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Biosurfactant Production By Soil Indigenous Free Living Diazobacteria

Sunil Radhakrishin Jagiasi
Associate Professor, Post Graduate Department of Microbiology
Seva Sadan's R.K. Talreja College, Ulhasnagar, Dist.- Thane, Maharashtra, India.

Abstract: Biosurfactants are surface active agents produced intracellular / extracellular by microorganisms. The biosurfactants have been reported for similar/better performance in comparison to synthetic surfactants. Their low toxicity, high biodegradability and functional at extreme pH and/or temperature make them green alternative to chemical counterparts. The yeasts, bacteria and some filamentous fungi are capable of producing biosurfactants with different molecular structures. These are generally consisting of fatty acids, glycolipids, lipopeptides, lipopolysaccharides and lipoproteins. Despite of many advantages, biosurfactants have not been fully commercialized due to expensive raw material and low yield. In current studies the efforts are taken to isolate and screen the biosurfactant producing free living diazobacteria followed by optimization of environmental factors for maximum biosurfactant production. The free living diazobacteria isolates were enriched using Burks Nitrogen Free media and screened for biosurfactant production by different techniques viz. hemolysis activity, Drop Collapse test, Oil spreading test and Emulsification Index by E24 % assay. The potent biosurfactant producing diazobacteria isolate was identified with reference to morphology, cultural, biochemical utilization followed by 16S rRNA gene sequencing as *Klebsiella singaporensis.* On analysing by standard methods, the composition of biosurfactant was found Glycolipid. The higher production of biosurfactant was seen at pH 7.0, 30º C and with 6% inoculum density in 5 days under static conditions. The pre-treated banana peels as a substrate in media had shown higher production of biosurfactant by isolate. The further studies are in progress to identify the biosurfactant using Mass Spectroscopy followed by its different applications for *In situ* bioremediation.

Index Terms: Biosurfactant, Bioremediation, Emulsification Index, Surface active agents.

I. INTRODUCTION

Biosurfactants are surface active agents produced intracellular / extracellular by microorganisms. The properties of biosurfactant viz. foaming, dispersion, emulsification etc. make them suitable candidate in remediation technologies applied for organic and metal contaminants (Nurul Hanisah Md Badrul Hisham *et al.*, 2019, Husam Sabah Auhim and Ahmed Isam Mohamed 2013 and Patel P.H. *et al.*, 2012). The biosurfactants have been reported for similar / better performance in comparison to synthetic surfactants. Their low toxicity, high biodegradability and functional at extreme pH and/or temperature make them green alternative to chemical counterparts. They have wide applications in agriculture, food, cosmetics, and petroleum industries as well as in bioremediation. They also get a candidature in many biomedical applications due to their antibacterial, antifungal, antiviral activities (Rabail Zulekha *et al.,* 2020, Elkhawaga M.A. 2017).

The biosurfactant producing microorganisms inhabit water (fresh, ground and sea water) and land (soil, sediment and sludge). The yeasts, bacteria and some filamentous fungi are capable of producing biosurfactants with different molecular structures. These are generally consisting of fatty acids, glycolipids, lipopeptides, lipopolysaccharides and lipoproteins. They may be different in their composition depending on microorganism, raw material and the conditions used for processing (Nurul Hanisah Md Badrul Hisham *et al.*, 2019, Elkhawaga M.A. 2017 and Patel P.H. *et al*., 2012). Despite of many advantages, biosurfactants have not been fully commercialized due to expensive raw material and low yield. The production media optimization requires selection of inducing nutrients from cheaper source and ideal microenvironment for producing microorganisms. The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels. This approach usually termed as one-factor-at-a-time (OFAT) technique (Rabail Zulekha *et al.*, 2020, Jamal Parveen *et al.*, 2014, Mona E. M. Mabrouk. e*t al.*, 2014 and Husam Sabah Auhim and Ahmed Isam Mohamed, 2013).

