JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2015. Vol. **9**(3), p. 2441-2450

Screening of Alkalitolerant Bacteria for Alkaline Enzymes and Amylase Production by Submerged Fermentation

Sonali Priyadarshini* and Pratima Ray

Department of Microbiology, C.B.S.H., Orissa University of Agriculture and Technology, Unit 7, Surya Nagar, Bhubaneshwar, Odisha - 751003, India.

(Received: 25 April 2015; accepted: 03 July 2015)

The alkalitolerant bacteria of red mud samples of Damanjodi, Koraput and sediment samples of Puri, Odisha were studied. A total of ten samples were collected and screened for the isolation of alkalitolerant bacteria at pH 10.3 by enrichment method followed by serial dilution technique. Maximum incidences of alkalitolerant bacteria were found to be present in red mud samples having bacterial load 4.4 × 10^7 CFU/gm. About 20 bacterial isolates were further studied and identified by their cultural-, physiological- and biochemical- characterization. The isolated alkalitolerant bacteria belonged to the genera of *Bacillus*, *Pseudomonas*, *Micrococcus*, *Cellobiosococcus*, *Xanthomonas*, *Staphylococcus* and *Virgibacillus*, which were tested for the production of alkaline extracellular enzymes like proteases, amylases, pectinases and lipases. Alkaline amylase activities at pH 8.0 were detected as the maximum than other enzymes. From quantitative assay, it was found that isolate RM2 showed maximum, i.e. 14.5366 IU/ml amylase production at 72 hr of incubation using starch as substrates by submerged fermentation. The potential of these alkalitolerant bacteria as a resource of enzymes have found their way into biotechnological and industrial applications.

Key words: Alkaline amylase, Alkalitolerant, Red mud, Sediments.

Habitats, whether acidic or basic select their own habitants to survive and function. Alkaliphiles are those microorganisms which grow optimally at pH value above 9.0, showing little or no growth at near neutral pH values. However, another group called alkalitolerants, capable of growing over a wider range of pH values, often at the acidic range, showing pH optima at near neutral pH[1] has been found. Alkaliphilic microorganisms are widely distributed in nature and can be found in almost all environments without the restriction of alkalinity. However, a few of the naturally-occurring alkaline environments, namely soda soils, lakes, and deserts, harbor a wide range of these types[2]. Others include the dilute alkaline springs, desert soils and oils containing decaying proteins or forest soil[3-6]. The pH values of these environments are commonly around 10 and above. The man-made alkaline environments were found to be the effluents from food, textiles, tanneries, potato processing units, paper manufactures units, calcium carbonate kilns, detergents and other industrial processes[3,7,8]. Highly saline, alkaline environments are relatively rare around the world compared with high saline, neutral environments. However, there is a possibility that such environments harbor a unique microbial population[8-10]. The best sources for halophilic alkaliphiles have been the extreme soda lakes of

* To whom all correspondence should be addressed.
Tel.: 91-8763349209; Fax: 0674-2397457;
E-mail: sonalisony.priyadarshini@gmail.com

the Wadi Natrum in Egypt and Lake Magadi in Kenya[11].

Many different kinds of alkaliphilic microorganisms, including bacteria belonging to the genera *Bacillus*, *Micrococcus*, *Pseudomonas*, and *Streptomyces* and eukaryotes such as yeasts and filamentous fungi, have been isolated from a variety of environments[12-17]. Therefore, for the present experiment, red mud samples from bauxite processing plant were taken having pH above 11.0, which increases the chances of getting more alkalitolerants than the normal soil. The viable count of alkaliphiles in normal soils is lower than that found in alkaline soils[18,19]. There are many physiological differences in the adaptive nature of alkaliphiles than the neutrophiles. The alkaliphiles have cell wall containing acidic polymers such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, and phosphoric acid[20]. However, they also have 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[21]. Plasma membranes maintain homeostasis by using the Na^+/H^+ antiporter system, the K^+/H^+ antiporter and ATPase-driven H^+ expulsion[19].

A large number of alkaline enzymes with industrial applications are available such as alkaline protease, amylase, pectinase, cellulase, alginase, pullulanase, catalase, glucanase, xylanase, penicillinase, maltose dehydrogenase, glucose dehydrogenase, uricase and polyamine oxidase. Amylases are among the most important industrial enzymes with biotechnological applications. Microbial production of amylase is easy and cost effective, which can be modified to obtain enzymes of desired characteristics. The alkaline amylase finds its application in pharmaceutical, fine-chemical industries, detergent formulation as well as in clinical research, medical chemistry, and starch analytical chemistry. They are also used in baking, brewing, textile, detergent, paper and distilling industries. Therefore, the present study was investigated upon the isolation and identification of alkalitolerant bacteria and their ability to produce alkaline extracellular enzymes and quantification of alkaline amylase by submerged fermentation method.

MATERIALS AND METHODS

Sample collection and characterization

Sediment and red mud samples were collected from Puri sea beach and Bauxite processing plant, NALCO Damanjodi, Koraput, Odisha, respectively in sterile polythene bags and stored at 4°C. The pH of all samples was determined using pH Meter (Systronics ì pH System 361)[22]. For the determination of moisture content, the method by Jackson, (1967) was followed[23]. The salinity of the samples was recorded using Salinometer (Salt Testr).

