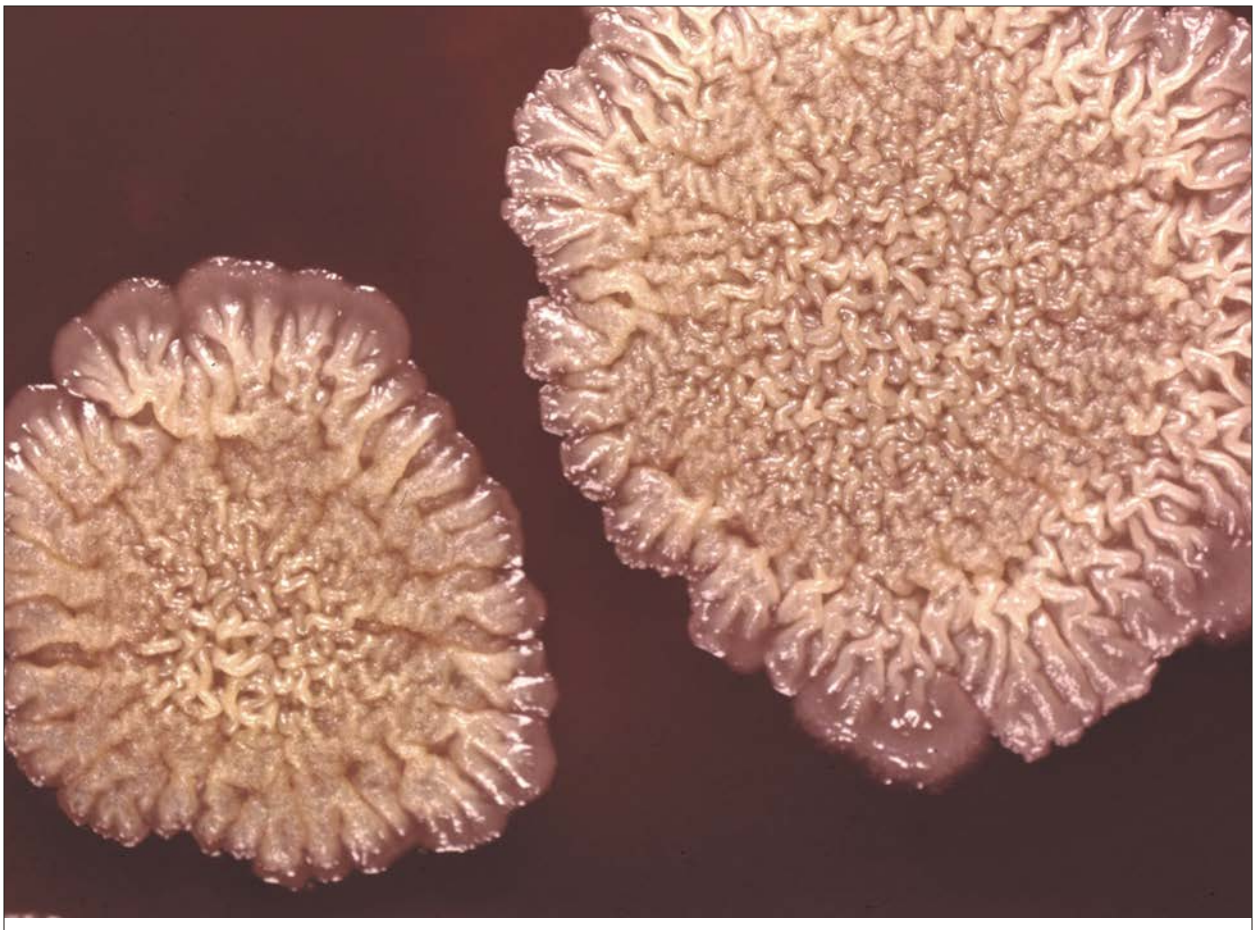

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Molecular Characterization of Marine *Streptomyces enissocaesilis* Capable of L-asparaginase Production

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ABSTRACT

Actinomycete strain producing L-Asparaginase was isolated from a marine sediment sample from Bay of Bengal collected near Cudallore of Tamil Nadu state, India. Morphological and physiological characteristics as well as chemotaxonomic features of strain AUBT-1404 were congruent with the description of the genus *Streptomyces*. The isolate, AUBT-1404, was identified by the polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strain AUBT-1404 belonged to the genus *Streptomyces*, with the highest similarity to *Streptomyces enissocaesilis* NRRL B-16365^T (99.86%). The isolate had cell-wall type I (LL-diaminopimelic acid and no diagnostic whole-cell sugars) and the diagnostic phospholipids were phosphatidylethanolamine and phosphatidylcholine. The predominant menaquinone was MK-9 (H4). The G+C content of the genomic DNA was 76.2 mol%. On the basis of polyphasic evidence, strain AUBT-1404 was recognized as a new strain of *Streptomyces enissocaesilis*.

