Research Article

Extremotolerant Gelatinase Enzyme Production by UCM4 Bacterial Strain Isolated from the Rhizospheric Soil of *Rhizophora mangle*

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Submission Date: 23-08-2022; Revision Date: 08-09-2022; Accepted Date: 17-10-2022.

ABSTRACT

Gelatinases are important metalloproteases which specifically act on gelatin. Gelatinase hydrolyze gelatin into polypeptides, peptides and amino acids which can cross the cell membrane to get utilized by the organisms. Gelatinase and collagenase are not only used in chemical and medical field but also in food and basic biological sciences. Since gelatinase has potential applications and high demands, the search for new isolates producing gelatinase with extremotolerant property is significant. In the present study we focus on potential extremotolerant gelatinase production from *Cellulosimicrobium funkei* strain UCM4 isolated from the rhizospheric soil of *Rhizophora mangle* from coastal regions of Dakshina Kannada, Karnataka, India. The isolate was tested for extremotolerant property with potential gelatinase production in extreme physiological conditions such as pH, salinity and temperature. The isolate showed 56,500 µg/ml of gelatinase production at its optimum growth conditions. The gelatinase production was increased with increase in pH from 7 to 11 which was between 56,500 µg/ml to 58,888.3 µg/ml. Gelatinase production was observed at temperature ranges from 30°C to 40°C which was between 57,777.2 µg/ml to 55,555 µg/ml, and salinity ranges from 0% to 5% NaCl (w/v) which was between 56,500 µg/ml to 49,999.5 µg/ml. The study revealed that the *Cellulosimicrobium funkei* strain UCM4 has ability to produce extremotolerant gelatinase and hence UCM4 has its potentiality for industrial scale production of gelatinase enzyme and the results will stand as a base line data for the production and application of gelatinase in future.

Keywords: Enzymes, Gelatinase, Halotolerant bacteria, Rhizospheric soil.

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INTRODUCTION

The marine environment has diversified biological and chemical properties which make their chemical compounds extremotolerant. These compounds gain more prominence in enzymes, pharmaceuticals, agrichemicals, cosmetics, nutritional supplements, molecular probes and fine chemicals.[1] The enzymes produced by terrestrial organisms are incompetent of tolerating unfavorable environment of industries when in fact marine organisms produce enzymes which retain its activity even at extreme physiological conditions such as high pH, temperature and salinity.[2] Till date the enzyme production is studied mostly on terrestrial organisms and inseveral on marine organisms.[1] Gelatinase is an extracellular metallo-endopeptidase or metalloproteinase which hydrolyze gelatin, pheromone, collagen, casein, proteoglycan, elastin, laminin, fibronectin and fibrinogen.[1,3,4] Gelatinase and collagenase are ingrained in chemical and medical filed, furthermore in food and basic biological sciences. Micro-organisms hydrolyze gelatin into polypeptides, peptides and amino acids which can cross the cell membrane to get utilized by the organisms.[1] In humans, gelatinase is involved in breakdown of extracellular matrix during embryonic development, reproduction and tissue remodeling and also in arthritis

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DOI: 10.5530/ajbls.2022.11.103

and metastasis.[5] Gelatinase is deeply ingrained in the production of drug since it has property of degrading connective tissue associated with tumor metastasis.[6,7] Considering potential applications and high demand of gelatinase, exploration of new isolates producing highly efficient, economical and extremotolerant gelatinase is crucial.[1] In the present study we focus on potential extremotolerant gelatinase production from *Cellulosimicrobium funkei* strain UCM4 isolated from the rhizospheric soil of *Rhizophora mangle* from the coastal regions of Dakshina Kannada, Karnataka, India. The isolate was tested for extremotolerant property with potent gelatinase production in extreme physiological conditions such as high pH, salinity and temperature.