Isolation of Alkalitolerant bacteria

Enrichment culture technique was used to isolate alkalitolerant bacteria from the Red Mud and sediment sample in Horikoshi II medium at 10.3 pH[22]. This was achieved by adding 2 g of each sample in 100 ml of enrichment media broth. The flasks were incubated on rotary shaker (120 rpm) at 37°C for 3 days. Serial dilution followed by spread plate technique was used to isolate alkalitolerant on nutrient agar plates maintained at 10.3 pH. Well-isolated and differentiated colonies were transferred to respective medium agar slants[24]. The isolates were then subjected to morphological, biochemical and physiological characterization.

Identification and characterization of alkalitolerants

Bacterial isolates were identified by cultural (colour, texture, margin, elevation, density and size), morphological (Gram variability) and biochemical characterization[25] and results were analysed by Bergey's Manual of Systematic Bacteriology[26] and PIBWin Software[27]. The bacterial isolates were characterized biochemically by conducting different tests, i.e. IMViC test, catalase, urease, oxidase, nitrate reduction, H_2S production and various sugar utilization tests. Physiological characterization was carried out in terms of bacterial growth on nutrient agar plates adjusted to various pH from 6.0 -13.0 using 1N HCl and 1N NaOH. Besides sodium salt (NaCl) tolerance, test was conducted using nutrient agar with addition of 2%, 5% and 10% of salts. Antibiotic susceptibility test was also performed for all the bacterial isolates against streptomycin, chloramphenicol, ampicillin and polymyxin B.

Screening for alkaline enzymes

All the bacterial isolates were screened for alkaline enzymes, i.e. amylase, protease, pectinase and lipase by using respective plate assay methods.

Screening for alkaline protease production

Skim milk agar plates maintained at pH 8.0 were prepared onto which all the bacterial isolates were spot inoculated and were incubated at 37°C for 72 hr. A clear zone around the colony on an opaque background indicated hydrolysis of the milk protein, casein[28].

Screening for alkaline amylase production

Starch agar plates were prepared using Nutrient agar supplemented with 1% (w/v) soluble corn starch maintained at 8.0 pH. All the spot inoculated plates were incubated at 37°C for 72 hr. After incubation all the plates were exposed to iodine for 5 - 10 min. A clear zone surrounding the microbial colonies indicates positive for starch hydrolysis[29].

Screening for alkaline lipase production

To test the lipolytic activity, the isolated bacteria were spot inoculated on tributyrin agar maintained at pH 8.0 and incubated for 72 hr and zone of clearance around the bacterial colonies was regarded as positive[30].

Screening for alkaline pectinase production

The ability of the bacteria to hydrolyse pectin into pectic acid was determined by taking nutrient agar with 1% pectin at pH 8.0, which were incubated for 72 hr. After incubation, 3.3% CTAB (N, N, N, N - cetyl trimethyl ammonium bromide) was flooded on assay plates and zone of clearance was determined[31].

Submerged fermentation for amylase production

Inoculum preparation

The bacterial isolates previously screened from the plate assay was inoculated in nutrient broth (pH 8.0) incubated at 37°C for 24 hr at 120 rpm. Freshly prepared inoculum was used for production of alkaline amylase.

Production medium

The submerged fermentation was carried out in 250 ml Erlenmeyer flask containing MSM medium[32] with slight modification KH_2PO_4, 0.1g; Na_2HPO_4, 0.25g; NaCl, 0.1g; $(NH_4)_2SO_4$, 0.2g; $MgSO_4$, 0.005g; $CaCl_2$, 0.005g; peptone, 0.2g; starch, 1.0g and maintained at 8.0 pH. About 1 ml of 24 hr grown culture was transferred to the flask containing the production medium. It was shaken in a shaker incubator at 37°C for 48 hr, 96 hr and 144 hr. After incubation, the fermented broth was centrifuged at 8000 rpm for 20 min. at 4°C[33]. The supernatant collected was regarded as crude alkaline amylase. From the above experiment, the potent alkaline amylase producer was selected and effect of the incubation period was evaluated from 24 - 168 hr.

Quantitative estimation of amylase production

Amylase was determined by the spectrophotometric method with slight modifications[34]. The assay mixture consisted of 1% starch prepared with 0.05M sodium phosphate buffer at 6.7 pH. About 0.1ml of crude enzyme was mixed with 0.9 ml of substrate and incubated for 20 min. at 37°C. After incubation, 1.5 ml of 3, 5 - dinitro salicylic acid reagent was added and boiled for 10 min. in water bath and after cooling absorbance were measured at 540nm in the spectrophotometer against maltose as standard. The amylase activity was determined in IU/ml/min and one international unit (IU) of enzyme is defined as the amount of enzymes releasing 1 µmole of reducing sugar as maltose per minute under assay conditions.

