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Biosurfactant Producing Bacteria from Groundnut Oil Cake and its Application in Pesticide Removal

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ABSTRACT

A member of the class of organophosphate pesticides that are commonly used in agricultural activities worldwide is ethion, also known by its chemical name, phosphorodithioate, or O, O, O', O'-tetraethyl S, S'-methylene bis. Its broad-spectrum insecticidal properties make it indispensable for controlling various pests in crops such as fruits, vegetables, and cereals. However, the indiscriminate use of ethion raises concerns due to its toxicity to non-target organisms, persistence in the environment, and potential adverse effects on human health. It was studied how bacteria that produce biosurfactants and were isolated from groundnut oil cake broke down ethion aerobically. In comparison to the FCO limitations of 5.77, 1.05, and 2.43 for Nitrogen, Phosphorus, and Potassium, the chemical analysis of groundnut oil cake revealed greater levels of these elements. Based on nutritional analysis, groundnut cake has low fiber content but high levels of carbohydrates (34.3%), moisture, starch, and proteins (5.33%, 16.7% and 36.12%, respectively), fat (13.67%), and ash (5.42%). The results of biosurfactant production screening showed that 17 isolates were positive for haemolytic activity, 11 were positive for Drop collapsed test and 2 isolates *B. niabensis* (B) and *B. endophyticus* (C) showed highest emulsification index 80% and 82% respectively. These two isolates *B. niabensis* and *B. endophyticus* were identified by 16S rRNA sequencing method. Both of them were found to produce glycolipid and lipopeptide after FTIR analysis. GC-MS analysis of degradation study showed significant degradation of ethion. The findings have consequences for how a bioremediation approach should be developed.

Keywords Groundnut oil cake, Biosurfactant producing bacteria, Pesticide removal, Ethion degradation, Bioremediation.

INTRODUCTION

In the economy of developing nations, groundnuts (*Arachis hypogea* L.) play an important role. The three main nations that produce groundnuts are China, India and USA. *Arachis hypogea* L., often known as groundnut, is a legume plant in the Fabaceae family. Common names for groundnuts include peanuts or Moong Phalli. Potassium, calcium, phosphorus and vitamins abound in groundnuts. Groundnut seeds are not only an essential food source in many countries but their abundance in high-quality dietary protein and oil has contributed significantly to reducing malnutrition in numerous developing nations (Arya

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et al. 2016). These are a great source of heart-healthy fats, fiber and protein that help regulate blood sugar, decrease appetite, and lower the risk of heart disease. It is also called the "king of edible oils" (Mudiraj *et al.* 2021). Although groundnut seeds are an essential food source in many nations, their abundance in high-quality dietary protein and oil has contributed to a decline in malnutrition in numerous developing countries. Bacteriological and nutritional analysis of groundnut cake was done to measure the amount of nutritional value and bacteriological value of Groundnut cake. *Salmonella*, *Escherichia coli*, *Pseudomonas* and *Bacillus* species isolated from groundnut.

Amphiphilic compounds with hydrophilic (attracting water) and hydrophobic (repelling water) sections are known as biosurfactants. This characteristic enables them to interact with organic substances, such as insecticides, and water. Biosurfactants can solubilize hydrophobic pesticides by forming micelles or emulsions, increasing their dispersibility in water and facilitating their removal. Biosurfactants can emulsify pesticide droplets, breaking them down into smaller droplets suspended in water. This process increases the surface area of the pesticide, enhancing its interaction with other remediation agents (e.g., microorganisms) and facilitating its degradation. Biosurfactants can also enhance the bioavailability of pesticides to microorganisms capable of degrading them. By increasing the solubility and dispersibility of pesticides, biosurfactants promote their uptake by microbial communities, accelerating their degradation and ultimate removal from the environment. Some biosurfactants have foaming properties, which can be utilized in soil washing or flushing techniques for pesticide-contaminated soils. Foaming agents help in the mobilization and removal of pesticides from soil pores and crevices, thereby improving the efficiency of remediation processes.

