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Biosurfactant Production and Characterization by *Bacillus altitudinis* Strain LCDB-BP3 Isolated from Pesticide-Contaminated Soil in Kwara State, Nigeria

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Abstract

Microbial biosurfactants have gained attention as promising alternatives for bioremediation, particularly for pesticide-contaminated soils, due to their biodegradability and non-toxic nature. These biosurfactants also hold potential for various industrial applications, including food, petroleum, and fertilizer industries, and may serve therapeutic roles as adjuvants. However, there remains a need to produce biosurfactants using indigenous bacterial isolates to address environmental pollution sustainably. In this study, biosurfactant was produced using *Bacillus altitudinis* strain LCDB-BP3, identified as a Lambda Cyhalothrin-Degrading Bacterium (LCDB-BP3) in a previous research. The biosurfactant-producing ability of the isolate was confirmed by oil displacement (14.32 cm), emulsification activity (70%), and beta-hemolytic activity on blood agar. Stability tests revealed the biosurfactant's resilience across a wide range of temperatures (up to 105°C), pH (3–13), and salinity (3–7%), making it suitable for diverse environmental conditions. Thin-layer chromatography (TLC) and Fourier-transform infrared (FTIR) spectroscopy further confirmed the presence of functional groups such as O-H, N-H, ester carbonyl, and C-H, supporting the lipopeptide nature of the biosurfactant. These findings highlight the potential of the biosurfactant produced by *Bacillus altitudinis* strain LCDB-BP3 for use in the bioremediation of pesticide-contaminated soils and offer a greener alternative to chemical agents in industrial settings.

Keywords: Biosurfactant, *Bacillus altitudinis*, Thin-Layer Chromatography, Fourier Transform InfraRed

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Introduction

Biosurfactants are amphiphilic compounds produced by microorganisms that possess significant potential in various applications, including agriculture, pharmaceuticals, food processing, and environmental management (Gayathiri *et al.*, 2022). In contrast to their synthetic counterparts, biosurfactants are biodegradable, non-toxic, and environmentally benign, making them an attractive alternative for use in bioremediation strategies (Goswami and Deka, 2019). Recent interest has surged in their potential to mitigate environmental pollution, particularly the contamination of ecosystems by hazardous chemicals such as pesticides. Pesticides, extensively used in agricultural practices, often lead to soil and water contamination, creating risks to ecosystems, human health, and wildlife (Ali *et al.*, 2021). The ability of specific bacteria to degrade these pesticides while simultaneously producing biosurfactants offers a promising approach for bioremediation, facilitating the detoxification of contaminated environments. This study

focuses on the production and characterization of biosurfactants by *Bacillus altitudinis* strain LCDB-BP3, isolated from pesticide-contaminated soil in Kwara State, Nigeria. Investigating this bacterium's ability to degrade pesticide residues and generate biosurfactants is crucial for enhancing bioremediation techniques, especially in regions severely affected by pesticide pollution (Lamilla *et al.*, 2021).

Soil contamination with pesticides remains a persistent environmental challenge, particularly in areas with intensive agricultural practices. In Nigeria, the widespread use of synthetic pesticides has led to the contamination of vast agricultural lands, negatively impacting soil fertility, biodiversity, and public health (Babafemi *et al*., 2022). While physical and chemical methods for pesticide removal are commonly employed, these strategies often prove to be costly, unsustainable, and may result in negative environmental consequences. Additionally, pesticide residues tend to persist in the soil, presenting long-term ecological challenges. These issues underscore the need for effective, environmentally friendly, and sustainable alternatives to address pesticide contamination. The use of microorganisms, especially bacteria capable of degrading pesticides and producing biosurfactants, offers a promising solution for bioremediation (Malik & Garg, 2024). However, there is a notable lack of research on indigenous bacterial strains from pesticide-contaminated soils in Nigeria that can effectively produce biosurfactants for the degradation of synthetic pesticides (Abdulsalam, 2023).

