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Screening the Antibacterial and Extended Spectrum of Betalactamases Inhibiting Activity of Seaweed Endophytic Bacillus Subtilis St-2

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A B S T R A C T

Seaweed associated organisms like bacteria and fungi colonize on the surface or inside of host. Endosymbiotic microorganisms are producing many bioactive compounds to protect the host and kill or inhibit the growth of pathogens. In this study, we have isolated totally 42 bacterial isolates from three *Sargassum* sp using Zobell marine agar. 10 isolates showed antibacterial activity against *Staphylococcus aureus* (ATCC 25922) *and E-coli* (ATCC 25923). ST-2, ST-4, ST-4o and S2-4, SW-11 filtrates inhibit cephalosporin resistant isolates also. ST-2, ST-4, ST-4o only produced zone against Esbl producing isolates like Ceftazidime/Clavulanic acid. Molecular identification of endophytic Bacillus subtilis ST-2 of *Sargassum* sp through partial 16S rRNA sequence and submitted the sequence to Genbank. Natural bioactive compounds of symbiotic marine microorganisms are considerable source to treat the Multidrug resistant organisms.

***Key words*:** Sargassum, Endophytes, Bacillus subtilis ST-2, Antibacterial, Esbl inhibiting activity

B-Lactam antibiotics are the widely prescribed antibacterial drugs against most common infections in healthcare settings like penicillin, Cephalosporins, Carbapenems and Monobactams [1]. Absolute resistance of this group antibiotics by violent, inappropriate use and used as growth promoters, animal care in developing countries [2]. Clinically significant gram-negative isolates hydrolysis betalactam antibiotics by producing betalactamases enzymes like Esbl, AmpC and Carbapenemases. Third generation cephalosporins (Ceftazidime, Cefotaxime and Ceftriaxone) introduced to treat the betalactamases producers especially in clinical settings. Esbl producing isolates hydrolysis the third generation cephalosporins and Monobactams. Infections due to this type resistant isolates minimize the drug choice; extend the hospital stay and mortality [3-8].

Marine environment is most important resource for the discovery of chemical compounds with essential applications worldwide. Seaweed is one of the macro algae have economical and industrially important products with antimicrobial activity in marine. Seaweed associated bacteria build a symbiotic mutualism with seaweeds to acquire the nutrients and shelter for the growth [9]. Symbiotic microorganisms protect the host from other infectious organisms by producing many compounds [10]. These bioactive compounds contain antibacterial, antifungal, antiviral, anti-inflammatory and anticancer activity especially *Bacillus* sp., produce novel antibiotics Polymixin-B, bacitracin and gramicidin. Seaweed associated bacterial and fungal compounds valued as efficient natural bioactive agents against Multidrug resistant and betalactamases producing gram negative organisms [11-14]. This study designed to isolation of endophytic bacteria bacillus subtilis ST-2 from *Sargassum* sp and screening the antibacterial, Esbl inhibiting ability and molecular identification of endophytic bacteria.

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MATERIALS AND METHODS

Isolation of endophytic bacteria

Three brown seaweed samples were collected from coast of Thondi, Rameshwaram, Tamil Nadu in sterile plastic bags, stored in a cooler box with ice packs and transported aseptically. Algal sample washed thoroughly with sterile water to remove debris then rinse with 70% ethanol and 4% Sodium hypochlorite subsequently. Allowed samples to dry then 1gm of sample homogenized and serially diluted with sterile sea water. 100µl of samples were inoculated by spread plate on Zobell marine agar and incubated at room temperature for 7 days. Obtained bacterial colonies were purified based on morphological characteristics for additional screenings [15].

Screening the antibacterial activity

Spot plate assay

Primarily the antibacterial activity of endophytes was screened by spot plate assay against control strains

Staphylococcus aureus (ATCC 25922) *and E. coli* (ATCC 25923). In detail the control strains swabbed on Mueller Hinton agar after few minutes spotted the endophytes on same agar plate. The plates were incubated for 24-48 hours at room temperature [16].

