ISOLATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCING BACTERIA FROM OIL CONTAMINATED SOIL

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CERTIFICATE

This is to certify that the thesis on **"ISOLATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCING BACTERIA FROM OIL SOIL"** is submitted by **AJEET KUMAR SONI (109CH0493)** to National Institute of Technology, Rourkela under my supervision and is worthy for the partial fulfillment of the **degree of Bachelor of Technology (Chemical Engineering)** of the Institute**.** He has fulfilled all the prescribed requirements and the thesis, which is based on candidate's own work, has not been submitted elsewhere.

DATE:

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ABSTRACT

Biosurfactants are surface active compound that reduce the interfacial tension between two liquids, or that between a liquid and a solid. Their unique property like nontoxic, easily biodegradable, eco-friendly and high stability, and wide variety of industrial application makes them highly useful group of chemical compound. Biosurfactants are produced from variety of microorganisms.

The objective of this study was to isolate and characterize the biosurfactant producing bacteria from oil contaminated soil and study of their growth kinetics at different temperature and pH. The isolation and Growth study was carried out in MSM composition using kerosene oil as sole carbon source for bacterial growth. Isolated strain, assigned B1 and B2, were found Gram's positive and in general Gram's positive bacteria are able to produce lipopeptides type biosurfactant. Isolated strains were bacilli type. The optimal conditions for bacterial growth were found to be pH 7 and temperature 30^{o}C for strain B1 and pH 8 and temperature 30^{o}C for strain B2.

Keyword: Biosurfactant, eco-friendly, microorganism, biodegradable

CHAPTER- 1

INTRODUCTION

1. INTRODUCTION

Surfactants are surface active compound that reduce the interfacial tension between two liquids, or that between a liquid and a solid. Surfactants are organic compound that contain both hydrophobic (head part of the surfactant) and hydrophilic (tail part of the surfactant) moieties. Thus surfactant contains both water insoluble i.e. water repellent group as well as water soluble i.e. water loving group. **Biosurfactants** are also surface active compound like chemical surfactants but unlike the chemical surfactant, biosurfactant are synthesized by microbes like bacteria, fungi and yeast. Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are usually non-toxic and biodegradable. Recently interest in biosurfactant has increased because of its diversity, flexibility in operation, and more ecofriendly then chemical surfactant (1) (2). Furthermore possibility of their production on large scale, selectivity, performance under intense conditions and their future applications in environmental fortification also these have been increasingly attracting the attention of the scientific and industrial community. These molecules have a potential to be used in a variety of industries like cosmetics, pharmaceuticals, humectants, food preservatives and detergents (1). But the production of biosurfactant on industry level is still challenge because of using high costly synthetic media for microbial growth. Biosurfactants are classified on the basis of diversity in their structure and their microbial origin. They contain a hydrophilic group, that contain an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic group of unsaturated or saturated hydrocarbon chains or fatty acids. Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition and their nature and the amount depend on the type of microbes producing a particular biosurfactant. And in resulting wide variety of biosurfactant can be produced accordingly to the demand and uses.

Table.1 Biosurfactant and microorganism involved (3)

Biosurfactant	Microorganism
Glycolipids Rhamnolipids Trehalose lipids Sophorolipids Mannosylerythritol lipids	*Pseudomonas aeruginosa* *Rhodococcus erithropolis* *Arthobacter* sp. *Candida bombicola, C. apicola* *C. antartica*
Lipopeptides Surfactin/iturin/fengycin Viscosin Lichenysin	*Bacillus subtilis* *P. fluorescens* *B. licheniformis* *Serratia marcescens*

Polymeric surfactants Emulsan Alasan Liposan Lipomanan	*Acinetobacter calcoaceticus* *A. radioresistens* *C. lipolytica* *C. tropicalis*

1.1 Classification of Biosurfactant:

Biosurfactants are classified in to two major group one is low molecular weight surface active agent call biosurfactant and high molecular weight substance called bio-emulsifier that is especially used as enhancement of emulsification of hydrocarbon. Further these two major group is divided in to six major group known as glycolipids, lipopolysaccharides, lipoproteins-lipopeptides, phospholipids, hydroxylated and cross linked fatty acids.

(a) Glycolipids:

Mostly biosurfactants are glycolipds. They are lipids with a carbohydrate attached. The connection is by means of either an ether or ester group. Among the glycolipids, the best known are rhamnolipids sophorolipids and trehalolipids.

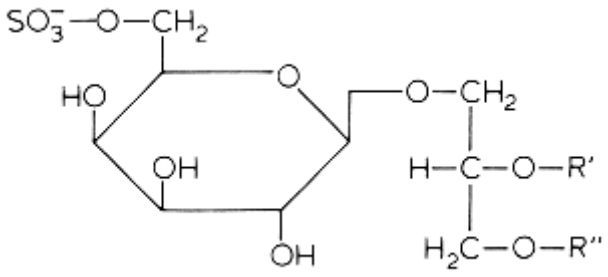


Fig 1

(b) Rhamnolipids:

Rhamnolipids is a group of biosurfactant that studied extensively. These are produced by many species of Pseudomonas and have tremendous antimicrobial activity against several common microorganisms, which is an essential property of all cosmetics due to the daily contamination of the product by the human touch (3). Rhamnolipids is a type of glycolipid biosurfactant that contain either a one or two molecule of β-hydroxydecanoic acid. In 1999 the structure of rhamnolipid produced by P. aeruginosa on mannitol and nepthalene by liquid chromotography was compared. And it was found that for maanitol the most common rhamnolipid contained two rhamnoes and two 3-hydroxydecaonic acid and whereas rhamnolipid produced by nepthalene contained two rhamnoes and one 3-hydroxydecaonic acid.

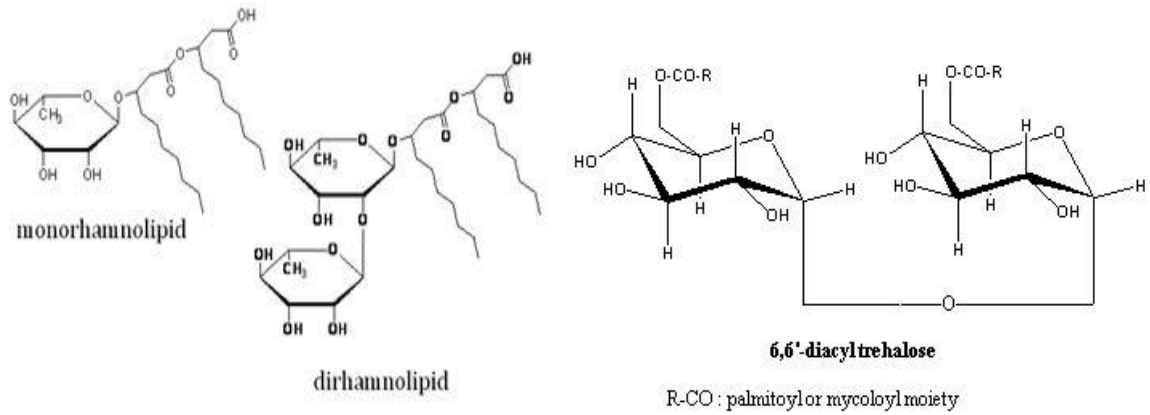


Fig.2 (a)Structure of mono and di rhamnolipid (b) Structure of Trehalose lipid

(c) Trehalolipids: It is another type of glycolipids that containing trehalose hydrophobic moieties. Such type of biosurfactant structure is diverse in hydrophobic moieties, varying from short fatty acid to long fatty acid chain. In general it is observed that the bacteria showing Gram's positive produces Trehalolipids biosurfactant (4).

(d) Sophorolipids: Such glycolipids are synthesized by yeast; these are the complex mixture of both free acid and lactone form. The acidic SL contain fatty acid as tail where as SL lactonic are formed by the esterification between carboxylic end of the fatty acid and 4" of the sophorose end. Generally lactonic SL has better surface tension lowering property whereas the acidic SL have better potential to form foam and solubility properties (5).

