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Lurking product from *Pseudomonas aeruginosa* act as Bio surface Active Agent for Removal of Reactive Dyes from Cellulosic Materials

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Abstract

The soaping treatment is very important for producing quality end products in textile finishing process. Textile materials are currently treated with chemical surfactant but they are harmful to aquatic species. The use of biotechnology offers interesting innovative attempts for solving this environmental problems. Biosurfactants are extracellular surface-active agent produced by several bacteria. In this study biosurfactant are produced by *Pseudomonas aeruginosa* using different substrate medium. The different shades of cotton dyed fabric were treated with biosurfactant using various concentrations and compared with chemical surfactant. This Biosurfactant acts as an antimicrobial agent and it has stable emulsification properties. The maximum production obtained using substrate (palm oil) was about 98g/L. The red, green and black dyed cotton fabric was treated with both chemical surfactant and biosurfactant (1-1.5 g/L). Biosurfactant acts as an effective surface-active agent for removal of reactive dyes from cellulosic material. It showed better wash fastness result (CF 4-5) in biosurfactant treated fabric when compared to chemical surfactant treated fabric. *Pseudomonas aeruginosa* producing biosurfactant as best alternative for treating the cellulosic fabric in textile industry and this paper reports the potential application of microbial biosurfactants in textile washing processes for removing the unfixed dyes.

Keywords: Biosurfactant, Rhamnolipid, Reactive dyes, Emulsification index, chromatography

1. Introduction

The textile industry, the world's oldest branch of consumer goods industry, is severely intertwined with environmental issues, especially in its wet processes, where dyes, auxiliaries, and finishing agents are consumed to convert the raw materials into finished products. Cotton is the most extensively used natural fiber and accounts for almost half of all the fibers used by the world's textile industry. Textile dyeing (especially exhaust dyeing) is thus such an important consumer of water and generator of contaminated wastewater because the dyeing processes are normally conducted in water-based dyeing baths and they involve the addition of dyes and dyeing auxiliaries (Yaseen *et al.,* 2019). One of the major classes of dyes for cellulose fibers are the reactive dyes, as they have good washing fastness, bright shades and very flexible batch and continuous dyeing methods (Debasreepaul *et al*., 2017). The hydrolyzed dye has to be removed after dyeing by thorough washing to ensure good washing fastness (Meric *et al.*, 2005 and Allegre *et al.*,

2005) and this process is vital for the final quality of the dyeing. The washing treatment is done by treating the fabric with chemical surfactants for removal of unfixed dyes. Synthetic surfactant was potential for environmental contamination and depletion of natural resources is also serious and moreover fabric treated with harsh chemicals is unsafe for human health. So alternative approaches must be considered to make the washing process environment friendly.

Biosurfactant are surface active compounds produced by *Pseudomonas aeruginosa*. It can reduce surface tension, nontoxic, biodegradable and environmentally friendly nature (Banat *et al*., 2000). The synthetic surfactants have become a major issue in textile industries. Optimization of biosurfactant production is one of the main fields of research owing to the low yields, high cost of raw materials and need for potential microorganism (Bhat *et al*., 2015). *Pseudomonas aeruginosa* is the preferred microorganism for the production of rhamnolipid type of biosurfactant utilizing glycerol, vegetable oil, palm oil, Castrol oil, ghee and coconut oil. *Pseudomonas aeruginosa* has the ability to metabolize a variety of substrates including n-alkanes, hexadecane and oils. Uptake of these hydrophobic substrates is speculated to rely on the production of rhamnolipids (Raza *et al*., 2008). The possibility of their production on large scale, selectivity, performance under intense condition and their future applications in environmental fortification also these have been increasingly attracting the attention of the industrial community. These molecules are potential alternative for chemical surfactant, but the production of biosurfactant on industry level is still challenge because of using high costly synthetic media for microbial growth. The present study is aimed to check the ability of potential surfactant produced from *Pseudomonas aeruginosa* and as an alternative surfactant for removing unfixed residual reactive dyes in textile industries.

