Isolation and Identification of *Bacillus Tequilensis* from Mangrove Soil and Their Antimicrobial Activity against Common Pathogens

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Abstract: Different mangrove soils were screened for bacterial cultures with antimicrobial activity against common pathogens. Out of 80 bacterial isolates obtained from mangrove soils from various regions of India, 20 cultures were found to inhibit most tested pathogenic microbial cultures, however only 3 cultures designated as JF6, MP15 (2) and JF14+1 were found to be non-toxic in sheep blood haemolysis test. Anti-microbial activity of these cultures was found to be extracellular and maximum activity was observed when grown at pH 6 at 30℃ and after 36 hr of incubation. The activity of the culture was stable even after treatment with proteinase K and temperature up to 80℃. By biochemical and molecular identification methods, these cultures were identified as *Bacillus tequilensis*.

Keywords: Bacillus, Mangrove soil, Antimicrobial.

I. INTRODUCTION

Antibiotics have been major part of the fight against pathogens and have saved innumerable human lives. However, recent decade has witnessed the emergence of multi drug resistant (MDR) and extreme drug resistant (XDR) bacteria, increasing the mortality and cost of healthcare. Resistance in pathogens takes place by various biochemical and physiological processes such as altering the target site so that antibiotic becomes ineffective, rapidly pump out antibiotics out of cell [1]. Major reasons for this scenario can be the indiscriminate use of antibiotics as well as non-availability of new antimicrobial molecules. This necessitate the control of antibiotic usage and identification of new antibiotic molecules [2].

Mangrove soils are highly dynamic habitat due to constantly varying conditions and can act as the source of microorganisms with diverse activity including antifungal and antimicrobial activity [3]. Mangrove soils offer a range of ecological niches for the development of different micro-organisms such as actuinomycetes, fungi, microbes, macro and micro algae with environmental recycling and secondary pharmaceutical metabolite processing capability. [4,5]. The present report highlights the isolation of Bacillus species from mangrove soils of Indian coast and its activity against common pathogenic micro-organisms.

II. MATERIALS & METHODS

Sample collection : Soil samples were collected from 5-10 cm depth in the rhizosphere of Avicennia marina a common mangrove plant from Navi-Mumbai (Panvel), Kharghar, Vashi, and Mansourwar (Mumbai). Sample were collected, after removing the debris it was transferred to the laboratory in sterile polyethylene bags.

Culture isolation: Soil sample (1g) were added in 100 ml of Sterile distilled water, mixed well and serially diluted. Dilutions (10-3 and 10-6) were then spread plated on starch casein agar (SCA) and incubated at 37℃ for 48h. Morphological distinct colonies were isolated, purified and inoculated in yeast manitol agar (YMA) for preservation. Cultures were grown in yeast manitol broth (YMB), incubated for 72 hr. and used for all experiments.

Haemolysis: Isolated cultures were spot inoculated on sheep blood agar medium and incubated at 37℃ for 24h., and observed for haemolytic pattern on sheep blood agar plate [6].

Screening of Antimicrobial activity: Isolates were screened for antimicrobial activity by using agar against different pathogenic microorganisms such as *B. subtilis*, *S. aureus* 2408, *S. typhi*, *S. paratyphi*, *P. auroginosa*, *E. coli* 3099, *Shegilla* spp. and *Xanthomonas* spp. Pathogenic cultures (100µl, 1 OD) were spread on Mueller Hinton agar (MH) plates and incubated at 37℃ for 30 min. Wells of 5mm were perforated in the agar, and 10 µl of supernatant culture was added and incubated for 48h at 37°C. Inhibition zone was also observed and measured [7].

Molecular identification: The DNA was isolated by means of genomic DNA isolation package (Sigma, USA) as defined by the manufacturer and used as a PCR template. ABI Prism BigDye Terminator Cycle Reaction Kit was used to sequence the pcr product for each reaction mélange with approximately 10 Ng of DNA; 2.5 mM MgCl2; 1 x PCR (Bangalore Genei, Bangalore, India) 200 µM each with dCTP, dGTP, dATP and dTTP; 2 PMol for each, forward and reverse primer. A combination of FDD2-RPP2 (1.5 kb fragment amplification universal primers for eubacteria) was used to sequence the practically completed gene. The data and analysis and template were purified as per the instructions of the manufacturer (Applied Biosystems). ABI Prism 3100 Genetic Analyzer samples were run, and DNA sequence analyzer software was used to analyse the sequence output. This sequence was matched using the BLAST algorithm with the National Biotechnology Information Center GenBank entries. [8,9]**.**

Optimization of growth parameters, pH and temperature : Specified culture was inoculated with varying pH in 100 ml broth (YMB) and incubated at 25^0C with the shaker incubator (120 rpm). Isolates were inoculated at medium optimised pH and incubated at different temperatures to verify the optimal temperature. Following the incubation operation of cultures the method of diffusion against pathogenic cultures was tested [10,11].

