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Production of bacterial biosurfactants using whey waste as a substrate

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Abstract

Surfactants of microbial origin offer significant value and versatility, and they are used in microbially enhanced oil recovery (MEOR) in the petroleum sector. Biosurfactant generation was investigated by isolating bacteria that were abundant in hydrocarbon-contaminated soil. Biosurfactants have gained admiration because of their low surface tension, biodegradability, high foaming, and ability to synthesis from renewable waste substrates, hence avoiding the need of non-renewable resources. Microbes can be studied on an experimental scale using a renewable, low-cost substrate. The biosurfactant activity was tested utilizing an uncommon source of nutrients that is cheese whey. Whey as a by-product of the cheese industry is typically dumped as desolate while it has lactose, the primary carbon source, and it is clearly suitable for microbial development. Microbes can be studied on an experimental scale using a renewable, low-cost substrate. In soil, surface active chemicals improve nutrient uptake by assisting microorganisms and crop yield in a variety of crops from varied regions. In contrast to previous examples, biosurfactants have several disadvantages in commercial manufacturing due to their low yield and high production costs.

Keywords: Surfactants, Cheese whey, Biosurfactant, Renewable substrate

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1. INTRODUCTION

In recent years, several causes such as industrial emissions, oil spills, unintentional leaks during transportation, and agricultural factors have had an impact on the environment [1]. Spillages of oil caused by environmental disasters that damaged marine habitats have wide and severe ecological and environmental consequences [2]. Pollutants and wastes produce negative outcomes in soil ecosystems by clogging the soil, resulting in less productive land in agricultural systems, which causes significant damage to plants and animals. These pollutants remain on the water's surface in the aquatic environment and harm aquatic organisms [3].

In multi-element polluted soil and groundwater, better approaches for identifying harmful chemicals permits evaluation or detection in parts per billion ranges [4]. The leakage of hydrocarbons into natural environments releases their constituents immediate. The management of these pollutants for environment and human benefit is a global concern [5]. Chemical industry processes have a substantial negative impact on the nature of the environment [6]. Surfactants known as biosurfactants have both non-polar and polar areas and are created by microorganisms such as bacteria or fungi from various substrates [3]. Bacillus, Pseudomonas,

Klebsiella, Acinetobacter, Alcanivorax, Halomonas, Pseudoalteromonas, Streptomyces, and Stenotrophomonas species have generated a lot of interest in recent years [2].

Whey is a by-product of the cheese industry that is typically dumped as a waste [7]. Whey is a suitable substrate since it contains lactose, the primary carbon source, and it is clearly suitable for microbial development [8]. Only half of the cheese whey waste is used for animal and human supplies. Over 100 million tonnes of cheese whey are generated worldwide each year. Some cheese whey is recycled, but the majority is a source of pollution for the environment due to its high Biological oxygen demand (BOD) [9]. The organisms that make use of lactose for production must be able to break it down into galactose or glucose. Modern research has unlocked the mysteries of whey proteins and other whey components, establishing a solid foundation for their nutritional and functional value, as well as preserving valuable whey components such as lactose, proteins, and minerals, resulting in a variety of products [10].

To eliminate hydrocarbon pollutants, biosurfactants have increased remediation effects in a cost-effective manner [2]. Biosurfactants have also anti-tumor, antibacterial, and antifungal properties, and production on low-cost renewable substrates is cost-effective and environmentally friendly [11]. On the one hand, bioremediation and cultures are utilized in various industries such as in household, pharmaceuticals, nourishment industry, fabric, dye, pelt, paper, metal removal, bioprocesses, and recently in energy-rectifiable technology [12]. Keeping in mind the preceding studies, the current research work aims to isolate bacterial efficiency to produce biosurfactant in the presence of a cheaper substrate as a carbon source, in order to overcome the problem of whey as waste by converting it into a value-added product in the form of bacterial biosurfactant.

