur (19.27 ° N, 84.92 ° E), Puri (19.81 ° N, 85.83 ° E) and Paradip (20.2654 ° N, 86.68 ° E). All the sediments were collected in sterile plastic polythene and were brought to the Department of Microbiology, O. U. A. T., BBSR (Odisha) for further study. After collection of samples, pH and moisture content were calculated accordingly.

Sediment pH was determined by taking about 10 g of samples, weighed into a 50 ml size beaker and 20 ml of 0.01 M $CaCl_2$ (1.47 g $CaCl_2.2H_2O$ in 1000 ml deionize water) was added to the sample [16]. The preparation was allowed to stand for 30 min with occasional stirring before determination of pH of the supernatant by digital pH meter 335 (Systronics, India).

For moisture content, about 10 g of sediment from each sample was taken and dried at 60±1 °C for 24-30 h in hot air oven and percentage of moisture content was determined [17]:

$$\text{Moisture content (\%)} = \frac{\text{Weight before drying} - \text{Weight after drying}}{\text{Weight before drying}} \times 100$$

Isolation of alkali-tolerant bacteria

For the isolation of alkaliphilic or alkali-tolerant bacteria, Horikoshi medium was used, which comprises (g/l: glucose, 10.0 g; peptone, 5.0 g; yeast extract, 5.0 g; KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.2 g; Na_2CO_3, 10.0 g; NaCl, 40.0 g) having 10.3 pH [18]. About 1 g sediment sample was inoculated in the above 100 ml medium. After 96 h, serial dilution was carried out in Ringer's solution and 0.1 ml of diluents was spread on the sterilized pre-cooled nutrient agar plate (pH 8) with Fluconazole (0.015% w/v). Then the plates were incubated at 37±1 °C for 24 h. The CFU/g of each sediment sample was calculated. The strains were maintained on nutrient agar slants (pH 8) at 4 °C for further study. Bacterial isolates were revived in an interval of 2-3 w.

Screening for alkaline protease production

For the production of alkaline protease, all the isolated bacteria were screened on skim milk agar (Hi-media) maintained at pH 6 and 10, to differentiate the nature of enzyme. Isolates were spot inoculated on the above agar plate incubated at 37±1 °C for 96 h. The zone of clearance around the colony was regarded as positive for enzyme production. Besides, another direct method was applied for isolation and screening of alkaline protease producing bacteria. For that the serially diluted sediment samples were directly applied to skim milk agar (pH 10) and the isolates showing zone of clearances at 37±1 °C were selected for further study.

Qualitative optimization of alkaline protease

Qualitative optimization was done in skim milk agar plates by using potent isolates. It was based on the solubilisation index (SI), which was calculated by subtracting the colony diameter from the total diameter. The optimizing parameters investigated were pH (4, 6, 8, 10 and 12) and temperatures (17, 27, 37, 47 and 57 °C).

Quantitative screening of alkaline protease

In addition to the plate assay method another new method was created for the quantitative determination of alkaline protease by "Hydrolytic Run (HR)" method. This method was conducted in tubes. Skim milk agar (Hi-media) was prepared having 10 pH and distributed equally to test tubes having 5 ml each. After autoclave the height of the stab was measured in each case and aseptically 0.1 ml bacterial isolates (prepared in nutrient broth having pH 8 and 24 h old) were applied and incubated at different temperatures of 27, 37 and 47 °C for 3 d. After every 24 h, HR% was calculated by the following:

$$\text{Hydrolytic Run percentage (HR\%)} = \frac{R - r}{R} \times 100$$

Where "R" denotes, the actual height of the assay stab medium and "r" denotes, the height left un-hydrolyzed. By this new method the potentiality of isolates were measured. The optimizing parameter investigated by using the above method was incubation temperatures (27, 37 and 47 °C).

Partial characterization of bacterial isolates

Afterward the screening of alkaline protease producers, isolates were subjected for partial characterization by cultural (colour, texture, margin, elevation, density and size), morphological (Gram variability), standard biochemical and physiological tests (pH and temperature). The results were analyzed in PIBWin Software for probabilistic identification of bacteria [19].

