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INFLUENCE OF CULTURAL CONDITIONS FOR EXTRACELLULAR LIPASE PRODUCTION BY A HALOTOLERANT BACTERIUM, *Bacillus* sp. ORS4

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ABSTRACT

During the course of survey of halotolerant bacteria from saline environments, 17 bacterial cultures were isolated in pure form from the multi-pond solar salterns of Odisha and West Bengal, India. The isolates were screened for their lipase production capability on basal MH-Tween 80 agar medium containing 10% NaCl and the isolate ORS4 having potential for high lipase production, was selected for further detailed studies. The ORS4 isolate showed most similarity to *Bacillus vallismortis* from genus *Bacillus* on behalf of the morphological, physiological and biochemical characteristics along with 16S rRNA gene sequence analysis. Time course of growth and lipase production by the isolate ORS4 showed maximum enzyme activity (35.79 U/ml) during the stationary phase of growth. Moreover, the optimum conditions for lipase production by the isolate in basal MH medium involved 1% Tween 80, 5% NaCl, 1% glucose, 1% tryptone with an inoculum density of 10^{12} cells/ml. The partially purified extracellular lipase of *Bacillus* sp. ORS4 showed tolerance to alkaline pH along with high salinity and temperature suggesting its potential for biotechnological applications in saline environments.

Keywords: Halotolerant-bacteria, hypersaline environments, lipase, 16S rRNA gene sequence, *Bacillus* sp., multi-pond solar salterns

INTRODUCTION

Lipases (triacyl glycerol acylhydrolases, EC 3.1.1.3) represent a diverse and versatile group of enzymes, which catalyzes the hydrolysis of long chain acylglycerols. They remain active in variety of organic solvents and can catalyze various other transformations like esterification, perhydrolysis, alcoholysis, interesterification, and aminolysis (**Margolin *et al.*, 1987**). Biotechnological potential of lipases is therefore, enormous and they have a high interest for food, agriculture, chemicals, pharmaceuticals, medical, dairy, detergent, cosmetics, oleo-chemical, fat processing, leather, textile and paper industries (**Houde *et al.*, 2004**). As most of the industrial processes are carried out under harsh physico-chemical environments, the microbial lipases showing optimum activities under wide range of stressed conditions like high salt concentrations, pH, temperature etc are on great demand.

Halotolerant and halophilic microorganisms growing in high salt concentrations (**Martin *et al.*, 2003; Khunt *et al.*, 2012**) have greater capacity to produce wide variety of salt and temperature tolerant enzymes like cellulases, amylases, proteases, lipases and xylanases (**Sanchez-Porro *et al.*, 2003; Ardakani *et al.*, 2012**). As against the lipases of halotolerant *Staphylococcus warneri* (**Kanlayakrit and Boonpan, 2007**) and *Bacillus pumilus* LV01 (**Guzman *et al.*, 2008**), the production of thermophilic lipases by moderate halophiles *Salinivibrio salina* (**Amoozegar *et al.*, 2008**) and *Marinobacter lipolyticus* (**Martin *et al.*, 2003**) are not uncommon. Conditions for production of lipase by moderately halophilic *Halomonas salina* isolated from little Rann of Kutch (**Khunt *et al.*, 2012**) and that of extremely halophilic bacterium *Salicola* sp. from Kerala, India ((**Anisha *et al.*, 2012**) have been optimized and their lipases showed optimal activities at high NaCl concentrations, pH and temperature. In studies dealing with lipases, the studies were mostly made with crude extracts, but **Li and Yu (2012)** have recently characterized a novel extracellular lipase from a moderately halophilic *Chromohalobacter* sp. LY7-8 isolated from Lake of Yuncheng. Though majority of the lipases so far reported were derived from the moderately halophilic bacteria but extremely halophilic archaea were not exceptional. **Boutaiba *et al.* (2006)** reported that lipase from *Natronococcus* sp. exhibited an optimum activity at pH 7.0, while **Ozcan *et al.* (2009**) reported optimal esterase activity of haloarcheal strains at variable conditions.

This study was aimed to screen halotolerant and moderately halophilic bacteria for the production of extracellular lipases isolated from soil and water samples from the coastal solar salterns of Odisha and West Bengal, India. Moreover, lipase production by the most potential isolate *Bacillus* sp. ORS4 has been optimized with variable nutritional and cultural parameters.

MATERIALS AND METHODS

Source of bacterial isolates

Soil and water samples collected from multipond solar salterns of Odisha and West Bengal, India, were analyzed microbiologically in order to isolate halotolerant and halophilic bacteria following dilution and plating method as well as by enrichment technique in MH medium (**Ventosa *et al.*, 1989**) supplemented with 10% NaCl. The medium contained (g/l): yeast extract (10), protease peptone (5), glucose (1), NaCl (100), $MgCl_2$, $6H_2O$ (7), $MgSO_4$, $7H_2O$ (9.6), $CaCl_2$, $2H_2O$ (0.36), KCl (2), $NaHCO_3$ (0.06), NaBr (0.026), pH 7.2. Phenotypically distinguishable halotolerant and moderately halophilic bacterial isolates were purified by dilution-streaking on MH agar medium and maintained on agar slants of the same medium until used for the screening of lipase production.