Statistical analysis

All the data were analyzed by statistical methods, i.e. Pearson correlation coefficient, ANOVA (analysis of variance) and T- test for significant variations by using Microsoft excel 2007.

RESULTS AND DISCUSSION

All the samples were analysed for the determination of pH, moisture content and salinity. Besides the microbiological analysis (alkalitolerant bacterial load) was also carried out. From Table 1, it was found that the samples collected from Damanjodi valley, i.e. red mud (pH 11.86 - 12.13) were more alkaline than the Puri samples (pH 7.84 - 8.25). The same pattern of maximum moisture percentage and salinity was found in case of red mud samples than Puri sediments. Besides alkalitolerant bacterial load was found to be more from red mud samples (4.05 - 4.4 $\times 10^7$ CFU/gm) indicating this habitat more suitable for alkalitolerants than the Puri sediments (1.42 - 1.8 $\times 10^3$ CFU/gm). The statistical analyses of all physico-chemical and microbiological parameters

were demonstrated in Table 2. There is a significant correlation among all the parameters which again is proved by determining T-test.

Different sampling sites have been chosen for isolation of alkaliphiles. Tiago and Pedersen revealed that all the cultivated strains isolated from alkaline ground water samples were alkalitolerants rather than alkaliphiles[35,36]. About 3 novel alkaliphilic bacteria belonged to *Bacillus vedderi* sp. nov. and *Bacillus* spp. were isolated from a bauxite- processing red mud tailing pond having 10.0 pH[37]. Water and sediment samples were collected from Lonar Lake[38], Christian and Quid-e-Azam industrial area of Kott Lakh- Patt, Lahore[39] and Dhobighat region of Mula Mutha River[40] for the isolation of novel alkaliphiles.

Horikoshi II medium at pH 10.3 was used to isolate the alkalitolerant bacteria[19,38]. A total of 20 isolates were taken for further characterization. Isolates from red mud samples and Puri sediments were designated as RM and PS respectively. Of the total isolates, Gram-positive rods were found to be maximum (65%), followed by Gram-positive cocci (25%) and Gram-negative rods (10%). Further all the isolates were subjected to biochemical tests as depicted in Table 3. All the isolates were negative for indole test, but positive for catalase and H_2S production, urease and mannitol motility. All the isolates were positive for nitrate reduction except RM4, RM8 and PS2**.** Sugar fermentation tests were carried out for all the bacterial isolates (Table 4). Almost all the isolates utilize all the sugars investigated except xylose.

Table 5 depicts the physiological characterization study of all bacterial isolates. From pH tolerance study, about only 10% isolates were able to tolerate 12.0 pH, and 80% at pH 11.0 whereas 100% can grow at pH 6.0, which confirms that the

Table 1. Physico-chemical and microbiological characterization of samples

Sampling sites	No. of samples	pH[a]	Moisture percentage (%)[a]	Salinity (ppt) [a]	Bacterial load (CFU/gm)[a]
Red mud (RM)	1	11.99	22.19	0.90	4.2×10^7
	2	11.86	21.74	0.79	4.1×10^7
	3	12.05	26.01	0.94	4.25×10^7
	4	12.13	20.22	0.99	4.4×10^7
	5	11.92	20.81	0.88	4.05×10^7
Puri sediment (PS)	1	7.95	13.92	0.30	1.62×10^3
	2	7.89	10.22	0.29	1.55×10^3
	3	7.84	16.83	0.27	1.42×10^3
	4	7.91	13.41	0.32	1.71×10^3
	5	8.25	15.25	0.32	1.8×10^3

[a]Results are the mean of three independent experiments.

Table 2. Correlation coefficient and t- test for significant test

Physico-chemical parameters	Soil pH	Soil moisture content (%)	Salinity (ppt)
Soil moisture content (%)	r = 0.893* t (18)= 4.789**, p<0.001		
Salinity (ppt)	r = 0.992* t (18)= 13.809**, p<0.001	r = 0.88* t (18)= 9.49**, p<0.001	
CFU/g	r = 0.999* t (18)= 2.998**, p<0.05	r = 0.89* t (18)= 2.998**, p<0.05	r = 0.991* t (18)= 2.998**, p<0.05

*correlation is significant at 0.05 level (two tailed)
**population mean is significant (two tailed)

Table 3. Biochemical characterization of the isolates

Isolates	In	MR	VP	Ci	Ca	Ur	Oxi	NR	Es	Mo
RM1	-	+	+	-	+	+	+	+	+	+
RM2	-	-	+	+	+	+	-	+	+	+
RM3	-	-	+	-	+	+	-	+	+	+
RM4	-	+	-	+	+	+	-	-	+	+
RM5	-	+	+	-	+	+	+	+	+	+
RM6	-	-	-	-	+	+	-	+	+	+
RM7	-	-	-	-	+	+	-	+	+	+
RM8	-	+	-	-	+	+	-	-	+	+
RM9	-	-	-	+	+	+	-	+	+	+
RM10	-	-	-	+	+	+	-	+	+	+
PS1	-	-	+	+	+	+	+	+	+	+
PS2	-	-	-	-	+	+	+	-	+	+
PS3	-	+	+	+	+	+	+	+	+	+
PS4	-	+	-	-	+	+	+	+	+	+
PS5	-	-	+	-	+	+	+	+	+	+
PS6	-	+	+	-	+	+	+	+	+	+
PS7	-	+	+	+	+	+	+	+	+	+
PS8	-	+	+	+	+	+	+	+	+	+
PS9	-	+	-	-	+	+	+	+	+	+
PS10	-	-	+	+	+	+	+	+	+	+

In- Indole production; MR- Methyl red test; VP- Voges Proskauer test; Ci- Citrate utilisation; Ca- Catalase; Ur- Urease test; Oxi- Oxidase; Es- Esculin hydrolysis; NR- Nitrate reductase; Mo- Motility test.