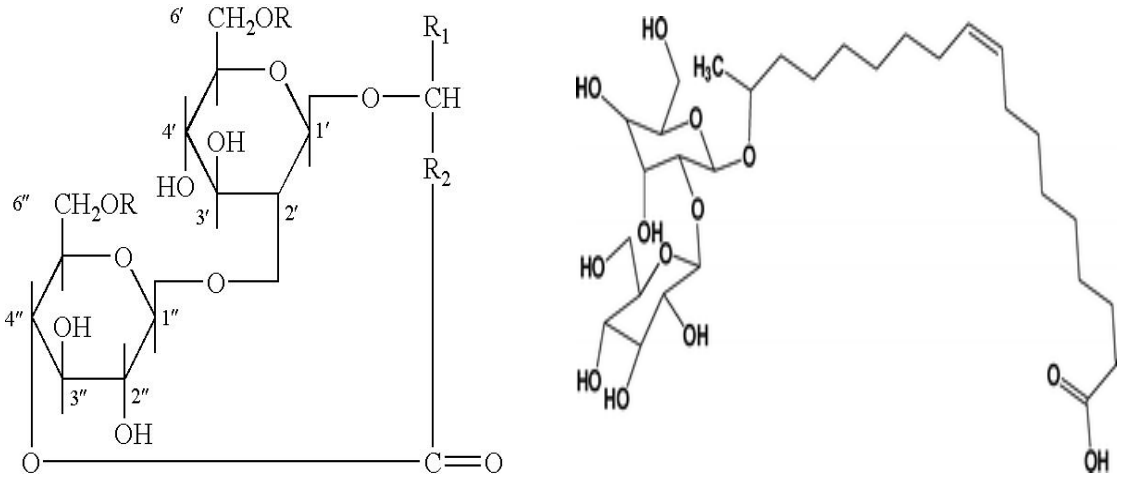


Fig.3. Structure of SL lactonized and acid Form.

1.2 Lipopeptides and lipoproteins:

Lipopeptide biosurfactants are cyclic compounds and they are mostly isolated from Bacillus and Pseudomonas type bacteria Lipopeptides mainly consist of hydrophilic peptides, generally they consist 7 and 10 amino acids long, linked to a hydrophobic fatty acid structure. Bacillus cyclic lipopeptides consist of three major groups known as the surfactin, iturin and fengycin families. Surfactin is the most commonly studied and it contains 7 amino acid cyclic sequences connected to a C13–C16 fatty acid (6).

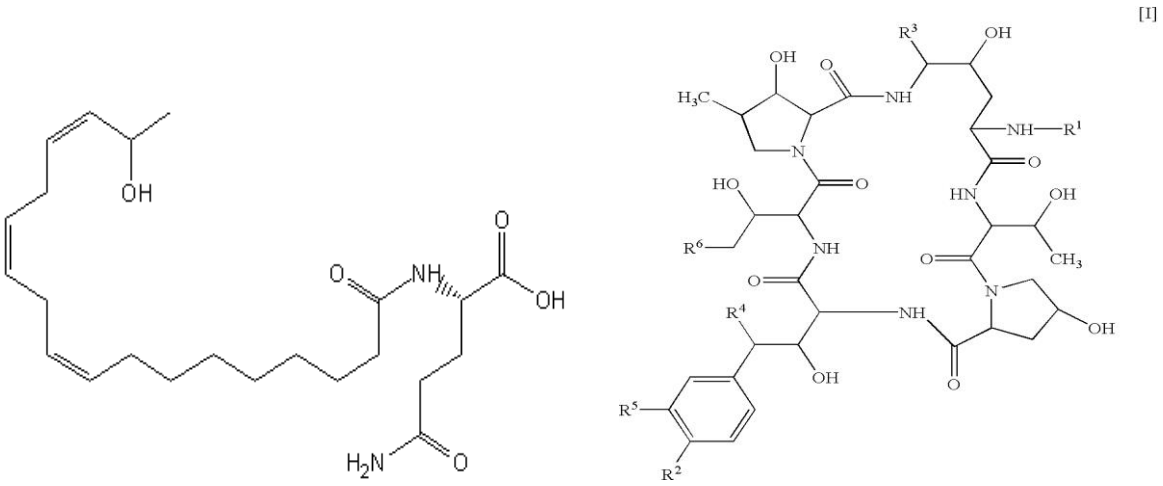


Fig.4 Structure of aminolipopetide and cyclic aminolipopetide

1.3 Polymeric Biosurfactant:

Polymeric biosurfactant are high weight molecular biopolymers which consist polysaccharides, proteins, lipopolysaccharides, lipoproteins or mixture of theses biopolymers. A wide variety of microbes produces polymer biosurfactant. Polymer biosurfactant exhibits properties like high viscosity, tensile strength, and resistance to shear. The following are example of different type of polymeric biosurfactant.

Table.2 Example of biosurfactant and microorganism involved (7).

Biosurfactant	Microorganism
Emulsan Alasan Liposan Lipomanan	*Acinetobacter calcoaceticus* *A. radioresistens* *C. lipolytica* *C. tropicalis*

1.4 Properties of Biosurfactant:

Biosurfactants are surface active compound that accumulate at the boundary between two immiscible fluids or between a fluid and a solid. By reducing surface (liquid-air) and interfacial (liquid-liquid) tension they reduce the repulsive forces between two different phases and allow them to mix and thus enhance the solubility properties like chemical surfactant. The most effective biosurfactants can reduce the surface tension of water from 72 to 30 $mN \cdot m^{-1}$ and the interfacial tension between water and *n*-hexadecane from 40 to 1 $mN \cdot^{m-1}$ (2, 10) Biosurfactant produces from *B. subtilis* is able to lower the surface tension of water to 25 mN/m and interfacial tension of water/hexadecane to <1 mN/m (8). Further more, biosurfactants are more effective and efficient and CMC of biosurfactant is about 10–40 times lower than that of chemical surfactants, so less amount surfactant is required to get a maximum decrease in surface tension as compare to the chemical surfactant (9).

In many cases it was found that biosurfactant activities are not influenced by environmental condition such as temperature and pH. In 1990 McInerney suggested that lichenysin produced by *B. licheniformis* was not affected by temperature (up to 50°C), pH (4.5–9.0) and by NaCl and Ca concentrations up to 50 and 25 g/l respectively. Apart from theses above properties biosurfactant can be easily degraded unlike the chemical surfactant and thus and they are chiefly suited for the environmental applications such as bioremediation, and dispersion of oil spills. The toxicity of biosurfactants is much lower and some of the researcher consider as these are non toxic compounds. Very few literature are available that describes the toxicity of biosurfactant and their direct bad impact on environment. Therefore they are appropriate for pharmaceutical, food and cosmetic uses. A study suggested that a synthetic anionic surfactant (Corexit) showed an LC50 (concentration lethal to 50% of test species) against Photobacterium phosphoreum ten times lesser than rhamnolipids, suggesting the larger toxicity of the chemically derived surfactant. In a particular study where toxicity of six biosurfactants was comparing with the toxicity of, four of the synthetic surfactants and two commercial dispersants, it was observed that mostly biosurfactants degraded quicker (10), except for a synthetic sucrose-stearate that showed structure homology to glycolipids and was degraded more rapidly than the biogenic glycolipids. A biosurfactant from *P. aeruginosa* was compared to a synthetic surfactant that is widely used in the industry, regarding toxicity and mutagenic properties. Both assays indicated a higher level of toxicity and mutagenic effect of the chemically derived surfactant, whereas the biosurfactant was considered to be slightly non-toxic and no mutagenic.

1.5 Advantages and Uses of Biosurfactant:

The unique properties of biosurfactant (Microbial Surface Active Agents) such as low toxicity, relative ease of preparation and widespread applicability, make it different from chemical synthetic surfactant and now it has become recently an important product of biotechnology for industrial and medical applications and they allow to replacement of chemical synthetic surfactant. They can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors such as, Petroleum and Petrochemicals, Organic Chemicals, Foods and Beverages, Cosmetics and Pharmaceuticals, Mining and Metallurgy, Agrochemicals and Fertilizers, Environmental Control and Management, and many others.

