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Formulation of Novel Culture medium for the cultivation of Biosurfactant producing Bacteria using Agrowastes and Crop residues

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Abstract

The present research was aimed to formulate the Novel culture medium for the Biosurfactant producing microorganisms like *Bacillus subtilis* (Surfactin, Subtisin, Iturnin and Fengycin), *Pseudomonas fluorescens* (Rhamnolipids, Viscosin and Arthrofactin), *Achromobacter* sp. (Iturin) and *Rhodococcus* sp. (Rhamnolipids). The Biosurfactant producing bacteria in Soil samples was isolated in the Nutrient agar plates by Serial Dilution Method (Pour plate method) and, identified by Microscopic examination (Gram staining, Endospore staining and Motility test), Platting in Selective medium and Biochemical tests. The isolated bacterial strains were screened for its ability to produce Biosurfactants, all the isolated bacterial strains have an efficiency to produce Biosurfactants. The Biosurfactant producers were cultivated in Agrowastes (Orange peel, Lemon peel, Banana peel, Sweet lime peel, Sugarcane bagasse, Cassava peel and Potato peel) and Crop residues (Rice bran) for lowering the cost of cultivation medium. In Solid medium, all the Biosurfactants producing bacteria showed good and luxuriant growth. In Liquid medium, Biosurfactants producing bacteria showed good growth in the form of Turbidity in the Liquid medium. In conclusion, utilization of Agrowastes and Crop residues for the Biosurfactant production reduces the waste accumulation in the environment and increases the Biosurfactant yield.

Key words : Biosurfactants, Bacteria, Novel culture medium, Agrowastes and Crop residues.

Surfactants are substances with surface activity that lessen the friction at the interface of two liquids or between a liquid and a solid. Surfactants are organic compounds with hydrophobic (the surfactant's head portion) and hydrophilic (the surfactant's tail portion) moieties. Therefore, a surfactant has both a water soluble, or water loving, group and a water insoluble, or water repelling, group. Biosurfactants are also surface-active substance

like synthetic surfactants but unlike the synthetic surfactant, biosurfactant are generated by microbes including bacteria, fungi and yeast. In general, biosurfactants are non-toxic and biodegradable and have the properties of lowering surface tension, stabilizing emulsions, and stimulating foaming. Lately, interest in biosurfactants has surged due to their diversity, operational flexibility, and environmental friendliness compared to commercial surfactants[11]. The majority of microorganisms that make biosurfactants are bacteria, fungus, and yeasts. And a vast range of biosurfactants can be created as a result, depending on the demand and usage[15]. There have been reports of several bacterial genera producing g biosurfactants, including *Acinetobacter, Arthrobacter, Pseudomonas, Halomonas, Bacillus, Rhodococcus, Enterobacter, and Serratia*[7], and fungal species such as *Saccharomyces cerevisiae*[12], *Fusarium fujikuroi*[17], *Candida tropicalis*[13], *Pseudozyma*[18] and *Xylaria regalis*[22]. One of the types of *Pseudomonas aeruginosa* that produces biosurfactant effectively[20].

Biosurfactants are less poisonous and less detrimental to the environment than chemically generated surfactants, making them decomposable. High foaming, careful selection of the most appropriate ingredient, certainty of potency, a wide range of capabilities, and the use of renewable materials in the production process are other distinguishing qualities of derived biosurfactants[19]. These substances have the potential to be used in emulsification and enhanced oil recovery because they lower surface tension and interfacial tension in hydrocarbon compounds and water solutions[16]. Due to their excellent efficiency in both ecological and industrial activities, biosurfactants have received a lot of attention recently Many studies have demonstrated that the best carbon source for encouraging the production of biosurfactants in various microbes is carbohydrates[14].