MATERIALS AND METHODS

Sample collection

The coastal regions of Dakshina kannada is ~43km from Thalappady (south) to Mulki (North). The sample collection region was ~36km from Someshwara beach to Sasihithlu beach. The entire sample collection region was equally divided into 6 sample collecting sites i.e., Someshwara beach (N 12° 47' 11.58", E 74° 51' 12.35"), Ullal delta (N 12° 50' 8.52", E 74° 49' 57.36"), Lion's beach park (N 12° 53' 58.2", E 74° 48' 44.64"), Hosabettu beach (N 12° 58' 22.8", E 74° 47' 50.64"), NITK beach (N 13° 0' 51.12", E 74° 47' 17.16"), Sasihithlu beach (N 13° 4' 14.52", E 74° 46' 36.12"). Samples were collected in the month of October and November, 2019. The rhizospheric soil of *Rhizophora mangle* growing in these regions was collected by excavating the roots with sterile shovel (Figure 1). The pH, temperature and salinity of the sample collecting site were determined. The samples were covered in a sterile polytene bag and transferred to the lab.

Figure 1: *Rhizophora mangle* growing at coastal regions of Dakshina Kannada.

Rhizophora mangle was growing at 200m away from the sea shore.

Isolation of halophilic bacteria

The root samples were moderately shaken to eliminate unwanted soil. The tightly adhering rhizospheric soil was taken for isolation of bacteria. The soil samples were serially diluted ten times and all the dilutions were inoculated into culture plates containing nutrient agar with different NaCl concentration ranging from 0 to 35% (w/v). The CFU of soil samples were calculated and each colony were subculture many times to get single colony.[8]

Colony characteristics

The colony characteristics such as pigmentation, colony elevation, opacity, shape, size, margin, and motility were studied.[9,10]

Gelatine liquefaction test (primary screening)

The isolates were tested for the production of gelatinase by stabbing the gelatine agar media with 24-hr old culture isolates. The tubes along with control was incubated at 32°C for 48hr and further kept in refrigerator at 4°C until the control tube solidified. The tubes checked for liquefaction of gelatine. The isolates which liquified gelatine were quantitatively tested.[1]

Quantitative estimation of gelatinase (secondary screening)

The concentration of culture broth adjusted to 2.0×10^6 CFU/mL from which 100µL was added to 30 mL of nutrient gelatine broth (HiMedia) and cultured at 37°C for 24-hr. 0.1ml of the sample (crude enzyme produced on the gelatine media) was added to the reaction mixture which contained 0.3 ml of (0.2%) gelatine in water (w/v), 0.2 ml of (150 mM) Tris-HCl, pH 7.5 containing 12mM $CaCl_2$. The reaction mixture was incubated at 30°C for 30 min and reaction was stopped by the addition of 0.6 ml (0.1 N) HCl. The mixture was tested for the amount of released free amino acids which is directly proportional to the quantity of gelatinase. The amount of free amino acids was tested using ninhydrin method (reagents required: standard amino acid stock solution i.e., 150µg/ml, ninhydrin reagent, 50% ethanol, distilled water) by taking 2µl of sample in 998µl distilled water, to that 1ml ninhydrin was added. The tubes were incubated in boiling water bath for 15min and 5ml of solvent was added and incubated for 10 min at room temperature and absorbance was measured at 570nm. The same mixture except gelatine was used as blank.[1]

The isolate with maximum gelatinase production was selected for identification.

Biochemical characterization, PCR amplification of 16S rRNA and sequencing of the PCR product

The isolate was subjected to various biochemical tests with all the tests being carried out in triplets.[9,10] The isolation of DNA and 16S rRNA amplification was performed according to Sahay *et al.*, 2011.[8] The PCR reactions were performed in a Bio-Rad thermal cycler. Primers 24F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' TACGGTTACCTTGTTACGACTT 3') were used to amplify the 16S rRNA. The nucleotide sequences were di-deoxy cycle sequenced with fluorescent terminators and run in 3130x1 Applied Biosystems ABI prism automated DNA sequencer. The identification of the isolate was done using the GenBank. The multiple sequence alignment and comparison studies were performed using Clustal Omega with default parameters and data were converted into PHILIP format. The phylogenetic tree was constructed using the neighbor-joining method in the program MEGA 7. The sequence was submitted to GenBank, and accession number was retrieved.[8]

