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SCREENING OF PESTICIDE DEGRADING AND BIOSURFACTANT PRODUCING BACTERIA FROM CHLORPYRIFOS CONTAMINATED SOIL

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ABSTRACT

Pesticides have played a significant role in food production, and in view of growing worldwide food demand. This has resulted in serious concern about environmental contamination. Many remediation technologies are used to remove the pesticides from the soil. The use of biosurfactant enhanced bioremediation of pesticides. Biosurfactants are valuable surface active molecules produced by wide variety of microorganisms. Due to its amphipathic nature, these biomolecules are capable of lowering the surface tension, interfacial tension and forming micro emulsion to enable mixing of two immiscible solutions. Such properties exhibit excellent detergency, emulsifying, foaming, and dispersing traits. Some of the features, which make them promising alternatives to chemically synthesized surfactants, are their lower toxicity, higher biodegradability, greater stability at wide range of pH and temperature, and better environmental compatibility. This research was conducted to investigate the chlorpyrifos degrading bacteria and the bacteria having the ability to produce surfactants (biosurfactants) isolated from the pesticide-contaminated soil. The isolate (MCAS01) was identified as *Pseudomonas stutzeri* by comparing its 16S rRNA sequence with those available in The National Centre for Biotechnology Information (NCBI) gene bank. The strain was selected for its ability to produce extra cellular products (biosurfactants) able to reduce surface tension. The objective of this study was to isolate and characterize the surface active components from the crude biosurfactant produced by *Pseudomonas stutzeri* MCAS01. To confirm the ability of this isolate in biosurfactant production, different screening methods including blood hemolysis, emulsification, methylene blue reduction assay, drop-collapse, Cetyl Tri Ammonium Bromide (CTAB) and determination of surface tension were assessed. The fraction rich in glycolipids was obtained by the fractionation of crude biosurfactant using acid precipitation method and further identified using Thin Layer Chromatography and FT-IR. FT-IR results confirmed it to be glycolipid type of biosurfactant. It showed stability on exposure to high temperature (up to 100^{o}C), the surface tension was reduced up to 30mN/m as well as the emulsification index E24 was 65%. Properties of the biosurfactant that was separated by acid precipitation were investigated. The biosurfactant activity was stable at high temperature with wide range of pH and salt concentrations. Thus the biosurfactant are required to make the field application competitive with chemical surfactants.

KEYWORDS: **Rhamnolipid, screening assays, surface tension, Thin Layer Chromatography**

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INTRODUCTION

Synthetic organophosphates are widely used to control various pests for agriculture and for public health protection and these accounts for approximately 38% of the total pesticides used globally. India is the second largest pesticide producer in Asia and globally, it occupies 12th rank position[1] with a value of US $ 0.6 billion, which is 1.6% of the global market. India is the second largest user of pesticides after China in Asia[2]. Continuous and excessive use of these compounds has led to the contamination of several ecosystems in different parts of the world. Organophosphate poisoning causes three million health problems and 20,000 deaths annually[3]. A recent study estimated that every day, around the world almost 700 people die and several thousands are affected by pesticide poisoning[4,5]. Pesticide pollution not only affects the human health, but also other ecological assets, such as soil surface, air and ground water and also affects the flora and fauna[6]. Microbial degradation of organophosphate pesticides is of particular interest because of the high mammalian toxicity of such compounds and their widespread and extensive use. Chlorpyrifos [O, O-diethyl *O*-(3, 5, 6- trichloro-2-pyridyl) phosphorothioate] is one of the most widely used commercial organophosphorus insecticides as a foliar spray and dormant spray. Chlorpyrifos has been registered in India in 1968 under insecticides Act of India for regular use in the country. It persists in soil for 60–120 days and degrades there primarily through microbial action. The common degradation pathway for chlorpyrifos involved the formation of TCP (3, 5, 6-trichloropyridinol) has a half-life from 65 to 360 days[7]. Considerable amount of work has been done on chlorpyrifos degradation by bacteria and fungi isolated from agricultural soil and other sources[8]. Pesticides and their additives also show toxic effect on the growth of soil bacteria and interrupts with the degradation of organic matter. Chlorpyrifos accumulates at the top soil and influence the biochemical properties of soil microorganisms. Chlorpyrifos shows a marked adverse effect on soil enzyme activity which get considered as an index of soil organic matter degradation. In the soil environment microbes are responsible for the degradation of pesticides[9]. Only specific microorganisms which are tolerant to pesticides may remain and further multiply. Microorganisms are important in maintaining soil fertility and are also important agents, which detoxify pesticides in soil[10]. Biodegradation is the only way to minimize such problem concerned with pesticide toxicity in agricultural land. Bacteria and fungi have the capacity to utilize virtually all naturally and synthetically occurring compounds as their sole carbon and energy source[11]. Biosurfactants are amphiphilic agents which, by accumulating at the interface between immiscible phases, can reduce surface and interfacial tensions produced by bacteria, fungi and yeast, which either adhere to the cell surfaces or are excreted extracellularly in the growth medium[12]. They belong to various classes, including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopolysaccharides. The most effective biosurfactants can reduce the surface tension of water from 72 to 30 Nm−1[13]. In recent years, interest in biosurfactants has generated due to their possible applications in environmental protection, crude oil drilling, and in the pharmaceutical and food processing industries. The significant reduction of interfacial tension caused by the biosurfactant increases the solubility and emulsification of the immiscible phases and bioavailability of the insoluble substrate for microorganism[14]. The large surface-to-volume ratio enables many bacterial species to produce several types of structurally diverse surface-active compounds, which having antimicrobial, antiviral, hemolytic, and anti tumoral activity. However, the application of biosurfactant is impeded due to the poor yield of many strains and the increased costs of substrates[15].Therefore, the search for new biosurfactant producers from different environmental sources with enhanced and novel properties is a continuing endeavour for the biodegradation of pesticides[16,17]. Hence, in the present study an attempt was made to screen new biosurfactant producing bacteria capable of chlorpyrifos degradation. The prime objectives of the present study includes: i. screening of chlorpyrifos pesticide degrading bacteria ii. Screening for biosurfactant production iii. Characterization of biosurfactants.