2. Materials and Methods

2.1. Isolation and Identification

Pseudomonas was isolated from goat droppings in a local goat farm at Erode. The goat droppings were serially diluted andpour plated on nutrient agar plates to obtain pure colonies. The pure colonies were streaked on selective cetrimide agar medium for screening *Pseudomonas*. The colonies were selected based on the characteristics of diffusible green pigment production and the selected isolate was subjected to biochemical tests and gene sequencing followed by BLAST analysis for species confirmation. Biochemical characterization was carried out based on *Bergey's Manual of Determinative Bacteriology*. The DNAwas extracted using High Pure PCR template kit (Roche). DNA is cut short and amplified using PCR primer set, 27F-5'AGAGTTTGATCMTGGCTCAG3' and 1492R-5'TACGGYTACCTTGTTACGACTT3'. This amplified product was sequenced using automated sequencer with the primer set 518F-5'CCAGCAGCCGCGGTAATACG3' and 800R-5'TACCAGGGTATCTAATCC3'. The sequence obtained is aligned and ran through BLAST to identify the organism, only sequence obtained with 1200 base pairs or above is considered to give assured result in BLAST.

2.2 Screening of potential surfactant strain

Oil displacement method

100µl of castor oil was placed on the surface of 30ml of distilled water in a petridish and 10µl of cell-free supernatant was added on the surface of oil film. The strain which caused oil spreading under visible light was considered positive.

Foaming activity

Isolated strain was inoculated on the nutrient medium and the flask was incubated at 37^{o}C in a shaking incubator for 48hrs. After incubation, based on foaming the culture was referred to be positive or negative.

Drop collapsing test

100µl of Castrol oil was placed on the surface of the slide and50µl of cell-free supernatant and distilled water (control) was added on the surface of oil. The surface area of the drop was observed under a microscope to conform biosurfactant production.

Blue agar plate method

Mineral salt agar medium supplemented with glucose (2%), cetyltrimethylammonium bromide (0.05%) and methylene blue (0.02%) were used for the detection of anionic rhamnolipid biosurfactant (S K Satpute *et al*., 2008). Wells of 4 mm diameter were made using cork borer on methylene blue agar plates and loaded with 30 µl of fresh culture of individual isolate. The plates were incubated at 37^{o}C for 48-72 hours. A dark blue

halo zone around the culture was considered positive for anionic biosurfactant production.

2.3 Factors influencing rhamnolipid production

Bacteria were grown in mineral salt media with different oil substrates for rhamnolipid production (S K Satpute *et al.*, 2008). Mineral salt media containing g/l: KH_2PO_4 – 1g, K_2HPO_4 – 0.5g, $MgSO_4$ – 0.5g, NaCl – 2g, $(NH_4)_2SO_4$ – 1g, $NaNO_3$ – 1g, Glycine – 1.5g, Yeast extract – 1g, Glucose – 2g, pH – 7. In this study, biosurfactant production increased using oil substrates such as Peanut oil, palm oil, sunflower oil, coconut oil, glycerol, castor oil and ghee. *Pseudomonas aeruginosa* was inoculated on different oil medium and incubated in shaker for 7 days at 37^{o}C to enhance rhamnolipid production (Rahman *et al.*, 2002).

2.4 Biosurfactant extraction

The culture broth was centrifuged at 10000rpm for 10 minutes. The cell-free supernatant was acidified to pH 2 by using 6N HCl. The precipitation occurs due to acidic pH and pellet was separated by centrifugation and dissolved in distilled water. The pH was adjusted to 8 with 1N NaOH.

2.5 Emulsification index (E24)

Emulsification index was measured by the cell-free supernatant. The culture was grown in nutrient medium for 24 hours. The culture was centrifuged at 10000rpm for 15minutes. To the 5ml of cell-free supernatant in test tube, 5 ml of diesel was added. Castrol oil and vegetable oil were homogenized by vortexing with high speed for 2 min and left undisturbed for several hours. After 24 hours, the emulsification activity was calculated (Tabatabaee *et al*., 2005).