While several studies have explored the potential of biosurfactant-producing bacteria in bioremediation, much of the existing research focuses on non-indigenous or laboratory-cultivated strains (Sah *et al*., 2022). Specifically, in Nigeria, there is a significant gap in the study of indigenous bacterial strains isolated from pesticide-contaminated soils for their biosurfactant production capabilities. Moreover, although *Bacillus altitudinis* is recognized for its biodegradation potential in various environments (Goswami and Deka, 2019), its ability to produce biosurfactants in pesticide-contaminated environments has not been sufficiently explored. This study aims to bridge this gap by isolating and characterizing *Bacillus altitudinis* strain LCDB-BP3 from pesticide-contaminated soil in Kwara State and evaluating its potential for biosurfactant production to enhance environmental remediation efforts.

The escalating levels of pesticide contamination in Nigerian agricultural soils call for the urgent development of sustainable, cost-effective remediation strategies. Traditional pesticide removal methods are often limited by their environmental toxicity and high costs (Rani and Shanker, 2018; Varsha *et al.*, 2022). Bioremediation through microbial agents, such as biosurfactant-producing bacteria, presents an environmentally friendly alternative for cleaning contaminated soils. The application of indigenous bacterial strains like *Bacillus altitudinis* strain LCDB-BP3 offers localized solutions for pesticide degradation while enhancing our understanding of bioremediation capabilities specific to the region. Additionally, harnessing the full potential of biosurfactants in promoting pesticide biodegradation could revolutionize current environmental cleanup practices in Nigeria and similar agricultural regions worldwide.

This research is critical for advancing bioremediation efforts in Nigeria, where pesticide contamination poses significant environmental challenges. By investigating the biosurfactant production and characterization of *Bacillus altitudinis* strain LCDB-BP3 (Goswami & Deka, 2019), this study will contribute to the development of sustainable bioremediation strategies. The findings will provide valuable insights into the potential of indigenous microorganisms in degrading pesticide residues and producing environmentally friendly biosurfactants for soil decontamination. Furthermore, this study will fill an essential research gap by focusing on local bacterial strains and their biotechnological

applications in the context of pesticide contamination. Ultimately, the outcomes of this research will support the promotion of cleaner agricultural practices with the production of biosurfactant, leading to improved soil health and environmental sustainability in Nigeria and other agricultural regions with similar challenges.

Materials and Methods

Chemicals

All chemicals and reagents used in this study were of analytical grade and were bought from the Central Research and Diagnostic Laboratory, Ilorin, Kwara State, Nigeria.

Subculturing of *B. altitudinis* strain LCDB-BP3

Pure culture of *B. altitudinis* strain LCDB-BP3 from our previous study was subcultured on nutrient agar and tested for viability (Abdulsalam *et al*., 2023). Broth culture of the isolate was then prepared in nutrient broth and standardized to McFarland (0.5). The standardized culture was used for this study.

Confirmation of Biosurfactant-Producing Ability of *B. altitudinis* strain LCDB-BP3

The biosurfactant producing ability of the isolate was confirmed using Oil Displacement, Emulsification capacity, and Haemolytic activity tests as described below.

Preparation of Cell Free Supernatant (CFS)

The isolate was inoculated into 100 ml mineral salt medium enriched with LC in separate 500 ml Erlenmeyer flask. The flask was kept in incubator shaker at 37 ºC for 7 days at 160 rpm. After 7 days of incubation, CFS was prepared from the culture broths. Broth from the flask was spinned in the centrifuge at 6000 revolution per minute for 15 minutes, then the supernatant was filtered using a millipore filter paper of 0.45 µm pore size. The CFS was used to carry out the biosurfactant-producing confirmation tests, all of which were performed in triplicates (Femi-Ola *et al*., 2015).

Oil Displacement Test (OD)

The OD test was carried out to determine the diameter of the clear zone. Forty milliliter (40 ml) of distilled water was added into a plastic Petri dish, then 10 ml of crude oil was gently added to the surface of the water. Finally, 10 ml of CFS was gently added to the oil-water surface (Alyousif *et al*., 2020). The displacement of oil and the formation of a clear zone indicates biosurfactant production in the CFS. The diameter of clear zone was measured after 30 seconds using a meter rule. Distilled water without surfactant was used as the negative control, while Triton X100 was used as the positive control (Adetunji and Olaniran, 2021).