2. MATERIALS AND METHODS

2.1 Isolation, screening and purification of bacterial isolates

Soil samples from diesel-contaminated soil and whey soil (whey thrown as garbage) were collected in Jhabran, Sheikhupura, Pakistan, for the isolation of crude oil-degrading bacteria. The material was collected in a sterile plastic container and brought to Lahore Garrison University's Biology Research Laboratory, where it was kept in a refrigerator at 4°C until further use. Soil sample (1 g) was serially diluted (10^{-6}) and spread on Nutrient agar media enriched with vegetable oil. The plates were then incubated for 24 hours at 37°C. Growth was detected after 24 hours.

2.2 Qualitative and Quantitative assays for Biosurfactant production

i. Oil spreading Assay

The ability of isolated bacteria to display oil displacement activity was tested using the method described by [13]. In the oil spreading test, 10 µl of hydrocarbons were poured onto the top of a petri plate until a thin layer formed. Each pure bacterial culture was injected in L-Broth and incubated at 37°C for 24 hours in a shaking incubator. Finally, the culture was harvested, and the supernatant was added to the oil layer's centre. The creation of a visible displacement zone of hydrocarbons confirmed the activity of the bio surfactant. The diameter of the oil displacement was examined and measured using metric ruler after 30 seconds.

ii. Emulsification capacity

The emulsification assay is determined by calculating the E-24 emulsification index. Following the process, 2ml of cell free supernatant was added to 4ml of cooking oil. For regular mixing, the liquid was allowed to display emulsification test was evaluated and measured using the following formula:

$$E24 = \frac{\text{Total height measurement of the emulsification layer}}{\text{Total height of the aqueous layer}} \text{ x } 100$$

iii. Blood Hemolysis Test

When allowed to develop on blood agar media, the bacterial isolates were examined for hemolysis activity. The newly formed bacterial colony was streaked on blood agar media. After that, these petri plates were given a 24-hour incubation period at 37°C. Blood hemolysis was observed as the emergence of distinct zones.

iv. Foaming activity

To allow separated bacteria to thrive, 100ml of Nutrient Broth Media was added to separate 250 ml flasks. Flasks were incubated in a shaking incubator at 37°C for 72 hours with centrifugation at 200 rpm and a time duration of 72 hours till foam appeared.

$$\text{Foaming index} = \frac{\text{Total height of foam layer}}{\text{Total height of the liquid layer}} \text{ x } 100$$

v. Drop collapsing assay

For screening, the drop collapse approach was utilized [13]. 2 µl of oil was placed to a 96 well microtiter plate, and 4 µl of supernatant was added to the surface of the oil and incubated for 1 hour at 37°C. Following the incubation period, the flattened and curved surfaces were examined to determine positive and negative results, respectively.

2.3 Screening of lipase producing bacteria

Qualitative screening for lipase activity

Using a plate assay, the isolates that produce biosurfactants were qualitatively screened for lipase activity. In this, colonies were observed through fluorescent halo zones when observed under UV light and opaque zones, respectively, on tributyrin agar and Rhodamine B agar plates, followed by incubation of 3-7 days.

Cheese whey collection and processing

Whey from a small dairy farm in Sheikhupura, Pakistan, was collected shortly after production. In a stainless-steel vessel, cheese whey was boiled for 10 minutes, cooled to 4°C, then centrifuged at 4000 g for 10 minutes. Whey supernatant was autoclaved for 20 minutes at 10 psi and utilized in the experiment. The inoculums were prepared using nutrient broth. The cultures were leaved to grow at 45°C for 8-10 hours.

Biosurfactant production

Inoculums were added to flasks holding 50 mL of cheese whey. For 72 hours, the cultures were incubated at 45°C without shaking. Optical densities (OD600) will be determined from whey supernatant to divulge that isolates growth in the cheese whey as a substrate. The results would be exemplified that isolates implanted in cheese whey showed highest cell densities of bacterial isolates.

Extraction t using Cheese Whey

Solvent extraction was performed [25]. Crude biosurfactants would be stored at 4°C and weight of crude surfactant would be measured using weighing machine.

3. RESULTS AND DISCUSSIONS

3.1 Isolation of microorganisms:

Different soil samples from the diesel and whey soils were taken. The purified bacteria that grew on oil-supplemented plates were given the DS1, DS2, DS3, WS1 and WS2 labels.

