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Extraction Of Biosurfactant From Isolated *Bacillus Pasteurii* And It's Applications

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Abstract: Biosurfactants can be produced by microorganisms. They contain both hydrophilic and hydrophobic domains. They act as emulsifying agent by solubilizing hydrophobic substrates. To improve oil recovery biosurfactant production may be an economic approach. This research aims to study active product extraction procedures, analytical terminologies used in this field and the application of biosurfactants. Petroleum-contaminated soil was used as a source of bacterial culture and MSM (Mineral Salt Medium) was used as a nutritious medium. The bacterial characterization test confirms that the isolated strain of bacteria was *Bacillus pasteurii*. Extracellular production of biosurfactants can be confirmed by using various screening methods. Various parameters of biosurfactants such as pH, temperature, carbon source and nitrogen source were optimized to obtain a higher yield of biosurfactant. Nuclear Magnetic Resonance (NMR) was performed to identify the particular type of biosurfactant. Rhamnolipid type of biosurfactant was produced and it is used for biosurfactant-mediated synthesis of silver nanoparticles.

Keywords **-** Biosurfactant, Bioemulsifier, Petroleum-contaminated soil, MSM, *Bacillus pasteurii*, Surface tension, Interfacial tension, Biosurfactant mediated nanoparticles, etc.

1.INTRODUCTION

Biosurfactants are amphiphilic molecules that contain both hydrophilic and hydrophobic moieties. Biosurfactants are mainly produced by microorganisms like bacteria, fungi and yeasts. They act as emulsifying agent by solubilizing hydrophobic substrates [(1)]. A carbohydrate, an amino acid, a phosphate group, or similar compounds whereas the hydrophobic moiety is mostly the fatty acid carbon chain are the hydrophilic moieties of the biosurfactant. This property helps to reduce the interfacial and surface tensions, making them potential candidates for enhancing oil recovery [(2)]. Biosurfactants or microbial surfactants of bacteria or fungi the extracellular or intracellular metabolites are divided into different structural and functional groups: lipopeptides, glycolipids, polysaccharide-protein complexes, phospholipids, neutral lipids, and fatty acids [(3)]. These molecules perform various natural roles for the growth and reproduction of microorganisms [(4)]. A large number of hydrophobic compounds continuously enter the environment, either as natural products originating from animals, plants and microorganisms such as steroids, terpenes and waxes or pollutants produced by anthropogenic activities such as hydrocarbons, petroleum and their derivatives. These substrates are degraded by microorganisms which can colonize almost all ecological niches as a result of their metabolic versatility and adaptability to different carbon sources [(5)]. Biosurfactants are low molecular weight compounds and have surface active properties which help to reduce surface tension and interfacial tension. Low molecular weight biosurfactants mainly include rhamnolipids and surfactin [(5)]. Various functional properties of

biosurfactants such as emulsification, wetting, foaming, cleansing, phase separation, surface activity, and reduction in viscosity of crude oil, make it feasible to utilize them for many application purposes [6]. Based on the structure of their hydrophilic part, biosurfactants are mainly classified into five categories: Glycolipids, lipopeptides, fatty acids, polymer type and particulate biosurfactant. Glycolipids: Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxy aliphatic acids. Among the glycolipids, the best known are rhamnolipids, trehalolipids and sophorolipids lipopeptides and lipoproteins: A large number of cyclic lipopeptides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) possess remarkable surface-active properties. Fatty acids, phospholipids, and neutral lipids: Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on n-alkanes. Polymeric biosurfactants: The best-studied polymeric biosurfactants are emulsan, liposan, mannoprotein and other polysaccharide-protein complexes. Particulate biosurfactants: Extracellular membrane vesicles partition hydrocarbons to form a microemulsion which plays an important role in alkane uptake by microbial cells.

