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(RESEARCH ARTICLE)

Optimization of bio-surfactant production by Azo-rhizobium strain isolated from oil-contaminated soil

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Abstract

Bio-surfactants are amphiphilic molecules possessing both hydrophilic and hydrophobic moieties. They are surface active agents that are produced extracellularly or as a part of the cell membrane by bacteria, yeast, and fungi. In the present study, the screening and optimization of bio-surfactant production were carried out using oil-contaminated soil sample. The cultural, morphological and biochemical tests as well as 16s rRNA analysis identified the most efficient bio-surfactant producer as *Azorhizobium* strain. The preliminary screening of bio-surfactant production was done with the help of oil displacement test, drop collapse test and observed hemolysis on superimposed blood agar plates. Optimization studies revealed that 2% inoculum size of test strain (1.0 O.D 530 nm) can be exploited for bio-surfactant production using 2% coconut oil and ammonium nitrate with a C: N ratio of 20:1 in MSM medium (pH 8.5) and incubation conditions of 30 ˚C for 96 h. The crude yield of bio-surfactant produced was estimated as 2.5 g/L. Further purification of crude bio-surfactant was carried out using acid hydrolysis and rotary vacuum evaporator. The bio-surfactant thus obtained successfully reduced the surface tension of the medium from 59 mN/m to 38 mN/m with E_{24} 60%. The characterization studies of the purified bio-surfactant carried out by FTIR analysis confirmed it to be lipopolysaccharide type of bio-surfactant. Thus our current study suggests useful application of bio-surfactant producing bacterial strain that may be helpful in the petroleum industry for the purpose of recovery of petroleum and other oils from oily sludge, cleaning of oil storage tanks and bioremediation of oil-contaminated sites.

Keywords: Bio-surfactant; Optimization; FTIR; *Azorhizobium*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are common environmental contaminants that result primarily from the incomplete combustion of organic matter associated with coal and crude oil processing. Many PAHs and other petroleum hydrocarbons are toxic, mutagenic and carcinogenic in nature [1], and hence pose serious health concerns for humans as well as the environment. One of the main approaches for eliminating these pollutants from contaminated sites involves the process of bioremediation using microorganisms [2].

Bio-surfactants are surface active agents produced by microorganisms when grown on water immiscible or oily substrates [3]. Many microbes degrade natural hydrocarbons viz., saturated and unsaturated alkanes, monoaromatic compounds and low-molecular-mass PAHs to produce bio-surfactants either as cell surface components or extracellular molecules [4, 5]. However, the biodegradation process of complex PAHs is more demanding due to its hydrophobic nature and the ability to adsorb strongly to soil particles, which results in their limited bioavailability [6]. In order to overcome this problem, recent research focuses on microbial strategies of bio-surfactant production. Presently, a structurally diverse group of surface-active bio-molecules are known. Rhamnolipids from *Pseudomonas aeruginosa*,

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surfactin from *Bacillus subtilis*, emulsan from *Acinetobacter calcoaceticus* and sophorolipids from *Candida bombicola* are some examples of microbial-derived surfactants [2, 7-9].

Surfactants can accumulate between fluid phases such as oil/water or air/water due to the presence of both hydrophobic (non-polar) and hydrophilic (polar) moieties in their structures. This reduces the surface and interfacial tensions forming emulsions [10]. Hence they are identified as one of the most versatile classes of chemical compounds.

The microbial-derived surfactants have gained popularity since the late 1960s as an improved alternative to chemical surfactants primarily because of their specific action, low toxicity, higher biodegradability, and effectiveness at extremes of temperature, pH, and salinity. In addition, their unique structures provide new properties that classical surfactants may lack. Hence they offer a variety of applications in enhanced oil recovery, medicinal field, food and cosmetic industries [2, 11].

