Optimization and Characterization of Biosurfactant Produced by *Pseudomonas Aeruginosa*

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ABSTRACT

Biosurfactant are amphiphilic molecule synthesized by variety of microorganisms as secondary metabolites. Due to non toxicity, biodegradability and eco-friendly nature, biosurfactant can be utilized in various sectors. Previous research studies reported that biosurfactant possess antimicrobial activity against human pathogens. On the circumstances of the increasing market of biosurfactant all over the world, the present study focused on Optimization and characterization of biosurfactant produced by *Pseudomonas aeuginosa*. Optimization of production media and physiochemical parameters for efficient biosurfctant production was carried out using MSM broth supplemented with 2% soyabean oil. Statistical analysis was carried out. Biosurfactant was characterized on the basis of TLC and Mass spectrometry. The results revealed that soyabean oil as carbon source, KNO_3 as nitrogen source, aeration rate of 25vvm, agitation rate of 200 rpm, p^H 8, 144 hrs. incubation periods, inoculum size 4% and temperature 30℃ was found to be statistically significant for the production of rhamnolipid by *Pseudomonas aeruginosa*. The finding of thin layer Chromatography showed two spots on silica gel plate after drying. The R^f value was found to be 0.53 and 0.67 respectively confirming presence of mono and di- rhamnolipid. The mass spectra showed the presence of both potassium adducts $(M+K)^+$ ions sodium adducts $(M+Na)^+$ ions confirming the presence of rhamnolipid. Hence, *Pseudomonas aeruginosa* isolated from oil contaminated soil could be utilized for rhamnolipid production.

Keywords: Biosurfactant, *Pseudomonas aeruginosa*, Rhamnolipid, Mass spectrometry

INTRODUCTION

Biosurfactant are natural surface active compounds synthesized by variety of fungi, yeast and bacteria(Chen *et al.,* 2007). Biosurfactant are amphipathic molecule containing hydrophilic and hydrophobic components. Hydrophobic portion contain either a long chain fatty acid, or a-alkyl-b-hydroxyl fatty acid and the hydrophilic section contain amino acid, carbohydrate, phosphate, a cyclic peptide, or alcohol, etc (Katemai *et al.,*2008).On the basis of molecular weight, biosurfactants are classified into two major categories low molecular weight biosurfactants which includes glycolipids and lipopeptides; and high molecular weight biosurfactants i.e., lipoproteins or lipopolysaccharide (Harshada 2014).Biosurfactant form a microemulsion by reducing surface tension of compounds with different phases (Sudhakar Babu *et al.,* 1996).Biosurfactant possess various properties which includes good detergency, high foaming, great emulsification, microbial growth enhancement, and oil recovering (Banat *et al.,*2014).Biosurfactant are environment friendly, biodegradable, less toxic, highly soluble, and potentially active compared to synthetic surfactants (Desai and Banat 1997).Various studies reported that Biosurfactant shows multipurpose therapeutic and biomedical Properties which include antimicrobial, immunomodulatory compounds and adhesive agent (Benincasa *et al.,*2004;Mulligan 2005). Due to thisproperties, biosurfactant are applied in various fields viz. food, cosmetic and pharmaceutical (Mandal *et al.,* 2013).

Microorganisms synthesized various classes of biosurfactant such as phospholipid, glycolipids, fatty acids, and lipopeptides (Cooper 1986; Cooper *et al.,* 1980; Velikonja and Kosaric 1993). Some biosurfactant synthesized

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by microorganisms produced on water soluble substrate such as glucose, ethanol and glycerol but majority of known biosurfactant is produced on water insoluble hydrocarbons (Haferburg *et al.,* 2005). Sophorolipids were the first microbiological biosurfactant introduced in the market. Up to now, the best studied groups of biosurfactant are phospholipids and glycolipid compounds (Sandoval *et al.,* 1999). Currently rhamnolipid have the great potential for becoming the next generation biosurfactant (Muller *et al.,* 2012). Several studies reported that biosurfactant have antimicrobial activity against bacteria, fungi and viruses. Due to wide broad spectrum antimicrobial activity biosurfactant can be used as a new alternative to synthetic chemical drug (Rahman *et al.,* 2002). Hence, present research study highlights optimization and characterization of biosurfactant produced by *Pseudomonas aeruginosa.*

METHODS

Pseudomonas aeruginosa used in this research work was isolated from oil contaminated soil sample from Washim, Maharashtra. The strain was maintained on nutrient agar slant for further use. A single colony from 24 hrs. old culture was inoculated in nutrient broth, 24 hrs old culture broths was used for further studies.