Surfactants are mainly classified as cationic surfactants, anionic surfactants, zwitterionic (Amphoteric) surfactants, and non-ionic surfactants. They play an important role as stabilizing agents for the non-toxic and non-hazardous synthesis of nanoparticles. Biosurfactants have a biodegradable nature hence they are more environment friendly than chemical surfactants. Biosurfactants include glycolipids, lipopeptides, and phospholipids, whereas the bio emulsifiers include polymeric and particulate biosurfactants such as emulsan and alasan [7]. Biosurfactants act as potential stabilizing agents and are emerging as a green alternative for the synthesis and stabilization of nanoparticles. Biosurfactants easily form a variety of liquids in aqueous solutions hence they are used for high-performance nanomaterial production [8]. Micelle-forming ability is one of the most important properties exploited in nanoparticle synthesis. The major concern in nanoparticle synthesis is tuning up these structures to obtain aggregates with desired morphology and properties. By changing the pH, temperature, surfactant concentration, and the ionic strength of the solution the morphology of these nanomaterials can be significantly varied [9]. The rate of reduction and adsorption mechanisms of capping agents with metal ions are some factors that can be controlled in the design of nanoparticles. To determine the final quality of the particles capping agents particularly play an important role. It essentially reduces the tendency of nanoparticles to agglomerate, by protecting the surface by either causing steric or electrostatic stabilization. In the biosurfactant-mediated synthesis of nanoparticles, biosurfactant acts as a capping agent and facilitates the uniform dispersion of the nanoparticles in the liquid medium [9]. Biosurfactant-mediated synthesis and stabilization of nanoparticles developing as a greener method is being considered to be a potential method for size-controlled synthesis of nanoparticles. Hence, biosurfactant-mediated AgNPs were produced, and their efficacy on biofilms was assessed [9]. Biosurfactant produced by microorganisms plays a very important role in the aggregation and stabilization process. The mode of action involved is through adsorbing onto metallic nanoparticles, surface stabilizing the nanoparticles, and then preventing subsequent aggregation. The mechanism of surfactant absorption depends on the type of surfactant and the thickness of the adsorbed layer [9].

Rhamnolipids, a class of biosurfactants mainly glycolipid-containing biosurfactants, are mainly produced by *Bacillus species* [10]. The structure of a rhamnolipid contains a combination of a polar head group and a hydrophilic tail. This type of biosurfactant contains one or two L-rhamnose moieties linked to one or two β-hydroxy fatty acids, production yield of rhamnolipid is higher than the other biosurfactants such as lipopeptides (surfactin, *etc.*) [10, 11]. For mass production due to their great physicochemical properties, high production yield and metabolizing various carbon sources rhamnolipids are the most promising candidates [10, 11]. Despite their abundant advantages to synthetic surfactants and their wide application in different industries, rhamnolipids could not compete with synthetic surfactants due to high production costs [12, 13].

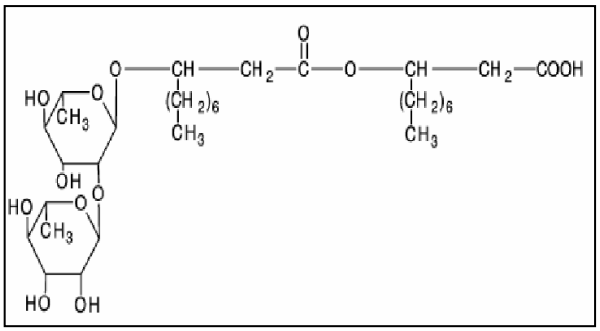

Figure 1. Structural representation of Rhamnolipid.

One of the main bottlenecks for future commercial applications is the low productivity of biomaterials. So, the scientists use methods such as gene manipulation of the microorganism and culture condition optimization to increase the yield of bio-products. Since economic production is one of the biggest concerns in BS production, the selection of optimal growth conditions is unavoidable. Micro emulsion is one of the significant applications, which helps in drug delivery systems. The use of biosurfactants in microemulsion increases the oral bioavailability of poor aqueous soluble drugs [15]. In drug delivery, gene delivery, vaccine production, antioxidant activity, personal care and hygiene, antiadhesive and antibiofilm activity anticancer activity, antitumor activity, antimicrobial activity, etc. biosurfactants play an important role [15].