Biosurfactants play a significant role in pesticide removal from contaminated environments. Pesticides, often used in agricultural practices, can contaminate soil and water sources, posing serious environmental and health risks (Bhatt *et al.* 2021). Pesticides, which are often used in agricultural practices, can contaminate soil and water sources, posing serious environmental and health risks. The effectiveness of biosurfactants can be influenced by environmental conditions such as temperature, pH and salinity, which can affect their stability and activity (Huang *et al.* 2020, Sajadi Bami *et al.* 2022). Additionally, the cost and scalability of biosurfactant production remain challenges that need to be addressed to facilitate their widespread application in bioremediation efforts (U *et al.* 2023). Despite these challenges, biosurfactants represent a sustainable and eco-friendly alternative to synthetic surfactants, with significant potential for environmental restoration.

Traditional methods of pesticide removal, such as chemical and physical treatments, can be expensive, energy-intensive, and may produce harmful by-products. Biosurfactant-based approaches offer a more sustainable and environmentally friendly alternative for pesticide remediation.

MATERIALS AND METHODS

Sample collection

A groundnut oil cake (GOC) waste sample of 1 kg was collected from the groundnut oil mill in Rajkot. Using hand glove, spatula, and sterile polythene bag, the GOC was collected and then moved to the microbiological laboratory where it was kept at 4ºC.

Groundnut oil cake analysis

Analysis of the chemical composition of groundnut oil cake with respect to nitrogen, phosphorus, potassium, moisture, ash, crude protein, crude lipid, crude fiber, carbohydrate, and starch content. Determination of nitrogen content by the Kjeldahl method, phosphorus content as per potassium content. The standard processes of analysis provided by the Association of Official Assessment of Chemistry researchers were used to perform the proximate analyses. Assessment of moisture, ash, crude lipid as per, crude protein, crude fiber and carbs.

Groundnut cake bacterial isolation

The three agar mediums that were used in the culture process were Mannitol salt agar, MacConkey agar, and Nutrient agar. The media were prepared

per the manufacturer's instructions and autoclaved for 15 minutes at 121ºC to sanitize them. The agar plate surfaces were dried in an oven prior to sample inoculation. To make buffered peptone water, 0.5 g of BPW was weighed and then dissolved in 500 ml of distilled water using an analytical weighing scale. It was then autoclaved at 121ºC for 15 minutes.

Sample collection and preparation

The sellers at the Samaru open market provided the groundnut cake powder that was utilized in the food samples. The market was split up into zones A, B, C and D based on the four cardinal points classification system. Samples were provided by three different sellers in each zone, they were subsequently homogenized and zone-specifically identified. An analytical weighing balance was used to aseptically weigh the homogenized samples from each zone, which were then dissolved in 225 ml of buffered peptone water in a 500 ml conical flask. The meal sample was well mixed in a shaker and diluted in a tenfold serial dilution up to the dilution of 10−6 using buffered peptone water as the diluent.

Sample inoculation

The nutrition agar plates were prepared using the pour plate method. Prior to incubation, 0.2 ml of the material was pipetted and put into a petri dish, and 15 ml of recently prepared molten nutritional agar was added. After swirling the liquid, it was allowed to solidify. The MacConkey and Mannitol salt agar plates were prepared using the spread plate method. The sample suspension was then distributed evenly over the surface of the plates after 1 ml of the dilution was applied using a sterile glass rod spreader to the dried agar surface. The spreader was heated using a Bunsen flame, cleaned by submerging it in 100% ethanol, and then given a 20-second cooling period.

Screening of biosurfactant-producing bacteria

The screening process for biosurfactant-producing bacteria was subjected to Minimal Salt Medium (MSM) with crude oil following the method (Ohadi *et al.* 2017). The modified composition of the medium per liter included: $MgSO_4$ (0.1 g), KH_2PO_4 (0.5 g), NH_4Cl (0.01 g), $FeSO_4.7H_2O$ (0.001 g), $NaHCO_3$ (1 g), and K_2HPO_4 (0.5 g), adjusted to pH 7.0. Additionally, 0.1% crude oil was included as an inducer to promote biosurfactant production.

Haemolysis test

Isolated specimens were streaked over blood agar medium containing 5% v/v human blood, and the mix was then cultured for 24 hrs at 37°C. After incubation, the zone that surrounded the culture developed. Haemolytic activity is a quantitative indicator of biosurfactant production.

Oil displacement test

40 mL of distilled water was placed into sterile glass petri plates. Add 20 µl of oil and 10 µl of cell-free supernatant. A positive test result was reported in the setup when a halo formed around the supernatant (Batool *et al.* 2017, Hassanshahian 2014).