Emulsification index (E24) = (he/ht) x100

he(mm)- height of the emulsion layer
ht(mm)- overall height of the mixture

2.6 Antibacterial activity by well diffusion method

The agar plate surface was inoculated by spreading 0.1ml of the microbial inoculum over the entire medium. Well was made with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer. The surfactant solution was prepared using chloroform and methanol (1:2) ratio and with a concentration of (200, 1000, 2000µg) was introduced into the well. Then, agar plates were incubated at room temperature. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain.

2.7 Analysis of rhamnolipid mixture by Thin-Layer Chromatography

The extracted rhamnolipids was dissolved using methanol and analyzed by thin-layer chromatography (TLC) according to (Schenk T *et al*., 2015). The TLC was carried out on silica gel coated aluminum sheets using the solvent system $CHCl_3$ /CH_3OH/CH_3COOH (81:17:2). The developed pattern was stained with 5% sulfuric acid in methanol followed by drying at 100°C for 15 minutes.

2.8 Textile application of biosurfactant

Soaping is the important process to remove the unfixed reactive dyes to provide the better quality and wash fastness of the material (Kesting W *et al*., 1996). The pretreated cotton fabric was dyed with red (1%, 3% and 7.69%), green (3.5%) and black (1% and 3%) reactive dyes with a liquor ratio of 1:10 (M: L) at 60^{o}C for 90 minutes. After dyeing the fabric was treated with biosurfactant and chemical surfactant with different concentration (1 and 1.5g/L). Fabric was treated with surfactant at 95^{o}C for 15 minutes. The surfactant treated fabric was tested with ISO washing property method. The results were compared with chemical surfactant.

3. Results and Discussion

3.1. Isolation and Identification

The green colored colonies were selected for biochemical characterization and subjected to gene sequencing for molecular identification. Table (1) shows biochemical test results for the isolate.

Table 1: Biochemical test result for the isolate

S. No	Biochemical / Morphology test	Triplicate	Result
1	Gram staining	-ve	Gram Negative
2	Capsule staining	-ve	Non- Capsulated
3	Shape	-	Rod Shaped bacteria
4	Pigment production	-	Green pigment diffusible in agar
5	Litmus milk	(-)(-)(-)	Negative
6	Lactose	(-)(-)(+)	Negative
7	Methyl red	(-)(-)(-)	Negative
8	Indole	(-)(-)(-)	Negative
9	Citrate test	(+)(+)(+)	Positive
10	Catalase test	(+)(+)(+)	Positive
11	Salt tolerance test	(+)(+)(+)	Positive
12	Urease test	(-)(-)(-)	Negative
13	Starch Hydrolysis	(+)(+)(+)	Positive
14	Gelatin Hydrolysis	(+)(+)(+)	Negative
15	Casein Hydrolysis	(+)(+)(+)	Positive
16	Voges Proskauer	(-)(-)(-)	Negative
17	Oxidase	(+)(+)(+)	Positive

In gene sequencing, a sequence of 1493 base pairs were obtained. And on running through BLAST, it was observed that for 100% query sequence, a 99% identity was observed for *Pseudomonas aeruginosa*. And on cross verification with the results obtained from biochemical tests, the microbe was affirmatively confirmed as *Pseudomonas aeruginosa.*