2. MATERIALS AND METHODS:

2.1 Isolation of Bacteria:

The petroleum-contaminated soil sample was collected from the Hindustan Petroleum soil Narhe, Pune. The collected petroleum-contaminated soil sample was inoculated in 100ml of sterilized Mineral Salt Medium (MSM). Incubated at 37^0C in shaker incubator at 120 rpm, for 24hrs. After a time of incubation, inoculate a loopful culture of bacteria on a nutrient agar plate containing crude oil (1ml) and incubate for 24 hrs at 37^o C and maintain the culture. A single colony was obtained and used for the characterization of isolated strains from petroleum-contaminated soil.

2.2 Characterization of isolated bacteria:

Isolated bacteria were characterized by using 'Bergey's manual' of the systematics of Archaea and Bacteria, identification flowchart. According to 'Bergey's manual' of the systematics of Archaea and Bacteria, identification flowchart biochemical tests were performed for bacterial characterization.

2.3 Extraction of Biosurfactant:

Bacteria were grown aerobically in 100ml of Mineral Salt Medium (MSM). Inoculate a loopful culture of bacteria in a culture flask containing 100ml of MSM containing crude oil (1ml), and incubate in a shaker incubator for 7 days, at 30^oC and 120 rpm. After 7 take out culture broth and centrifuged it at 6000 rpm at 4^oC for 15 min. Collect supernatant and subject to filtration through membrane filter assembly. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1M H_2 SO_4. Then equal volume of chloroform: methanol (2:1) was added. This mixture was shaken well and left overnight for evaporation, white coloured sediment obtained is biosurfactant. Dry it in a hot air oven at 60 ^{o} C for 30 minutes. After drying take a dry weight of biosurfactant.

2.4 Screening methods for detection of biosurfactant:

A) Oil spreading test: To study the surface activity, an oil spreading test was performed. 20 ml of distilled water was added to a plastic petri plate, 20µl of crude oil was added on the surface of the water in a Petri plate and 10µl of cell-free culture broth was added to the crude oil surface in a petri plate. If biosurfactant is present in the cell-free culture broth, the oil will be displaced with an oil-free clearing zone and the diameter of this clearing zone indicates the surfactant activity. Triton X-100 is used as a positive control and distilled water with no surfactant is used as a negative control [17].

B) Tilted glass slide test: To check the decreased level of surface tension tilted glass slide test was performed. Isolated culture is grow on nutrient agar plate for 24 hr. A droplet of 0.9% NaCl was mixed with an isolated colony at one end of a glass slide. The slide was tilted and droplets were collapse down. Biosurfactant producers are detected by observation of droplets collapsing down [18]. This technique effectively modifies the drop collapse method.

C) Bacterial Adhesion To Hydrocarbon (BATH) assay: To check the cell surface hydrophobicity, a BATH assay was performed. The cell pellets were washed twice and then suspended in a buffer salt solution containing 16.9 g/L of K_2HPO_4 and 7.3 g/L of K_2HPO_4. The suspension was diluted using the same buffer solution to achieve an optical density (OD) of approximately 0.5 at 610 nm. 100µl of crude oil was added to 2ml of the cell suspension in test tubes and the mixture was vortex-shaken for 3 minutes. After shaking, the crude oil and aqueous phases separated for 1 hour. The OD of the aqueous phase was then measured at 610nm using a spectrophotometer. Finally, the percentage of cells attached to the crude oil was calculated from the OD values using the following formula [19].

Cell surface Hydrophobicity (H %) = 1- (OD of shaken with oil / OD of original) ×100

Where OD is shaken - OD of a mixture containing cells and crude oil. OD original- OD of cell suspension in the buffer solution.

D) Emulsification assay: To study the emulsifying ability of biosurfactants, an emulsification assay was performed. 3mL of supernatant was mixed with 0.5mL of crude oil and vortexed vigorously for 2 minutes. The mixture was left undisturbed for 1 hour to separate the aqueous and oil phases. Uninoculated broth was used as a blank. The absorbance of the aqueous phase at 400nm was measured using a spectrophotometer. Emulsification activity was calculated in EU/ml using the following formula [20].

