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Influence of Vegetable Oils on Biosurfactant Production by *Serratia* species Isolated From Soil

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KEYWORDS

Biosurfactant, *Serratia* sp, Vegetable oils, Emulsification

A B S T R A C T

Biosurfactants are the surface active molecules synthesized by microorganisms. The present study was focused on production of biosurfactant using various vegetable oils using *Serratia* sp. isolated from soil. The isolated bacterial strain was screened for biosurfactant production by oil spreading method, drop collapse test and emulsification test. Different vegetable oils at various concentrations were used individually as sole carbon source for the production of biosurfactant. The best carbon source achieved was gingely oil at a concentration of 1.0ml/100ml which was measured by dry weight of biosurfactant (0.44g/100ml). The obtained biosurfactant from gingely oil medium had a good emulsification activity with kerosene and also had higher activity with toluene.

Introduction

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively. Biosurfactants possess the characteristic property of reducing the surface and interfacial tension using the same mechanisms as chemical surfactants (Praveesh *et al.*, 2011). Biosurfactants are a structurally diverse group of surface active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, which makes them potential candidates for enhancing oil recovery and deemulsification

processes (Pruthi *et al.*, 2000). They are synthesized by microbial strains of different genera, especially when they grow in the presence of low water soluble substrates like petroleum hydrocarbons and waste oils (Moreno *et al.*, 2011). Most biosurrfactants are classified in to glycolipids, lipopeptide, phospholipids, fatty acids, neutral lipids etc., (Muthusamy *et al.*, 2008). Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH, and salinity and the ability to be synthesized from renewable feedstocks (Pruthi *et al.*, 2000).

During the past few years, biosurfactant production by various microorganisms has been studied extensively. Also various aspects of biosurfactants, such as their biomedical and therapeutic properties, natural role, production on cheap alternative substrates and commercial potential have been recently reviewed.

Microorganisms are able to grow on vegetable oils or fats and produce new products with potential industrial application such as lipase, biodiesel and used olive or sunflower cooking oil as carbon source for biosurfactant production (Soniyambi *et al.*, 2011). Various studies with plant-derived oils have shown that they are effective and cheap raw materials for biosurfactant production; for example, rapeseed oil, Babassu oil and corn oil. Similarly vegetable oils such as sunflower and soybean oil were used for the production of rhamnolipid, sophorolipid and mannosylerythritol lipid biosurfactants by various microorganisms Apart from various vegetable oils, oil wastes from vegetable-oil refineries and the food industry were also reported as good substrates for biosurfactant production.

The use of low-cost agro-industrial waste for alternative nutrients substrates would significantly improve the economic problem of biosurfactant production. The main challenge using such waste is obtaining substrates with the proper balance of nutrients to support cell growth, as well as suitable and consistent product yields. The availability of agro-industrial wastes is usually locally confined and access difficulty to these wastes at large enough quantities is a handicap for large-scale production of biosurfactants. Additionally, agro-industrial wastes have variable components so the actual concern is sustainability of same wastes with same ingredients for production (Kaskatepe and Yildiz, 2016).

Furthermore, various waste oils with their origins at the domestic level, in vegetable-oil refineries or soap industries were found to be suitable for microbial growth and biosurfactant production In this sense, the present work was aimed to evaluvate the suitable vegetable oil carbon source for biosurfactant production using *Serrtia* sp.

Materials and Methods

Microorganism

The microbial strain used in this study is *Serratia* sp. was isolated from soil and maintained in nutrient agar slants at a refrigeration temperature of 4°C.

Screening of biosurfactant producing organisms (Karthik *et al.*, 2010)

The isolated bacterial strain was screened for biosurfactant production by three methods,

Oil spreading method
Drop collapsing test
Emulsification test

Oil spreading method

50ml distilled water was taken in petriplates. 20µl of oil was added in th e centre of the petriplate containing distilled water. Then, 10µl of culture supernatant was added to the centre of the oil and observed for displacement of oil. The culture supernatant which displaces the oil was indicated as positive.