3.2 Morphological and biochemical characterization of bacterial isolates

Bacterial isolates were purified and described. Colony characteristics were observed. According to the results, isolate DS 1 had off white mucoidy colonies with regular edges and flat elevation, whereas isolate DS 2 had white convex colonies with whole margins and non mucoid texture. Yellow elevated colonies with uniform margins and non-mucoid texture are found in isolate DS 3. Off-white colonies with uniform margins, level elevation, and non-mucoid consistency were found in WS 1. Yellow colonies with uniform margins, convex elevation, and non-mucoid consistency were observed in WS 2. Using several staining techniques, the morphological behaviour of bacterial isolates was examined. Bacterial isolate DS 1 was a rod-shaped isolate with gram-negative behaviour and spore formation. The remaining four isolates were rod-shaped, gram-positive, and non-spore forming. Biochemical studies revealed that DS 1 was negative for the catalase test, indicating that no oxygen was produced, and that the VP test was also negative. As a result of the Benedict test, all isolates DS 1, DS 2, DS 3, WS 1 and WS 2 revealed an orange color, indicating that isolates reduce the sugar content of cheese whey.

3.3 Results of Qualitative and Quantitative Biosurfactant assays:

The activities of the assays are mentioned in Table 1.

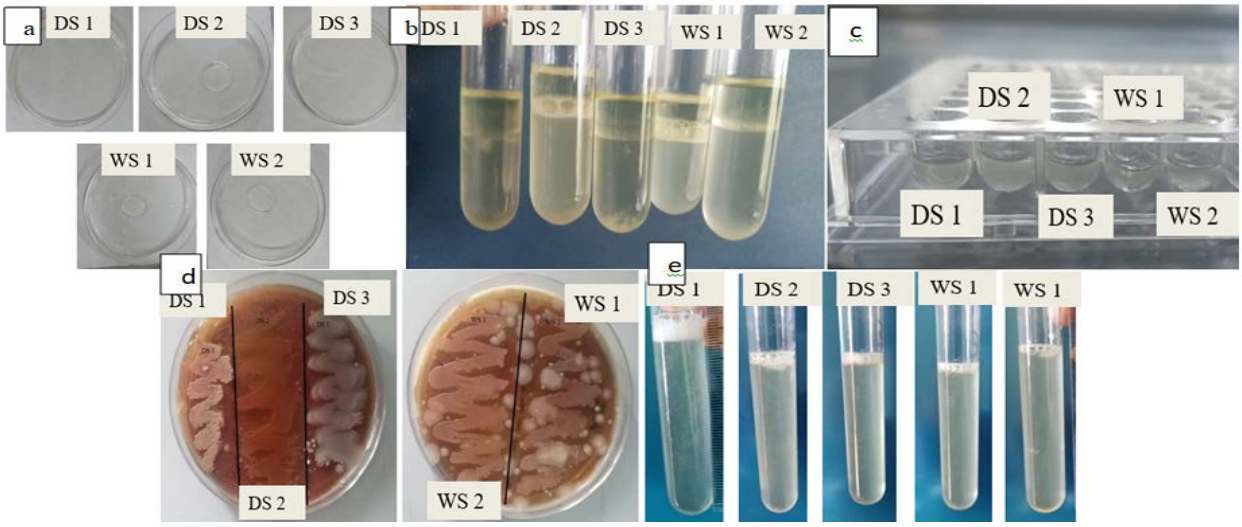

Fig. 1. (a) Oil Spreading Assay (b) Emulsification Assay (c) Drop Collapsing Assay (d) Blood Hemolysis Test (e) Foaming Activity

Results of screening of lipase producing bacteria

Plate test was used to perform qualitative lipase activity screening of biosurfactant producing isolates. After 3-7 days of incubation, the bacterial isolates DS 1, DS 2, DS 3, WS 1 and WS 2 showed lipolytic activity on tributyrin agar and Rhodamine B agar plates, but WS 1 and WS 2 showed no zone on Rhodamine agar plate. Under UV light, halo and opaque zones were found.

Table 1. Bacterial isolates showing oil spreading assay, emulsification, foaming activity, drop collapse test and blood hemolysis results.