Emulsification Assay (EU/ml) = Absorbance × Dilution factor

E) Emulsification Index: Emulsification activity is measured by calculating EI. Kerosene is added to culture broth (1:2 v/v), vortexed for 2min and allowed to stand for 24 hrs. The height of the emulsion is measured by taking the layer formed between the aqueous and kerosene layers. EI is calculated by using the following formula [21, 22].

Emulsification Index (EI) = (Height of emulsion layer/Total height) ×100

2.5 Optimization of Biosurfactant produced by *Bacillus pasteurii*:

1. Effect of pH: Five pH values were selected, 5.0, 6.0, 7.0, 8.0 and 9.0 for optimization of pH. By adding 1% glucose as the sole carbon source MSM was prepared. The activated culture of *B. pasteurii* was inoculated in MSM media and incubated at 37°C for 7 days in a shaker incubator at 150 rpm.
2. Effect of Temperature: For optimization of temperature, 20°C, 30°C and 40°C are three temperature values were selected. The activated culture of *B. pasteurii* was inoculated and incubated at different temperatures for 7 days in a shaker incubator at 150 rpm.
3. Effect of Carbon: Three carbon sources were taken sucrose, starch and kerosene for optimization of carbon. The activated culture of *B. pasteurii* was inoculated and incubated at 37°C for 7 days in a shaker incubator at 150 rpm.
4. Effect of Nitrogen: Five nitrogen sources were selected, ammonium nitrate, ammonium sulphate, ammonium chloride, potassium nitrate and peptone for nitrogen optimization. The activated culture of *B. pasteurii* was inoculated and incubated at 37°C for 7 days in a shaker incubator at 150 rpm.
Analysis for optimization conditions: At the end of each optimization process, the bacterial cells were for 15 min at 6000rpm at 4°C and the supernatant was collected for emulsification activity. By calculating emulsification activity the optimal growth conditions were confirmed.

2.6 Synthesis of biosurfactant-mediated Silver nanoparticles (AgNPs):
Supernatant was added to 0.05M Silver nitrate ($AgNO_3$) in equal volume and vigorously stirred for 5 minutes. Then Sodium Borohydride ($NaBH_4$) of 0.1M was added to the above mixture and stirred well. The colour changed from white to brown within five minutes indicating the production of AgNPs.

3. RESULT AND DISCUSSION:

3.1 Characterization of isolated bacteria: According to 'Bergey's manual' of the systematics of Archaea and Bacteria, identification flowchart, bacteria isolated from the petroleum-contaminated soil is *Bacillus pasteurii.*

Table 1: Biochemical tests for characterization of isolated bacteria.

Sr. No.	Biochemical Test	Result	Discussion
1.	Gram staining	Gram-positive	Rod-shaped bacteria were observed.
2.	Endospore staining	Positive	Green spores were observed in pink bacterial cells.
3.	Strict anaerobes	Negative	No bacterial growth was observed at anaerobic conditions.
4.	Starch hydrolysis	Negative	No clear zone was observed around the bacterial growth.
5.	Catalase test	Positive	Effervescences was observed.
6.	Nitrate reduction test	Positive	A red colour was observed.

3.2 Extraction of Biosurfactant: *Bacillus pasteurii* produces extracellular biosurfactants in the MSM medium. 0.34g biosurfactant was extracted from *Bacillus pasteurii.*

3.3 Screening methods for detection of biosurfactants: All the characterization tests for the detection of biosurfactants showed positive results. Biosurfactant was produced by *Bacillus pasteurii.* The emulsification index obtained is 62.5% which is greater than 50% which means it showed the presence of biosurfactant.

Table 2.1: Characterization test for detection of biosurfactant.

Sr. No.	Test	Result	Description
1.	Oil spreading test	Positive	Displacement of oil with an oil-free clearing zone was observed.
2.	Tilted glass slide	Positive	The drop collapsed down.
3.	Bacterial adhesion to hydrocarbon (BATH) assay	Positive	Cell surface hydrophobicity obtained was 14%.
4.	Emulsification assay	Positive	The emulsification activity obtained was 3.2 EU/ml.
5.	Emulsification Index	Positive	Emulsification obtained was 62.5%, which was greater than 50%.