Drop collapsing test

Drop collapse test was carried out using 96 well microtitre plate containing 2µl of mineral oil which was equilibrated for an hour at room temperature. 5µl of culture supernatant was added to the surface of the oil in the well. The drop shape on the oil surface was observed after 1 minute. The culture supernatant that collapsed the oil drop was indicated as positive.

Emulsification test

Sterile screw capped tube was taken, to which 2ml of kerosene and 1ml of culture broth was added. The tube was vortexed for 2 minutes and allowed to stand for 24 hours. After incubation period, the height of the emulsion is measured as follows,

$$\text{Emulsification Activity} = \frac{\text{Height of emulsion layer}}{\text{Total height of mixture}} \times 100$$

Production of biosurfactant (Anyanwu *et al.*, 2010)

The screened isolates were further used for production of biosurfactant. The fresh overnight culture was used as an inoculum for production of biosurfactant. To the production medium different vegetable oils such as olive oil, sunflower oil, gingely oil and coconut oil were added at various concentration of about 0.5ml, 1.0ml, as carbon sources.

The production medium was prepared and 100ml was distributed in each of 250ml conical flasks aseptically and 5% of inoculum was transferred into the production medium. The pH of the production medium was adjusted to 7.0 and incubated at 25°C for 3 days in a rotary shaker at 150 rpm. All experiments were carried out in triplicates

Extraction of biosurtactant (Anandraj *et al.*, 2010)

The obtained biosurfactant was extracted from the production medium. After incubation period, the culture broth was centrifuged at 5000 rpm for 20 minutes at 4°C. The supernatant was adjusted to pH 2.0 using 1M sulphuric acid. Then 2ml of chloroform and 1ml of methanol was added and shaken well for proper mixing and left overnight for evaporation and observed for the formation of precipitate using rotary evaporator.

Determination of dry weight of biosurfactant (Anandraj *et al.*, 2010)

The dry weight of biosurfactant precipitate was estimated. A sterile petriplate was taken and the weight of the plate was measured. The obtained precipitate was poured on the petriplates.

The plates were kept in the hot air oven for drying at 100°C for 30 minutes. After drying of the precipitate, the plates were weighed. The dry weight of the biosurfactant was measured as follows,

Dry weight of biosurfactant = weight of plate after drying – weight of the empty plate.

Assay oy emulsifying activity (Anyanwu *et al.*, 2010)

The obtained biosurfactant was assayed for its emulsification activity. The emulsification activity was done using various hydrocarbon oils such as kerosene, xylene, toluene and benzene. Sterile screw capped tube was taken, in which 3ml of different hydrocarbon oil was added to each tubes. Then, 2ml of the biosurfactant solution was added to the tubes and vortexed for 2 minutes. The emulsion layer was measured as follows,

$$\text{Emulsification Activity} = \frac{\text{Height of emulsion layer}}{\text{Total height of mixture}} \times 100$$

Statistical Analysis

All analyses were performed in triplicates and expressed as mean ± standard deviation (SD). Stastical significance was achieved by Analysis of variance (ANOVA)

Results and Discussion

In the spread plates from serial dilution tubes the colonies were enumerated and listed in Table.1, the number of colonies enumerated in the plates was 261. 5 $\times 10^{-3}$ CFU/ml. Three different colonies were identified morphologicaly and the results were compared with Bergey's manual, it showed that the identified organism was *Serratia* sp.(Plate 1)

Screening of biosurfactant producing organisms

Oil spreading method

The bacterial isolates were screened for its biosurfactant production. The isolates S1, S2 and S3 showed the zone of displacement in oil. Among the three isolates, S3 showed maximum zone of displacement in oil, choosen further for biosurfactant production under soild state fermentation. (Table.1).