Drop collapse test

A single oil droplet and its supernatant were placed on a cleaned glass slide. To find out if a droplet of supernatant collapsed or kept beading, it was carefully studied. The collapsed supernatant drop was considered as "+" and it shows the presence of biosurfactant.

Emulsification index assay

In the emulsification assay, 2 mL of the hydrocarbon (crude oil) was combined with 2 mL of the cell-free supernatant (obtained after centrifugation at 10,000 g for 5 min of the culture). The mixture was vortexed for 2 minutes to ensure thorough homogenization. Emulsification activity was then assessed after a 24h incubation period at 30±2°C. This activity was determined using a specific formula: 2 mL of the cell-free supernatant (obtained after centrifugation at 10,000g for 5 min of the culture) was mixed with 2 mL of the hydrocarbon (crude oil) in the emulsification experiment. For two minutes, the mixture was vortexed to ensure complete homogeneity. After incubating for 24 hrs at 30±2°C, the emulsification activity was evaluated. We used the following formula to determine this activity :

$$EI\ 24 = \frac{\text{Total height of emulsified layer}}{\text{Total height of liquid layer}} * 100$$

Identification of bacterial isolates by using molecular techniques

The sanger sequencing 16S rRNA technique was used to identify the bacterial strain. We used 16S rRNA universal primers to do single-pass sequencing on each template which included Reverse primer 1492R and Forward primer 27F. Ethanol precipitation was used to distinguish fluorescently labelled fragments from unincorporated terminators. The 3500 genetic analyzer (Applies biosynthesis) was then used to evaluate the samples after they had been suspended in distilled water.

The DNA of the isolated bacterial strain was extracted using a standard protocol. Following the acquisition of pure DNA, it was amplified via Polymerase Chain Reaction (PCR), wherein it was mixed with a PCR reaction mixture comprising forward and reverse primers, Taq Polymerase, and deionized water. The temperature ranges for PCR were 93°C to 95°C for denaturation, 50°C to 70°C for primer annealing, and 72°C for elongation. After a purity assessment, 16S rDNA sequencing was used to analyze the amplified DNA and characterize it molecularly. To identify the selected isolates, 16s RNA and gene sequences were submitted to GenBank and assigned accession numbers.

Production and extraction of biosurfactant

The biosurfactant was prepared and extracted following (Abbasi *et al.* 2012), with a few modifications. Inoculating the culture required 300 milliliters of MSM broth and the addition of 2% (v/v) crude oil. At 120 rpm and 37°C, the culture was shaken for five days. Using centrifugation at 10,000×g for 15 minutes at 4°C, cells were removed from the culture broth after incubation. Using 6 N HCl to acidify it to pH 2, the cell-free supernatant was then stored at 4°C for the duration of the night. As a result, the biosurfactant precipitation was enhanced. Centrifugation (15,000×g, 15 min, 4°C) was used to separate the precipitate, which was extracted many times at room temperature using ethyl acetate. The solvent evaporated completely after drying at ambient temperature. Crude biosurfactant was found to be a viscous dark brown substance upon extraction.

FTIR-based identification of biosurfactant

Using fourier transform infrared spectroscopy (FTIR) to determine the functional groups and chemical bonds included in the biologically active fraction of the biosurfactant, the chemical composition of the fraction was determined. The measurements of the spectrum were done in the absorbance mode. The partially purified biosurfactant was assessed and its functional groups were determined using Bruker Alpha II Fourier Transform Infrared spectrophotometer (FTIR) spectroscopy. A spectrum spanning from 400 cm^{-1} to 4,000 cm^{-1} was utilized.

Removal of pesticides by bacteria producing biosurfactants

Three distinct flasks—one for the control and two samples—each holding 250 mL of MSM, 2.5 mL of ethion (at 5.5 µg/100 ml), and 1 mL of cultures of *B. endophyticus* and *B. niabensis* with 0.6 O.D. were used in the degradation investigation. The broth that had not been infected was used to suspend the control flask. The mixture was incubated in the rotary shaker for seven days at a temperature of 30±2°C and 120 rpm. Following the seventh day of incubation, 3 mL of the extracted material was pipetted out of the three flasks that had been treated to chloroform and subjected to GC-MS analysis.

