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RESEARCH ARTICLE

Evaluate the antimicrobial activity of Biosurfactant against ESBL producing bacterial isolates

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ABSTRACT: In this present study, the biosurfactant producing ability of the microorganisms isolated from the soil samples was investigated by blue agar plate, oil spreading test and emulsification index. Among the 15 of them, the best isolate was identified as *B.cereus* based on microscopic and biochemical analysis. Our aim of the present study was to evaluate the antimicrobial activity of biosurfactant against ESBL producing *P.aeruginosa* isolates. Susceptibility tests were performed by the agar well diffusion method. Among the 5 ESBL isolates, 3 of them were inhibited by biosurfactant. The zone of inhibition ranged from 12 mm to 22 mm and MIC was 10mg/ml. This effect is comparable to antibiotics and therefore the future use of this biosurfactant as broad spectrum antibiotics are highly promising.
Keywords: Biosurfactant, *B.cereus*, emulsification index, ESBL, *Pseudomonas aeruginosa*

INTRODUCTION

Antibiotic resistances are a public health problem in all parts of the world. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. This phenomenon was mostly promoted by incorrectly prescribed antibiotics, duration of antibiotic therapy also contribute the development of resistance.

In most cases, antibiotic-resistant infections require prolonged and/or costlier treatments, extend hospital stays, necessitate additional doctor visits and health care use, and result in greater disability and death compared with infections that are easily treatable with antibiotics. In this situation, urgently we need to investigate for newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. Concerning the above facts, it is worthwhile to screen microbes which have the above properties to synthesize new drugs (Nascimento *et al.,* 2000).

The production of antimicrobial substances seems to be a general phenomenon for the bacteria, this meritorious array of microbial defense systems are produced, including broad-spectrum classical antibiotics metabolic by-products (Motta *et al.,* 2004). In addition, microbes produce surface active agents, which possess a number of advantages such as low toxicity, effective on a wide range of pH values and temperatures (Christova *et al.,* 2007). This surface active agent is also called as Biosurfactant. This biosurfactant may have one of the following structures: mycolic acid, glycolipids, polysaccharide–lipid complex, lipoprotein, or lipopeptide and phospholids (Karanth, 1999).

Among the various categories of biosurfactants the glycolipid biosurfactants "rhamnolipids" stand apart. Rhamnolipids biosurfactants show several useful properties for the processing industries. This Biosurfactant possess that capable of inhibiting the microbes such as bacteria and fungi (Magalhae, 2013). Number of authors reported the antimicrobial activity of Biosurfactant against gram negative and positive bacteria but no one inhibit the ESBL producing isolates with *P.aeruginosa* producing Biosurfactant.

Against these backdrops, this study was aimed at isolating and screening of biosurfactants producing microorganisms from oil contaminated soil samples and its active against ESBL producing isolates.

MATERIALS AND METHODS

Pathogenic isolates

The Multidrug resistance isolates of UTI causing *Pseudomonas aeruginosa* were procured from Microtech, Microbiology Laboratory and Coimbatore and used for the study. All isolates were confirmed to UTI chromogenic agar media.

PCR amplification for detection of beta lactamase genes from food samples

All isolates were screened for the resistance genes SHV, TEM, CTX-M, and OXA by a multiplex PCR assay using Hong Fang *et al.,* (2008) procedure. The plasmid DNA was separated according to procedure of Sathasivam and Manickam (1996). PCR

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amplification reactions were performed in a volume of 25 µl containing 12.5 µl of 2x Promega PCR Master Mix (USA), 0.2 µM concentrations of each primer (1 µl), 2 µl of plasmid DNA template and make up 25 µl with molecular grade water. The cycling parameters were as follows: an initial denaturation at 95°C for 15 min; followed by 30 cycles of 94°C for 30 s, 62°C for 90 s, and 72°C for 60 s; and with a final extension at 72°C for 10 min. The amplified PCR products were subjected to electrophoresis at 1.5% agarose gel in 1XTBE buffer. A 100 bp ladder molecular weight marker was used to measure the molecular weights of amplified products.

