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RESEARCH ARTICLE OPEN ACCESS

Halophilic and Halotolerant Actinomycetes of Sambhar Salt Lake, India: Screening and Optimization of Cellulolytic Activity

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Abstract

Actinomycetes **are Gram-positive filamentous bacteria well known for the production of bioactive compounds. Recently, many halophilic habitats have been explored for isolation of** ***actinomycetes*** **that exhibit biotechnological potentials. In this investigation, a saline habitat of Rajasthan, Sambhar Salt Lake (SSL) was selected to study the** ***actinomycetes*** **population and Carboxy Methyl Cellulase (CMCase) production by native isolates. A total of sixteen actinomycete isolates, halotolerant and moderately halophilic, were obtained using culture-dependent methods and characterized morphologically and biochemically. They were identified as members of** ***Streptomyces, Nocardiopsis, Pseudonocardia, Saccharospolyspora*****, and Microbispora.** ***Streptomyces*** **was the most dominating genus, followed by** ***Nocardiopsis.*** **Agar plate assay was used for screening the isolates for CMCase production. Thirteen were found to produce the enzyme, apparent by hydrolysis observed on media plates. The highest relative activity of 22.04 was shown by isolate SSL 14 identified as** ***Nocardiopsis*** **sp. by 16S rDNA sequencing studies and thus selected for further optimization studies. Maximum enzyme (1.08 ± 0.09 U/ml) was produced using medium containing Carboxy Methyl Cellulose (Carbon source) and yeast extract (nitrogen source) at 12% NaCl and pH 9.0, incubated at 30 °C for 96 h. Maximum CMCase production at high salt concentration and pH suggests that** ***Nocardiopsis*** **SSL 14 can be used for industrial processes that operate under excessive saline and alkaline conditions.**

Keywords: CMCase, Halophiles, International *Streptomyces* Project (ISP), *Nocardiopsis*, Saltern

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INTRODUCTION

Actinomycetes are the most economically and biotechnologically valuable prokaryotes used to produce a wide variety of bioactive compounds.[1] They also contribute to the cycling of nutrient elements by producing extracellular hydrolytic enzymes. One such hydrolytic endoglucanase enzyme is CMCase, which acts on the internal amorphous region and randomly hydrolyses long cellulose chains. It acts synergistically with exo-(1,4)-β-D-glucanase to hydrolyse cellulose.[2] CMCases are used for enhancing fabric feel, look, and colour in textile processing.[3] They decrease pulp viscosity and improve pulp beatability of the paper.[4] Besides this, they are also used in food,[5] beverages,[6] detergent,[7] biofuel industries,[8] drug delivery,[9] and wound healing.[10] The demand for CMCase functional at high pH, temperature and salt concentrations has increased gradually. This emphasizes the need for the exploration of CMCase producing actinomycetes from extreme habitats. In order to suffice this demand, a saline habitat of Rajasthan (India), Sambhar Salt Lake, was selected which forms a unique ecosystem possessing a wide range of salinity in its water and salt formation ponds. It is India's largest inland saline lake that produces salt for domestic and industrial needs. The present study was conducted to isolate actinomycetes and investigate CMCase production from halophilic and alkaline habitats of Sambhar Salt Lake, Rajasthan.

MATERIALS AND METHODS

Study Area: Sambhar Salt Lake

Sambhar Salt Lake is the India's largest saline lake lying at a latitude and longitude of 26°55'12"N and 75°12'00"E, respectively. It is situated off National Highway No. 8, about 64 km northeast of Ajmer and 96 km southwest of Jaipur, extending up to Nagaur district in Rajasthan

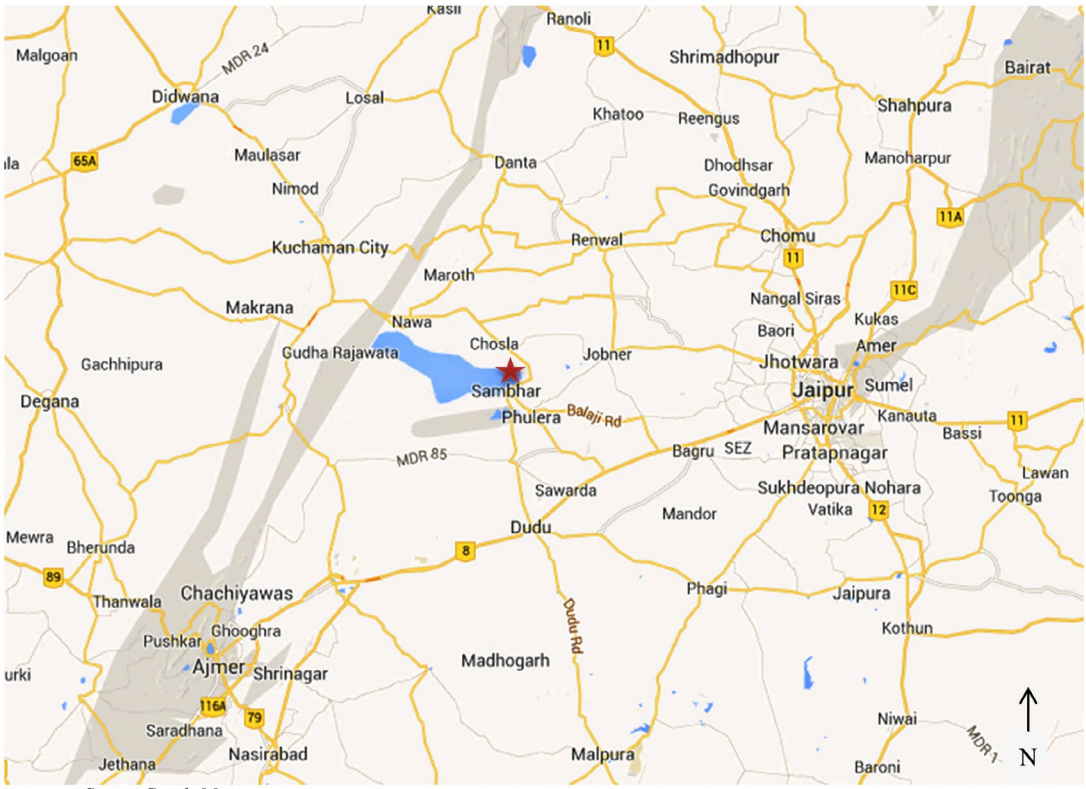


Figure 1. Map of Sambhar Salt Lake, Rajasthan, INDIA.