Statistical analysis

The data recorded during the investigation was subjected to Pearson's correlation coefficient determination (r), Student's T-test and Analysis of Variance (ANOVA), and the conclusions were drawn accordingly.

Table 1: Physico-chemical parameters and alkaliphilic bacterial load of different sediment samples

Sampling sites	S. No.	pH	Moisture content (%)	Bacterial load (CFU/g)× 10^3
Chandipur	1	7.17±0.21	26.7476±1.07	0.267±0.033
	2	7.71±0.11	16.9576±2.1	1.133±0.767
	3	7.31±0. 1	21.6344±1.11	0.4±0.2
	4	7.73±0.13	30.0343±2.41	1.533±0.567
	5	8.12±0.12	27.4632±1.77	3.267±1.133
Chilika	6	7.72±0.1	55.0202±0.57	25.7±4.2
	7	6.75±0.04	21.0175±0.59	0.4±0.1
	8	7.21±0.02	47.6811±1.12	5.2±2.5
	9	7.53±0.2	46.7435±1.67	2.767±1.633
	10	8.11±0.08	45.5714±1.1	9.667±1.433
Gopalpur	11	7.37±0.2	13.482±2.13	0.367±0.233
	12	7.45±0.12	15.4483±1.87	0.867±0.033
	13	7.11±0.11	14.0412±1.25	0.3±0.1
	14	7.31±0.1	19.6983±2.01	0.567±0.233
	15	7.19±0.2	20.5549±2.88	0.333±0.067
Puri	16	7.13±0.22	26.1396±1.09	0.267±0.233
	17	7.21±0.16	17.812±2.04	0.533±0.266
	18	7.16±0. 1	21.6088±1.71	0.333±0.166
	19	7.33±0.3	27.7011±0.88	0.5±0.2
	20	7.48±0.1	27.4922±1.7	0.733±0.166
Paradip	21	7.55±0.14	10.6704±1.92	0.967±0.333
	22	7.47±0.11	17.4273±1.11	0.633±0.166
	23	7.64±0.06	18.0421±2.19	1.067±0.233
	24	7.16±0.1	37.3013±1.01	0.233±0.166
	25	7.51±0.04	36.4901±2.1	1±0.5

All values are mean±SD, sample size, n=3

Table 2: Pearson correlation coefficient and T-test for significant study

Parameters	Moisture content (%)	pH
pH	r = 0.288 t = 7.966**	
Bacterial load (CFU/g)	r = 0.678* t = 2.209**	r = 0.415* t = 2.228**

*correlation is significant (p<0.05), **population mean is significant (p<0.05, 2-tailed)

RESULTS AND DISCUSSION

Sample characterization

A total of 25 sediment samples were investigated for physicochemical properties like pH and moisture content percentage. The pH values of sediment samples were near neutral to slight alkaline ranged from 6.75±0.04 to 8.12±0.12 and moisture content varied with sampling sites, ranged from 10.6704±1.92 to 55.0202±0.57% (table 1). Microbiological analysis revealed 25.7±4.2 × 10^3 CFU/g bacterial load at pH 10.3 from Chilika sediments. The minimum (0.233±0.166 × 10^3 CFU/g) bacterial load was found from Paradip sediments. About 80 isolates from five sites were selected on the basis of their macro-morphology on agar medium and stored for further screening.

From statistical analysis, it is observed that there is a positive and significant correlation between the bacterial load with both moisture content and pH (p<0.05). The t-test revealed that there is a significant variation between all the population means of all variables (table 2). There is a non-significant correlation found among pH and moisture content (p>0.05), which corroborates the results of [20]. Alkaline bacteria from coastal regions of Odisha showed a significant correlation between pH and moisture content [21]. The similar significant correlations among the bacterial load with pH were also reported [21-22].

