

Characterization and extracellular enzyme activity of predominant marine *Bacillus* spp. isolated from sea water of Orissa Coast, India

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ABSTRACT

Bacillus species are ubiquitous and diverse both in the terrestrial and marine ecosystems. In this investigation, predominant *Bacillus* species from sea water of three different sites of Orissa Coast were isolated and identified. In total, 16 *Bacillus* species were identified using morpho-physiological and biochemical characterisation. These identified bacterial strains include *B. fastidiosus* (CMB1), *B. alvei* (CMB2), *B. coagulans* (CMB3), *B. marinus* (CMB5), *B. mycoides* (CMB8), *B. coagulans* (PMB1), *B. circulans* (PMB2), *B. cereus* (PMB3), *B. subtilis* (PMB4), *B. alcalophilus* (GMB1), *B. licheniformis* (GMB2), *B. polymyxa* (GMB3) and *B. pumilus* (GMB4). The isolates CMB4, CMB6 and CMB7 were identified only up to genus level. These isolates were further screened for their salt tolerance and growth under varied temperature and pH conditions. Ability of these strains to produce extracellular enzymes such as protease, amylase, lipase, gelatinase, casein hydrolase, lecithinase, chitinase and pectinase were also screened and found that most of the *Bacillus* spp. possess extracellular enzymes.

Keywords: marine bacteria, Orissa coast, extracellular enzymes

INTRODUCTION

Marine bacteria play a decisive role in the cycle of matter in water, as they are able to breakdown all natural components from which they have originated (Rheinheimer, 1980). Besides, marine bacteria are known to produce wide range of compounds, which have potential applications as bioactive compounds, probiotics and nutritional supplements (Watanabe *et al.*, 1996). Marine bacteria are screened for secondary metabolites like antibiotic production which has not been observed from terrestrial microorganisms (Jensen and Fenical, 1996). The genus *Bacillus* comprised a phylogenetically and phenotypically diverse species; they are ubiquitous in terrestrial and fresh water habitat and are also widely distributed in sea water (Ruger, 1989). Systematic studies of *Bacillus* have always focused on the terrestrial *Bacillus*, although marine Bacilli are noted for their ability to produce different antibiotics, glucanases and cyclic acylpeptides (Oguntoyinbo, 2007). Besides their enzyme production, marine Bacilli are also well known for the reduction of toxic heavy metals.

The state of Orissa lies in the east coast of India has a coast line of over 480 kms along the Bay of Bengal. So far no study has been done on bacterial diversity especially in *Bacillus* group from sea water of Orissa coast. Marine microorganisms known to have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with novel enzymes and

they are also source of secondary metabolites and therapeutics (Das *et al.*, 2006). Hence, in the present study an attempt has been made to isolate and characterize some of the predominant marine *Bacillus* species from water samples of three marine sites viz. Chandipur, Puri and Gopalpur with a view to identify the strains and to evaluate their enzyme production potentials.

MATERIALS AND METHODS

Site description and sample collection

Orissa is situated from 17° 49' to 22° 34' north latitude and from 81° 29' to 87° 29' east longitude. Sea water samples were collected from sea beaches of Chandipur, Puri and Gopalpur of Balasore, Puri and Ganjam districts respectively. The samples were collected in 1 L sterile plastic bottles with proper level. The samples were immediately stored in a refrigerator for further microbial work. pH, water temperature and conductivity were measured the sampling site. pH of the water sample varied from 8.4-8.6, similarly the electrical conductance (Eh) of the samples varied from 24.9-61.8 (ms/ppt).

Isolation of bacteria

A 1 mL of water sample was mixed with 9 mL sterile distilled water, diluted logarithmically up to 10⁻⁵ level. A 1 mL suspension of each water sample was inoculated

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separately in 20 mL nutrient broth medium containing 5% NaCl (w/v). The samples were grown on a shaker at 100 rpm at 30 ± 1 °C for 72 h and 20 μ L of each culture broth were plated separately on nutrient agar plates containing equivalent concentration of NaCl (Holt, 1984). The plates were incubated at 30 ± 1 °C for 48 h. Colonies with distinct morphology were aseptically subcultured into fresh nutrient agar medium until pure cultures were obtained.

Physiological and biochemical characters

The characters of the organisms were studied following the standard microbiological methods. Morphology, vegetative cell and spore characters were observed under a phase contrast microscope (100X objective) from 12 h old culture grown on a rotary shaker at 100 rpm, 30 ± 1 °C. The physiological and biochemical characters viz. indole production, oxidase, catalase, urease hydrolysis, acid from glucose, mannitol, arabinose, xylose, citrate, and propionate utilization and tyrosine hydrolysis were studied.

Antibiotic resistance

Response of the organisms to different antibiotics was tested on nutrient agar medium. Nutrient agar plates were surface seeded with 2 μ L of 10^8 bacterial suspension/mL. Different antibiotic discs with effective concentrations were placed over the plates. Inhibition of growth depicted by a clear zone formation around the discs indicated sensitive reaction otherwise the organism was resistant to the antibiotic. Diameter of the inhibition zone was measured with an antibiotic zone scale.