Growth pattern and optimization of growth conditions of the identified isolate

The kinetic study on growth of bacterial isolates were done using nutrient broth medium (HiMedia) with 1% NaCl (w/v). The concentration of culture broth adjusted to 2.0×10^6 CFU/mL and 100µL was added to 30 mL of nutrient broth (HiMedia) and cultured at 37°C for 24-hr. The isolates were incubated at 36°C for 48 hr with shaking at 200 rpm. The cell density was measured at 600nm for every 2-hr interval.[11] The effect of physiological parameters on bacterial growth was determined at various pH, temperature, salinity, carbon and nitrogen sources. For obtaining pH from 5.0-6.0, 50mM sodium acetate; for 7.0-8.0, Tris-HCL; and for pH from 9.0-11.0 and 12-13 glycine-NaOH were used.

Temperature ranges from 20°C-50°C and NaCl concentrations from 0%-30% (w/v) were tested [11]. Different carbon sources such as, glucose, lactose, maltose, mannitol, and potato starch and starch rich flours such as rice, wheat, maize, sorghum and different nitrogen sources such as peptone, gelatine, beef extract, yeast extract, casein, skim milk powder, ammonium chloride, and urea were tested. The carbon in 1% (w/v) and nitrogen sources at concentration of 0.8% (w/v) were added to the mineral salt medium.[11]

Antibiotic susceptibility test

The identified isolate was tested for antibiotic susceptibility against 19 antibiotics (HiMedia). The tests were conducted according to disc diffusion or 'Kirby-Bauer' method, using Muller Hinton agar (HiMedia).[12]

Effect of physiological parameters on gelatinase production

The culture concentration of the isolate was adjusted to 2.0×10^6 CFU/mL, 100µL was added to 30 mL of nutrient gelatine broth (HiMedia) and cultured at 37°C for 24-hr. The effect of pH, temperature and salinity was tested on gelatinase production. pH from 7 to 11, temperature from 30°C to 40°C and salinity from 0% to 5% was tested.[11]

Location of the enzyme

To determine whether gelatinase produced is intracellular or extracellular, the culture concentration of the isolate was adjusted to 2.0×10^6 CFU/mL, 100µL was added to 30 mL of nutrient gelatine broth (HiMedia) and cultured at 37°C for 24-hr. The broth sample was centrifuged for 5min at 5000G. The supernatant was used for estimation of extracellular gelatinase. The pellet was added with lysis buffer containing PMSF (phenylmethylsulphonyl fluoride) and the lysis carried out at frequency of 20 kHz for 30 sec in sonicator. The lysed extract was used to estimate gelatinase.[13]

RESULTS

Characteristics of sample collection sites

The *Rhizophora mangle* plants were growing at 200m away from sea shore. The soil was blackish brown in colour, pH 7.8, temperature 39°C, salt level 31 (ms/m).

Isolation of halophilic bacteria

A total of 24 bacteria were isolated. The color of the colonies was yellow, pale yellow, white, pink, orange and red.

Gelatine liquefaction test (primary screening)

Out of 24 isolates, 2 isolates showed gelatine liquefaction in nutrient gelatine media (Figure 2a). The two isolates were named UCM4 and UCM8.

Quantitative estimation of gelatinase (secondary screening)

The quantitative production of gelatinase showed highest production in UCM4 i.e., 56,500 µg/ml and UCM8 showed 30,500 µg/ml of gelatinase (Figure 2b and 2c).

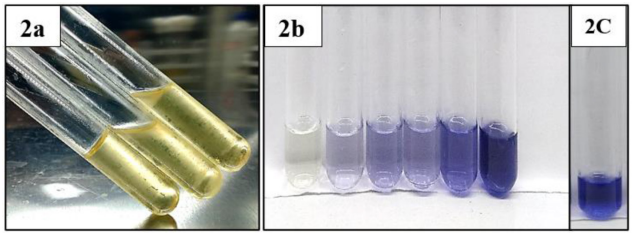

Figure 2: 2a) gelatin liquefication test of UCM4 and UCM8. 2b) standard reactions of ninhydrin test, 2c) protein estimation in UCM4 by ninhydrin method.