Sr no	Isolates Name	Oil spreading assay Distance (cm)	Emulsification (%)	Foaming Activity (%)	Drop collapse test	Blood Hemolysis
1.	DS 1	2	20	10.7	Positive	Alpha
2.	DS 2	1	16	7.6	Positive	Gamma
3.	DS 3	1.5	13	6.1	Positive	Beta
4.	WS 1	1.5	10	3.07	Positive	Beta
5.	WS 2	0.5	10	1.53	Positive	Beta
Drop collapse test; Positive=plan surface; Negative=curved surface						

Table 2. Lipolytic activity on tributyrin agar and Rhodamine B agar.

Sr no	Isolate name	Tributyrin agar	Rhodamine B agar
1.	DS 1	Positive	Positive
2.	DS 2	Positive	Positive
3.	DS 3	Positive	Positive
4.	WS 1	Positive	Negative
5.	WS 2	Positive	Negative
Tributyrin agar Positive=clear zone around the bacterial growth; Negative=absence of a clear zone Rhodamine B agar Positive= oil displacement zones under UV light; Negative=No zones			

Production and extraction of biosurfactant using Cheese Whey

Collection and processing of Cheese whey

As soon as the cheese was produced in Jhabran, Sheikhupura, Pakistan, the whey was collected for the cultivation of crude oil-degrading bacteria seen schematically in figure 2.

Biosurfactant production

Optical densities (OD_{600}) were determined from whey supernatant to find out biosurfactant production. The results revealed that isolate WS 2 grew the fastest compared to DS 1, DS 2, DS 3, and WS 1. At 600 nm, DS 1 had a growth of 0.90, DS 2 isolation had a growth of 0.89, DS 3 had a growth of 0.94, WS 1 had a growth of 0.92, and WS 2 isolate had a growth of 1.21. The results are shown in Figure 3 and demonstrate that isolates implanted in cheese whey had the highest cell densities of bacterial isolates.

Extraction of crude biosurfactant

Solvent extraction was performed following [25]. Crude biosurfactants was stored at 4°C. The weight of crude surfactant was measured for DS 1, DS 2, DS 3, WS 1 and WS 2.

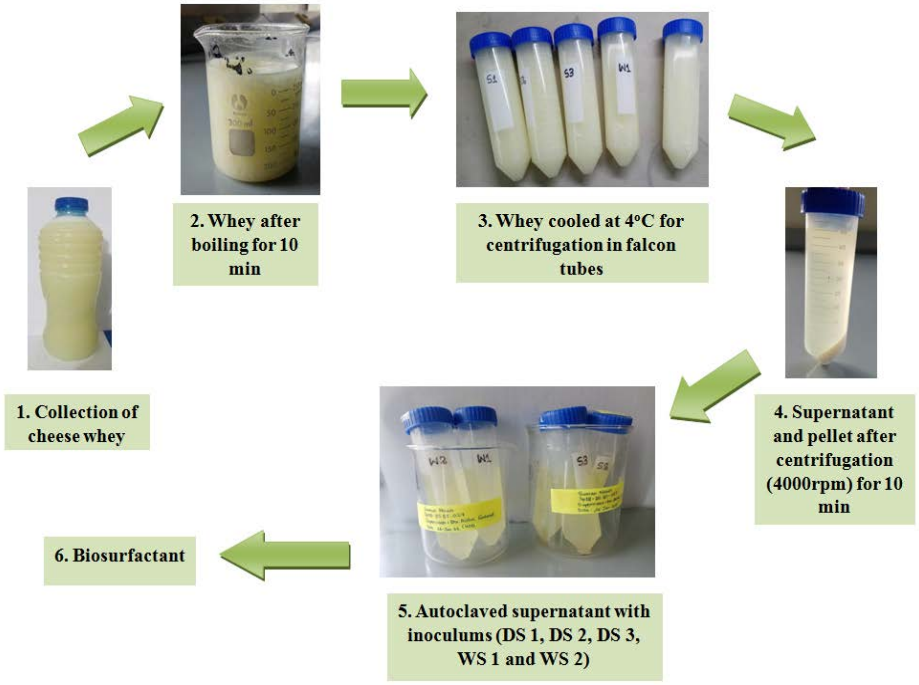

Fig. 2. Whey as a substrate for bacterial biosurfactant synthesis is depicted schematically.