NaCl and pH tolerance

Growth of the organisms on nutrient agar medium supplemented with different concentration of NaCl 3-15% (w/v) was checked. Highly diluted suspensions of the organisms were spotted on the plates, incubated at 37 °C for 72 h and the growth of the organism was checked. pH tolerance of the organisms was also checked on same medium maintained at different pH (3-13). The organisms were spotted on the plates and incubated at 37 °C for 72 h and growth was checked.

Analysis of extracellular enzymes

The extracellular enzymes production viz. amylase, protease, lipase were analyzed through plate test and quantitative method by growing these individual bacterial isolates in starch agar (amylase), gelatin agar, chitin agar, lecithin agar (protease) and peptone agar media (lipase). After 72 h of incubation at 37 °C, culture plates were tested for enzyme activity by adding iodine solution in amylase plates, HgCl₂ (10%) in protease plates. The clear zone formation around the growing colony was considered as positive. The lipase activity of bacterial isolates were determined using two lipid sources viz., Tween 80 and cholesterol on lipase test medium and the formation of

opaque whitish zone around the growing colony was considered as positive (Booth, 1978).

RESULTS AND DISCUSSION

In the present study sixteen possible strains of bacteria have been isolated from three sites from sea water of Orissa coast and designated as Chandipur marine bacteria (CMB1-8), Puri marine bacteria (PMB1-4) and Gopalpur marine bacteria (GMB1-4). The isolated microorganisms were stained Gram-positive and formed both elliptical and round endospores. All strains were found to be rod shaped; some grew in short chain and some in long chains. Previous researchers (Priffner *et al.*, 1986; Bock *et al.*, 1994) reported that most of the microorganisms from hyper saline environment are rod shaped. The isolates CMB4, CMB6 and CMB7 are identified only up to genus level because the phenotypic characters of these isolates did not match with any known species of *Bacillus* so they may belongs to new species. Anaerobic and catalase test showed that all strains except CMB7, CMB8 and PMB1 were able to grow under anaerobic condition but all strains were found catalase positive. These findings are in good agreement with previous studies which reported that aerobic organisms present in the saline environment are predominated by Gram-positive, facultative and spore forming rod bacteria (Belyaev *et al.*, 1993).

Based on the biochemical and morphological tests (Table 1) according to the Bergey's manual (Holt, 1984) all strains were identified as genus *Bacillus*. However, the organisms differed in some physiological and biochemical characters among themselves but the less distinguishing characters like oxidase, anaerobic growth, acid and no gas formation from different sugars, protease, amylase, lipase, chitinase, NO₃ production, fermentation of organic carbon sources, antibiotic sensitivity test etc. (Table 1, Table 3) also confirm to the characters of *Bacillus* spp. Further some confirmative tests were also done to identify the isolates up to species level. Taking all the above characters into consideration the isolates were tentatively identified as *B. fastidiosus* (CMB1), *B. alvei* (CMB2), *B. coagulans* (CMB3), *B. marinus* (CMB5), *B. mycoides* (CMB8), *B. coagulans* (PMB1), *B. circulans* (PMB2), *B. cereus* (PMB3), *B. subtilis* (PMB4), *B. alcalophilus* (GMB1), *B. licheniformis* (GMB2), *B. polymyxa* (GMB3) and *B. pumilus* (GMB4). The isolates CMB4, CMB6 and CMB7 were identified only up to genus level.

Molecular analyses such as 16S rRNA gene sequencing, FAME analysis etc. are also required for proper identification of any newly isolated strains. Bacteria are also identified on serological reactions, analysis of the components like amino acids, lipids, phage typing etc. (Holt, 1984; Sneath, 1986; Garrity, 2001). However, all these methods have both inherent merits and demerits and sometimes one method may not be sufficient to classify all of the new isolates. Therefore, biochemical grouping is widely accepted for classification of bacteria

Table 1: Identification scheme of the bacterial isolates up to species level based on morpho-physiological characters from sea water of Orissa coast

Characteristics	CMB1	CMB2	CMB3	CMB4	CMB5	CMB6	CMB7	CMB8	PMB1	PMB2	PMB3	PMB4	GMB1	GMB2	GMB3	GMB4
Cell diameter > 1 µm	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spores round	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	-
Sporangium swollen	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
VP test	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+
pH in VP broth:																
pH <6	NG	NG	NG	+	-	-	-	-	ND	+	+	+	+	+	+	+
pH >7	-	+	-	+	+	+	+	NG	NG	+	+	+	+	+	+	+
Acid from:																
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- xylose	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-
D- mannitol	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+
Gas from glucose	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+
Hydrolysis of:																
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Strach	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
Propionate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6.8 pH in NB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.7 pH in NB	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+
Growth on NaCl >5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on NaCl >10%	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+

Identified species	Growth at 50 °C	Growth at 40 °C
<i>B. fastidiosus</i>	-	+
<i>B. alvei</i>	-	+
<i>B. coagulans</i>	-	+
<i>Bacillus sp.</i>	-	+
<i>B. marinus</i>	-	+
<i>Bacillus sp.</i>	-	+
<i>Bacillus sp.</i>	-	+
<i>B. mycoides</i>	-	+
<i>B. coagulans</i>	-	+
<i>B. circulans</i>	-	+
<i>B. cereus</i>	-	+
<i>B. subtilis</i>	-	+
<i>B. alcalophilus</i>	-	+
<i>B. licheniformis</i>	-	+
<i>B. polymyxa</i>	-	+
<i>B. pumilus</i>	-	-

+ = Positive result, - = negative result, ND = not detected, NG = no growth

and other methods are being used as supporting information (Garrity, 2001).