In spite of the benefits offered by bio-surfactants, currently, they are outweighed by the disadvantages faced with its applications. One of the major set-backs regarding the use of bio-surfactants is the requirement of raw material (i.e., crude oil) in large quantities for its production. Moreover, the productivity is very low and there occurs intense foaming during the bio-surfactant production [12]. This makes the process expensive and labor extensive. However, the impediment to research progress in the field of bio-surfactant production may be overcome by utilization of waste substrates as raw material to reduce the cost of the process. The development of oxygenation strategies may be applied to reduce the formation of foam [13]. Finally and most importantly, the optimization studies w.r.t growth conditions for production of bio-surfactants may be subjected to genetic modifications, use of combined omics analysis and computational modeling [14]. These efforts will allow the bio-surfactants to be used in large quantities and as preferred replacements for synthetic surfactants in several industrial processes [13, 15]. Successful application of bio-surfactant producers in oil-contaminated sites may also help in overcoming problems associated with bioremediation [1, 5, 16-17].

The interest shown by industrialists and scientists in the field of bio-surfactant production can be substantiated with the fact that, in spite of the high cost (3-10 times) of bio-surfactants as compared to chemical surfactant, its production in the global market is expected to reach 4.7×10^5 tons, which is worth 2.2 billion USD, in 2018 from an initial worth of 1.7 billion USD in 2011 [18].

The current study was carried out with an aim to optimize bio-surfactant production from *Azorhizobium* species and characterize the same in order to obtain high yields.

2. Material and methods

2.1. Enrichment and isolation of bio-surfactant producers

In our study, the contaminated soil samples were obtained from oil mills and enriched for isolation of bio-surfactant producers. The enrichment was carried out by inoculating 1 g of the soil sample in 100 ml Nutrient Broth (NB) containing 2% coconut oil as an inducer and incubating it at 30 °C for 4-5 days under shaker (150 rpm) conditions. Isolation of bio-surfactant producers from the enriched medium was carried out on Nutrient Agar (NA) plates incorporated with the inducer and incubated for 4-5 days at 30 °C.

2.2. Primary screening of bio-surfactant producers

Screening for bio-surfactant production was carried out by qualitative methods viz., Oil displacement test, Blood hemolysis test and Drop collapse test using the supernatant obtained by centrifuging pre-grown culture (in NB for 48 h at 30 °C) medium at 8000 rpm for 20 min.

In oil displacement test, 15 µl of crude oil was added on the surface of 40 µl of distilled water in a petri-dish to form a thin oil layer. To this layer, 10 µl of culture supernatant was gently placed at the center. The surfactant activity which correlates to the diameter of the clearing zone on the oil surface was visualized under visible light and recorded after 30 sec [19].

The hemolytic activities of the strains were tested by plating it onto Superimposed Blood Agar (SIBA) and incubating it at 37 °C for 24-48 h [20]. Hemolysis was observed as a zone of clearance around isolated colonies after incubation.

Drop-collapse test was performed in a micro-titer well plate. In this method, 20 µl of culture supernatant was added to 7 µl mineral oil that was left in the micro-titer well for 24 h at 30 °C and the extent of collapse in the drops was examined visually after 1min [21].

2.3. Identification of the bio-surfactant producing organism

Preliminary identification of the bio-surfactant producers was carried out on the basis of morphological, cultural and biochemical tests in our laboratory, as described in the Bergey's Manual of Determinative Bacteriology, 8th edition [22]. Molecular confirmation of the strain was done by 16s rRNA sequence analysis, which was carried out at Yaazh Xenomics Navi, Mumbai, India.

2.4. Screening of media for the bio-surfactant production

Three different media were used for screening of bio-surfactants in our study. The media and its components (in g/L) are listed below.

1. *MSM medium [Glucose (20); Na2HPO4 (2.2); KH2PO4 (1.4); MgSO4.7H2O (0.6); FeSO4. 7 H2O (0.01); NaCl (0.05); CaCl2 (0.02); Yeast extract (0.02); KI (0.66); ZnSO4. 7H2O (2.32); MnSO4.4H2O (1.78); CuSO4.5H2O (1.0); CoCl2.6H2O (0.42); H3BO3 (0.56); Na2MoO4 (0.39); EDTA (1.0)] [23].