(Figure 1). The lake is surrounded by Aravali mountain ranges.

Sampling Sites

Eastern part of the lake is divided into solar salt pans (kyars) called salterns, where salt is produced for industrial and human consumption. Surface water samples were collected from five major stages (1 to 5) of salt production in various seasons, i.e., monsoon (A1-E1), winter (A2-E2), and summer (A3-E3) in the year 2012-13 (Figure 2a, b). The density of water in salterns was measured on-site by hydrometer. The pH and salinity of samples were measured using microprocessor water and soil analysis Kit Model 1160E.

Isolation and Characterization of Actinomycetes

Actinomycetes were isolated on Actinomycetes Isolation Agar (AIA) medium containing antifungal agents nystatin and cycloheximide (50 µg/ml). For all first isolations, medium was prepared in sterile sample water and for subsequent inoculations medium was supplemented with NaCl (determined from salinity of samples). AIA plates were incubated at 28°C for 5-20 days for the appearance of actinomycete colonies and maintained on AIA at 4°C. Purified colonies were characterized by their morphological and physiological features as per the International *Streptomyces* Project (ISP).[11] Micro morphological features were studied by the Coverslip culture method.[12] The observed structures were compared with those described in Bergey's manual[13] to identify isolates up to the genus level. Various biochemical tests, including substrate degradation and utilization of different sugars as sole carbon source, were also studied.[14,15]

Screening for CMCase Production

All selected isolates were inoculated on carboxy methyl cellulose (CMC) agar medium and incubated at 28°C for 15 days, after which they were stained by flooding them with Congo red solution (1% w/v) for 15 min, followed by destaining with 1 M NaCl for 15 min.[16]

Selection of Potent Isolate for Optimization Studies

Relative enzyme activity of the isolates was measured from the screening assays using the formula D^2- d^2/d^2, where d and D represent the widths of growth and hydrolysis, respectively.[17] The isolate with maximum relative activity was selected for further optimization studies.

CMCase (Endoglucanase) Activity Assay

CMC containing broth (100 ml) was used for cellulase production. It was inoculated with 1% inoculum grown for 48 h at 30°C and 150 rpm. The culture was centrifuged at 10,000 rpm and 4°C for 10 min. The CMCase activity was determined in the crude supernatant in terms of reducing sugars released, which was quantified by dinitro salicylic acid (DNS) method.[18] One unit (U) was defined as the amount of enzyme that released 1 mg of glucose per minute under experimental conditions.

Optimization of Culture Conditions for Enzyme Production

Culture conditions for CMCase production were optimized by following One Factor at a Time (OFAT) approach.[19]

Effect of Incubation Time

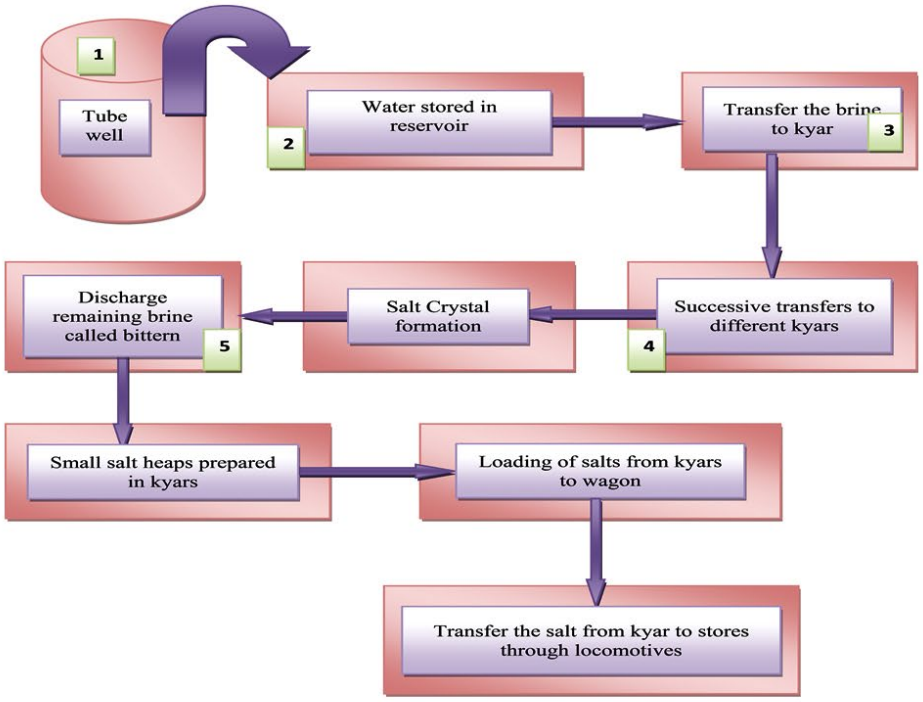
The production media was inoculated with selected actinomycete isolate (1%) and incubated at 30°C for different time intervals (24-144 h) at 150 rpm. CMCase production was estimated after every 24 h for 7 days by the method described by.[18] Total biomass was measured and expressed as mg dry mass per 50 ml of culture medium.[20]