Screening of isolates for lipase production

Screening of bacterial isolates for lipase production was carried out on basal MH agar medium supplemented with 10% NaCl and 1% Tween 80 as substrate (**Gonzalez *et al.*, 1978**). The plates were inoculated with bacterial isolates in form of small streaks and incubated at 32°C for 4 days. Formation of halo precipitation zone around the colony of the isolate indicated the production of lipase.

Physio-biochemical characterization of the selected isolate

Morphological, physiological and biochemical characteristics of the selected bacterial isolate were determined on basal MH medium supplemented with 5% NaCl. Gram-staining, motility, color and shape of the colonies and IMVIC tests were performed as recommended by **Smibert and Krieg (1994**). Optimum pH and temperature for growth and tolerance to NaCl (%, w/v) were also routinely performed. Fermentation of different carbohydrates was also tested by using phenol red medium supplemented with 1% (w/v) carbohydrate (**Gerhardt *et al.*,**

1994). The inhibitory effect of different antibiotics was determined by disc-diffusion method. The antibiotic containing discs (Himedia) were placed on MH agar medium seeded with test isolate, incubated for 24 h at 32°C and the diameter of the inhibition zone was measured to nearest mm (**Gerhardt *et al.*, 1994**).

Molecular identification of the selected isolate

Molecular identification of the selected bacterial isolate was carried out by 16S rRNA gene sequence analysis. Chromosomal DNA of the bacterial strain was isolated and purified according to a modified method of **Marmur (1961**) and used as template for polymerase chain reaction (PCR) to amplify 16S rRNA gene using bacterial universal primers (Forward Primer 8F : 5'-AGAGTTTGATCCTGGCTCAG-3; Reverse Primer 1492R: 5'-GGTTACCTTGTTACGACTT-3' (**Turner *et al.*, 1999**). The PCR amplified product was purified and sequenced in ABI 377 automated DNA sequencer. Sequencing data were analyzed by ABI version 3.0.1 b3 software and compared with the available databases by using the BLAST search programs (BLASTN), (**Altschul *et al.*, 1997**). Multiple sequence alignments were carried out by using the program package Clustal W (**Thompson *et al.*, 1994**) and used for phylogenetic analysis by neighbor joining method using MEGA 5.

Growth kinetics and lipase production

Lipase production during growth of the potent isolate ORS4 was monitored in basal MH and Davis-Mingioli's medium supplemented with 1% Tween 80. The medium was inoculated with freshly grown culture and incubated on a rotary shaker (120 rpm) at 32°C for 6 days. Samples were withdrawn at defined time interval and bacterial growth was determined by measuring optical density at 540 nm. Lipase activity of the culture filtrate was measured following the method of **Winkler and Stuckman (1970).**

Assay of lipase activity

Lipase activity of the culture filtrate was assayed (**Winkler and Stuckman, 1979**) using *p*- nitrophenolpalmitate (*p*-NPP) as the substrate. The reaction mixture (1 ml) contained 20 µl of *p*-NPP (10 mM prepared in 2- propanol), 100 µl of culture filtrate in phosphate buffer (pH 7.0) and 880 µl of Tris-HCl buffer (50 mM, pH 9.0). The mixture after thorough mixing, was incubated at 50°C for 20 min in a water bath and the reaction was stopped by adding 330 µl of chilled (-20°C) acetone and ethanol mixture (1:1). A control was maintained separately using heat inactivated culture filtrate in the same way.
Absorbance (A_{420}) of *p*-nitrophenol released as a result of the catalytic reaction was measured and the corresponding concentrations were determined from the previously prepared calibration curve of *p*-nitrophenol. One unit (U) of lipase activity was defined as the amount of enzyme that liberates one micromole (µmole) of *p*-nitrophenol by hydrolysis of *p*-NPP per minute by 1 ml of enzyme at 50°C under the standard assay conditions.

Influence of culture parameters on growth and lipase production

To optimize the conditions for growth and lipase production, the isolate was grown in basal MH medium with various substrates, having different concentrations of NaCl, carbon source, nitrogen source, initial pH and inoculum density by incubating at 32°C under shaking conditions (120 rpm) for 5 days.

Partial purification of the enzyme

Under optimized cultural conditions, the isolate was grown for 5 days and the culture was centrifuged (10000×g) at 4°C for 15 min. The obtained supernatant was treated with solid ammonium sulphate to 80% saturation and stirred overnight at 4°C. The precipitate was collected by centrifugation (12000×g at 4°C), resuspended in minimal amount of 0.1M Tris-HCl buffer (pH 7.0) and dialyzed overnight at 4°C against the same buffer. The desalted fractions were used as the partially purified lipase.

Characterization of the lipase

The effect of pH on enzyme activity of the partially purified lipase was determined by incubating the enzyme in buffers over a pH range of 6.0-9.0 at 32°C for 1 h. The 0.2 M citrate (pH 4.0-5.0), phosphate (pH 6.0-7.0) and Tris-HCl (pH 8.0-9.0) buffers were used to determine the most effective pH for lipolytic activity. The optimum temperature for enzymatic activity was determined by incubating the crude enzyme in 0.2 M phosphate buffer (pH 7.0) for 1 h over a temperature range of 25-50°C. Effect of NaCl concentration on lipase activity was routinely performed by incubating the crude lipase in the presence of 0-20% NaCl at pH 7.0 and incubation temperature at 32°C.