Screening for alkaline protease

All the isolates were screened for alkaline protease production in skim milk agar plates at 10 pH. From this, it was found that only 22 of 80 isolates (27.5%) were positive for alkaline protease production and designated as AP1-AP22. Further investigation revealed that, 11 isolates (50%) of 22 showed a maximal zone of clearances at pH 6 than respective plates of 10 pH. Therefore, for further analysis rest 11 isolates were taken. Table 3 depicts the effect of different pH of skim milk agar for the production of protease enzymes by the above 11 isolates. All the isolates showed a zone of clearance at pH 8–12, while 63.64% and 9.09% isolates were positive for protease hydrolysis at pH 6 and 4 respectively. Fig. 1 depicts the zone of clearances by isolates at pH 8. Optimum zone of clearance was found at alkaline pH, which denotes the alkalinity nature of the protease produced by the isolates.

Table 3: Effect of pH for protease production by isolates

Isolates code	Solubilization indexes at different pH on 48 h of incubation				
	4	6	8	10	12
AP1	0	0	8±2	10±2	12±2
AP2	0	0	15±1	17±1	11±1
AP3	1±0	9±1	10±2	14±2	17±3
AP5	0	0	9±1	17±1	5±1
AP7	0	6±2	9±1	10±2	9±1
AP8	0	0	9±1	14±2	11±3
AP9	0	2±0	17±1	15±3	14±2
AP10	0	15±1	15±1	16±2	9±1
AP12	0	9±1	10±2	11±1	8±2
AP13	0	7±1	15±1	16±2	11±1
AP21	0	12±2	12±2	12±2	10±2

All values are mean±SD, ANOVA revealed that there is a significant differences found among the SI with respect to various pH investigated (p<0.05), sample size, n=3

Table 4: Qualitative and quantitative analysis of alkaline protease at pH 10 by isolates at 37 °C

Isolates code	Inoculum (600 nm)	HR% at different incubation period (h)**		SI at different incubation period (h)*				
		24	48	24	48	72	96	120
AP1	1.386±0.026	42.308±3.846	61.539±7.143	2±0	7±1	9±1	12±2	20±2
AP2	1.546±0.007	12.5±7.692	16.667±3.704	13±3	16±2	21±1	26±2	30±2
AP3	0.856±0.026	34.615±4.167	65.385±15.3846	6±2	14±2	16±2	18±3	20±2
AP5	1.451±0.002	34.615±3.704	50±3.571	7±3	17±1	19±1	21±3	22±2
AP7	1.548±0.013	17.857±3.846	28.571±3.846	7±1	9±1	11±1	14±4	16±2
AP8	0.831±0.026	37.037±4.167	48.148±3.571	4±2	9±1	15±1	20±0	30±2
AP9	1.726±0.007	32±4.167	60±4.167	12±2	15±3	17±3	26±0	26±2
AP10	1.420±0.026	15.385±7.143	34.615±3.571	11±1	13±1	16±2	25±1	28±2
AP12	1.707±0.013	21.429±3.571	32.143±4.167	6±0	8±2	12±2	18±2	23±1
AP13	1.779±0.026	39.286±3.571	53.571±4.167	10±2	12±2	14±2	28±0	30±2
AP21	1.531±0.026	35.714±3.571	64.286±7.143	9±1	11±1	16±2	21±1	26±2

All values are mean±SD, *ANOVA revealed that there is a high significant difference found in SI along the incubation period (p<0.001), **ANOVA revealed that there is a significant difference found in HR% of all isolates along the incubation period (p<0.05), Sample size, n=3

