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ISOLATION AND IDENTIFICATION OF BACILLUS Sp. FROM SEAWEED LIQUID FERTILIZER (SLF)

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Abstract

Totally 15 different bacterial isolates (RR1- RR15) were obtained by screening the 100% SLF. The isolates RR2, RR8, RR9 and RR10 are identified as *Bacillus sp.* by staining and biochemical techniques. Genomic identification of the *Bacillus* sp. was identified by 16S rRNA. All the fifteen different bacteria isolated from the *K. alvarezii* SLF were screened for the production of IAA. Among them, the isolate RR2 was identified as *Bacillus* sp. produced high amount of IAA (8.5 µg/ml). Optimization of *Bacillus* sp. RR2 for IAA production in different carbon sources at different time intervals (12,24,36 hrs) were done. Phylogenetic tree was constructed using the neighbour-joining method with the help of MEGA3 package. The sequence of the selected bacterium has showed higher homology with the genus *Bacillus*. The bacterium finds its closest match (99%) with the *Bacillus cereus* 770 (*EU430093*).

Keywords: Antibiofilm, Anticancer, Marine Organisms, Metabolites, Diversity

INTRODUCTION

The marine macroalgae *Kappaphycus alvarezii* (Doty) Doty ex Silva (Fig. 1) belonging to the family *Solieriaceae* of the class Rhodophyceae, is an economically important tropical red seaweed with a high demand worldwide for its cell wall polysaccharide, kappa carrageenan, (Bixler,1996) which is widely used in the food and pharmaceutical industries. Owing

to its high commercial value, *K. alvarezii* is widely cultivated in Philippines, Indonesia, Vietnam and Tanzania. In India too, *K. alvarezii* has been successfully cultivated in Mandapam region of South India (Eswaran *et al.*, 2002, 2006). However, the biostimulant and fertilizer potentials of the alga are poorly understood for exploitation in agriculture.

In India, nearly 70% of the total population lives in rural areas and agriculture is their only source of income. Modern day intensive crop cultivation requires the use of chemical fertilizers which are not only in short supply but are also very expensive in developing countries like India. Therefore, there is a need to utilize the possibility of supplementing chemical fertilizers with organic ones such as Seaweed Liquid Fertilizers (SLFs) which are cost effective and within the reach of the poor farmers. Seaweeds are one of the most important marine resources of the world and have been nurtured for several years as fertilizer additives with beneficial results (Booth, 1965).

MATERIALS AND METHODS

The seaweed Liquid Fertilizer (SLF) prepared at pH 7.2 and 4.0 from the red seaweed *Kappaphycus alvarezii* (Doty) Doty ex Silva obtained from a multinational company, PepsiCo. Pvt. Ltd., Tuticorin in December 2005 were used for the present investigation.

Seaweed extracts are a source of PGRs that exhibit multiple functions. The PGRs regulate plant growth, increase plant resistance to various environmental stresses, such as drought, salinity, and low temperature. Seaweed extracts possess cytokinin-like and auxin-like properties which can stimulate endogenous cytokinin activities of plants (Jayaprakash *et al.,* 2017). Application of seaweed extracts enhances root growth, delays senescence, improves crop quality (Schmidt, 1990; Crouch, 1990, Nabati *et al.*, 1991), and regulate cell membrane components of plants or crops during drought (Yan et al., 1993).

Apart from the effect of SLF on the growth of higher plants, there are also a few studies on the effect of SLF on micro organisms. The effects of SLF were studied for the growth and dynamics of soil microbial community, especially those concerned with biological nitrogen fixation and their host leguminous plants (Thevanathan and Dinamani, 2005).

Isolation and Identification of the microorganisms from 100% SLF

Serial dilution plating technique was followed to isolate the bacteria present in the 100% SLF at pH 7.2 (SLF at pH 4.0 did not support microbes). One mL of the sample was diluted with 9 mL glass distilled water and this was further diluted up to 10^{-7}.

Hundred micro liters from each of the dilutions were taken and spread plated on nutrient agar (NA). The inoculated plates were incubated at room temperature for 3 days. The bacterial colonies appeared on NA plates were sub cultured to purity and used for further experiments.

The bacterial antagonists performed synergistically in glasshouse experiment were subjected to various biochemical and staining techniques as described by Cappuccino and Sherman (2004) and the results were interpreted with the key provided in the Bergy's Manual of Determinative Bacteriology (Holt *et al.*, 2004). In addition, 16s rRNA sequencing was done to confirm the identity of bacterial strains.

Screening of bacterial isolates for the production of IAA

The isolated bacteria were grown in nutrient broth at room temperature for two days. After, the bacterial culture was subjected to the IAA extraction procedure as described previously. The extract was then run on TLC for the presence of IAA bands which indicates the ability of the respective bacterium to produce IAA. The IAA was also quantified by using the procedure described earlier.

Growth study of *Bacillus* sp.

The growth study was conducted on for *Bacillus* sp. for two days. At every 12 h interval its absorbance was read at 600 nm.

Effect of different carbon sources on IAA production of *Bacillus* sp.