RESULTS

Screening of halophilic bacteria for lipase production

A total of 17 phenotypically distinguishable halotolerant and moderately halophilic bacterial isolates obtained from soil and water samples of solar salterns of West Bengal and Odisha, India, were tested for their lipase production on basal MH agar medium (pH 7.0) containing 1% Tween 80 as substrate. The lipid hydrolyzing activity of the isolates was detected by the formation of halo precipitation surrounding the colonies using caliber (mm). Among the tested isolates, four of them were found to be negative, while the remaining thirteen isolates showed different amounts of lipid hydrolysis as evident from the formation of precipitation zones surrounding the growth. It was also found that the bacterial isolate ORS4 was characterized by the highest lipolytic activity as revealed by the widest zone of precipitation (Figure 1). However, bacterial isolates BKS210, BKS201, BKS402, DPS501 and DPS502 were found to be moderate producers. Thus, on the basis of its growth and extent of lipid hydrolysis on solid medium, the bacterial isolate ORS4 was selected for further studies.

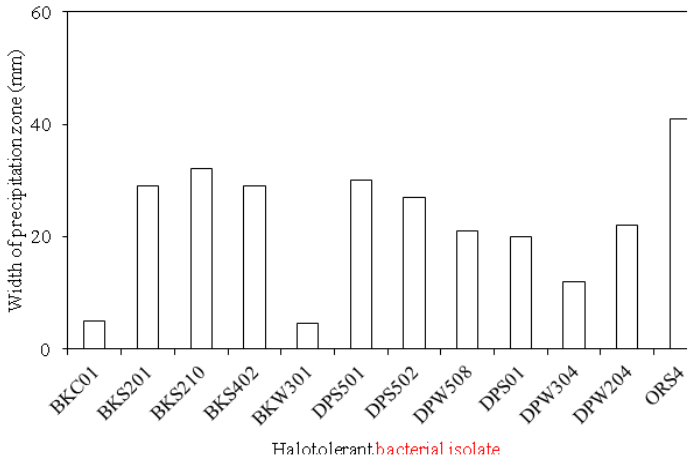

Figure 1 Screening of halotolerant bacterial isolates for extracellular lipase production

Characterization and identification of the isolate ORS4

Morphological and biochemical characteristics of the selected halotolerant isolate ORS4 were evaluated following standard microbiological methods and the results are shown in Table 1. Cells are aerobic, Gram-positive, motile rods and produced endospores. The isolate produced white, gummy colonies on MH agar medium with no diffusible pigment. Cell surface was smooth as revealed by scanning electron microscopy (SEM). The isolate can tolerate 15% (w/v) NaCl in the medium, a pH of 11.0 and temperature of 45°C. Along with the lipase, it produced H_2S, indole, asparaginase, glutaminase, cellulase, gelatinase, caseinase, xylanase, inulinase, pullulanase, pectinase, catalase and nitrate reductase, but showed negative response towards citrate utilization, production of decarboxylases (lysine, ornithine and arginine), deaminases (phenylalanine and tryptophan) and MR-VP test. The isolate was able to utilize as well as ferment all the carbon sources tested except maltotriose. Antibiotic susceptibility profile showed that the isolate was resistant to a number of antibiotics like bacitracin, cycloserine, chlortetracycline, gentamycin, kanamycin, methicillin, netillin, neomycin, polymixin B and tetracycline, but was sensitive to amoxycillin, ciprofloxacin, chloramphenicol, erythromycin, fusidic acid, norfloxacin, novobiosin, trimethoprime and vancomycin. The morphological and physio-biochemical characteristics of the isolate ORS4 as outlined in Table 1 were compared with those of halophilic genera described in Bergey's Manual of Determinative Bacteriology (**Holt *et al.*, 1994**) and the isolate was tentatively identified as a member of the genus *Bacillus.*
An almost complete 16S rRNA gene sequence of ORS4 (1456 nucleotides) was determined and compared with the existing sequences in the databases. Phylogenetic analysis demonstrated that the strain belonged to the genus *Bacillus* and the relationship between ORS4 and the closest relatives is presented in Figure 2. Similarity index analysis after neighbor-joining analysis indicated that ORS4 showed closest relations with *B. vallismortis* DSM11031 (99%), *B. subtilis* NRRL B-23049 (99%), *B. amyloliquefaciens* (98%) *B. licheniformis* (98%), *B. pumilis* (97%) and *Virgibacillus halodenitricans* (95%). On the basis of morphological, physio-biochemical properties and 99% similarity with *B. vallismortis* on phylogenetic analysis of 16S rRNA gene sequences, the isolate ORS4 was found to be the most similar to *Bacillus vallismortis*. The nucleotide sequence of the 16S rRNA gene of the isolate ORS4 has been deposited to NCBI with GenBank accession number of KJ933395.