Effect of Incubation Temperature, pH, and Salt Concentration

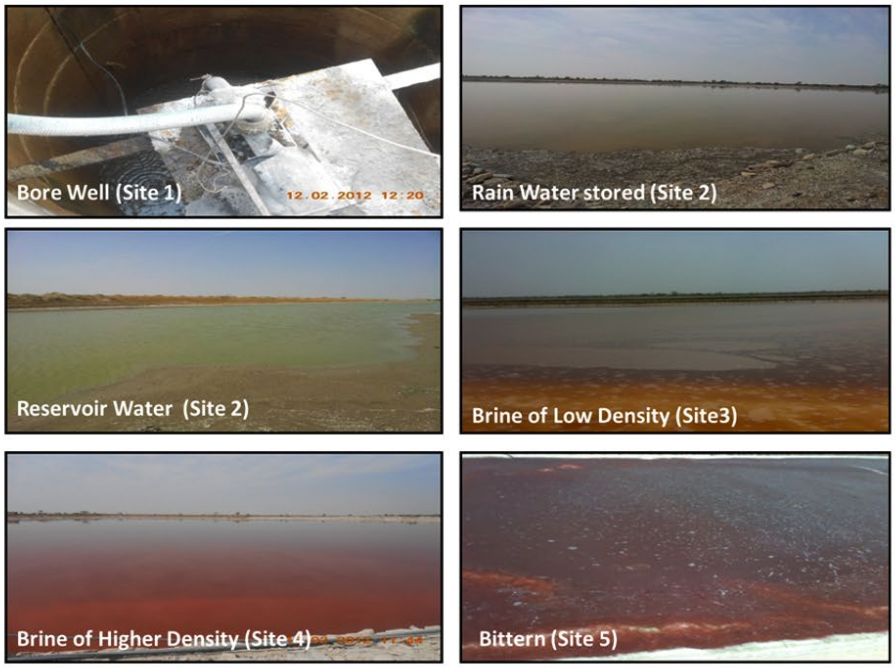
The selected isolate was incubated at different temperatures (25-60°C); pH (5.0-10.0) and NaCl concentrations (1-13%) underdetermined optimum conditions.

Effect of Carbon and Nitrogen Sources

In order to maximize enzyme production effect of different carbon sources (1% w/v) and nitrogen sources (1% w/v) were studied by incorporating them into production media and incubating them under determined optimum conditions.



(a)



(b)

Figure 2. Salt production process and sample collection sites (1 to 5) at Sambhar Salt Lake, Rajasthan (a) Flow chart of salt production, (b) Sampling Sites (1-5).

Identification of Selected Isolate by 16S rDNA Sequencing

Isolate showing maximum enzyme production was grown in nutrient broth medium and DNA was isolated in the form of a single discrete PCR amplicon band on agarose gel. 8F and 1492R primers were used for carrying out forward and reverse DNA sequencing reaction of PCR amplicon using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. With the help of aligner software, a consensus sequence of 16S rDNA gene was generated, and the sequences which were similar to it were searched by BLAST. First ten sequences selected based on maximum identity score were then aligned using Clustal W. RDP database was used to generate distance matrix, the evolutionary history of the taxa was inferred by the Neighbour-Joining method,[21] and MEGA 4 was used to construct phylogenetic trees.[22]

Statistical Analysis

The data for all experiments were expressed as mean ± standard deviation. The experiments for enzyme assays were set with three replicates, and three aliquots were taken from each replicate for all parameters, except for biomass determination. The data were analysed by

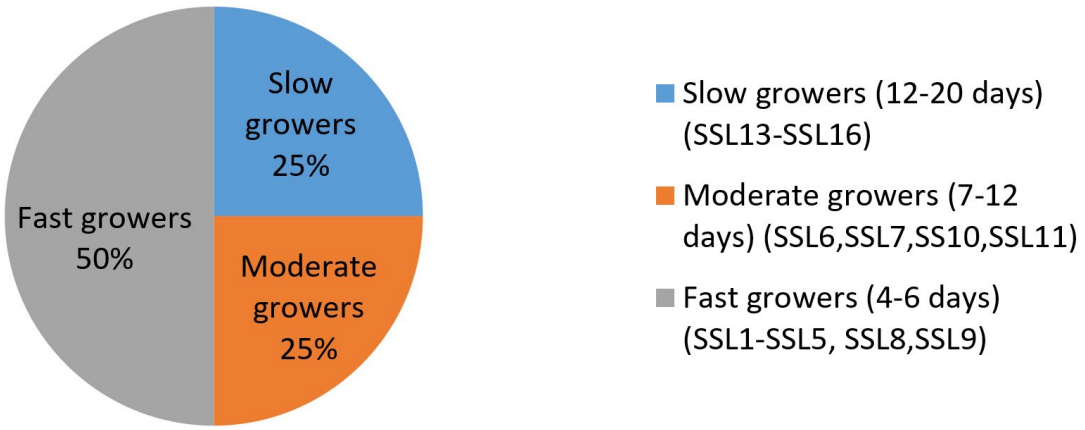


Figure 3. Growth Characteristics of SSL Isolates on AIA Medium.

Table 1. Physico-chemical analysis of water samples collected in different seasons

Rainy Season					
Water Samples	A1	B1	C1	D1	E1
Salt Production Stage	Rain water stored	Reservoir	Brine	Brine	Brine
Density (°Be)	1	13	20	26	17
Salinity (g/l)	30.959 ± 1.04	101.961± 1.906	186.640 ± 2.30	294.521 ± 2.355	205.563 ± 1.467
pH	12.0 ± 0.91	12.5 ± 0.264	11.2 ± 0.86	9.0 ± 0.14	11.0 ± 0.55
Winter Season					
Water Samples	A2	B2	C2	D2	E2
Salt Production Stage	Brine	Brine	Reservoir	Brine	Bittern
Density (°Be)	25	23.5	6	15.5	28
Salinity (g/l)	293 ± 3.168	334 ± 2.0	82 ± 1.87	120 ± 1.896	354 ± 2.104
pH	10.2 ± 1.3	11.8 ±0.2	11.0 ±0.264	10.5 ± 0.40	10.8 ± 0.692
Summer Season					
Water Samples	A3	B3	C3	D3	E3
Salt Production Stage	Reservoir	Brine	Brine	Brine	Bittern
Density (°Be)	6	7.6	25	27	29
Salinity (g/l)	82 ± 1.81	147 ± 1.63	299 ± 1.575	275 ± 2.68	357 ± 1.70
pH	11.0 ± 0.264	10.3 ± 0.346	10.0 ± 0.529	9.6 ± 0.346	10.9 ± 0.40

one way analysis of variance (ANOVA) followed by LSD post hoc test. All the analysis was performed using the IBM SPSS software (Version 20).

RESULTS

The study was divided into three different seasons during which water samples were collected and analysed for their pH and salinity content. The results revealed that Sambhar Lake water has an alkaline pH ranging from 9.0-12.5 and high salinity from 30.959 -357.0 g/l (Table 1).