Collection of soil samples

Surface sediment samples were collected from oil contaminated sites in Namakkal area. Soil samples were collected from10 different points in the oil contaminated site using a sterile stainless steels spatula. The collected samples were pooled and transferred to sterilized plastic container and transported at 4°C to the laboratory and maintained at 4°C until analysis.

Screening of bacterial isolates from oil contaminated soil

The serially diluted soil samples were spread on BHA plates overlaid with 100 µl of Hydrocarbon (Crude oil) and were incubated at 25°C for 14 days. Any isolate which grew on BHA plate were confirmed as degraders. After, such colonies were inoculated into NA plates and incubate at 24hrs to 48hrs and store at 4°C for further work. (Mamitha *et al.,* 2013).

Selection of efficient isolates

Isolates were subjected to blue agar plate method. Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.2 mg/ml) and methylene blue (MB: 0.2 mg/ml) were used to detect extracellular anionic glycolipid production (Siegmund and Wagner, 1991). Biosurfactants were observed by the formation of dark blue halos around the colonies.

Oil spreading assay

The culture suspension was screened for biosurfactant production (Anandaraj and Thivakaran, 2010). Twenty millilitre (20ml) of distilled water was taken in the Petri plate. One milliliter (1ml) of crude oil was added at the centre of the plates containing water. Then twenty micro litre (20µl) of the supernatant of the culture was added to the centre. If biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clearing zone and diameter of this clearing zone indicates the surfactant activity, also called oil displacement activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed.

Emulsification index (E24)

The emulsifying capacity was evaluated by an emulsification index (E24). The culture sample was determined by adding 2 ml of crude oil and 2 ml of the cell-free broth in a test tube, vortexed at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of the emulsified layer (cm) divided by the total height of the liquid column (cm). The percentage of emulsification index was calculated by using the following equation (Cooper and Goldenberg, 1987).

$$\text{Emulsification index (E24)} = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

Production and extraction of biosurfactant (Anandaraj and Thivakaran, 2010)

The culture was inoculated in 50ml of bushnell haas broth with 1ml of crude oil. The culture was incubated at 27°C for 8 days with shaking condition. After incubation the bacterial cells were removed by centrifugation at 5000rpm, 4°C for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1M H_2SO_4. Now add equal volume of chloroform: methanol (2:1). This mixture was shaken well for mixing and poured into a sterile Petri plate, then left overnight for evaporation. White colored sediments were obtained as a result, i.e., the Biosurfactant. They were placed in the hot air oven for drying at 100° C for 30 minutes. After drying the plates were weighed. The dry weight of the biosurfactants was calculated by the following formula:
Dry weight of biosurfactant = (Weight of the plate after drying -weight of empty plate).

Antibacterial assay

The MHA plates were inoculated with freshly prepared overnight inoculums which were swabbed over the entire surface of the medium, rotating the plate 60 degrees after each application by using a sterile cotton swab, to ensure the spread of the tested microbes (10^8 CFU/ml) on the surface of the plate completely. The 6mm diameter of the well was made with borer on the agar plates. The DMSO

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dissolved biosurfactant was filled in well with the help of micropipette. Fifty µl of Ampicillin (10µg/ml) was used as positive control and 100µl of DMSO was added in to well, which was negative control.

RESULTS AND DISCUSSION

Antibiotic resistance is a serious problem in the worldwide and emerged as one of the foremost problems in public health concerns of the 21st century. Among the different types of resistant mechanism, Beta-lactamases are the most important mechanism of drug resistance among Gram-negative bacteria (Serife, 2013). This fact was due to the selective pressure imposed by inappropriate use of third generation cephalosporins.