Drop collapsing test

The bacterial isolates were screened for drop collapsing test. All the three isolates collapsed the coated oil in the microtitre plate. (Plate 2)

Emulsification test

The emulsification stability (E24) is another characteristic of biosurfactants and it was evaluated against the kerosene oil. The highest E24 value was observed in cultures S3 (52%) comparing to S1 and S2 48.75% and 41.25%, respectively (Table.2)

Production and extraction of biosurfactant

The isolate S3 inoculated in the production medium with various vegetable oil was centrifuged, the supernatant was mixed with chloroform: methanol. The white precipitate was obtained while placed in rotor.(Plate 3)

Determination of dry weight of biosurfactants

The dry weight of the biosurfactant was measured and estimated. The production medium supplemented with 1.0ml of gingely oil was found to give maximum yield of biosurfactant (0.44±1.8g/ml) (Table). Sunflower oil medium also gave a maximum yield (0.36±1.6g/ml)

An moderate yield was obtained in the medium containing 1.0ml of coconut oil carbon source (0.26±1.2g/ml), whereas the lowest yield was obtained in olive oil medium (0.22±2.6g/ml) (FIG 1)

Assay of emulsifying activity of biosurfactant in 0.5ml of carbon sources (%) (Plate 4)

Emulsification activity of the biosurfactant with kerosene

The highest emulsification activity was obtained with kerosene (64 ±0.2%) when using gingely oil as carbon source. Furthermore the maximum emulsification activity of the biosurfactant from the medium supplemented with sunflower oil was also obtained (62 ± 0.8%).

It was also observed that, the obtained biosurfactant from coconut oil carbon source gave a good emulsification activity (58 ± 2.2%). Olive oil substrate gave a minimum emulsification activity with kerosene (46 ±2.7%).

Emulsification activity of the biosurfactant with toluene

Emulsification capacity of the biosurfactant obtained from the medium supplemented with gingely oil was found to be maximum with toluene (62 ± 0.8%).

Biosurfactant from sunflower oil medium showed good emulsification activity than coconut (52 ± 2.2%) and olive oil (40 ±2.8%).

Emulsification activity of the biosurfactant with benzene

Benzene showed maximum emulsification activity with biosurfactant from the medium supplemented with gingely oil (56 ±2.2%). Biosurfactant from sunflower oil medium also showed a good emulsification activity with benzene (54±0.8%) compare to the biosurfactant from coconut oil (50 ±2.2%) and olive oil medium (43 ± 0.2%).

Emulsification activity of the biosurfactant with xylene

Biosurfactant from the medium supplemented with gingely oil showed the maximum emulsification activity (54 ±0.2%) than the other carbon sources. It was observed that sunflower oil medium also showed good emulsifying activity(50 ± 2.8%) whereas minimum activity was seen in biosurfactant from coconut oil (44 ± 2.9%) and olive oil medium(38 ± 2.9%).

Assay of emulsifying activity of biosurfactant in 1.0ml of carbo sources (%) (Fig 2)

Emulsification activity of the biosurfactant with kerosene

Emulsification activity of the biosurfactant with kerosene showed maximum activity (95 ± 0.8%). It was found that the relative activity was seen with biosurfactant obtained from sunflower oil medium (88 ± 2.2%). Similarly the emulsification capacity of biosurfactant from coconut oil (76 ± 2.2%) and olive oil medium (68 ± 0.0%) was also found to be maximum and effective.

Emulsification activity of the biosurfactant with toluene

An effective emulsification activity was seen in biosurfactant solution obtained from gingely oil (86 ± 2.4%). The emulsifying activity of the produced biosurfactant from sunflower oil medium was also maximum with toluene oil (76 ± 2.2%).

The moderate emulsification activity was seen in biosurfactant obtained from medium supplemented with coconut oil (62 ± 2.2%). Minimum emulsification activity was observed with biosurfactant obtained from

medium incorporated with olive oil (68 ± 0.0%).