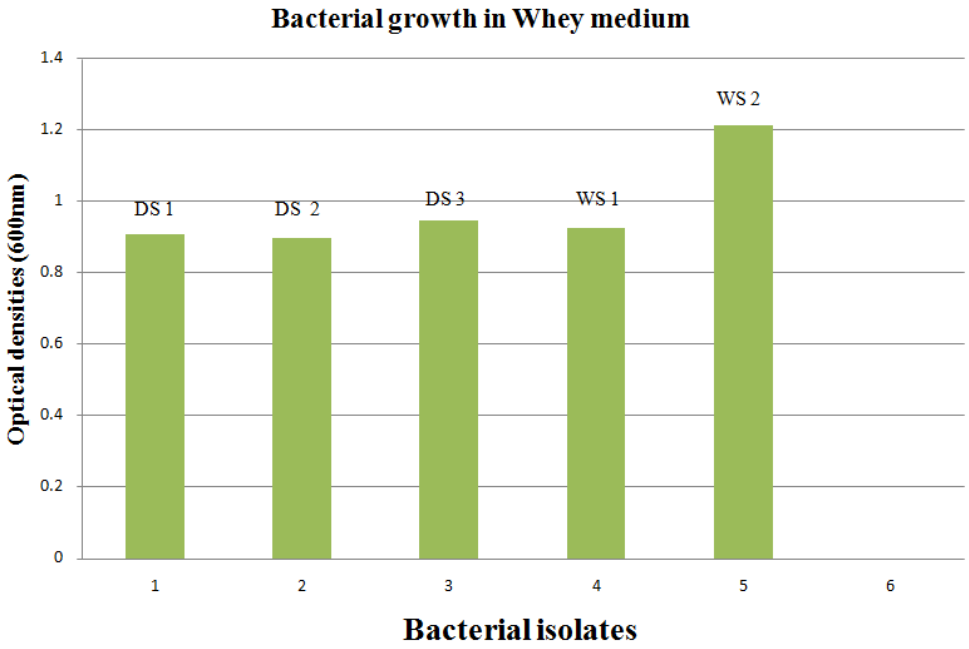

Fig. 3. OD of whey inoculated with DS1, DS2, DS3, WS1 and WS2.

3.4 DISCUSSION

The current study measured the lipolytic and biosurfactant activities of bacteria that break down hydrocarbons. Petroleum and its by products are widely used as a energy source in industry and daily life, but leakage can happen when the fuel is stored, transported, explored, refined, or produced. The cost of production and intended uses for biosurfactants will determine their future [14]. It has also been documented that *P. aeruginosa* may produce rhamnolipids utilizing whey wastes as substrates [15]. In a study on the improvement of the media composition for lactic acid bacteria (*L. lactis* 53 and *S. thermophilus A*) to create biosurfactants, it was shown that the mass of produced cell-bound biosurfactant (milligram) per gram of dry cell weight increased by almost two times [16]. There is evidence that nitrogen sources are crucial to the formation of biosurfactants. Several low-cost sources were investigated in order to lower the cost of producing biosurfactants. The study of production of biosurfactants using cheese whey as the carbon source is an intriguing and affordable option.

Five isolates were taken during the course of the current research project from two soil samples (diesel and whey dirt) and processed at Lahore Garrison University. In a different investigation, bacteria were isolated from samples (10 g or ml) taken from hot springs (45 to 67°C), oil reservoirs, deserts, and samples of fermented food [17]. In the current investigation, biosurfactant and lipolytic activities of bacterial isolates were examined. All five isolates produced large amounts of biosurfactant (qualitative and quantitative). The four separate *P. aeruginosa* strains that were recovered were effective in producing Rhamnolipid biosurfactant, according to the results of another study [18]. The little variances between the current data and earlier investigations are caused by variations in the bacterial isolates and the type of hydrocarbon employed.