2. *SM medium [Glucose (20); NH4NO3 (3.3); K2HPO4 (2.2); KH2PO4 (0.14); MgSO4.7H2O (0.6); FeSO4. 7 H2O (0.2); NaCl (0.01); CaCl2 (0.04); Yeast extract (0.1); KI (0.66); ZnSO4. 7H2O (2.32); MnSO4.4H2O (1.78); CuSO4.5H2O (1.0); CoCl2.6H2O (0.42); H3BO3 (0.56); Na2MoO4 (0.39); EDTA (1.0)] [23].

3. Kay's medium [$NH_4H_2PO_4$ (2.2); K_2HPO_4 (1.4); $MgSO_4.7H_2O$ (0.6); $FeSO_4$. 7 H_2O (0.01); NaCl (0.05); CaCl2 (0.02); Yeast extract (0.02)] [24].

*The MSM and SM medium used in our study have the same composition with a slight change in concentration of individual components.

The screening and selection of the optimum medium for maximum bio-surfactant production were done on the basis of microbial growth, and maximum surface tension reduction observed.

2.5. Optimization of culture condition for maximum bio-surfactant production

The media optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed at a specific set of conditions. These varying factors included pH (6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0), temperatures (30 °C, 37 °C, 40 °C and 55 °C), incubation period (24, 48, 72, 96, 120, and 144 h), NaCl concentrations (0.5%-3%), carbon sources used as an inducer (Glucose, Glycerol, Sunflower oil, Coconut oil, Palm oil, Soyabean oil) and nitrogen sources (Peptone, Yeast extract, Urea, Ammonium chloride, Ammonium nitrate, Sodium nitrate). In addition, the optimization of C:N ratio was carried out (10:1, 15:1, 20:1, 25:1, 30:1, 35:1 and 40:1) with coconut oil and Ammonium nitrate (i.e., optimized carbon and nitrogen sources) for achieving maximum bio-surfactant production [21, 25, 26]. All the fermentations were carried out in 250ml Erlenmeyer flasks with 50 ml media and 2%v/v inoculum adjusted to 1.0 O.D at 530 nm and all incubations were carried out under shaker (150 rpm) condition. The enriched broth obtained by growing the isolate under test conditions were subjected to centrifugation at 8000 rpm for 20 min and the cell-free supernatant was tested for surface tension reducing ability and emulsification activity as indicated below.

2.5.1. Surface tension reduction

The surface tension of the aqueous solution was measured by using Du Nouy ring-type tensiometer and was carried out after dipping the platinum ring in a solution for a while in order to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. For calibration of the instrument, the surface tension of the double distilled water was measured, before each set of experiments, which lies between 70 mN/m to72 mN/m [23].

2.5.2. Activity characterization by determining emulsification index

The ability of the bio-surfactant to emulsify some liquid hydrocarbons as substrates, such as diesel oil, kerosene, and sunflower oil was determined. The crude bio-surfactant dissolved in 2 ml distilled water was added to each test tube containing the substrate (2 ml). The content of the tubes was vortexed at high speed for 2 min and left undisturbed for 24 h. The emulsion index (E24) was determined by using the formula

$$\text{Emulsion index} = \frac{a}{b} \times 1000$$

Where "a" is the total height of the emulsion layer and "b" is the total height of the liquid layer [23].

2.6. Extraction of the bio-surfactant

For extraction of bio-surfactant from the test isolate, it was first allowed to grow in 100 ml MSM (pH 8.5) for 4 days at 30 °C. The cell-free supernatant obtained by centrifugation of the medium at 8,000 rpm for 20 min was adjusted to pH 2.0 using 6 N HCl and kept at 4 °C overnight in order to precipitate the bio-surfactant. The precipitate was recovered by centrifugation at 10,000 rpm for 20 min. This crude bio-surfactant was dried and further purified by dissolving it in distilled water (pH 7). The bio-surfactant thus obtained was dried at 60 °C and extracted with Chloroform: Methanol (1:2) solution; filtered and concentrated using a rotary vacuum evaporator [27].

2.7. Characterization of bio-surfactant by Fourier transform infrared spectroscopy

The characterization of bio-surfactant obtained in our study was carried out by Fourier transform infrared spectroscopy (FTIR) analysis using Agilent technology Cary 630 instrument. The Spectral scan was normally acquired over the range of 400-4000 cm^{-1} [28].

