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Moderate halophilic bacterial community in excreta of wild ass (*Equus hemionus khur*)

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Abstract

Twenty-four moderate halophiles were isolated from excreta of wild ass, designated as Mk-1 to Mk-24. All the organisms were Gram's positive and non-capsulated. Biochemical characterization was performed on the basis of Bergey's Manual of Systematic Bacteriology and all the isolates were belongs to *Bacillus* genera. Organisms were able to grow optimally at pH 5-6, 30-40˚C temperature and 10-15% NaCl (w/v) while no growth below 5% NaCl indicated moderate halophilic nature of isolates. All were grown in the presence of bile salt, indicated intestinal origin. Organisms were further explored for extracellular hydrolytic enzyme production such as lipases, proteases, amylases, cellulases and chitinase, apart from first aim to focus on biodiversity studies.

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Introduction

The Wild Ass sanctuary is located in the Little Rann of Kutch, Gujarat, India. Wild ass is a protected species of the Indian Wildlife Protection Act, 1972. Sanctuary covers an area of 4954 km². The sanctuary is named after a wild ass (Equus hemionus khur), highly endangered species of mammal. Persistent threats of poaching, habitat fragmentation and disease outbreaks are causing a rapid decline in their numbers (Moehlman 2002). The Khur are mainly distinguished by their pale, chestnut color and the dark chocolate fringe of hair on their back. They are very swift animals, and can reach and maintain maximum speeds of up to 50 km per hour (Menon 2003). Little Rann of Kutch is a typical ecological system with saline desert climate having unique floral and faunal diversity.

Organisms that could tolerate harsh conditions like extreme pH, temperature, salt etc. are categorized as extremophiles. Moderately halophilic bacteria are a group of micro-organisms that grow optimally in media containing 3-15 % NaCl (Ventosa et al., 1998). For haloadaptation, moderate halophilic bacteria must have certain mechanisms to cope up with external osmolarity. Main strategy for haloadaptation in moderate halophiles is accumulation of organic compounds, compatible solutes, which function as osmoprotectants. These solutes can be taken up from external environment (e.g. choline, betain) or synthesized by the cell (e.g. ecotine) (Nieto and Vargas, 2002).

Extremophilic biodiversity is widely studied around the world, since they produce enzymes able to work under extreme conditions and can be used in biotechnological and industrial potential applications (Hashim, 2007, Ventosa et al., 1998). Halophiles are the group of extremophiles that could tolerate high salt concentration. Halophilic bacteria are found in different environments such as salt lakes, saline soils and salted foods (Ventosa et al., 1998). Moderate halophiles are important at an industrial scale because they accumulate high cytoplasmic concentrations of compatible solutes that may be used as osmoprotectants and stabilizers of enzymes and cells (Galinski, 1993); some of them are used for the degradation of polluting industrial residues or toxic chemicals and for enhanced oil-recovery processes (Ventosa and Nieto, 1995). Moreover, moderately halophilic bacteria produce extracellular salt and thermo tolerant enzymes of great interest for biotechnological processes (Onishi, 1972). Halophilic microorganisms have greater capacity to produce salt and thermo tolerant enzymes like cellulases, amylases, proteases, lipases and xylanases (Sánchez-Porro et al., 2003; Govender et al., 2009; Rohban et al., 2009).

Little Rann of Kutch is a wetland- saline biome, plant and grass grows on saline environment and turns to halophyte. As wild ass is herbivore, its intestinal microflora have adapted to saline conditions. Work is focused on biodiversity study of bacteria in comparative unexplored habitat i.e. wild ass intestine, its role in ecosystem, adaptation towards changing environment and possible biotechnological applications through extracellular enzyme production.

Materials and methods

Collection of samples

Samples (Excreta of wild ass) were collected from little Rann of Kutch, from wild ass sanctuary near Dhrangadhra, Gujarat, India [Latitude- 22°98'4.181"N and Longitude- 71°51'0.242"E].

Enrichment and isolation of halophiles

Halophiles were enriched in halophilic broth (Himedia) containing (gm/lit); Casein acid hydrolysate-10, Yeast extract- 10, Protease peptone-5, Trisodium citrate- 3, Potassium chloride- 2, Magnesium sulfate- 25, Sodium chloride- 50-150, pH- 7.0-7.4 as well as complete media broth containing (gm/lit); Glucose- 10, Potassium dihydrogen phosphate- 10, Yeast extract- 5, Peptone- 5, Sodium chloride- 50-150, pH- 7.0-7.4. From enriched 15% NaCl (w/v) halophilic broth and complete media broth organisms were streaked on respective agar media by four sector method for the purpose of isolation into pure culture. Total 24 isolates were obtained, designated as Mk-1 to Mk-24 and preserved on N-agar slant at 4˚C for further studies.