The soil indigenous heterotrophic free living diazobacteria are well known for their role in increasing soil fertility and maintaining soil sustainability. They are also documented for their role in bioremediation of tannery effluents and herbicide degradation. Hence, in current studies the efforts are taken to isolate and screen the biosurfactant producing free living diazobacteria followed by optimization of environmental factors for maximum biosurfactant production.

II. MATERIALS AND METHODS

The soil sample was collected from nearby areas of Ulhas nallah of Ulhasnagar, which is waste dumping centre for many small scale industries and municipal. This soil sample was used for isolation of biosurfactant producing free living diazobacteria. All the media and chemicals used were of AR grade and purchased from Hi media Pvt. Ltd., Mumbai.

1. Hydrocarbon acclimatization of sample

The microflora present in soil sample were acclimatized with 20% increasing concentration of cooking oil at interval of 5 days for period of 15 days. The initial concentration of cooking oil in Burks Nitrogen free media used was 100 mg/lit. The cooking oil was used as cheaper source of hydrocarbon to adopt and induce the biosurfactant production in diazobacteria.

2. Enrichment and isolation of free living diazobacteria

The enrichment of free living diazobacteria was carried out using sterile Burks Nitrogen Free media with 1% of acclimatized microflora soil sample as inoculum and incubated at Room Temperature (30º C) for five days under static conditions. Three enrichments at interval of five days were given using above mentioned media and 1% of pre-enriched inoculum with same conditions of incubation. The discrete well developed colonies different in appearance were picked up and maintained on Burks Nitrogen free media slants at 4º C for further studies.

3. Screening of potential biosurfactant producing diazobacteria isolates

3.1. Hemolytic activity

All the preserved diazobacteria isolates were assessed for their haemolytic activity by streaking growth on Super Imposed Blood Agar (SIBA) plate. The plates were incubated at 30º C for 5 days. The biosurfactant producing bacteria showed the hemolysis activity with clear colorless zone around the colonies on SIBA plate (Ijeoma Vivian Nwaguma *et al.*, 2016).

3.2. Drop Collapsing test

The test was carried out using 96 wells microtitre plate. Briefly, Mineral oil was added in microtitre well and equilibrated at 37º C for one hour. The respective culture supernatant of isolates was added separately to well carrying the Mineral oil. The shape of the oil surface was

noted after 1 minute. The positive results were indicated by collapsed oil drop in one minute. The negative control was run in parallel using distilled water in place of culture supernatant (Patel P. H. *et al.,* 2012).

3.3. Oil spreading test

To perform this test, 50ml of distilled water was taken in petri dish having 15 cm diameter followed by overlaying of 20 µl of castor oil to form thin layer on water surface. In the center of oil layer surface, gently load the 10 µl of culture supernatant. The addition of culture supernatant results in oil displacement by forming clear zone. The zone size of oil clearance greater than 5 mm was considered as positive (Mariam Hassan *et al.,* 2014).

3.4. Emulsification test

The test was performed by vortexing for 2 minutes the mixture of 2 ml of culture supernatant (biosurfactant) and 2ml of hydrocarbon substrate in test tube. The cooking oil was used as hydrocarbon substrate for study. After vortexing, the mixture was kept undisturbed for 24 hrs. at 30º C. The control was run in parallel carrying 2 ml of sterile distilled water instead of culture supernatant. The emulsification activity was calculated by dividing height of emulsion layer to total height and expressed in percentage (Patel P. H. *et al.,* 2012).

4. Identification of potent biosurfactant producing diazobacteria isolate

The potent isolate was identified on the basis of morphology, cultural and biochemical utilization with reference to Bergey's Manual of Systematic Bacteriology 9th ed. (2000) followed by 16S rRNA gene sequencing.

5. Extraction and quantification of biosurfactant produced by potent isolate

Biosurfactant extraction was done by cold acetone precipitation method. Briefly, refrigerated culture broth was centrifuged at 4000 rpm for 30 minutes to settle the cells, followed by clear supernatant filtration using sterile Whatman No. 1 filter paper. The 2 ml of chilled acetone was added in filtrate and allowed to stand for 10hrs. at 4º C. The precipitate was collected by centrifugation followed by evaporation to remove acetone traces. The dried precipitate was re-dissolved in sterile distilled water for further use (Patel P. H. *et al.,* 2012).