Emulsification Index (EI): The emulsifying capacity of strain LCDB-BP3 was estimated using the EI test. Crude oil (1.5 ml) was mixed with 1.5 ml of CFS in a test tube. It was then vortexed shaken at high speed for 2 minutes using Stuart auto vortex mixer (AE-11D, Great Britain, and left undisturbed for 24 hours. The emulsification index percentage was calculated using the equation: EI = (Height of emulsion formed / Height of solution) × 100 (Barakat *et al*., 2017).

Haemolytic Activity test: The isolate was streaked on blood agar and the plate incubated at 28 ºC for 48 hours. The plate was inspected visually for zone of clearance (haemolysis) around the colonies. Isolate that had ability to lyse red blood cell and form a clear zone around the colonies was noted as biosurfactant producer and recorded as positive (+) while those that could not form halo zones are recorded as non-biosurfactant producer (Lamilla *et al*., 2018).

Biosurfactant Production

The LCDB-BP isolate was used for biosurfactant production in the laboratory. The isolate was inoculated into a sterile mineral salt medium enriched with LC in 500 ml Erlenmeyer flask. The flask was kept in incubator shaker at 37 ºC for 12 hours at 160 rpm. Then, 1ml of the 12 h old culture was transferred into 100 ml of freshly prepared mineral salt medium containing 1ml of LC. The mixture was incubated at 25 ºC for 7 days with shaking at 200 rpm using flask shaker (Meena *et al*., 2021).

Biosurfactant Extraction

Extraction of biosurfactant was done using acid precipitation method. The bacterial isolate was removed after 7 days of incubation by centrifugation at 6000 g, at 4

^{0}C for 30 minutes. The CFS was acidified with 1M of freshly prepared H_2SO_4 to obtain a pH of 2.0. The acidified CFS was then used for the extraction of the biosurfactant. To the 50 ml of the acidified CFS, 50 ml of mixture of chloroform: methanol in the ratio of 2:1 (v/v) was added. The mixture was allowed to react for 30 seconds. It was shaken vigorously until two phase separation appeared. Then the supernatant was decanted and the lower layer containing the biosurfactant was concentrated using a rotary evaporator. The solvent was then evaporated and the leftover sediment was poured into a test tube. This was washed three times by centrifugation to obtain the biosurfactant which was the whitish sediment (Ibrahim *et al.,* 2013; Lee *et al*., 2018).

Stability Test of the Biosurfactant

The stability of biosurfactant produced by *B. altitudinis* strain LCDB-BP3 was studied under diverse conditions such as temperature, pH, and salinity. The methods of Ahmad *et al*. (2021), was used for the stability tests. Briefly, the CFS was kept at temperatures (25 ^{0}C, 35 ^{0}C, 45 ^{0}C, 55 ^{0}C 65 ^{0}C, 75 ^{0}C, 85 ^{0}C, 95 ^{0}C, 105 ^{0}C and 115 ^{0}C), pH (3.0, 5.0, 7.0, 9.0, 11.0 and 13.0) and salinity (3 %, 4 %, 5 %, 6 % and 7 %) for 1 h, and emulsification activity was then measured. All experiments were conducted in three replicates (Goswami and Deka, 2019).

Characterization of Biosurfactant

Thin Layer Chromatography

The CFS was analyzed by TLC using silica gel plate (G60, Merck, Darmstadt, Germany) was activated at 160 ^{0}C using the hot air oven for an hour. Two points were located at a distance of 2 cm away from the base of the TLC plate). A drop of CFS was placed on the labelled point using a capillary tube and allowed to dry for 10min. Then it was placed inside a TLC tank flooded with an organic solvent (chloroform-methanol-water) at 70:10:0.5 (v/v/v) and then covered. The test sample and solvent travelled along the TLC plates and after a while the movement stopped. The plate was removed and allowed to air dry for 10 min and were viewed under a UV light (Model UVGL-15). The spots were marked, and then sprayed with Ninhydrin solution and Anthrone (Joy *et al*., 2017).