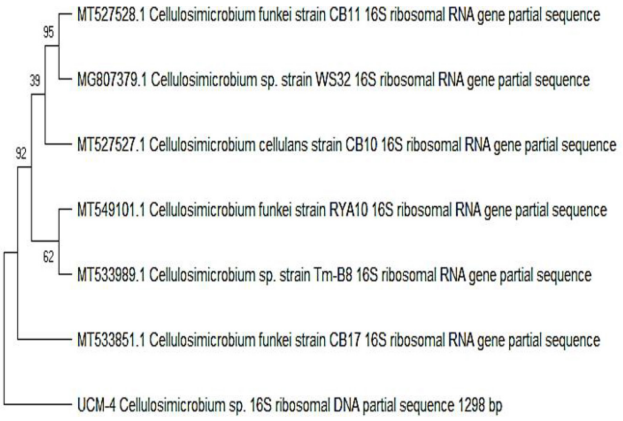

Figure 3: Phylogenetic tree of UCM4.

Biochemical characterisation, identification and phylogenetic analysis of UCM4

UCM4 was yellow coloured colony which was gram positive cocci with no motility. The biochemical properties are given in Table 1. The 16S rRNA analysis was done to identify the halotolerant bacterial isolate UCM4. The PCR product was ~1250bp in length. The 16S rRNA amplicons of the bacterial isolate UCM4 were sequenced on both strands using the primers 27F and 1492R. The sequences obtained were subjected to BLAST analysis. The BLAST and phylogenetic analysis showed that the isolate UCM4 belong to the genus *Cellulosimicrobium*. UCM4 showed 98.10% similarity to *Cellulosimicrobium funkei* strain CB17 (Figure 3). The isolate was identified and named as *Cellulosimicrobium funkei* strain UCM4. The sequences were submitted to GenBank, and Accession numbers was assigned as *Cellulosimicrobium funkei* strain UCM4 - MW883186.

The Phylogenetic tree showing the halophilic isolates position, based on the partial 16S rRNA sequence comparison, is calculated with the neighbor-joining method. Bootstrap values are indicated on the branches.

Table 1: Biochemical characterization of UCM4.

Strains	UCM4
Cell morphology	Cocci
Motility	-
Pigmentation	Yellow
Shape	Irregular
Gram reaction	+
Catalase	+
Amylase	-
Gelatinase	+
Urease	+
Oxidase	+
Indole	+
Methyl red	+
Voges Proskauer test	-
Citrate utilization	+
Carbohydrate fermentation	+

Various biochemical tests such as, gram staining, motility test for catalase, amylase, gelatinase, urease, oxidase and indole test, methyl red and VP tests, citrate utilization test, the carbohydrate fermentation test were performed in triplets.

Growth curve and optimal growth parameters of UCM4

The growth pattern of UCM4 was initial 4 hr of lag phase, 6 to 20 hr of log phase and 22 to 32 hr of stationary phase and 34 to 48 hr of decline phase. The isolate was able to grow up to pH 11.0 and no growth found below pH 7.0, which indicates alkaliphilic nature of the isolate. UCM4 was able to grow at temperature 30-40°C and 0-5% salinity which shows halotolerant and thermotolerant property. UCM4 showed growth in all the carbon, starch rich flours and nitrogen sources tested except mannitol, casein and skim milk.

The optimum growth of the isolate was at pH 7, 35°C and 2% NaCl (w/v) (Figure 4a).

Antibiotic susceptibility test of UCM4

The zone of inhibition was measured using ruler (Figure 4b). According to CLSI guidelines, resistant, susceptible, intermediate resistance property of the identified isolates were determined (Table 2).