Cross streak assay

The endophytes were seeded by a single streak in the corner of the Mueller Hinton agar plate and incubate the plates for 24 hours. *Staphylococcus aureus* (ATCC 25922), *E. coli* (ATCC 25923) and Cephalosporin resistant isolates were streaked one by one perpendicular to the endophytes.

Agar well diffusion

S2-4, SW-11, ST-2, ST-4 and ST-4o isolates were seeded in 100ml of Zobel marine broth for 72 hours at 37°C in shaking incubator. Culture broth centrifuged at 7,000 rpm for 30mts then 25, 50, 75 and 100µL supernatant of each strain were added into well (5 mm in diameter) after swabbing control and cephalosporin resistant isolates to evaluate the minimum inhibitory concentration (MIC) of supernatant. Allow plates 30mts to diffuse the broth into medium and incubate for 24 hours [17].

Phenotypic detection of Esbl producers

ESBL producers were confirmed by the double-disk synergy test using third generation cephalosporins and amoxicillin-clavulanic acid, combined disc synergy test using Cefotaxime and Ceftazidime with and without clavulanic acid placed gently on Mueller Hinton agar plate previously inoculated with 8 hours fresh cephalosporins resistant isolates. *Escherichia coli ATCC 25922* negative control strains used in this study [18].

E-Test

Commercially available Improved Esbl detection Ezy MIC™ strip (MIX+/MIX) himedia were used in this study. The mixture of Ceftazidime, Cefotaxime with Clavulanic acid coated in the end of MIX+, then mixture of Ceftazidime, Cefotaxime without Clavulanic acid coated in another end of MIX. Cephalosporins resistant isolates inoculated on Mueller Hinton agar plate then placed the E-strip gently [18].

ESBL inhibition activity of culture filtrate

Add 20µl filtrate with commercially available Ceftazidime antibiotic disc and allow disc to dry. Esbl producing isolate swabbed on Mueller hinton agar palte then place the Ceftazidime, Ceftazidime with culture filtrate disc. Ceftazidime/clavulanic acid disc used to confirm the Esbl production.

Identification of potential strain

DNA extraction and PCR amplification

DNA extracted from pure colonies of ST-2 QIAmp DNA Minikit (Qiagen) according to the manufacturer's instructions: 1µl of DNA extract amplified with 1µL of 10µM primers, 3.5µLof GoTaq Green Master mix (Promega) and 3.5 µL of nuclease free water. Then 5 min at 94°C to denature the DNA, followed by 30 cycles of denaturation at 94°Cfor 30 sec, primer annealing at 55°C for 10 second strand extension at 72°Cfor 30 sec on a Eppendroff thermal cycler. PCR products were separated on a 2% agarose gel and DNA bands were visualized with ethidium bromide.

PCR sequencing and sequence analysis

PCR product 1µL purified DNA templates were sequenced in the forward and reverse direction in separate reactions. Each reaction was heated to 96°C for 1 min, followed by 35 cycles at 96°C for 10 sec, 55°C for 5 sec and 60°C for 1 min. Sequenced product homogenized with 0.5µL of 0.5M EDTA and washed using 21µL of ethanol precipitation mix, centrifuged at 4000 rpm for 15 min. Incubate the pellet at room temperature, dried under vacuum condition. Dried DNA pellet denatured at 90°C and cooled. Load denatured DNA pellet into a 3730xl Genetic Analyzer Capillary Array for detection (Applied Biosystems) to complete the reaction. The bacterial sequence was identified by matching the maximum identity score from the known 16S rRNA gene sequences in GenBank and constructs the phylogeny, Submit ST-2 sequence to GenBank of National center of biotechnology information.

RESULTS AND DISCUSSION

Isolation and screening the antibacterial activity

A total of 42 bacterial isolates were isolated from the seaweed samples, 12 isolates from *Sargassum-1*, 18 from *Sargassum*-2 and 12 from *Sargassum* 3 were purified and subcultured for the screening of antibacterial activity (Fig 1) 10 bacterial isolates showed inhibition zone against control strains namely S2-3, S2-4, S3-5, SW-1, SW-11, ST-2, ST-3, ST-4, ST-4_0 and ST-9 by dot plate assay and cross streak assay (Fig 2).

