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ORIGINAL ARTICLE

Identification of Biosurfactant producing Bacterial strains and its application as a consortium for the removal of used Engine oil

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ABSTRACT
Biodegradation of used engine oil with biosurfactant producing bacterial consortium is considered as the most effective way in terms of its high success. Here, removal of the used engine oil (UEO) by a consortium of Bacillus cereus and Serratia marcescence was evaluated. The UEO degradation and production of biosurfactants was analysed by Fourier Transform Infrared Spectroscopy (FT/IR). The work reported that the bacterial strains used for the formulation of consortium produced glycolipid biosurfactants with rhamnose ring and showed 72% of removal of UEO after 10 days of incubation. The FT/IR spectra of treated UEO sample showed the absence of many corresponding peaks indicated the effective degradation of UEO.
***Keywords**: Bacterial consortium, Biodegradation, Biosurfactants, Fourier Transform Infrared Spectroscopy, Used engine oil.*

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INTRODUCTION

Environmental pollution with used engine oil (UEO) or used motor oil has been recognised as one of the most serious problems nowadays. UEO is a liquid mixture consisting of different components like aliphatic and aromatic hydrocarbons, polychlorinated biphenyls, different lubricating additives, heavy metal contaminants etc. A huge amount of used engine oil is generated in every year from different sources and is dumped in our environment without proper recycling. Accumulation of such toxic materials affects the quality of our environment and would contribute to chronic health problems including mutagenicity and carcinogenicity [1]. So it is needed to find out efficient methods for the removal of such toxic chemicals from our ecosystem [2]. In recent time different methodologies like mechanical, chemical and biological methods are used for the removal of used engine oil. Treatment methods like incineration, burial in landfills reduce such pollution, but are expensive, time consuming and non eco-friendly. But bioremediation which exploits the ability of microorganisms is found out the most efficient, economic, affordable and ecofriendly method for the removal of used engine oil from our ecosystem [3].

The used engine oils are hydrophobic and less water soluble in nature. Hence the pollutant becomes less available to microorganisms. The compounds are slowly released into the water phase and that could result in taking more time for biodegradation process [4].This difficulty can be overcome by the application of surfactants which made the pollutant available for the microorganisms to degrade. Surfactants are surface active compounds that reduce the interfacial tension between two liquids or that between a liquid and solid. Biosurfactants are synthesised by microorganisms like bacteria, fungi and yeast and are non-toxic and biodegradable. Biosurfactants have got increased interest because of its diversity, flexibility and eco-friendly nature [5, 6, 7] and recently the application of biosurfactants produced by microorganisms are adopted to speed up the biodegradation process of used engine oil. Various of microorganisms like *Pseudomonas sp* [8,9], *Bacillus sp* [10, 11], *Acenetobacter sp* [12], *Alcaligenes sp* [13], *Serratia sp* [14, 15], *Candida sp* [16], *Enterococcus sp* [17], *Lactic acid bacteria* [18, 19, 20] etc have been reported as biosurfactant producers and produce different types of biosurfactants such as glycolipids, fatty acids, lipopeptides and lipoproteins, glycoproteins and neutral lipids. UEO is a mixture of different hydrophobic components and so it is difficult to remove all such components with the action of a single type of microorganism. So the effective degradation of used engine oil needs the cooperative action of different microbial strains with known degradative capacities.

The present study was aimed to construct a biosurfactant producing bacterial consortium for the enhanced removal of used engine oil from the oil containing sample and also investigate the structural and functional components of biosurfactants.

MATERIAL AND METHODS

Soil sample

Oil spilt soil sample was used as a sample for the screening of biosurfactant producing bacterial strains for the degradation of used engine oil (UEO). The sample was collected from an oil logging area located near Aluva, Ernakulam, Kerala. The sample was collecting after removing the surface layer and transferred to the laboratory for the isolation of bacterial strains.