MATERIALS AND METHODS

Sample collection and screening of Chlorpyrifos degrading bacteria

Soil samples were collected from farm lands of Rasipuram, North Tamilnadu, which had a long time usage of Chlorpyrifos for pest and insect control. The soil samples were then transferred to the laboratory, and 5 g of the samples were suspended in 500-ml flasks containing 100 ml of mineral salt medium (MSM) enriched with Chlorpyrifos (200 mg/l) as a carbon source. The MSM had the following composition in (g/l): NH_4NO_3 (4.0), Na_2HPO_4 (5.9), KH_2PO_4 (4.1), NaCl (0.5), $MgSO_4$ (0.096), $CaCl_2$ (7.74×10^{-4}) and Na_2EDTA (1.48×10^{-3}) at pH 7. The flasks were incubated at 180 rpm for 7 days at 30°C. At each 24 hr intervals, a loop full of samples from the flasks were streaked onto mineral agar plates supplemented with 200 mg/l Chlorpyrifos and the plates were incubated at 30°C for 3 days. The single bacterial colonies were picked up and sub cultured on mineral agar plates containing Chlorpyrifos of the same concentration until pure cultures were obtained. The growth of isolates was further checked with different concentration of chlorpyrifos pesticide described by Bhagubaty and Abdul Malik[18].

Biodegradability Assay

Reduction of tetrazolium chloride by the bacterial isolates for determining the biodegradability of chlorpyrifos was performed as described by Bhagobathy *et al*[18], with some modifications. The bacterial isolates were inoculated with 200mg/L of chlorpyrifos as a sole carbon sources in Minimal media. After 24 hrs incubation, add one millilitre of 0.02% of tetrazolium chloride and incubate for 4 hrs. White colour of minimal medium was changed into red shows positive result.

Estimation of chlorpyrifos

Amount of chlorpyrifos in the media after incubation was estimated by spectrophotometric analysis at 520 nm was described by Rokade and Gajanan[19]. Percent degradation was calculated using the given formula.

Percent degradation = $[(C_0-C_t)/ C_0] * 100$

Where C_0 = initial concentration and C_t = concentration at time't' i.e. 24 hr.