5.1. Chemical analysis of Biosurfactant

The extracted biosurfactant was chemically analysed to determine its composition using standard methods. The carbohydrate content of the biosurfactant was determined by Phenol Sulphuric acid method (Dubois *et al.,* 1956). The protein content was determined by Lowry *et al*., (1951) method. The lipid content of biosurfactant was determined by method given by Folch *et al*., (1956).

6. Effect of pH, Temperature and Inoculum density on biosurfactant production

The sterile Mineral Salt Medium supplemented with 1gm/lit. of cooking oil, 0.1% glucose and 0.2% Yeast extract was used to study the effect of environmental factors on biosurfactant production by isolate. The addition of glucose and Yeast extract in media was to initiate the bacterial growth. For study effect of environmental factors, the One Factor At a Time (OFAT) was considered. To study effect of pH, the initial pH of media was adjusted in range of 5 – 9, for effect of temperature the inoculated flasks were incubated at different temperatures 0º C, 10º C, 30º C, 37º C and 55º C. To study effect of inoculum size on biosurfactant production, inoculum size ranges from 2-10 % was used. All inoculated flasks were adjusted (except where conditions are specified) to pH 7.0 and incubated at 30º C with 2% inoculum, incubated for 5 days under static conditions to study the effect of environmental factors.

7. Effect of different substrates on biosurfactant production

The use of waste as a substrate for production of biosurfactant is a best waste reduction at source and also results in low cost and eco-friendly process. The different waste materials used as substrates for study were Corn Steep Liquor (CSL), Banana peels, Rice straw, Soyabean oil refinery waste, and Grease. These different substrates were given pre-treatment to make utilizable substrate available to isolate for biosurfactant production (Mohanty S. S. *et al.*, 2021). The sterile Mineral Salt Medium with 1% of these pre-treated substrates supplemented with 0.1% glucose and 0.2% yeast extract with initial pH of media adjusted at 7.0, inoculated with 6% inoculum, incubated at 30º C under static conditions for 5 days. The biosurfactant production was analysed chemically to determine the effect of different substrates on its production.

III. RESULTS AND DISCUSSION

The acclimatization of free living diazobacteria was carried out to adopt and induce the biosurfactant production in microflora. As mentioned in Materials and Methods, acclimatization of microflora was done with increasing concentration of cooking oil in media after every 5 days. On enrichment and isolation, total 32 isolates were selected based on their varied cultural characteristics (Figure -1). All the isolates were designated as DA1, DA2, DA3, DA4 and so on.

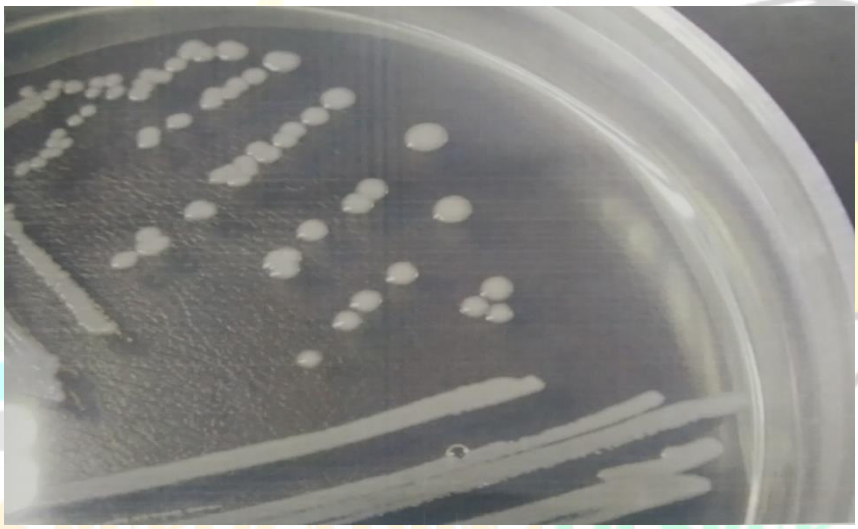

Figure 1- free living diazobacteria growth on burks nitrogen free media.