The most commonly isolated uropathogen was *Escherichia coli,* second most were *Klebsiella* spp and *Enterobacter* spp and followed other Gram negative organisms, such as *Acinetobacter baumannii, Proteus* spp, and *Pseudomonas aeruginosa.* Mostly, those diagnosed UTI isolates are positive for ESBL (Pooja *et al.,* 2017). In complicated UTIs, *Pseudomonas aeruginosa* was more common with antibiotic resistance (Getenet and wondewosen, 2011).

The increased prevalence of ESBL producing bacteria make an unavoidable phenomenon, Worldwide, UTIs due to ESBL-producing bacteria are an important part of this problem (John *et al.,* 2013). These ESBL-producing bacteria are difficult to resolve due to various reasons, difficulty in detecting ESBL production and inconsistencies in reporting (Steward *et al.,* 2000). Different risk factors are associated with ESBL strains (Ejaz *et al.,* 2009). Therefore action must be taken against this problem through a newer antimicrobial substance.

In this present study, totally 25 UTI isolates of *P.aeruginosa* were collected from clinical laboratory and subjected to determination of ESBL genes by PCR method. Among the 4 types of ESBL genes, TEM gene was observed on most of the isolates, second most was OXA and 2 isolates had CTXM and SHV genes. In this study, 4 isolates had 2 or more genes. These isolates were utilized for the inhibition activity (Plate.1).

The results of the present study were near to Omar *et al.,* 2015, who reported four types of ESBL genes observed from clinical isolates of *P.aeruginosa*. Recently Agnieszka (2017) observed OXA gene from most of the clinical isolates. Different risk factors are associated with ESBL strains such as prolonged hospitalization, extended ICU stay and severity of illness (Ejaz, 2009). Therefore, investigations shall be carried out to battle against the development of antimicrobial resistant.

This study aimed to determine the ESBL producing *P.aeruginosa* and prompt the inhibitory activity with Bacillus producing biosurfactant. In our study, 15 bacterial isolates were isolated from petrochemical contaminated soil samples collected in Namakkal. The ability of 15 isolates to grow on medium containing Crude oil, which eventually proved their ability for biosurfactant production and any isolate which grow on NA plates containing hydrocarbons but failed to grow on the NA plate were confirmed as non-degraders. Those 15 isolates were carried out for determination of efficient isolates by blue agar, oil spreading test and Emulsification test. Among the 15 isolates, 6 were positive for blue agar plate method. The formation of insoluble ion pair precipitates in the agar plate containing methylene blue exhibited dark blue color against the light blue background. While using the oil spreading technique, 7 isolates were able to displace the oil, it indicates that the bacterial isolates produce biosurfactant extracellularly. This oil spreading method was also studied by Priya *et al.,* (2009), Anandaraj *et al.,* (2010) and Jaysree *et al.,* (2011).

Emulsification activity is one of the criteria to determine the potential of biosurfactant. Emulsifying activities (E24) determined the productivity of bio emulsifier (Santhini and Parthasarathi, 2014). In our study, the highest Emulsification index (E24) value showed in SA12 isolate and followed by SA14 isolate. Therefore, SA12 isolate was selected as a potent isolate for biosurfactant production, this isolate showed positive results for Blue agar and oil spreading test. The extracted, biosurfactant was utilized for the antibacterial activity against UTI isolates of *P.aeruginosa.* Simultaneously, the best isolate of SA12 was identified as *B. cereus* based on microscopic and biochemical analysis according to Bergey's manual of determinative bacteriology.

Several biosurfactants exhibit antibacterial, antifungal and antiviral activities, which make them relevant molecules for applications in combating many diseases and infections. For the last decades, a usage of biosurfactant was increased in the medical field. In 2009, Falagas and Makris were applying the biosurfactant on the medical devices (such as catheters and other medical insertional devices) for decreasing colonization by microorganisms responsible for nosocomial infections.