Biochemical and molecular identification of the selected isolate

The bacterial isolate grown on Chlorpyrifos containing agar plate was subjected to biochemical and morphological analysis. Biochemical identification of selected isolate was done using standard biochemical test. For molecular identification, the selected bacterial isolate was referred for 16S rRNA gene sequence analysis. Chromosomal DNA extraction was performed from 2 ml bacterial culture collected at the mid-exponential growth phase using CTAB-DNA extraction method. PCR amplification of 16S rRNA with universal eubacterial primers 8f (5_ CACGGATCCAGA CTTT GATY MTGG CTCAG 3_, forward) and 1512r (5_ GTGAAGC TTACG GYTAGC TTGTTAC GACTT 3_, reverse)[7] by following standard cycle sequencing protocol was conducted in a reaction mixture containing 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM of each primer, and 1 U of Taq DNA polymerase. After denaturation of chromosomal DNA at 95°C for 5 min, 16S rRNA was amplified during 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 s, and extension at 72ºC for 30 sec. Sequencing of the PCR product was carried out with ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA) using mentioned primers. The National Centre for Biotechnology Information BLAST tool was employed for identification of the species which is closed to the isolated bacterial strain.

Construction of the phylogenetic tree

Sequences representing a broad range of closely related to the microorganisms from this study and from the literature were retrieved from the NCBI 16S rRNA gene database and downloaded in FASTA format. Sequences were aligned using ClustalX version 1.81. This alignment software to create large gap regions in the sequences, which would have created biases in subsequent phylogenetics analysis. Finally, the tree was constructed using unweighted pair group method with arithmetic mean (UPGMA) by Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0 based on 16S rRNA sequence data[20].

Primary and secondary screening for biosurfactant activity

Biosurfactant activity of the pure culture of MCAS 01 was determined by five different methods: (1) Haemolysis, (2) Drop collapsing test, (3) Lipase production, (4) Blue agar method, and (5) Emulsification measurement. Primary screening included haemolysis, drop collapsing test, lipase production, and Blue agar method, while the secondary screening included emulsification measurement.

Haemolytic activity

Haemolysis was carried out on plates of blood agar medium supplemented with human blood (5%). The blood agar plates were inoculated and incubated at 30 ^{0}C for 24 hr. Plates were examined for clear zone around the colonies[21].

Drop collapsing test

Biosurfactant production was determined using the qualitative drop-collapse test described by Youssef *et al*[22]. Mineral oil was added to 96-well microtiter plates and MCAS 01 culture supernatant was added to the surface of the oil in the well and plates were incubated for 1 hr at 37^{0}C. The shape of drop on the oil surface was observed after1 min. The culture supernatant makes the drop collapsed, it indicates as a positive result and if the drops remain intact, it indicates as a negative result. Distilled water was used as control treatment.

Lipase production

Lipase produced by the pure culture was determined using tributyrin agar plates. Plates were inoculated and incubated at 30^{0}C for 24 hrs and the plates were examined for clear zone around the colonies[23] and positive CTAB test[24,25].

Extraction and Purification of the Produced Biosurfactants

The pH of the remaining supernatant was adjusted to pH 2.0 with 1 N HCl to precipitate rhamnolipid. The precipitate was harvested by centrifugation (9000 g, 20 min) and was then extracted three times with ethyl acetate at room temperature. The organic phase was collected and the solvent was removed in a rotary evaporator, allowing the yield of viscous honey-colored rhamnolipid product[26]. The extracted viscous honey-coloured rhamnolipids product was collected for further purification using chromatographic procedures. The fractions that demonstrated the oil displacement test were further separated by Thin Layer Chromatography (TLC) using aluminium silica gel 60 F254 plates and a chloroform:methanol:20% aqueous acetic acid (65:15:2) solvent system[27]. The plate was developed with the solvent system, chloroform: methanol: acetic acid in the ratio 65:15:2 (v/v/v) and air dried. The mono rhamnolipids and dirhamnolipids were detected (Koch *et al*., 1991) by combining two methods to ensure the perfect rhamnolipids by their relative *Rf* values[27]. The first method is spraying with p-anisaldehyde [anisaldehyde: sulphuric acid: glacial acetic acid 0.5:1:50] to spot carbohydrates and the second method is by spraying the plates initially with 1% α-naphtol solution and later by sulphuric acid: ethanol in the ratio 1:1 to spot fatty acids. The plates were heated in an oven at a temperature of 120-150^{o}C for 15-20mins for development of the colour. Later the plates were observed for the colored spots, and their *Rf* was calculated.