3.4 Analysis of biosurfactant using Nuclear Magnetic Resonance (NMR):
NMR spectrum showed the presence of various functional groups based on their chemical shift. Hydrocarbon chain (0.8-1.4 ppm), Rhamnose ring in rhamnolipid (3.0 - 5.5 ppm), Methyl group ($-CH_3$) (0.8767 ppm), Rhamnose moiety (-CH-OH) (3.363 ppm), Sugar moiety (3.493- 4.865 ppm), Rhamnose moiety (-CH-O-C-) (5.268 ppm). 1H NMR spectrum using Dimethyl Sulfoxide (DMSO) as a solvent contains a long hydrocarbon chain (1.262 ppm), rhamnose ring in rhamnolipids (3.363 ppm) and sugar moiety (3.493ppm-4.865 ppm). All these molecules showed that the Rhamnolipid type of biosurfactant was produced.

3.5 Optimization of biosurfactant produced by *Bacillus pasteurii*: pH **7**, temperature **30ºC,** carbon source **starch** and nitrogen source **peptone** show the highest emulsification activity than the others and hence these parameters are optimized for biosurfactant produced by *Bacillus pasteurii.*

Table 3: Optimization of biosurfactant.

Sr. No.	Parameters	Variations	Emulsification Assay (EU/ml)
1.	pH	5	1.32
		6	1.38
		7	**3.88**
		8	0.60
		9	0.47
		Control	0.73
2.	Temperature	20°C	5.74
		30°C	**5.88**
		40°C	4.30
		Control	4.36
3.	Carbon	Sucrose	1.43
		Starch	**1.49**
		Kerosene	1.34
		Control	4.36
4.	Nitrogen	Ammonium nitrate	1.14
		Ammonium chloride	1.69
		Ammonium sulphate	1.17
		Potassium nitrate	0.95
		Peptone	**4.82**
		Control	0.71

4. APPLICATION OF BIOSURFACTANT:

4.1 Synthesis of biosurfactant mediated Silver nanoparticles (AgNPs):

The formation of a brown colour indicates silver nanoparticles were synthesized from biosurfactant extracted from *Bacillus pasteurii.* A UV-visible spectrum of biosurfactant-mediated silver nanoparticles (AgNPs) showed a pointed peak at 365nm. Here silver nanoparticles were synthesized by using a rhamnolipid type of biosurfactant.

1. Isolation of Bacteria:

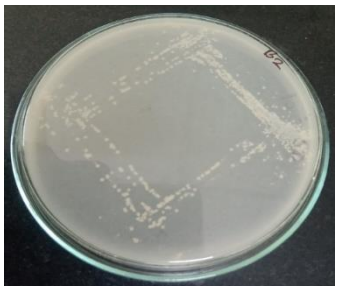

Figure 1. Isolated colonies of *Bacillus pasteurii.*

2. Characterization of Bacteria:

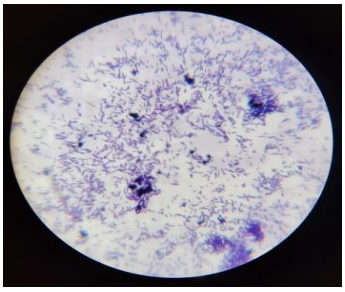

Figure 2.1. Gram positive rods.

Figure 2.2. Starch hydrolysis test.

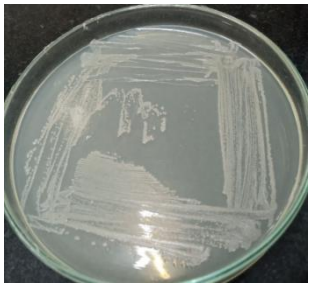

Figure 2.3. Catalase test.

2. Characterization of Bacteria:

Figure 2.4. Nitrate reduction test.