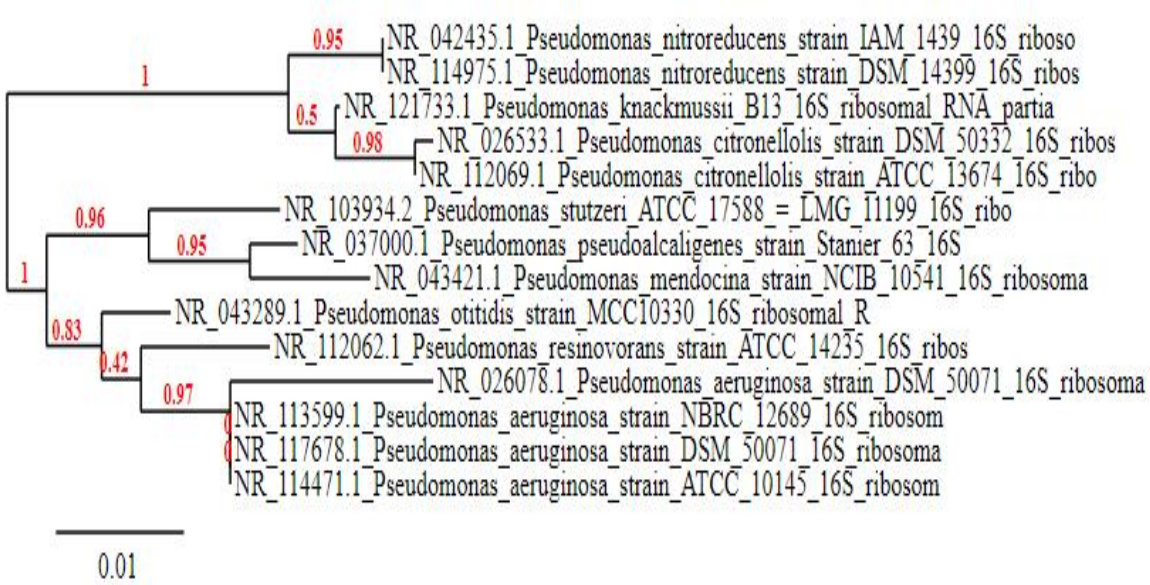


Fig- 1.Phylogenetic tree

3.2 Screening of potential surfactant strain

The rhamnolipid bio surfactants are predominantly produced by *Pseudomonas aeruginosa* utilizing varied sources of nutrition available in nature. The *Pseudomonas aeruginosa* exhibited varied results in rhamnolipid biosurfactant specific parameters such as of oil displacement, drop collapse and blue agar methods. The oil spreading was observed clearly under normal visible light. The dark blue halo zone in methylene blue agar plate supplemented with cetyltrimethylammoniumbromide (CTAB) confirmed the production of rhamnolipid biosurfactant. The selection of potential surfactant strain is shown in Table (2).

Table 2: Selection of potential surfactant strain

S. No	Methods	Results
1	Foaming activity	+++
2	Oil displacement method	+++
3	Drop collapse method	+++
4	Blue agar test method	+++

3.3 Factors influencing rhamnolipid production

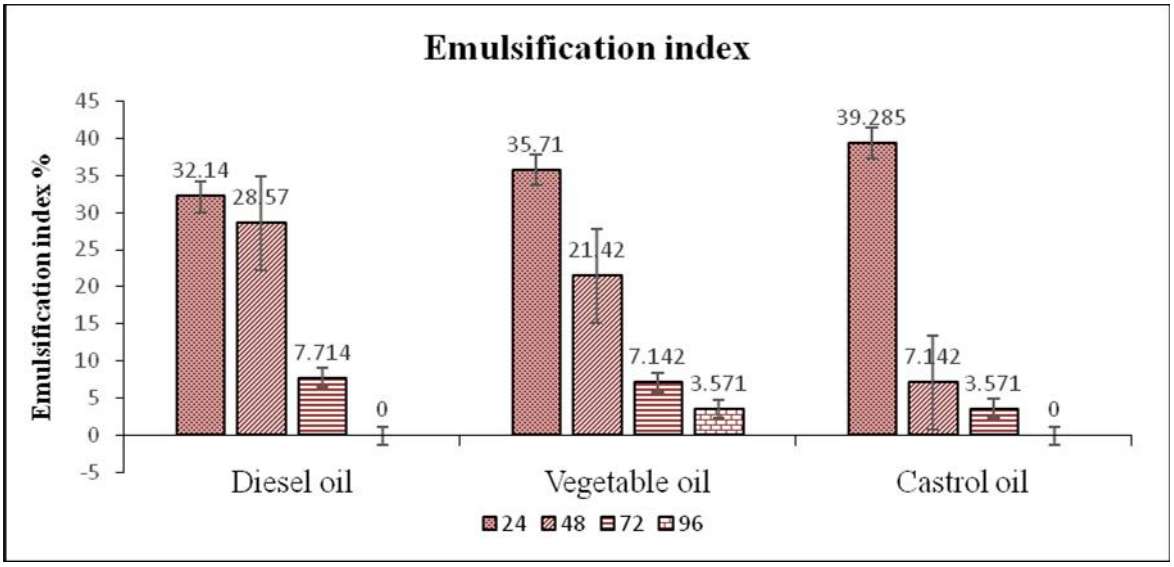
Bacterial culture was grown in mineral salt medium with different oil substrates for rhamnolipid production. The rhamnolipid yield increases by adding different substrates such as palm oil, peanut oil, ghee, glycerol, coconut oil and castor oil. Palm oil shows high yield and it was the best substrate for the rhamnolipid production from *Pseudomonas aeruginosa*. Substrate optimization for biosurfactant production are represented in Table (3).