Table 1 Comparison of morphological and physio-biochemical characteristics of isolate ORS4 with *B. vallismortis* NRRL B-14890^T

Morphological and physiological characters	Results	Biochemical characters	Results
Colony character	White, circular, rough	Catalase	+
Gram-staining	+	Amylase	+
Cell size (µm)	0.8×1.4 - 0.8	Lipase	+
Cell shape	Rods	Asparaginase	+
Arrangement of cells	Single	Glutaminase	+
Endospore formation	+	Cellulase	+
Motility	+	Caseinase	+
Range of pH for growth	5.0-11.0	Xylanase	+
Optimum pH for growth	7	Pectinase	+
Range of temp. for growth (°C)	22-45	Inulinase	+
Optimum temp. for growth (°C)	37	Urease	-
Tolerance of NaCl (%)	0-15	Nitrate reduction	+
Optimum NaCl for growth (%)	5	Lysine decarboxylase	-
Growth on McConky agar	-	Ornithine decarboxylase	-
H_2S production	+	Arginine decarboxylase	-
MR test	-	Phenylalanine decarboxylase	-
VP test	-	Tryptophan deaminase	-
Indole production	+	Citrate utilization	-
Fermentation of		Susceptibility to	
Glucose	+	Amoxycillin(Am^{30})	21 (I)
Fructose	+	Bacitracin(B^{10})	- (R)
Maltotriose	-	Cycloserine(Cy^{30})	8 (R)
Sucrose	+	Ciprofloxacin(Cf^{30})	- (R)
Maltose	+	Cloramphenicol(C^{30})	25 (S)
Fumarate	+	Clorotetracycline(Ct^{30})	10 (R)
Rhamnose	+	Erythromycin(E^{15})	17 (R)
Acetate	+	Gentamycin(G^{10})	25 (S)
Lactose	+	Kanamycin(K^{30})	32 (S)
Mannose	+	Methicillin(M^{5})	11 (R)
Citrate	+	Netillin(Nt^{10})	- (R)
Arabinose	+	Neomycin(N^{30})	10 (R)
Glycine	+	Norfloxacin(Nx^{10})	- (R)
Galactose	+	Novobiosin(Nv^{30})	6.5 (R)
Meso-inositol	+	Polymixin B(Pb^{10})	25 (S)
Xylose	+	Penicillin G(P^{10})	- (R)
Mannitol	+	Streptomycin(S^{10})	- (R)
Na-benzoate	+	Tetracycline(TE^{30})	21 (I)
Salicin	+	Trimethoprime(Tr^{30})	14 (I)
Glycerol	+	Vancomycin(Va^{30})	- (R)

Results: +, positive reaction; −, negative reaction; ND, not detected

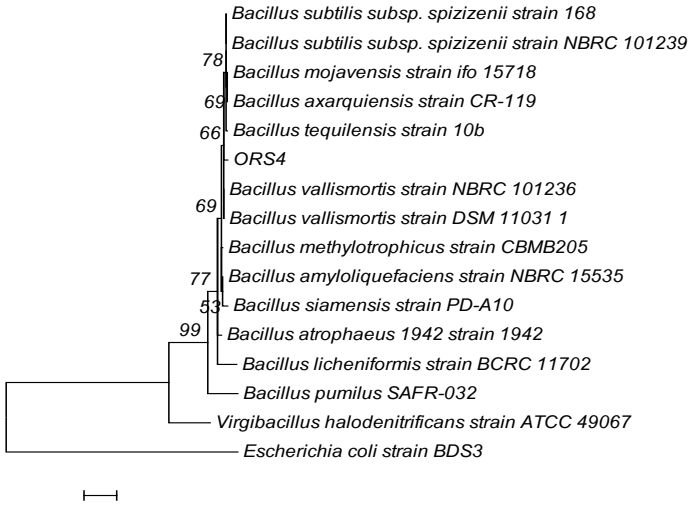

Figure 2 Phylogenetic tree based on 16S rRNA gene sequence showing the relationship of the isolate ORS4 with other members of the halophilic bacteria. The tree was constructed using the neighbor joining method. Bootstrap values >52, based on 1000 replications are indicated at nodes

Growth kinetics and lipase production

The isolate ORS4 was grown in basal MH and Davis-Mingioli's media supplemented with 1% Tween 80 at 32°C, 120 rpm for 6 days. Samples were withdrawn at regular interval and growth as well as, the lipase activity of the culture filtrate was determined following the methods as described earlier. Results as presented in Figure 3A and 3B, indicated that the growth of isolate, both in complex and synthetic media attained maximum at 72 h, while lipase production increased gradually till the culture reached to late log phase of growth. At this stage, lipase activity in basal MH medium was doubled (35.79 U/ml) when compared with synthetic Davis Mingioli's medium (17.75 U/ml).