Fourier Transform Infra-Red (FTIR) Analysis

This analysis was carried out to determine the functional groups in the biosurfactant. One milligram of the extracted biosurfactant was grounded with 100 mg of KBr pressed with a silver coated hand presser at 7500 kg for 30 seconds to obtain translucent pellets. The pellet was inserted into Fourier Transform infrared Spectrophotometer (FTIR-8400S, Shimadzu, Japan) where the infrared spectra were recorded within the range of 4500-500cm-1 wave number. All measurements consisted of 500 scans, and KBr pellet was used as background reference (Muñiz-González *et al*., 2021). Respective peaks were compared with standard FTIR spectra and interpreted with a correlation table and literature (Yaraguppi *et al.,* 2020).

Statistical Analysis

The variability of the data was expressed in terms of their standard error (σ) of mean value. The data were further subjected to DUNCAN analysis and the differences among the mean values were established using the Least Significant Difference (LSD) test at significant level of 5 %.

Results

The biosurfactant-producing ability of the isolate was confirmed from the positive oil displacement diameter of 14.32cm and an emulsification index of 70 %. The isolate also lysed red blood cells showing a beta heamolysis on the blood agar plate as shown in Table 1.

The biosurfactant was found to be stable at high temperatures up to 105 ^{0}C, after which it stated reducing. There was significant change in the emulsification activity of biosurfactant at temperatures 45 and 55 ^{0}C (Figure 1). Furthermore, the effect of pH was evaluated between 3 and 13, there were significant changes observed in the activity of biosurfactant especially at pH 7 and 9. The average emulsification index at pH 3–13 was 69.7 % (Figure 2). Salinity variations between 3.0 and 7.0 % also showed significant changes in biosurfactant activity

and maximum EI (72.0 %) was observed at 3 % salinity (Figure 3).

The CFS of *Bacillus altitudinis* strain LCDB-BP3 analyzed on the TLC plate revealed a lipopeptide type of biosurfactant in nature, indicating that protein and lipid were present in the biosurfactant. This was confirmed from the positive ninhydrin test, whereas the anthrone test was negative. The bacterium produced a yellow spot with a retention factor (Rf) of 0.79 which is quite similar to lipopeptides.

The FTIR analysis of the CFS from the bacterium (Figure 2) showed a broad strong peak at 3458.58 cm^{-1} which indicates the presence of O-H and N-H stretching due to the occurrence of the free hydroxyl group. A strong sharp peak was observed at 2923-2850 cm^{-1}, showing the CH stretching due to the presence of alkane aliphatic hydrogen. The peak observed at 1634.83 cm^{-1} was due to the presence of ester carbonyl group typical of the peptide components of the biosurfactant. The peak at 1307.36 cm^{-1} was due to the presence of a strong nitro group (N-O). A peak around 1090.24 cm represents the presence of the C-O stretch primary alcohol. A medium peak at 982.50 – 919.26 cm^{-1} speculates the presence of alkene bending, while the peak at 669.13 cm^{-1} suggested the presence of (C-Br) halo-group (Figure 4). Therefore, the biosurfactant produced by the bacterium was expected to contain fatty acids and peptide moieties, revealing the lipopeptide nature of the biosurfactant. The interpretation of the infra-red spectra with the compounds present in the biosurfactant was summarized in Table 2.

Table 1: Confirmation of Biosurfactant-producing Ability of *Bacillus altitudinis* strain LCDB-BP3

Tests	***Bacillus altitudinis* strain LCDB-BP3**
Oil Displacement (cm)	14.80 cm
Emulsification Index (%)	70 %
Heamolytic Activity	Positive