Different carbon sources: glucose, sucrose, fructose, lactose, sorbitol, manitol and glycerol were amended separately in nutrient broth at 1% at pH 7.0 and inoculated the bacterium and kept at room temperature for 48 h.

Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from overnight grown cultures of *Bacillus* sp. using QIAGEN DNA isolation kit (Qiagen, Valencia, CA), suspended in 100 µl of elution buffer (10 mM/L^{-1} Tris-Cl, pH 8.5) and quantified by measuring OD at 260 nm. PCR amplification was performed using a 20 µL reaction mixture containing 100 ng of template DNA, 20 µmol of 16S rRNA primers, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1U of *Taq* DNA polymerase (MBI Fermentas) and 2 µl of 10x *Taq* polymerase buffer. The sequences of 16S rRNA primers used were as follows.

FP 5′-AGAGTTTGATCCTGGCTCAG-3′

RP 5′ -ACGGCTACCTTGTTACGACTT -3′

Amplification was carried out with an initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 1 minutes and final extension at 72°C for 5 minutes using a thermo cycler (iCycler; Bio-Rad Laboratories, CA). PCR products were

analyzed on 1% agarose gel for 16S rRNA amplicons in 1x TBE buffer at 100 V.

Cloning and sequence analysis of PCR products

The 16S rRNA amplified fragments were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) from the agarose gel and ligated into the pGEM®-T Easy vector (Promega Corporation, Madison, USA), transformed into *E. coli* strain DH5α and plated on Luria Bertaini agar medium contained ampicillin (50 µg/mL), X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; 20 µg/mL) and IPTG (isopropyl-β-D-thiogalacto pyranoside; 0.1 mM/mL) (Sambrook *et al.*, 1989). The presence of insert DNA encoding for 16S rRNA in the recombinants was confirmed by PCR amplification and sequencing using automated DNA sequencer (Model 3100, Applied Biosystems, USA). The sequences were analysed using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analysis

The sequences of these 16S rRNA genes were compared against the sequences available from GenBank using the BLASTN program (Altschul *et al.*, 1990) and aligned using CLUSTAL W software (Thompson *et al.*, 1994). Distances were calculated according to Kimura's two-parameter correction (Kimura, 1980). Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was done based on 1000 replications. The MEGA3 package was used for all analyses (Kumar *et al.*, 2004).

RESULTS

Identification of bacteria isolated from SLF

Totally 15 different bacterial isolates (RR1-RR15) were obtained by screening the 100% SLF (pH 7.2). The isolates were subjected to various biochemical and staining techniques and identified using Bergeys Manual (2004). The isolates are shown in Fig. 2.

RR1, RR3, RR4, RR5, RR6 and RR7

All the above isolates are gram negative; motile with one or several polor flagella, aerobic, oxidase positive or negative, catalase positive and produce water soluble fluorescent pigments. These isolates are identified as those of *Pseudomonas* sp.

RR2, RR8, RR9 and RR10

All these isolates are gram positive and are motile by peritrichous flagella. Cells are rod shaped and straight with rounded and squared ends. Produce oval or round or cylindrical endospores. Aerobic or facultative anaerobic and catalase positive. They are identified as *Bacillu* sp.

RR11, RR12 and RR13

All these isolates are gram positive, motile, irregular rods and non- filaments. As growth proceeds the rods segment into small cocci, arranged singly, in pairs, and in

irregular clumps. Non-spore forming, non acid-fast, arerobic and catalase positive. They are clearly indicated that these isolates are *Arthrobacter* sp.

RR14 and RR15

All these isolates are gram negative, motility occurs by peritricus flagella, and are aerobic. Large ovoid cells, pleomorphic, ranging from rods to coccoid cells that occur singly or in pairs, or as irregular clumps, and sometimes in chains of varying length. Do not produce endospores, but form cysts. They are catalase positive. These isolates are *Azotobacter* sp.

Screening of IAA production bacteria from the isolates

All the fifteen different bacteria isolated from the *K. alvarezii* SLF were screened for the production of IAA. Out of fifteen isolates only five (RR2, RR4, RR5, RR6 and RR7) produced IAA (Fig. 3). Among them, the isolate RR2 which was identified as *Bacillus* sp. produced high amount of IAA (8.5 µg/ml).

Optimization of *Bacillus* sp. RR2 for IAA production in different carbon sources at different time intervals

IAA production at 12 h

The isolate RR2. i.e. *Bacillus* sp. inoculated in the Basal medium amended with glucose (1%) and sucrose (1%) produced 13.5 µg/mL and 13.4 µg/mL of IAA respectively. The organism produced less amount of IAA when grown in sorbital and mannose amended medium and much lesser IAA in media amended with maltose and lactose (Fig. 4).

IAA production at 24 h

As similar to the previous observation, the bacterium inoculated in the medium amended with glucose and sucrose enhanced the production of IAA to a maximum 11.5 µg/mL and 11.0 µg/mL, respectively. The bacterium grown in the medium amended with lactose and sorbital produced each 9.9 and 10.2 µg/mL IAA whereas maltose and mannitol amended medium supported only 8.0 µg/mL and 6.5 µg/mL of

of IAA (Fig. 4).