Isolation and Characterization of Actinomycetes Isolates

A total of sixteen isolates was recovered from Sambhar salterns and were designated by numbers 1 to 16 and prefixed with "SSL" as an abbreviation of Sambhar Salt Lake. They were classified based on incubation time required for complete growth on AIA medium as slow, moderate, and fast growers (Figure 3). Four isolates (SSL 6, SSL 7, SSL 10 and SSL 11) showed optimum growth at 8% NaCl and three isolates (SSL 14, SSL 15, SSL 16) at 12% NaCl hence classified as moderate halophiles. The remaining nine were classified as halotolerant as they grew well in the absence of salt and also tolerated salinity in the range of 5-8% NaCl. The colonies of thirteen isolates were tough, leathery, or powdery in appearance with the presence of aerial (AM) and substrate mycelium (SM), whereas three isolates were non-filamentous. All the isolates were Gram-positive. The growth was more rapid on ISP 2 and ISP 6 media, along with the production of diffusible pigment by many isolates. AM of many isolates was white coloured on different ISP media, and SM was of varied colours such as cream, yellow, orange, and brown (Table 2). The nature of AM and SM and their fragmentation in AIA medium helped in identifying the isolates up to the genus level (Table 3). *Streptomyces* was found to be the

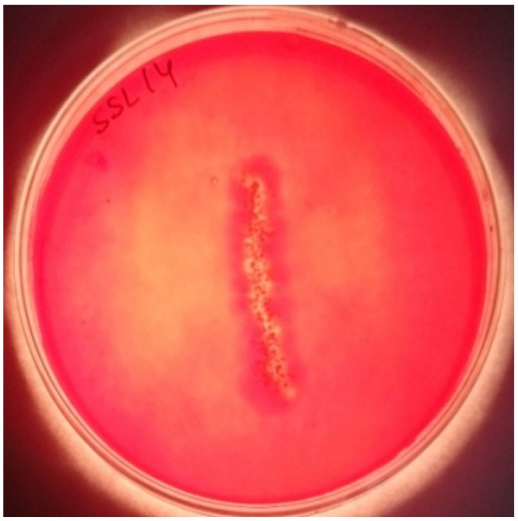


Figure 4. Carboxy Methyl Cellulose hydrolysis by SSL 14

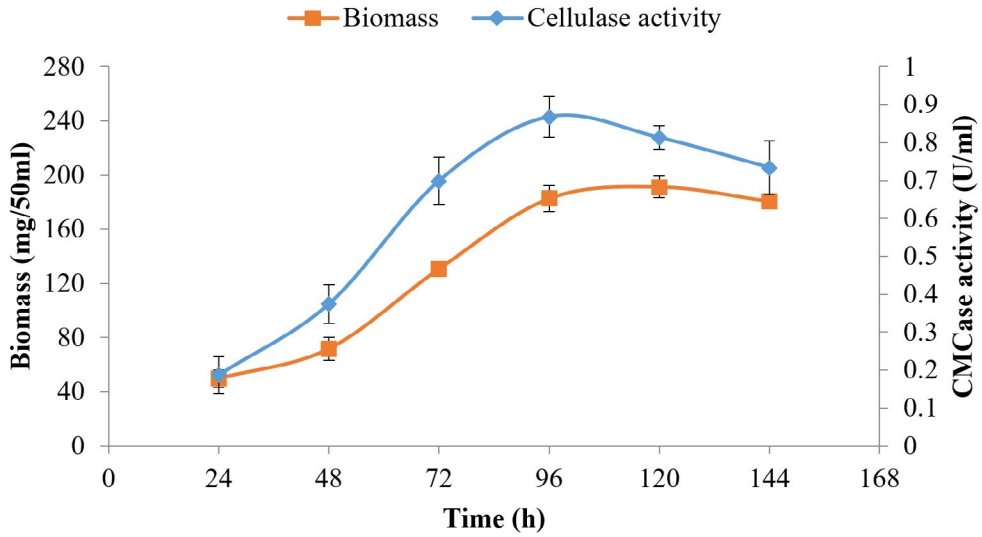


Figure 5. Fermentation Time Course for Cell Growth and Extracellular CMCase Produced by Isolate SSL 14

Table 2. Characteristics of Actinomycete Isolates on ISP Media

Isolate	ISP2			ISP3		ISP4		ISP5		ISP6		
	AM	SM	Diffusible pigment	AM	SM	AM	SM	AM	SM	AM	SM	Diffusible pigment
SSL1	White	Bright orange	+	White	White	Light Cream	Light Cream	Creamish white	Creamish white	White	Dark orange	-
SSL2	Greyish white	Brown	+	White	Dark brown	White	Light brown	White	Light Cream	White	Orange	-
SSL3	White	Dark Yellow	-	White	Chocolate brown	White	Yellowish cream	White	Creamish White	White	Orange	-
SSL4	Cream	Cream	-	Greyish white	Greenish brown	White	Yellowish cream	White	Light cream	White	Light orange	-
SSL5	Grey	Dark Yellow	-	Greyish white	Greenish brown	White	Light yellow	White	Light Cream	White	Orange	-
SSL6	White	Bright orange	+	White	Light Cream	Cream	Cream	No Growth	No Growth	White	Brownish orange	+
SSL7	White	Orange	+	cream	Light Yellow	Cream	Cream	no AM	Light Cream	White	Orange	+
SSL8	No AM & SM, orange-coloured colonies		-	No AM & SM, light cream growth		No Growth		No Growth		No AM & SM, Yellow colonies		-
SSL9	No AM &SM, cream-coloured colonies		-	No growth		No Growth		No Growth		No AM &SM Cream colonies		-
SSL10	White	Orange	+	Cream	Light Yellow	White	Light Yellow	No AM	White	White	Light orange	-
SSL11	White	Bright orange	+	Cream	Brown	Creamish white	Light brown	No Growth	No Growth	White	Orange	+
SSL12	No AM & SM, Yellowish cream		-	Peach		No Growth		No Growth		No AM & SM, orange colonies		-

Table 2. Cont...