2.8. Statistical analysis

The experimental data are represented in terms of the arithmetic average of at least three replicates, and the standard deviations are indicated by error bars. The data analysis was done using Microsoft Excel 2007.

3. Results

3.1. Isolation, primary screening, and identification of bio-surfactant producers

Amongst the six isolates obtained from the enriched medium, isolate 3 appeared to be the most efficient bio-surfactant producer as observed in Table 1. It was identified as Azorhizobium strain on the basis of morphological cultural, biochemical and 16s rRNA analysis.

Table 1 Qualitative screening of Bio-surfactant producers

Test isolates	A*	B*	C*
1	+++	+	+
2	++	++	+
3	+++	+++	+++
4	+++	+++	++
5	+	++	++
6	+++	++	++

*A (Blood hemolysis test), +: incomplete hemolysis (D < 1 cm); ++: complete hemolysis (D= 4-8 cm); +++: complete hemolysis (D= 9-13 cm).
*B (Oil displacement test), +: D< 30 mm; ++: D=30-50 mm; +++: D≥50 mm.
*C (For Drop collapse test), +: slightly collapsed; ++: partially collapsed; +++: fully collapsed.

While screening the most efficient bio-surfactant producer, it is necessary to consider more than one qualitative test, keeping in mind the limitations it may hold. For instance, in our study, hemolysis test cannot be relied upon since lytic enzymes can also lead to clearing zones [29]. However, a concurrent indication of positive results by multiple tests increases the reliability of observations. The drop collapse test is more conclusive comparatively [17]. It relies on the destabilization of liquid droplets by surfactants. In presence of surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop, and the hydrophobic surface is reduced. However, in absence of surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension [17, 29].

3.2. Screening of media for the bio-surfactant production

Figure 1 represents the three different media used for screening of bio-surfactant production in our study. Maximum reduction of surface tension i.e., 33 dynes/cm was observed in the MSM medium and hence it was used as a production media for further optimization studies. A recent investigation carried out with *Acinetobacter junii* B6 also reported optimum bio-surfactant production in MSM medium supplemented with 1% Iranian light crude oil [30].

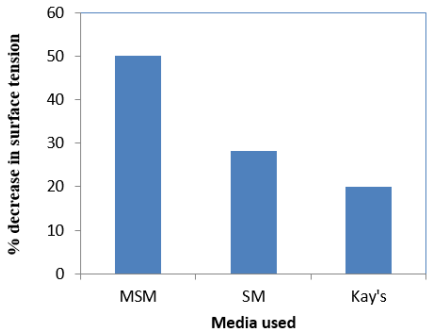

Figure 1 Screening of media for bio-surfactant production

3.3. Optimization of bio-surfactant production

Optimization of physicochemical factors is essential for any biological process since they have direct effects on cellular growth and activity. Figures 2-8 represent the optimum parameters required for bio-surfactant production. In the current study, the optimum bio-surfactant production was obtained using 2% inoculum size of Azorhizobium species (1.0 O.D at 530nm) in MSM medium (pH 8.5) containing 2% coconut oil and ammonium nitrate with a C: N ratio of 20:1, and incubation conditions of 30˚C for 96 h.

A reduction in surface tension by 51% and emulsification activity of 42% was observed at pH 8.5 in our study (Figure 2). The gradual increase observed in the bio-surfactant production with an increase in the pH of the culture medium is due to the dependence of enzymatic processes on the same [27]. In a similar study, *P. aeruginosa* RS29 also required an optimum pH between 8 and 9 for bio-surfactant production [31]. Another study indicated a significant bio-surfactant production by *Bacillus brevis* in the pH range of 4-9 [32]. The rhamnolipids produced by *P. aeruginosa* was found to be tolerant to variations in pH (6-12) hence making it a good candidate for industrial use [33].