Analysis by gas chromatography and mass spectrometry (GC–MS)

The extracted sample was taken for analysis from the deterioration. A Gas Chromatography–Mass Spectrometry (GC–MS) analyzer (Sicart, Anand, Gujarat) with the model number SHIMADZU, QP2010 PLUS was used for the analysis. Dichloromethane was used as the solvent in the chromatographic analysis. Gas chromatography was performed using an ion source temperature of 250°C and an interface temperature of 300°C. The column oven's temperature was kept at 75°C for five minutes, and then it was raised to

280°C for ten minutes at a rate of 10°C per minute. Peaks for the roughly 39-minute mass spectrometry analysis were noted. The procedure for the control sample was the same.

RESULTS AND DISCUSSION

Groundnut oil cake proximate analysis

The chemical analysis of groundnut oil cake showed higher amounts of Nitrogen, Phosphorus and Potassium than FCO limits, which were found to be 5.77, 1.05, and 2.43 respectively (Table 1).

Table 2 presents a nutritional analysis that takes into account the level of moisture, ash, crude fat, crude protein, crude fiber, carbohydrates and starch.

The results of the nutritional analysis showed that groundnut cake has a low fiber content but is high in fat (13.67%), ash (5.42%), carbohydrates (34.3%), moisture, starch, and proteins (5.33%, 16.7% and 36.12%, respectively). A study was conducted by group researchers which showed nutritional and chemical analysis of groundnut oil cake which showed similar results.

Bacterial screening for biosurfactant production

Using primary, secondary and tertiary screening techniques, all 30 isolates were examined for their ability to produce biosurfactants (Table 3).

The results of screening showed that 17 isolates showed positive results for hemolytic activity, 11 showed positive results for drop collapsed test and 2 isolates A2 and A4 showed the highest emulsification

Table 1. Chemical analysis groundnut oil cake.

Sl. No.	Tests	Limits as per FCO	Results
1	Nitrogen	Min 4.5	5.77
2	Phosphorus	Min 1.0	1.05
3	Potassium	Min 1.0	2.43

Table 2. Nutritional analysis groundnut oil cake.

Sl. No.	Tests	Results (%)
1	Moisture content	5.33
2	Ash content	5.42
3	Crude lipid	13.67
4	Crude protein	36.12
5	Crude fiber	5.16
6	Carbohydrates	34.3
7	Starch content	16.7

Table 3. Biosurfactant screening.

Isolates	Biosurfactant producing bacteria		
	Haemolytic activity	Drop collapse test	Emulsification index (%)
A 1	+	-	-
A 2	+	+	80
A 3	-	-	-
A 4	+	+	82
A 5	-	-	-
A 6	-	-	-
A 7	+	+	40
A 8	-	-	-
A 9	+	+	21
A 10	+	-	-
A 11	+	+	35
A 12	+	-	-
A 13	-	-	-
A 14	-	-	-
A 15	-	-	-
A 16	+	-	-
A 17	+	+	33
A 18	+	+	25
A 19	-	-	-
A 20	-	-	-
A 21	-	-	-
A 22	+	+	30
A 23	+	-	-
A 24	+	-	-
A 25	+	+	22
A 26	+	+	48
A 27	-	-	-
A 28	-	-	-
A 29	-	-	-
A 30	+	+	44

Note: '+' = Positive test. '-' = Negative test.

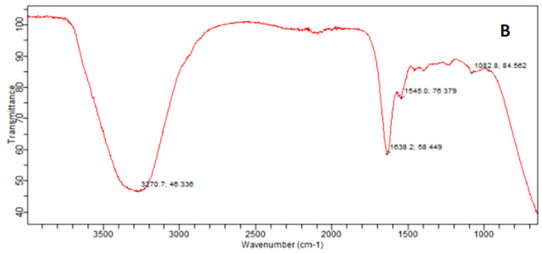

Fig. 1. FTIR analysis of sample B.

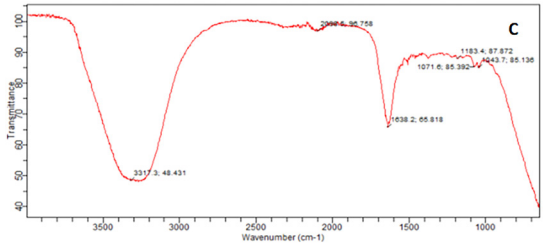

Fig. 2. FTIR analysis of sample C.

index 80% and 82% respectively. Oil displacement was found to be higher in strains with stronger emulsification activity. In order to verify that bacteria are capable of producing biosurfactants, the hemolysis test is the main qualitative screening technique. Similarly, surfactants destabilize liquid droplets, which