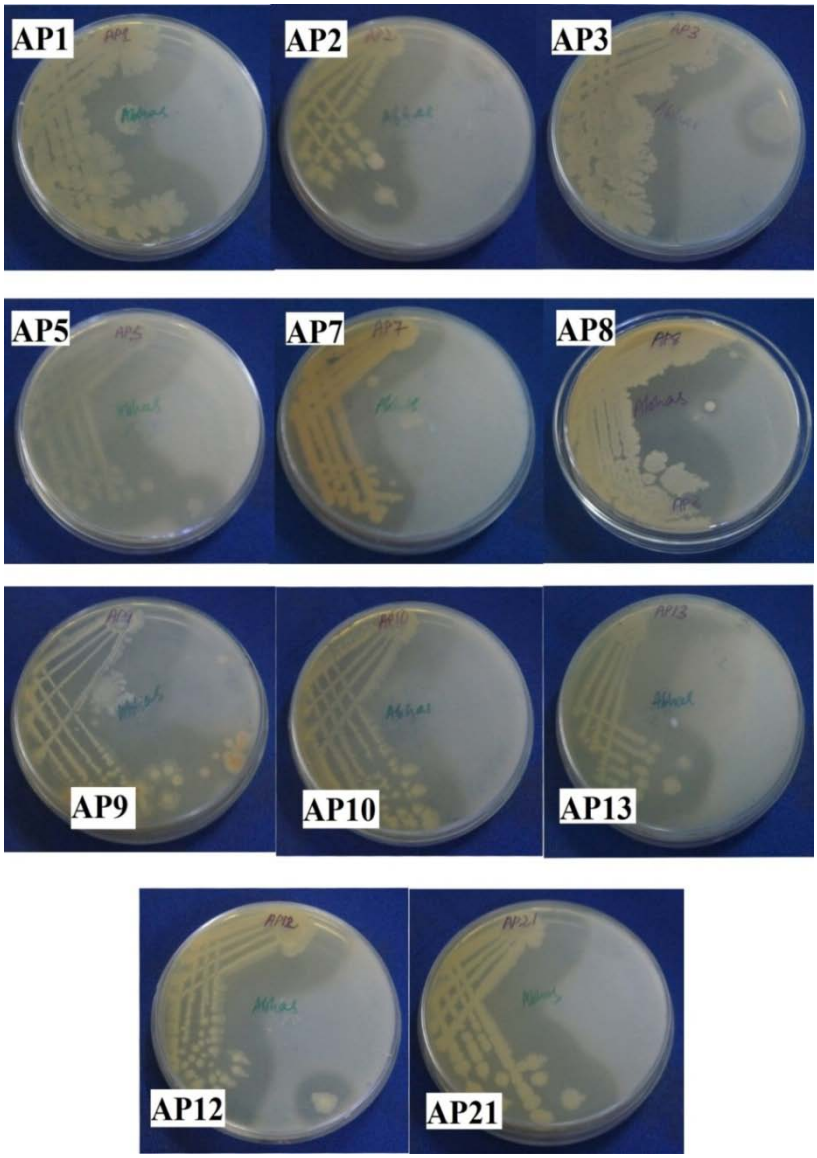

Fig. 1: Alkali-tolerant isolates showing zone of clearances at pH 8.0 at 37 °C

Quantitative analysis of alkaline protease

Table 4 revealed that from 11 isolates maximum zone of clearance at 10 pH was shown by 3 isolates (SI = 30±2) by AP2, AP8 and AP13, while the minimum by AP7 (SI = 16±2) after 5 d of incubation.

Nevertheless, the quantification experiment result was somehow different from the above qualitative test, which is depicted in table 4. The maximum HR% was seen by isolate AP3, i.e. 65.385±15.3846% while the minimum by the isolate AP2 (16.667±3.704%). From qualitative experiment, the potent producers were AP2, AP8 and AP13 while by quantitative experiment, the potent producer was AP3. There is no report on the hydrolytic run of any isolates as it is a new methodology that gives information regarding the "isolates" for vertical hydrolysis of substrates.

Effects of temperature for alkaline protease

All the isolates were screened for protease by taking skim milk agar maintaining at different incubation temperatures (table 5 and 6). Among all the isolates about 18.18% were competent to produce alkaline protease at 57 °C at pH 10, whereas 81.82% could capable of showing zone of clearances at 17 °C while incubating for 8 d. If maximum solubilisation index was considered about 45.46% isolates at 27 °C, 45.46% at 37 °C and 9.09% at 47 °C showed more than 20 SI. Isolate AP8 showed maximum solubilisation index of 39±1 at pH 10 while incubating at 47 °C.