Isolation of biosurfactant producing bacterial strains

Biosurfactant producing bacterial strains for UEO degradation was isolated through the soil enrichment method. 10 grams of soil sample was added to 100 mL mineral salt (MS) medium with 2.5 mL used engine oil as the only carbon source in a 250 mL Erlenmeyer flask. The MS medium was formulated by mixing 1g KH_2PO_4, 1g $(NH_4)_2SO_4$, 0.5 g Mg $SO_4.7H_2O$ and 0.001g $CaCl_2$ in 1 litre of the distilled water. The sample was incubated at 37 ^{0}C for 2 days at 150 rpm. The pH of the medium was adjusted to 7.0 ± 0.2. The enrichment was initiated at the concentration of 2.5 mL of UEO and the soil extract was enriched up to a maximum of 10 mL of UEO. The soil extract was kept for 2 days for each concentration of UEO, ie, 5, 7.5 and 10 mL and the enrichment was finished with 8 days. After enrichment, the soil extract was pour platted with nutrient agar medium. The plates were incubated at 37 ^{0}C for 24 h and the individual isolates were selected.

Screening of biosurfactant producing strains

Screening of biosurfactant producing strains was done by growing the selected individual strains in the screening medium composed of 20 g glucose, 10 g peptone, 8 g meat extract, 4 g yeast extract, 2 g di-potassium hydrogen phosphate, 2 g di-ammonium hydrogen citrate, 5 g sodium acetate, 0.2 g magnesium sulphate and 0.05 g manganese sulphate and 1 g Tween-80 in 1 litre of distilled water [20]. Isolation of surfactant producing bacterial strains was conducted using the streak plate method in the nutrient medium after 5 days of incubation.

Extraction of biosurfactants

The experiments were carried out in 250 mL Erlenmeyer flasks with 100 mL screening medium and the individual strains were inoculated and incubated at 37 ^{0}C for 5 days. After incubation, the supernatant was collected by centrifuging the medium at 10,000 rpm for 10 min. The supernatant was acidified to pH 2 using 6N HCl and stored at refrigerator overnight. After overnight storage, the sample was extracted with diethyl ether and the production of biosurfactants was monitored by FT/IR analysis at the Department of Chemical Sciences, MG University, Kottayam.

Identification of biosurfactant producing strains

The identity of biosurfactant producing strains was confirmed 16S rDNA sequence analysis. Polymerase chain reaction (PCR) was carried out in Mycycler™ (Bio-Rad, USA) using forward primer sequence (5´-AGA GTT TGA TCM TGG CTC-3´) and the reverse sequence (5´-AAG GAG GTG WTC CAR CC-3´). PCR products were sequenced at Scigenome labs, Pvt Ltd, Cochin, Kerala.

Formulation of a consortium with biosurfactant producing strains

Individual bacteria were grown in nutrient broth supplemented with 5 mL of UEO and the flasks were incubated overnight at 37 ^{0}C at 150 rpm. From the culture the cells were harvested by centrifugation and re-suspended in sterile saline to yield an absorbance reading of 1 at 540 nm. The consortium was constituted by mixing equal proportions, ie, 3 mL of individual strains which produce biosurfactants.

Biodegradation of UEO

Mineral Salt medium with 10 mL of UEO was used as the sample for biodegradation studies. The MS-UEO medium was inoculated with 5 mL of formulated bacterial consortium and carried out the biodegradation at 37 ^{0}C for about 10 days. After biodegradation, bacterial cells were removed by centrifugation at 10,000 rpm for 10 min. The collected supernatant was subjected to FT/IR for analysing the biodegradation efficiency. The biodegradation efficiency of the formulated consortium, when compared with individual strains, was also analysed by calculating the % removal of used engine oil from the medium.

$$\% \text{ removal of UEO} = \frac{\text{Initial OD at 391nm - Final OD at 391nm}}{\text{Initial OD at 391nm}} \times 100$$

where λmax of control MS-UEO medium was considered as initial OD. The spectral scanning of the samples was conducted between 200-800 nm using Hitachi spectrophotometer.