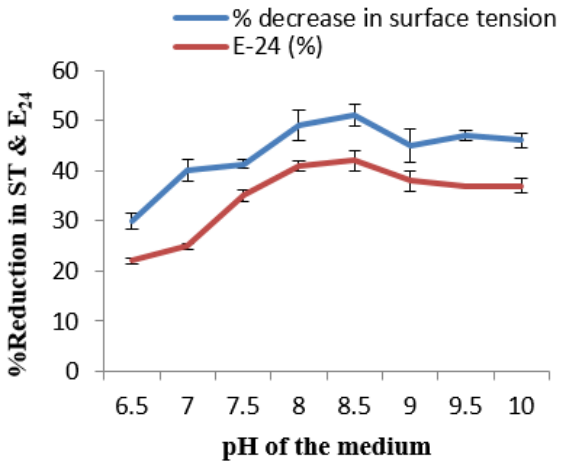

Figure 2 Optimization of pH for production of bio-surfactant by *Azorhizobium* strain

The optimum temperature for bio-surfactant production was observed to be 30 °C by Azorhizobium strain in our study. It showed surface tension reduction of the media by 50% and an emulsification activity of 40% (Figure 3). Similar results were obtained by Padmapriya et al. who reported optimum bio-surfactants production at 40 °C by Candida species [34].

Thermally stable bio-surfactant production was observed by *Bacillus brevis* in the temperature range of 30 -80 °C [32]. Bio-surfactant production has also been reported at low temperatures i. e 20 °C by Aspergillus species [35].

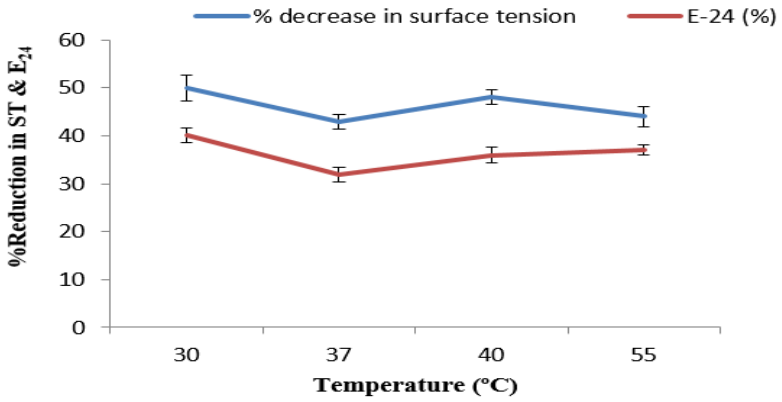

Figure 3 Optimization of temperature for production of bio-surfactant by *Azorhizobium* strain

The production of bio-surfactant increased considerably after incubation of 96 h and was found to be stable up to 144 h in our study (Figure 4). The surface tension reduction and emulsification activity were found to be 49% and 50.3% respectively. Initial lag in bio-surfactant production is due to the adaptation of culture to the nutrient media. In certain cases, an increased surface tension is reported in the medium after crossing the optimum temperature range. This is due to the poor bio-surfactant activity observed at critical micelle concentrations. At these concentrations, the surfactant monomers associate into structured aggregates such as micelles, vesicles, and lamellae spontaneously due to weak chemical interactions [36]. Maximum bio-surfactant production has been reported after 7 and 10 days by Brevibacterium species and *Bacillus brevis* respectively [19, 32].

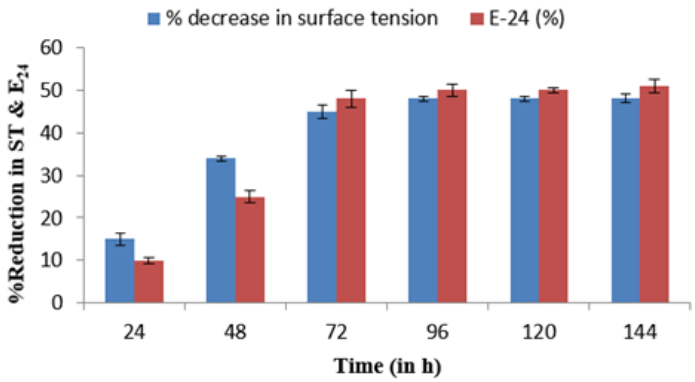

Figure 4 Optimization of the incubation period for production of bio-surfactant by Azorhizobium strain