Morphological and biochemical characterization

Morphological characterization of moderate halophiles was performed by Gram's staining and capsules staining. Biochemically isolates were analyzed on the basis of Bergey's Manual of Systematic Bacteriology.

Screening of extracellular lipase producers

Lipase producers were screened on Tributyrin agar containing (gm/ 100ml) Tributyrin- 1ml, Yeast extract- 1, peptone-1, NaCl- 10, Agar- 3 and pH- 7.2. Colonies showing clear zone surrounding at 30°C after 3 days was taken as evidence of lipolytic activity.

Screening of extracellular amylase producers

Amylase producers were determined by method described earlier (Amoozegar, 2008) on starch agar containing (gm/ 100ml); Starch- 0.2, Yeast extract- 0.5, Peptone- 1, NaCl- 10, Agar- 3 and pH- 7.2. After incubation for 2 days at 30°C, iodine solution (gm/100ml; Iodine- 0.33, KI- 0.66) was added. Clear zone surrounding the colonies against blue background was evidence of amylolytic activity.

Screening of extracellular protease producers

Isolates were screened on gelatin agar containing (gm/ 100ml) Gelatin- 3, Peptone- 1, yeast extract-1, NaCl-10, Agar- 3 and pH 7.2. Protease production was monitored by adding Frazier's reagent (gm/100 ml); $HgCl_2$- 15g; Concentrated HCl 20 ml in all the plates previously incubated at 30°C for 3 days. Colonies showing clear zone surrounding was considered to be a protease producer.

Screening of extracellular cellulase producers

Cellulolytic activity was determined on Dubo's agar medium containing (gm/100 ml); Cellulose- 1, K_2HPO_4- 0.1, $NaNO_3$- 1, KCl- 0.05, $MgSO_4{\cdot}7H_2O$- 0.05, $FeSO_4{\cdot}7H_2O$- 0.001, Agar- 3, NaCl- 10 and pH 7.2. After 5 days at 30°C, colonies showing clear zone surrounding were cellulase producers.

Screening of extracellular chitinase producers

Chitinase producers were screened on Chitin agar plates containing; (gm/100 ml); Chitin- 1, Yeast extract- 0.5, Peptone- 0.5, NaCl- 10, pH 7.2 and Agar- 3. After incubation period of 3 days at 30°C, colonies showing clear zone surrounding were chitinase producers.

Results and discussion

Isolation and characterization

The samples collected from different regions of wild ass sanctuary approximately weighed 10-15 grams each and with blackish color. Samples were collected during the month of May when the little Rann of Kutch appears to be a salt desert.

All together 24 moderate halophiles were obtained from excreta sample. Isolates were named as Mk-1 to Mk-24. The isolates could tolerate 10-15% NaCl but fails to grow below 5% NaCl indicated moderate halophilic nature of the organisms, similar to *Halomonas pantelleriensis* isolated from Pantelleria Island, Italy (Romano et al., 1996). This result was surprising as such a high salt concentration tolerance by intestinal organism is unusual. It might be due to presence of salt in grass that is utilized by wild ass as a food. Moderate halophiles were growing optimally at temperature 30-40˚C, similar to *Salinivibrio* SA2, isolated from salty environment of Iran (Amoozegar, 2008) and pH 5-6, lower than *Salicola* sp. IC10 (De Lourdes et al., 2009). Additionally all the isolates were able to tolerate bile salt (Sodium deoxycholate-Himedia), indicative of their truly intestinal origin.

Table 1. Biochemical characterization of moderate halophiles.

Sr. No.	Name of the Test	Mk-1	Mk-2	Mk-3	Mk-4	Mk-5	Mk-6	Mk-7	Mk-8	Mk-9	Mk-10	Mk-11	Mk-12	Mk-13	Mk-14	Mk-15	Mk-16	Mk-17	Mk-18	Mk-19	Mk-20	Mk-21	Mk-22	Mk-23	Mk-24
1	Gram's stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Capsule Stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	Methyl Red test	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
5	Voges-Proskaur test	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
6	Starch hydrolysis	-	-	-	-	-	+	+	-	+	-	-	+	-	-	+	+	+	-	+	-	+	-	-	-
7	Casein hydrolysis	+	-	-	+	-	+	+	-	-	-	+	+	-	-	+	-	+	+	+	-	-	-	+	+
8	Gelatin hydrolysis	+	-	-	+	-	+	+	-	-	-	+	+	-	-	+	-	+	+	+	-	-	-	+	+
9	Citrate utilization	-	+	+	-	-	-	-	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-
10	Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	Motility	+	-	+	+	-	-	+	+	+	+	-	+	-	+	+	+	+	+	-	+	+	-	-	+
12	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	Phenyl-alanine Deaminase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	Nitrate reduction	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	-	+	+
15	Growth in media containing NaCl (%, w/v)																								
	2% NaCl	-	-	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-
	5% NaCl	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	9% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	Growth at pH																								
	pH 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	pH 9	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