Optimization of physiochemical parameter and media components for efficient biosurfactant production by *Pseudomonas aeruginosa*

The optimization study was carried out with modified mineral salt medium containing 2% soyabean oil as a carbon source and efficient strain of *Pseudomonas aeruginosa* for rhamnolipid production. While testing the effect of carbon source, soyabean oil was not added in production medium. Different carbon sources viz. Soyabean oil, glucose, diesel, petrol, kerosene were added in the concentration of 2 % were analyzed. The Nitrogen sources tested were Yeast extract, sodium nitrate, Peptone, ammonium chloride, potassium nitrate, ammonium sulphate and ammonium nitrate. Three agitation rates were tested viz. 100,150 and 200 rpm. The aeration rate was measured using various ranges of production media volume 25, 50, 75, 100, 125, 150 and 175 mL in 250 ml conical flask corresponding to volumetric oxygen percentage of 90,80,70,60,50,40,30 % respectively. Effect of inoculum size was studied at various inoculum percentages viz 1, 2, 3 upto 8%. Effect of temperature on rhamnolipid production was done at different temperatures ranging from 25° to 55° C. Effect of pH on rhamnolipid production was carried out at different pH ranging from 3.0-9.0. Effect of incubation period on rhamnolipid production was studied at various time intervals ranging from 24 to 192 hrs. All flasks were inoculated with 5% overnight culture at an initial pH 7 and incubated for 72 hours, 200 rpm on rotary shaker at 37°C. After incubation, the supernatant was collected and subjected for surface tension reduction, rhamnose concentration, determination of dry cell weight and Emulsification activity. Further Statistical analysis was carried out. All the experiments were performed in triplicate, and the results were expressed as means $\pm$ standard deviation (SD), with a 95% confidence interval. Further the characterization of rhamnolipid was done by thin layer chromatography (TLC) and mass spectrometry (MS) adapting standard methods (Housseiny *et al.,* 2020; Jacques *et al.,* 2008).

RESULT

Optimization of physiochemical parameter and media components for efficient biosurfactant production by *Pseudomonas aeruginosa*

A) Effect of Carbon Source on rhamnolipid production

In the present research study, several carbon sources (2%) were tested. Maximum dry cell weight of 2.4 g/l was observed in soyabean oil followed by glucose (1.8), diesel and kerosene (1.6) and petrol (1.5). Maximum surface tension reduction (49 mNm^{-1}) was noted in soyabean oil followed by glucose (38 $mNm^{-1)}$, diesel (34 mNm^{-1}) and kerosene (32 $mNm^{-1)}$. Lowest surface tension reduction (31 mNm^{-1}) was noted in petrol. Highest emulsification index and rhamnose concentration was observed in soyabean oil. Soyabean oil enhanced biosurfactant production also soyabean oil is cost effective alternative to other expensive oils. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.68 ± 0.55 with a 95% confidence interval of 0.69. Hence soyabean oil was found to be optimum carbon source for rhamnolipid production.

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B) Effect of Nitrogen Source on rhamnolipid production

In the present study, various organic and inorganic nitrogen sources (0.3%) were tested. Ammonium nitrate, potassium nitrate and peptone enhanced the biosurfactant activity, of which KNO_3 decreased the surface tension by 46% and also gave E24 value of 54% but as KNO_3 showed highest surface tension reduction than peptone, hence, it was considered superior nitrogen source for the production of rhamnolipid by *Pseudomonas aeruginosa* strain PA02. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.7 ± 0.35 with a 95% confidence interval of 0.32.

C) Effect of aeration on Rhamnolipid production

It is found that maximum dry cell weight (2.1g/l) was observed at aeration rate 25vvm.Aeration rate is inversely proportional to dry cell weight because as the aeration rate increases dry cell weight gradually reduces. Maximum surface tension reduction (%) was found at 25vvm after which it shows gradual decrease 50, 75 and 100 vvm respectively. Similarly, maximum emulsification index was noted at 25 vvm followed by 50, 75 and 100 vvm respectively. The Rhamnose concentration of 2.4 g/l was obtained at aeration rate 25 vvm which is the highest concentration followed by 2 g/l at 50 vvm, 1.8 g/l at 75 vvm and 1.5 g/l at 100 vvm aeration rate. Hence 25 vvm is the optimum aeration rate for rhamnolipid production. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.9 ± 0.37 with a 95% confidence interval of 0.60.