RESULTS AND DISCUSSION

Biodegradation of used engine from the polluted area could be done either by physical, chemical or biological methods. But biological methods get more attention now and are mainly by the activities of different microorganisms. Bioremediation is having more important because of its low environmental impacts, low cost and the possibility of beneficial use of treated pollutants. Many previous studies isolated different varieties of microorganisms [21, 22, 23] and have shown better oil degradation efficiency.

Isolation of biosurfactant producing bacterial strains

The microbial strains which have the capacity of oil degradation were isolated from contaminated soil through enrichment techniques. In the present study, oil spilt soil sample was enriched with used engine oil, initiated at a concentration of 2.5 mL and enriched up to a maximum of 10 mL. After progressive enrichment, a total of 7 bacterial colonies were isolated through pour plating on nutrient agar medium.

Screening of biosurfactant producing strains

All the 7 colonies isolated through soil enrichment were individually incubated in 100 ml screening medium and incubated at 37 ^{0}C for 5 days. Only two isolates were grown in the specific screening medium after 5 days of incubation. These isolates were denoted as sample 1 and sample 2 and selected as biosurfactant producing strains. The strains were stored for the analysis of biosurfactant production and biodegradation studies.

Individual strains degrade only a limited range of components present in a pollution mixture like engine oil. So many researchers focussed their studies on mixed microbial strains for better degradation [24]. A microbial consortium of *Pseudomonas sp, Acinetobacter sp* and *Bacillus sp* was reported for their high efficiency in used engine oil degradation [25]. A Degradation rate of 90% of the used engine was reported by a bacterial consortium formulated by *Pseudomonas aeruginosa, Serratia marcescens* and *Bacillus licheniformis* [26]. Along with consortia formulation, the production of biosurfactants also enhances the degradation of used engine oil. Biosurfactants are considered as the most suitable emulsifiers for the degradation of UEO because it enhances the bioavailability of hydrophobic components to microorganisms [27]. Many bacterial genera were reported for the production of biosurfactants and their involvement in petroleum oil degradation [28, 29]. A reported consortium of *Stenotrophomonas maltophilia*, *Bacillus cereus* and *Bacillus pumilus* effectively degraded used engine oil [30]. A biosurfactant producing bacterial consortium of *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2 was reported for their better efficiency in UEO degradation [31]. The current study formulated a bacterial consortium with *Bacillus cereus* and *Serratia marcescence* which showed efficient biodegradation of UEO. The *Bacillus* sp is a versatile species which exhibits extensive production of biosurfactants [32, 33, 34].

FT/IR analysis of biosurfactants

The preliminary analysis for the production of biosurfactants was conducted using FT/IR analysis. Many researchers used FT/IR as one of the most useful analytical methods for the elucidation of functional groups present in biosurfactant [35, 36]. The FT/IR analysis of biosurfactants produced in the present study indicated the presence of important absorbance peaks which represent glycoprotein moieties. This result is in agreement with many previous studies [35, 37, 38].

After acidification and extraction with diethyl ether, the supernatant was subjected to FT/IR analysis for the confirmation of biosurfactant production. The FT/IR spectra (Fig 1 A, B) showed many absorption peaks, which was produced by the presence of specific functional groups in the samples. In sample 1 (Fig 1A) the most important peak at 3332.17 represented the O-H and C-H stretching. Peaks at 2920.16, 2851.46, 1459.69 and 1376.13 represented the presence of C-H stretching of the aliphatic group. Absorbance at 1727.49 and 1632.94 indicated the presence of ester (C=O) and carbonyl groups (COO^-) and peaks at 1119.20 and 1.69.71 indicated the presence of C-O-C group, which represents the rhamnose ring in the compound. Corresponding absorption peaks were observed in the IR spectrum of sample 2 except the peak at the 3600-3100 region (Fig 1B). The FT/IR spectral analysis clearly indicated the production of biosurfactants by the isolated bacterial strains. The chemical structure of biosurfactants produced by both the strains (Sample 1 and 2) was identical to glycolipids with rhamnose ring.