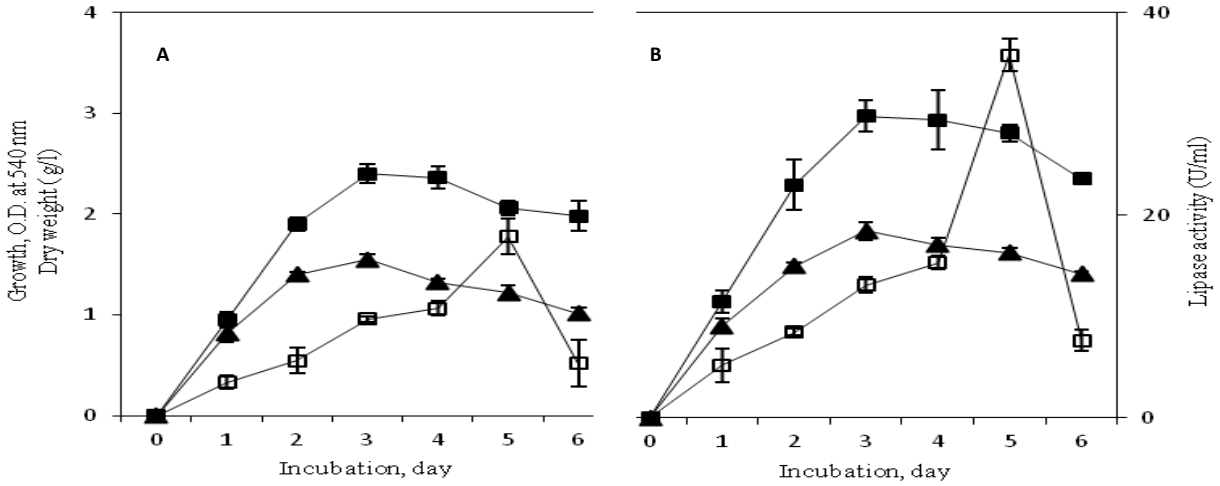

Figure 3 Time course of growth in terms of OD (□) and dry weight (▲), and lipase production (■) by *Bacillus* sp. ORS4 in Davis Mingioli's (A) and MH (B) media

Effect of substrates

Influence of different substrates on lipase production was tested using olive oil, mustard oil, sunflower oil, soybean oil, coconut oil, castor oil and Tween 80. A control set was also supplied without a lipid source. Amongst the different substrates used, Tween 80 showed the best lipase production (38.79 U/ml) in basal MH medium supplemented with 10% NaCl (Figure 4A). Growth of the isolate in oils emulsified with polyvinyl alcohol (1:3) was lesser than the control (without oil supplement), but lipase production was higher with coconut and sunflower oil which showed better lipase activity (16.93 U/ml) than olive oil, castor oil, soybean oil and mustard oil. Tukey's test also revealed that the production of lipase along with growth was most significant (Sig. 0.000<0.001) with Tween 80 as substrate. When basal MH broth was supplemented with different concentration of Tween 80 (0.5-6%), maximum lipase activity (40.71 U/ml) was expressed with 1% Tween 80 supplemented medium. The concentrations above this caused a gradual decrease of the lipase activity (Figure 4B) and this was supported by the mean difference value.

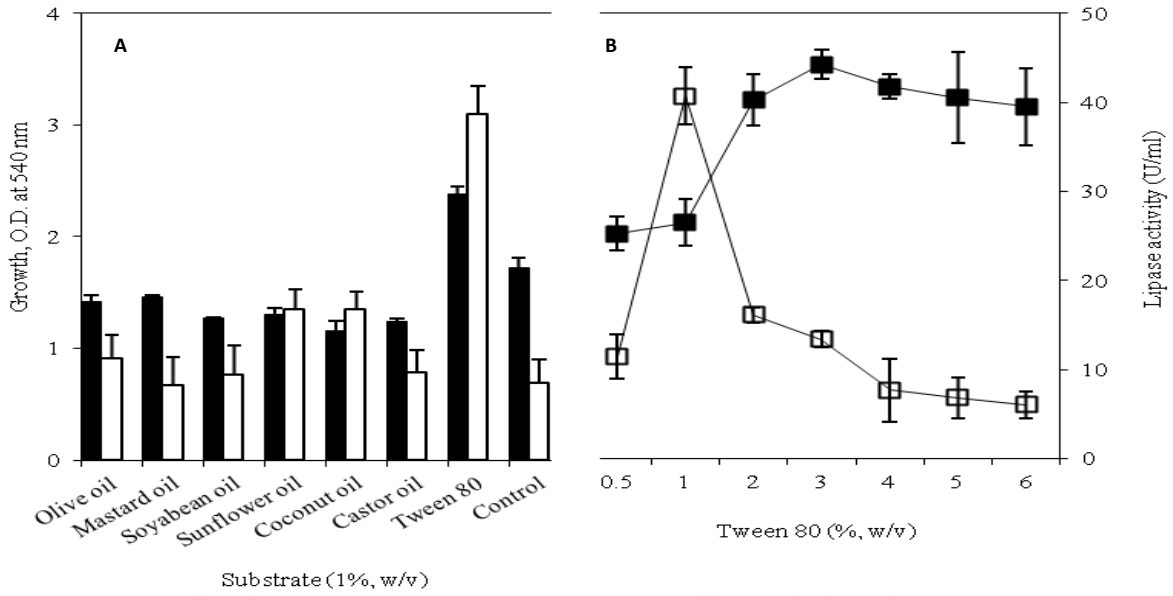

Figure 4 Effect of substrates (A) and Tween 80 (B) on growth (■) and lipase production (□) by *Bacillus* sp. ORS4

Effect of carbon and nitrogen source

Effect of carbon sources in the growth media showed different effects on both the growth and the lipase production of the isolate. Maximum lipase activity (43.98 U/ml) was achieved in glucose (Sig. 0.000<0.001) containing basal MH medium followed by fructose and sucrose (Figure 5A). It was also observed from this experimental analysis that though starch was not inhibitory to growth, it reduced the lipase production significantly. When the isolate ORS4 was grown in basal MH medium with different organic nitrogen sources such as peptone, tryptone, yeast extract and beef extract, it was evident that the growth of the isolate was more or less comparable in all the organic nitrogen sources tested. However, a significant difference in lipase production was recorded with variation of nitrogen sources. Lipase production was most significant (38.79 U/ml) in the presence of tryptone as nitrogen source followed by peptone, yeast extract and beef extract (Figure 5B) according to their significance value and mean difference.