Surfactants can be found in nature in a wide range of chemical forms, such as fatty acids, neutral lipids, neutral peptides and proteins, phospholipids, polymeric and particulate lipids, and glycolipids. Biosurfactants are non-toxic, more potent, and environmentally benign than synthetic surfactants. The microbiological surfactants can be manufactured utilizing a range of inexpensive agro-based raw materials, unlike the chemical surfactants, which are often made from petroleum feedstock. Their resistance to changes in pH, salinity, and temperature make them economically stronger to their chemically manufactured competitors. These characteristics are desired in a variety of industrial processes, including bioremediation of the environment, pharmaceutical formulation, food processing, and increased oil recovery. Biosurfactants have also been reported to exhibit antibacterial, antifungal, anticancer, antimycoplasmic, and antiviral activities in addition to the traditional applications[11].

Collection of soil samples :

Soil samples were collected in clean sterilized plastic bags from soil source in Tirupattur district of Tamil Nadu. After sampling of soil samples, the collected samples were transported to laboratory for Physico-chemical and microbiological testing. The soil samples were collected for the isolation of Biosurfactant producing microorganisms[21].

a) Petrol contaminated soil was collected for the isolation of *Pseudomonas fluorescence* that release Viscosin and Arthrofactin.

b) Tomato rhizosphere soil for the isolation of *Bacillus subtilis* which release Surfactin, Subtisin, Iturnin and Fengycin.

c) *Achromobacter* sp. isolation tomato rhizosphere soil was collected which release Iturin.

d) Lubricant contaminated soil was collected for the isolation of *Rhodococcus* sp. which release Rhamnolipids.

Isolation of Biosurfactant producing Bacteria :

The Biosurfactant producing bacteria in Soil samples was isolated in the Nutrient agar plates by Serial Dilution Method (Pour plate method). The isolated colonies were maintained in Nutrient agar slants and preserved in the Refrigerator at 4°C [10].

Identification of Biosurfactant producing Microorganisms :

The bacteria isolated from the soil samples will be identified by

a) Microscopic examination (Gram staining, Endospore staining and Motility test).

b) Plating on Nutrient agar, MacConkey agar and Selective medium (*Bacillus* Differentiation Agar, King's B Agar and Pseudomonas Isolation Agar).

c) Biochemical Tests (Carbohydrate fermentation test, Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test, Oxidase test and Urease test)[9].

Screening of Biosurfactant producing efficiency of Bacteria :
Hemolytic activity :

Blood agar media containing 5 % v/v human blood was streaked with isolated strains and incubated at 37°C for 24 hrs. After incubation, zone formation around culture was observed. Hemolytic activity is qualitative indication of biosurfactant production[8].

Oil spreading assay :

Forty ml of distilled water was taken in clean glass petri plates, 20 µl oil and 10 µl cell free supernatant was added. System was monitored carefully for formation of halo around supernatant, indicative of positive test result[5].

Oil coated agar plate test :

Surface of Nutrient agar media plates were coated with oil. Plates were streaked with isolated strains and incubated at 37°C for 7 days. Plates were observed for presence of emulsification halo around culture growth, indicative of biosurfactant activity[1].

Drop collapse assay :

A single droplet of oil and supernatant was taken on a clean glass slide. Droplet of supernatant was monitored carefully to notice whether it remained beaded or collapsed[4]. Collapsed supernatant drop was scored as positive "+," indicative of biosurfactant presence.

Tilted glass slide assay :

A single droplet of 0.9 % NaCl was

taken on a clean glass slide angled at 45 degrees and a single colony of isolated was transferred to it. Colony was not mixed in 0.9 % NaCl droplet. Droplet was monitored carefully to observe whether it remained beaded or collapsed[18]. Beaded drop was scored as negative "-," indicative of biosurfactant absence.

Emulsification activity :

Two ml of volume of supernatant and oil were mixed vigorously for 2 min and then left undisturbed for 24 hrs. After 24 hrs, height of emulsified layer and total height of mixture was observed[6].

Emulsification index was calculated as follows:

Emulsification Index (E24) = Height of Emulsifier layer/Total weight × 100

Foaming activity :

The isolated bacterial 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 Shaking incubator (200 rpm) for 72 hrs. Foam activity is detected as duration of foam stability, foam height and foam shape in the graduated cylinder[4].