Determination of the Critical Micelle Concentration (CMC)

The CMC of the biosurfactant was determined by standard methods as reported by Haba *et al*[28]. The surface tension of the biosurfactant was measured by

the Du-Nouy ring method at room temperature. The concentration at which micelles began to form was represented as the CMC and a sudden change in surface tension was observed. The CMC was determined by plotting the surface tension as a function of the biosurfactant concentration.

Determination of the Emulsification Index

A mixture of 2 mL supernatant and 3 mL kerosene (or diesel) was vertically stirred for 2 min and the height of the emulsion layer was measured after 24 hr to determine the emulsification index[29,30]. The equation used to determine the emulsification index (E24 (%)) is as follows:

The E24 (%) = (height of emulsion layer/ height of total solution) *100%

Determination of stability of the biosurfactant

Determination of the effect of temperature, pH and sodium chloride on the activity of the biosurfactant was studied[31]. The thermal stability of the biosurfactant was determined by maintaining the supernatant at constant different range of temperature from 30–100 ^{0}C for 15 min and cooled at room temperature. To determine the effect of pH on activity, the pH of the cell free broth was adjusted to different values using 1 N NaOH or 1 N HCl. The effect of addition of different concentration of NaCl on the activity of the biosurfactant was investigated.

Characterization of biosurfactant

Fourier transform infrared spectroscopy (FTIR) is widely used method for identifying the types of chemical bonds (functional groups). It can be used to elucidate some components of unknown mixture. The molecular characterization was performed using one milligram of freeze dried partially purified biosurfactant which was ground with 100 mg of KBr and pressed with 7500 kg for 30 sec to obtain translucent pellets. Infrared absorption spectra were recorded on a Thermo Nicolet, AVATAR 330 FTIR system with a spectral resolution wave number accuracy of 4 and 0.01 cm-1, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as a background reference[32].

RESULTS AND DISCUSSION

Screening and identification of Chlorpyrifos degrading bacteria

The enrichment of microbial populations present in different soil samples grown on minimal agar supplemented with chlorpyrifos as a sole carbon source. One morphologically distinct strain was selected on the basis of their ability to degrade chlorpyrifos and to produce biosurfactant. The potential isolated strain was identified by biochemical tests and partial16S rRNA sequencing using universal primers (B8F and B1500 R).Based on biochemical tests the isolate MCAS01 was tentatively identified as *Pseudomonas* spp. Based on nucleotide sequence and phylogenetic tree analysis, MCAS01 was identified as *Pseudomonas stutzeri*. The nucleotide sequence of *Pseudomonas stutzeri* was deposited in NCBI gene bank with accession numberKT757902.The phylogenetic trees were constructed using MEGA 7.0 software, shown in Figure 1. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions with less than 95% site coverage were eliminated.

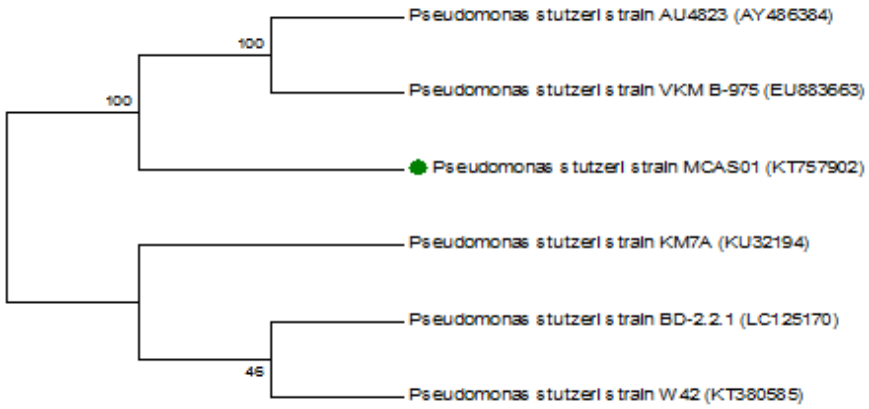

Figure 1

Phylogenetic tree of 16S rRNA sequences of Pseudomonas stutzeri MCAS01. The scale bar represents 0.05 substitutions/base position. Bootstrap values >50 for 1000 resamplings (≥50% of the trees support the node) are shown for each node. Black circles delineate samples sequenced in this study. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions with less than 95% site coverage were eliminated. *Pseudomonas stutzeri* MCAS01 were grown in liquid culture medium containing chlorpyrifos as the sole carbon source. The time course of chlorpyrifos metabolism by *Pseudomonas stutzeri* MCAS01 is shown in Figure 2 where complete disappearance of 50 mg chlorpyrifos/l was observed within 7 days.