Key words: *Streptomyces enissocaesilis* sp., marine sediment, polyphasic taxonomy, L-asparaginase

INTRODUCTION

Current research suggests there is a tremendous diversity and novelty among the marine actinomycetes present in marine environments which are emerging as a promising new source of novel antibiotic and anticancer agents with unusual structured and properties (Jensen *et al*., 2005). Among actinobacteria, *Streptomyces* are recognized as a rich biotechnological resource, as the members of this genus are still the most promising sources of industrially significant compounds that find applications as anti-infectives, anti-cancer agents or other pharmaceutically useful compounds (Miao and Davies, 2010). These filamentous bacteria are well adapted to the marine environment and are able to break down complex biological polymers. Of late, the improved classification based on a polyphasic approach has provided an invaluable tool for the recognition of additional species from diverse substrates and has led to a rapid increase in the number of described species. As such, it is evident that *Streptomyces* species should be identified using judicious combinations of genotypic and phenotypic data.

In recent years, the potential of L-Asparaginase as an anti carcinogenic agent has been widely acknowledged. Its antineoplastic activity is associated with the property of depleting the circulating pool of L-asparagine by the asparaginase catalytic activity (Jayaramu *et al.*, 2010). Some of the commercially available L-asparaginase are A-ase, ASN-ase, Colaspase, Crasnitin, Elspar, Crisantas, Pasum, PEG-asparaginase and Pegasparagasum. These drugs are used in the formulation along with other chemotherapeutic agents such as Methotrexate. The production of L-asparaginase from marine *Streptomyces* may form the basis of novel therapeutic drugs which may be less toxic to humans, as the saline conditions of the human blood are closer to the marine environment.

The objective of the present study was to establish the taxonomic position of a marine sediment isolate strain AUBT-1404 which was found to exhibit L-asparaginase activity and antimicrobial activity. The organism was subjected to polyphasic taxonomic study.

MATERIALS AND METHODS

Isolation and cultivation of strain AUBT-1404: The actinomycete strain AUBT-1404 was isolated from a marine sediment sample collected from Cudallore (Latitude 12°11.718N and Longitude 80°15.246E), a coastal region along the south east coast of India, at a depth of 53.26 m. This strain was isolated from the sample by the standard serial dilution method (Jensen *et al.*, 1991) using glycerol asparagine agar plates (Shirling and Gottlieb, 1966) which were incubated at 28±2°C for 7-45 days. The colonies of this strain were purified by subculturing on ISP 5 medium (Shirling and Gottlieb, 1966) and maintained on the same medium as a working culture. Strain AUBT-1404 was deposited in Microbial Type Culture Collection and GeneBank, India (MTCC) as strain NRRL B-16365(T).

Screening for L-asparaginase production: The isolate was subjected to plate and broth preliminary screening assays for the extracellular L-asparaginase production. The isolate was streaked on Asparagine Dextrose Agar (ADA) medium (Saxena and Sinha, 1981) and Asparagine Dextrose Salts (ADS) broth incorporated with 0.009% w/v phenol red indicator. On incubation, phenol red appears yellow at acidic pH and turns pink at alkaline pH (Gulati *et al.*, 1997). The color change of the medium from yellow to pink due to release of ammonia is an indication of the extracellular L-asparaginase production by the isolate.

Production of L-asparaginase was carried out by adopting shake flask fermentation method using asparagine dextrose salts broth. L-asparaginase activity was measured by using Nesslerization which is based on the determination of ammonia liberated from L-asparagine by enzyme (Wriston, 1971). L-asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The liberated ammonia was quantified on reaction with Nesslers reagent and measurement of OD at 450 nm with the help of UV-Visible spectrophotometer. The enzyme activity was expressed in International Unit (IU); one IU being the amount of enzyme which liberates 1 μM of ammonia per mL per min (μM mL^{-1} min^{-1}).