3. Extraction of biosurfactant:

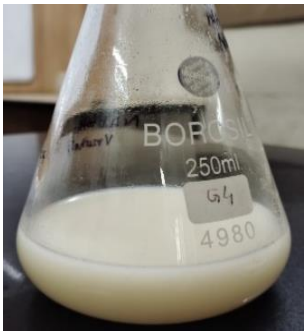

Figure 3.1. Extracellular production of biosurfactant from *Bacillus pasteurii.*

Figure 3.2. Extracted biosurfactant from *Bacillus pasteurii.*

4. Characterization test for detection of biosurfactant:

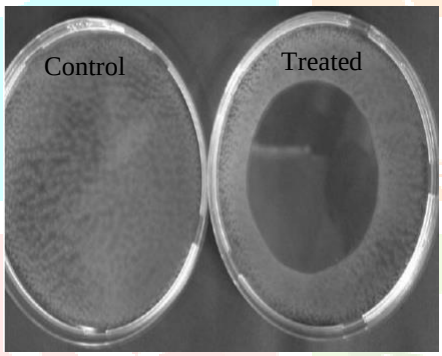

Figure 4.1. Oil spreading test.

Figure 4.2. Tilted glass slide test.

Figure 4.3. BATH assay.

Figure 4.4. Emulsification Index.

5. Analysis of biosurfactant using Nuclear Magnetic Resonance (NMR):

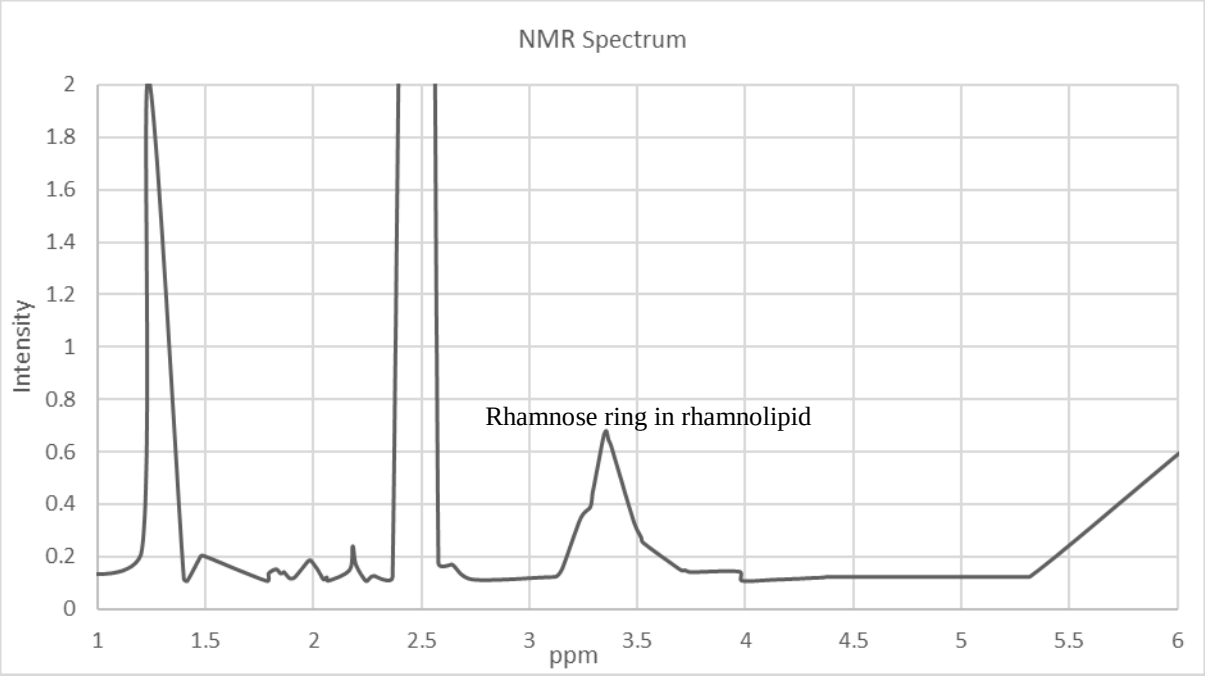

Figure 5.1. Graphical representation of ^{1}H-NMR spectrum of biosurfactant extracted from *Bacillus pasteurii.*