Table 4. Sugar utilization tests of the isolates

Isolates	Gl	Su	La	Ma	Mn	Mo	Is	Fr	Ar	Ce	Ga	Sa	Ra	Xy
RM1	-	+	+	+	+	+	-	+	+	+	+	+	+	-
RM2	-	-	-	-	+	-	-	+	-	+	-	+	-	-
RM3	-	-	-	-	+	+	-	+	+	+	+	+	+	-
RM4	+	+	-	+	+	+	-	+	+	+	+	+	-	-
RM5	+	+	+	+	+	-	-	+	+	+	+	+	+	-
RM6	+	+	+	-	+	+	-	+	-	+	-	+	+	-
RM7	+	+	+	+	+	+	+	+	-	+	-	-	-	-
RM8	+	+	-	+	+	+	-	+	-	+	-	-	+	-
RM9	+	+	-	+	+	+	+	+	+	-	+	-	+	-
RM10	+	+	+	+	+	+	+	+	+	+	-	+	+	-
PS1	+	+	+	-	+	-	-	+	-	-	-	-	-	-
PS2	+	-	-	-	+	+	-	+	+	+	+	+	+	-
PS3	+	-	-	-	+	-	-	+	+	+	-	-	+	-
PS4	+	-	-	-	+	-	-	+	+	-	+	+	+	-
PS5	+	+	+	+	+	+	+	+	+	+	-	+	+	-
PS6	+	-	+	+	+	-	-	+	-	-	-	-	-	-
PS7	+	-	-	-	+	+	+	+	-	+	+	-	-	-
PS8	+	+	+	+	+	-	-	+	-	+	+	-	+	-
PS9	+	+	+	+	+	+	+	+	-	+	-	+	-	-
PS10	+	+	+	+	+	+	+	+	-	+	+	+	+	-

Mn- Mannitol; Ce- Cellobiose; Ma- Maltose; Gl- Glucose; Su- Sucrose; La- Lactose; Fr- Fructose; Is-Inositol; Mo-Mannose; Ar- Arabinose; Ga- Galactose; Sa- Salicin; Ra- Raffinose; Xy- Xylose

studied bacterial isolates were alkalitolerants rather than alkaliphiles. About 80% isolates can grow at 10% NaCl concentration and 65% can tolerate 50°C. All the isolates can grow under anaerobic conditions, which denote them as facultative anaerobic bacteria. Besides, antibiosis revealed that 100% bacterial isolates were susceptible to Streptomycin and Chloramphenicol where as 10% showed resistant to Ampicillin and 20% to Polymyxin.

Based on biochemical tests and PIBWin software analysis the isolates were analysed, which belonged to *Bacillus* sp., *Pseudomonas cepacia*, *Bacillus cereus*, *Micrococcus varians*, *Cellobiosococcus* sp., *Xanthomonas* sp., *Bacillus smithii*, *Staphylococcus* sp., Taxon 24, *Bacillus carotarum*, *Virgibacillus* sp., *Bacillus licheniformis* showing above 90% PIBwin scores. There are many reports of getting such alkalitolerant bacteria by researchers[30,35,36]. The dominant genera reported were belonged to Gram-positive type, i.e. *Agrococcus*, *Bacillus*, *Clavibacter*, *Microbacterium*, *Micrococcus*, *Rhodococcus* and *Staphylococcus* isolated from Portugal and Jordan[35,36]. About 20 alkaliphilic bacteria were isolated and belong to genera *Alcaligenes*, *Escherichia*, *Natronobacterium*, *Aeromonas*, *Pseudomonas*, *Marinococcus*, *Neisseria*, *Micrococcus*, *Sporosarcina*, *Pleisomonas* and *Cupriavidus*[39]. From the saline and swampy mangrove ecosystem, *Bacillus* spp. were isolated, which includes *B. subtilis, B. sphaericus, B. polymyxa, B. pasteurii, B. cereus, B. Macquariensis, B. smithii and B. licheniformis*[41]. Wagale and co-workers reported that majority of the isolates were Gram-positive, motile rods with few cocci and actinomycetes that were strict alkaliphiles and grew at pH 11.0 only[40]. Besides morphological and biochemical characterization of those isolates indicated that most of the bacterial isolates belonged to the genus *Bacillus*[40]. Kanekar and co-workers reported bacteria, i.e. *Halomonas campisalis, Alkalibacillus haloalkaliphilus, Dietzia natronolimnaea, Vagococcus carniphilus, Exiguobacterium aurantiacum, Bacillus horikoshii, Cellulosimicrobium cellulans, Thermoactinomyces thalpohilus, Paenibacillus* spp., *Marinobacter excellens, Marinobacter*