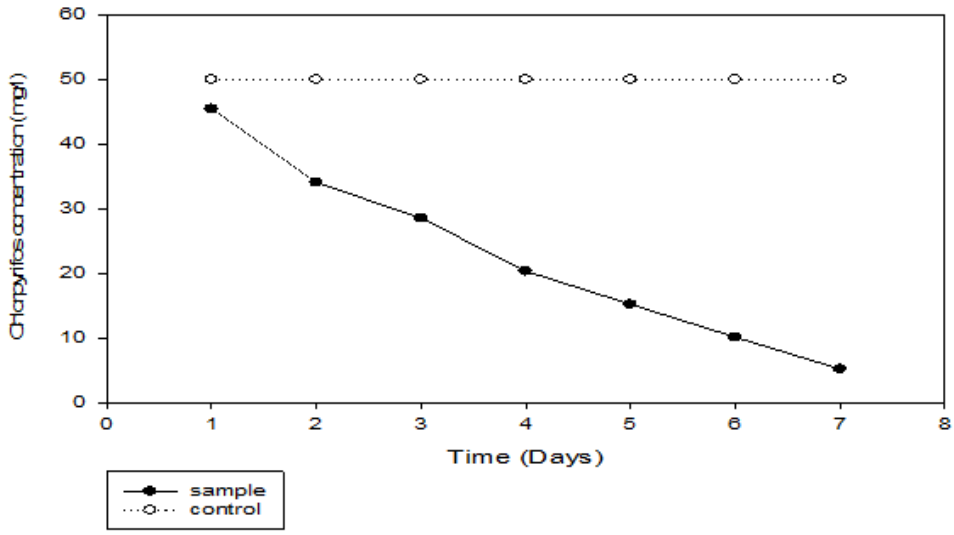

Figure 2
Bacterial growth and degradation of chlorpyrifos (50 mg/l) by Pseudomonas stutzeri MCAS01 cultivated in mineral salts medium. Values are means ± standard deviations of three replicates.

Screening for biosurfactant activity

Screening of biosurfactant production by testing the Haemolytic activity of *Pseudomonas stutzeri* MCAS01showed the zone of inhibition (Figure 3). In the present study, a significant correlation was established between the haemolytic activity and biosurfactant production. According to Carrillo *et al*[21] and Banat[33], biosurfactant production of the new isolates was preliminary screened by haemolytic activity. Blood–agar lysis has been used to quantify surfactant and rhamnolipids. Carrillo et al found an association between haemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen biosurfactant production. From the above observation, it was confirmed that the *Pseudomonas stutzeri* MCAS01 was a potent biosurfactant producer. Both the techniques have several advantages such as small volume of samples was required, rapid and easy to carry out and also do not require specialized equipment. This also confirmed by comparing with previous report[34].

Figure 3
Pseudomonas stutzeri MCAS01showed the zone of inhibition on Blood–agar plate

Biosurfactant production and recovery

The surface-active molecules produced by *Pseudomonas stutzeri* was recovered by acid precipitation. The biosurfactants extracted was characterized by using TLC. The biosurfactant belonged to the class of Rhamnolipid where the sugar moiety was specifically Rhamnose. Similar results were also reported of rhamnolipid from *Pseudomonas aeruginosa* by TLC analysis[35]. In this system, mono-rhamnolipid has an R_f value of 0.71 and di-rhamnolipid has an R_f value of 0.44.

Temperature Stability

The stability of biosurfactant was tested over a wide range of temperatures 55°C-100°C. Rhamnolipidsproduced by *Pseudomonas aeruginosa* strain F23 was shown to be thermostable in nature which could be attributed to the presence of fatty acid tails in its structure. Biosurfactants from *P. aeruginosa* R2[36] , *Brevibacterium aureum* MSA13 were reported to be thermostable. Biosurfactants exhibit thermostability[31,37] but synthetic surfactants such as SDS exhibit a significant loss of emulsification activity at 70°C[38].