Isolate	ISP2			ISP3		ISP4		ISP5		ISP6		
	AM	SM	Diffusible pigment	AM	SM	AM	SM	AM	SM	AM	SM	Diffusible pigment
SSL13	Light Grey	Light Brown	-	White	Light Cream	White	White	No Growth	No Growth	White	Orange	-
SSL14	White	Bright orange	+	White	Sandy Yellow	Light yellow	Light yellow	No AM	White	White	Dark orange	+
SSL15	White	Bright orange	+	White	Sandy Yellow	Yellow	Yellow	White	Light Yellow	White	Light orange	+
SSL16	White	Bright orange	+	Cream	Cream	Cream	Cream	White	Light Yellow	Brownish white	Light orange	-

(+) Diffusible pigment produced; (-) Diffusible pigment not produced.

most dominant genus. Biochemical features of isolates are represented in Table 4.

Screening for CMCase Production and Relative Enzyme Activity Determinations

All filamentous isolates formed a clear zone of hydrolysis when CMC agar plates were flooded with Congo red and NaCl. Three non-filamentous actinomycetes isolates SSL 8, SSL 9 and SSL 12 did not show the production of CMCase. Relative enzyme activity was calculated for all positive isolates (Table 5), and highest was shown by SSL 14 (Figure 4), and thus, it was selected for enzyme optimization studies.

Optimization of Culture Conditions

Effect of Incubation Time

The majority of the actinomycetes are slow-growing. CMCase production by SSL14 was found to be maximum at 96 h of growth (0.868 ± 0.054 U/ml), and thus, the other parameters were optimized at the same incubation time. A significant difference in CMCase activity was observed between 96 and 120 h ($p < 0.05$). Growth analysis showed that enzyme was produced during log and stationary phase (Figure 5).

Effect of Incubation Temperature, pH, and Salt Concentration

Maximum activity (0.877 ± 0.055 U/ml) was observed at 30°C (Figure 6a), which is also the optimum growth temperature for actinomycetes.[23] As the temperature was increased from 40°C to 60°C, enzyme activity significantly declined. Among, the different pH tested, highest CMCase activity occurred at pH 9.0 (1.063 ± 0.045 U/ml) (Figure 6b) however no significant difference between pH 8.0 and 9.0 ($p > 0.05$) was observed. This observation revealed the alkaliphilic nature of CMCase produced.

Results presented in Figure 6c showed that CMCase production was least at 0.5% NaCl, and it increased gradually with the increasing salt concentration, with maximum occurring at 12% NaCl (1.055 ± 0.046 U/ml). These results suggested the moderately halophilic nature of the isolate.

Effect of Carbon and Nitrogen Sources

From the different carbon sources tested, the CMCase production was maximum (1.015 ±

0.051 U/ml) in the medium supplemented with CMC as a carbon source and minimum in the presence of dextrose (Figure 7). It was found that yeast extract gave maximum activity (1.08 ± 0.09 U/ml). Also, the enzyme activity was comparable when the medium was supplemented with beef extract, peptone, and ammonium nitrate (Figure 7).

Identification of Isolate SSL 14 by 16S rDNA Sequencing

The phylogenetic analyses based on BLAST search using 16S rDNA gene sequence of the isolate SSL 14 revealed 100% similarity to *Nocardiopsis* sp. BCEAA 7603 (Figure 8), and thus, it was designated as *Nocardiopsis* sp. SSL 14 (GenBank Accession number KU860459). These results were also supported by its morphological features (Figure 9a-c) as described above.

DISCUSSION

Salt lakes and multi-pond solar salterns are well-known hypersaline habitats having high salt concentration and alkaline pH.[24] They possess enormous potential in terms of microbial

Table 3. Genera of Actinomycetes of Sambhar Salt Lake Tentatively Identified on the Basis of Micro-morphological Features (Holt et al., 2000)

Isolate	Characteristics of Mycelium	Tentative Genera
SSL 1	AM and SM present. AM fragments in to short filaments. Spore chain was of rectiflexibiles (RF) nature.	Nocardiopsis
SSL 2	SM extensively branched that does not fragment. The AM forms rectiflexibiles (RF) spore chains at maturity.	Streptomyces
SSL 3	Extensively branched non fragmenting SM and AM. AM carried spiral spore chain with coccoid shaped spores.	Streptomyces
SSL 4	Both mycelia were well-developed. AM carried long spore chain of Retinaculiaperti type and SM was stable.	Streptomyces
SSL 5	Extensively branched and stable SM and AM with long chains of spores (spirales type) on latter one.	Streptomyces
SSL 6	SM stable and branched. AM sparse, often produced in tufts in older parts of colony and produced only at optimum growth temperature. Spore chain was of straight rectus type.	Saccharopoly-spora
SSL 7	Segmented AM and SM. Long chains of cylindrical shaped spores of unequal sizes are present on AM. Spore chain morphology was of rectiflexibiles type.	Pseudonocardia
SSL 8	AM and SM absent. Colonies were of cream colour. Rod shaped cells were present usually arranged in chain.	Georgenia
SSL 9	AM and SM absent. Cream colonies with brown tint present. Cocci shaped cells are usually arranged in singlets.	Kokuria
SSL 10	Extensively developed AM and SM absent. AM forms short chain of spores of rectiflexibiles type at maturity that fragments in rod shaped spores.	Streptomyces
SSL 11	Well-developed SM, hyphae are long and densely branched. Abundant AM that forms long chain of spores of rectiflexibiles type at maturity.	Nocardiopsis
SSL 12	AM and SM absent. Cream colonies with brown tint present. Cells are cocci shaped usually arranged in singlets.	Kokuria
SSL 13	Compact SM that grows into and on top of agar and does not bear spores. AM was stable and produces spores formed in longitudinal pairs present on short sporophores. The AM forms bud at the side and later the bud or occasionally the tip of the side branch swells up producing two conidia separated by cross walls.	Microbispora
SSL 14	Well-developed SM and AM, hyphae are long and densely branched. AM at maturity fragments to form long chain of spores. Spore chain was of rectiflexibiles type.	Nocardiopsis
SSL 15	Branched and stable SM. Aerial hyphae carried spore chain of rectiflexibiles type. Rod shaped spores were present in long chains.	Streptomyces
SSL 16	SM well developed and branched. AM sparse and had rectiflexibiles type of spore chain segmented into bead like chain of spores.	Saccharopoly-spora