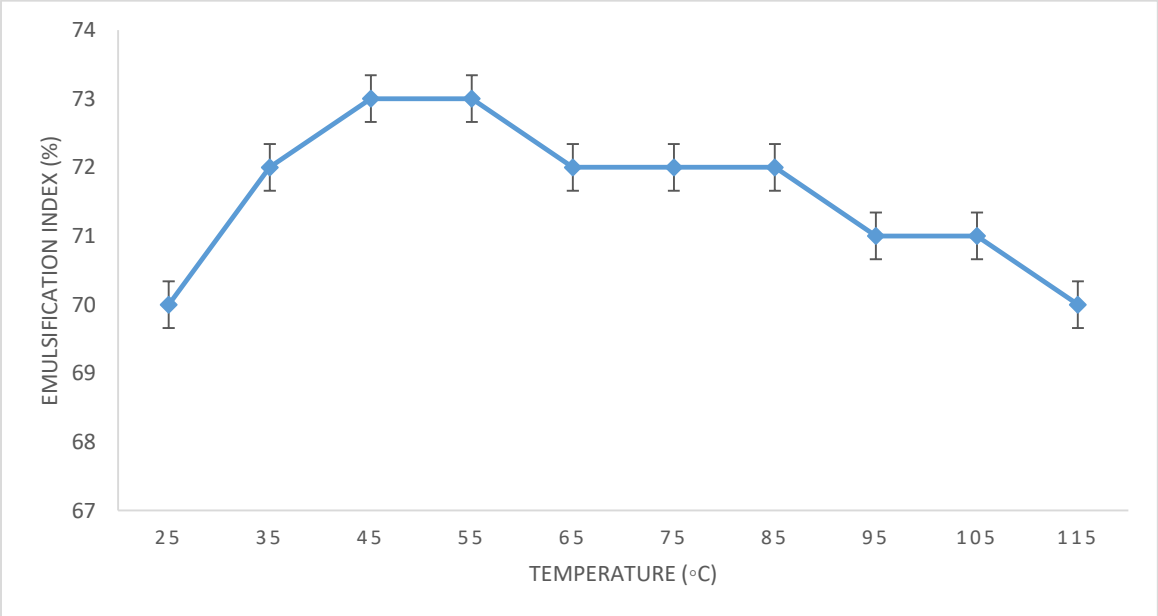

Figure 1: Effect of temperature on the stability of biosurfactant produced by *Bacillus altitudinis* Strain LCDB-BP3.

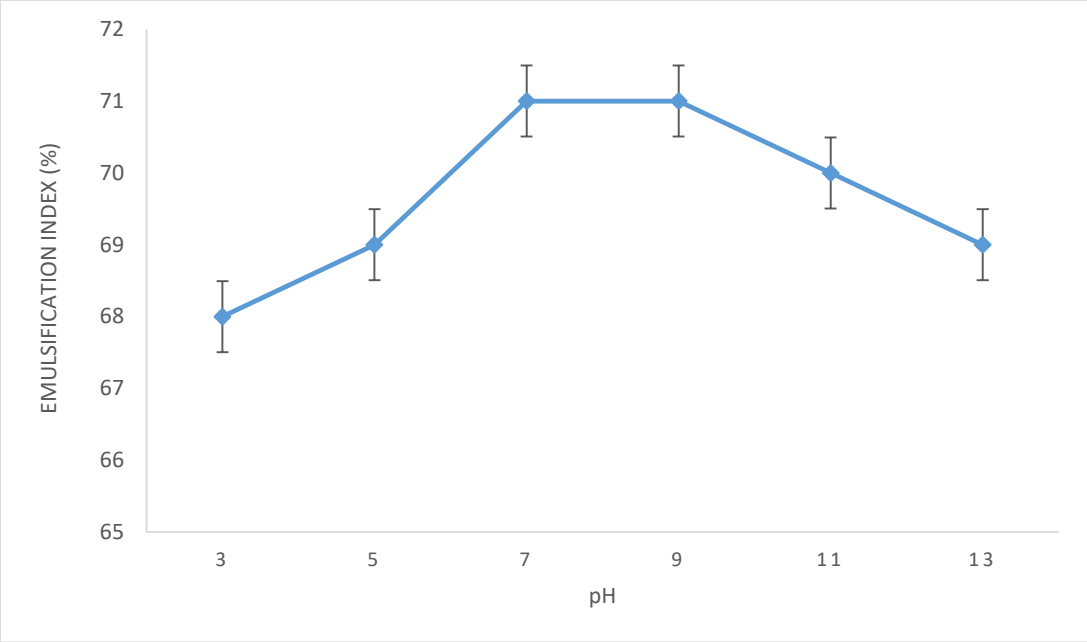

Figure 2: Effect of pH on the stability of biosurfactant produced by *Bacillus altitudinis* Strain LCDB-BP3.

