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RESEARCH ARTICLE

Screening and molecular characterization of *Serratia marcescens* VITSD2: A strain producing optimum serratiopeptidase

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Abstract The current work was attempted to isolate and characterize the serratiopeptidase producing *Serratia* sp. Among the 10 bacterial isolates 7 strains were identified as *Serratia* sp. Out of 7 strains one showed potent proteolytic activity and selected for further studies. Based on the morphological, biochemical and molecular characterization, the potent isolate (RH03) was identified as *Serratia marcescens* (GenBank accession number: KC961637) and the strain was designated as *Serratia marcescens* VITSD2. The production of serratiopeptidase was carried out in trypticase soya broth and the enzyme was partially purified using ammonium sulfate precipitation and dialysis. The specific activity was determined by casein hydrolysis assay and was found to be 12.00, 21.33, and 25.40 units/mg for crude, precipitated and dialysed samples. The molecular weight of the protease was determined by SDS-PAGE and it was found to be 50 kDa. The antibacterial activity of the produced serratiopeptidase showed moderate activity against *Pseudomonas aeruginosa* MTCC No. 4676 (12 mm) and *Escherichia coli* MTCC No. 1588 (15 mm).

Keywords Serratiopeptidase, *Serratia marcescens* VITSD2, antibacterial activity

Introduction

Serrapeptase is a proteolytic enzyme available for clinical use more than a decade. Serrapeptase, also known as serratia-peptase, serratiopeptidase, serratia E-15 protease, serralysin, serratiapeptidase or serratiopeptidase, a protease enzyme produced by *Serratia* sp. E-15 (Nakahama et al., 1986). This bacteria was isolated in the late 1960s from silk worm *Bombyx mori* L. (Miyata et al., 1970). It was originally considered to be an innocuous, saprophytic water organism and it was often used as a biological marker because of its easily recognizable red colonies (Miguel and Carrascosa, 2013). *Serratia marcescens* produces many extracellular serine and thiol proteases and metalloproteases (Aiyappa and Harris, 1976; Decedue et al., 1979). Serratiopeptidase binds to alpha-2-macroglobulin in the blood in the ratio of 1:1, which helps to mask its antigenicity but retains its enzymatic activity and is slowly, transferred to site of inflammation. The heterogeneity of the proteases produced is used as a diagnostic tool for taxonomic classification and clinical identification of strains (Grimont et al., 1977). Serrapeptase was far more effective than any other enzyme in repressing fibrinolysis and its unique biological phenomenon of the enzyme led researchers to study clinical applications of the enzyme. Despite the serratiopeptidase tablets having minimum side effects (Valeria et al., 2003; Trumbore et al., 2005; Nirale and Menon, 2010). The less pathogenic strain of *Serratia marcescens* than the ones presently used can be substituted for production. *Serratia marcescens* also possesses a red pigmentation known as prodigiosin which is capable for advancement in cancer studies. Obtaining the most potent enzyme producing strain can provide the ability to improve the results in cancer research. Hence the current study involves the identification of a potent serratiopeptidase producing *Serratia* sp.

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Materials and methods

Isolation of *Serratia* sp. from soil samples

Seven soil samples were collected in sterile bags from different locations of Vellore, Chennai and Kochi, India. 1 g

of the soil samples were serially diluted and inoculated by pour plate method. The plates were incubated at 37°C for 24 h (Weissmann, 2006). Individual red colonies were selected and the pure cultures were maintained on nutrient agar plates and stored at 4°C.

Primary screening

Casein hydrolysis method

Skim milk agar medium was prepared and sterilized. A single line streak inoculation of the 7 isolates was made from their respective pure culture plates onto the labeled sterile skim milk agar. The plates were incubated for 24 h at 37°C and observed for the zone of clearance.

Secondary screening

Secondary screening was performed with the culture filtrates of the 7 isolates using well diffusion method. The isolates were grown in trypticase soy broth. After two days of incubation, the cultures were harvested, centrifuged at 10000 r/min for 15 min at 4°C and the supernatant was collected. 100 μL culture filtrate of each isolate was placed in each well and incubated at 37°C for 24 h. After incubation the plates were flooded with coomassive brilliant blue staining solution for 30 min and it was destained by adding destaining solution. The development of clear zone around the well was observed and noted.