According to the study, E coli spp., Bacillus spp., and Enterobacter spp. were the most common bacterial species found in the isolates based on their morphological and biochemical properties. According to the results, 50% of the bacterial isolates were very similar to Bacillus spp. In another study, the cellular morphology revealed that 55% of the isolates were cocci and the remaining 49% were rods. However, the Gram responses revealed that 70% of the isolates were gram-negative and 30% were gram-positive [19]. After incubating the culture in cooking oil for 72 hours, the oil spreading assay was performed for the current investigation. Significant oil displacement areas were present in every bacterial isolate. According to prior studies, the oil displacement method is a simple way to assess the activity of biosurfactants. In a different investigation, oil spreading test findings revealed that 11 isolates had positive results with relative diameters of 2 cm, 1 cm, 1.5 cm, 1.5 cm, and 0.5 cm [20]. The assay for measuring blood hemolysis was also used to check for biosurfactants. Four of the five bacterial isolates had a significant ability to produce hemolysis. Biosurfactant screening was also done using the emulsification test. All five bacterial isolates were

considerably able to create an emulsification layer in order to determine the emulsification index. One crucial component of a surfactant is its ability to emulsify substances. Isolated biosurfactant demonstrated significant emulsification indices with gasoline, n-hexadecane, kerosene, hexane, and benzene in a different investigation [21]. The data from this investigation is largely consistent with previous considerations; the slight differences are caused by bacterial isolates and hydrocarbon consumption. Drop collapse assays were also carried out in this work to test for biosurfactants. All five bacterial isolates were considerably able to produce good results. Three of the six strains tested for the drop collapse assay in another investigation had good results [22]. Three of the five isolates in the investigation demonstrated complete lipolytic activity using two qualitative techniques (Rhodamine B agar and Tributyrin agar). The current data on bacterial isolates is similar to previous research.

In addition, the increasing the surface area of water-insoluble substrates through emulsification, surfactants also increase the bioavailability of hydrophobic substrates, bind heavy metals, play a role in pathogenesis, have antimicrobial properties, and control how microorganisms adhere to and detach from surfaces. In addition to the anticipated commercial uses for biosurfactants, the use of whole cell broth or lower purity biosurfactant preparations in the oil sector is another possibility [23]. The biosurfactant-producing bacterium was found in soil samples taken from hydrocarbon-contaminated soil. According to many studies, bacterial biosurfactantin hydrocarbon-contaminated locations should be isolated and dispersed. Although bacterial biosurfactants are present everywhere, they are primarily found in environments that are contaminated with hydrocarbons. Lipase activity, hemolytic test, oil spreading, emulsification index (E24), foaming activity, and drop collapse assay were the screening methods used [24]. When optical densities (600) from whey supernatant were checked, isolate WS 2 grew most rapidly compared to DS 1, DS 2, DS 3, and WS 1. The findings demonstrated that isolates inoculated in cheese whey displayed the highest cell densities. Bacterial isolates with bio surfactant activity may be useful in the cheap and wasteful breakdown of hydrocarbons from soil.

According to a review of the above investigations, the bacterial isolates (E. coli spp., Pseudomonas spp., Bacillus spp., and Enterobacter spp.) have verified biosurfactant activity in several biosurfactant procedures (both qualitative and quantitative). Different microbiological techniques were used to identify these bacterial isolates. These methodologies are useful for considering hydrocarbon degradation from soil.

4. CONCLUSIONS

Biosurfactants have a number of intriguing qualities that could be used in oil extraction, environmental improvement, agriculture, pharmaceuticals, food, and other industries. Petroleum products are widely used in modern life. They pose a risk of contaminating the soil through spills, accidents, industrial emissions, or leaks at gas pumps, parking lots, and roads, which are carried by rain to other locations. The current study found that several gram negative and positive bacteria might exhibit biosurfactant and lipolytic capabilities, as well as biofilm formation. Different hydrocarbons, hydrocarbons, and substrates can all influence their activity. These processes, rather than typical physical approaches, contribute to the successful degradation of oil contaminations. The current study is based on a small number of samples and microorganisms; more research is required to obtain more accurate results. Bacteria that produce biosurfactant activity will be a valuable source of significant ecologically friendly biosurfactants for future and are substitution of chemically manufactured surfactants in this study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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