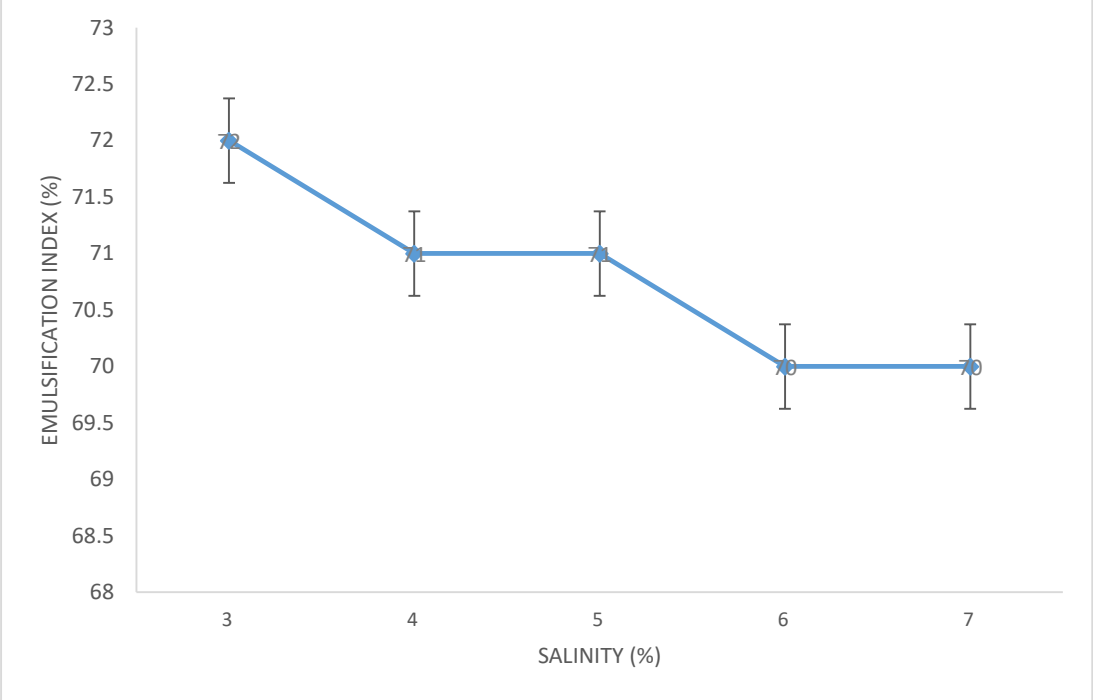

Figure 3: Effect of salinity on the stability of biosurfactant produced by *Bacillus altitudinis* Strain LCDB-BP3.