IAA production at 36 h

The production of IAA decreased after 24 h of growth and produced 10.8 µg/mL and 9.5 µg/mL of IAA in the media amended with glucose and lactose. The test organism inoculated in sucrose, maltose, and mannitol amended medium produced less amount of IAA at 36 h (Fig. 4).

Molecular taxonomy of *Bacillus* sp. RR2

The 476 bases of the 16S rRNA sequence of the test bacterium were as follows

CCATTTGTTTTAAATTTGAATCAACGGAAAA
ACTTTCCAGGTTTTGACAATATTGAAAACCC
AAGAAATAGGTGGTCTTCTTCGGGAGTAGA
GTGACAGGTGGTGCATGGTTGTTTTCAGTT
CGTTTTTTGAGATGTTGGGTTAAGTCTGGCT
CGGACGCACCCCTTGATTTTAGTTGCCTTC

ATTTAGTTGGGCACTTTAAGGTGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGG
CTACACACGTGCTACAATGGACGGTACAAA
GAGCTGCAAGACCGCGAGGTGGAGCTAAT
CTCATAAAACCGTTCTCAGTTCGGATTGTA
GGCTGCAACTCGCCTACATGAAGCTGGAAT
CGCTAGTAATCGCGGATCAGCATGCCGCG
GTGAATACGTTCCCGGGCCCTTGTTCACCC
CGCCCCGTCCACCCACCCCCAAAGAA

Phylogenetic tree was constructed using the neighbour-joining method with the help of MEGA3 package. The sequence of the selected bacterium has showed higher homology with the genus *Bacillus*. The bacterium finds its closest match (99%) with the *Bacillus cereus* 770 (*EU430093*).

DISCUSSION

Seaweeds offer living surfaces that harbor interesting microbial systems and have a unique community of microorganisms on their thallus surface (Armstrong *et al.*, 2000; Moore *et al.*, 2002). The number of bacteria on seaweeds determined by Scanning Electron Microscopy ranged from 3 to 15 x 10^7 cm^2 with no significant temporal pattern except in the case of *Fucus serratus* and *F. spiralis* (Armstrong *et al.*, 2000). The bacterial strain, *Pseudomonas* sp. identified through 16S rRNA analysis from the Japanese seaweed *Diginea* sp. has a symbiotic relationship with dinoflagellates such as *Amphidinium* sp. (Wimolpun Rungprom *et al.*, 2008). Bacteria that are surviving on the surfaces of marine algae live in a highly competitive environment where space and nutrients are limited. The phylogenetic analysis using 16 S rDNA sequences identified 7 out of 10 strains belonging to the genus *Bacillus* while the other three to *Microbacterium, Psychrobacter*, and *Vibrio*. Manmadan *et al.*, (2008) investigated the diversity of epibiotic bacteria isolated from nine species of red algae such as *Pachymeniopsis lanceolata, Plocamium telfairiae, Gelidium amansii, Chondrus oncellatus, Grateloupia filicina, Ceramium kondoi, Lomentaria catenata, Schizymenia dubyi* and *Porphyra yezoensis* and identified their phylogenetic position. Majority of the bacteria isolated from these algae belonged to the genus *Bacillus*.

In the present study, a total of fifteen different bacteria were isolated from *Kappaphycus alvarezii* SLF (pH 7.2) and they were screened for IAA production. Among them the isolate *Bacillus* sp. RR2

Fig. 1. *Kappaphycus alvarezii* (Doty) Doty ex Silva

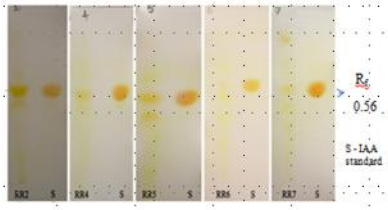

Fig. 3. IAA from Bacterial isolates

Fig. 2. Bacterial isolates from *K. alvarezii* SLF

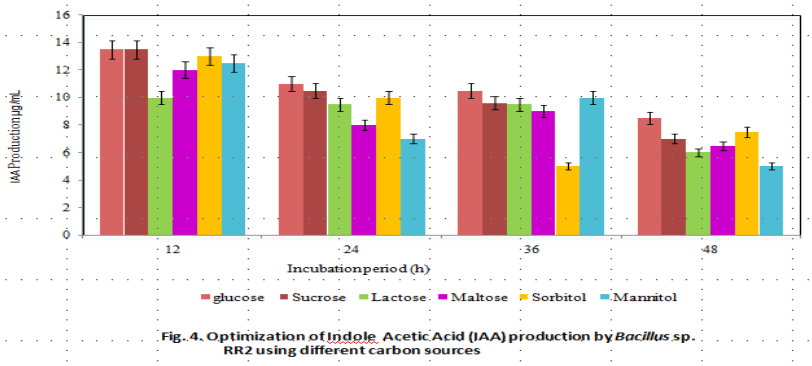

Fig. 4. Optimization of Indole Acetic Acid (IAA) production by *Bacillus* sp. RR2 using different carbon sources

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