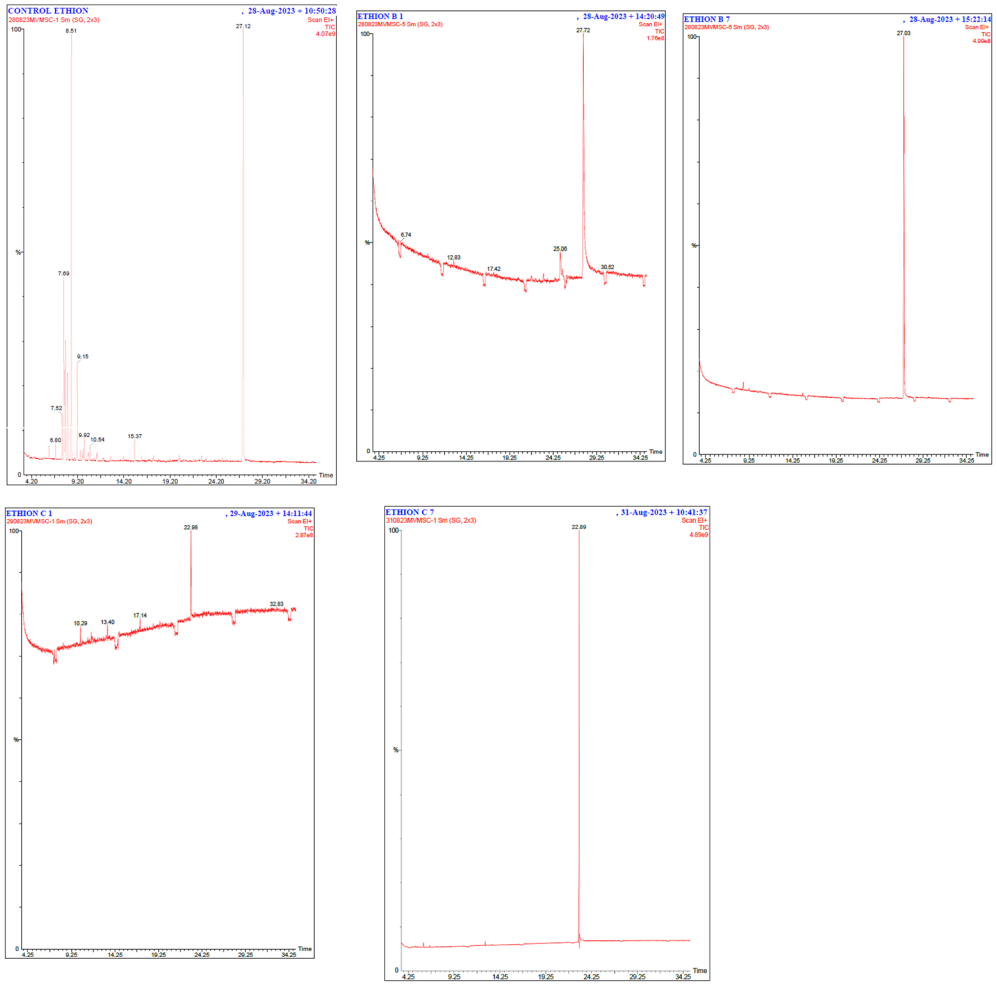

Fig. 3. GC-MS analysis of control, A2, A4.