There are many advantages of biosurfactant as compare to chemically synthesized surfactants. Some of those are:

1. Biodegradability: Easy to biodegradable as compare to the chemical surfactant (10).
2. Low toxicity
3. Biocompatibility and digestibility, that allows their application in cosmetics, pharmaceuticals and food seasonings.
4. Easily availability of raw material: The raw material need for production of biosurfactant are easily available, biosurfactant producing microorganism can be isolated from the industrial waste like oil contaminated soil, petrol pump spilled, and also can be isolated from municipal waste.
5. Use in environmental control. Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and lowering the toxicity of industrial discharges and in bioremediation of polluted soil.
6. Specificity in their action, since biosurfactant has specific organic functional group and often specific in their action. This is particularly used in lowering the toxicity of the pollutant, used in enhancing the emulsification property, used as raw material in cosmetic, medicinal and foodstuff applications.

CHAPTER -2

LITERATURE REVIEW

2. LITERATURE REVIEW

Biosurfactants have unique property like biodegradability, low toxicity, and more ecofriendly, large flexibility in operation etc. But production on huge industry level is still challenge reason is low economical than chemical surfactant, because of using synthetic nutrient media is expensive than natural media. To overcome with this problem associated with biosurfactant production, researchers mainly focus on uses of industrial waste for fermentation process like using of agro waste; molasses etc. and using of optimize bioprocess like optimum temperature, pH, and other parameters. Every year tons of hazards and non-hazards wastes are generated that needs to proper utilization to prevent the world from pollution and other hazards impact. Residues obtain from agriculture such as peels, hull, sugar beet, sweet potato, residue from coffee processing unit, residue from oil industries such as oil cake; can be used as substrate for biosurfactant production (1).

Table.3 Substrate for microbial surface agent (1)

Source	Substrate	End product
Cassava	Flour	Biosurfactant
Soybean oil	Seeds	Rhamnolipid
Sugar beet	Peels	Biosurfactant
Sweet Potato	Peels	Biosurfactant
Sweet Sorghum	Peels	Biosurfactant
Rice and wheat bran	Stem Husk	Biosurfactant
Sugarcane Bagasse	Stem Husk	Biosurfactant
Cashew Apple juice	Pomace	Biosurfactant
Dairy Whey	Whey	Biosurfactant

Ghayyomi Jazeh, M et. al (2012) isolated biosurfactant producing bacteria from petroleum contaminated soil and they observed that 160 strains were able to producing biosurfactant, in which 59 strains showed positive blood hemolysis test, 45 strains showed positive oil spreading technique. They found that emulsion and foaming activity was maximum at 7 pH and 37°C temperature. For the isolation culture media was synthesized in lab by Banat method (Rahman *et al*., 2002a; Rasooli *et al*., 2008) (11).

In 2008, Kevin B. Cheng et.al has studied the emulsion properties of bacterial biosurfactant, they isolated three unknown biosurfactant producing bacteria and their emulsification activity was compared with the two artificial surfactant, SDS and Triton X-100. They were used Lee *et al* (2008) to measured the emulsification activity and stability.4ml of each biosurfactant was dissolved in 1ml of diesel oil or hexadecane and mixture was vigorously shaken in vortex mixer for 2 min. And mixture was stand for 10 min and then reading was noted down. And it was found that two biosurfactant (L1 and L2) had batter emulsion activity in hexadecane as compare to the synthetic surfactant, whereas in diesel the EA of artificial surfactant was better than all extracted biosurfactant (L1, L2 and L3) (13) .

In another research in which *P. aeruginosa,* was isolated from oil contaminated sea water and it was seen that it was able to break down the hydrocarbon such as hexadecane, heptadecane, octadecane and nonadecane in sea water up to 47, 53, 73, and 60 % respectively (14).

Eduardo J. Gudi˜na et.al (2011), in particular research i.e production of biosurfactant from lactobacilli, they were found that the production of biosurfactant not only depends on the type of microorganism but also depend on the composition of mineral salt media. It was noted that lactobacilli produce lower amounts of biosurfactants as compare with other microorganisms, such as *Bacillus subtilis* or *Pseudomonas aeruginosa*, and also they consume many nutritional, they constitute a promising source of biosurfactant, because these microorganisms are usually considered GRAS and are already used in many food manufacturing and industrial process. Furthermore, it was noted that the yield of biosurfactant production can be increased through the optimization of culture condition.

In this research it was reported that yeast extract is an essential component for the bacterial growth, whereas the peptone is for the biosurfactant production. And the combination of yeast and meat extract resulting in higher yield of biosurfactant (15).

Wen-Jie Xia et, al (2011), in that research they were isolated three biosurfactant producing bacteria from reservoir formation water, B. subtilis, P. aeruginosa, and R. erythropolis, by using these three bacteria three biosurfactant was extracted and studied using crude oil as a carbon source. P. aeruginosa was noted that the overall biosurfactant production rate, resistance and stability are extremely well than rest two bacteria. This also attained emulsion index 80% for crude oil and also reduce the surface tension of medium from 71.2 to 22.6 mN/m. P. aeruginosa showed14.3% oil recovery after water flooding, in results of biosurfactant flooding experiment (16).

Biosurfactants were used in large scale site by Kosaric, In Canada and the Middle East, many contaminated sites were bioremediated with biosurfactant, and these sites were contaminated with heavy hydrocarbons. Bioremememediation was accelerated by addition of glycolipid biosurfactant.

2.1 REMARKS:

From the literature review, it can be concluded that biosurfactant can be produced from verities of microorganisms. Such type of bacterial strain has been isolated from various industrial wastes like agrowaste, coffee processing waste, petrochemical waste etc and it was found that the production of biosurfactant depends on bacterial growth. It was noted that the production of biosurfactant is still challenge on industry level because of high cost of bacterial nutrient, to overcome this problem, many of researcher isolated bacteria from industrial waste as mentioned above. It was also studied that the production rate of biosurfactant depends on bacterial growth, so the bacterial growth should be in optimal conditions like optimum temperature, and media pH.

2.2 Aim & Objectives:

My study was based on the isolation of biosurfactant producing bacteria from oil contaminated soil and studied of their growth kinetics for found out the effect of temperature and pH on their growth that was carried by following consideration:

1. Isolation of Biosurfactant Producing Bacteria from Contaminated oil soil.
2. Screening of isolated bacteria.
3. Characterization of bacterial strain.
4. Study of their growth kinetics.

CHAPTER-3

MATERIALS

&

METHOD

MATERIALS & METHOD

3.1 Isolation of biosurfactant producing bacteria:

The entire experiment comprises following steps:

1. Isolation of bacteria.
2. Screening of isolated strains
3. Characterization of isolated bacteria.
4. Growth Kinetics.

Materials Requirement:

1. Sample of oil-soil.
2. Autoclave, laminar flow hood, Flask (different size), Pipet, Petri disk, Test tube, hot-air oven, and chemicals for different tests.
3. Incubator and shaker.

(a) Sample collection:

Soil sample was collected from Haldia Petrochemical limited, Haldia.

(b) Isolation of bacterial colonies:

5g of soil sample was inoculated in 100 ml of Mineral Salt Medium (MSM) with 3ml kerosene oil added to the conical flask having capacity of 250 ml, as the carbon sources and then it was incubated for 72 hours at 30°C temperature. After then 1ml of incubated culture was streaked on the petriplates. The samples then were serially diluted up to 10^{-6} dilution. 1 ml of 10-6 time dilution was transferred to nutrient agar for spread culture. The plate was inverted and incubated at 30° C, for 72 hours. After incubation, morphologically five distinct colonies were selected for further studies.