FTIR analysis

Through the FT-IR spectrum of the pure biosurfactant produced by *Pseudomonas stutzeri* MCAS01 (Figure 4), the presence of a rhamnolipid structure, which is composed of rhamnose rings and long hydrocarbon chains, is clearly indicated by the absorbance bands at the wave numbers of 3448.1 cm-1, 2934.1 cm-1, 1638.7 cm-1, 1089.58 cm-1 and 991.714 cm-1. The strong absorbance at 1,638.7cm”1, which is considered to be the characteristic peak of biosurfactants by many researchers[39] must be assigned to the C–H stretching vibrations of the hydrocarbon chain positions. However, the strong absorbance in the range of 1,300–991.714 cm”1 indicates the presence of bands formed between carbon atoms and hydroxyl groups in the chemical structures of rhamnose rings. The findings of this study were in accordance with Rahman *et al*[40] and Saravanan and Vijayakumar[41] were reported the biosurfactant production by Pseudomonas aeruginosa. Similar characteristic bands were also observed in FTIR spectrum for rhamnolipid produced by various *Pseudomonas* strains by many researchers[36,38,42,43].

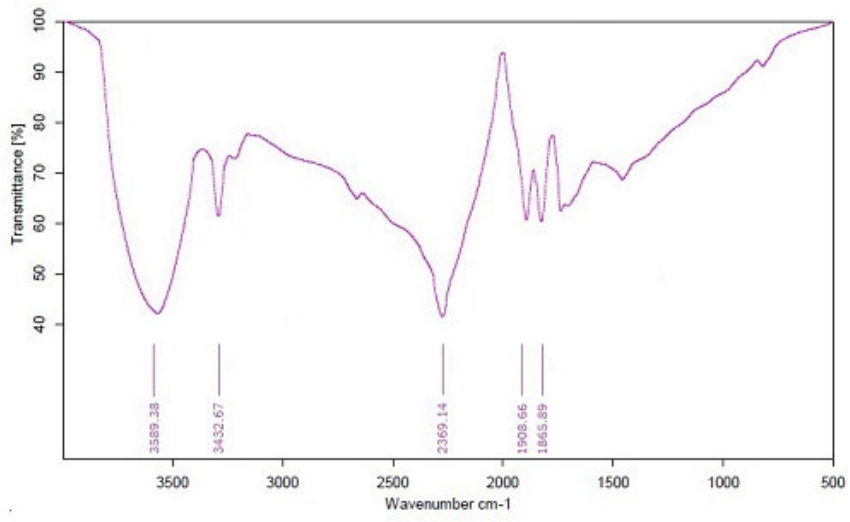

Figure 4

Characterization of the dried biosurfactant from Pseudomonas stutzeri MCAS01 by FT-IR spectrum profile.

CONCLUSION

In the basis of the study, one of the chlorpyrifos degrading isolate was obtained from soil taken from contaminated soil, which has long history of chlorpyrifos usage. It was concluded that the identified organism, *Pseudomonas stutzeri* MCAS01 was able to degrade Chlorpyrifos under laboratory conditions. Microorganisms are a major component of the ecosystem and play a considerable role in the degradation of pesticides. *Pseudomonas stutzeri* MCAS01 shows an immense capability of biodegradation of chlorpyrifos pesticide. So use of this bacterial strain to degrade the pesticide like chlorpyrifos will be helpful to make toxic free agriculture practices. The biosurfactant produced by this organism was found to be specific to organophosphates and it also plays a major role in enhancing the degradation processes of organophosphates. The excellent surface tension reducing property, CMC and the absence of toxicity suggest the possibility of the use of this biosurfactant in a wide variety of industrial, environmental and biotechnology applications.

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CONFLICT OF INTEREST

The authors confirm that this article has no conflict of interest.

REFERENCES

1. WHO. Health implications from monocrotophos use: a review of the evidence in India. World Health Organization, Regional Office for South-East Asia, New Delhi. 2009. Available on: http: //203.90.70.117/PDS_DOCS/B4293.pdf.
2. Shetty PK, Murugan M, Hiremath MB and Sreeja KG. Farmers' education and perception on pesticide use and crop economies in Indian agriculture. Journal of Experimental Sciences. 2010; (1):3-8.
3. Singh PB, Sharma S, Saini HS, and Chadha BS. Biosurfactant production by Pseudomonas sp. And its role in aqueous phase partitioning and biodegradtion of chlorpyrifos. Letters in Microbiology. 2009; 49, 378-383.
4. Kumar S, Pathak A and Mangal HM. Trends of Fatal Poisoning in Saurashtra Region of Gujarat. J. Indian Academic Forensic Med. July-September. 2011; 33(3):197-199.
5. Sharma DR, Thapa RB, Manandhar HK, Shrestha SM, and Pradhan SB. Use of pesticides