Table 4. Biochemical Characterization of Actinomycete Isolates

Isolates	Catalase Test	Oxidase Test	Gelatin lique-faction Test	Casein Hydrolysis Test	Xanthine Hydrolysis Test	Hypoxanthine Hydrolysis Test	Tyrosine Hydrolysis Test	Carbohydrate Utilization Test						
								Glucose	Maltose	Xylose	Lactose	Fructose	Sucrose	Mannitol
SSL 1	+	+	-	+	-	-	-	+	+	+	+	+	+	+
SSL 2	+	+	-	++	+	++	-	+	+	+	+	+	+	+
SSL 3	+	+	-	+++	++	-	-	+	+	+	+	+	+	+
SSL 4	+	+	+	+++	-	+++	-	+	+	+	+	+	+	+
SSL 5	+	+	+	++	-	-	-	+	+	+	+	+	+	+
SSL 6	+	+	-	+	++	+	++	+	+	+/-	+	+	+	+
SSL 7	+	+	-	+	+++	-	-	+	+	+/-	+/-	+	+	+
SSL 8	+	+	-	-	+	-	-	+	+/-	+/-	+	-	-	+
SSL 9	+	+	-	-	+	-	-	-	-	-	-	+/-	+/-	-
SSL 10	+	+	-	+	+	-	++	+	+	+/-	+	+	+	+
SSL 11	+	+	+	+	++	-	+++	+	+	+	+	+	+	+
SSL 12	+	+	-	-	-	+++	-	-	+/-	-	-	+/-	+/-	-
SSL 13	+	+	-	+	-	++	-	+	-	-	-	-	-	-
SSL 14	+	+	-	+	+++	+++	+	+	+	+/-	+	+	+	+
SSL 15	+	+	-	+	+	+++	+++	+	+	+/-	+	+	+	+
SSL 16	+	+	-	+	-	-	-	+	+	+/-	+	+	+	+

(+) Positive test; (-) Negative test; (+/-) Indecisive utilization of carbohydrates;
(+ + +) Strong Hydrolytic Activity; (+ +) Moderate Hydrolytic Activity; (+) Weak Hydrolytic Activity.

diversity that can be exploited for many industrial applications. One such hypersaline habitat, Sambhar Salt Lake, India, was studied for its actinomycetes population and their bioactive potential. The analysis of pH and salinity of lake water in different seasons revealed its alkaline and saline nature. Many previous studies also reported the same nature of Sambhar Salt Lake.[25,26] Similar values for alkalinity and salinity were also reported for other athalassohaline habitats like El- Djerid Salt Lake in southern Tunisia.[27] Actinomycetes from such halophilic habitats are composed of heterogeneous physiological groups of different genera. In this study, a total of 16 actinomycetes

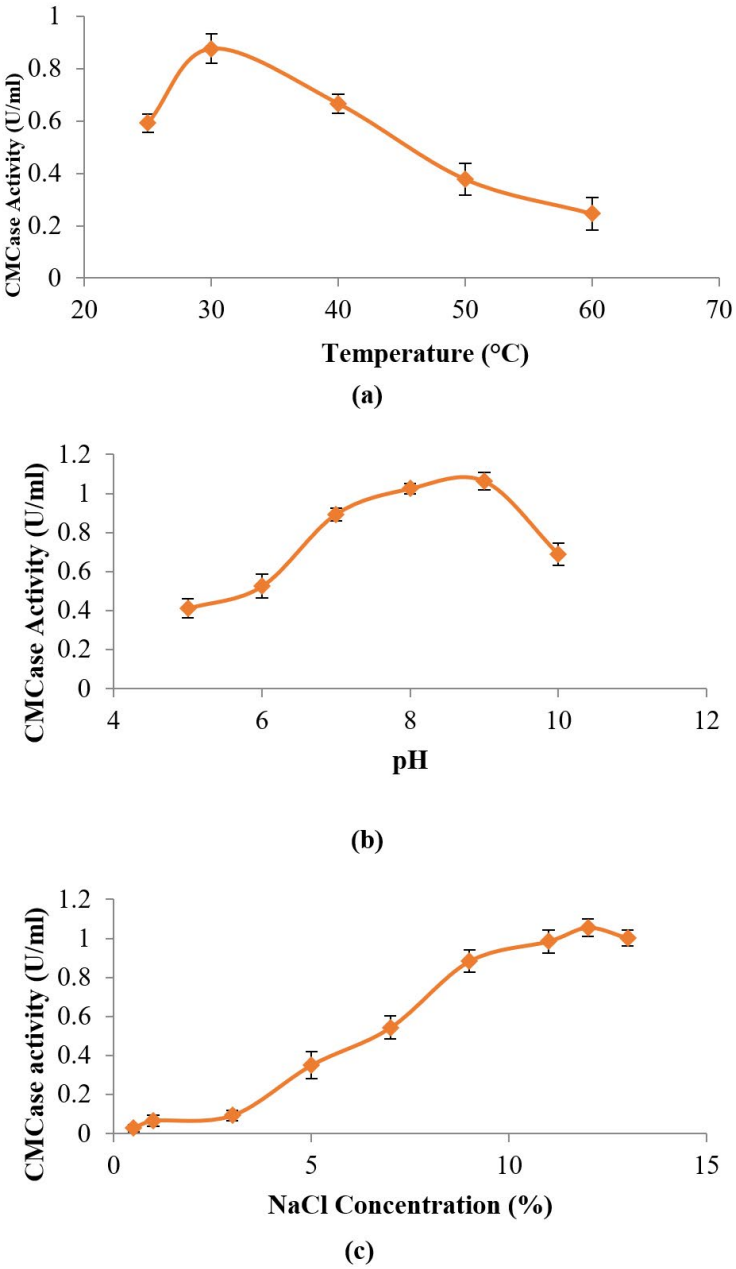


Figure 6. Optimization of (a) Temperature, (b) pH, (c) NaCl for CMCase Production by Isolate SSL 14.

isolates (SSL 1-SSL 16), including halotolerant and moderate halophiles, were recovered from solar salt pans of Sambhar Salt Lake, India. Other hypersaline environments studied for their presence were crystallizer ponds of solar salterns from Tuticorin in Bay of Bengal,[28] and the saline desert of Kutch, India.[29] Halotolerant and halophilic actinomycetes have been reported from other Salt Lake habitats.[30,31] To prevent osmotic lysis of cells in the presence of salt, most of the halophiles and halotolerant microorganisms accumulate compatible solutes in high concentrations within the cytoplasm as a part of their adaptive strategy.[32]