Emulsification activity of the biosurfactant with benzene

It was observed that the maximum emulsifying activity was seen with biosurfactant obtained from medium supplemented with gingely oil. (76 ± 2.0%). Similarly the emulsification of the biosurfactant from sunflower oil carbon source was also found to be highest (68 ± 0.2%). The moderate emulsification was seen in biosurfactant from coconut oil (62 ± 2.2%) and the minimum activity was seen in olive oil medium (56 ± 2.4%).

Emulsification activity of the biosurfactant with xylene

Xylene showed its maximum emulsification capacity with biosurfactant obtained from medium supplemented with gingely oil (84 ± 2.4%). In relation, biosurfactant from sunflower oil carbon source also showed good emulsification activity (78 ± 0.2%). A moderate emulsifying activity was seen in biosurfactant from coconut oil medium (68 ± 1.6%) and the minimum activity was observed in biosurfactant solution form olive oil carbon source (62 ± 1.8%).

In the last few decades, tremendous interest has swelled in the production of biosurfactant using different microorganisms. Recently more attention has been focussed on the biosurfactant production since they occupy an important position with respect to their application in environmental and commercial field. Microorganisms represent an excellent source of biosurfactant owing to their broad biochemical diversity and susceptibility to genetic manipulation. Further microbial surfactants possess almost all characteristics and thus, the microbes serve as preferred source of surface active agent because of their rapid growth, limited space required for their cultivation and the ease with which they can be genetically manipulated to generate a new biosurfactant with altered properties that are desirable for various applications.

Table.1 Screening of biosurfactant production by oil spreading method

S No	CULTURE SAMPLE	ZONE FORMATION 9mm)
1	S1	5 ± 0.2
2	S2	4 ±2.2
3	S3	6 ± 1.6

Note: S1, S2, S3 : *Serratia* sp., mean values of triplicates.

Table.2 Screening of biosurfactant production by emulsification test

S No	CULTURE SAMPLE	EMULSIFICATION INDEX (%)
1	S1	48± 2.2
2	S2	41 ± 1.6
3	S3	52 ± 2.6

Note: S1, S2, S3 : *Serratia* sp., mean values of triplicates

Table.3 Emulsifying activity of biosurfactant in 0.5ml of carbon sources (%)

S No	CARBON SOURCES	KEROSENE	TOULENE	BENZENE	XYLENE
1	SUNFLOWER OIL	60 ± 2.5	58 ± 2.5	54 ± 0.8	50 ± 2.8
2	OLIVE OIL	46 ± 2.7	40 ± 2.8	43 ± 2.0	38 ± 2.9
3	COCONUT OIL	58 ± 2.2	52 ± 2.2	50 ± 2.2	44 ± 2.9
4	GINGELY OIL	64 ± 0.2	62 ± 0.8	56 ± 2.2	54 ± 0.2

Note : mean values of triplicates.

Table.4 Emulsifying activity of biosurfactant in 1.0ml of carbon sorces (%)

S No	CARBON SOURCES	KEROSENE	TOLUENE	BENZENE	XYLENE
1	SUNFLOWER OIL	88 ± 2.2	76 ± 2.2	68 ± 0.2	78 ± 0.2
2	OLIVE OIL	68 ± 0.0	68 ± 0.0	56 ± 2.4	62 ± 1.8
3	COCONUT OIL	76 ± 2.2	74 ± 2.4	62 ± 2.2	68 ± 1.6
4	GINGELY OIL	95 ± 0.8	86 ± 2.4	76 ± 2.0	84 ± 2.4

