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Research Article

Isolation, Screening and Characterization of Potent Biosurfactant producing Bacteria from Oil Contaminated Site

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Abstract

Biosurfactant from microorganisms was proven to be an alternative source over chemical surfactant due to its structural and functional similarity and biodegradability properties. The biosurfactant producing bacteria were isolated from oil contaminated site. The oil contaminated soil samples were collected from Varachha, Surat. Total 09 morphological distinct colonies were obtained and were screened using different test such as drop collapse test, oil displacement test, emulsification activity and surface tension. Isolate T2 was screened and evaluated to be more potent to reduce the surface tension upto 37.71 dyne/cm^2. The molecular identification of T2 isolate was carried out by 16S rRNA sequencing; identified as *Pseudomonas stutzeri.* The T2 isolate produces biosurfactant measuring 6.6 mg/ml in a medium supplemented with waste fried oil. Thus, this study opens an insight not only for mass production of biosurfactant using waste fried oil, but also its application in waste oil cleanup. Thus it may contribute to solve environmental problems and reduce pollution load due to waste oil.

INTRODUCTION

Our daily routine activities are mostly dependent on the use of some kind of surfactants or emulsifiers including toothpaste, personal hygiene, cosmetic products, and other pharmaceutical product and by-products. The market for such surfactant/emulsifier is great in demand and day by day goes increasing. However, due to the non-biodegradability, ability to accumulate and toxicity of chemical based surfactant to the environment, there arise a need to find alternative source that can replace chemical surfactant (Satpute *et al.*, 2010a,b; Marchant and Banat, 2012a,b). Biosurfactant are mainly of microbial origin and generally more environmental friendly products. Biosurfactant is a substance that tends to reduce surface tension produced by different microorganism (Vigneshwaran *et al.*, 2016). This molecules have ability to aggregate between the interfaces of fluids with different polarity i.e., water and oil and therefore reducing the interfacial tension between them (Banat, 1995). Moreover, they can be used as moistening agents, dispersing agents, emulsifiers, foaming agents, beneficial food elements and detergents in many industrial regions such as organic chemicals, pharmaceuticals, cosmetics, beverages and foods, metallurgy, mining, petroleum, petrochemicals, biological control and management and many others (Banat *et al.*, 2000; Perfumo *et al.*, 2010; Vedaraman and Venkatesh, 2011). As the world production of fats and oils is about 120 million tones, 81% of which are from plant sources (Brackmann and Deutschland, 2004). Most of the oils and fats are used in the food industry, which produces large amounts of waste frying oils.

The disposal of frying oil waste is a serious question mark for environment ecosystem hazard; hence present research explains the increasing interest in the use of waste frying oils for microbial transformation (Vedaraman and Venkatesh, 2011). This study was conducted to produce biosurfactant from microbial source using waste fried oil as substrate. The bacterial isolate thus obtain serves as a biofactory for production of industrially important biosurfactant. It also provide an insight for utilization of waste fried oil as cheaper alternative substrate for biosurfactant production (Oliveira *et al*., 2013) thereby contributing to waste minimization and reduction in pollution load due to fried waste oil disposal (Emmanuel *et al,* 2009; Oliveira *et al.,* 2013).

MATERIALS AND METHODS

Sample Collection

About 10g of Oil contaminated soil samples were collected from local garage located at Trikamnagar, Varachha Road, Surat, India with the help of sterile spatula. All samples were placed into sterile polythene bags and stored at 4ºC until further use.

Enrichment and Isolation of bacteria

1g of soil sample were inoculated in Bushnell Hass mineral salt medium as enrichment medium (Himedia laboratory Pvt. Ltd., Mumbai) supplemented with 2% w/v waste fried oil and incubated at 30°C for 24-48 hrs. After incubation, the aliquots were prepared upto 10^{-6} and each aliquot were plated onto nutrient agar plate by spread plate techniques (Anandraj and Thivaran, 2010). Each colony with different morphological characteristics was picked and pure culture of isolates was prepared. All Pure culture obtained were preserved in nutrient slants at 4°C (Phalke *et al*., 2017).