Location of enzyme and effect of physiological parameters on gelatinase production

The location of enzyme was purely extracellular. The production of gelatinase was observed at pH 7-11, temperature 30°C-40°C and salinity 0%-5% NaCl (w/v). UCM4 produced, 56500 µg/ml of gelatinase at pH 7, 57775 µg/ml at pH 8, 58888.3 µg/ml at pH 9, 58888.3

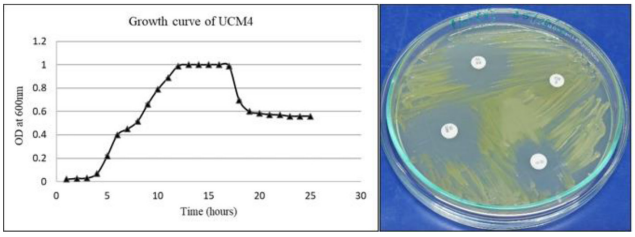

Figure 4: a) Growth curve of UCM4 at optimum physiological conditions. b) Antibiotic susceptibility test in UCM4.

Table 2: Antibiotic susceptibility test in UCM4.

Sl. No.	Antibiotic	Code	Conc (mg)	Antibiotic susceptibility of UCM4
1.	Chloramphenicol	C	30	I
2.	Azithromycin	AZM	30	R
3.	Ciprofloxacin	CIP	5	S
4.	Erythromycin	E	15	S
5.	Penicillin-G	P	2	I
6.	Ampicillin	AMP	10	S
7.	Cefazolin	CZ	30	R
8.	Tobramycin	TOB	30	I
9.	Clindamycin	CD	2	R
10.	Nalidixic acid	NA	S	I
11.	Cefadroxil	CFR	30	S
12.	Cephalothin	CEP	30	I
13.	Bacitracin	B	8	S
14.	Oxacillin	OX	5	I
15.	Ampicillin/ Cloxacillin	AX	10	I
16.	Amoxyclav	AMC	10	R
17.	Gentamicin	GEN	50	S
18.	Vancomycin	VA	10	S
19.	Streptomycin	S	S	S

µg/ml at pH 10 and 58888.3 µg/ml at pH 11. UCM4 produced 57777.2 µg/ml of gelatinase at 30°C, 56500 µg/ml at 35°C and 55555 µg/ml at 40°C temperature. Gelatinase production by UCM4 at 0% NaCl (w/v) was 56500 µg/ml, at 1% NaCl was 56500 µg/ml, at 2% NaCl (w/v) was 56500 µg/ml, at 3% NaCl (w/v) was 58888.3 µg/ml, at 4% NaCl (w/v) was 55555 µg/ml and at 5% NaCl (w/v) was 49999.5 µg/ml. The maximum gelatinase production was at pH 9, 10, 11 (Figure 5a), 30°C (Figure 5b) and 3% salinity NaCl (w/v) (Figure 5c and 5d).

DISCUSSION

In the present study, 24 bacterial isolates were isolated from the rhizospheric soil of *Rhizophora mangle* out

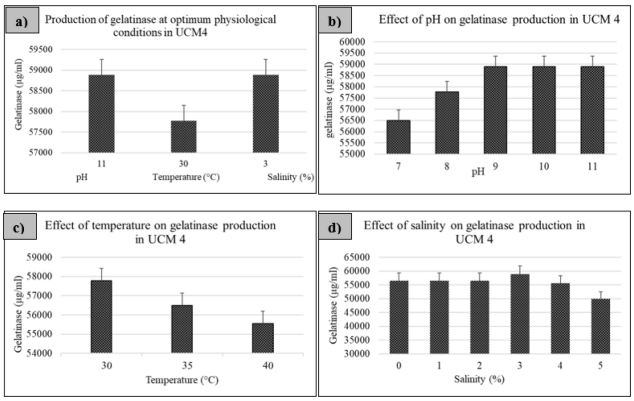

Figure 5: a) maximum production of gelatinase at optimum physiological conditions in UCM4, b) effect of pH on gelatinase production in UCM4, c) effect of temperature on gelatinase production in UCM4, d) effect of salinity on gelatinase production in UCM4.