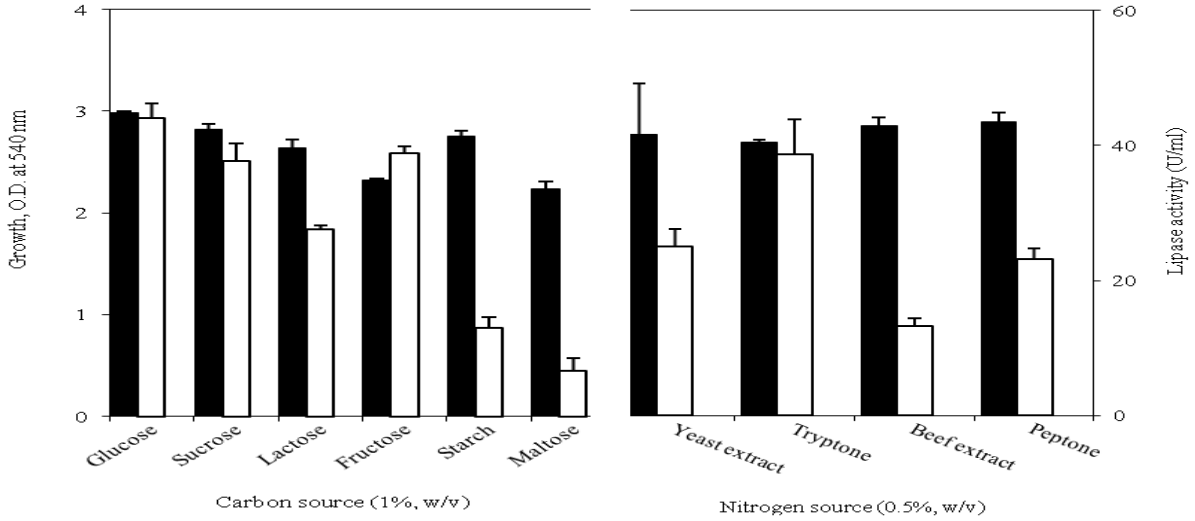

Figure 5 Effect of different carbon (A) and nitrogen source (B) on growth (■) and lipase production (□) by *Bacillus* sp. ORS4

Effect of NaCl, pH, and inoculum dose

The influence of NaCl concentration on growth and lipase production by the isolate ORS4 was tested in basal MH medium supplemented with 0-20% NaCl. The optimum concentration of NaCl for growth, as well as, lipase production was recorded at 5% NaCl with a lipase activity of 44.26 U/ml (Figure 6A). Growth as well as the lipase production by the isolate was severely affected at concentrations higher than 5% NaCl in the medium, which appeared to be best fitted and significant. The isolate ORS4 was grown in basal MH broth having different pH (4.8-8.8) and the maximum lipase activity (40.71 U/ml) was recorded at pH 7.8 (Figure 6B). The growth of the isolate was maximum at pH 6.8. Also, by varying initial inoculum, it was revealed that as the initial inoculum was increased, both growth and lipase activity was increased gradually. The highest lipase activity (45.62 U/ml) was recorded with initial inoculum of 4% (10^{12} cells/ml) when the growth was also maximum (Figure 6C). However, an inoculum dose of 6% was inhibitory to both growth and lipase production. The optimized inoculum dose and pH was also supported by the Tukey's test which derived the mean difference and significance values.

Characterization of partially purified lipase

The effect of temperature, different concentrations of NaCl and pH on the activity of the partially purified lipase was tested. The lipase showed best activity at a temperature of 40°C (60.01 U/ml, pH of 8.0 (59.83 U/ml) and in the presence of 15% NaCl (44.26 U/ml) (Figure 7A, 7B and 7C). However, under the defined optimum conditions the maximum activity of the lipase could not exceed 62.02 U/ml.

DISCUSSION

Survey of lipase producing microorganisms isolated from different environmental niches and their lipases have been made by several authors (**Senthilkumar and Selvakumar, 2008; Kumar *et al.*, 2012**). Moreover, lipases resistant to high salt concentration or temperature values have distinct advantages over the classical lipases and are capable of tolerating different organic solvents like benzene, isoamyl alcohol, acetonitrile, hexane, decane etc (**Bhatnagar *et al.*, 2005; Uttatree *et al.*, 2010**). In view of these findings, there is a growing interest to find halophilic lipases from moderate to extremely halophilic bacteria. **Sanchez-Porro *et al.*** (**2003**) have demonstrated that only 23% of 892 bacterial strains produced lipase on Tween 80 agar plate, while about 66% of 53 halobacterial isolates hydrolyzed Tween 20-Tween 80 at 4M NaCl in agar plate assay (**Gonzalez and Gutierrez, 1970**).