Strain identification

Morphological and biochemical characterization

The potent producer of serratiopeptidase producing isolate RH03 was selected for this study. Morphological and biochemical characterization was done for the isolate RH03 and identified according to the Bergey's manual of determinative bacteriology (Holt, 1994).

Molecular characterization

16S rRNA gene amplification and sequencing

16S rRNA gene sequencing of *Serratia* sp. was amplified using forward primer 27 F 5′-AGAGTTTGATCMTGGCTCAG-3′ and reverse primer 1792 R (5′-TACGGYTACCTTGTTACGACTT-3′). The sequences were subjected to homology search using BLAST program of the National Centre for Biotechnology Information (NCBI). (Altschul et al., 1990). The 16S rRNA gene sequences determined were aligned along with the sequences of the type strains using the CLUSTAL W program version 2.1 (Chenna et al., 2003). The phylogenetic tree was constructed by neighborhood joining method (Saitou and Nei, 1987). Individual branches in phylogenetic tree were determined by bootstrap analysis based on 1000 samplings (Felsenstein, 1985).

RNA secondary structure prediction

The gene sequence was transcribed to RNA sequence to identify the r-RNA secondary structure using the RNA fold server from Vienna RNA website (Gruber et al., 2008). RNA secondary structure can be predicted by free energy minimization with nearest neighbor parameters to evaluate stability (Ding and Lawrence, 2003). It was used to predict the minimum free energy (MFE) secondary structure of single sequences using the dynamic programming algorithm (Zuker and Stiegler, 1981) and equilibrium base-pairing probabilities are calculated using John McCaskill's partition function (PF) algorithm (McCaskill, 1990).

Serratiopeptidase production

Growing cultures of *Serratia marcescens* VITSD2 was transferred to 100 mL sterile trypticase soy broth. The broth culture was monitored for an OD in the range of 02–0.3 at 610 nm.When the OD value of the broth culture was in the desired range, 10 mL of the inoculum was transferred to 90 ml sterile production broth and incubated at 32°C for 24 h in a shaker at 120 r/min. The broth was centrifuged at 10000 r/min for 20 min at 4°C. The supernatant was filtered through Whatmann No. 1 filter paper and stored at 4°C (Mohankumar and Raj, 2011).

Partial purification of enzyme

Precipitation and dialysis

Ammonium sulfate was added to the culture supernatant in small quantities with constant stirring in order to achieve saturation. The supernatant was then concentrated by precipitation with ammonium sulfate to 60%–70% levels. The precipitates were dissolved in 50 mM phosphate buffer (pH 7) and dialyzed over night against the same buffer. The resultant dialysates were regarded as partially purified serratiopeptidase and were used for further studies.

Total protein estimation

Total protein content of the crude, precipitated and the dialysed samples were estimated by standard method (Lowry et al., 1951).

Enzyme assay

A total of 0.5 mL of culture supernatant was added to 2 mL of casein solution 2.0% (w/v) dissolved in Tris-HCl (pH 8.0) and incubated at 37°C for 10 min. The reaction was terminated by the addition of 2.5 mL of 0.1 M trichloroacetic acid (TCA) and further incubated at 37°C for 30 min. It was centrifuged at 10000 r/min for 10 min at 4°C. To 2.5 mL of supernatant, 3 mL of 0.5 M Na_2CO_3 and 0.5 mL of 1N Folic Ciocalteu reagent was added, mixed well and incubated for 10 min. Absorbance was measured at 660 nm. The standard curve was prepared using different concentrations of tyrosine. One unit of enzyme activity was defined as the amount of enzyme

required to liberate 1μmol of tyrosine per min under the defined assay conditions (Subbaiya et al., 2011).

SDS PAGE

The partially purified enzyme was subjected to sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) (Machielsen et al., 2006) with lower separating gel (pH 8.8), upper stacking gel (pH 6.8) and 12% acrylamide concentration. A broad range protein molecular weight marker was used to compare the protein bands with the standards, for the confirmation of the enzyme.

HPLC analysis

The high performance liquid chromatographic (HPLC) separation of the partially purified serratiopeptidase enzyme was carried out on a LC-10 AT vp model HPLC using 250 × 4.60 mm Rheodysne column (C-18). The proteins were eluted with 70% (v/v) acetonitrile as the mobile phase at the flow rate of 1.0 mL/h at 280 nm with C18 column (3.0 mm × 300 mm).