Morphological and biochemical identification

Morphology: Strain AUBT-1404 was grown on ISP medium No. 5 at 28°C for 14 days, then observed by light microscope equipped with an 40X objective (OIC; Olympus). Motility was determined by observing drops of an H_2O suspension of aerial mass from 2-week-old cultures incubated at 28°C, under light microscopy at 30 min intervals for 2 h (Busti *et al.*, 2006). Spore arrangement and spore surface ornamentation of 3-week-old cultures on ISP medium 5 were

examined by using a scanning electron microscope (model JSM-6610 LV; JEOL, Ltd., USA). For electron microscopy, agar block with growth was fixed with 1% osmium tetraoxide in 0.1 M phosphate buffer (pH 7.2), dehydrated through a graded series of ethanol (Lee, 2006) and dried with a critical point dryer (model EMITECH K850). It was then placed onto a stub bearing adhesive and spatter-coated with platinum under vacuum.

Cultural and physiological characteristics: The International *Streptomyces* Project media (Shirling and Gottlieb, 1966) were used to investigate the cultural features after 14 days incubation at 28°C. The degree of growth, the colour of mycelium and the presence of diffusible pigments of the organisms were recorded on all tested media. Color determination was performed using color chips from the ISCC-NBS color charts (standard samples, No. 2106) (Kelly, 1964). A range of physiological and biochemical characteristics were examined according to the standard protocols of Goodfellow (1971) and Williams *et al.* (1983). In addition, acid production from various carbohydrates was determined after 3 days incubation at 30°C, using bromocresol purple as an acid/base indicator (Gordon *et al.*, 1974). Tests for the utilization of various substrates as sole carbon (Shirling and Gottlieb, 1966) and sole nitrogen (Williams *et al.*, 1983) sources were carried out and the utilization results were observed over a period of 1 week. Growth was tested over a range of temperature (4-55°C), pH values (4.0-10.0) and NaCl concentrations (0-10%, w/v) as described by Xu *et al.* (2005).

Chemotaxonomy: Freeze-dried biomass for chemotaxonomic studies was obtained from one week culture in glycerol asparagine agar plates. Standard analytical procedures were used to extract and analyze the isomeric forms of diaminopimelic acid (Hasegawa *et al.*, 1983), whole-organism sugars (Staneck and Roberts, 1974), isoprenoidquinones (Minnikin *et al.*, 1984), polar lipids (Minnikin *et al.*, 1984) and fatty acids (Sutcliffe, 2000).

Molecular analysis: Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were performed as described by Li *et al.* (2007). The sequence analysis of the almost-complete 16S rRNA gene sequence (1449 bp) of strain AUBT-1404 was conducted using BLAST (Altschul *et al.*, 1997). Multiple alignments with sequences of the most closely related actinobacteria were carried out using CLUSTAL_X (Thompson *et al.*, 1997). The phylogenetic tree was inferred for the test strain and its nearest neighbors using the maximum-likelihood (Felsenstein, 1981) tree-making algorithm from the PHYLIP software package. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. Phylogenetic tree display, editing and printing were carried out using the Dendroscope software (Huson *et al.*, 2007).

DNA extraction was prepared according to the CTAB method described by (Ausubel *et al.*, 1992). The G+C content of the genomic DNA was determined spectrophotometrically using the thermal denaturation method (Marmur and Doty, 1962).

Nucleotide sequence accession number the 16S rRNA gene sequence of strain AUBT-1404 determined in this study has been deposited in GenBank under the accession number KC 698126.

Antimicrobial activity: The antimicrobial assay was conducted against *S. aureus* (MTCC 3160), *B. subtilis* (MTCC 441), *B. cereus* (MTCC 430), *P. aeruginosa* (MTCC 424), *E. coli* (MTCC 443) and *P. vulgaris* (MTCC 426) using the plate diffusion method. The plates were incubated at 28°C for 24 h and analyzed for zone formation.

Comparison of L-asparaginase production by *Streptomyces enissocaesilis* with reference bacterial strain *E. coli* MTCC 443: The L-asparaginase producing reference strain *E. coli* MTCC443 was obtained from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The *S. enissocaesilis* and *E. coli* 443 were cultured in Asparagine Dextrose Salts (ADS) broth, pH 7.0 and incubated at 28°C with shaking at 120 rpm for 4 days. At every 24 h intervals, the cultures were harvested and the L-asparaginase activity in the cell free supernatants were determined by quantitative enzyme assay as described above.