D) Effect of agitation rate on rhamnolipid production

From the figure it is found that dry weight was 1.7 g/l at 100 rpm, 2.3 g/l at 150 rpm, 2.5 g/l at 200 rpm and 2.2 g/l at 250 rpm agitation. Maximum dry weight was obtained at 200 rpm after that it started decreasing. Surface tension reduction (%) was noted at 100 rpm (11 mN m), at 150 rpm (21 mN m), at 200 rpm highest surface tension reduction was noted (34 mN m) and at 250 rpm surface tension reduction start decreasing (28 mNm).Highest Emulsification index was observed at 200 rpm. Emulsification index 15% was observed at 100 rpm, 34 % was observed at 150 rpm and 36 % at 250 rpm. Highest Rhamnose concentration 2.5g/l was observed at 200 rpm followed by 1.9 g/l at 250 rpm, 1.5 g/l at 150 rpm and 1.2 g/l at 100 rpm. Hence an agitation rate of 200 rpm was used in further studies. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.7 ± 0.56 with a 95% confidence interval of 0.89.

E) Effect of inoculum size on rhamnolipid production

It is found that maximum surface tension reduction and Emulsification index found at 4% v/v inoculum size after which it gradually decreases. Maximum dry cell weight was obtained at 4% v/v inoculum size. Also Maximum Rhamnose concentration was obtained at 4% v/v inoculum size. As the inoculum size increases Rhamnose concentration was decreases. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.7 ± 0.28 with a 95% confidence interval of 0.24.

F) Effect of Temperature on Rhamnolipid production

From the figure it is observed that highest rhamnolipid production was obtained at 30° C temperature. Maximum dry cell weight was observed at 30°C temperature. Similarly maximum surface tension reduction of 45% was noted at 30°Ctemperature after which it decline. Highest Emulsification index of 55% was observed at temperature 30°C. Hence, 30°C temperature was found to be optimum temperature for rhamnolipid production. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.5 ± 0.87 with a 95% confidence interval of 1.08.

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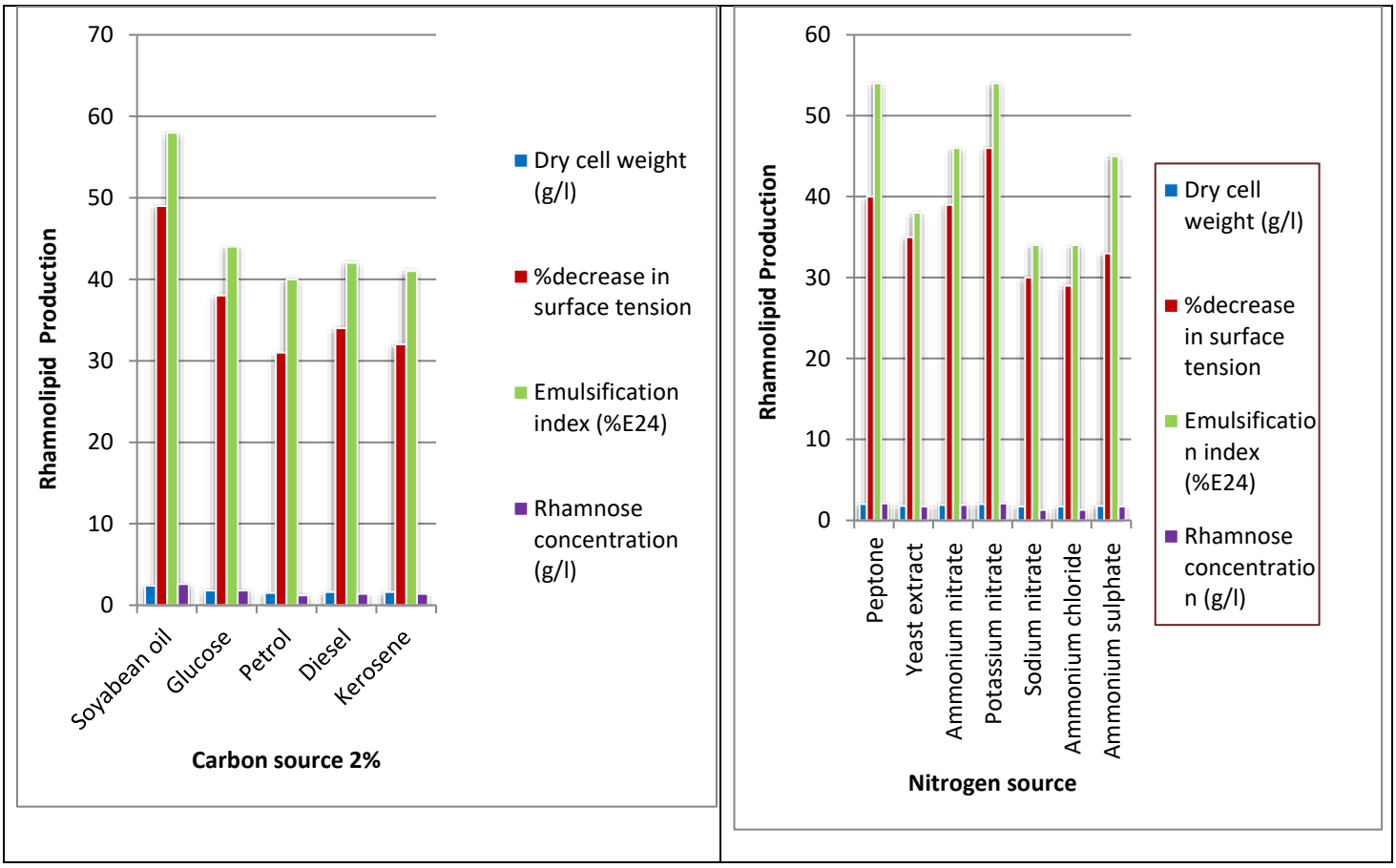
G) Effect of pH on rhamnolipid production