All strains could grow at temperature ranging from 37 °C to 65 °C, however optimum temperature varied from 40 °C to 45 °C (Table 2). *B. circulans* and *B. pumilus* were tolerating up to 9% and 12% NaCl (w/v) respectively (Table 2). All of these isolates were able to grow over a broad pH range (3-11). However, these isolates grew well between pH 7.5-9.0.

Table 2: Physiological characterization of bacterial isolates from sea water of Orissa coast

Strains	Optimum temperature (°C)	Growth in NaCl (w/v)	pH of culture
CMB 1	40	6	3-11
CMB 2	40	6	5-11
CMB 3	40-45	6	3-11
CMB 4	40	6	5-11
CMB 5	40-45	6	3-11
CMB 6	40-50	6	3-11
CMB 7	40-45	6	5-11
CMB 8	40	6	3-11
PMB 1	40-50	6	5-11
PMB 2	40	9	5-11
PMB 3	40-45	6	5-11
PMB 4	40-50	6	5-11
GMB 1	40	6	5-11
GMB 2	40	6	5-11
GMB 3	40	6	5-11
GMB 4	40	12	5-11

The antibiotic sensitivity of the isolates showed that all isolates were sensitive to penicillin G, ampicillin, chloromphenicol, streptomycin except the antifungal antibiotics nystatin (Table 3). Antibiotics are specific in nature, being effective against certain microorganism, and not against others. Microorganisms vary in their susceptibility to different antibiotics basing on which they can be put into different groups.

Extracellular enzyme activity of the isolates revealed that all of them are able to produce amylase and protease. Interestingly it was also found that *B. coagulans* and one species of *Bacillus* (CMB4) could able to produce pectinase, chitinase and lecithinase (Table 4). Production of enzymes tested was more common among endospore-forming bacteria. Though the *Bacillus* is an endospore forming organism it may be one reason for production of such diverse extracellular enzymes (Krystyna and Henrique, 2007). The bacterial strains isolated in the present study were able to produce more diverse extracellular enzymes. Combined hydrolytic activities have been detected in various bacterial strains that could be used for biotechnological purposes. Proteases have a long history of application in food and detergent industries with the detergent alkaline proteases holding the largest share of the enzyme market (Venugopal and Saramma, 2007). Proteolytic activity was the most common among isolates, since 93% of mesophiles and all thermophiles

produced extracellular proteases (Krystyna and Henrique, 2007). Many reports showed that actinomycetes, bacteria of genera *Bacillus* and *Pseudomonas* are able to degrade chitin (Krystyna and Henrique, 2007).

The result is encouraging as bacteria with usefull enzyme production potential are found in the sea water of Orissa coast. The present study is a preliminary screening report of diversity of *Bacillus* species and their enzyme producing potential from Orissa coast. This study revealed a high taxonomic diversity among the *Bacilli* isolated from Orissa coast. Besides that the results also indicated a distinct distribution of the *Bacillus* species among the marine sites. It is known that *Bacillus* from saline environment has greater biotechnological potential compared to other group of bacteria (Oguntoyinbo, 2007). Isolation of microbes from saline environment would also provide ample scope to assess their biotechnological potential. Attempt should be made for proper evaluation and exploration of these microbes for the biotechnological applications.

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Table 3: Antibiotic assay of bacterial isolates from sea water of Orissa coast

Antibiotics	CMB1	CMB2	CMB3	CMB4	CMB5	CMB6	CMB7	CMB8	PMB1	PMB2	PMB3	PMB4	GMB1	GMB2	GMB3	GMB4
Ampicillin (10 μ g)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Penicillin G (10 U)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Nystatin (10 μ g)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Chloromphenicol (30 μ g)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin (10 μ g)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

S = Sensitive, R = Resistance

Table 4: Extracellular enzymatic activities of bacterial isolates from sea water of Orissa coast

Test	CMB1	CMB2	CMB3	CMB4	CMB5	CMB6	CMB7	CMB8	PMB1	PMB2	PMB3	PMB4	GMB1	GMB2	GMB3	GMB4
Protease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pectinase	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-
Chitinase	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-
Lecithinase	+	+	+	+	+	-	-	-	+	-	+	-	-	-	-	-
Lipase:																
Tween 80 hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cholesterol hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Positive result, - = Negative result

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