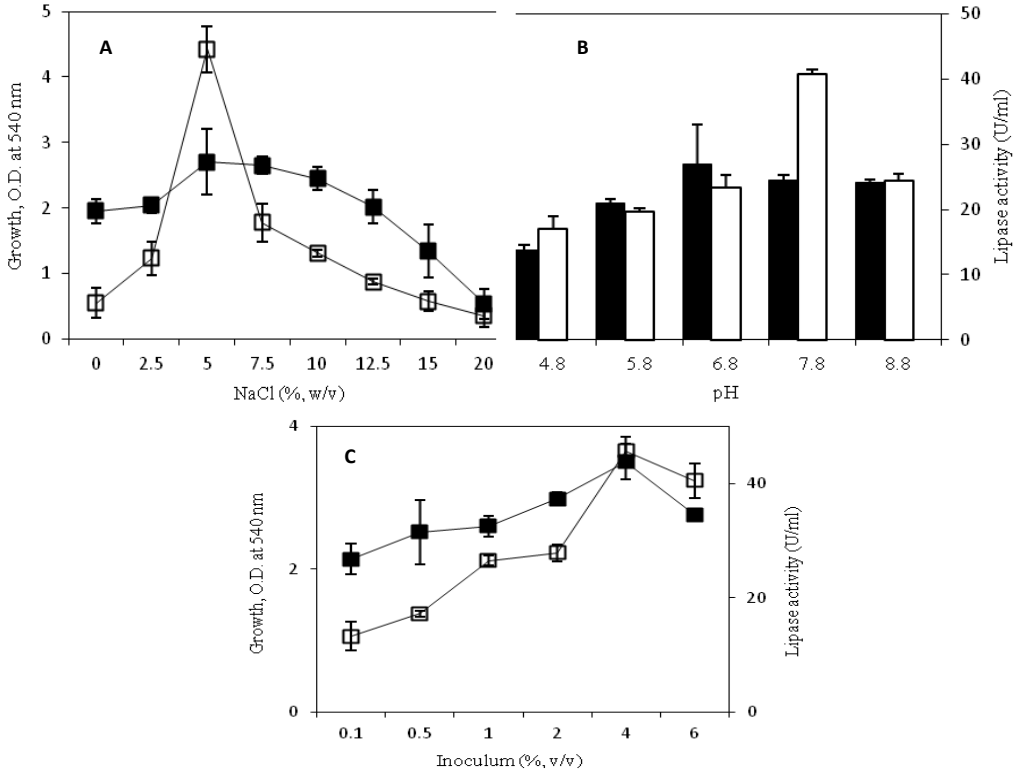

Figure 6 Influence of NaCl (A), pH (B) and inoculum dose (C) on growth (■) and lipase production (□) by *Bacillus* sp. ORS4

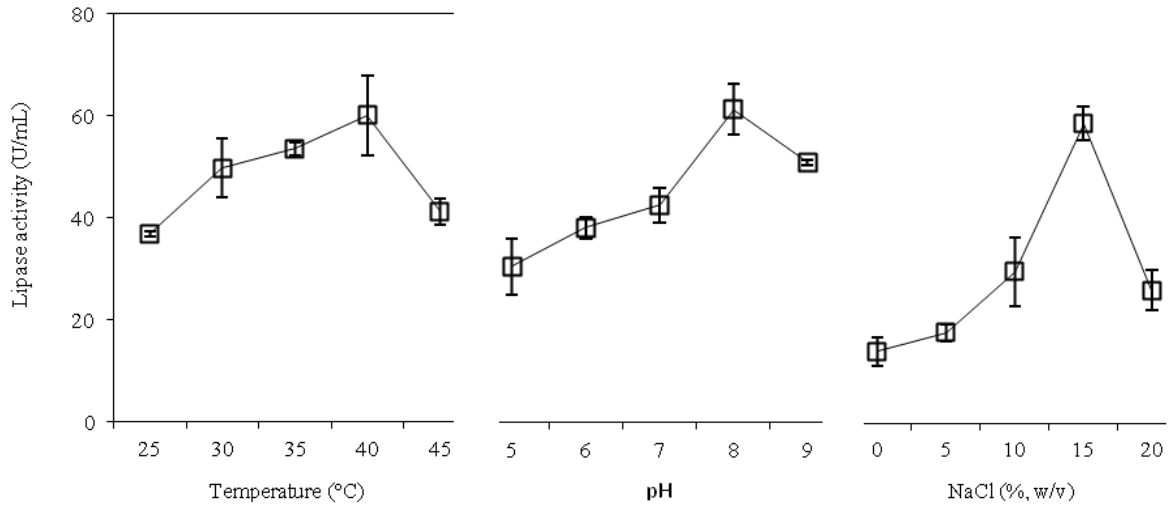

Figure 7 Enzyme activity of the partially purified lipase in different temperature (A), pH (B) and NaCl (C)