From the result it is observed that rhamnolipid production was started at pH 4 and gradually increases upto pH 8. The result on dry cell weight showed that maximum dry cell weight of 2.0 was noted at pH 8. Maximum surface tension reduction of 47% was observed at pH 8 after which it declines. Similarly maximum emulsification index of 55% was observed at pH 8. Hence, pH 8 was found to be optimum pH for rhamnolipid production. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.1$\pm$0.88 with a 95% confidence interval of 0.81.

H) Effect of Incubation period on rhamnolipid production

It is found that highest rhamnolipid production was obtained at 144 hrs. of incubation period. The emulsification activity and surface tension reduction showed gradual increase after 24 hrs. incubation period and attain highest value on 144 hrs. After 144 hrs. it showed gradual decrease. Similarly, rhamnose concentration show highest value at 144 hrs. incubation period after which it showed gradual decrease. Hence, 144 hrs. incubation period was found to be optimum for the rhamnolipid production using *Pseudomonas aeruginosa PA02*. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The mean $\pm$ SD value is 1.8$\pm$0.29 with a 95% confidence interval of 0.25.

Figure: Effect of different parameters on Rhamnolipid production



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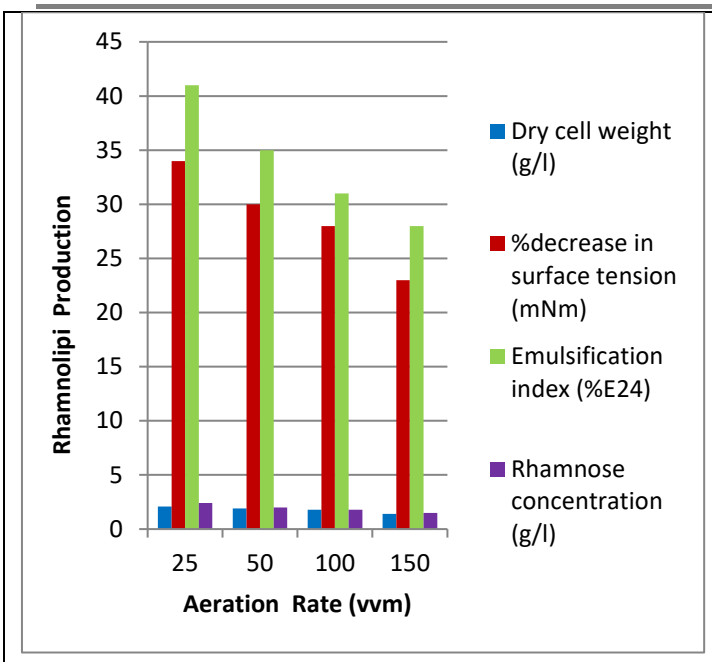