Identification of biosurfactant producing strains

PCR products were sequenced and the sequence similarities were analysed by sequences available in the National Centre for Biotechnology Information (NCBI) database using BLAST (Basic Local Alignment Search Tool) analysis. 16S rDNA analysis revealed that the bacterial sample 1 showed 98% similarity with *Bacillus cereus* and sample 2 showed 96% similarity with *Serratia marcescence*.

Biodegradation of UEO

FT/IR analysis of ether extracted Mineral Salt- used engine oil (MS-UEO) medium degraded with formulated bacterial consortium was conducted after 10 days of incubation. When compared with the IR spectrum of uninoculated MS-UEO medium (Fig 2). The FT/IR spectrum of the treated sample (Fig 3) showed many differences. Its showed the absence of many peaks present in the control sample and also showed a decrease in peak sharpness at the region between 3000-500 cm^1. These changes were a strong indication of degradation and the removal of many components present in the UEO sample.

The spectroscopic analysis indicated that the control MS-UEO medium showed an absorbance maximum of 3.12 at 391 nm, which was considered as the initial OD to calculate the % removal of engine oil. During biodegradation with individual strains, *Bacillus cereus* showed 55% and *Serratia marcescence* showed 50% removal of UEO. But the application of formulated consortium showed 72 % of UEO removal during treatment. This indicated the efficiency of formulated consortium in UEO degradation when compared with individual strains.

Further detailed studies like Nuclear Magnetic Resonance (NMR), Gas Chromatography-Mass Spectroscopy (GC-MS) etc are needed to illustrate the actual pathway involved in the biodegradation process, metabolites produced and the chemical structure of biosurfactant produced during treatment.

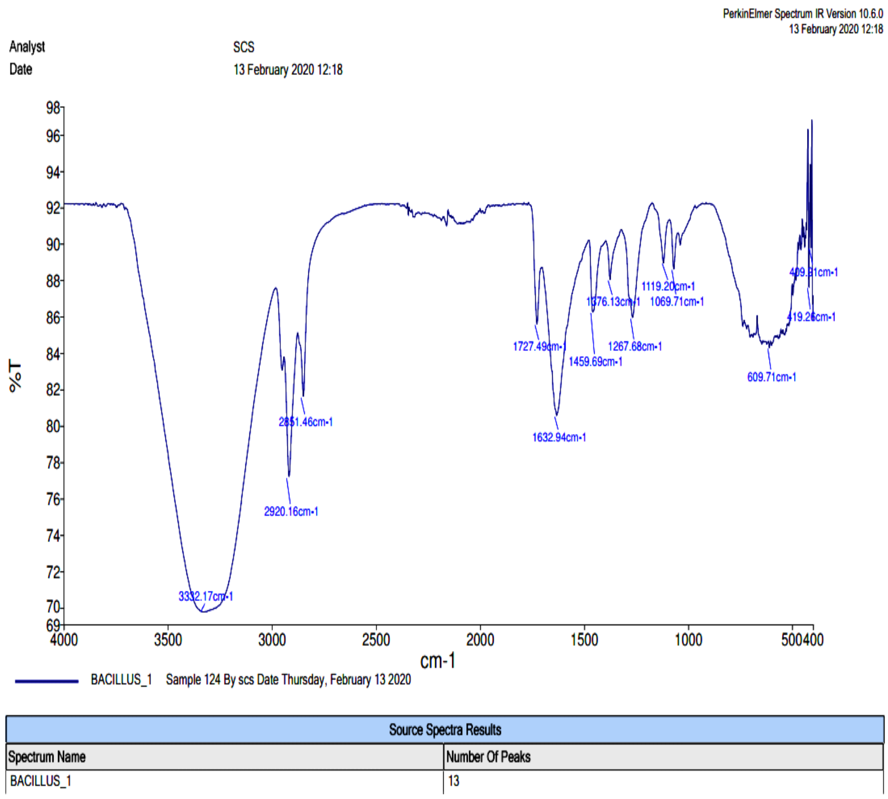
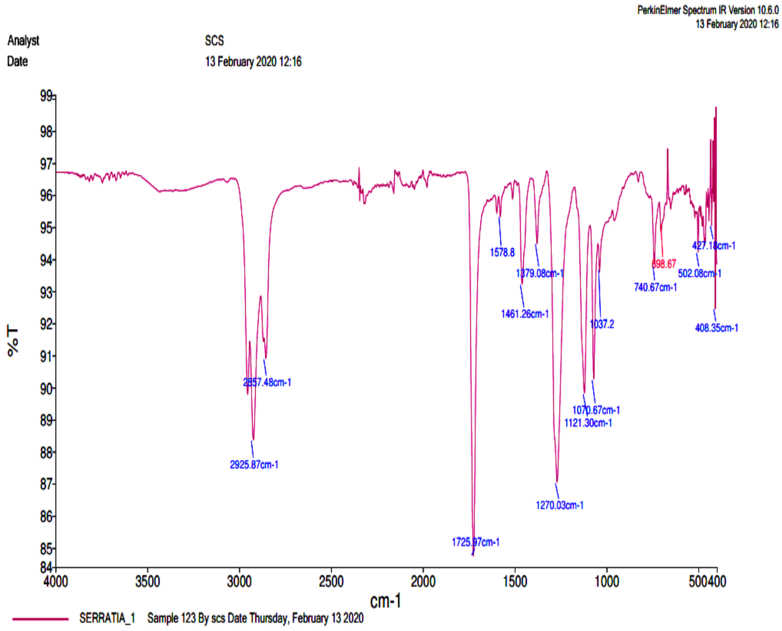