Tabl.4 (Composition of MSM)

Components	Weight (in gm)/ per liter of water
KH_2PO_4	1
$MgSO_4$	0.5
$FeSO_4$	0.01
$NaNO_3$	1.5
$CaCl_2$	0.002
$(NH_4)_2SO_4$	1.5

3.2 Screening of Biosurfactant producing bacteria:

The isolated colonies were taken and tested for the confirmation of biosurfactant bacteria that comprises following test:

(a) Oil spreading technique:

In oil spreading assay for oil displacement activity of surfactants was done as per the method described by Morikawa et al. (1993). The principle of this method was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. The surface pressure of the biosurfactant displaced the oil. In this method 10µl of kerosene oil was added to the surface of 50 ml distill water in a petri dish, oil form a thin layer then 10µl cultured supernatant gently placed on the center of oil layer. If the oil displaces and clear zone forms then it shows the presence of biosurfactant. The displaced diameter is measured after 30 second. This is also known as oil displacement activity. Measured area is express in BS unit, known as biosurfactant unit. One biosurfactant unit (BS unit) was defined as the amount of surfactant forming 1 cm^2 of oil displaced area (Thaniyavam et al.. 2003).

(b) EMULSIFICATION ASSAY:

Emulsification activity was calculated by emulsification index known as E_{24}. Emulsification assay was carried by adding 2ml kerosene oil in 1ml cell free supernatant which was obtained after the centrifugation, and then it was vortexed for 5 minutes confirm regular mixing of both the liquids. The emulsification activity was observed after 24 hours and it was calculated by using the formula:

E_{24} = ***Total height of the emulsion layer/height of the aqueous layer * 100***

(c) FOAMING ACTIVITY:

Isolated strains were grown separately in 250 mL Erlenmeyer flasks, each containing 100 mL of nutrient broth medium. The flasks were incubated at 37°C on a shaker incubator (200 rpm) for 72 h. Foam activity is detected as duration of foam stability, foam height and foam shape in the graduated cylinder.

(d) Blood Hemolysis Test:

This test is done to determine the ability of bacterial colonies to induce hemolysis when grown on blood agar. It is used to classify the certain microorganism. There are three types of hemolysis α, β, and γ. A hemolysis is said when the agar under the colony become dark and greenish, β is said when it becomes lighten yellow and transparent and when no any change then it is said γ hemolysis.

Fresh isolated colony was taken and streaked on blood agar plate and incubated for 72 hours under 30°C temperature. After then it was observed, and compare, presence of clear zone indicate the biosurfactant producing bacteria (17).

3.3. Physical characterization of bacterial isolates:

(a) GRAM STAINING:

Gram staining is a method that differentiates bacteria in two large group gram positive and gram negative. This method differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, in gram positive it is present as a thick layer. A Gram positive results in a purple/blue color while a Gram negative results in a pink/red color.

First bacteria smear was prepared taken from pure culture, bacteria colony and distilled water was properly mixed until just slightly turbid on dry and clean glass slide using sterile loop or needle. Then it was heats fixed using spirit lamp and allows cooling at room temperature. Then it was treated by crystal solution and kept for 1 minute and then it was washed using distilled water. The slide was then flooded with gram's iodine kept for 1 minute and washed. Then put 1 to drops of gram's decolorizer (95% alcohol and 5% acetone) on the smear and kept for 30 second and washed properly. Then put counter stain (safranin) for 60 second and rinsed with distilled water and allow to air dry. The slide was then observed under the light microscope at 100x objective.

(b) Cell Morphology:

The Gram stained cells were observed and the shape and color of the cells were determined.

3.4 Bio-Chemical Characterization of isolated bacteria:

(a) Catalase Test:

It is a common enzyme found in living organisms exposed to the oxygen. It has one of the maximum turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. The reaction of catalase in the decomposition of living tissue:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

The main objective of this test is to determine whether microbes are able to produce catalase enzymes or not, it is a protective enzyme i.e. able to the dangerous chemical hydrogen peroxide. A pure culture, growth from the overnight was smeared on glass slide and then added 3% hydrogen peroxide and observed. If rising bubbles are observed then it shows positive else negative.

(b) Citrate Test:

The **citrate test** notices the capability of an organism to consume citrate as the sole source of carbon and energy for growth and metabolism. The principle is based on the breaks down of citrate to oxaloacetate and acetate. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator such as bromothymol blue. This medium also comprises inorganic ammonium salts, which are utilized source of nitrogen. Bacteria decompose the citrate and convert in to the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further decomposed in to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH. This results in a change of the medium's color from green to blue.

(c) Urease Test:

The main objective of this test is to determine the ability of bacteria to degrade urea by means of urease enzymes. Culture was inoculated in urease broth for 48 hrs and maintains temperature 37°C and then observed the color, if a color change from light yellow to pink then test is positive.

(d) Methyl Red Test:

Methyl red test is used to find out the ability of bacteria producing acid by mechanism of mixed acid fermentation. Culture was inoculated in MR-VP broth for 3 days at temperature of 37°C after the incubation added 2-3 drops of Methyl red and observed the color change that take place in few seconds.

Positive: Yellow to Red color

Negative: No color change

(e) Nitrate Test:

The objective of this test is to determine the ability of production of a enzyme called nitrate redcatase by bacteria, resulting reduction of nitrate (NO_3). Incubated the bacterial strain in nitrate broth at the optimal temperature (30 or37°C) for 48 hours further added few drops of nitrate reagent and see the reaction within the minute or less, if there is no evolve of N_2 or nitrate gas then there is need to add a bit powdered zinc.

Pink red color: Nitrate negative reduction

No pink form: Positive nitrate reduction

(f) Indole Test:

The test determines the bacterial capability to breakdown the indole from tryptophan molecule. 24 hours pure culture was inoculated in tryptone broth. It was incubated for 24 hours at 30°C and then removed 2ml media and added in to the empty sterile test tube and added few drops of Kovac's reagent and agitate the tube for few minutes.

Positive: Within few minute cherry red color form

Negative: No color changes

(g) Gelatin Test:

The objective of the test is to determine bacterial production of gelatins enzymes that liquefy the gelatin. Pure culture was inoculated in nutrient gelatin media and then it was incubated for 24 hours at 30°C temperature.

Positive: Media is liquefied

Negative: No liquefaction occur

(h) Starch Hydrolysis:

Some bacteria can use starch as source of carbon energy, starch hydrolysis test shows whether bacteria can use starch as sole carbon energy or not. The pure culture is streaked on starch agar plate and the inoculated plate is incubated for 24 hours and temperature is maintained 30 -35 °C. After then iodine reagent is added to flood the growth.

Positive: Presence of clear halo surrounding colonies

3.5 GROWTH STUDY OF ISOLATED STRAINS

Growth study was conducted for the isolated strains (B_1 & B_2) from oil soil. The study was done in 250ml flask using 100ml of mineral salt media kept in shaker at 30°C temperature for 144 hours and reading was taken at regular interval of 12 hours at absorbance 600 nm. Growth study of both strains was done at three different pH (6, 7, and 8) and three different temperatures (25, 30 and 35°C) compare to find out the optimum growth condition of bacterial strain.

CHAPTER-4

RESULT

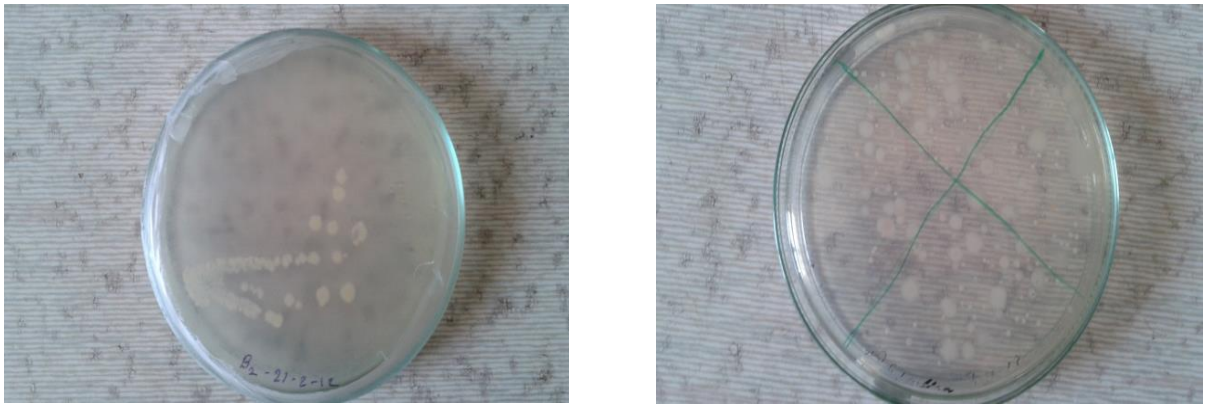
&

DISCUSSION

RESULT and DISCUSSION:

4.1 Isolation of Bacteria:

Five strains observed different colony morphology were streaked on nutrient agar plate to obtain pure culture and they were taken for further study.