Halophilic and halotolerant species have been reported for the genera *Streptomyces, Pseudonocardia, Streptosporangium, Nocardiopsis, Salinispora,* and *Streptomonspora*.[33] The presence of *Streptomyces* as a predominant genus in halophilic environments of both marine and non-marine origin has been reported in many studies,[28,34,35] and a similar pattern was noticed in the present study also. *Nocardiopsis* was the second most abundant actinomycete isolated in the present study, and its presence was also observed in Kovalam, Puthalum, and Thamaraikulam salt works, India.[36] Member of a rare genus *Pseudonocardia* was also recovered in the present study. Its presence has also been noticed in Bay of Bengal's hypersaline environments, India,[37] and in the South China Sea.[38] Rajagopal and Kannan[39] recently reported isolation of *Actinoalloteichus* sp. from sediments of the Havelock Island of Andaman, India.

Table 5. Relative Enzyme Activity of Actinomycete Isolates

No.	Isolates	Relative CMCase activity
1.	SSL 1	16.488
2.	SSL 2	19.25
3.	SSL 3	11.96
4.	SSL 4	6.249
5.	SSL 5	12.69
6.	SSL 6	6.41
7.	SSL 7	4.861
8.	SSL 10	7.028
9.	SSL 11	20.496
10.	SSL 13	10.56
11.	SSL 14	22.04
12.	SSL 15	14.391
13.	SSL 16	17.063

Sufficient studies were not available on actinomycetes population of Sambhar Lake water till 2012 but in 2013, Yadav et al.[40] published reports on actinomycetes diversity from Sambhar Lake water which showed the presence of the same actinomycetes genera.

Actinomycetes are studied most extensively for antibiotic production, but less focus is on enzyme production, especially those isolated from halophilic habitats.[41] In this study, 81% of the isolates were found to be positive for CMCase production. The Enzyme produced by isolate SSL 14 identified as *Nocardiopsis* sp. was optimized following the OFAT approach. Similar

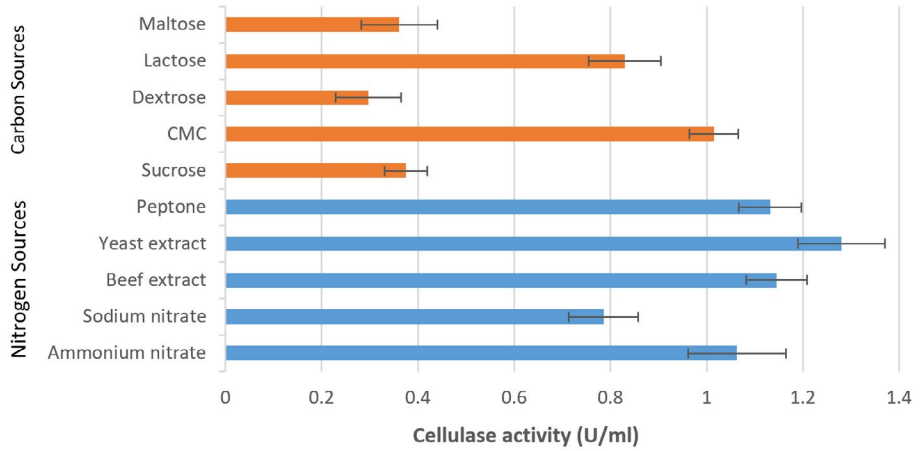


Figure 7. Optimization of Carbon and Nitrogen Sources for CMCase Production by Isolate SSL 14.

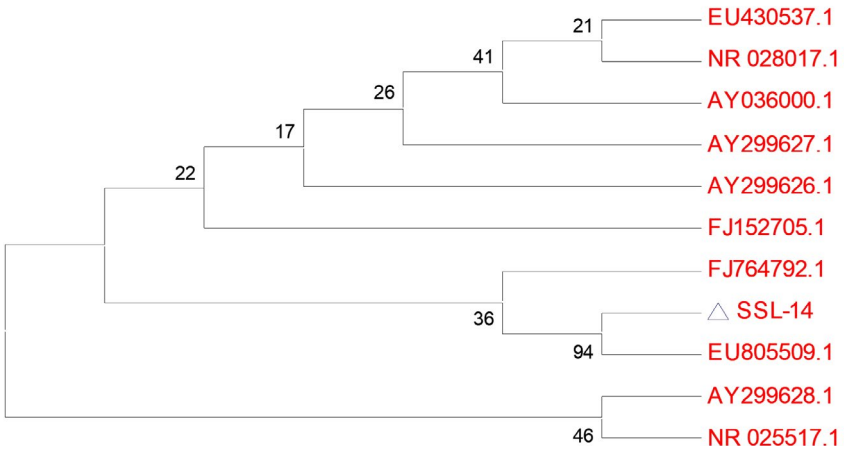


Figure 8. Neighbor-joining tree showing the position of isolate *Nocardiopsis* SSL14 to first ten similar sequences. Values are the branch lengths reflecting the actual distances between the sequences. EU805509.1: *Nocardiopsis* sp. BCEAA 7603); FJ764792.1: *Nocardiopsis* sp. E-143; EU430537.1: *Nocardiopsis exhalans* strain VTT E-063001; FJ152705.1: Uncultured bacterium clone TX1A_153; AY299626.1: *Streptomyces* sp. YIM 80125; AY299627.1: *Nocardiopsis* sp. YIM 80129; AY036000.1: *Nocardiopsis exhalans*; NR_028017.1: *Nocardiopsis exhalans* strain ES10.1; AY299628.1: *Nocardiopsis* sp. YIM 80130; NR_025517.1: *Nocardiopsis metallicus* strain R2A