The screening results for potential biosurfactant producing free living diazobacteria isolates are shown in Table 1. Among 32 diazobacteria isolates, 14 were able to haemolyse the human RBCs and showed the haemolysis on SIBA plate. These 14 haemolytic isolates along with 18 non haemolytic isolates were assessed for Drop Collapsing Test. In Drop Collapsing test, 6 haemolytic isolates showed positive results in Drop Collapsing Test. These isolates were further tested for oil spreading test, where only three isolates showed zone of clearance by oil displacement more than 5 mm (pre-defined criteria for positive test). On checking the Emulsification activity of isolates by E24%, isolates showed the Emulsification in the range of 34% – 84%. The free living diazobacteria isolate DA17 showed comparatively higher Emulsification value of 84% in the form of E24%, with cooking oil as hydrocarbon substrate used for assay. Hence, potent biosurfactant producing isolate DA17 was identified and used for further studies.

Table 1- screening of potential biosurfactant producing free living diazobacteria isolates

Sr. No.	Isolate	Haemolysis	Drop Collapse Test	Oil Spreading test (zone of oil displacement)	Emulsification test (E24%)
1.	DA1	H	+ve	2 mm	34%
2.	DA2	NH	-ve	--	--
3.	DA3	H	-ve	--	--
4.	DA4	NH	-ve	--	--
5.	DA5	H	-ve	--	--
6.	DA6	NH	-ve	--	--
7.	DA7	NH	-ve	--	--
8.	DA8	H	+ve	5 mm	76%
9.	DA9	NH	-ve	--	--
10.	DA10	NH	-ve	--	--
11.	DA11	H	-ve	--	--
12.	DA12	NH	-ve	--	--
13.	DA13	H	+ve	3 mm	62%
14.	DA14	H	-ve	--	--
15.	DA15	NH	-ve	--	--
16.	DA16	NH	-ve	--	--
17.	DA17	H	+ve	6 mm	84%
18.	DA18	NH	-ve	--	--
19.	DA19	NH	-ve	--	--
20.	DA20	NH	-ve	--	--
21.	DA21	H	-ve	--	--
22.	DA22	NH	-ve	--	--
23.	DA23	NH	-ve	--	--
24.	DA24	NH	-ve	--	--
25.	DA25	H	-ve	--	--
26.	DA26	H	+ve	6 mm	78%
27.	DA27	NH	-ve	--	--
28.	DA28	NH	-ve	--	--
29.	DA29	H	-ve	--	--
30.	DA30	H	-ve	--	--
31.	DA31	NH	-ve	--	--
32.	DA32	H	+ve	3 mm	67%

Key: H – Haemolysis, NH – No Haemolysis, +ve – Oil Drop Collapse, -ve – No oil Drop Collapse

The isolate DA17 was identified with reference to morphology, cultural and biochemical utilization as per Bergey's Manual of systematic Bacteriology, 9th ed. (2000) followed by 16S rRNA gene sequencing as *Klebsiella singaporensis* (Figure - 2).