The present study revealed that among the 17 halotolerant and halophilic bacterial isolates obtained from solar salterns, 70% showed Tween 80 degrading activity (Figure 1). Of those, the isolate ORS4 was found to be the most potential lipase producer. The isolate was Gram-positive, motile spore forming rod and was able to tolerate a wide range of pH and temperature. Along with lipase, the isolate has the potential of producing different industrially important enzymes characteristic of halotolerant and halophilic microorganisms (**Ardakani *et al.*, 2012**) (Table 1). In addition, the isolate has the ability to utilize and ferment a wide variety of carbon sources and also showed multiple antibiotic resistances (Table 1). Based on these morphological, physiological and biochemical characteristics along with 16S rRNA gene sequence analysis (Figure 2) the isolate ORS4 was found to be most related to type strain *Bacillus vallismortis.* When the phenotypic characters of the isolate ORS4 was compared with most of the *B. vallismortis* strains so far reported (**Roberts *et al.*, 1996**), the results differed by means of fermentation of lactose, utilization of citrate, phenylalanine and tyrosine decomposition, indole production and MR-VP tests (Table 1).

While optimizing the conditions for growth associated with lipase production, the isolate showed maximum enzymatic activity (35.79 U/ml) during stationary phase of growth in Tween 80 supplemented basal MH medium (Figure 3). This corroborates the findings of **Kanlayakrit and Boonpan** (**2007**) and **Khunt *et al.*** (**2012**) for the production and isolation of lipase from *Staphylococcus warneri* PB233 and *H. salina* Ku19, respectively. Non halophilic *Staphylococcus* spp. present in oil contaminated soils were found to be good lipase producers (**Sirisha *et al.*, 2010; Tembhurkar *et al.*, 2012; Kumar *et al.*, 2012**). **Ghasemi *et al.*** (**2012**) also identified a moderately halophilic strain *B. vallismortis* BCCS007 which produced 3.41 U/ml of lipase. Optimization of nutritional conditions is one of the most important parameter for large scale production of metabolites. The isolate ORS4 utilized different oil emulsions as substrates for lipase production, but Tween 80 was considered to be the most suitable one (Figure 4A and 4B), which supports the findings of **Adrakani *et al.*** (**2012**). Production of carboxyl ester hydrolase by *Halobacterium* sp. NRC1 was studied and the physicochemical conditions required for growth and the production of the enzyme were established (**Camacho *et al.*, 2010**). This haloarcheaon produced lipase with 1.64 U/ml of enzyme activity at 4.6M NaCl, pH 6.0 at 30°C. Lipase production by moderately halophilic bacteria has also been supported by tributyrin (**Khunt *et al.*, 2012**) and palm oil (**Papapavaraskevas *et al.*, 1992**) while the non-halophiles are no exceptions in this regard (**Sharma *et al.*, 2001**). As carbon and nitrogen sources, glucose and tryptone favored the lipase production by the isolate ORS4 (Figure 5A and 5B) and thereby, they seem to justify the requirement of easily consumable carbon and nitrogen source for growth associated production of essential metabolites. Moreover, higher level (4%) of inoculum density promoted the lipase production by the isolate ORS4 (Figure 5C). The halotolerant nature of the isolate ORS4 was further confirmed by its NaCl requirement for maximum lipase production (Figure 6A), however this was comparatively lower than the NaCl requirement of *H. salina* Ku 19. The optimum pH (7.8) for the lipase production indicated its tolerance to alkaline condition (Figure 6B). Recent studies on screening of the lipase producing bacteria isolated from oil spilled soil samples revealed a potential lipase producing strain *Pseudomonas gessardii* which showed a lipase activity of 168.7 U/ml at 37°C, pH 7.0 with 1% protease peptone (**Veerapagu *et al.*, 2013**).

Further the partially purified lipase produced by *Bacillus* sp. ORS4 showed maximum activity at higher temperature values which could be categorized as a moderately thermostable one showing temperature optima of 40°C (Figure 7A). Optimal pH for this enzyme was found to be 8.0indicating its stability under alkaline condition (Figure 7B). The partially purified lipase also showed maximum activity under high salinity (15% NaCl) (Figure 7C), which marked it as an interesting extremozymes for future investigations. Similarly, **Boutaiba *et al.*** (**2006**) reported that lipase from *Natronococcus* sp. exhibited an optimum activity at pH 7.0, while **Ozcan *et al.*** (**2009**) reported a range of pH 8.0-8.5 for optimal esterase activity of haloarcheal strains. **Sharma and Rathore** (**2010**) studied on the lipase purification and demonstrated that higher purified lipases showed more enzyme activity which supports this study.

CONCLUSION

The present study was to explore the halophilic strains with potential lipase activity obtained from the multi-pond solar salterns of Odisha and West Bengal, India. In screening of 17 halophilic and halotolerant isolates, the most lipolytic isolate ORS4 was selected for further studies. Morphological, physiological and biochemical characteristics along with 16S rRNA gene sequence analysis revealed that the isolate ORS4 belonged to the genus *Bacillus* and was most related to *Bacillus vallismortis*..Time course of growth and lipase production by *Bacillus* sp. ORS4 have been optimized and the partially purified lipase showed maximum activity at alkaline pH, high salinity and temperature suggesting it's potential for exploitation in extreme environments.

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