in Nepal and impacts on human health and environment. The Journal of Agriculture and Environment. 2012; 13: 67-74.
6. Gulati K, Thakur S and Jindal T. Chlorpyrifos toxicology and persistence in environment: An Indian Perspective. International Journal of Multidisciplinary Research and Development. 2015; 2: 7, 1-6
7. Singh BK, Walker A, Morgan JAW and Wright DJ. Biodegradation of chlorpyrifos by Enterobacter strain B-14 and its use in bioremediation of contaminated soils. Appl Environ Microbiol 2004; 70, 4855–4863.
8. Lakshmi CV, Kumar M and Khanna S. Biodegradation of chlorpyrifos in soil by enriched cultures. Curr Microbiol. 2008; 58, 35–38.
9. Naphade SR, Annika A, Durve, Meeta Bhot, Jossy Varghese and Naresh Chandra. Isolation, characterization and identification of pesticide tolerating bacteria from garden soil. European Journal of Experimental Biology, 2012; 2 (5):1943-1951
10. Harishankar MK, Sasikala C. and Ramya M. Efficiency of the intestinal bacteria in the degradation of the toxic pesticide, chlorpyrifos 3 Biotech. 2013; 3:137–142.
11. Barathidasan K, Reetha D, John Milton D, Sriram N, and Govidammal M. Biodegradation of chlopyrifos by co-culture of *Cellulomonas fimi* and *Phanerochaete chrysosporium.* African Journal of Microbiology Researh. 2014; 8(9), 961-966.
12. Volkering F, Breura, AM and Rulkens WH. Microbiological aspects of surfactant use for biological soil remediation. Biodegradation. 1998; 8, 401–417.
13. Bellingsley KA, Backus SM and Ward OP. Effect of surfactant solubilization on biodegradation of polychlorinated biphenyl congeners by *Pseudomonas LB400*. Appl Microbiol Biotechnol. 1999; 52, 255–260.
14. Noordman WH, Wachter JHJ, De Boer GJ and Janssen DB. The enhancement by surfactants of hexadecane degradation by *Pseudomonas aeruginosa* varies with substrate availability. J Biotechnol. 2002; 94, 195–212.
15. Rodrigues L, Banat IM, Teixeira J and Oliveira R. Biosurfactants: potential applications in medicine. J. Antimicrob. Chem. 2006; 57:609-618.
16. Thavasi RS, Jayalakshmi, Balasubramaian T, Banat IM. Biosurfactant production by *Pseudomonas aeruginosa* from renewable resources. Indian J Microbiol. 2011; 51: 30-36.
17. Gomathy C and Senthilkumar R. Production of rhamnolipid biosurfactant from a Marine *Pseudomonas aeruginosa.* International Journal of Research in Environmental Science and Technology. 2013.
18. Bhagobathy RK and Abdul malik. Utilization of chlorpyrifos as a sole source of Carbon by bacteria isolated from Waste water irrigated Agricultural soils in an industrial area of Western Uttar Pradesh, India. Research Journal of Microbiology. 2008; 3 (5): 293-307.
19. Rokade KB and Gajanan, VM. Biodegradation of chlorpyrifos by pseudomonas desmolyticum ncim 2112. Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 609 – 616.
20. Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 2013; 30(12):2725–2729.
21. Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giulietti AM. Isolation and selection of biosurfactant-producing bacteria. World J Microbiol Biotechnol. 1996; 12:82–84
22. Youssef NH, Duncan KE, Nagle D, Savage KH, Knapp R and McInerney MJ. Comparison of methods to detect biosurfactant production by diverse microorganisms. *J Microbiol Methods*. 2004; 56: 339-47
23. Gandhimadhi R, Sehgalkiran G, Hema TA, Selvin J, and RajeethaRaviji T. Production and characterization of Lipopeptide biosurfactant by a sponge associated marine actinomycetes *Nocardio psisalba* MSA10. Bioprocess Biosyst. Eng. 2009; 32 (16), 825-835.
24. Siegmund I, and Wagner F. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. Biotechnol. Tech. 1991; 5:265-26
25. Satpute SK, Bhawsar BD, Dhakephalkar PK. and Chopade BA. Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Ind. J. Mar. Sci. 2008; 37: 243–250.
26. Chen SY, Wei YH and Chang JS. Repeated pH-satisfied batch fermentation for rhamnolipid production with indigenous Pseudomonas aeruginosaS2. Appl Microbiol Biotechnol. 2007; 76(1): 67-74.
27. Koch AK, Kappeli O, Fiechter A, and Reiser J. Hydrocarbon assimilation and biosurfactant production in Pseudomonas aeruginosa mutants. J Bacteriol. 1991; 173:4212–4219
28. Haba E, Espuny MJ, Busquets M and Manresa A. Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. J Appl Microbiol. 2000; 88, 379–387.
29. Cooper, D.G., C.R. Macdonald, S.J.B. Duff and N. Kosaric, 1981. Enhanced production of surfactin from Bacillus subtilis by continous product removal and metal cation additions. Appl Environ Microbiol, 42: 408-412.
30. Das P, Mukherjee S and Sen R. 2009. Substrate dependent production of extracellular biosurfactant by marine bacterium. Bioresource Technology. 100(2009), 1015-1019.
31. Khopade A, Biao R, Liu X, Mahadik K, Zhang L, et al. (2012) Production and stability studies of biosurfactant isolated from marine *Nocardiopsis* sp. B4. Desalination 285: 198-204.
32. Thaniyavaran J, Roongsawang N, Kameyama T, Haruki M, Imanaka T, Morikawa M and Kanaya S. Production and Characterization of Biosurfactant from *Bacilluslicheniformis* F2.2. Biosci. Biotechnol. Biochem. 2006; 67: 1239-1244.