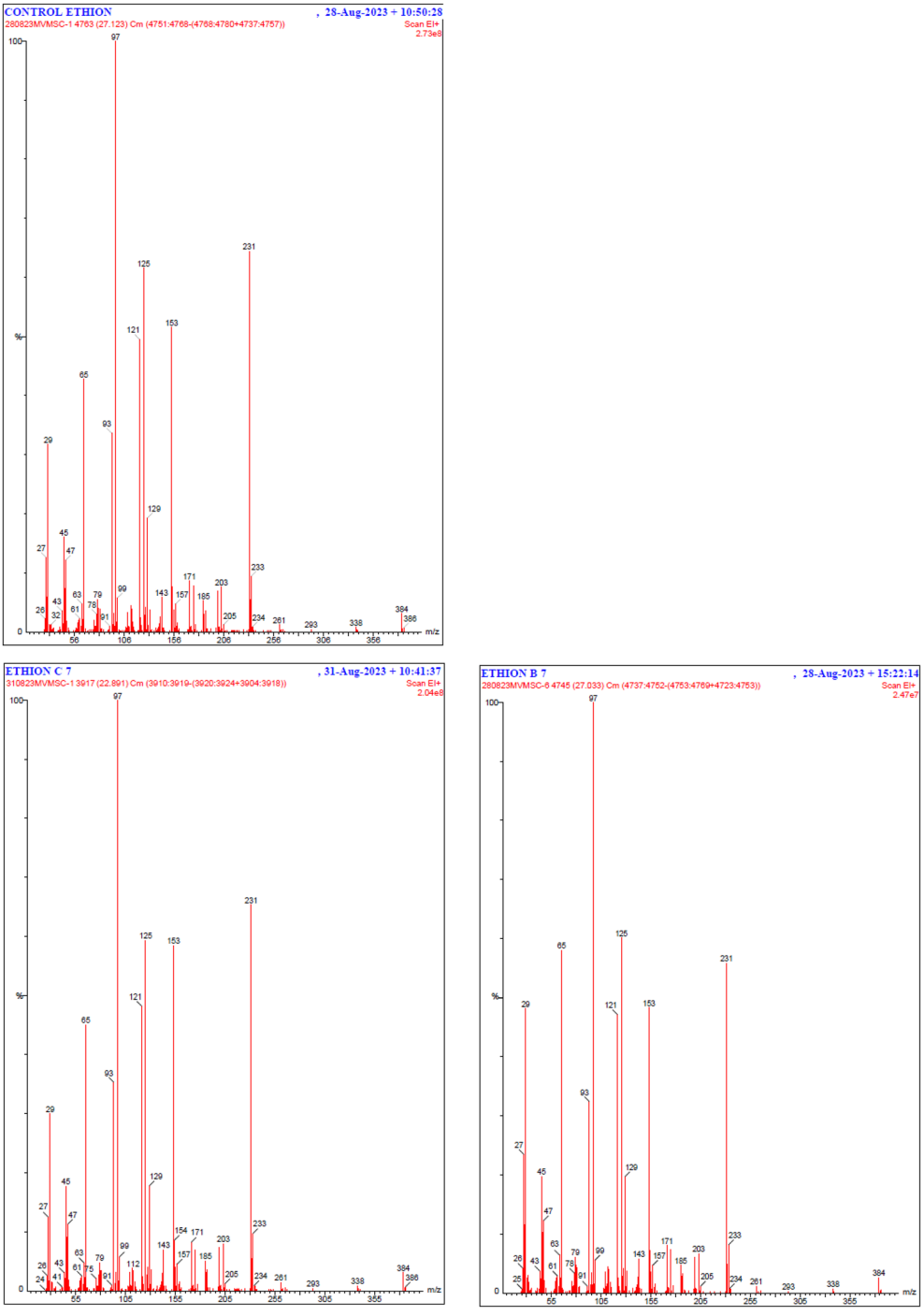

Fig. 4. Spectral peak of control, B and C.

is the basis for the drop collapse test.

This aligns with recent findings by Bhatt *et al.* (2021), who emphasized the importance of identifying novel microbial consortia for enhanced pesticide removal. The high biosurfactant yield observed in our study surpasses previously reported yields, such as the 76.3 mg/L reported for *B. subtilis* SDNS strain, indicating the superior efficiency of our isolated strains. Recent work by Kumar *et al.* (2024) has shown that optimizing fermentation conditions can further enhance biosurfactant production up to 12 g/L, suggesting potential for even higher yields in our system.

Molecular identification of isolates

First, the 16S rRNA sequences were examined for chimeric pieces using the CHIMERA-CHECK software version 2.7. Subsequently, reference sequences from the GenBank Nucleotide Sequence Database were compared with the sequences using the FASTA approach. Accession numbers OQ89229 and OQ799125, respectively, have been assigned to the sequencing data for isolates *B. niabensis* (B) and *B. endophyticus* (C) in the GenBank database.

Production of biosurfactant

After 5 days of incubation, *B. niabensis* and *B. endophyticus* produced a maximum yield of 8 g/L and 6 g/L biosurfactant following solvent extraction. The generation of lipopeptide biosurfactant from the *B. subtilis* SDNS strain was reported to produce 76.3 mg/L in a similar study.