Note: mean values of triplicate

Fig.1 Estimation of dry weight of biosurfactant

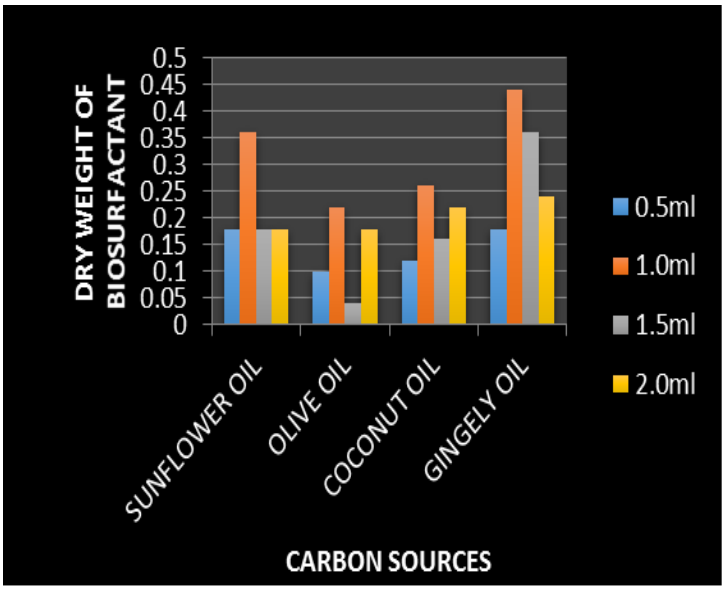


Fig.2 Assay of emulsifying activity of biosurfactant in 0.5ml of carbon sources (%) 1 ml of carbon sources (%)