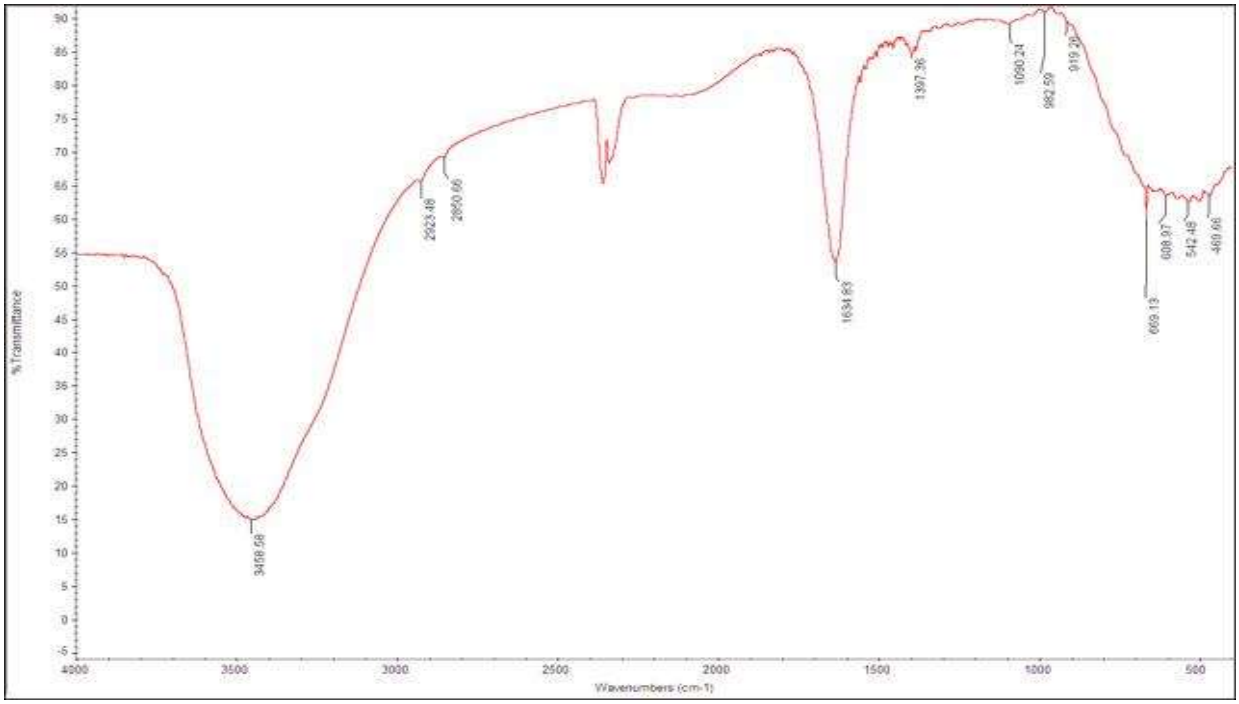

Figure 4: FTIR of biosurfactant produced by *Bacillus altitudinis* strain LCDB-BP3.

Table 2: Infra-Red Interpretation of Biosurfactant from *Bacillus altitudinis* strain LCDB-BP3.

S/N	Wave Number Range (cm^{-1})	Functional Groups
1	669.13	C-Br Stretching
2	982.50-919.26	C-C
3	1090.24	C-O
4	1307.36	N-O
5	1634.83	Ester carbonyl group
6	2923.48-2850.66	C-H
7	3458.58	O-H and N-H (alcohols and amines

Discussion

Biosurfactant production is an important survival strategy for microorganisms because it facilitates their attachment, and adhesion to natural substrates (Shafieia *et al*., 2013). In this study, the isolate *Bacillus altitudinis* strain LCDB-BP3 gave clearance zone diameter of 14.30 cm with crude oil. This is in line with the range of 10.00-14.20 cm diameter observed in a *Bacillus* species reported by Al-Yousif *et al*. (2020). The isolate *Bacillus altitudinis* strain LCDB3 can thus be consider a very good biosurfactant producer because of its high displacement zone diameter. However, the diameters were smaller than 31.20 cm which Abubakar *et al.* (2020) observed in *Bacillus* species, but higher than 4.90 cm obtained for *Bacillus subtilis* by Umar *et al*. (2021) and 9.22 - 9.65 cm obtained for different *Bacillus* species by Ilusanya *et al.* (2020). However, to Roy (2017), the clearance zone diameter is directly proportional to the concentration and activity of biosurfactant. The OD test is more sensitive than other methods of identifying biosurfactant producers and more suitable since it can detect low levels of biosurfactant production (Jayasree and Latha, 2018).

The formation of stable emulsions with crude oil by the isolate further affirms that it was a good biosurfactant producer. The high value of 70.0 % E.I. can be regarded as an excellent biosurfactant producer. The emulsification index study which agrees with the

emulsification index study of Nawazish *et al*. (2019), who reported an emulsification index of 66.4 % for *Bacillus* species with crude oil. Higher values of emulsification indices for crude oil by *Bacillus subtilis* have also been reported by other researchers (Adamu and Ibrahim, 2021; Wu *et al*., 2022). a strain of *Bacillus cereus* showed a high emulsification ability and thus have a valuable role in the bioremediation of contaminated environments (Gupte and Sonawdekar, 2015). The positive haemolytic activity of isolate is a good preliminary indicator of biosurfactant production by microorganisms as described for both fungi and bacteria (Ferreira *et al*., 2020). Studies have reported different biosurfactant-producing *Bacillus* species with high hemolytic activities (Diez *et al*., 2022; Deosthali *et al*., 2024).