Characterization of biosurfactant by FTIR

The broad absorbance peak with wave numbers 3270 cm^{-1} that was identified in the FTIR analysis of B (Fig. 1) is predicted to represent the O–H stretching vibrations of hydrocarbon chain locations. The stretching bond of unsaturated esters can be seen at a peak of 1638 cm^{-1}. The aliphatic chain of the C-H group can be identified by an absorption band at 1545 cm^{-1}.

The ester's C-O-C stretch is indicated by the prominent absorbance peak that was seen at 1082 cm^{-1}. The glycolipid nature of biosurfactants, as reported by numerous scientists, is similar to the observed peaks (Xu *et al.* 2020).

The FTIR spectrum of C revealed (Fig. 2) a large absorption peak about 3317.36 cm^{-1}, which is predicted to be the N-H stretching vibrations of amines or peptides. A sharp peak in absorbance at 1638 cm^{-1} indicates the presence of aliphatic chains (-CH3- and -CH2-). The ester's C-O-C stretch is indicated by the strong absorbance peak that was seen at 1071 cm^{-1}. Similar findings on the lipopeptide nature of *Bacillus* sp. biosurfactants have been documented (Nayak *et al.* 2020).

These molecular characteristics are particularly significant as they contribute to the enhanced solubilization and subsequent degradation of ethion. The presence of specific functional groups, such as O-H stretching vibrations and C-O-C stretches, confirms the amphiphilic nature of the biosurfactants. Recent studies by Yang *et al.* (2024) have demonstrated that these specific molecular structures can enhance pesticide degradation efficiency by up to 45% compared to conventional methods.

Degradation of ethion

The Total Ion Chromatography (TIC) analysis of the ethion pesticide control sample (Fig. 3) using GC-MS indicated the presence of ethion at retention time: 27.12. The degradation study was conducted for 7 days.

On days 1 and 7, samples were extracted in solvent and checked for ethion degradation. GC-MS analysis of samples B and C showed that at day 1 and 7, B Day 7 and B Day 7 showed a significant reduction of ethion as it shows a reduction in the size of the peak when compared to the control, B Day 7 and C Day 7 graph (Fig. 4).

Moreover, new peaks were found which indicates the breakdown of the compound and the formation of new compounds. Scientists have found that *B. amyloliquefaciens* C11 produces a biosurfactant that increases pesticide bioavailability, which may enhance the efficacy of bioremediation processes (Schalchli *et al.* 2023).

The synergistic effect of multiple functional groups in the produced biosurfactants, as recently demonstrated by Rodrigues *et al.* (2022).

These findings are particularly relevant in the context of sustainable agriculture, as highlighted by Sajadi Bami *et al.* (2022), who emphasized the role of biosurfactants in environmental remediation. Emerging research by Thakur *et al.* (2024) has shown that biosurfactant-based remediation can reduce soil pesticide residues by up to 85% within 14 days, while simultaneously improving soil health parameters.

Recent economic analyses by Karlapudi *et al.* (2018) suggest that biosurfactant-based remediation can reduce treatment costs by up to 40% compared to conventional chemical methods, while providing additional environmental benefits. The integration of waste utilization and environmental remediation makes this approach particularly promising for sustainable agricultural practices.

Furthermore, recent studies by Rizvi & Verma (2024) have demonstrated the potential for scaling up such systems for commercial application, suggesting a viable pathway for widespread implementation of this technology.

CONCLUSION

The degradation of ethion by biosurfactant-producing bacteria derived from groundnut oil cake presents a promising and environmentally sustainable solution for pesticide remediation. Through this study, it has been demonstrated that these bacteria possess the capability to utilize ethion as a carbon source for growth while simultaneously producing biosurfactants that aid in the solubilization and enhanced degradation of the pesticide. This dual action not only accelerates the degradation process but also mitigates the potential environmental impact of ethion residues in soil and water systems. Furthermore, the utilization of groundnut oil cake as a substrate for biosurfactant production not only adds value to an agricultural by-product but also underscores the potential for utilizing locally available resources in bioremediation efforts. This not only reduces the cost associated with biosurfactant production but also promotes sustainable practices within agricultural communities. Overall, the findings of this study highlight the potential of harnessing the capabilities of biosurfactant-producing bacteria from groundnut oil cake for the efficient degradation of ethion, thereby offering a viable and eco-friendly strategy for pesticide remediation in contaminated environments. Further research and application of this approach could significantly contribute to addressing pesticide pollution and promoting sustainable agricultural practices.

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