RESULTS AND DISCUSSION

Strain AUBT-1404 exhibited macroscopic and microscopic characteristics typical of most species of the genus *Streptomyces*. The strain was gram-positive, aerobic and it formed raised, wrinkled, opaque circular colonies that had creamish-white substrate mycelium and grey aerial mycelium on glycerol asparagine agar, with no diffusible pigment (Fig. 1a). Morphological observation of the 7-14 days old culture of strain AUBT-1404 revealed that the substrate mycelia were well developed and aerial mycelia produced compact spiral spore chains. Each spore chain consisted of 15-20 turns with greyish white and tuberculate spores showing short rod morphology (Fig. 1b). Spores were 1.08-1.55 µm long with a mean diameter of 0.905 µm and did not show motility. The isolate grew well on ISP3 and 5 and Nutrient agar media. It exhibited moderate growth on ISP 6 and 7. Poor growth was observed on ISP2 and 4 and Czapek's agar. Grey aerial mycelium and creamy-white substrate mycelium were produced on most of the tested media. Brown diffusible pigment was produced on some of the test media (Table 1).

Growth of strain AUBT-1404 occurred in the pH range 6-12 and 0.5-6% NaCl (w/v), with optimum growth at pH 8-9 and 4-5% NaCl (w/v). The temperature range for growth was 15-42°C, with the optimum temperature being 28°C. The isolate was catalase and oxidase positive and gave a negative indole reaction. Detailed results of the physiological characterization of the isolate are given in the Table 2.

Chemotaxonomic tests showed that the cell wall contained LL-diaminopimelic acid and traces of glycine, indicating the presence of cell-wall chemotype I (Lechevalier and Lechevalier, 1970a).

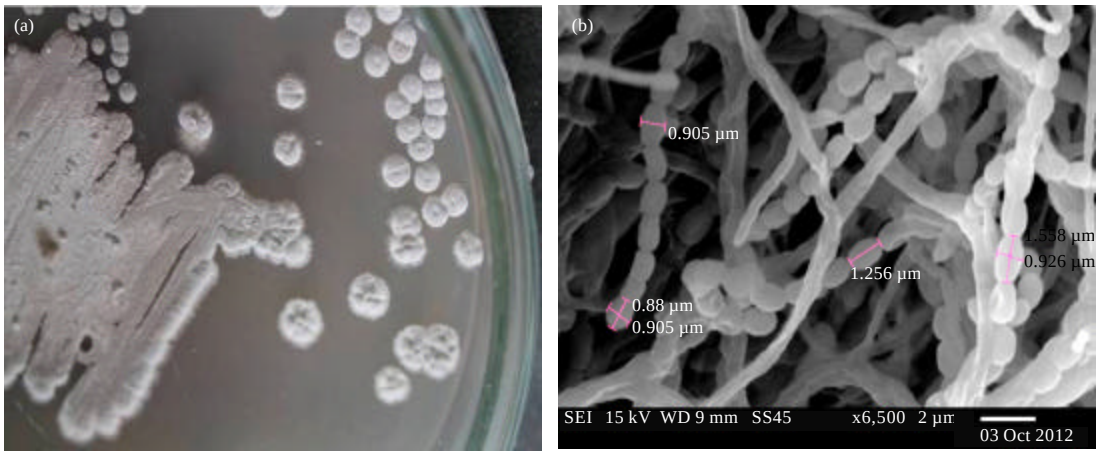

Fig. 1(a-b): (a) Micrograph of strain *Streptomyces enissocaesilis* on glycerol asparagine agar and (b) Scanning electron micrograph indicates the spore chain morphology of *Streptomyces enissocaesilis* grown on ISP medium 5 for 2 weeks at 28°C. Bar 2 µm

Table 1: Cultural characteristics of strain *Streptomyces enissocaesilis* on various ISP media

Medium	growth	sporulation	diffusible pigment	colony colour	
				Aerial mycelium	substrate mycelium
Yeast extract agar ISP2	poor	poor	--	--	white$_{(263)}$
Oatmeal agar ISP3	good	good	brown$_{(59)}$	grey$_{(265)}$	white$_{(263)}$
Inorganic salts-starch agar ISP-4	poor	moderate	--	white$_{(263)}$	pale yellow$_{(89)}$
Glycerol-asparagine agar ISP-5	good	good	--	grey$_{(265)}$	creamy white$_{(101)}$
Peptone-yeast extract iron agar ISP-6	moderate	moderate	reddish brown$_{(41)}$	light grey$_{(189)}$	white$_{(263)}$
Tyrosine agar ISP-7	moderate	moderate	brown$_{(59)}$	dark grey$_{(266)}$	white$_{(263)}$
Czapek's agar	poor	poor	--	--	creamy white$_{(101)}$
Nutrient agar	good	good	brown$_{(59)}$	grey$_{(265)}$	creamy white$_{(101)}$