Among the different carbon and nitrogen sources studied for bio-surfactant production, coconut oil and ammonium nitrate showed maximum surface tension reduction (55%, 53%) and emulsification activities (60.2%, 58%) respectively (Figures 5 and 6). A recent study showed that kitchen waste oil can be used as raw material for bio-surfactant production by *P. aeruginosa* over other carbon sources. They further reported a higher optical density of 2.33 and lower interfacial tension of 0.57 mN/m. [33]. Another study has previously reported that Pseudomonas species is capable of utilizing different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins, and vegetable oils to produce rhamnolipid-type bio-surfactants [37]. Other studies have reported ammonium nitrate [38], sodium nitrate [37, 39] and corn steep liquor as an optimum nitrogen source for bio-surfactant production by *Pseudomonas aeruginosa* [37]. A 15% whey concentration is also shown to positively influence the bio-surfactant production (8.9 mg/L) and reduce surface tension by about 18.1 mN/m [40].

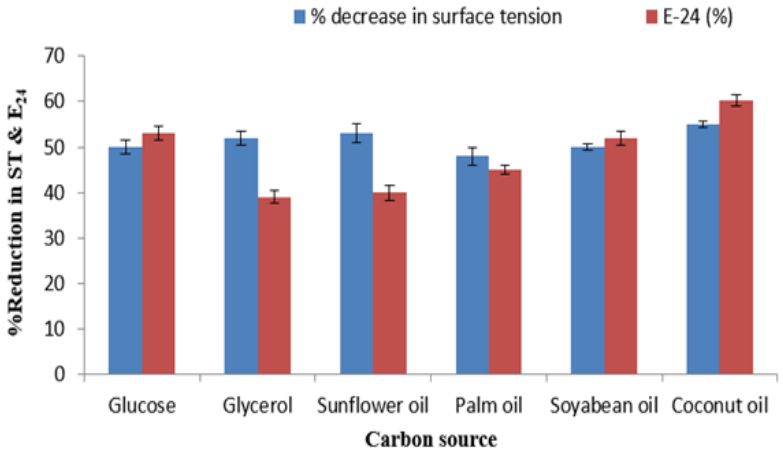

Figure 5 Optimization of carbon source for production of bio-surfactant by *Azorhizobium* strain

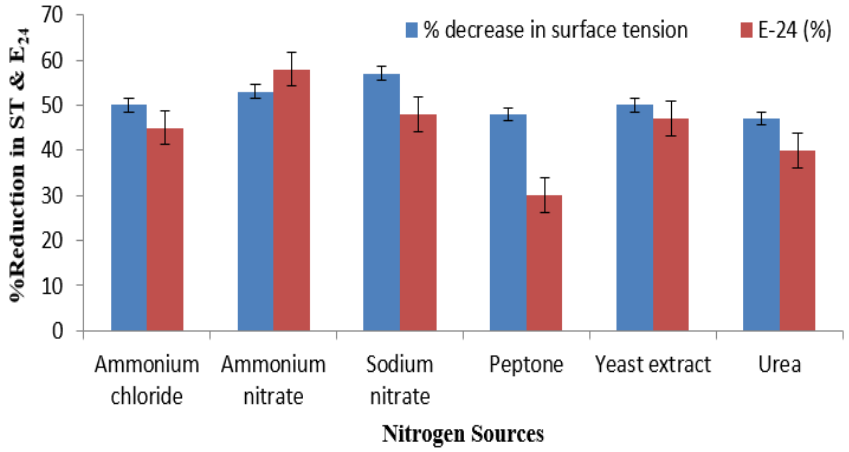

Figure 6 Optimization of nitrogen source for production of bio-surfactant by *Azorhizobium* strain

In order to obtain high concentrations of bio-surfactant, it is necessary to maintain the carbon to nitrogen ratio. The optimum C: N ratio suitable for bio-surfactant production was found out to be 20:1 in our study (Figure 7). It supported a reduction in surface tension by 55.3% and showed emulsification activity value of 52.26%. The concentration of nitrogen is reported to be a rate-determining factor during the biosynthesis of rhamnolipid and nitrogen limitation may promote lipid accumulation [41]. In contrast to this, Guerra-Santos et al. showed maximum rhamnolipid production after nitrogen limitation at a C: N ratio of 16:1 to 18:1 and no surfactant production below a C: N ratio of 11:1 [42]. Abouseoud et al. reported the use of frying oil and sodium nitrate as carbon and nitrogen source respectively at C: N ratio of 10:1 for effective production of bio-surfactants [43].