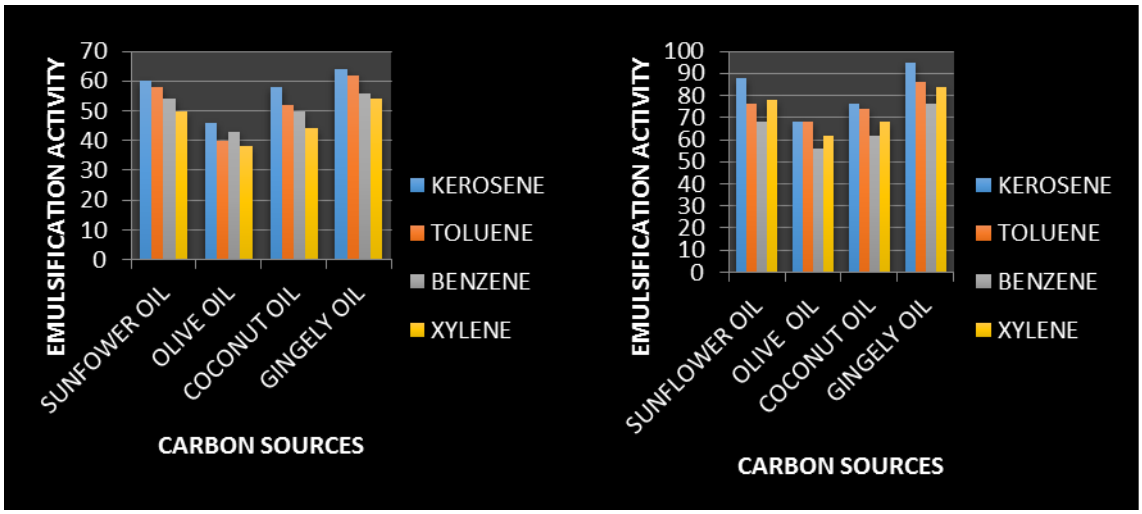


Plate.1 *Serratia* Species

Plate.2 Drop collapse test on nutrient agar

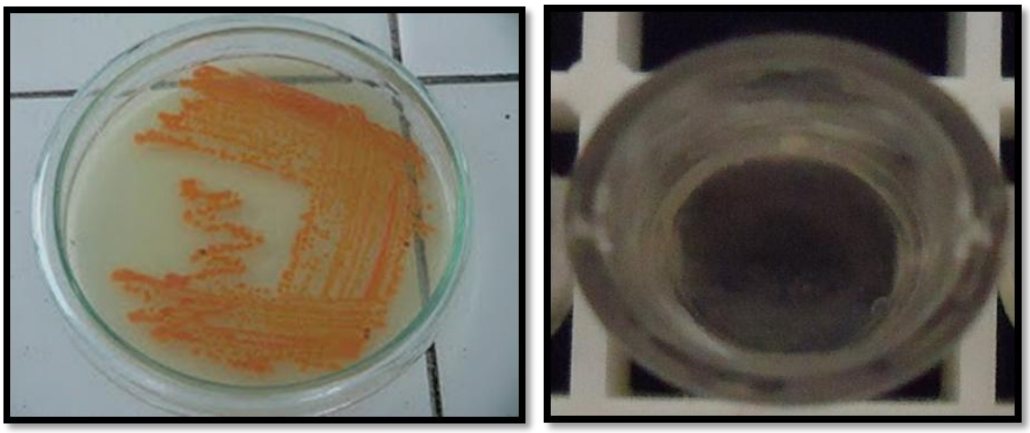
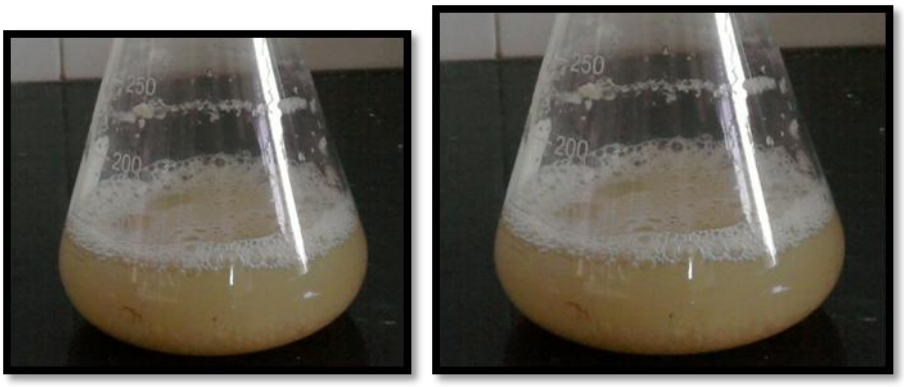