Table 2: Morphological, physiological and biochemical properties of *Streptomyces enissocaesilis*

Reaction	Result
Spore chain morphology	Spiral
Colony colour on ISP 5	Creamy white-grey
Spores per chain	15-20
Spore surface	Tuberculate
Production of diffusible pigment	-
Production of melanoid pigment	
ISP-1	-
ISP-6	-
ISP-7	-
Growth temperature range	15-42°C
pH	6-12
NaCl tolerance	0.5-6%
Starch hydrolysis	+
Casein hydrolysis	+
Citrate utilization	+
Gelatine liquefaction	+
H_2S production	-
Methyl red	-
Voges-proskauer	+
Nitrate reduction	+
Indole	-
Catalase	+
Oxidase	+
Urease	+
Utilisation of carbon sources	
Glucose	+
Arabinose	+
Sucrose	+
Xylose	-
Inositol	+
Mannitol	+
Fructose	+
Rhamnose	-
Raffinose	+

Table 2: Continue

Reaction	Result
Acid production from	
Adonitol	-
Sorbitol	-
Dextrose	+
Fructose	+
Inositol	-
Lactose	+
Maltose	+
Mannitol	+
Raffinose	-
Rhamnose	-
G+C content mol%	76.2%

+: Utilization, -: Non-utilization

Sugars were not detected in whole-cell hydrolysates. The predominant menaquinones were MK-9 (H4)-71.5%, MK-9 (H8)-17.3% and MK-9 (H6)-11.2% and the phospholipids were phosphatidylethanolamine and phosphatidylcholine. Fatty acid analysis showed that strain AUBT-1404 contained straight-chain and iso and anteiso-branched components. The major components of the fatty acid profile included iso-branched hexapentanoic acid (iso-C15: 0)-20.88%, anteiso-branched hexapentanoic acid (anteiso-C15: 0) - 17.65% and iso-branched hexadecanoic acid (iso-C16: 0)-13.75%. The detailed fatty acid profile of strain AUBT-1404 is given in Table 3. Chemotaxonomic methods have long been used to distinguish streptomycetes from other actinomycetes (Lechevalier and Lechevalier, 1970b). The salient features of the genus *Streptomyces* having straight chain, iso- and anteiso-branched chain fatty acids with a carbon chain-length of 14-18 atoms (Anderson and Wellington, 2001) and the presence of LL isomer of 2, 6-Diaminopimelic acid (LL-DAP) and absence of any diagnostic sugar in the cell wall is a salient feature of the genus *Streptomyces* (Xu *et al*., 2009). Thus, chemotaxonomic and phenotypic data showed that strain AUBT-1404 should be to the genus *Streptomyces*.

The G+C content of the DNA of the strain AUBT-1404 was determined as 76.2%. A BLAST search of the GenBank database using the 1471 bp 16S rRNA gene sequence of strain AUBT-1404 showed its similarity to that of many species of the genus *Streptomyces*. The 16S rRNA gene sequence of strain AUBT-1404 showed levels of homology of 99.864% (2 nucleotide differences out of 1,470) to *Streptomyces enissocaesilis* NRRL B-16365^T (GenBank Accession No. DQ026641), 99.863% (2 nucleotide differences out of 1,463) to *S. plicatus* NBRC 13071^T (GenBank Accession No. AB184291), 99.863% (2 nucleotide differences out of 1,461) to *Streptomyces rochei* NBRC 12908^T (GenBank Accession No. AB184237) and 99.795% (3 nucleotide differences out of 1,463) to *Streptomyces geysiriensis* NBRC 15413^T (GenBank Accession No. AB184661). The phylogenetic tree was constructed based on 16S rRNA gene sequences to illustrate the evolutionary relationship between strain AUBT-1404 and most closely related type strains of species of the genus *Streptomyces*. The phylogenetic tree indicated that this strain formed a distinct branch with *Streptomyces rochei* NBRC 12908^T, within the *Streptomyces enissocaesilis* NRRL B-16365^T (the top BLAST result) clade (Fig. 2). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain AUBT-1404 as a new strain of *Streptomyces enissocaesilis*.