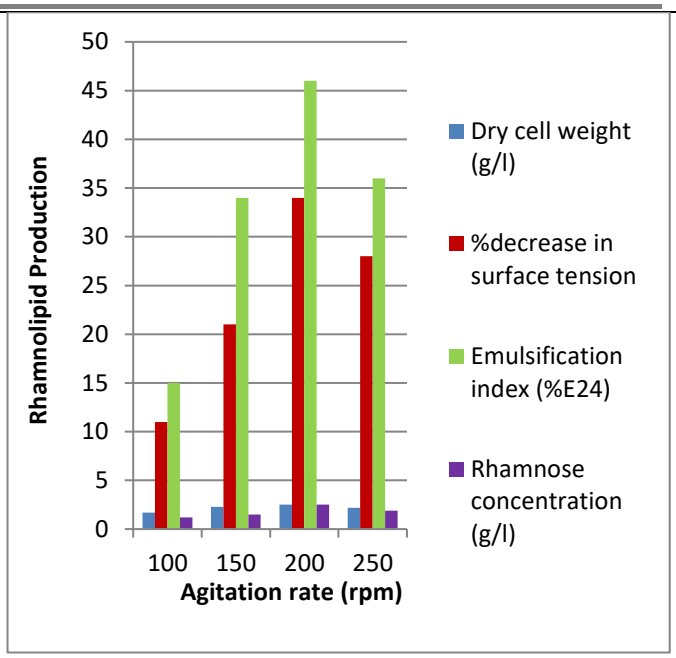

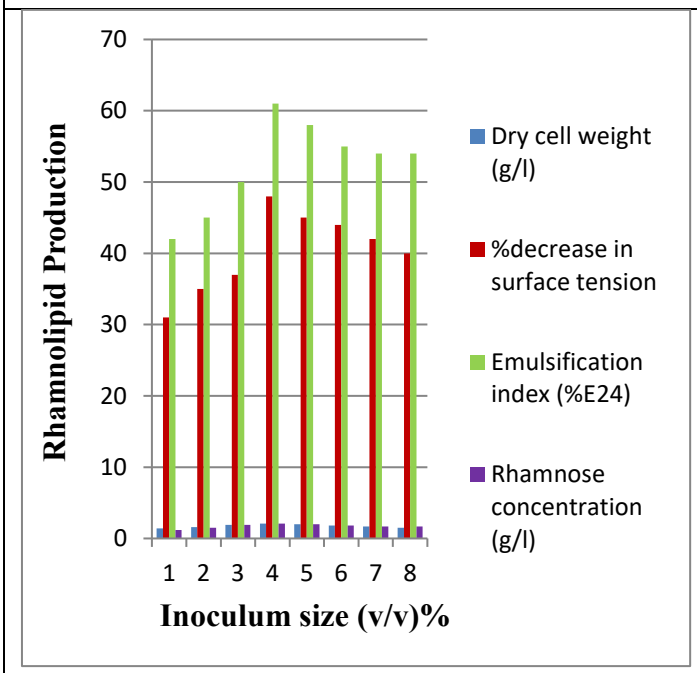

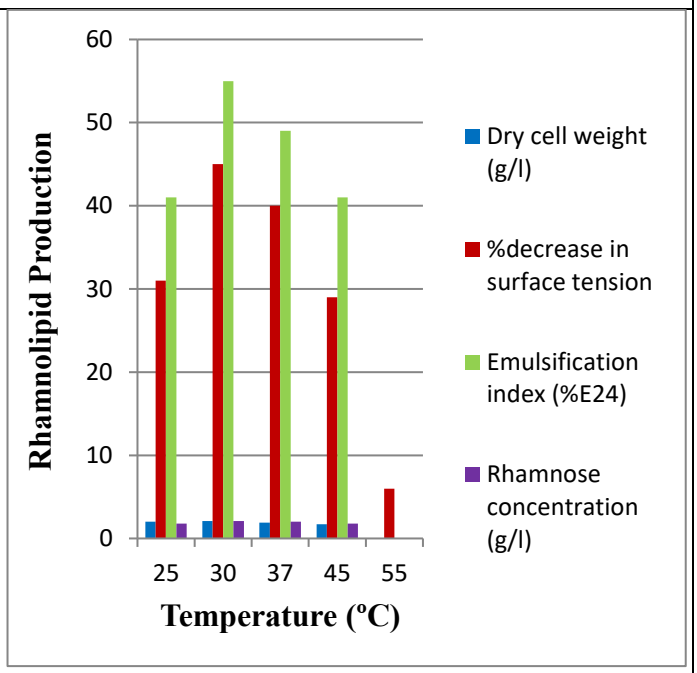

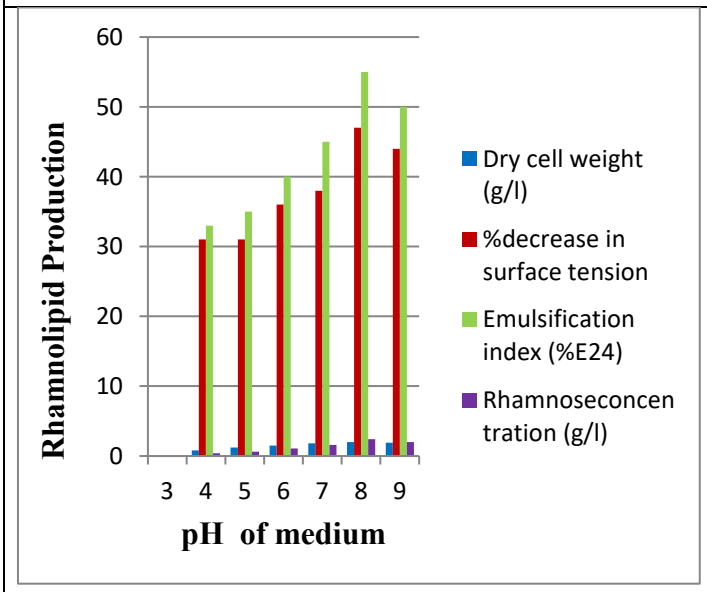

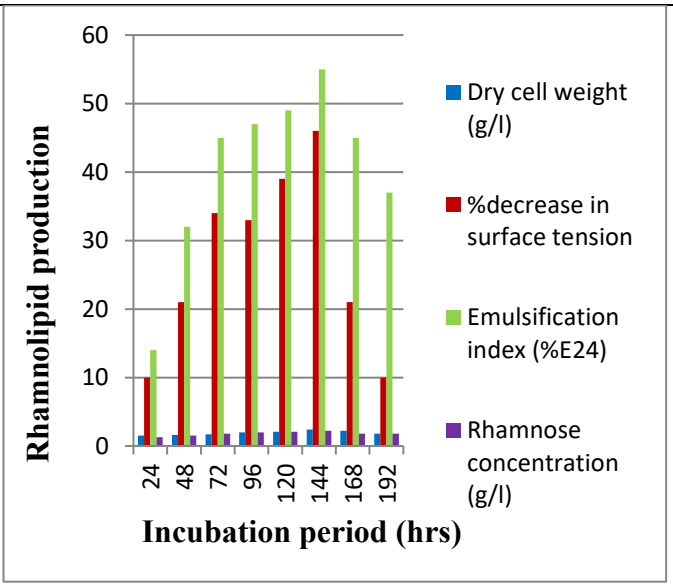

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Characterization of Rhamnolipid

Thin layer Chromatography

The finding of thin layer Chromatography showed two spots on silica gel plate after drying. The R^f value was found to be 0.53 and 0.67 respectively. Hence, the rhamnolipid produced by *Pseudomonas aeruginosa* is combination of mono and di rhamnolipid.

MASS Spectrometry

The mass spectra showed the presence of both potassium adducts $(M+K)^+$ ions sodium adducts $(M+Na)^+$ ions confirming the presence of rhamnolipid. The most abundant ions are mono-rhamnolipid corresponding to $(M+Na)^+$ ions at *m/z* 526.13 and di-rhamnolipid corresponding to $(M+K)^+$ ions at *m/z* 672 respectively.

CONCLUSION

The results on optimization study revealed that soyabean oil as carbon source, KNO_3 as nitrogen source, aeration rate of 25vvm, agitation rate of 200 rpm, p^H 8, 144 hrs incubation periods, inoculum size 4% and temperature 30℃ was found to be significant for the production rhamnolipid by *Pseudomonas aeruginosa*. From the statistical evaluation it is found that the experimental results were statistically significant. The mean difference is significant at 0.05 level. The finding of thin layer Chromatography showed two spots on silica gel plate after drying. The R^f value was found to be 0.53 and 0.67 respectively confirming presence of mono and di- rhamnolipid. The mass spectra showed the presence of both potassium adducts $(M+K)^+$ ions sodium adducts $(M+Na)^+$ ions confirming the presence of rhamnolipid. The most abundant ions are mono-rhamnolipid corresponding to $(M+Na)^+$ ions at *m/z* 672 and di-rhamnolipid corresponding to $(M+K)^+$ ions at *m/z* 526.13 respectively. Hence, *Pseudomonas aeruginosa* isolated from oil contaminated soil could be utilized for rhamnolipid production.

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