Table 5. Physiological characterization of the isolates

Isolates	pH							NaCl (%)			Temp. (±1°C)		Anaerobic		Antibiosis		
	6	7	8	9	10	11	12	2	5	10	37	50	growth	Str	Chl	Amp	Poly
RM1	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	S
RM2	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	S
RM3	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	S
RM4	+	+	+	+	+	-	-	+	+	+	+	+	+	S	S	S	S
RM5	+	+	+	+	+	-	-	+	+	-	+	+	+	S	S	R	S
RM6	+	+	+	+	+	+	-	+	+	+	+	-	+	S	S	S	S
RM7	+	+	+	+	+	+	-	+	+	+	+	-	+	S	S	S	S
RM8	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	S
RM9	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	S
RM10	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	S
PS1	+	+	+	+	+	+	-	+	+	+	+	-	+	S	S	S	S
PS2	+	+	+	+	+	+	+	+	+	+	+	-	+	S	S	S	S
PS3	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	S
PS4	+	+	+	+	+	+	-	+	+	+	+	-	+	S	S	S	S
PS5	+	+	+	+	+	+	-	+	+	+	+	-	+	S	S	S	S
PS6	+	+	+	+	+	+	+	+	+	-	+	+	+	S	S	S	S
PS7	+	+	+	+	+	-	-	+	+	+	+	+	+	S	S	S	R
PS8	+	+	+	+	+	+	-	+	+	+	+	-	+	S	S	S	R
PS9	+	+	+	+	-	-	-	+	+	+	+	+	+	S	S	R	R
PS10	+	+	+	+	+	+	-	+	+	+	+	+	+	S	S	S	R

Str- Streptomycin; Chl- Chloramphenicol; Amp- Ampicillin; Poly- Polymyxin B; S- Susceptible; R- Resistant.

alkaliphiles, Roseinatronobacter monicus, Rhodobacteriaceae bacterium, Alkalimonas delamerensis and Rhodobaca bogoriensis from Lonar Lake[38].

All the isolates were screened for alkaline amylase, protease, pectinase and lipase enzyme production at pH 8.0 and the results are shown in Table 6. Of all the isolates, 25% were positive for alkaline amylase, 70% for alkaline lipase, 55% for alkaline protease and 10% for alkaline pectinase. Among the total alkaline enzyme production test, it was found that the isolates were maximum producers of alkaline amylase than other enzymes, which were evaluated upon the basis of comparing zone of clearance. Between 5 alkaline amylase producers *Pseudomonas cepacia* (RM1), *Bacillus* sp. (RM2), *Cellobiosococcus* sp. (RM7), *Bacillus smithii* (RM9) and *Staphylococcus* sp. (RM10), RM2 showed the highest zone of clearance (13 mm). Then all the 5 isolates were taken for production of alkaline amylase, using submerged fermentation by taking starch as substrates. There are many reports of getting more than one alkaline enzymes at 11.0 pH[40]. Kanekar and co-workers tested all the alkaliphilic isolates for the production of extracellular enzymes viz. protease, amylase, lipase and cellulase by growing them in the nutrient mediums at pH 10.0 containing casein, starch, tributyrin and carboxymethylcellulose as substrates respectively and among all the isolates, 34 could produce lipase, 23 isolates could produce amylase and 10 isolates could produce protease while 6 isolates produced enzyme cellulase at alkaline pH[38]. Other reports on *Bacillus amyloliquefaciens* P-001 and *Bacillus* sp. AB 04 suggested the production of alkaline amylase[42,43]. For the submerged fermentation, fresh cultures of RM1, RM2, RM7, RM9 and RM10 were prepared and inoculated into the MSM medium at 8.0 pH. They were subjected to alkaline amylase assay as described before. RM2 was found to be the potent producer of alkaline amylase among 5 bacterial isolates as illustrated in Figure 1. ANOVA revealed that there is a significant difference between the incubation period and isolates for enzyme production at $P<0.05$. Therefore, RM2 was selected and effect of the incubation period on the production of alkaline amylase was investigated. The results shown in Figure 2 demonstrated that maximum production was at 72 hr (14.5366 IU/ml) of incubation and thereafter a decrease in production was found. Similar reports were recorded by other researchers[41,44,45]. T-test revealed there is a significant difference between the incubation period and amylase production at $P<0.05$. Generally, enzyme production depends upon many factors, which are directly proportional to the growth of the microorganisms and many other molecules that show feedback inhibition of enzyme production in the production medium[32]. The reduction in enzyme yield after an optimum period is probably due to depletion of nutrients or production of inhibitors to the medium[32]. Behal and co-workers reported production of alkaline α amylase (535 IU/ml/min) at 48 hr of incubation by *Bacillus subtilis* GCUCM-25 at 40°C[43]. The same pattern was also seen by other researchers[42,45-47]. Tigue *et al.*,[48] reported a novel extracellular amylase of alkalophilic *Bacillus* sp. IMD 370 displaying maximum enzyme production at pH 10.0 and 40°C at 24 hr (5.4 U ml^{-1}). Zhang and co-workers reported maximal amylase activity by *Bacillus* sp. JCM 9141, to be 45 U/ml during cultivation with a starch medium[49]. *Bacillus* sp. from dhal industry waste which produced 0.0648