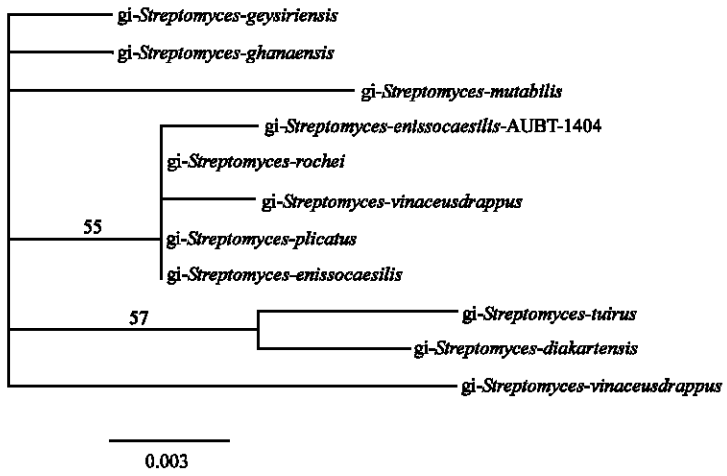

Fig. 2: Phylogenetic maximum-likelihood tree based on 16S rRNA gene sequences showing the relationship between *Streptomyces enissocaesilis* and related members of the genus *Streptomyces*. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at nodes. Bar 0.03 substitutions per nucleotide positions

Table 3: Cellular fatty acid content of *Streptomyces enissocaesilis*

Fatty acid	Total (%)
15:0 iso-pentadecanoic acid	15.91
15:0 anteiso-pentadecanoic acid	29.68
16:0 iso-hexadecanoic acid	12.50
Sum in feature 3	3.37
16:0 hexadecanoic acid	8.19
17:0 iso-heptadecanoic acid	5.26
17:0 anteiso-heptadecanoic acid	14.04
18:1 2OH-octadecanoic acid	11.07

Summed feature 3 comprises of 16:1 7°C/15ISO 2OH

Based on the phenotypic, phylogenetic and chemotaxonomic analysis, it is evident that the strain AUBT-1404 is different from previously described *Streptomyces enissocaesilis*. Therefore, strain AUBT-1404 represents a new type strain of *Streptomyces enissocaesilis*.

L-asparaginase production: The Asparagine Dextrose Salts (ADS) broth exhibited pink coloration (Fig. 3) after 48 h of incubation period. This indicates the hydrolysis of L-asparagine into aspartic acid and ammonia were released by L-asparaginase synthesized by the isolate. Upon submerged fermentation, the strain exhibited maximal enzyme production at 48 h. The strain exhibited crude L-asparaginase activity of 2.27 μm mL^{-1}. Results indicated that the L-asparaginase production approximately parallels growth of the cells and is accompanied by an increase in the pH of the medium (Savitri and Azmi, 2003).

Antimicrobial activity: The antimicrobial activity of *Streptomyces enissocaesilis* against 6 bacterial species was tested using the plate diffusion method. The results depicted in Table 4 reveal that the isolate was more active against gram positive bacteria, *Bacillus subtilis* (Fig. 4) and

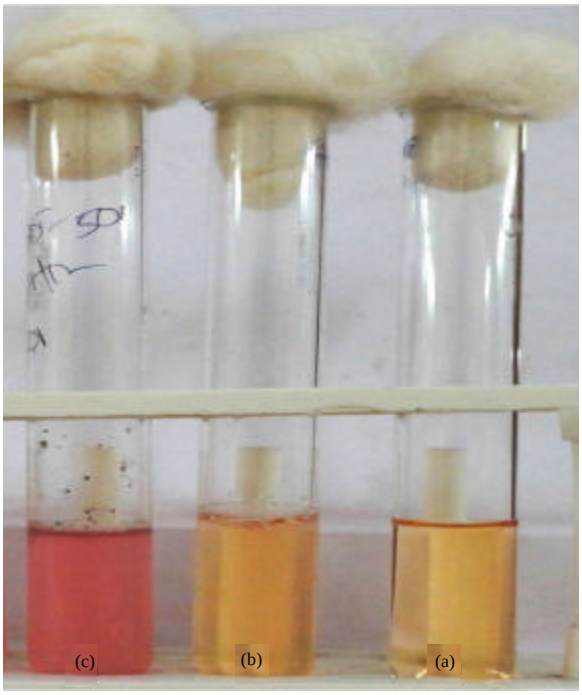

Fig. 3: Test tubes showing L-asparaginase activity using the Asparagine Dextrose Salts (ADS) broth (a) Control broth, (b) Negative reaction showing no colour change and (c) Positive reaction of *Streptomyces enissocaesilis* showing colour change from yellow to pink