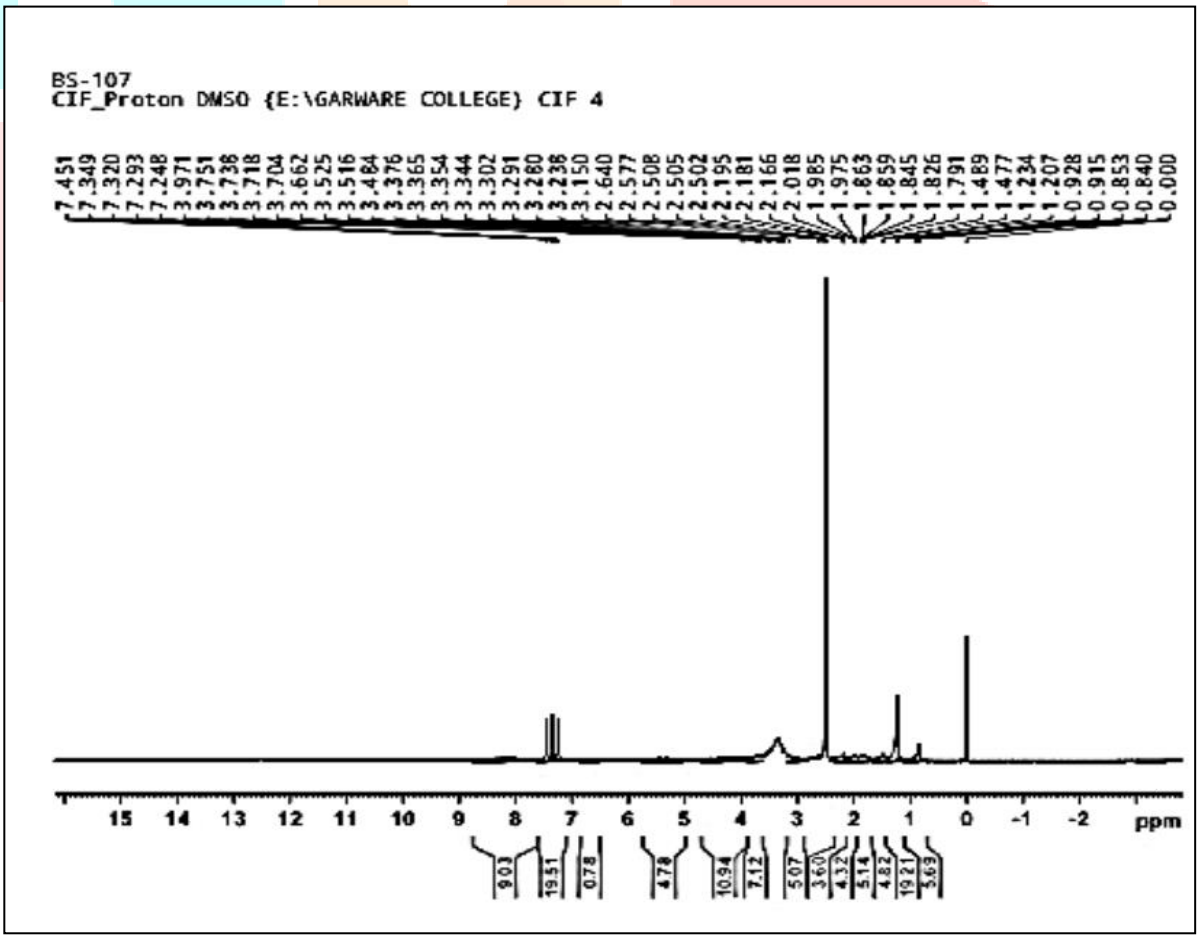

Figure 5.2. Graphical representation of ^{1}H-NMR spectrum of biosurfactant extracted from *Bacillus pasteurii.*

7. Synthesis of biosurfactant mediated silver nanoparticles:

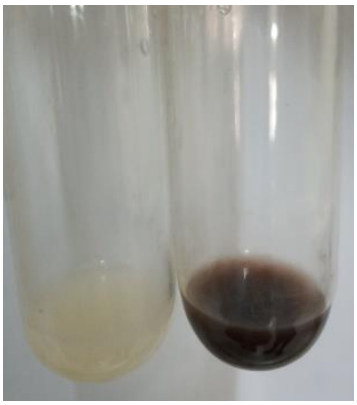

Figure 7.1. Synthesized biosurfactant mediated silver nanoparticles (AgNPs) by chemical reduction method.