Screening for biosurfactant production by Isolates

The quantitative screening for biosurfactant production was carried out by oil displacement test, drop collapse test, emulsification activity and measurement of surface tension. The strain which showed the lowest surface tension value was selected for a further study (Bodour and Maier, 1998; Youssef *et al.,* 2004; Sriram *et al.,* 2011).

a. Drop collapse test

A drop of the culture supernatant was placed carefully on an oil coated glass slide and observed after one minute. If the drop of supernatant collapsed and spread on the oil coated surface, it indicates positive test for biosurfactant. This test was simultaneously carried out on distilled water as control (Jain *et al.,* 1991).

b. Oil Displacement Test:

15 µl of used engine oil were placed on the surface of 40 ml distilled water in a petri dish with 90 × 10 mm in diameter. 10 µl of the culture supernatant were gently put on the center of the oil film. Formation of clear halo suggests the presence of biosurfactant. Sodium Decodyl Sulphate was used as positive control and uninoculated Bushnell Hass mineral salt medium as negative control (Morikawa *et al.,* 1993).

c. Emulsification Index Measurement

Emulsification Index was determined by adding 4 ml of culture supernatant with 4 ml of n-hexadecane, vortex at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity is defined as the ratio of height of the emulsion layer with the total height expressed in term of percentage (Cooper and Goldenberg, 1987).

$$\text{Emulsification index (E24) \%} = \frac{\text{Height of the emulsion}}{\text{Total height}} \times 100$$

d. Surface Tension Measurement (Stalagmometer)

The surface tension of supernatant broth containing biosurfactant was measured at 25°C using a stalagmometer (Stalagmometer Rohr B Abgew) by drop method (Caykara and Birlik, 2005). The surface tension of the biosurfactant containing broth was calculated using the following equation (Langmuir, 1917):

$$\gamma 0 = \gamma\ (n/n0),$$

where γ0 and γ are the surface tensions of the reference solvent (for water, γ0 = 72 dyne/cm^2) and biosurfactant containing broth solution, and n0 and n are the drop numbers of the reference solvent and biosurfactant containing solution, respectively. The reference solution was taken as uninoculated Bushnell Hass mineral salt broth.

Morphological, Colonial and Biochemical characterization of bacterial Isolate: The isolate was identified according to Gram staining, colonial characteristics and biochemical characterization (Rakesh and Kiran Patel, 2004; Kamble *et al.,* 2012).

Molecular Identification of biosurfactant producing Isolate:

a. Extraction of bacterial genomic DNA

Selected isolate was grown in Bushnell Hass mineral salt broth supplemented with 1% glucose for 24 hrs at37 ^{0}C. For DNA extraction,precipitation

precipitation and purification, HiPer Bacterial Genomic DNA Extraction Kit (Himedia Laboratory Pvt. Ltd. Mumbai) was used. DNA from isolate was electrophoreses in 0.8 % Agarose gel and visualized on a Gel Documentation System (Genie, Bangalore).

b. 16S rRNA sequencing for identification of Isolate:

The selected isolate was send to Gujarat State Biotechnology Mission (GSBTM), Gandhinagar, Gujarat, India, for 16S rRNA sequencing. The sequence thus obtain was subjected to similarity search using nucleotide BLAST 2.2.31 (blastn) software available at NCBI. (Johnson *et al.*, 2008). The identification was made by evaluating total score, maximum score, query coverage, E-value and percentage (Relman, 1993).

c. Phylogenetic position of isolate with respect to their closely related sequence:

The closely related sequence obtain from blast were downloaded and multiple sequence alignment were conducted using Clustal Omega tool available at EMBL-EBI. Phylogenetic tree was constructed to determine taxonomic position of the isolate using Neighbor Joining algorithm (Sievers *et al.*, 2011).