Antibacterial activity

The *in vitro* antibacterial activity of serratiopeptidase was determined by agar well diffusion method. Exponential phase cultures of *Pseudomonas aeruginosa* MTCC No. 4676 and *Escherichia coli* MTCC No. 1588 were used to determine the antibacterial activity of the enzyme. The pathogens were overlaid onto the surface of Mueller-Hinton agar plates. Wells were dug on the agar plates and 100 μL of the crude enzyme, 60% precipitated and dialysed enzyme samples were added and the plates were incubated at 37°C for 24 h.

Results

Isolation and screening

A total of ten different soil samples were collected from different locations such as Kochi, Chennai, and Vellore. Isolates were selected based on the colony color and elevation (Tables 1, 2). Out of 10 soil isolates, only 7 showed red pigmentation (Fig. 1) and these isolates were further subjected to primary screening for the production of serratiopeptidase. All the 7 isolated strains showed a clear zone, indicating the presence of serratiopeptidase (Fig. 2).

Further all the seven bacterial strains were selected for secondary screening. A clear zone was formed around the wells containing the crude enzyme. The extracted supernatant of all the strains were found to have proteolytic activity. Interestingly, the isolate obtained from the rhizosphere soil, which was designated as RH03 remarkably hydrolyzed the substrate and produced a prominent and maximum zone of clearance (24 mm) when compared to other strains isolated (Table 3). Determination of SP enzyme from *Serratia* was confirmed by adopting Commasie brilliant blue staining. The formation of halo zone indicates the production of extra-cellular enzyme (Fig. 3). The potent strain RH03 was selected for further studies.

Morphological and biochemical characterization

Morphological and biochemical assessment of the isolated potent strain (RH03) confirmed the characteristic properties of *Serratia* sp. (Fig. 4, Table 4).

Molecular charecterisation

Sequence result of 16S rRNA was exported to the database and checked for the homologous alignment. Based on the alignment results strain RH03 was found as *Seratia marcescens* which showed 99% similarity and the strain name was designate as *Serratia marcescens* VITSD2. The partial 16S rRNA sequences were deposited in GenBank under the accession number KC961637. The phylogenetic tree based on 16S rRNA of the strain *Serratia marcescens*-VITSD2 was shown in Fig. 5.

RNA secondary structure prediction

The secondary structure was predicted using RNAfold server (using MFE) with a minimum free energy of −339.13 kcal/mol. The centroid secondary structure was predicted with a minimum free energy of −291.60 kcal/mol. Based on the energy of the predicted models, the secondary structure predicted by MFE gives the more stable structure with a minimum energy. Hence MFE model can be considered as the optimal secondary structure of the rRNA. The mountain plot is also derived from RNA fold server which represents a secondary structure in a plot of height versus position, where the height is given by the number of base pairs enclosing the base at position i.e. loops correspond to plateaus (hairpin loops are peaks), helices to slopes (Fig. 6).

Table 1 Soil samples collected from different regions of India

No.	Place collected	Number of samples	Soil type
1	Thevera, Kochi, Kerala, India	3 soil samples	Garden soil
2	VIT University, Vellore, Tamil Nadu, India	3 soil samples	Rhizosphere
3	Chennai, Tamil Nadu, India	1 soil sample	Garden soil

Table 2 Colony morphology of the bacterial isolates

Organism name	Morphology	Sample
RH01 (Kochi, Kerala)	Elevated red colonies	
RH02 (Kochi, Kerala)	Elevated red colonies	
RH03 (Kochi, Kerala)	Elevated red colonies	
RH04 (Vellore, Tamilnadu)	Elevated red colonies	
RH05 (Vellore, Tamilnadu)	Irregular, pale red colonies	
RH06 (Vellore, Tamilnadu)	Elevated red colonies	
RH07 (Chennai, Tamilnadu)	Elevated red colonies	

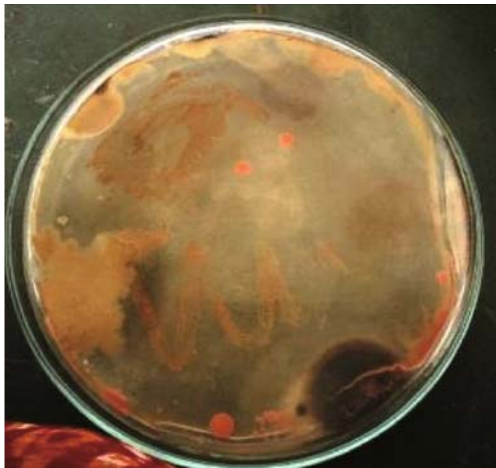


Figure 1 Red color colonies on nutrient agar.