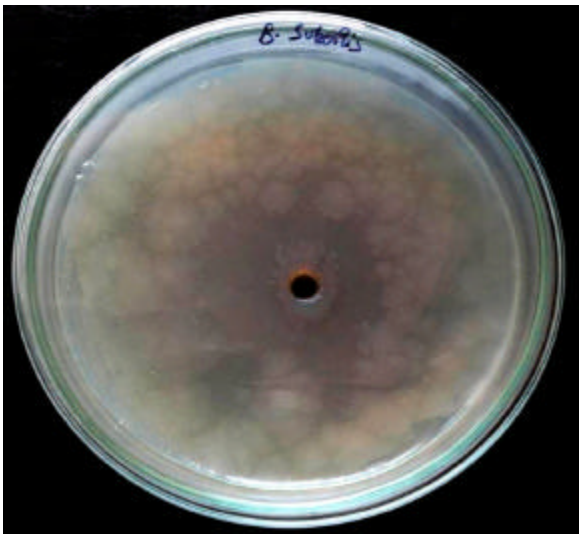

Fig. 4: Antimicrobial activity of *Streptomyces enissocaesilis* against *Bacillus subtilis*

Staphylococcus aureus than gram negative bacteria. The reason for different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane

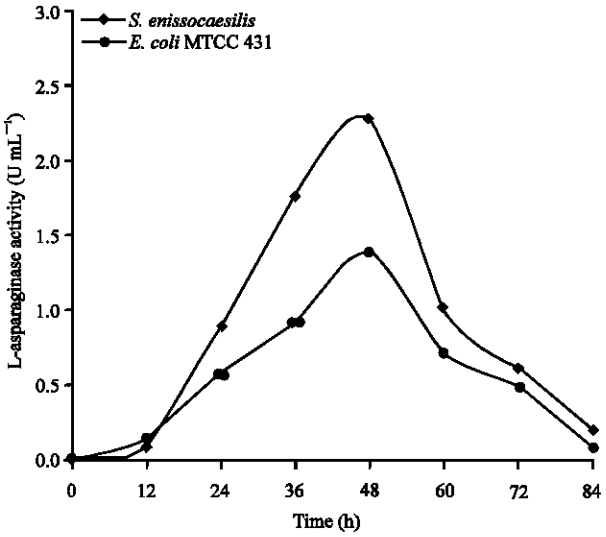

Fig. 5: Comparison of L-asparaginase production by *Streptomyces enissocaesilis* with reference bacterial strain *E. coli* MTCC 443

Table 4: Antimicrobial activity shown by *Streptomyces enissocaesilis*

Test organism	Antimicrobial activity of the isolate (AUBT-1404)
Bacillus subtilis (MTCC 441),	++
Staphylococcus aureus (MTCC 3160)	++
Bacillus cereus (MTCC 430)	+
Pseudomonas aeruginosa (MTCC 424)	+
Escherichia coli (MTCC 443)	±
Proteus vulgaris (MTCC 426)	--

--: No clear zone, +: Clear zone with diameter 1-10 mm, ++: Clear zone with diameter 11-20 mm, ±: Foggy zone

carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, The gram positive should more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt, 1971).

Comparison of L-asparaginase production by *Streptomyces enissocaesilis* with reference bacterial strain *E. coli* MTCC 443: Results illustrated in Fig. 5 showed that the *S. enissocaesilis* new strain exhibited higher L-Asparaginase activity of 2.27 μm mL^{-1} at 48 h compared to reference bacterial strain, *E. coli* MTCC 443 which showed an activity of 1.38 μm mL^{-1} at 48 h. These results established the increased potential of the new strain to produce L-asparargina se compared to the reference bacterial strain.

CONCLUSION

This is the first report of marine *Streptomyces enissocaesilis* producing L-asparaginase. The present study revealed the high potential of this strain for the production of L-asparaginase enzyme. Futher studies on the enzyme relating to purification and characterisation would reveal the enormous scope of the enzyme's applications in the healthcare industry.

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