(a) (b)

Fig 5 Isolated bacterial strain and colony on agar plate.

4.2 Screening of Isolated strains.

All the isolated strains were screened for the confirmation of biosurfactant producing bacteria in which two strains showed good result and these two (B1 and B2) were taken for the further study.

(a) Oil Spreading Technique:

Supernatant of isolated strains were added to the plate containing kerosene oil. It was added to the center of oil layer. The strains B1 and B2 displaced the oil and showing a clear zone. The results were noted down. Bacterial strain B1 and B2 was chosen for the further study.

Table.5 Result of oil spreading technique.

Bacterial Strain	Diameter of clear zone (in cm)	INTERPRETATION
B1	2.2	Positive
B2	3.0	Positive
B3	0	Negative
B4	No result	Negative
B5	0	Negative

(b) EMULSIFICATION ASSAY*:*

The isolated and strain showed positive result were tested for their abilities emulsify crude oil and in this study kerosene was taken was for the study of emulsification assay. Test was done by the adding 2ml of kerosene oil and 1 ml of supernatant and kept overnight. After then results were noted down.

Table.6 E_{24} index of bacterial strain.

Bacterial Strain	Emulsified layer (cm)	Total liquid layer (cm)	E_{24} (%)
B1	1.00	1.8	52.6
B2	0.8	1.9	42.1

(c) Foaming Activity:

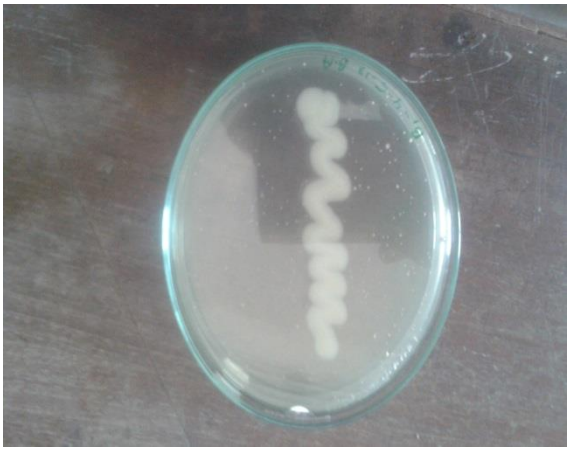
Isolated strains were grown separately in 250 mL Erlenmeyer flasks, each containing 100 mL of nutrient broth medium. The flasks were incubated at 30°C on a shaker incubator (200 rpm) for 72 h.

(a) (b)

Fig.7 foaming activity of both bacterial strain

(d) Blood Hemolysis Test:

Both the strain was streaked on blood agar plate, in which strain B1 showed β hemolysis, whereas strain B2 showed γ hemolysis.



(a) **(b)**

Fig.7 Result of blood hemolysis test

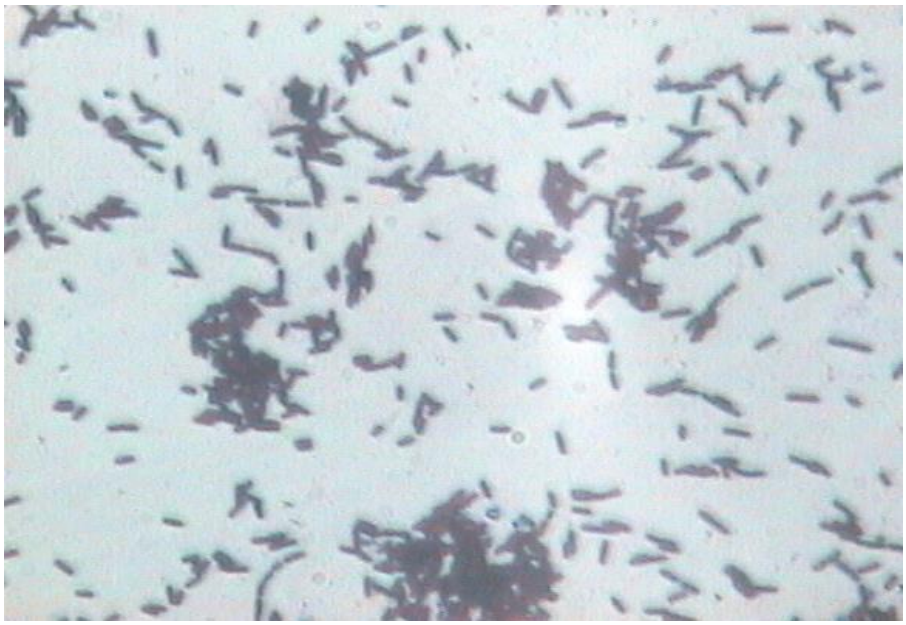
4. 3 CHARECTERIZATION OF ISOLATED BACTERIA:

(a) Colony Morphology:

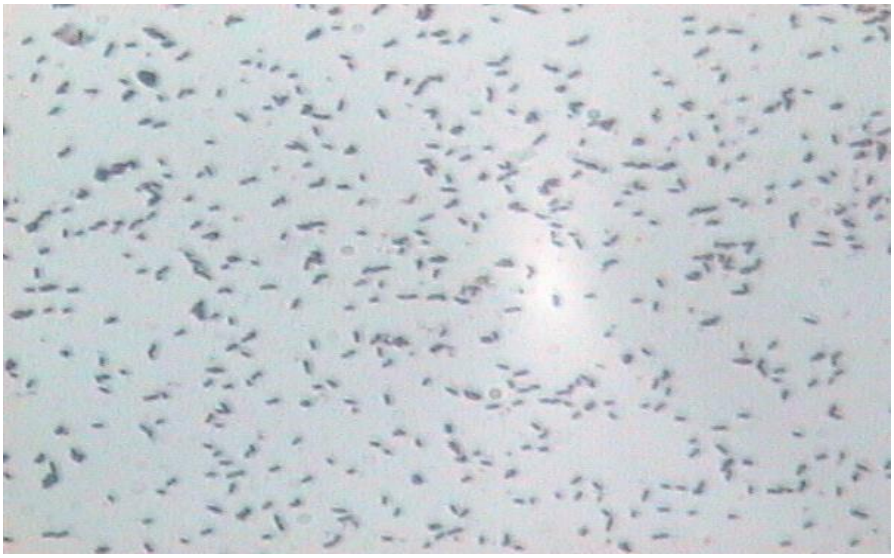
The shape, size, elevation, margin and cooler of the colony were observed in the culture plates with Nutrient Agar used as the nutrient medium. The observations were noted down.

(b) Gram's staining:

Morphological characterizations of isolate strains were done by Gram's staining. And it was found that both strains were Gram positive Bacillus.