Fig 1 Showing growth of isolated endophytic bacteria Zobell marine agar plate

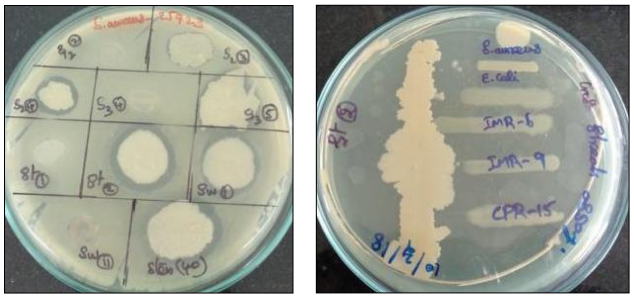

Fig 2 Showing antibacterial activity of endophytic bacteria

Niken dharmayanti *et al*. [19] isolated 14 symbiotic bacteria from *Turbinaria conoides* brown seaweed, 7 isolates inhibit growth of *Staphylococcus aureus* and 1 isolate inhibit *S. aureus* and *Escherichia coli* similar to this study. ST-4, ST-4_0 and ST-2 were producing notable inhibition zone against *Staphylococcus aureus* and *Escherichia coli*, *S*2-3, ST-3, SW-11 moderately active against *Staphylococcus aureus* and *E. coli*, S2-4, S3-5 and SW-1 inhibits *Staphylococcus aureus* only, ST-9 showing the low activity against *Escherichia coli* only. ST-2, ST-4, ST-4_0, S2-4, SW-11 active against Cephalosporin resistant isolates also (Table 1).

20 clinical isolates confirmed as Esbl producing pathogens by E-test and disc diffusion methods (Fig 3). Strain S2-4 and SW-11 active against *Staphylococcus aureus* and not

active against Esbl producers. ST-2 and ST-4 against control strain as well as Esbl producers. ST-4o was moderately active against Esbl producers. Amraoui *et al*. [20] screened marine derived 32 isolates from Seaweed, Seawater, invertebrates and sediments. 76% antibacterial activity against Gram positive and 62% against gram negative bacteria. ST-2, ST-4 and ST-4o were showing good activity against Esbl producers at the 25 µl concentration in agar well diffusion method (Fig 4).

Fig 3 Detection of Esbl producing isolates by E-test and Disc diffusion method

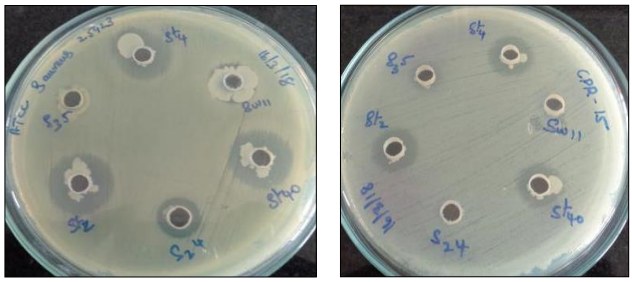

Fig 4 Antibacterial activity of endophytic bacteria

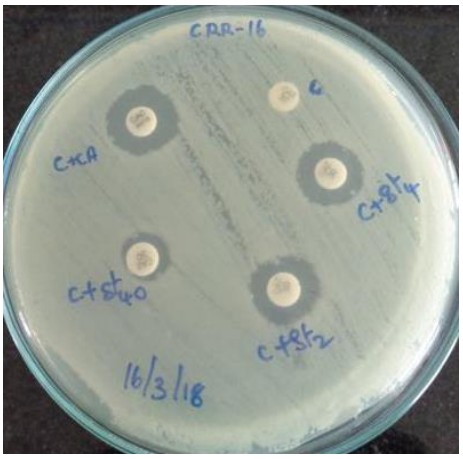

Fig 5 Esbl inhibiting activity of endophytes C- Ceftazidime, C+Ca- Ceftazidime/clavulanic acid, St2, St4, St4o culture filtrates