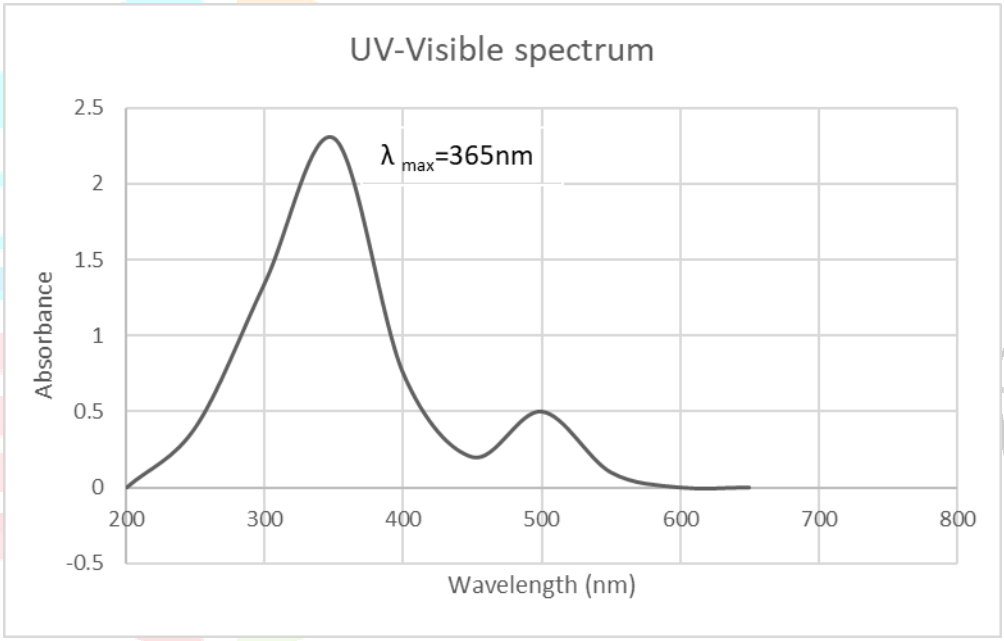

Figure 7.2. UV-Visible spectrum of synthesized silver nanoparticles (AgNPs).

5. CONCLUSION: *Bacillus pasteurii* was isolated from petroleum-contaminated soil. 'Bergey's manual' of the systematics of Archaea and Bacteria, identification flowchart was used for the characterization of isolated *Bacillus pasteurii*. Biosurfactant was extracted from *Bacillus pasteurii* and characterized by using various screening methods and confirmed by using the NMR spectrum. Screening methods for the detection of biosurfactants show emulsification activity greater than 50% which is a significant value for biosurfactant detection. Analysis of biosurfactant by using 1H-NMR spectrum showed that the rhamnose ring in rhamnolipid (3.363 ppm), long hydrocarbon chain (1.262 ppm) and sugar moiety (3.493ppm-4.865 ppm) was present in biosurfactant and it confirmed that it is rhamnolipid type of biosurfactant. pH 7, temperature 30^{o}C**,** carbon source starch and nitrogen source peptone show the highest emulsification activity and give a higher yield of biosurfactant on optimization.

Biosurfactant-mediated silver nanoparticles were synthesized as an application of biosurfactant and characterized by using UV-visible spectrum analysis. λ_{max} =365nm confirmed the synthesis of silver nanoparticles.

PROSPECTS: Biosurfactants can be used in advanced drug delivery systems. Biosurfactant used as penetration enhancer in Transdermal Drug Delivery System (TDDS). Surfactants can play an important role in cerumen removals. Niosomes (non-ionic) surfactants can play a vital role in gene therapy. To avoid biofilm formation in bacteria biosurfactant mediated silver nanoparticles can be coated. Because of their antimicrobial, antioxidant, and antitumor activity, they can used in pharmaceutical products and can act as an emulsifying agent.

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