(a)



(b)

Fig .9 shows the microscopy view of Gram's Staining of Strain B1 and B2

Table.7.Result of cell and colony morphology

Strains	Gram Staining	Cell Shape	Colony Shape	Pigment(Colony)	Oxygen Requirement
B1	+ve	Red shape Bacili	Circular, wet, smooth, Concave	Colored	Aerobic
B2	+ve	Red shape Bacili	Circular, dry, smooth, flat and irregular with lobate marins	Colored	Aerobic

(c) BIOCHEMICAL CHARECTERIZATION

From the Gram's staining result, it was found that both the strains are positive so for further characterization, different bio-chemical tests were performed to identify the species of strains. For the Identification and characterization of isolate bacterial strains Bergey's manual of determinative bacteriology was used and on the basis of this manual the species was predicted as bacillus species of bacteria. And it was noted that bacillus type bacteria are able to produce lipopeptides type biosurfactant (7).

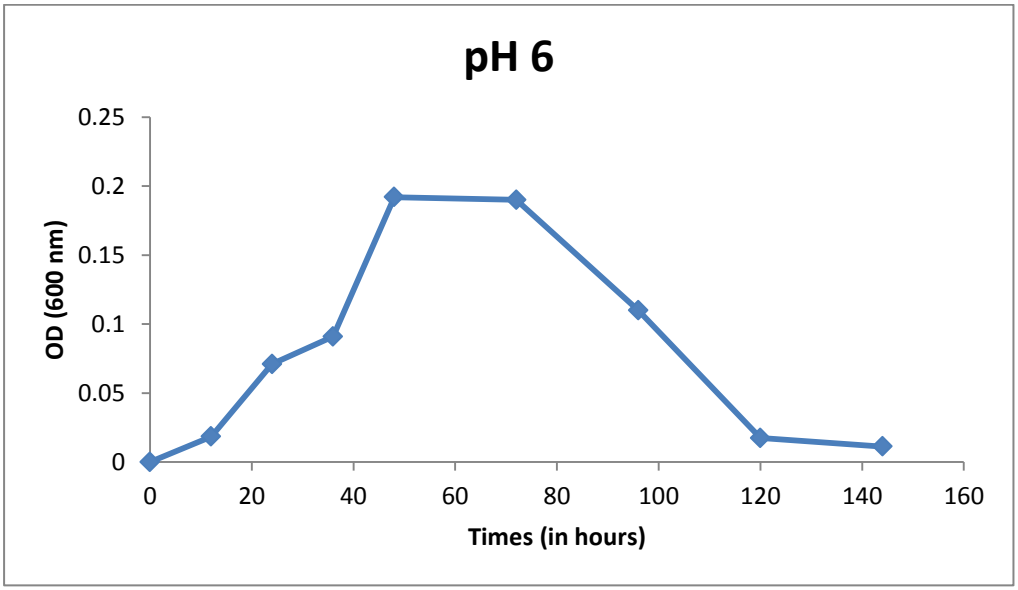
Table.8 Result of biochemical Test

Biochemical Test	Result (B1)	Result (B2)
Indole	Negative	Negative
Catalase	Positive	Positive
Citrate	Positive	Positive
Methyl Red	Negative	Positive
VP	Negative	Positive
Starch Hydrolysis	Positive	Positive
Gelatin Hydrolysis	Positive	Negative
Motility	Positive	Positive
Urease	Negative	Positive
Nitrate	Positive	Negative

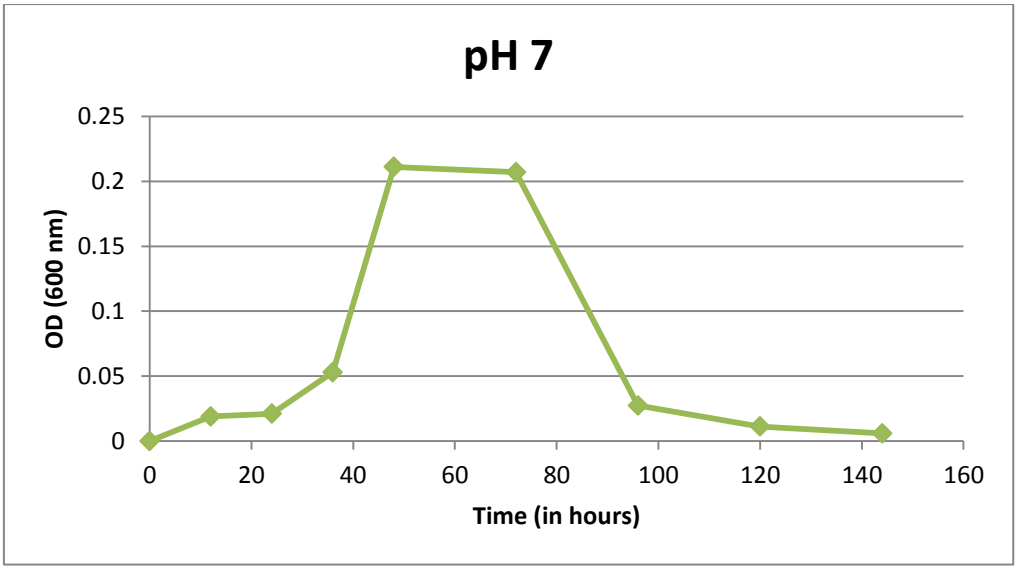
4.5 GROWTH STUDY:

Growth study of freshly inoculated strains was studied under different pH and different temperature condition to find out the optimal condition for the growth of isolates bacterial strains. The readings were plotted against time from 0 hour to 144 hour and reading was taken on interval of 12 hours.

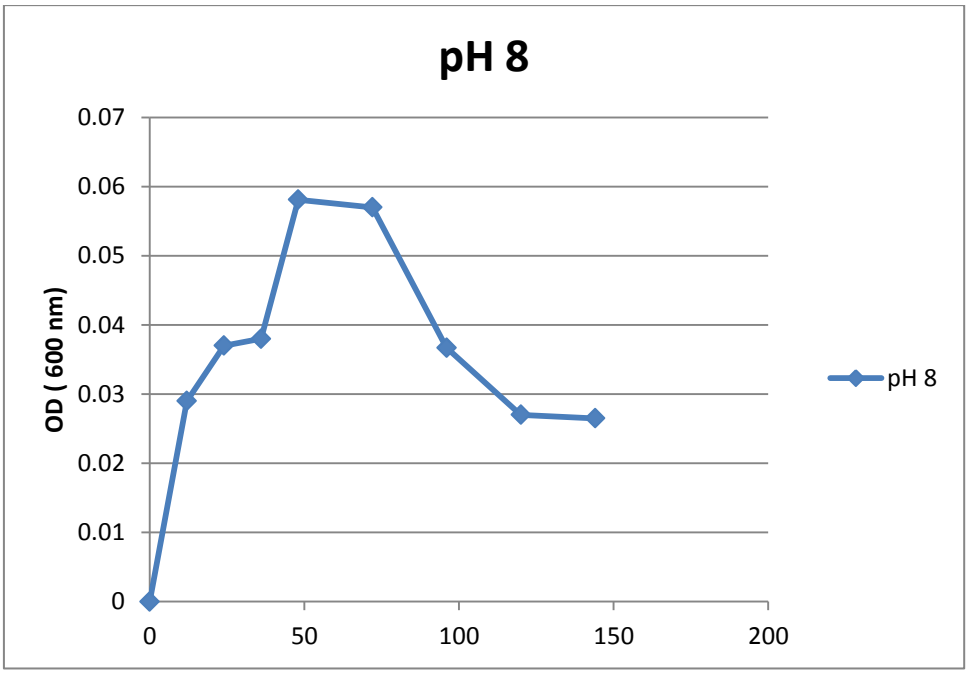
(a) Growth study of strain B1 at pH 6, 7 and 8.