Table 6. Screening for alkaline enzymes performed at pH 8.0

Isolates	Amylase	Lipase	Protease	Pectinase
RM1	+++	-	++	-
RM2	+++	++	++	-
RM3	-	+++	+++	-
RM4	-	++	-	-
RM5	-	++	+++	-
RM6	-	++	++	-
RM7	+++	++	+	-
RM8	-	+	-	-
RM9	+++	++	+	-
RM10	++	++	+	-
PS1	-	-	-	-
PS2	-	-	-	+
PS3	-	++	-	-
PS4	-	-	-	-
PS5	-	+	++	-
PS6	-	-	+	-
PS7	-	++	+	-
PS8	-	+	-	-
PS9	-	+	-	+
PS10	-	-	-	-

>10mm (+++); 5-9mm (++); <5 (+); - negative.

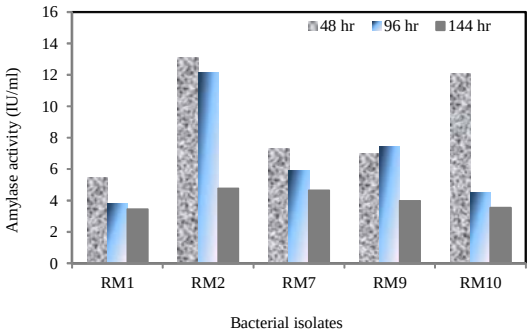

Fig. 1. Alkaline amylase production by alkalitolerant bacteria using submerged fermentation

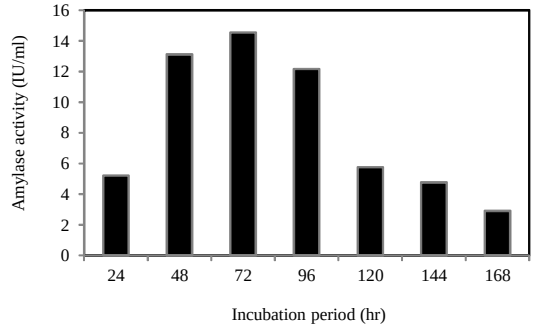

Fig. 2. Effects of incubation period on the production of alkaline amylase by isolate RM2

µ/min/mL using starch was reported[50]. Khan and co-workers reported β amylase production using wheat bran 0.08 U/min/mL followed by vegetable waste 0.06 U/min/mL, banana peel 0.05 U/min/mL and rice husk 0.047 U/min/mL by *Aspergillus niger*[51]. Behal and co-workers reported an alkaline α amylase by *Bacillus* sp. AB04 which showed maximum activity at pH 8.0 and was stable even at 10.0 pH[52].

The present work indicates that alkalitolerant bacteria exist in the red mud and sediment samples of Puri. The isolates studied are good producers of many alkaline enzymes, which find vast applications in the food, medicine, and detergent industries. Further research work can be carried out by taking RM2 for better production of alkaline amylase, and its purification followed by characterization.

ACKNOWLEDGEMENTS

Authors are very grateful to HOD, Department of Microbiology, OUAT, BBSR, India for providing laboratory support.

REFERENCES

1. Grant, W.D.: Cultivation of aerobic alkaliphiles. In: *Methods in Microbiology* (Rainey, FA, Oren, A ed). Academic Press, San Diego, 2006; **35**: 439-449.
2. Kumar, C.G., Tiwari, M.P., Jany, K.D.: Screening and isolation of alkaline protease producers from soda soils of Karnal, India. *Proceedings of First National Symposium on Extremophiles*, Hamburg, Germany, 1997.
3. Grant, W.D., Tindall, B.J.: The alkaline saline environment. In: *Microbes in Extreme Environments* (Herbert RA, Codd GA, ed). London: Academic Press, 1986; 22-54.
4. Langsworthy, T.A. Microbial life in extreme pH values. In: *Microbial Life in Extreme Environments* (Kushner DJ, ed). New York: Academic Press, 1978; 318-68.
5. Horikoshi, K., Akiba, T.: Alkalophilic Microorganisms: A New Microbial World. Tokyo, Japan: Japan Scientific Societies Press and Berlin, Germany: Springer-Verlag, 1982.
6. Durham, D.R. Utility of subtilisin GX as a detergent additive. *J. Appl. Bacteriol.*, 1987; **63**: 381-86.
7. Gee, J.M., Lund, B.M., Metcalf, G., Peel, J.L. Properties of a new group of alkalophilic bacteria. *J. Gen. Microbiol.*, 1980; **117**: 9-17.
8. Joshi, A., Ball, B. Extracellular alkaline enzymes of facultative bacteria of $CaCO_3$ kilns near Jabalpur. *Indian. J. Microbiol.*, 1993; **33**: 179-83.
9. Lodwick, D., Ross, H.N.M., Walker, J.A., Almond, J.W., Grant, W.D. Nucleotide sequence of the 16s ribosomal RNA gene from the haloalkalophilic archaeon (archaebacterium) *Natronobacterium magadii* and the phylogeny of halobacteria. *Syst. Appl. Microbiol.*, 1991; **14**: 352-57.
10. Kostrikina, N.A., Zvyagintseva, I.S., Duda, V.I. Ultrastructural organization of the extremely haloalkalophilic archaebacteria *Natronobacterium pharaonis* and *Natronococcus ocultus. Microbiol.*, 1991; **59**: 710-13.
11. Grant, W.D., Tindall, B.J. The isolation of alkalophilic bacteria. In: *Microbial Growth and Survival in Extremes of Environments* (Gould GW, Corry JCL, ed). London, New York: Academic Press, 1980; 27-36.
12. Horikoshi, K.. Microorganisms in alkaline environments. Kodansha- VCH, Tokyo, Japan. *Sci*. 1991.