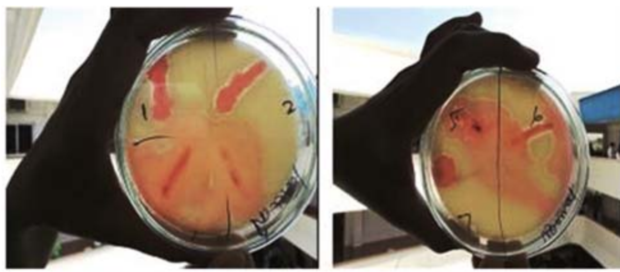


Figure 2 Isolates showing zone of clearance on skim milk agar.

Table 3 Screening of *Serratia* sp. for Serratiopeptidase enzyme

Strain No.	Primary screening	Secondary screening
	Zone of clearance(mm)	Zone of clearance (mm)
RH01	21	21
RH02	21	21
RH03	23	24
RH04	20	20
RH05	22	22
RH06	22	22
RH07	20	19

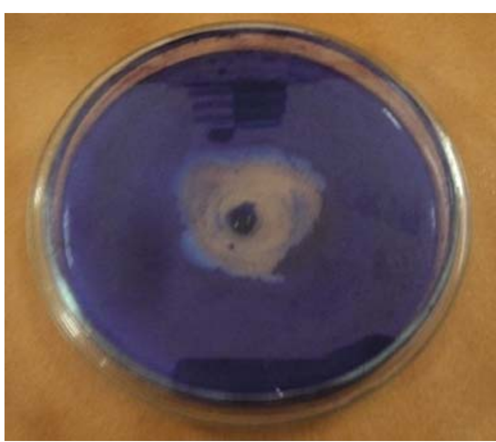


Figure 3 Caseinolytic activity of crude enzyme (RH03).

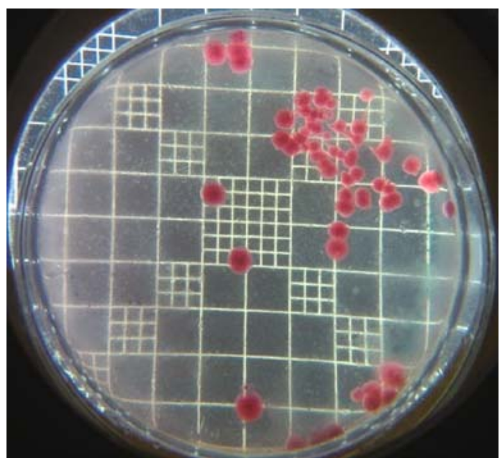


Figure 4 Pure culture of *Serratia* sp. (RH03) on nutrient agar.

Total protein estimation and enzyme assay

Results of lysate, precipitated and dialysed sample showed differences in OD readings as it was due to its variable presence of total protein content. In crude the enzyme activity was determined with the protein content as 2.8 mg/mL, the precipitated protein content as 2.3 mg/mL and the dialyzed with 1.7 mg/mL. Serratiopeptidase activity was determined colorimetrically by detecting the amount of protease released. The specific enzyme activity of the crude supernatant was found to be 12.00 U/mg whereas the specific enzyme activity increased after partial purification and determined as 21.33 and 25.40 units/mL for precipitated and dialysed samples. Casein assay results showed the precipitated and the dialysed sample has better enzyme activity and more specific enzyme activity (Table 5).

SDS-PAGE

The molecular weight of the partially purified peptidase was found to be 50 kDa which was comparable by the banding patterns along with the protein marker and this further confirms the peptidase produced by *Serratia marcescens*-VITSD2 (Fig. 7).

HPLC analysis

HPLC chromatogram for partially purified enzyme produced from *Serratia marcescens* VITSD2 is shown in Fig. 8. Peak 1 indicates the presence of serratiopeptidase in the extract. Retention time of the peak was found to be 3.45 min.