RESULTS AND DISCUSSIONS

Enrichment and Isolation of Bacteria

Total 09 bacterial isolates were obtained from oil contaminated soil samples. They were further screened for biosurfactant production by oil displacement test, drop collapse test, emulsification activity and measurement of surface tension. Out of 09 isolates, 04 isolates were screened positive. The results are depicted as Table 1.

Table 1 Summary of the screening methods for biosurfactant producing bacteria

Bacterial Isolates	Oil Displacement Test	Drop Collapse Test	Emulsification Activity	Surface Tension (dyne/cm2)
T2	++++	+++	48%	37.71
T5	+++	+++	-	45.98
T7	++	-	41%	52.10
T8	++	+	38%	-

keys: (-) = no result; (+)= weak result; (++)= average result, (+++)= good result, (++++)= best result.

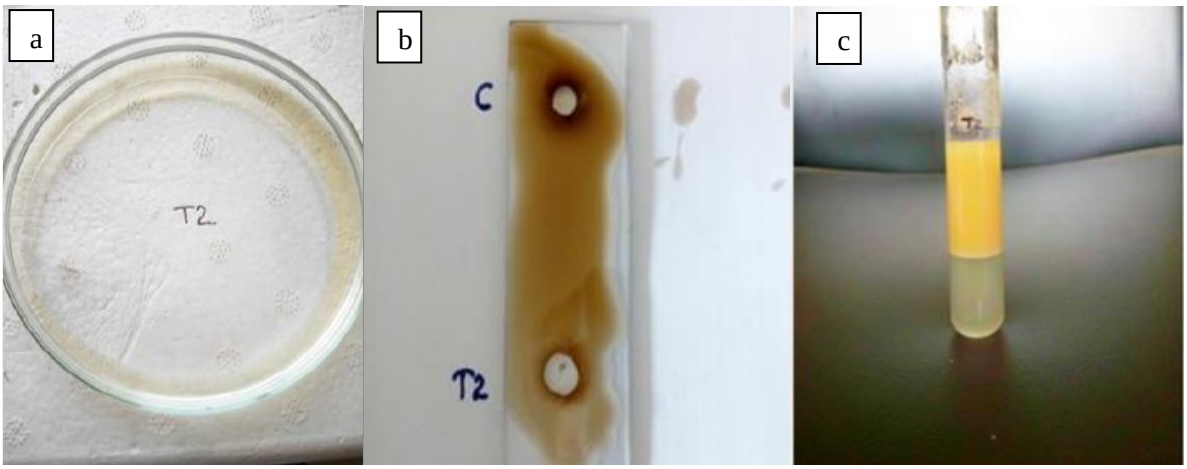

Figure1. Screening result of T2 isolate (a) Oil displacement, (b) Drop collapse and (c) Emulsification activity

The isolate T2, T5, T7 and T8 show oil displacement test positive. The results shows that T7 and T8 isolate had average, T5 isolate had moderate and T2 isolate had higher activity with respect to oil displacement. The isolate T2 and T5 show drop collapse test positive. The drop collapse test and oil displacement test are indicative of surface wetting activity (Youssef *et al.*, 2004). Thus, isolate T2 and T5 may be considered as potential isolates for biosurfactant production. The results are in accordance with different authors (B. Anandaraj *et al*., 2010; Youssef *et al.*, 2004; Jain *et al.*, 1991).

The Emulsification index of T2 isolate was 48%, T7 isolate was 41% and T8 isolate was 38%. However, T5 isolate were screened negative.

Emulsification index aids in quantitative screening of biosurfactant producers (Satpute *et al.*, 2008). The best result in decreased surface tension of 37.71 dyne/cm^2 by supernatant containing biosurfactant was observed in T2. The results of screening test suggest that out of 04 isolates, T2 isolates is more potent and can be used for further investigation. The screening tests of T2 isolate were shown as Figure 1. The isolate T2 was able to degrade waste fried oil and produce potent biosurfactant in great quantity of about 6.6 mg/ml which result during production of biosurfactant.