Table 6 revealed the hydrolytic run percentage of the isolates at different temperatures. Maximum HR% was found by AP1 while incubating at 27 °C (65.517±3.846%) and AP3 at 37 °C (65.385±7.143%) and at 47 °C, AP5 showed the maximum (53.846±0%).

Partial characterization of alkaline protease producers

All the protease producing isolates again characterized partially to know the behavior and identification to their genus level. The biochemical results were depicted in table 7. Among all the isolates only 9.1% were positive for motility, 54.6% for citrate utilization, 90.9% for catalase test whereas about 18.2% were negative for esculin hydrolysis and nitrate reduction test. All isolates except one (9.1%) were positive for anaerobic growth. Table 8 represents about 20 sugar tests, where all the isolates were negative for lactose, 18.18% were positive for inulin and sorbitol. The micro-and macromorphological characteristics were given in table 9. All were bacilli except AP7, which is a Gram-positive coccus. Identification was done by putting appropriate results in the matrix available in PIBWin software, where only the identification is probabilistic as denoted in scores in the parenthesis (table 9). The isolated alkali-tolerant bacteria belonged to the genera of *Bacillus, Virgibacillus* and *Micrococcus*. There are many reports of getting alkaline proteases from bacterial origins like *Bacillus cereus* [23-24], *Bacillus smithii* [25], *Virgibacillus* [26] and *Micrococcus luteus* [27].

Table 5: Effect of incubation temperature for alkaline protease production by isolates

Isolates code	Solubilization indexes at varied incubation temperature (°C) at pH 10				
	17 (8 d)	27 (4 d)	37 (4 d)	47 (4 d)	57 (4 d)
AP1	0	13±1	7±3	16±2	0
AP2	2±0	25±3	12±2	1±0	0
AP3	0	14±2	10±2	16±2	0
AP5	6±2	12±2	15±1	9±1	7±1
AP7	2±0	17±1	17±1	9±1	0
AP8	13±1	20±2	22±2	39±1	11±3
AP9	9±1	23±1	22±2	12±2	0
AP10	9±1	19±1	23±1	11±1	0
AP12	11±1	18±0	13±1	7±1	0
AP13	3±1	24±0	30±2	10±2	0
AP21	2±0	21±1	26±2	6±0	0

All values are mean±SD; ANOVA revealed high significant differences between the SI of isolates at varied temperatures ($p<0.001$), Sample size, n=3

Table 6: Effect of incubation temperature on HR% at pH 10 by isolates

Isolates code	Hydrolytic Run (%) at different temperatures for varied incubation period (h)								
	47 °C			37 °C			27 °C		
	24	48	72	24	48	72	24	48	72
AP1	0	20±3.846	28±7.143	0	42.308±0	61.538±3.704	0	34.483±4.167	65.517±3.846
AP2	0	0	0	0	12.5±7.143	16.667±7.143	0	24.138±0	37.931±0
AP3	19.231±0	30.769±3.846	34.615±3.846	0	34.615±3.846	65.385±7.143	0	37.931±3.846	58.621±3.846
AP5	15.385±0	26.923±7.143	53.846±0	0	34.615±4.167	50±3.846	0	33.333±0	43.333±3.704
AP7	4±0	28±3.846	48±0	0	17.857±4.167	28.571±4.167	0	21.429±3.846	35.714±3.846
AP8	0	0	52±3.846	0	37.037±0	48.148±3.571	0	27.586±7.143	31.034±4.167
AP9	0	26.923±7.143	42.308±4.167	0	32±7.143	60±0	0	32.143±0	50±0
AP10	0	0	0	0	15.385±3.704	34.615±3.704	0	25.926±7.143	40.741±0
AP12	0	0	0	0	21.429±3.846	32.143±3.846	0	29.63±3.846	40.741±7.143
AP13	0	0	0	0	39.286±7.143	53.571±3.846	0	17.857±0	42.857±0
AP21	0	0	0	0	35.714±4.167	64.286±3.704	0	25.926±3.846	40.741±7.143