Antibacterial activity of serratiopeptidase

The serratiopeptidase produced by *Serratia marcescens* VITSD2 were found to exhibit antibacterial activity against *Pseudomonas aeruginosa* (12 mm) and *E. coli* (15 mm). The maximum activity was found when dialysed sample was used against the test pathogens. The diameters of zone of inhibition are shown in (Table 6, Fig. 9).

Table 4 Morphological and biochemical characterization of the strain RH03

Gram staining	Motility	Margin	Capsule	Indole	MR	VP	Citrate	Lysine	Urease	Nitrate reduction	H_2S	Glucose	Adonitol	Lactose
-ve rods	+	Smooth and raised	+	-	-	+	+	+	-	+	-	+	+	-

Figure 5 Phylogenetic tree of the strain *Serratia marcescens*VITSD2

Discussion

Serratiopeptidase is known very well for its anti inflammatory properties, anti bacterial properties and anti cancer properties. It is used in the clinical treatment of pain and inflammatory disorders due to arthritis, trauma, surgery, sinusitis, bronchitis. Screening of *Serratia* sp. producing serratiopeptidase was done by casein hydrolysis method. The maximum zone of clearance was noted by isolate RH03 (23 mm). A similar study was completed by Jo et al. (2008) in which protease producing *Serratia marcescens* strains were isolated from soil samples and it was screened by growing the isolates on LB agar plates containing 1%(w/v) skim milk at 30°C for 3 days and the isolates that showed a zone of clearance were grown on 1%(w/v) skim milk agar plates. *Serratia marcescens* FS-3 isolate showed maximum zone of clearance and was found to have specific activity of 60.7 U/mg. The medium used in this study for the production of serratiopeptidase by RH03 was trypticase soya broth. The data clearly showed that RH03 (VITSD2) produces serratiopeptidase in trypticase soy broth. Subbaiya et al. (2011) used casein or cysteine as a substrate for the production of serratiopeptidase. The specific activity

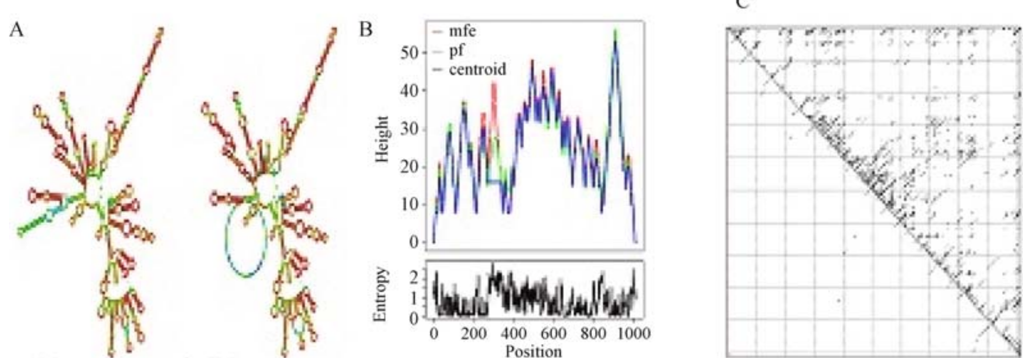


Figure 6 (A) RNA secondary structure, (B) Mountain plot representation, (C) Base pair probabilities of Dot plot

Table 5 Determination of SP activity and yield of enzyme from isolate *Serratia marcescens* VITSD2

Fraction	Volume	Protein content (mg/mL)	Total protein (mg/mL)	Tyrosine (μg/mL)	Activity units	Total activity (units/mL)	Specific activity(units/mg)	Yield(%)	Purification fold
Lysate	100	2.8	280	80.6	33.6	3359.4	12.00	100.00	1
50%	2	2.3	4.6	117.8	49.1	98.1	21.33	2.92	1.8
Dialysed	2	1.7	3.4	103.6	43.2	86.4	25.40	2.57	2.1