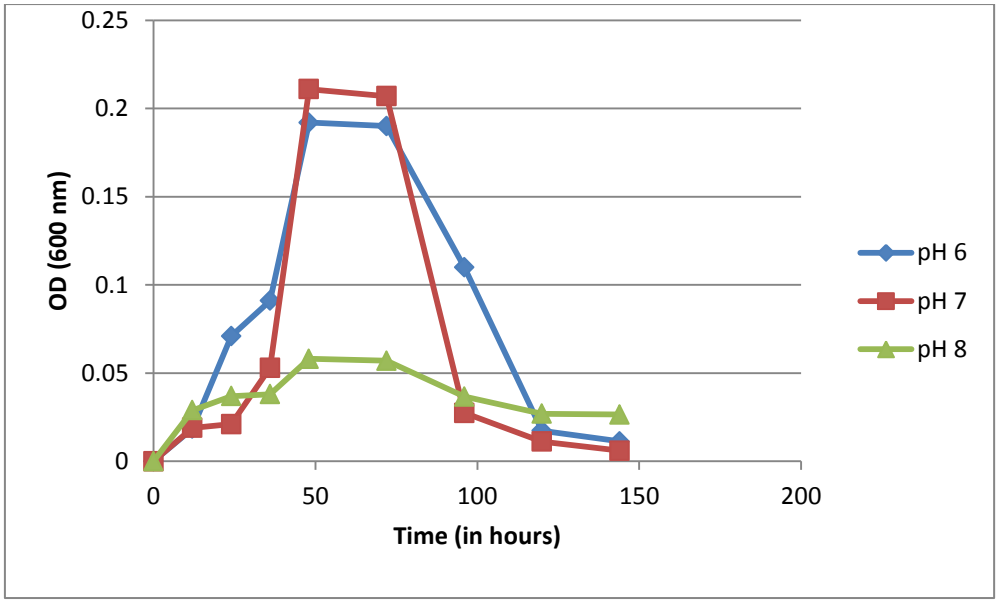
(a)


pH 7
0.25
0.2
0.15
0.1
0.05
0
OD (600 nm)
0
20
40
60
80
100
120
140
160
Time (in hours)

(b)


pH 8
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
OD (600 nm)
0
50
100
150
200
pH 8

(c)

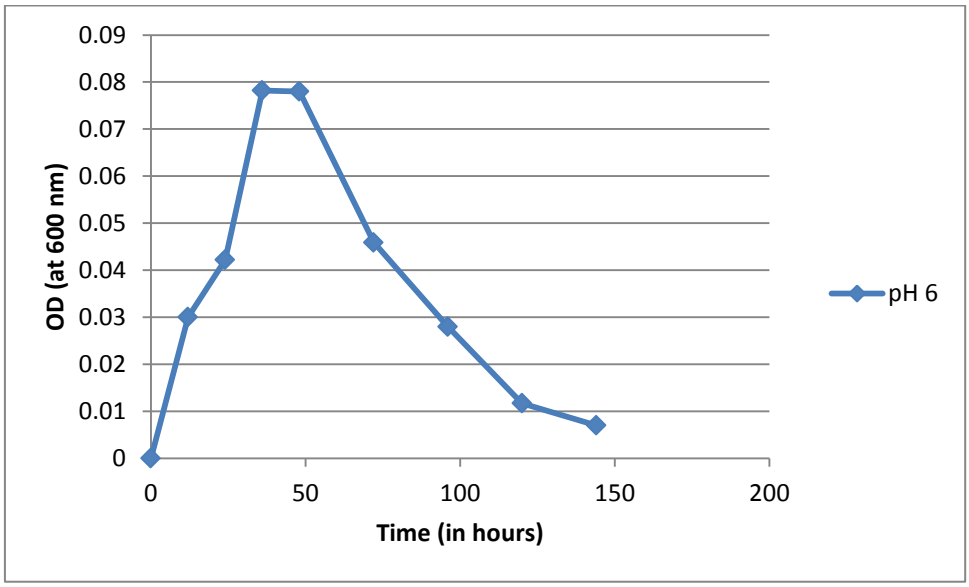


(d)

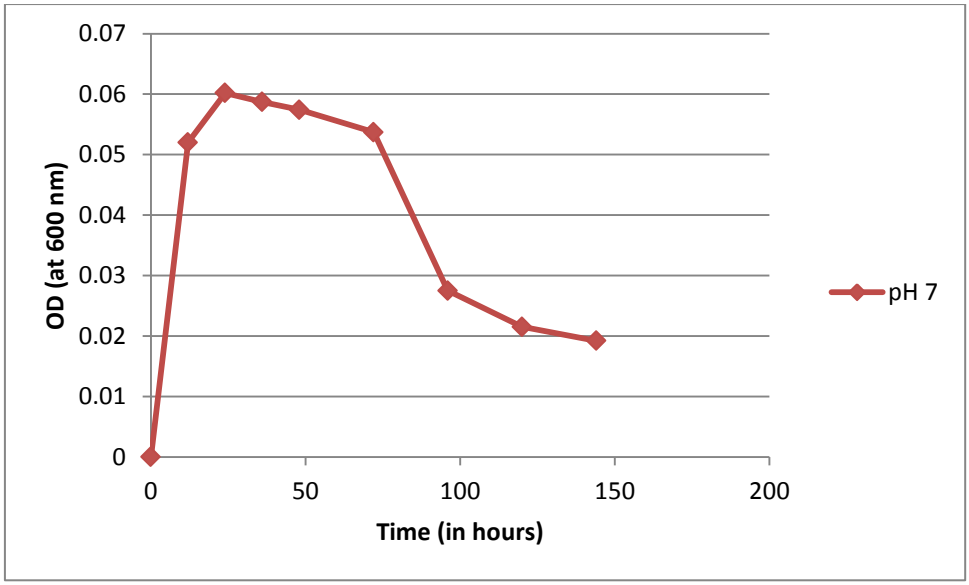
Fig.10 growth curve of bacterial strain B1 at pH 6, 7 and 8 respectively

From the growth curve, it was observed that the growth of bacterial strain highly depends on pH and Strain B1 has maximum growth at pH 7 and it was obtained after 48 hours, after 48 hours the growth remains constant i.e. the stationary phase then death phase is until 140 hours and the growth was almost negligible after 140 hours. pH 6 had compressional growth as in 7 but the growth at pH 8 is much lesser than 7 & 8. Maximum growth was around 0.06 as compare to the 0.211 at pH 7, so the pH 7 can be set as optimum pH condition for growth of strain B1.

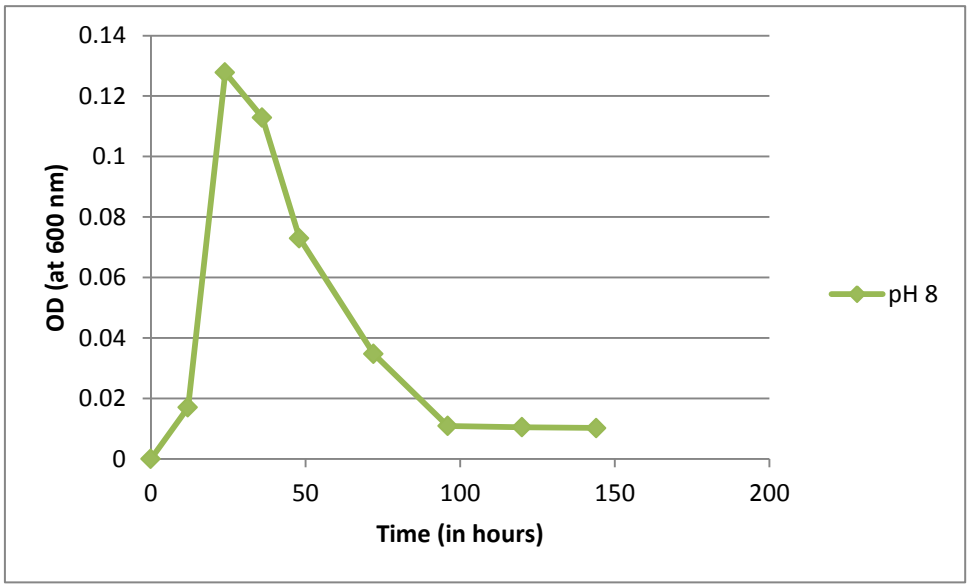
(b) Growth Study of Strain B2 at different pH:



(a)