All values are mean±SD; ANOVA revealed high significant differences between the HR% of isolates at different temperatures for varied incubation period ($p<0.001$), Sample size, n=3

Table 7: Biochemical characterization of alkaline protease producing isolates

Isolates code	Mo	MR	VP	In	Ci	Ca	Ox	Es	Ur	PS	NR	AN
AP1	-	-	+	-	-	+	+	+	+	-	+	+
AP2	-	-	-	-	-	+	-	-	+	-	-	+
AP3	+	-	+	-	-	+	+	+	+	-	+	+
AP5	-	+	-	-	+	+	+	+	+	-	+	+
AP7	-	-	-	-	-	+	+	-	+	-	-	+
AP8	-	+	-	-	+	+	-	+	+	-	+	+
AP9	-	+	-	-	+	+	-	+	+	-	+	+
AP10	-	-	+	-	+	+	-	+	+	-	+	+
AP12	-	+	-	-	+	+	-	+	+	-	+	+
AP13	-	+	-	-	+	+	-	+	+	-	+	+
AP21	-	-	-	-	-	-	+	+	-	-	+	-

Mo: Motility test; MR: Methyl red test; VP: Voges-Proskauer test; In: Indole production; Ci: Citrate Utilization; Ca: Catalase; Oxi: Oxidase; Es: Esculin Hydrolysis; Ur: Urease test; PS: Phosphate solubilization; NR: Nitrate reductase; AR: Anaerobic growth; (+): Positive; (-): Negative.

Table 8: Sugar utilization tests of alkaline protease producing isolates

Isolates code	Sugars investigated																			
	Mn	Ce	Ma	Gl	Su	Me	La	Fr	Rh	Is	Ga	Xy	Mo	Ar	Du	De	Tre	In	So	Sa
AP1	+	+	-	+	+	-	-	-	-	+	-	-	+	-	+	+	+	+	-	+
AP2	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
AP3	+	+	-	+	+	-	-	-	-	-	+	-	+	-	+	+	+	+	+	+
AP5	+	+	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	-	+
AP7	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-
AP8	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
AP9	-	+	+	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	-	-
AP10	-	+	-	+	-	-	-	+	-	-	-	+	-	+	+	+	-	-	-	-
AP12	+	-	+	-	+	+	-	+	-	+	-	-	+	+	-	+	-	-	-	-
AP13	+	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-	-	+
AP21	-	-	-	-	-	-	-	+	-	-	+	+	-	+	-	+	-	-	+	-

Mn: Mannitol; Ce: Cellobiose; Ma: Maltose; Gl: Glucose; Su: Sucrose; Me: Melibiose; La: Lactose; Fr: Fructose; Rh: Rhamnose; Is: Inositol; Ga: Galactose; Xy: Xylose; Mo: Mannose; Ar: Arabinose; Du: Dulcitol; De: Dextrose; Tre: Trehalose; In: Inulin; So: Sorbitol; Sa: Salicin; (+): Positive; (-): Negative.

Table 9: Micro-and macro-morphological study and probabilistic identification of isolates