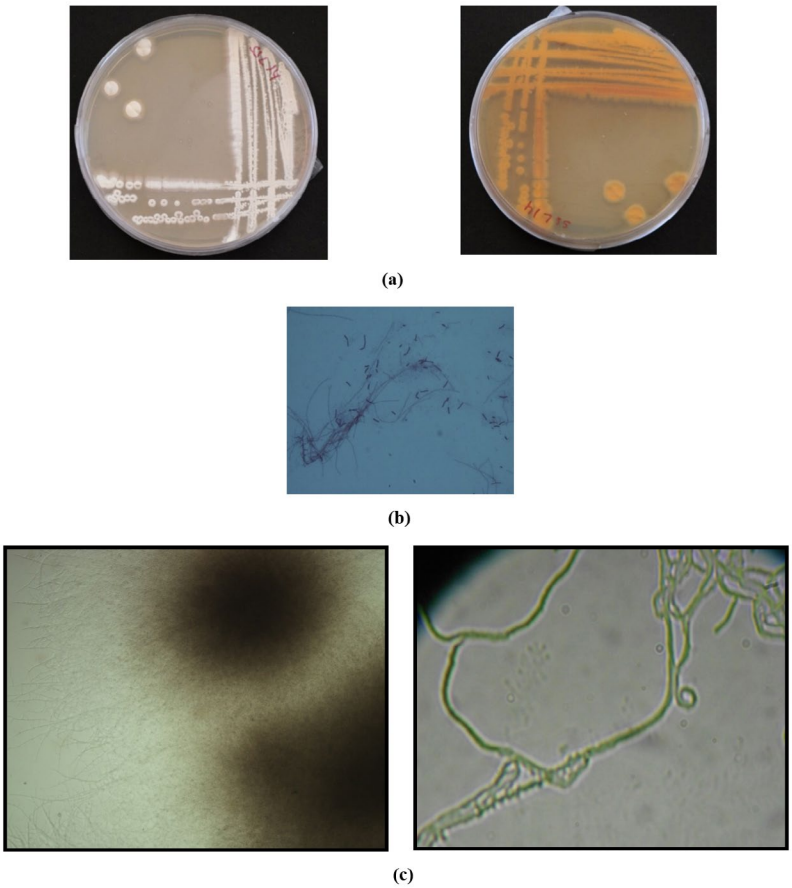


Figure 9. Morphological features of SSL14 (a) AM and SM on AIA medium, (b) Grams Staining and (c) Micro morphological features (Colony View and AM).

enzyme optimization studies using the OFAT approach were performed for cellulase production by *Streptomyces* sp. B-PNG23 and by *Fomitopsis* sp. RCK 2010.[42,43]

It has been reported that enzyme production by actinomycetes starts in the early log phase, increases in the late log phase, and continues till the onset of the stationary phase, after which it declines.[44] The results of the present study were found to be in accordance with this theory. CMCase production by *Nocardiopsis* SSL 14 was highest at 96 h of incubation. A novel actinomycete, *Nocardiopsis* sp. KNU was found to produce maximum cellulase in the late log phase that continued up to the stationary phase,[45] whereas non-growth associated CMCase production was observed in *Streptomyces drozdowiczii*.[46] In another study on *Streptomyces* sp., maximum enzyme production was observed after five days of incubation.[47] It has been reported in the literature that end product act as an inhibitor to hydrolysis of cellulose,[48,49] and therefore is responsible for the decline in activity after a few days. The depletion of nutrients in the medium can also adversely affect enzyme production after a few days of incubation.[50]

The effect of temperature on the enzyme productions was related to growth temperature, maximally produced at 30°C. Enzymes are not stable at very high or very low pH values as they get, denatured and thus, these kinds of pH values adversely affect enzyme production by microorganisms.[51] This fact is supported by the finding of the present study, where the production of CMCase was very low at lower pH values. However, maximum enzyme production at alkaline pH was observed and can be attributed to the origin of isolate and thereby its adaptation to saline habitat of Sambhar Lake. Different species of *Streptomyces* were reported to show maximum enzyme production at a broad range of pH from 5.5 to 8.0.[52-55]

Nocardiopsis SSL 14 showed the highest activity when the medium was supplemented with 12% NaCl. This adaptation of proteins in a media with high salt concentrations is attributed to the presence of a large number of acidic residues on protein surface supported by electrostatic interactions and an increased number of salt bridges.[56]

Synthesis of cellulase is subjected to induction and catabolite repression in both bacteria and fungi. Cellulose and its derivatives act as inducers, and soluble sugars like glucose, maltose, etc., act as a repressors.[57] Consistent with these theories, in the present study also, maximum enzyme production occurred in the presence of CMC and decreased significantly in the presence of glucose and maltose. Thus, CMC was easily metabolized by SSL 14 and also acted as an inducer for CMCase production. In a study on *Streptomyces* sp., it was found that CMC (1%) was better than glucose and other carbon sources for endoglucanase production.[58]

Yeast extract was found to be the best source for CMCase production by SSL 14, similar to that reported for *Streptomyces* sp. F2621,[59] and *Streptomyces drozdowiczii*.[46] Inorganic sources such as ammonium nitrate and peptone did not show a significant difference in enzyme activity. A similar finding has also been reported in *Streptomyces* sp. T3-1 by Jang and Chen.[58] Wang, in 1982, reported that ammonium ions could be absorbed to the mycelium resulting in a lowering of the pH, thereby inhibiting cellulase production.[60] This could be responsible for lowering of CMCase production in the presence of ammonium salt.

CONCLUSION

In a nutshell, the findings of the present investigation have shown that CMCase was produced by *Nocardiopsis* SSL 14 under high salt concentrations (12% NaCl) and alkaline pH 9.0. This property is advantageous in the bioremediation of cellulosic materials and the production of bio-energy. The enzyme can be used with ionic liquids (ILs) in the pre-treatment of lignocellulosic biomass to produce renewable products in an eco-friendly way.[61-62] The enzyme can be further purified and characterized for its ability to function at high pH and salt concentrations and can be tested for its use in commercial detergent formulations. The production of protease by actinomycete isolates of Sambhar Lake and their application as a detergent additive has been studied.[63] Thus, it is also suggested that these actinomycetes might also produce other enzymes and products that could prove beneficial for different biotechnological applications.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

CS conceptualized the study, acquired the funding, investigated and performed the experiments. PC performed the data curation and formal analysis. PC and PM visualized the study. CS and PC wrote the original draft. PB and NM performed the supervision. PB, NM and PM reviewed and edited the final manuscript for publication.

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DATA AVAILABILITY

16S rDNA sequence of *Nocardiopsis* sp. SSL 14 is available in GenBank with Accession number KU860459. All other datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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