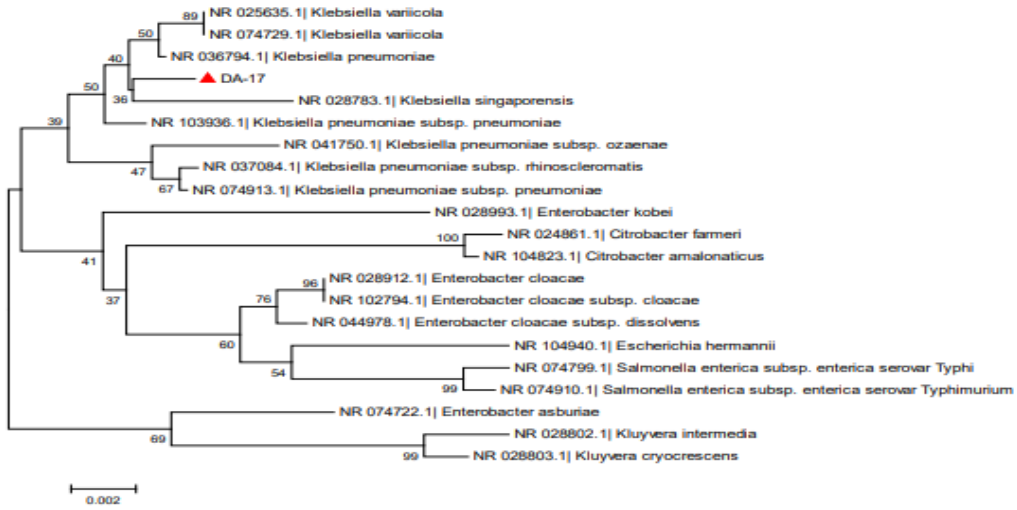

Figure 2- phylogenetic tree for potent biosurfactant producing free living diazotroph isolate by 16S rRNA gene sequencing

Extraction and quantification of biosurfactant produced by potent isolate was carried out by precipitation method using chilled acetone. On quantification the content of biosurfactant for carbohydrate, protein and lipid content by standard methods mentioned in materials and methods, 73% of carbohydrate and 37% of lipid content was found. Hence, the biosurfactant produced by *Klebsiella singaporensis* was presumed as of Glycolipid type.

The higher biosurfactant production was seen at pH 7.0, temperature 30º C with 6% inoculum in 5 days incubation under static conditions. The pH of the media plays an important role in transportation of nutrients inside the cell and ultimately controls cell growth and biosurfactant production.

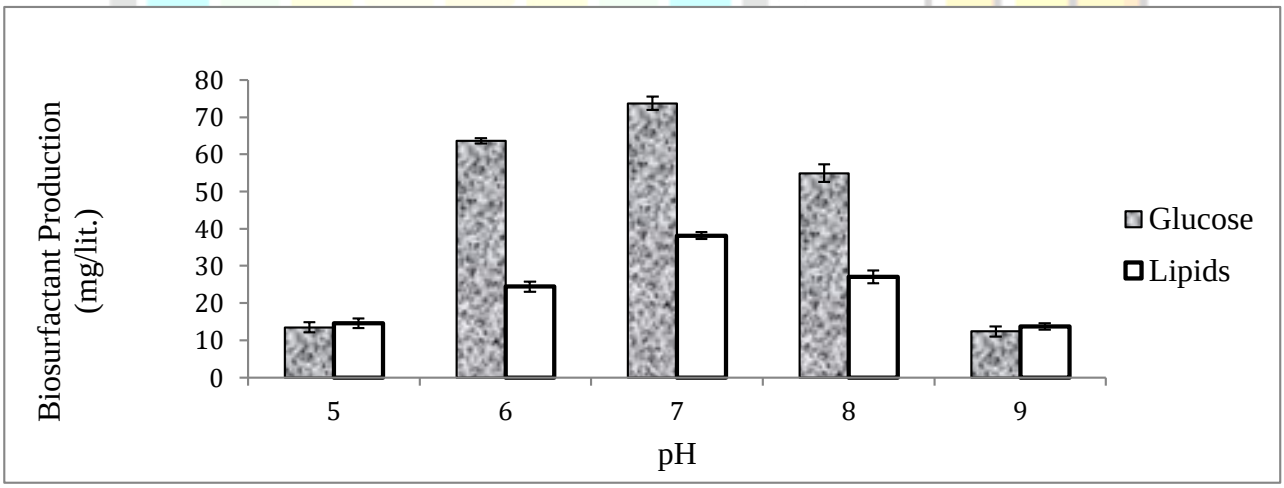

Figure 3- effect of pH on biosurfactant production by *klebsiella singaporensis*

As shown in figure-3, the higher production of biosurfactant by isolate was seen at pH 7.0 when the optimized nutrients transportation across the cell membrane is expected and thus supports the higher biosurfactant production at neutrality. At 30º C followed by 37º C, the higher biosurfactant production was seen, could be due to the optimum temperature for growth of isolate (figure-4). At 0º C, neither isolate growth nor biosurfactant production was seen whereas, at 10º C and 55º C insignificant production of biosurfactant was seen. At low temperature, growth of isolate is insignificant whereas, at high temperature the responsible enzymes for biosurfactant production get denatured. Hence, at extreme temperature the biosurfactant production gets decreased.