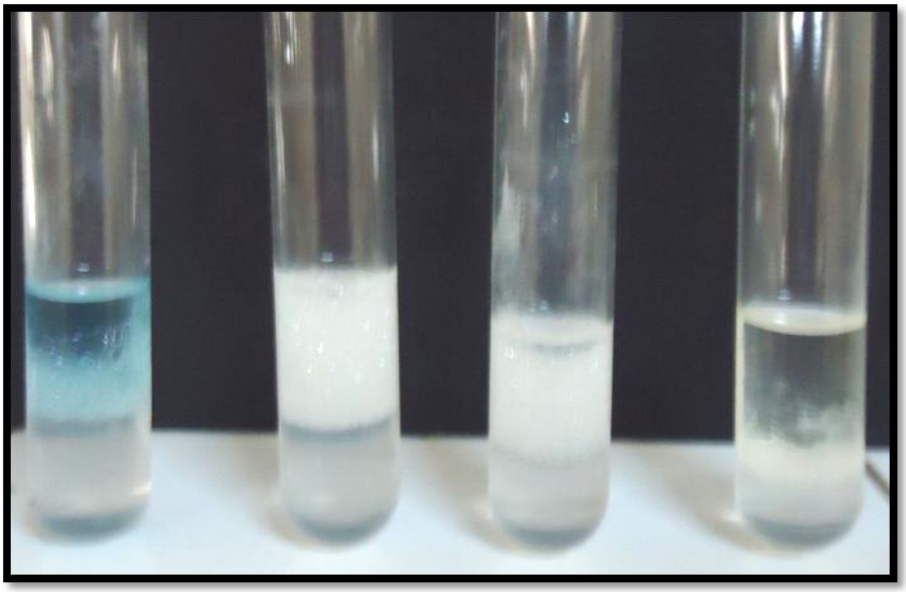
Plate.3 Production of biosurfactant

Control **Gingelly oil** **Olive oil**



Sunflower oil **Coconut oil**

Plate.4 Emulsification test with various hydrocarbon oils



KEROSENE **TOLUENE** **XYLENE** **BENZENE**

The present investigation highlights the capacity of the vegetable oils like sunflower oil oil, olive oil, coconut oil and gingely oil as carbon and energy source for production biosurfactant using *Serratia* species isolated from soil.

The vegetable oil was used in different concentrations like 0.5ml and 1.0ml. The aim of this study is to detect the excellent substrate for biosurfactant production and their emulsification activity with hydrocarbon oils. Similar studies was also done by Soniamby; Cipinyte *et al.*, 2011.

Three bacterial strains were isolated from soil sample by serial dilution method. Based on the morphological characteristics and biochemical test described in Bergey's Manual of determinative bacteriology the isolates S1, S2 and S3 was identified as *Serratia* sp. These isolates were further screened for their ability to produce biosurfactant by three methods such as oil spreading method, drop collapsing method and emulsification test. Oil spreading method was followed by Karthik *et al.*, 2010 to detect the biosurfactant producing marine actinobacteria from marine soil samples.

The result of the present study also suggests that oil spreading method is good to quantify the biosurfactant. In the present study, isolate S3 displaces the oil to form a zone of 6 mm in diameter whereas the isolates S1 and S2 displaces the oil to form zone of 5mm and 4 mm in diameter. The oil spreading method was also followed by Cipinyte *et al.,* 2011.

Bodour *et al.,* 2003 suggested that Drop collapsing test is an excellent screening method for biosurfactant producers. The isolates S1, S2 and S3 were further tested for its ability to produce biosurfactant.

All the isolates were capable of collapsing the oil within 1minute in a microtitre plate. Thus the isolates S1, S2 and S3 was capable to destabilize the tension between oil-coated surface and culture droplet within a minute, followed by a total collapse of the oil surface and therefore, the drop-collapsing test found that these isolates could be the potential biosurfactant-producing bacteria and also correlates with the studies of Karthik *et al.,*2010.

The biosurfactant production was also detected by emulsification test. The isolate S3 showed the highest emulsification activity among the three isolates. The isolate S3 emulsified the kerosene oil of about 52.25% whereas isolates S1 and S2 showed emulsification activity of about 48.75% and 41.25%. The emulsification test was also studied by Karthik *et al.,* 2010 for the screening of biosurfactant producing marine actinobacteria. The emulsification test was also followed by Aparna *et al.,* 2011.

Thus, the culture S3 was choosen to be the most potential biosurfactant producers among the other two isolates due to their ability to displace the oil, collapsing the oil and high emulsification capacity.

The culture S3 was further used for biosurfactant production under solid state fermentation. In the present study, the biosurfactant production was studied by using various vegetable oils as carbon source in a different concentration and incubated in a rotary shaker at 150rpm for 3 days where the pH of the medium is 7.0. In early work, Ferraz *et al.,* 2002 studied the influence of vegetable oils on biosurfactant production by *Serratia marcescens.* In relation, many works have been done using vegetable oil as substrate followed by Sidnei *et al.,* 2010 who used different vegetable oil and glycerol for biosurfactant production by *Pseudomonas* strain. Moussa *et al.,* 2006 also carried biosurfactant production by *Nocardia amarae* with different commercial oils like castor oil, corn oil, olive oil, sunflower oil, sucrose and glycerin. Similar study was also done by Soniyamby *et al.,* 2011 where edible oil was used as carbon source for rhamnolipid production which gave a yield of about 7.6g/ L.