Incubation Time: Cultures were introduce into a culture medium and samples were collected at various incubation time till 120 h. Cultural development was calculated by optical density at 600nm and surnatant behaviour was regulated simultaneously by the well diffusion.

Partial purification of active compound: Isolates (1 OD, 1 percent) were inoculated at YMB and incubated at 35 ° c at 120 rpm for 48 h. Culture supernatant was collected at 4°C by centrifugation. The supernatant was vigorously combined with various solvents (methanol, acetone, chloroform and ethyl acetate) from 1:1 v/v and held at 20°C for 2 hours. Solvent was evaporated at 50°C in order to precipitate. Then it was immersed in 1 ml of purified water before antibacterial property was confirmed.

III. CHARACTERIZATION OF THE ANTIMICROBIAL COMPOUND

Sensitivity to temperature and Proteinase K : Precipitate (1mg) obtained after solvent extraction were exposed to various temperature (40 to 80ºC) for 20 min. followed by checking the activity on MH agar plate against the pathogens. Similarly, the precipitate was exposed to Proteinase K (1mg/ml) for 30 min in phosphate buffer. Supernatant without treatment and buffer or Proteinase K were used as the control. The plates were incubated at 37℃ for 48 h. Zone of inhibition was observed and calculated [12].

IV. RESULTS

Isolation of yeast cultures, Isolation and Screening of antimicrobial activity : In different mangrove soil samples, a total of 80 morphological colonies were isolated. Moreover these cultures were used for well-diffusion regulation of antimicrobial activity. Only 20 cultures designated as MP1(1), MP1(2), MP6(2), MP15(1), MP15(2), JF6, JF7, JF8, JF9, JF10, JF12, JF13, JF14+1, Va1(1), Va1(2), Va1(5), Va2(5), Fe2po3, p2b5 and p09(1) Inhibitor activity seen against most pathogenic microorganisms were studied.

Hemolysis: Out of 20 bacterial cultures 3 cultures were found to be non-hemolytic designated as JF6, MP15 and JF14+1.

Molecular identification : In 16s rRNA sequencing Culture JF6, MP15 (2), JF14+1 was identified as *Bacillus tequilensis* with Accession no. with 99.7%, 99.93% and 99.4% similarity.

Optimization of inhibitory activity , Optimization of pH, Temperature and incubation time:

Optimum culture activity was observed after 36 hours of incubation. at pH 6.0 and temeprature 30ºC under shaking conditions. (Fig. 1, 2, and 3).

Purification of active compound: JF6, MP (15) 2, MP14+1 culture was further screened for antimicrobial activity with different solvent extracts, in that methanol extract was found to be best against all pathogenic microorganisms (Table 1 and Fig. 1).

Effect of Proteinase K and temperature: Culture supernatant after treatment with proteinase K, retained its activity almost similar to that of supernatant (Fig. 2), however, it almost lost its activity after heat treatment at 80℃ (Fig. 4).

Table: 1 Antimicrobial activity of the extracts by well diffusion methods

Pathogens	Jf6	MP15(2)	JF14+1
B. subtilis	0.6 cm	0.4 cm	1.1cm
S. aureus	0.7 cm	0.6 cm	0.8 cm
S. typhi	0.8 cm	0.6 cm	0.9 cm
S. paratyphi	0.7 cm	0.8 cm	0.9 cm
P. aeruginosa	0.7cm	0.7 cm	0.8 cm
E.coli	1 cm	0.8 cm	0.8 cm
Shigella	1.9	0.5 cm	0.8
Xanthomonas	0.6cm	1.3cm	2.4cm