Isolates code	Micro-morphological characterization	Macro-morphological characterization on NA at pH 8	Bacteria identified (PIBWin Score)
AP1	Gram+ve, small rods, central spore	Big colony, irregular margin, creamy, flat surface, opaque, dry and hyper growth at pH 8.0 within one-day	*Bacillus cereus* (0.99993)
AP2	Gram+ve, small rods, short chain, central spore	Big, round, creamy, convex, raised, entire, opaque and aggregation at middle	*Bacillus cereus* (0.99732)
AP3	Gram+ve, tiny rods to coccobacilli	Big, irregular, rhizoidal, creamy to white, flat, opaque, sticky, hyper growth at pH 8.0 within one day and somehow same as AP1	*Bacillus cereus* (0.89744)
AP5	Gram+ve, thin rods	Medium, round, regular, creamy, opaque, grooved at middle and raised at the border.	*Bacillus cereus* (0.89336)
AP7	Gram+ve, cocci	Tiny, round, regular, lemon yellow, convex, and opaque	*Micrococcus luteus* (0.90346)
AP8	Gram+ve, small rods, streptobacilli, central spore	Big, round to oval, irregular, creamy, flat, opaque and wheel like colony	*Virgibacillus* (0.81499)
AP9	Gram+ve, tiny, coccobacilli	Big, round to oval, flat, regular, creamy, smooth, opaque and lambda like marks on colony	*Bacillus carotarum* (0.95797)
AP10	Gram+ve, tiny, coccobacilli, in short chain	Medium, round, regular, yellowish white, convex, opaque and circular rings on colony	*Bacillus carotarum* (0.91842)
AP12	Gram+ve, tiny, coccobacilli	Medium, round, irregular, creamy, rough, flat, opaque and some clumps at middle of colony	*Bacillus smithii* (0.71708)
AP13	Gram+ve, long rods, short chain, central spore	Medium to big, round, regular, creamy, raised, flat to convex and not sticky as AP21	*Bacillus cereus* (0.99826)
AP21	Gram+ve, long chain, streptobacilli, central spore	By some means same as AP13, but slightly change in furrow patterns, and sticky, creamy, round, regular, raised, distinct furrows like triangular shape and opaque	*Virgibacillus* (0.99425)

Physiological characterization of isolates

For physiological tests, isolates were investigated for its tolerance to various pH and salt concentrations, which are depicted in fig. 2 and fig. 3, respectively. All the bacterial isolates investigated were alkali tolerant having pH optimum near to alkaline. However, among the isolates AP5, AP7 and AP21 have optimum growth at alkaline pH.

Johnvesly and Naik reported an alkaline and thermostable protease from *Bacillus* sp. JB-99, which showed maximum production at pH 9 and the purified product, was stable at pH 9 and more active in 11 pH [28]. The similar findings were observed while working on alkaline and thermostable protease by *Bacillus stearothermophilus* F1 [29]. *Virgibacillus pantothenticus* reported an extracellular thermostable serine protease having pH optima at 10 and temperature at 50 °C [26]. Similarly, there was a report about protease from a halophilic *Virgibacillus* sp. EMB13 [30]. Production of alkaline protease by *Bacillus cereus* was reported in new industrial culture mediums, which had pH optima at 7 and temperature for optimum production was at 60 °C [23]. Protease production capabilities of *Micrococcus luteus* and *Bacillus* sp. isolated from abattoir environment were reported [27]. They reported the optimum temperature and pH for *Micrococcus luteus* was 37 °C and pH 7 while in case of *Bacillus* sp. it was 47 °C and 9 pH. Whatever it may be the above bacterial spp. showed the maximum alkaline protease production at near to 7 while could tolerate 12 pH.

From the salt tolerance test, it was observed that all the isolates needed additional salts for their optimal growth, which was confirmed by analyzing the control lacking of extra salts from growth medium (fig. 3). All isolates have optimum growth at 2% (w/v) NaCl except AP5, which in turn has been optimum at 4%. However, all showed growth at 12% NaCl, which confirms them as halo-tolerants. It is apparent because all the isolates were from marine sources.