Collection of Agrowastes and crop residues:

Agrowastes like Potato peel waste, Cassava waste, Lemon peel waste, Orange peel waste, Sweet lime waste, Banana peel waste, and Sugarcane bagasse and Crop residues, Rice bran were collected in and around Tirupattur. The collected wastes were Grinded into powder for further use[2].

Preparation of Low-cost Culture medium for the cultivation of Biosurfactant producers:

Sterilization of wastes :

The collected Agrowastes were grinded and sterilized in Autoclave for 121°C for 15 - 20 minutes.

Carbohydrate analysis by Molisch's *test :*

The Agrowaste sample was taken in a test tube and Molisch's reagent was added to the test solution. After that, 1 ml of Concentrated H_2SO_4 was pipetted on the side of the tubes so that distinct layer is formed. Appearance of purple colour indicates the presence of Carbohydrate.

Qualitative analysis of Lipid by saponification test :

The Agrowaste sample was taken in a test tube and Strong Alkali NaOH was added, and boiled the solute ion in a water bath for 5 minutes. At last, Ethanol was added to the solution. Positive result will be appearance of froth in the test sample.

Protein estimation by Bradford *method :*

The Protein content in the Agrowaste samples were estimated by Bradford method developed by Marion Bradford[20].

Qualitative Growth analysis of Biosurfactant producing Bacteria (Broth and Agar medium):

The bacterial isolates which was isolated and screened for Biosurfactant activity

were grown on Agrowastes (Orange peel, lemon peel, Banana peel, Sweet lime peel, Sugarcane bagasse, Cassava peel and Potato peel) and Crop residues (Rice bran). The Agrowaste culture medium was prepared as both Broth and Agar. The grinded Agrowastes and Crop residues were taken and measured about 2.8 gm for 100 ml with 1 % oil in it as supplement for bacterial growth. The pH of the medium was adjusted for about 7 - 7.2.

Identification of Biosurfactant producing Bacteria from soil samples :

The Biosurfactant producing bacteria was isolated from different sources and the characteristics of the isolated bacteria is given in the Table-1. The *Bacillus* sp. was confirmed as *Bacillus subtilis* by Gram staining, Endospore staining, Motility test, Biochemical test and Colony morphology on *Bacillus* Differentiation Agar (colour change from violet to yellow). The *Pseudomonas* sp. was confirmed as *Pseudomonas fluorescens* by Gram staining, Endospore staining, Motility test, Biochemical test and Colony morphology on King's B medium (fluorescent green under UV light). *Achromobacter* sp. and *Rhodococcus* sp. were identified by Gram staining, Motility test and Biochemical test.

Table–1. Identification of Biosurfactant producing Bacterial strains

Tests	*Bacillus subtilis*	*Pseudomonas fluorescens*	*Achromobacter* sp.	*Rhodococcus* sp.
Gram staining	+ rod	- rod	- rod	+ cocci
Motility test	Motile	Non-motile	Non-motile	Non-motile
Endospore staining	Endospores were observed in Green colour	Non - spore	Non - spore	Non – spore
Catalase test	+	+	+	+
Oxidase test	-	-	+	-
Indole test	-	-	-	-
Methyl red test	-	-	+	+
Voges Proskauer test	+	-	-	-
Citrate utilization test	+	+	+	+
Urease	-	+	-	
Triple sugar iron test	-	Alkaline slant, Alkaline butt, No gas and No H_2S	-	-
Starch hydrolysis test	+	-	+	-
Casein hydrolysis test	+	-	-	-
Gelatin liquification test	+	+	-	-

(+ Positive; - Negative)

Screening of Biosurfactant producing efficiency of Bacteria :

All the isolated bacterial strains were screened for its ability Biosurfactant production. All the isolated bacterial strains have the efficiency to produce Biosurfactants.

Haemolytic activity :

All the isolated bacterial strains were patched on the Blood agar, in which *Bacillus subtilis, Pseudomonas fluorescens, Achromobacter* sp. showed γ hemolysis (Gamma hemolysis), and *Rhodococcus* sp. showed β hemolysis (Beta hemolysis).