In this present study, potential isolates of *Bacillus cereus* was utilized for the production of biosurfactant and evaluated the antimicrobial activity. Among the 5 isolates of *P.aeruginosa*, 3 (60%) of were inhibited by biosurfactant and two of were resistance. The zone of inhibition ranged from 12mm to 22mm. In this study, different concentration of biosurfactant was utilized for determination of MIC; the 10mg/ml of concentration was inhibited to two isolates of *P.aeruginosa*. The inhibitory concentrations of sophorolipid, as shown in Table 1 and plate 2 were comparable to standard antibiotic. However, no inhibition of the cell growth for *P.aeruginosa* was observed.

Several biosurfactants that exhibited antimicrobial activity have been previously described. However, there are few reports about the antimicrobial activity of biosurfactants isolated from Bacillus species and active against UTI isolates of *P.aeruginosa*.

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Most of the studies obtained from other species showed antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus agalactiae,* the minimum inhibitory concentration (MIC) was achieved from 25mg/ml to 30mg/ml (Gomaa, 2012). In 2013, Salman *et al* observed that the crude biosurfactant isolated from *S. thermophilus* showed an inhibitory effect against *Pseudomonas aeruginosa*. The previous studies of Gudina *et al.,* (2010) also determined the antibacterial activity of biosurfactant against to UTI causing bacterial isolates.

The bacterial microbes were inhibited by different mechanisms of biosurfactant, such as disturb the membrane structure (Lotfabad *et al.,* 2013), deterioration in the integrity of cell membranes, break down the nutrition cycle and prevent the protein synthesis (Gomaa *et al.,* 2012). Chaisson 2003 reported that the biosurfactant inhibits the initiation of transcription by preventing the synthesis of RNAs larger than dinucleotides.

CONCLUSION

The antimicrobial activity of biosurfactants has not been reviewed vast, especially against to UTI pathogenic bacteria. The present study shows that biosurfactants are a suitable alternative to the medical field for applications against ESBL producing UTI isolates of *P.aeruginosa.*

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Table .1
Antibacterial activity of biosurfactant against ESBL producing *P.aeruginosa*

S.No	Isolates	Different concentration Zone of inhibition in mm					
		10mg	15mg	20mg	25mg	DMSO	AMP
1.	Isolate 1	-	-	-	-	-	-
2.	Isolate 2	12	15	18	22	-	-
3.	Isolate 3	12	15	17	19	-	-
4.	Isolate 4	-	-	-	-	-	-
5.	Isolate 5	-	10	13	15	-	-

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Plate 1

Amplification of ESBL genes from *P.aeruginosa*

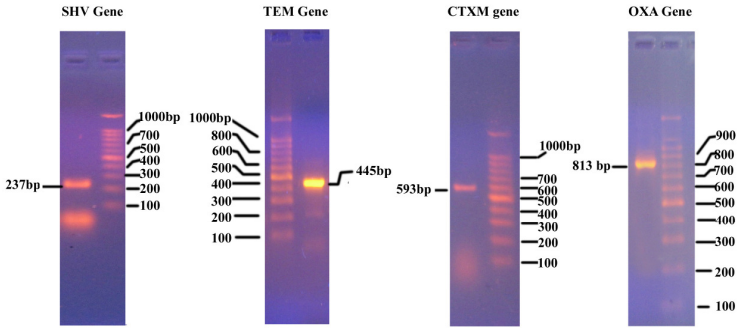
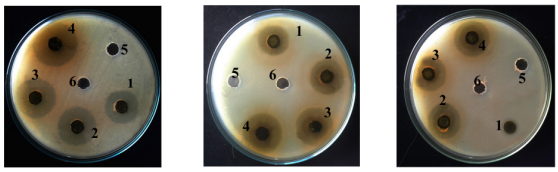


Plate 2

Antimicrobial activity of Biosurfactant



1-10mg, 2-15mg, 3-20mg, 4-25mg, 5- Negative control (DMSO), 6-Positive control (Ampicillin)

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CONFLICTS OF INTEREST
"The authors declare no conflict of interest".