13. Duckworth, A.W., Grant, W.D., Jones, B.E., Vansteenbergen, R. Phylogenetic diversity of soda lake alkaliphiles. *FEMS Microbiol. Ecol.*, 1996; **19:**181-191.
14. Groth, I., Schumann, P., Rainey, F.A., Martin, K., Schuetze, B., Augsten, K. *Bogoriella caseilytica* gen. nov., sp. nov., a new alkaliphilic actinomycete from a soda lake in Africa. *Int. J. Syst. Bacteriol*., 1997; **47:** 788-794.
15. Maharana, A.K., Ray, P. Application of Plackett-Burman design for improved cold temperature production of lipase by psychrotolerant *Pseudomonas* sp. AKM-L5. *Int. J. Curr. Microbiol. App. Sci*., 2014; **3**: 269-282.
16. Maharana, A.K., Ray, P. Low temperature degradation of various substrates by psychrotolerant *Fusarium* spp. isolated from soil of Jammu city. *J. Adv. Microbio*., 2014; **1**: 52-56.
17. Maharana, A.K., Ray, P. Screening of psychrotrophic micro-fungi for cold active extracellular enzymes isolated from Jammu city, India. *J. Pure. App. Microbiol*., 2014; **8**: 2369-2375.
18. Horikoshi, K. Alkaliphiles from an industrial point of view. *FEMS Microbiol. Rev*., 1996; **18**: 259-270.
19. Horikoshi, K. Discovering novel bacteria with an eye to biotechnological applications. *Current. Op. Biotech.*, 1999; **6**: 292-297.
20. Aono, R., Horikoshi. K. Chemical composition of cell walls of alkalophilic strains of *Bacillus. J. Gen. Microbiol*., 1983; **129**: 1083-1087.
21. Boone, D.R., Mathrani, I.M., Liu, Y., Menaia, J.A.G.F., Mah, R.A., Boone, J.E. Isolation and characterization of *Methanohalophilus portucalensis* sp. nov. and DNA re-association study of the genus *Methanohalophilus*. *Int. J. Syst. Bacteriol*., 1993; **43**: 430-437.
22. Akpor, O.B., Okoh, A.I., Babalola, G.O. Cultural microbial population dynamic during decomposition of *Theobroma cacao* leaf litters in tropical soil setting. *J. Bio.*, 2006; **6**(4): 768-774.
23. Jackson, M.L. Soil chemical analysis, Prentice Hall of India, Pvt . Ltd., New Delhi, 1967; 498.
24. Horikoshi, K. Discovering novel bacteria with an eye to biotechnological applications, *Current. Op. Biotechnol.* 1995; **6**: 292-297.
25. Collins, C.H., Lyne, P.M. Microbial methods. University Park Press, Baltimore, USA, 1970; 234-235.
26. Holt, J.G., Bergey, D.H., Krieg, N.R. Bergey's Manual of Systematic Bacteriology (2). Williams and Wilkins, Baltimore, USA. 1984.
27. Bryant, T.N. PIBWin-software for probabilistic identification. *J. app. Micro.*, 2003; **97**(6): 1326-1327.
28. Wang, H.Y., Liu, D.M., Liu, Y., Cheng, C.F., Ma, Q.Y., Huang, Q., Zhang, Y.Z. Screening and mutagenesis of a novel *Bacillus pumilus* strain producing alkaline protease for dehairing. *Lett. Appl. Microbiol*., 2007; **44**: 1-6.
29. Vihinen, M., Mantsala, P. Microbial amylolytic enzymes. *Crit. Rev. Biochem. Mol. Biol.* 1989; **24**: 329-418.
30. Maharana, A.K., Ray, P. Isolation and screening of cold active extracellular enzymes producing psychrotrophic bacteria from soil of *Jammu City. Biosci. Biotech. Res. Asia*., 2013; **10**(1): 267-273.
31. Rodriguez, K., Echer, F.M.K., Salamoni, P.S., Cofre, B.V., Van Der, S.S. Perfil da atividada enzimatica de actinomicetos isolados de proceso de compostagem. Sinaferm XVI, BAM 0626. 2007.
32. Maharana, A.K., Ray, P. Optimization and characterization of cold active endoglucanase produced by *Aspergillus terreus* strain AKM-F3 grown on sugarcane bagasse. *Turk. J. Biol*., 2015; **39**: 175-185.
33. Singh, A.K., Maharana, A.K., Masih, H., Kumar, Y., Mishra, S.K. Production, optimization and purification of bacterial cellulase by solid state bio-processing of agro biomass. *Res. J. Pharm. Biol. Chem. Sci.*, 2012; **3**: 977-989.
34. Fisher, E., Stein, E.A. α amylase from human saliva. *Biochem. Prepar*., 1961; **8**: 27-33.
35. Tiago, I., Chung, A.P., Verissimo, A. Bacterial diversity in a non-salaine alkaline environment: heterotrophic aerobic populations. *Appl. Environ. Microbiol*., 2004; **70**: 7378-7387.
36. Pedersen, K., Nilsson, E., Arlinger, J., Hallbeck, L., O'Neill, A. Distribution, diversity and activity of microorganisms in the hyperalkaline spring waters of Maqarin. *Extremophiles*., 2004; **8**: 151-164.
37. Agnew, M.D., Koval, S.F., Jarrell, K.F. Isolation and characterization of Novel alkaliphiles from bauxite processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. *System. Appl. Microbial*., 1995; **18**: 221-230.
38. Kanekar, P.P., Joshi, A.A., Kelkar, A.S., Borgave, S.B., Sarnaik, S.S. Alkaline Lonar Lake, India- A treasure of alkaliphilic and halophilic bacteria. Proceedings of Taal 2007: The 12th World Lake Conference: 2008; pp 1765-1774.
39. Ali, S.S., Habib, I., Riaz, T. Screening and characterization of alkaliphilic bacteria from industrial effluents. *Punjab Univ. J. Zool.*, 2009; **24** (1-2): 49-60.