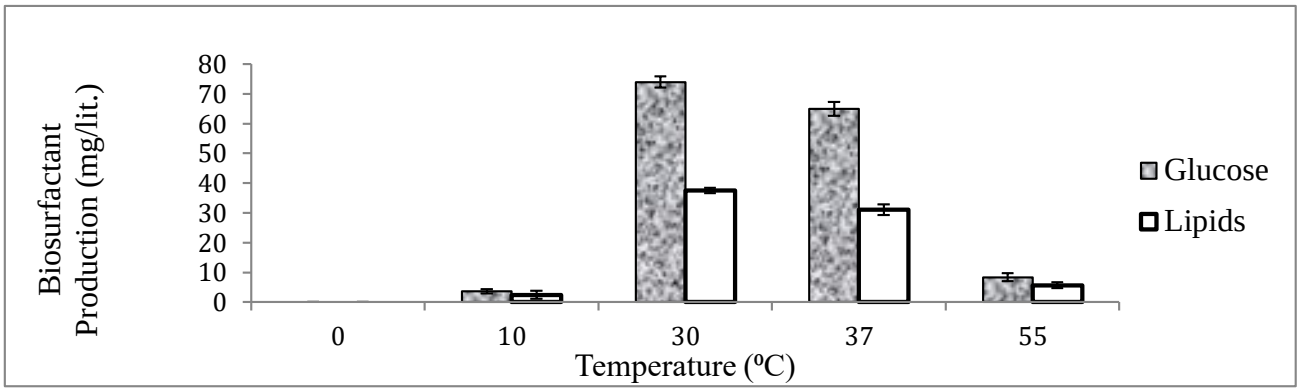

Figure 4- effect of temperature on biosurfactant production by *klebsiella singaporensis*

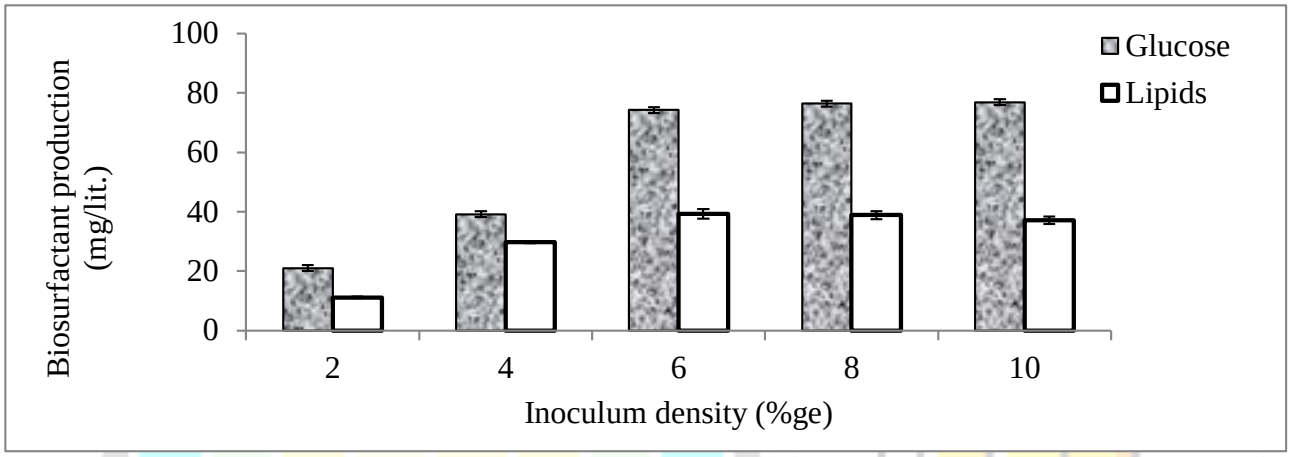

Figure 5- effect of inoculum density on biosurfactant production by *klebsiella singaporensis*