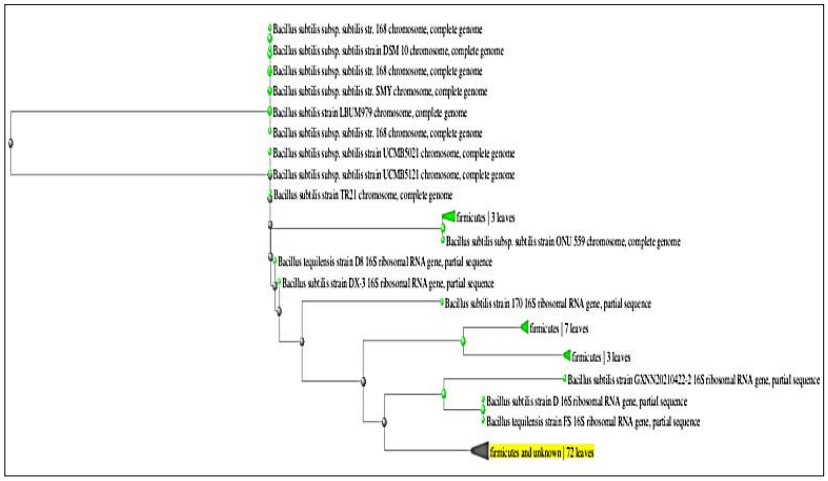

Fig 6 Shows Phylogenic construction of *Bacillus subtilis ST-2*

Jamal *et al*. [21] revealed that Fucus serratus associated *bacillus licheniformis* producing (30.7 kDa) protein having activity against vancomycin-resistant enterococci, MRSA and Listeria monocytogenes. Maria *et al*. [22] screened 15 bacteriogenic *Bacillus licheniformis* or *pumilus* from *Ascophylum nodosum, F. serratus, F. vesiculosus, P. palmata, P. lanosa, U. lactuca and Ulva spp* with antimicrobial activity against control strains. Ceftazidime disc with cell filtrate of ST-2, ST-4 and ST-4o were producing induced zone up to 15-20 mm as Ceftazidime/clavulanic acid (Fig 5).

Esbl inhibiting *Bacillus subtilis* STEN-2 confirmed by 16S rRNA sequencing with similarity of >98.65% and Sequence were submitted to GenBank with Accession number OM31545 (Fig 6). Minimal *et al*. [23] identified the EPS producing *Bacillus cereus* by 16S rRNA sequencing from *Sargassum wightii*. Susilowati *et al*. [24] identified *Sargassum sp*., associated *Bacillus subtilis* inhibit the growth of MRSA, identified *Bacillus subtilis* LC002977 with 95% similarities which sustain the significance of present study. Rajasree *et al*. [25] investigated that antifouling activity of exopolysaccharides of *Sargasum wightii* associated *Bacillus sp*. ICN-SS01against biofilm forming bacteria. Several prior researches acknowledged the Seaweed associated endophytic *Bacillus sp*. as novel source for the production of bioactive compounds with numerous pharmaceutically important activity.

CONCLUSION

Bacillus subtilis ST-2 Sargassum associated bacterial isolates were screened for antibacterial activity against gram positive and gram-negative pathogens by dot plate and cross streak assay. 25µl culture filtrates of ST-2 able to inhibit the cephalosporin resistant pathogens. Ceftazidime with 20µl culture filtrates of ST-2 inhibiting Esbl production as Ceftazidime/clavulanic acid. Findings of this study enlighten the capability of bioactive compounds from marine symbiotic microorganisms with antibacterial, antifungal activity. Betalactamases inhibiting activity of *Bacillus subtilis ST-2* will be examined by incorporate with other betalactam antibiotics against various betalactamases producing clinically important pathogens.

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