In this study, the ability of *Bacillus altitudinis* strain LCDB-BP3 to produce biosurfactant suggests that, it can be used as emulsifying agent for many industrial applications. This also allow its direct applications in environmental remediation of contaminated soil. The stability of biosurfactant produced by *Bacillus altitudinis* strain LCDB-BP3 at extreme environmental conditions such as temperature, pH, and salinity on the emulsification index further affirms that the isolate was a good biosurfactant producer. Jiang *et al*. (2022), reported that a lichenysin produced by *Bacillus licheniformis* was very stable at a wide range of pH, temperature, and salt concentration. Studies have also demonstrated highly stable biosurfactants from *Bacillus megaterium*, *Bacillus amyloliquefaciens* and *Bacillus* sp. SS15 (Ali *et al*., 2021; Zargar *et al*., 2022; Zeng *et al*., 2022). Rawat *et al*. (2024), also reported a *Bacillus* sp G6 from oil-contaminated soil, which was very stable at extreme temperatures, pH and salinity. The estimation of biosurfactant stability is more important before their application in diverse fields. This finding showed that the biosurfactant produced by *Bacillus altitudinis* strain LCDB-BP3 was very stable under diverse range of environmental factors, therefore, this product can be employed under different environmental conditions to bioremediate the pesticide-contaminated environment.

In this study, the result of the TLC revealed that the developed spots retained a yellow colour and an Rf value of 0.79, indicating positive reaction for lipopeptides. This finding is in line with the study of Joy *et al*. (2017), who reported similar pattern of TLC with an Rf value of 0.72 and 0.55 for lipopeptide biosurfactants produced by *Bacillus* sp. SB2. Ramyabharathi *et al*. (2018) also reported two lipopetides produced by *Bacillus subtilis* Bbv57, which were confirmed on TLC with Rf values of 0.3 and 0.7. Furthermore, Yánez-Mendizábal *et al*. (2012), reported two different lipopeptides with Rf values 0.3 and 0.7. Another *Bacillus nealsonii* S2MT has been demonstrated to produce lipopeptide with different spots at Rf values 0.25, 0.35 and 0.75 (Phulpoto *et al*., 2020).

In this study, the result of the FTIR analysis revealed a peak range at 982.50-919.26 cm^{-1} indicates the C bonding of alkenes and a peak around 669.13 cm^{-1} indicating the presence of C-Br halo groups speculated according to the standard FTIR spectra correlation table. Based on FTIR spectrum analysis, it was observed that the biosurfactant synthesized by *Bacillus altitudinis* strain LCDB-BP3. was a surfactin. According to Sharma *et al*. (2018), the FTIR spectra of lipopeptides obtained in this study also showed similarities with previously reported lipopeptides produced by *B. amyloliquefaciens* SAS-1 and *B. subtilis* BR-15.

Conclusion

In conclusion, *Bacillus altitudinis* strain LCDB-BP3 demonstrated significant biosurfactant-producing ability, as evidenced by its positive oil displacement activity and emulsification index. The biosurfactant exhibited remarkable stability across a broad range of temperatures, pH, and salinity, with optimal activity observed at specific conditions. The biosurfactant was identified as a lipopeptide, with both protein and lipid components, confirmed through TLC and FTIR analysis. The infrared spectral data further supported the presence of key

functional groups indicative of fatty acid and peptide moieties, reinforcing the lipopeptide nature of the biosurfactant. These findings suggest that *B. altitudinis* strain LCDB-BP3 produces a stable and effective biosurfactant with potential applications in various environmental and industrial processes, particularly in bioremediation efforts. Further research could explore its application in large-scale bioremediation of contaminated environments.

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