Morphological, Colonial and Biochemical characterization of Bacterial isolate T2.

Morphological, colonial and biochemical characterization aids in partial identification of microorganism. Morphological and colonial characteristics of T2 isolate were shown as Table 2. Biochemical characteristics of T2 isolate were shown as Table 3. The colony of T2 isolate on nutrient agar and macConkey agar plate are shown as Figure 2. The T2 isolate was characterized as gram negative rods and lactose non-fermentor.

Table 2 Characterization of T2 Isolate	
Isolate	T2
Morphological characteristics	
Gram reaction.	Gram negative
Shape and arrangement	Rods shape bacteria occurring singly
Motility	Non-motile
Colonial characteristics	
Shape	Irregular
Colony Size	Small
Margin	Undulate
Pigmentation	Pale yellow
Elevation	Raised
Surface	Punctate
Consistency	Dry
Opacity	Opaque

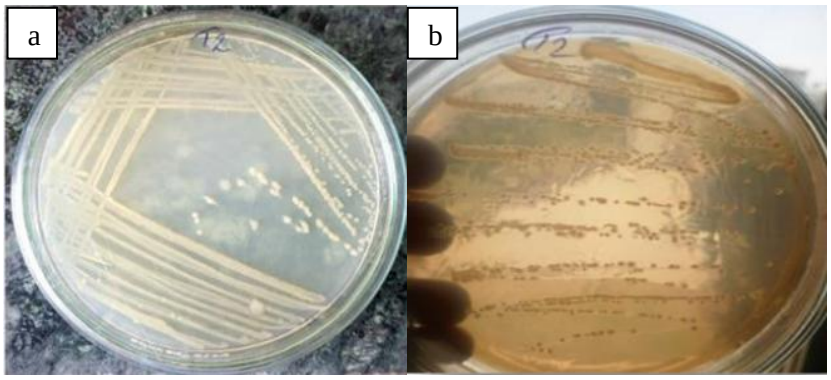

Figure 2. Isolate T2 colony on (a) Nutrient agar and (b) MacConkey agar plate

Table 3. Biochemical analysis for biosurfactant producing bacterial isolate T2			
Name of Test	**Result**	**Test**	**Result**
Indole production	-	Glucose	+ (A, G)
H_2S production	-	Fructose	+ (A)
Citrate utilization	+	Maltose	+ (A)
Methyl red test	-	Sucrose	+ (A, G)
Voges-Proskauer test	-	Mannitol	-
Nitrate reduction	-	Lactose	-
Urea hydrolysis	-	Xylose	+ (A)
Keys:(-) =Negative result; (+)=Positive result, A=Acid production, G=Gas production			

Molecular basis Identification of biosurfactant producing bacteria

DNA isolation were performed and validated by agarose gel electrophoresis. The 16S rRNA sequencing was conducted using 16S rRNA primer (8F: 5'-AGAGTTTGATCCTGGCTCAG-3'). By analyzing the resulting sequence, the bacterium was identified as *Pseudomonas stutzeri.* The past work also determined that *Pseudomonas spp.* had ability to produce biosurfactant (Tayfun *et al.*, 2014). Phylogenetic tree of the strain of *Pseudomonas stutzeri* with closest similarity using neighbor joining method was analyzed by clustal omega (Sievers *et al*., 2011) is shown as Figure 3. The 16S rRNA sequence was published at NCBI with accession number **KY941131.1** is shown as Figure 4.