Table 3: Biosurfactant yield with different substrates

S. No	Substrate with (2%)	Biosurfactant yield (g/L)
1	Palm oil	98.38
2	Peanut oil	37.08
3	Ghee	7.24
4	Glycerol	5
5	Coconut oil	4.14
6	Castor oil	3.74

3.4 Emulsification index

Examination of emulsification index reveals that the emulsion formation and stability of biosurfactant produced by *Pseudomonas aeruginosa*. The stability of emulsion was observed, emulsion formed with vegetable oil (96hrs) are more stable than emulsion formed with diesel oil and Castrol oil.The results observed in this study reveals that this strain shows the positive indication of emulsification activity. The graphical representation of emulsification stability for biosurfactant is shown in Figure 2.



Figure– 2. Emulsification activity of Biosurfactant

3.5 Agar well diffusion method

The Agar well diffusion method was the best plating procedure for detecting the antibacterial activity. The zone of inhibition observed in Muller Hinton agar plates for biosurfactant against *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus*, *Salmonella* spp, *Bacillus subtilis* and *Serratia marcescens* are shown in table. Biosurfactant showed high antibacterial activity against gram-negative *Salmonella* and *Serratia marcescens.*The antimicrobial activity of biosurfactant is shown in Figure (3).

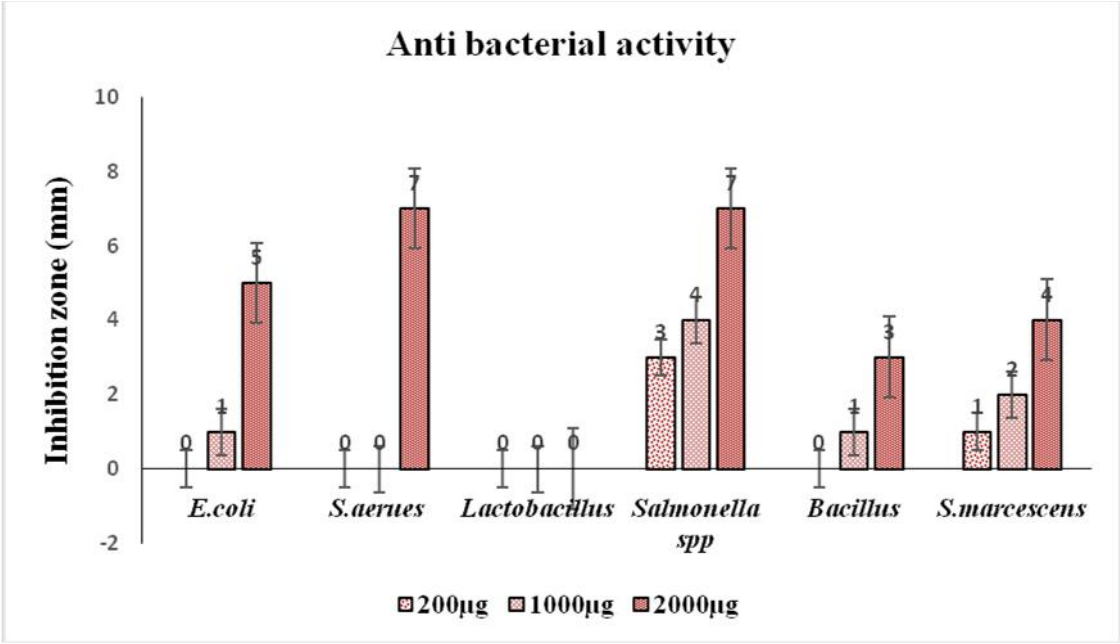


Figure- 3. Antimicrobial activity of biosurfactant

3.6 Thin layer chromatography

The surfactant extracted using chloroform and methanol was run in TLC sheet using chloroform, methanol and acetic acid. From analysis, it is verified that the extracted surfactant was rhamnolipid as the retention factor was 0.73 (solvent front = 6.3 cm and solute front = 4.6 cm).