of which 2 isolates were observed for the gelatinase production which were named as UCM4 and UCM8. The gelatinase production was identified by gelatine liquefication test. The quantitative gelatinase estimation showed maximum production in UCM4. UCM4 was biochemically characterised and 16S rRNA sequence analysis was carried out and identified as *Cellulosimicrobium funkei* strain UCM4. The gelatinase production at optimum growth physiological conditions was 56,500 µg/ml. The gelatinase production was purely extracellular. The gelatinase production was increased with the increase pH from 7-11. At pH 8 there was maximum gelatinase production i.e., 5888.3 µg/ml which was constant up to pH11. Previous reports showed gelatinase activity is optimum at, pH 7-7.2 [4] maximum at pH 8 and showed activity up to pH 10.5[14] thus this report present new information on pH optimum and pH tolerance. At 30°C, UCM4 produced 57777.2 µg/ml of gelatinase. Although Gelatinase production was slightly decrease with further increase in temperature, gelatinase production was seen up to 40°C which was 55555 µg/ml. There are other reports where the gelatinase activity was seen up to 65°C and maximum production at 50°C.[14] Other works showed gelatinase characterisation such as, *Mrakia* sp (36°C and pH 7.0),[15] *E. faecalis* (30°C and pH 7.5–8.0),[16] *Bacillus* strains (50°C and pH 7),[17] *Brevibacillus agri* (40°C and pH 7.2),[18] and *Salinicoccus roseus* (37°C and pH 8).[19] This shows that the enzyme discovered in this work has traits in common with those of other enzymes and points to its possible use in the hydrolysis of gelatine. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium perfringens* and *Serratiamarcescens* are some of the bacteria which express gelatinase.[1] In this report gelatinase production was seen from 0-5% NaCl (w/v) and maximum production

was at 3% NaCl (w/v) i.e., 58888.3 µg/ml. The maximum gelatinase production was at pH 9 to 11, 30°C temperature and 3% salinity NaCl (w/v). As per our knowledge this is the first report to characterise halotolerant bacterial gelatinase activity against salinity.

The main sources of gelatine utilized in the food, beverage, cosmetics, and pharmaceutical industries are porcine, bovine, and fish. Because some individuals view these sources as halal, these varieties of gelatine were identified in the products using a variety of approaches, including DNA analysis and protein-based analysis. These methods have their limits. In order to identify porcine gelatine, scientists have reported gene expression of microbial gelatinase activity.[20]

In the hunt for biomolecules with industrial value, marine microbes are being researched more and more. According to estimates, marine habitats have a higher level of biological richness than tropical rain forests.[21] There are studies where bacterial (*Bacillus*) gelatinase used for degradation of animal hoof which majorly made up of gelatine[14] and gelatine's functional, textural, and nutritional qualities are enhanced through enzymatic hydrolysis. In recent years, gelatine hydrolysates have proven valuable in a variety of food composition applications.[22] Despite the fact that gelatinases are common in nature, there aren't many findings from microbial sources.[23,24]

CONCLUSION

Considering practical and beneficial objectives, the enzyme sources derived from microbes have advantages include quick growth and affordable growth and smaller spaces are needed for its large-scale cultivation. Furthermore, the study revealed that the isolated strain *Cellulosimicrobium funkei* has its potentiality for industrial scale production of gelatinase enzyme and the results will stand as a base line data for the production and application of gelatinase in future. Future industry and technology focus on marine enzyme biotechnology.

ACKNOWLEDGEMENT

The authors are thankful to Mangalore University for the financial support and the Principal of University College Mangalore. Thanks to Dr. Malathi Shekhar and Girisha, Scientists at Fisheries College Mangalore, for providing infrastructural support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Cite this article: Deepa PBK, Nanjundaiah SM. Extremotolerant Gelatinase Enzyme Production by UCM4 Bacterial Strain Isolated from the Rhizospheric Soil of *Rhizophora mangl*e. Asian J Biol Life Sci. 2022;11(3):769-75.