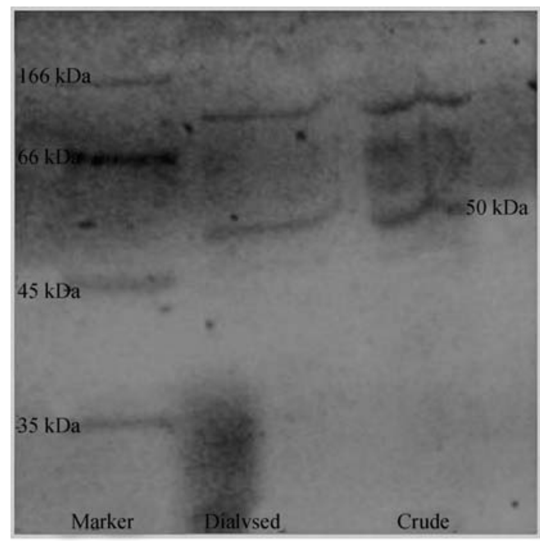


Figure 7 SDS PAGE

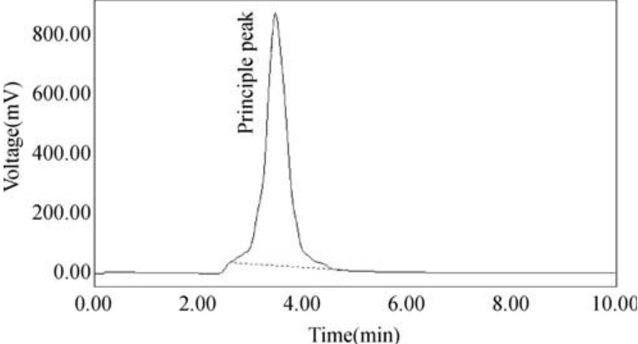


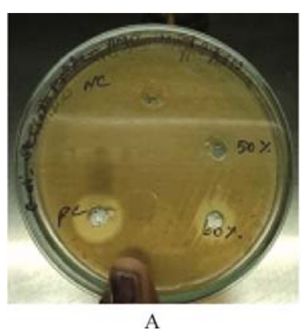
Figure 8 HPLC chromatogram of the partially purified serratiopeptidase enzyme

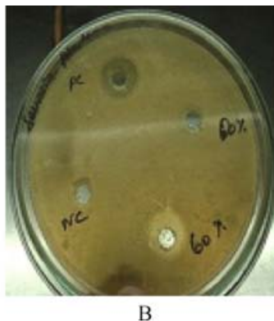
of the crude extract, 60% ammonium sulfate precipitated and dialysed enzyme was 12.00 U/mL, 21.33 U/mL, and 25.7 U/mL. A similar experiment conducted by Subbayia et al. (2011) produced serratiopeptidase from *Serratia marcescens* showed a specific activity of 0.65 U/mL in filtered solution. The specific activity of RH03 (VITSD2) was found to be more compared to the serratiopeptidase produced by *Serratia marcescens* in Subbayia et al. (2011). However, serratiopeptidase produced by *Serratia marcescens* FS-3 isolate showed more specific activity (60.7U/mg) compared to the serratiopeptidase produced by RH03(VITSD2) (25.7U/mL).

By SDS-PAGE, the molecular weights of the serratiopeptidase was found to be 50 kDa. Machielsen et al. (2006) demonstrated that the molecular weights of serratiopeptidase was 52 kDa by using SDS-PAGE. The molecular weights were comparable and this further confirms that the exoprotease produced by RH03 (VITSD2) was serratiopeptidase. Hence the present study justifies the potent producer of serratiopeptidase from *Serratia marcences* VITSD2 isolated from soil source. Further studies into utilization of peptide sequencing may reveal the new finding and help to identify and cleave a distinct peptide bond within the specific protein.

Table 6 Antibacterial activity of partially purified enzyme

Organisms	Zone of inhibition (mm)	
	(Reference drug) Chloramphenicol 25μg/mL	Partially purified enzyme(60%)
Escherichia coli MTCC No. 1588	18mm	15mm
Pseudomonas aeruginosa MTCC No. 4676	12mm	12mm





A B

Figure 9 Zone of inhibition of serratiopeptidase against *E. coli* and *Pseudomonas aeuroginosa.* (A) *Escherichia coli* MTCC No. 1588 (15mm); (B) *Pseudomonas aeruginosa* MTCC No. 4676 (12mm).

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Compliance with ethits guidelines

Subathra, Renuka, Harini, Jemimah and Mohanasrinivasan declare that they have no conflict of interest.

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

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