The biosurfactant production medium with different oil was extracted by centrifugation using chloroform and methanol extraction method. The most widely used technique is solvent extraction with a variety of solvents at several different ratios. The choice is dependent on cost and effectiveness. Solvents used for this purpose include chloroform-methanol mixture (2:1).

In my study the biosurfactant was extracted using chloroform and methanol and the mixture was left overnight to obtain the white coloured precipitate called as biosurfactant, by evaporating in a rotor. The early worker, Anandraj *et al.,* 2010 also used this method to extract biosurfactant produced by *Pseudomonas* sp. This extraction method was also followed by many workers like Anyanwu *et al.,* 2011 who studied lipopeptide production by

Serratia marcescens NSK -1 strain isolated from petroleum contaminated soil.

The extracted biosurfactant was estimated for its dry weight by drying the obtained precipitate in the hot air oven at 100°C for 30 minutes. This method was also carried out by Anandraj *et al.,* 2010 who studied biosurfactant production by *Pseudomonas* sp isolated from oil spilled area. In the present study the maximum biosurfactant produced were 0.44g/100ml in production medium containing 1.0ml of gingely oil as carbon source. The lowest yield of biosurfactant was observed in medium containing 0.5ml of coconut oil as carbon source where the dry weight was about 0.10g/ml.

The ability of biosurfactant to emulsify different hydrocarbon oils like kerosene, benzene, toluene and xylene was examined in the present study. The emulsification activity was greater with kerosene which showed 95% and toluene of about 86% in biosurfactant solution obtained from medium containing 1.0ml of gingely oil as substrate compared to the other oils in various concentrations, while benzene showed the least activity of about 76%. The overall highest emulsification activity with kerosene was examined in biosurfactant obtained from the medium containing gingely oil (1.0ml) followed by sunflower oil whereas least activity was seen in olive oil and the moderate activity was seen in coconut oil. Apart from kerosene the emulsification activity was higher in toluene. The emulsification study with different hydrocarbon oils and vegetable oils was also done by Kokare *et al.,* 2007 where toluene showed the highest activity when *Streptomyces* sp. S1was used for bioemulsifier production.

However, as many industries and research organizations concern to the environmental approach, they are currently attempted to find new ways of producing surfactants. There are two new strategic approaches that are taken into account in developing new surfactant, which are i) the impact of the surfactant to the environment and ii) the functionalities of the surface-active molecules. Synthetic surfactants exhibit a low rate of biodegradation and a high potential to aquatic toxicity. For these reasons, biosurfactants are seen to be the promising alternative for many purposes even though their performance could be slightly inferior or their prices are more expensive.

In the present study the biosurfactant production was higher when 1.0ml of gingely oil used as carbon source which gave a yield of about 0.44g/ml.The emulsification activity was greater in kerosene followed by toluene in production medium containing 1.0ml of gingely oil and the biosurfactant production yield was higher in the medium cointaining 1.0ml of gingely oil as carbon source. Several studies with the plant derived oils have shown that they can act as an effective & cheap raw materials for biosurfactant production. Sunflower, Soybean, Rabassu & Corn oil respectively, have been frequently focused as an excellent substrate for biosurfactant production (Deshmukh & Kulkarni, 2015)

Therefore different concentration of gingely oil had adverse effect on the production of biosurfactnt and the emulsification of various hydrocarbon oils. The increase in gingely oil concentration caused increase in amount of biosurfactant till gingely oil reached 1ml/100ml after that the amount of biosurfactant decreased with increasing in gingely oil concentration. Thus from this study it was proved that water insoluble commercial oil gives high biosurfactant production equal to the other renewable

resources and could be used as an emulsifying agent.

From this study it was concluded that the biosurfactant production was maximum when uing1.0ml/100ml of gingely oil as carbon source in production medium with *Serratia* sp. comparing to the other different carbon source at 0.5ml concentration. From this study it was proved that various commercial oils as carbon source can also give a good yield of biosurfactant which showed maximum emulsification activity with kerosene and toluene.

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