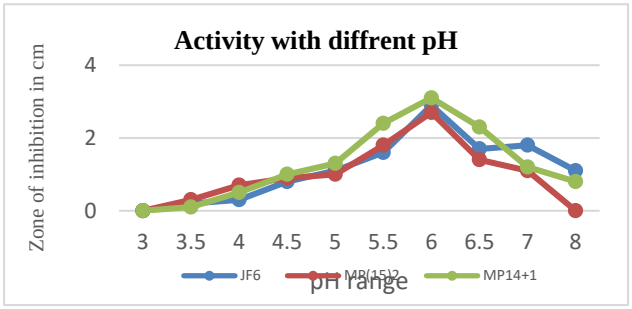

Figure 1. Optimization of pH

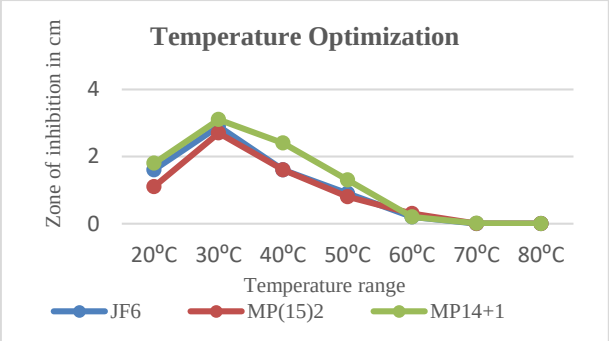

Figure 2. Optimization of temperature

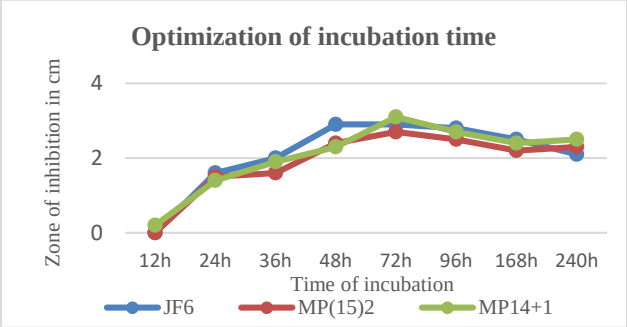

Figure 3. Optimization of inhibition zone

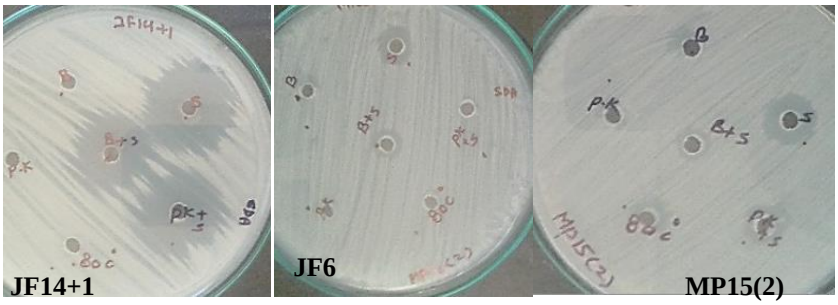

Figure 4. Effect of Proteinase K and temperature on activity of the extract.

V. CONCLUSION

Currently the need of new antimicrobial molecules has forced scientists around the world to explore different sources and organisms. The microbial profile of mangrove soil is rich in diversity, amongst them Bacillus spp. being second most dominant genus after actinomycetes [6]**.** Bacillus genus of bacteria are known to produce antibacterial as well as antifungal compounds that are helpful in fight against pathogenic microorganism. One of the important characteristic of any microbial culture is its non-toxic nature, the isolates were first screened for haemolytic activity on sheep blood agar [5] and non-hemolytic cultures were screened for antimicrobial activity on pathogens such as *E.coli, S. aureus, S. typhi, B. subtilis, Shigella*. Only 3 cultures designated as JF6, JF14+1, MP15(2) had the ability to inhibit the growth of above mentioned pathogens. Molecular identification of JF6, JF14+1, MP15 (2) reveal the isolates identity as *Bacillus tequilensis* as performed by 16S rRNA sequencing. There are reports of microbial community in mangrove soil possessing antimicrobial and antifungal properties [1,13,14]**.** Further, optimization of isolates JF6, JF14+1 and

MP15(2) for antimicrobial ability was checked and showed maximum activity at pH of 6, temperature of 30ºC and incubation period of 36-96 hr. Isolates were further screened for partial purification of active compound and supernatants of isolates were treated with different solvents [15,16] and found that the fraction with methanol extraction only showed antimicrobial activity. When the extract treated with proteinase K it did not lost the activity, however lost complete activity when heated at 80ºC. It indicates non-protein nature of the antimicrobial compound.

Mangrove soils are a rich source of different microorganisms. Three Bacillus strains were extracted from mangrove soils on the Indian coast have been shown to be non-hemolytic and may inhibit typical pathogenic bacteria. Maximum cultures antimicrobial activity was detected as cultures were incubated for 36 hr at 30ºC in medium with pH 6.0. Activity was found to be in extracellular fraction and it was purified using methanol extraction. The extracted molecules were found to tolerant to proteinase K activity and sensitive to temperature at 80ºC. This indicates that the active fraction in nature is non-proteinous. Since species of Bacillus are capable of producing large quantities of extracellular factions, the crops mentioned are an ideal for further antimicrobial characterization.

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