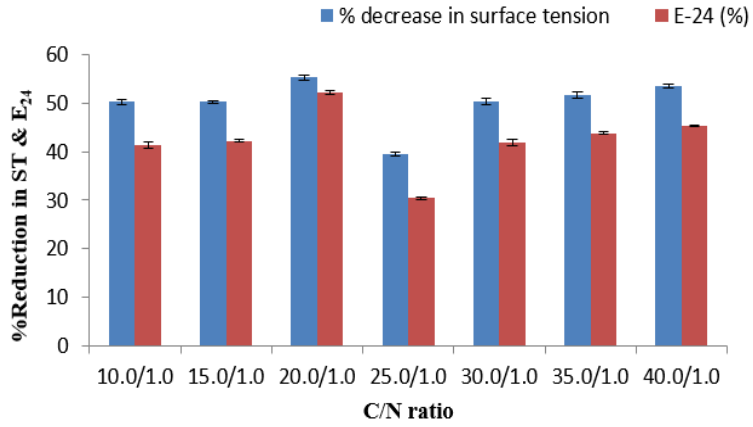

Figure 7 Optimization of carbon: nitrogen ratio for production of bio-surfactant by *Azorhizobium* strain

The optimum salt concentration was observed to be 1.5% in our study with 50% reduction in surface tension and emulsification activity of 36.4% (Figure 8). Kiran et al. found that the bio-surfactant produced by the marine *Brevibacterium aureum* MSA13 was stable at high NaCl (up to 5%) [19]. A high degree of tolerance was also observed by rhamnolipid producing *P. aeruginosa* against the salinity levels (2–20% w/v) [33].

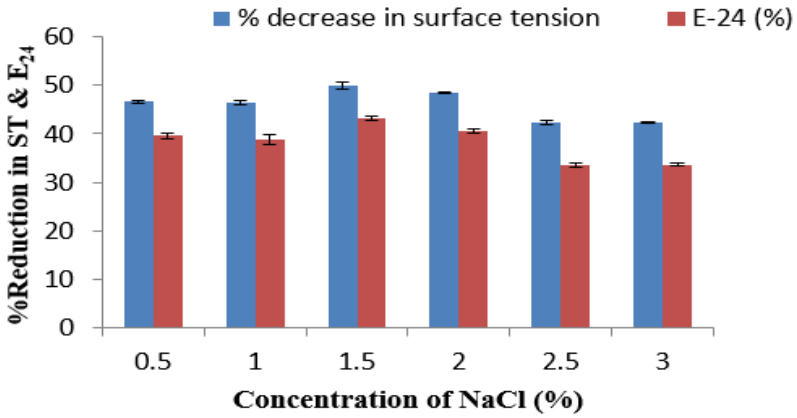

Figure 8 Optimization of NaCl concentration for production of bio-surfactant by *Azorhizobium* strain

3.4. Extraction

The extraction of bio-surfactant using optimized culture conditions was carried out using acid hydrolysis method and a dry yield of 2.5 g/L was obtained. In addition to significant reduction in surface tension (i.e., from 59 mN/m to 38 mN/m), Azorhizobium species was checked for its ability to emulsify crude oils viz., Diesel, kerosene, sunflower and coconut oil. Our study indicated diesel oil and kerosene to be the best substrates for emulsification activity that was calculated as 60% and 55% respectively. A significant emulsification activity was also observed with sunflower oil (50%) and coconut oil (48%). Similar to our study, a 55% emulsification activity against diesel was observed by the test culture [23]. In another study, the highest emulsification activity of the bio-surfactant for diesel oil (55%) was produced from isolate B5 using corn oil [44]. Lima et al. reported production of 2.2 g/L of rhamnose when ammonium nitrate (0.56%) was used as the nitrogen source for *P. aeruginosa* cultivated in 2.2% residual soybean oil [45]. Another study reported olive oil as the best carbon source for the production of bio-surfactants by *Pseudomonas fluorescence* when compared to hexadecane and glucose. In their study, fermentation of olive oil reduced the surface tension of the media to 38 dyne/cm and showed an emulsification activity of 49%. However, in presence of hexadecane as a carbon source, only 10% emulsification activity was observed with a significant reduction in surface tension, and no bio-surfactant production was observed in presence of glucose [43].