Oil displacement assay :

On a plate with Olive oil, supernatant from isolated strains was applied. It was subjected in the middle of the oil layer. The oil displacement was measured in mm. The strains Bacillus subtilis, Pseudomonas fluorescens, Achromobacter sp. and Rhodococcus sp. displaced the oil and showed a clear zone. Here, Pseudomonas fluorescens have the largest displacement diameter (70 mm in dm) than other bacteria. The results of Oil displacement assay were shown in Table-2.

Oil coated agar plate :

The Biosurfactant producing bacterial strains *Bacillus subtilis, Pseudomonas fluorescens, Achromobacter* sp. *and Rhodococcus* sp. showed clear zone around the colonies after incubated for 72 hrs in Oil coated nutrient agar plate.

Drop collapse assay :

The Drop collapse method is based on the idea that an oily surface will cause a drop of liquid containing a biosurfactant to collapse and spread. The diameter of the sample and the concentration of the biosurfactant are directly correlated, while the absence of the Biosurfactant causes the drop to remain beaded due to the hydrophobicity of the oil surface, which leads to droplet aggregation. As expected, there was no action found for distilled water in the drop collapse assay. In this investigation, the isolate *Pseudomonas fluorescens* displayed the largest Drop collapse compared to other bacteria, demonstrating that the biosurfactant droplets do result in a collapsed droplet.

Tilted glass slide assay :

Water flow over the inclined slide's surface was observed to be positive. Even

Table–2. Oil Displacement Assay by Biosurfactant producing bacteria

Bacterial strains	Diameter of Oil Emulsion	Diameter of Oil Displacement	Interpretation
Bacillus subtilis	20 mm in dm	43 mm in dm	Positive
Pseudomonas fluorescens	40 mm in dm	70 mm in dm	Positive
Achromobacter sp.	25 mm in dm	55 mm in dm	Positive
Rhodococcus sp.	35 mm in dm	60 mm in dm	Positive

though the slide was slanted, water retention over the surface was regarded as undesirable. All the bacterial isolates showed positive reaction.

Emulsification activity :

Olive oil was used for studying the Emulsification activity since the isolated bacteria produced positive results when evaluated for their capacity to emulsify crude oil. The test was conducted by adding 2 ml of Olive oil and 1 ml of Supernatant, aggressively mixing for 2 minutes, and leaving the mixture undisturbed for 24 hours (Table-3).

Table–3. Emulsification activity (E24 Index) of bacterial strains

S. no	Bacterial strains	Emulsified layer (mm)	Total liquid layer (mm)	E24 %
1	*Bacillus subtilis*	20	28	62.8
2	*Pseudomonas fluorescens*	15	29	78.5
3	*Achromobacter* sp.	21	30	73
4	*Rhodococcus* sp.	22	30	51.7

Foaming activity :

For studying the Foaming activity, the isolated Biosurfactant producing strains were cultivated in 250 ml Erlenmeyer flasks with 100 ml of Nutrient broth medium in each. The flasks were incubated for 72 hrs at 30 °C in a Shaker incubator and spinning at 200 rpm. All the bacterial isolates showed foaming activity after incubation.

Molisch *test for Carbohydrates :*

All the agrowastes and crop residue which were collected showed positive result, as it is indicated by Purplish layers.

Saponification test for Lipids :

The agrowastes like Banana peel, Orange peel, Sweet lime peel and Lemon peel showed Froth formation when it reacts with the alkali NaOH than Cassava peel, Rice bran, Sugarcane bagasse and Potato peel wastes.

Bradford *method for Protein estimation :*

The agrowastes and crop residues were tested for Protein by Bradford Method, estimated under 600 nm in Spectrophotometer. The protein content was high in Cassava followed by Lemon, Sweet lime, Sugarcane bagasse, Orange and Banana, Potato. Low protein content was recorded in Rice bran (Table-4).