33. Banat I M, Franzetti A, Gandolfi I, Bestetti G, Martinoti M G, Fracchia L, Smyth T J, and Marchant R. Microbial biosurfactants production, applications and future potential. Appl. Microbiol. Biotechnol. 2010; 87. 427-444.
34. Chandankere R, Jun Y, Choi MMF, Masakorala K, and Chan Y. An efficient biosurfactant-producing and crude-oil emulsifying bacterium *Bacillus methylotrophicus* USTBa isolated from petroleum reservoir. Bio chem. Eng. J. 2013; 74: 46 53.
35. Anandaraj B. and Thivakaran P. Isolation and production of biosurfactant producing organism from oil spilled soil. J Biosci Tech. 2010; 1(3): 120 126.
36. Karkera K, Pendse A and Aruna K. Studies of Biosurfactant production by *Pseudomonas aeruginosa* R2 isolated from oil contaminated soil sample. Asian Journal of Bio Science. 2012; 7(2):123-129.
37. Aparna A, Srinikethan G, Hegde S Isolation, screening and production of biosurfactant by *Bacillus clausii* 5B. Res in Biotechnol. 2012; 3: 49-56.
38. Abouseoud M, Macchi R, Amrane A, Boudergua S, and Nambi A. Evaluation of different carbon and nitrogen sources in production of biosurfactant by Pseudomonas fluorescens. Desalination. 2008; 223, 143-151.
39. Pornsunthorntawee O, Arttaweeporn N, Paisanjit S, Somboonthanate M, Abe, Rujiravanit R and Chavadej S. Isolation and comparison of abiosurfactants produced by Bacillus subtilis and Pseudomonas aeruginosa SP4 for microbial surfactant enhanced oil recovery. Biochemical Engineering, J., 2008; 42: 172-179.
40. Rahman KSM, Rahman TJ, McClean S, Marchant R and Banat I.M. Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost raw materials. Biotechnol Prog. 2007; 18:1277-1281.
41. Saravanan V and Vijayakumar S. Isolation and screening of biosurfactant producing microorganisms from oil contaminated soil. J. Acad. Indus. Res. 2012; 1(5), 264-268.
42. 6. Zhang Y and Miller RM. Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl Environ Microbiol. 1994; 60, 2101–2106.
43. Xia W, Zhi-Bin L, Han-Ping D, Li Y, Qing-Feng C. and Yong-Qiang. Biosynthesis, Characterization, and Oil Recovery Application of Biosurfactant Produced by Indigenous *Pseudomonas aeruginosa* WJ-1 Using Waste Vegetable Oils. Appl Biochem Biotechnol. 2012; 166:1148–116