3.5. Fourier transform infrared spectroscopy analysis

Figure 9 represents the FTIR spectrum of purified bio-surfactant obtained in our study. In this figure, we can observe the absorbance of C-H stretching bond at 3004 cm^{-1}, aliphatic chain-CH at 2824 cm^{-1}, C=O(ketones) at 1710 cm^{-1}, carboxylates at 1420 cm^{-1} and peptide bond at 1092 cm^{-1}. The absorbance bands at the wavenumbers of 3004-2824 cm^{-1}, 1710 cm^{-1}, 1200-1500 cm^{-1} and 900-1200 cm^{-1} in FTIR spectrum clearly indicate the presence of fatty acids, amides, mixed components, and lipopolysaccharides respectively. The relative position of axial and equatorial (OH) side groups influence the main band frequency positions at 1092 cm^{-1} and the maximal assigned to ring and side group vibrations can be related to the peptide spectra. The characterization of bio-surfactant revealed its chemical nature to be of lipopolysaccharides type. The molecular composition of the bio-surfactant determined by FTIR and nuclear magnetic resonance confirmed the presence of carbohydrates, lipids and proteins, defining this molecule as a glyco-lipopeptide [40].

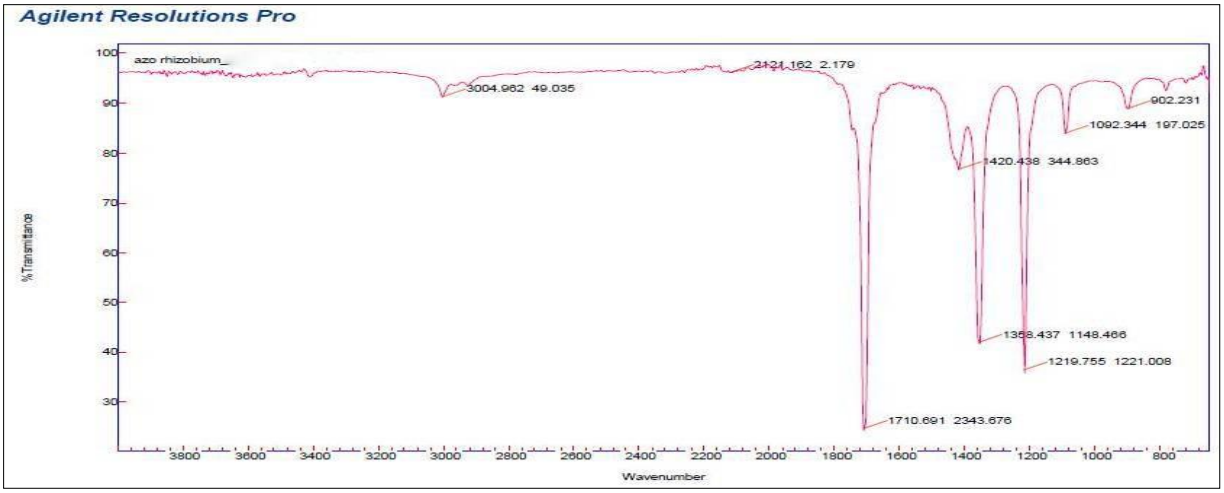

Figure 9 FTIR spectrum of purified biosurfactant

4. Conclusion

A large number of studies carried out for use of microbial bio-surfactants highlight its extensive potential in industries. In this regard, our study provides challenging outputs with high yielding (2.5 g/L) strain of Azorhizobium species. With further optimization studies and molecular insights, it can help in overcoming problems associated with oil recovery and bioremediation of oil-contaminated sites.

Compliance with ethical standards

Disclosure of conflict of interest

All authors declare that they have no conflict of interest.

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