40. Wagale, A., Berde, N.P., Jape, A.A., Sapre, V.R. A study to isolate the alkaliphiles in Mula Mutha River of Pune. *Asian J Multidiscip. Stud.*, 2013; 1.
41. Lakra, P.B., Mohapatra, S.B., Ray, P. Quantitative estimation of α amylase of *Bacillus spp*. isolated from water and sediment of Bhitarkanika mangrove ecosystem. *J. pure appl. Microbiol.*, 2014; **8**(4): 3147-3153.
42. Deb, P., Talukdar, S.A., Mohsina, K., Sarker, P.K., Abu Sayem, Deb, S.M. *et al*. *SpringerPlus.*, 2013; **2**:154.
43. Behal, A., Singh, J., Sharma, M.K., Puri, P., Batra, N. Characterization of alkaline α amylase from *Bacillus sp*. AB04. *Int. J. Agri. Biol.*, 2006.
44. Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K.M., Pandey, A. α-Amylase production by *B. amyloliquefaciens*. *Food Technol. Biotechnol*., 2006; **44**(2): 269-274.
45. Riaz, A., Qadar, S.A.U., Anwar, A., Lqbal, S., Bano, S. Production and characterization of thermostable α-amylase from a newly isolated strain of *Bacillus subtilis* KIBGE-HAR, The *Int. J. Microbiol*., 2009; **6**(1).
46. Dash, T., Ray, P. Characterization and quantification of amylase from *Bacillus* Dash *stearothermophilus. J. Pure. App. Microbiol*., 2010; **4**(1): 303-308.
47. Dash, T., Ray. P. Production and characterization of amylase from *Bacillus subtilis*. *Asian. J. Microbiol. Biotech. Env. Sc.*, 2013; **15**(1): 143-147.
48. Tigue, M., Kelly, C., Doyle, E., Fogarty,W. The alkaline amylase of the alkalophilic *Bacillus* sp. IMD 370. Department of industrial Microbiology, University College Dublin, Betfield, Dublin, Ireland.
49. Zhang, C., Xing, X., Liu, M. Production of multi enzymes consisting of alkaline amylase and cellulase by mixed alkalophilic culture and their potential use in the saccharification of sweet potato. *Biochem. Eng. J.*, 2004; **19**: 181-187.
50. Thippeswamy, S., Girigodwa, K., Mulimani, V. Isolation and Identification of α amylase producing *Bacillus* sp from dhal industry waste. *Ind. J. Biochem. Biophy*., 2006; 295-298.
51. Khan, J.A., Yadav, S.K. Production of alpha amylases by *Aspergillus niger* using cheaper substrates employing solid state fermentation. *Int. J. Pl. An. and Env. Sci*., 2011; **1**(3).
52. Behal, A., Singh, J., Sharma, M.K., Puri, P., Batra, N. Characterization of alkaline α- amylase from *Bacillus sp*. AB04. *Int. J. Agri. Biol*., 2006; 80-83.