Table–4. Protein content of Agrowastes

S. no	Agrowastes	Protein content (mg)
1	Rice bran	0.66
2	Sugarcane bagasse	1.27
3	Cassava	2.0
4	Potato	0.62
5	Banana	0.71
6	Lemon	1.90
7	Sweet lime	1.37
8	Orange	0.86

Qualitative Growth analysis of Biosurfactant producing Bacteria (Agar medium) :

Potato peel waste :

In Potato peel waste agar medium, all the bacterial isolates showed growth after 24 hrs incubation at 37°C, especially *Pseudomonas fluorescens* showed Fluorescent green color.

Cassava peel waste :

In the Cassava peel waste agar medium, all the three bacterial isolates *Bacillus subtilis, Pseudomonas fluorescens and Achromobacter* sp. showed good and luxurious growth. The bacteria *Rhodococcus* sp. did not show any growth on Cassava peel waste agar medium.

Lemon peel waste :

In the Lemon peel waste medium, all the four bacteria did not show any growth. Presence of Citric acid in the Lemon peel waste completely inhibits the bacterial growth in the medium.

Orange peel waste :

In Orange peel waste medium, *Pseudomonas fluorescens* showed Fluorescent green color colonies with vigorous growth than any other bacteria.

Sweet lime waste :

In Sweet lime waste agar medium, all the bacterial isolates showed good and luxuriant growth, except *Bacillus subtilis*. The *Pseudomonas fluorescens* showed Fluorescent green colour in the Sweet lime waste agar medium.

Banana peel waste :

Surprisingly in Banana peel waste medium, all the bacterial isolates, *Bacillus*

Table–5. Growth of Biosurfactants producing Bacterial isolates in Agrowastes and Crop residues

S. no	Agrowastes and Crop residues	Biosurfactant producing Bacteria			
		Bacillus subtilis	*Pseudomonas fluorescens*	*Achrom-obacter* sp.	*Rhodoco-ccus* sp.
1	Orange peel	++	++	++	++
2	Lemon peel	+	+	+	+
3	Banana peel	++	++	++	++
4	Sweet lime peel	++	++	++	++
5	Sugarcane bagasse	+	+	+	+
6	Cassava peel	++	++	++	++
7	Potato peel	++	++	++	++
8	Rice bran	+	+	+	+

(+ Moderate growth; ++ Good and luxurious growth)

subtilis, *Pseudomonas fluorescens, Achromobacter* sp. and *Rhodococcus* sp. showed good and luxurious growth.

Sugarcane bagasse :

In the Sugarcane bagasse waste medium, all the four bacteria did not show any growth.

Rice bran :

In the Rice bran waste medium, all the four bacteria did not show any growth.

Qualitative Growth analysis of Biosurfactant producing Bacteria (Broth medium) :

The Biosurfactant producers were cultivated in Agrowastes (Orange peel, Lemon peel, Banana peel, Sweet lime peel, Sugarcane bagasse, Cassava peel and Potato peel) and Crop residues (Rice bran) for lowering the cost of cultivation medium. All the bacterial isolates *Bacillus subtilis, Pseudomonas fluorescens, Achromobacter* sp. and *Rhodococcus* sp., showed growth in the form of Turbidity in the liquid medium (Table-5).

Due to high manufacturing costs and low yields, Microbial Biosurfactants are now more expensive than chemical surfactants. Thus, they have not been heavily commercialized. Process optimization needs to be enhanced at the biological and engineering levels in order to develop biosurfactants that are economically viable. Although additional considerable advancements are needed, the production method for biosurfactants has already allowed for 10 – 20 times increase in productivity. The utilization of less expensive substrates like Agrowastes (Orange peel, Lemon peel, Banana peel, Sweet lime peel, Sugarcane bagasse, Cassava peel and Potato peel), and Crop residues (Rice bran) are the good substrates for the cultivation of Biosurfactant producing bacteria *Bacillus subtilis, Pseudomonas fluorescens, Achromobacter* sp. and *Rhodococcus* sp. In conclusion, utilization of Agrowastes and Crop residues for the Biosurfactant production reduces the waste accumulation in the environment and increases the Biosurfactant yield.

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