Fig 1 A: FT/IR spectrum of biosurfactant produces by *Bacillus cereus* (Sample 1). Spectra indicated the presence of biosurfactant which was a glycolipid with rhamnose ring



Source Spectra Results	
Spectrum Name	Number Of Peaks
SERRATIA_1	12

Fig 1 B: FT/IR spectrum of biosurfactant produces by *Serratia marcescence* (Sample 2). Spectra indicated the presence of biosurfactant which was a glycolipid with rhamnose ring.

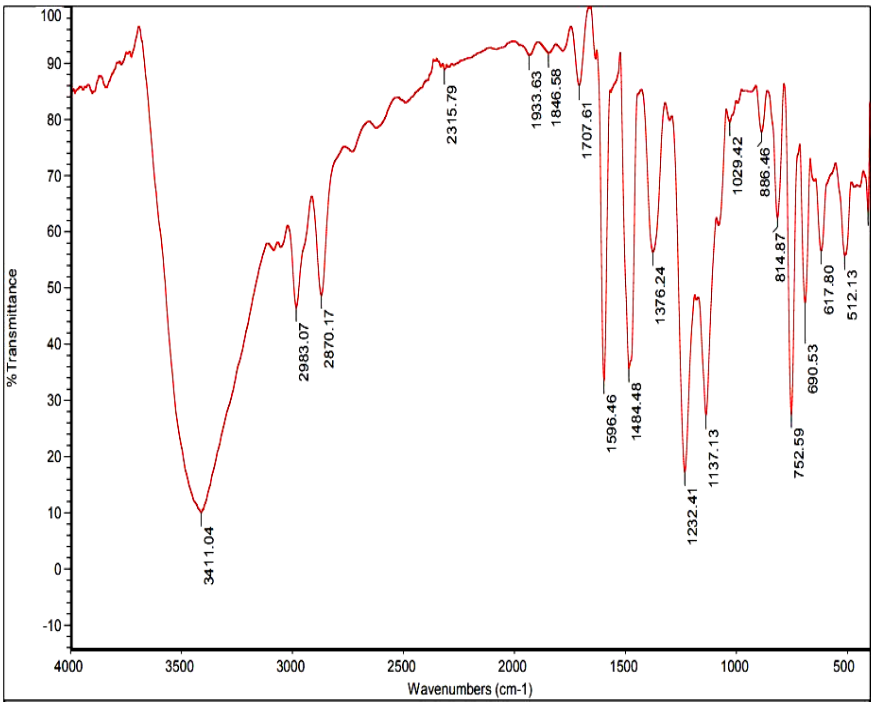


Fig 2: FT/IR spectrum of uninoculated MS-UEO medium. Spectrum indicated the presence of specific absorbance peaks which indicated the different components of used engine oil.