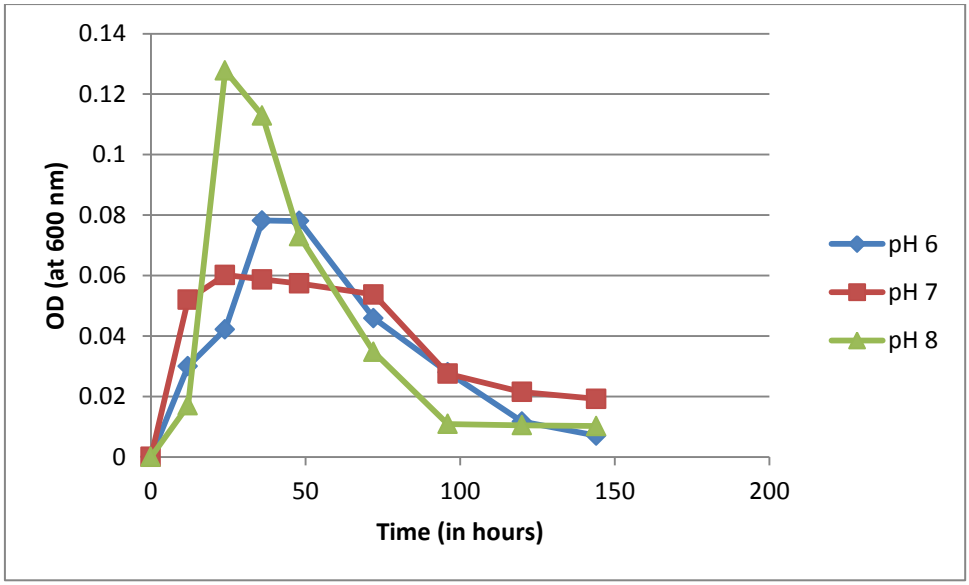


(b)



(c)

Fig.11 growth curve of bacterial strain B2 at pH 6, 7 and 8 respectively



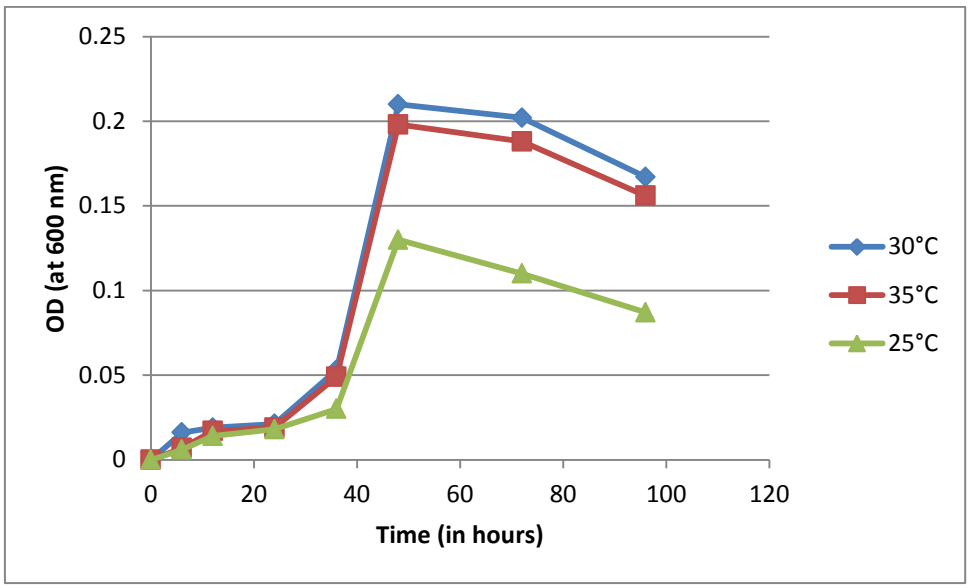
(d)

Fig.11 Growth curve of bacterial strain B2 at different pH

From the growth curve, it was observed that the bacterial strain B2 has optimum growth at pH 8 and it was obtained at time 24 hours, after 24 hours the death phase of bacteria started until 100 hours. Bacterial strain B2 has relatively low growth rate as compare to the B1.

4.6 Growth Study of Bacterial Strain B1 and B2 at different Temperature:

4.6.1 Growth study of bacterial strain B1 at temperature 30, 35 and 25°C

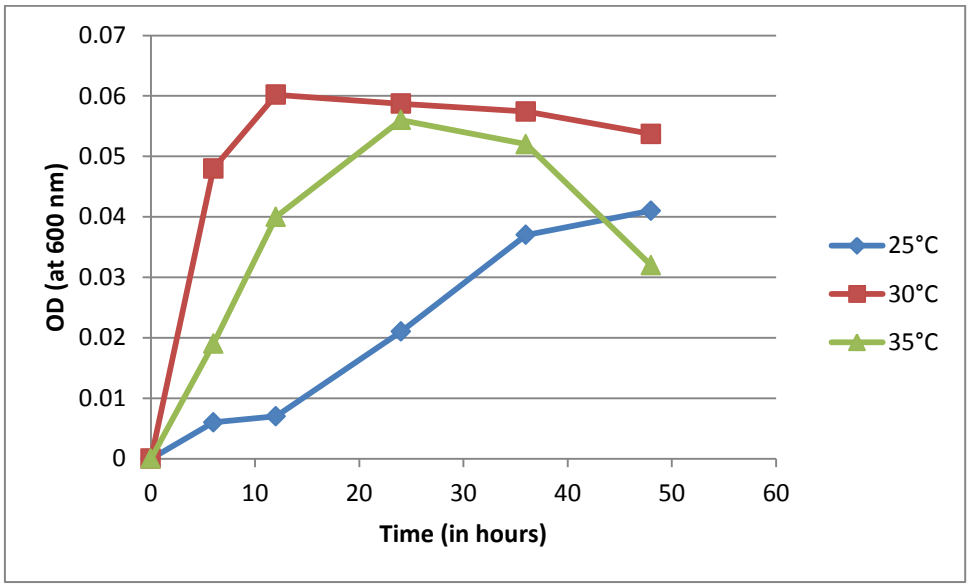


(a)

Fig.12 Growth Curve of strain B1 at different temperature

From the growth curve, maximum growth was obtained at temperature 30^{o}C and it was obtained after 40 hours. So temperature 30^{o}C can be set as optimum temperature for strain B1. At temperature 30^{0}C, the stationary phase was obtained after 40 hour and it remain almost same up to 60 hours then death phase started until 100 hours after then growth almost stop.

(b) Growth study of bacterial strain B2 at temperature 30, 35 and 25°C



(b)

Fig.12 Growth study of strain B2 at different temperature

From growth study curve of strain B2, maximum growth was obtained at temperature 30^{o}C, and it can be set as optimum temperature condition for B2.

CHAPTER-5

CONCLUSION

Biosurfactants producing bacterial strain were isolated and screened from oil soil using kerosene oil as sole carbon source. Oil spreading technique, blood hemolysis test, foaming activity, and emulsification activity were performed for the screening of the biosurfactant producing bacteria, and found positive oil spreading technique in bacterial strain B1 and B2. Strain B1 has high emulsifying activity and it was 52 %, where as in B2 it was around 42 %. Strain B1 showed β blood hemolysis test and in B2 it was γ hemolysis. It was observed that foaming stability in B1 is higher than B2.

Both the isolated bacterial strains B1 and B2 showed Gram's positive, and they are in rod and circular shape respectively. From the result of various biochemical characterization and cell morphological characterization the isolated strain may be bacillus type bacteria. From literature review it was found that Bacillus type bacteria and bacteria showing Gram's positive are able to produce lipopeptides type biosurfactant (7).Lipopeptides type biosurfactant can be extracted by using isolated bacteria.

From growth kinetics study, it was observed that the growth of bacterial strain depend on the pH and as well as on incubation temperature. Maximum growth was found at pH 7 and temperature of 30°C for bacterial strain B1 and in B2 it was found at pH 8 and temperature was 30°C. From literature review, it was found that the growth of bacterial strain like bacillus is obtained optimum at pH 7-8 and temperature 30-35 °C.

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