17	Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
18	Growth at Temperature																								
	4°C	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	50°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+
19	Acid production from																								
	Glucose	+	+	+	+	-	+	+	-	+	-	+	-	-	+	+	+	+	+	+	-	+	-	+	+
	Maltose	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+	-	+	-	-	-	+	-	-	-
	Fructose	+	+	+	+	-	+	-	-	+	-	-	-	-	-	+	+	+	+	-	-	+	-	-	+
	Sucrose	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	+
	Xylose	-	+	+	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	+	-	+	+
20	Growth on MacConky's agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	Growth on EMB agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	Bile salt tolerance	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

All the organisms were Gram's positive and non-capsulated. Until now, majority of moderate halophiles studied in detail belongs to Gram negative group and information regarding Gram positive moderate halophiles is rare (Ventosa et al., 1998). Even, there was versatility among colony morphology, size, shape and arrangement of all the isolates indicating rich biodiversity. Additionally, none of organisms from excreta were able to grow on EMB and MacConkey's agar, again indicative of elimination of Gram negative intestinal bacteria and colonization of Gram positive in wild ass intestine.

Biochemical identification

All the organisms have capacity to produce enzyme catalase, suggesting their aerobic nature. The majority of organisms could not utilize urea and tryptophan suggesting the absence of enzyme urease and tryptophanase in them. Similar types of results were also obtained by various investigators (Muntyan et al., 2002, Romano et al., 2005). None of isolates have capacity for indole production. Isolates were highly versatile for sugar utilization, again indicating rich biodiversity (Table 1).

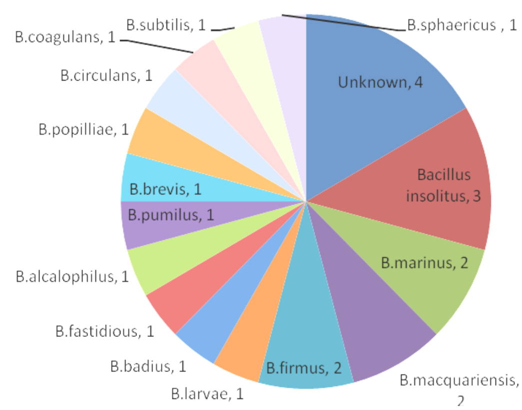

Fig. 1. Diversity of *Bacillus* from wild as excreta.

Moderate halophiles were biochemically identified according to Bergey's manual of systematic bacteriology. All the isolates were found to be Gram positive *Bacillus*, the result was also confirmed by Gram's staining and colony morphology. Diversity at species level is represented in Fig. 1.

Extracellular enzyme secretion

Halophilic organisms are important economically for their ability to produce of salt and thermotolerant enzymes (Kamekura, and Onishi, 1978). Extracellular enzymes like lipases, proteases, amylases, cellulases have their own importance in different fields of chemical industries (Kamekura et al., 1982, Sanchez-Porro et al., 2003, Govender et al., 2009). Halophiles can produce industrially important enzymes such as amylase (Amoozegar et al., 2003), protease (Vidyasagar et al., 2009) xylanases (Prakash et al., 2009) etc. Moderate halophiles have diversity for enzyme production. 7 produces lipase, 8 produces amylase, 12 produces protease, 2 produces chitinase while only 1 isolate produces cellulase. Majority of organism produces one or two enzymes, while six isolates were not able to produce any of the five enzymes. All the 24 isolates have different potential of enzyme production and have ability for the production of different quantity of enzymes judged on the basis of zone ratio (Fig. 2).

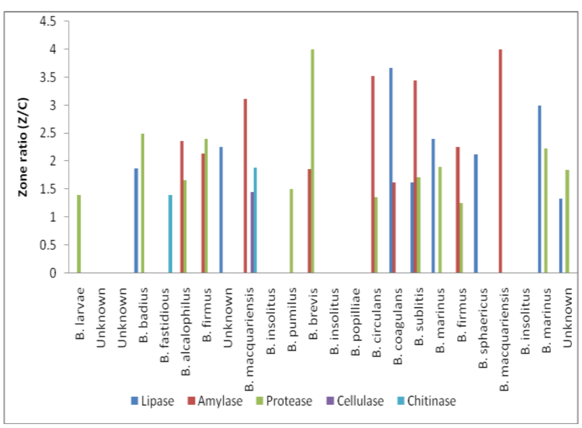

Fig. 2. Enzymatic profile of isolates.

In the present study, moderate halophiles were isolated from comparative unexplored ecological niche i.e. intestine of wild ass. All the isolates obtained from above ecological niche have rich biodiversity in terms of morphological, biochemical, cultural characteristics and extracellular hydrolytic enzymes production.

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