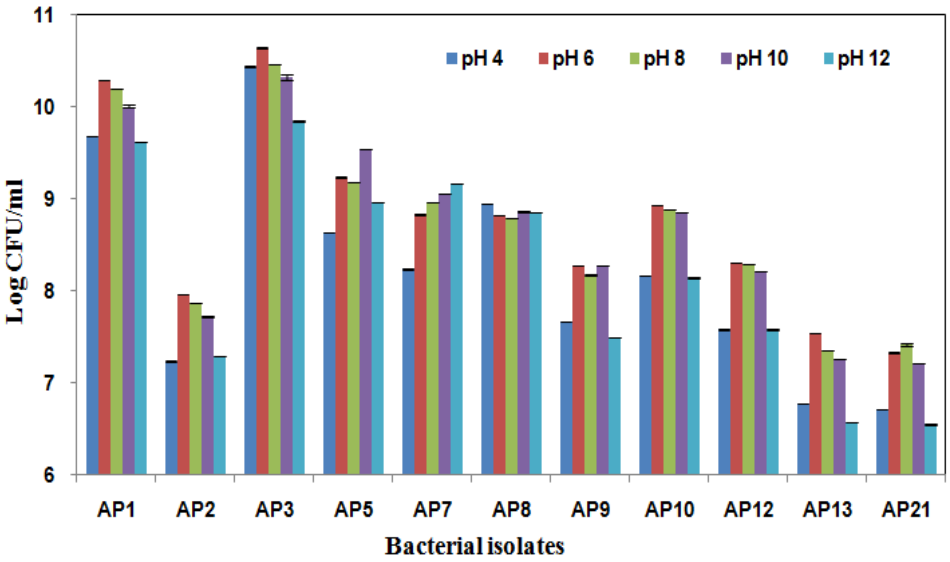

Fig. 2: Effect of initial pH on growth of isolates, and values are mean±SD for three distinctive experiments, n=3, ANOVA test revealed that there is a high significant difference in growth among the isolates along varied pH of the medium (p<0.001)

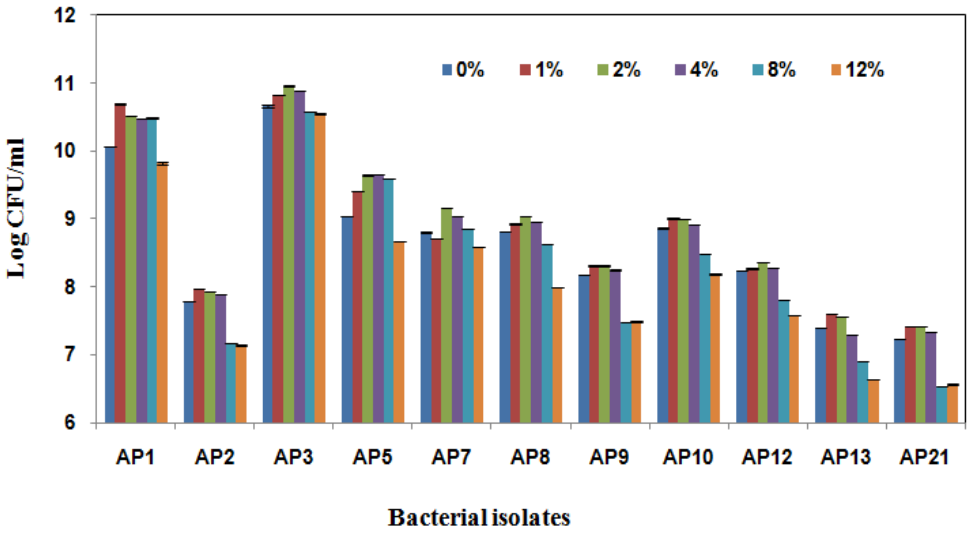

Fig. 3: Effect of salt concentrations (% w/v) on growth of isolates, and values are mean±SD for three distinctive experiments, n=3, ANOVA test revealed that there is a high significant difference in growth among the isolates along varied concentrations of NaCl (p<0.001)

CONCLUSION

From the above study, it can be concluded that marine sources are the reservoir of various alkali-tolerant bacteria, which are the good sources of alkaline protease. In our study, isolates found from marine sources were mostly Gram positives. The isolates were not only active in alkaline pH but also can tolerate 12% NaCl, which makes them as halo-alkali tolerant bacteria. The alkaline proteases produced from the above isolates have immense applications in various fields of sciences and industries. The new methodology adapted here for quantification of alkaline protease can strengthen the results found from qualitative and shows the real potentiality of the isolates.

ACKNOWLEDGEMENT

Authors would like to thank the DST, Govt. of Odisha for the financial support in the project having F. No. ST-11-(SC)-34/2014-2371/ST and also grateful to HOD, Department of Microbiology, Orissa University of Agriculture and Technology, Bhubaneswar, Odisha, India for providing laboratory support.

CONFLICTS OF INTERESTS

All authors have none to declare

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