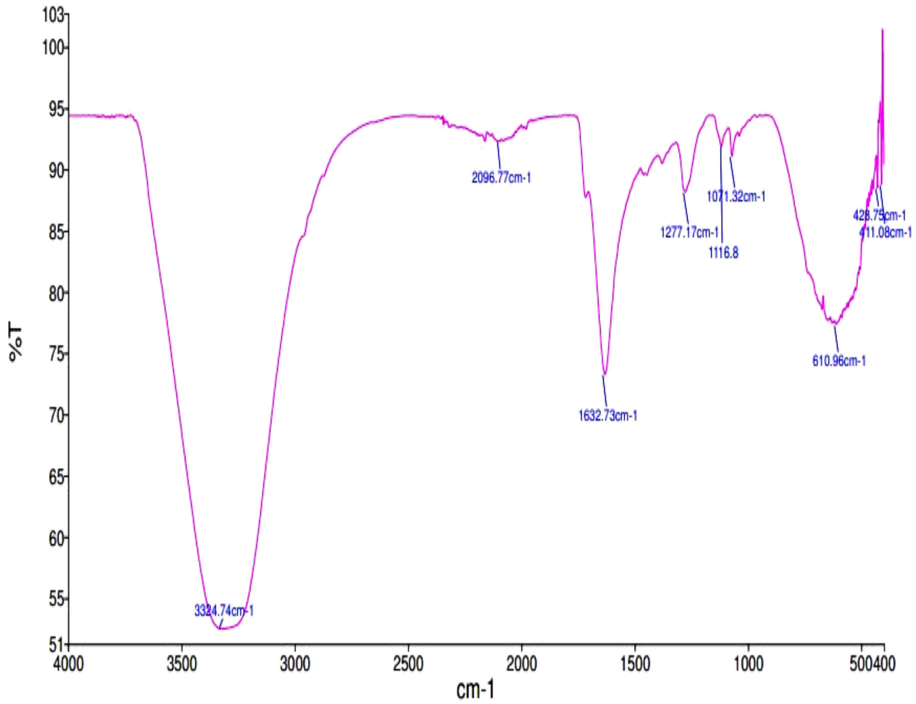


Fig 3: FT/IR spectrum of treated MS-UEO medium. Spectrum indicated the absence of specific absorbance peaks which indicated the different components of used engine oil.

CONCLUSION

In this study, used engine oil contaminated soil sample was collected for the isolation of biosurfactant producing strains and for the formulation of bacterial consortium for the better removal of UEO from the medium. *Bacillus cereus* and *Serratia marcescence* were selected for the formulation of the consortium, showed 72% removal of UEO after 10 days of incubation, which was higher than that of biodegradation rate by individual strains. The selected strains produced glycolipid biosurfactants with rhamnose ring. The formulated bacterial consortium used in the study will open up a new way for the degradation of used engine oil from polluted sites. The methodologies proposed in the present study will open up a new possibility for the removal of used engine oil from the polluted sites.

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AUTHOR'S CONTRIBUTIONS STATEMENT

The author's of the manuscript has made substantial contributions and the work is an accurate representation of the trial results.

COMPLIANCE WITH ETHICAL STANDARDS

We do not have any financial relationship with any type of organization, funding agencies and project sponsors.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The author's declared that they have no conflict of interest in the publication.

ETHICAL APPROVAL: RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS

This article does not contain any studies with human participants and/or animals performed by the author.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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