3.7 Soaping efficiency

Wash fastness of biosurfactant and chemical surfactant treated fabric were tested by ISO method [10]. The biosurfactant concentration (1 and 1.5g/L) has better wash fastness quality and result is shown in table (4). Red, green and black dyed fabric was treated with biosurfactant showed equal efficiency to chemical surfactant. The *Pseudomonas aeruginosa* produced biosurfactant was the best alternative for chemical surfactant and they are biologically degradable, nontoxic and eco-friendly washing of textile material. These bacterial biosurfactant has effective washing properties in cotton fabric.

Table 4: Wash fastness result

Trial no	Dyeing shade with	Surfactant and Concentration	Staining value	Colour change value
1	Red (7.69%)	Chemical surfactant (1.5g/L)	4-5	4-5
		Biosurfactant (1.5g/L)	4-5	4-5
2	Green (3.5%)	Chemical surfactant (1.5g/L)	4-5	4-5
		Biosurfactant (1.5g/L)	4-5	4-5
3	Red (1%)	Chemical surfactant (1g/L)	4-5	4-5
		Biosurfactant (1g/L)	4-5	4-5
4	Red (3%)	Chemical surfactant (1g/L)	4-5	4-5
		Biosurfactant (1g/L)	4-5	4
5	Black (1%)	Chemical surfactant (1g/L)	4-5	4-5
		Biosurfactant (1g/L)	4-5	4-5
6	Black (3%)	Chemical surfactant (1g/L)	4-5	4-5
		Biosurfactant (1g/L)	4-5	4

3.8 Color value measurement:

CIELa*b* colorimetric framework was utilized to decide the shading estimation on texture. Shading was resolved utilizing a Color Scan Machine (Premier Color Scan) shading distinction photometer which recorded the range of mirrored light and changed over it into a lot of shading arranges (L, an and b esteems). Factors of L*, a*, b* or E* are spoken to as delta L*, delta a*, delta b* or delta E*, where delta E* = (delta L*2+delta a*2+delta b*2). It speaks to the size of the distinction in shading, however doesn't show the bearing of the shading contrast. Biosurfactant treated texture shading esteems were estimated against substance surfactant treated texture. The delta E esteems are firmly coordinated with compound surfactant treated cotton texture. Concoction and biosurfactant treated red (3%) and dark (3%) texture were firmly coordinated against substance surfactant of delta E esteems are 0.39 and 0.32. *Bacillus mesophilus* creating biosurfactant are the best option in utilizing substance surfactant.

Table 5: Color values

Trial no	DL	Da	Db	Dc	DH	DECMS
1	-0.15	1.18	-0.24	0.40	-0.49	0.65
2	-0.64	-0.58	0.47	0.41	-0.50	0.91
3	-0.52	0.61	0.97	0.17	0.54	0.77
4	0.06	0.37	0.68	0.14	0.36	0.39
5	1.68	-0.0	-0.06	0.05	0.02	0.45
6	-0.20	0.03	0.23	-0.24	-0.02	0.32

4. Conclusion

The rhamnolipid produced from *Pseudomonas aeruginosa* was cultured in mineral salt medium with different oil substrates. This Biosurfactant acts as an antimicrobial agent against both several gram positive and negative bacteria and it has stable emulsification properties. The maximum production obtained using substrate (palm oil) was about 98g/L. The red, green and black dyed cotton fabric was treated with both chemical surfactant and biosurfactant (1-1.5 g/L). Biosurfactant acts as an effective surface-active agent for removal of reactive dyes from cellulosic material.It showedbetter wash fastness result (CF4-5) in biosurfactant treated fabric when compared to chemical surfactant treated fabric. This biosurfactant should be commercialized for industrial applications and this study has great significance of microbial biosurfactants are considered safer alternative to chemical surfactant.

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