The increase in inoculum density from 2-6%, results in increase of biosurfactant production also, but later on with further increase in inoculum density had not shown any significant increase in biosurfactant production could be due to cell aggregates / heap formation at high cell density (Figure -5) . Chanika Saenge Chooklin *et al.,* (2014) also reported the higher production of biosurfactant at neutral pH and temperature 30º C-35º C during their studies on biosurfactant production from *Deinococcus caeni* PO5 using jackfruit seed as a substrate. Our findings were also coincides with results obtained by Husam Sabah Auhim and Ahmed Isam Mohamed (2013) during their studies on effect of different environmental and nutritional factors on biosurfactant production from *Azotobacter chrococcum.*

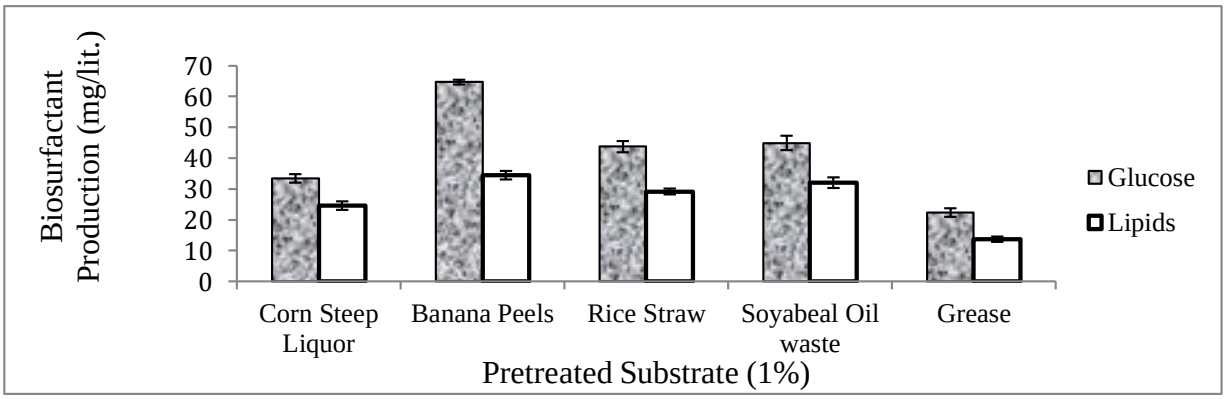

Figure 6- effect of pre-treated wastes used as substrates for biosurfactant production by *klebsiella singaporensis*

As mentioned in methodology, the use of waste material for production of biosurfactant will reduce the pollution at source and also decrease the cost of production by using cheaper source of substrate. During studies, pre-treated banana peels as substrate used for biosurfactant production had shown the higher production of biosurfactant by *Klebsiella singaporensis* (Figure-6).

IV. CONCLUSION

The role of diazobacteria is well known for maintaining soil fertility and its sustainability. It is also well documented for its bioremediation role for tannary effluents and herbicide degradation. In present studies, efforts were taken to isolate and screen the biosurfactant producing indigenous free living diazobacteria from soil. The potent biosurfactant producing isolate was enriched, isolated, screened and identified as *Klebsiella singaporensis* on the basis of morphology, cultural characteristics and biochemical utilization followed by 16S rRNA gene sequencing. The biosurfactant was analysed as Glycolipid having around 63% carbohydrate and 37% lipid content. The higher biosurfactant production was seen at pH7.0, 30º C with 6% inoculum in 5 days under static conditions. The isolate had shown comparatively higher biosurfactant production with pre-treated banana peels used as substrate in media. The further studies are in progress for biosurfactant analysis using LC-MS and to assess its role in *In situ* bioremediation of recalcitrant synthetic pollutants.

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