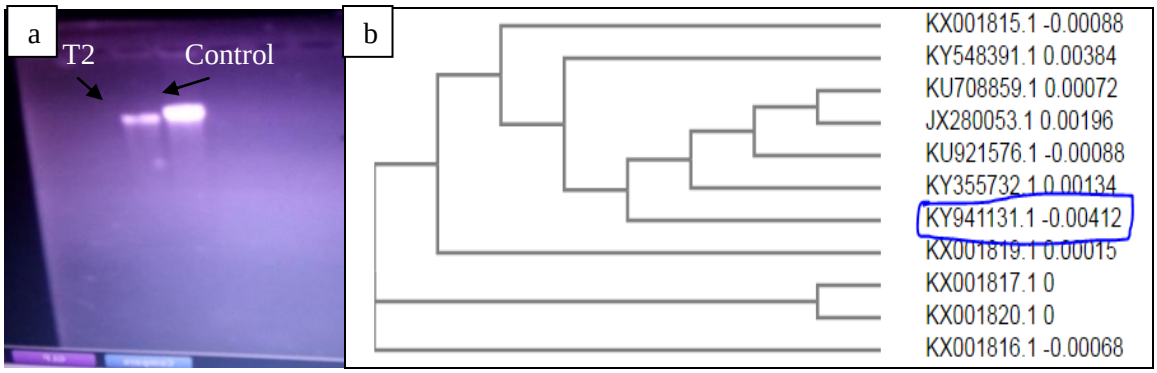

Figure 3 (a) Agarose gel electrophoresis of Genomic DNA of isolates T2, (b) *Pseudomonas stutzeri* phylogenetic view using NJ method

Pseudomonas stutzeri strain T2 16S ribosomal RNA gene, partial sequence

GenBank: KY941131.1

FASTA Graphics

Go to:

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LOCUS       KY941131                725 bp    DNA     linear   BCT 21-APR-2017
DEFINITION  Pseudomonas stutzeri strain T2 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KY941131
VERSION     KY941131.1
KEYWORDS    .
SOURCE      Pseudomonas stutzeri
  ORGANISM  Pseudomonas stutzeri
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 725)
  AUTHORS   Suthar,M.P., Hajoori,M.A. and Chaudhari,R.R.
  TITLE     Isolation and Screening of Potent Biosurfactant Producing Bacteria
            from Oil Contaminated Site
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 725)
  AUTHORS   Suthar,M.P., Hajoori,M.A. and Chaudhari,R.R.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-APR-2017) Biotechnology, Bhagwan Mahavir College of
            Science & Technology, Bharthana, Surat, Gujarat 395003, India
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Figure 4 16S rRNA sequence of *Pseudomonas stutzeri*

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REFERENCES

Anandaraj B and Thivakaran P, 2010. Isolation and Production of Biosurfactant producing organism from oil spilled soil. *J. Biosci. Tech.*, **1**:120-126.

Banat IM, Makkar RS and Cameotra SS, 2000. Potential commercial applications of microbial surfactants. *Appl. Microbiol. Biotechnol.*, **53**(5):495–508.

Banat IM., 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review. *Bioresour. Technol.*, **51**: 1-12.

Bodour AA and Miller-Maier RM, 1998. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. *J. Microbiol. Methods*, **32**:273–280.

Brackmann B and Deutschland C, 2004. *Proceedings 6th world surfactant congress CESIO*. Berlin, Germany, 112:21-23.

Caykara T and Birlik G, 2005. Swelling and adsorption properties of hydrophobic poly[(n- (3-(dimethylamino)propyl)methacrylamide)-co-(lauryl acrylate)] hydrogels in aqueous solutions of surfactants. *Macromol. Mater. Eng.*, **290**:869–874.

Cooper DG and Goldenberg BG, 1987. Surface-Active Agents from Two Bacillus Species. *Appl. Environ. Microbiol*. **53**(2):224–229.

Emmanuel OA, Kessington O, Obahiagbon and Mudiakeoghene, 2009. Biodegradation of vegetable oils: A review, *Scientific Research and Essay*, **6**: 543-548.

Jain D, Collins-Thompson D and Lee H, 1991. A collapsing test for screening surfactant- producing microorganisms. *Journal of Microbiology Methods*, **13**(4):271-279.

Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. 2008. NCBI BLAST: a better web interface. *Nucleic Acids Res.*1-(36-Web Server issue):W5-9.

Kamble KD, Bidwe PR, Muley VY, Kamble LH, Bhadange DG and Musaddiq M, 2012. Characterization of L-Asparaginase producing Bacteria from Water, Farm and Saline Soil. *Bioscience Discovery*, **3**(1):116-119.

Marchant R and Banat IM, 2012a. Biosurfactants: A sustainable replacement for chemical surfactants. *Biotechnol. Lett*., **34**:1597–1605.

Marchant R and Banat IM, 2012b. Microbial biosurfactants: challenges and opportunities for future exploitation. *Trends. Biotechnol.*, 30:558–565.

Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y and Imanaka T, 1993. A new lipopeptide biosurfactant produced by *Arthrobacter sp*. strain MIS38. *J. Bacteriol.*, **175**:6459–6466.

Oliveira, Juliana Guerra de, and Garcia-Cruz, Crispin Humberto, 2013. Properties of a biosurfactant produced by *Bacillus pumilus* using vinasse and waste frying oil as alternative carbon sources. *Brazilian Archives of Biology and Technology*, **56**(1), 155-160.

Phalke VS, Pawar BT and Gulve R M, 2017. Halophilic bacteria in salt stressed soils of Aurangabad district (MS) India. *Bioscience Discovery*, 8(1):55-60

Perfumo A, Smyth TJP, Marchant R and Banat IM, 2010. Production and roles of biosurfactants and bioemulsifiers in accessing hydrophobic substrates. In: Timmis, Kenneth N. (Ed.). In: Handbook of Hydrocarbon and Lipid Microbiology. *Springer UK*, **47**(7):501–1512.

Rakesh P and Kiran P, 2004. Identification of bacteria. *Experimental Microbiology-I.* Aditya Publication, Ahmedabad India, Pp 95-101.

Relman DA, 1993. Universal bacterial 16S rDNA amplification and sequencing. *In Diagnostic Molecular Microbiology*, 489–495.

Satpute SK, Banat IM, Dhakephalkar PK, Banpurkar AG and Chopade BA, 2010a. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. *Biotechnol.Adv.*, **28**: 436–450.

Satpute SK, Banpurkar AG, Dhakephalkar PK, Banat IM and Chopade BA, 2010b. Methods for investigating biosurfactants and bioemulsifiers: A review. *Crit. Rev. Biotechnol.*, **30**:127–144.

Satpute SK, Bhawsar BD, Dhakephalkar PK and Chopade BA, 2008. Assessment of different screening methods for selecting biosurfactant producing marine bacteria. *Ind. J. Mar. Sci.*, **37**: 243–250.

Siegmund I and Wagner F, 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnol. Tech.*, 5:265-268.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M , Söding J, Thompson JD and Higgins DG, 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, *Molecular Systems Biology*, **7**: 539.

Sriram MI, Kalishwaralal K, Deepak V, Gracerosepat R, Srisakthi K and Gurunathan S, 2011. Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced by heavy metal tolerant strain *Bacillus cereus* NK1. *Colloids. Surf. Biointerfaces.*, **85**: 174–181.

Tayfun K, Belma A and Ergin K, 2014. Production of biosurfactant by *Pseudomonas spp.* isolated from industrial waste in Turkey. *Turkish Journal of Biology*, **38**: 307-317.

Vedaraman N and Venkatesh N, 2011. Production of surfactin by *Bacillus subtilis* MTCC 2423 from waste frying oils. *Brazi. J. Chem. Eng.*, **28**(2):175–180.

Vigneshwaran C, Jerold M, Vasantharaj K, Sivasubramanian V, 2016. A Review on Biosurfactants and its Environmental Applications. *IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)*, **10**(8):152.160.

Youssef NH, Duncana KE, Naglea DP, Savagea KN, Knappb RM and McInerney MJ, 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. *J. Microbiol. Methods*, **56**:339–347.

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