Journal of Research in Biology

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Original Research paper

An International Online Open Access Publication group

Screening and isolation of potent biosurfactant producing *Bacillus subtilis* CS14 from contaminated soil samples of Kanchipuram, India

Authors:
Balasubramanian Ramesh[1,2], Gopalakrishnan Suresh[1], Nagaiya Ravichandran[1], Ganesan Vijaiyansiva[1*]

Institution:
[1] Unit of Environmental Biotechnology, Department of Biotechnology, University of Madras, Maraimalai Campus, Chennai, India – 600 025.
[2] Department of Biotechnology, Sri Sankara Arts and Science College, Enathur, Kanchipuram, India – 631 561.

Corresponding author:
Ganesan Vijaiyansiva

Email:
gvsbio@gmail.com

Phone No:
9840304888

Web Address:
http://jresearchbiology.com/Documents/RA0047.pdf.

ABSTRACT:

A good biosurfactant must reduce the surface tension of the water from 72 to 35 milli-Neuton/meter (mN/m) and should show a surface activity of at least 37 mN/m. An extensive screening with 13 contaminated soils and one oil refinery sludge was carried out to isolate a potential strain.. Totally 3662 colonies were obtained from all 14 contaminated soil samples over a period of three months from which 212 morphologically different colonies were tested for biosurfactant production and 22 positive strains were identified. All the isolates were tested quantitatively and the isolate CS14 showed maximum surface activity of 51.38 mN/m. Taxonomic identification of the biosurfactant producing strain CS14 was performed using 16s rDNA studies and was identified as *Bacillus subtilis.*

Keywords:
Biosurfactant, contaminated soil, *Bacillus subtilis* CS14, 16s rDNA studies.

Article Citation:
Balasubramanian Ramesh, Gopalakrishnan Suresh, Nagaiya Ravichandran, Ganesan Vijaiyansiva.
Screening and isolation of potent biosurfactant producing *Bacillus subtilis* CS14 from contaminated soil samples of Kanchipuram, India.
Journal of research in Biology (2011) 2: 140-147

Dates:
Received: 16 Jun 2011 /**Accepted:** 21 Jun 2011 /**Published:** 23 Jun 2011

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, which are reducing surface and interfacial tension at the surface and interface respectively. They are structurally diverse group of surface active molecules synthesized by microorganisms (Muthusamy *et al*., 2008).

The Biosurfactants are a diverse group of biomolecules, which share the same properties as synthetic surfactant, and in some cases, they are superior in creating water-in-oil or oil in water emulsions (Ashtaputre and shah, 1995; Jacobucci *et al.,* 2009). Microbial derived surfactants have special advantages over their chemically manufactured counterparts because of their lower toxicity, biodegradable nature and effectiveness at extreme temperature and pH values (Jacobucci *et al.,* 2009). When properly stimulated, Biosurfactants producing microorganisms can aid in the bioremediation of oil contaminated soil and hydrocarbon contaminant in the environment (Carrillo *et al.*, 1996; Jacobucci *et al.,* 2009).

A variety of microorganisms, including bacteria, fungi, and yeasts, have been reported to produce biosurfactants. Several of these biosurfactants are well described chemically ad categorized into high- and low-molecular-mass compounds. The low-molecular- mass biosurfactants include glycolipids and lipopeptides, such as rhamnolipids and surfactin. The high-molecular mass compounds include proteins and lipoproteins, or complex mixtures of these polymers (De-souza *et al.*, 2003).

In recent years, there has been a growing interest in the isolation and identification of new microbial surfactant the might have application in enhanced oil recovery processes. Biosurfactants are of interest because of their broad range of potential industrial applications, including emulsification, phase separation, wetting, foaming, emulsion stabilization and viscosity reduction of heavy crude oils (Ochsner *et al*., 1994). Potential application envisaged in several industries such as agriculture, food, textiles, cosmetics, petrochemical and petroleum production (Reiling *et al*., 1986). Biosurfactants are used in the remediation of organic and metal contaminated sites, enhanced oil recovery and as cosmetic additives (Bodour *et al*., 2003). They are powerful natural emulsifiers capable of reducing the surface tension of water from roughly 76 milli-Newton/meter (mN/m) to 25-30 mN/m. This biosurfactant activity makes them excellent candidates for assisting in the breakdown and removal of oil spills. Biosurfactants also demonstrate antibacterial and antifungal activities, suggesting possible roles in the medical and agricultural fields (Gunther *et al*., 2005).

The present study is in continuation of our previous finding that Indian soils are rich in biosurfactant producing bacteria and their population is higher in contaminated soils than in undisturbed soils (Ramesh *et al*., 2010).

MATERIALS AND METHODS

Collection of soil samples:

The soil samples were collected from different places in and around Kanchipuram (12° 50'23"N 79°42'0"E) using the procedure described by Bodour *et al*., in 2003 as shown in Table 1. Totally 13 soil samples were collected from metal, oil and petro-products contaminated sites. One oil refinery sludge sample was also collected from Chennai Petroleum Corporation Limited refinery (CPCL), Manali, Chennai. These samples were designated as SS1-SS14 and screened for potent biosurfactant producing isolates.

Enumeration of soil microbial counts:

The total number of culturable, aerobic, bacteria per gram of sample was determined by using nutrient agar plates (Huang *et al.,* 2008). The bacterial populations were enumerated as colony-forming units (CFU) from a serial dilution of the soil suspensions. The colonies were counted after incubation for 3 days at 30°C.

Screening for biosurfactant producing isolates:

Soils were screened for biosurfactants producing isolates by using the following procedure (Bodour *et al*., 2003). A 5 g sample of each soil was placed into a 250 ml flask containing 50 ml of sterile tap water and incubated at 23°C on a shaker at 200 rpm for 21 days. On days 3, 7, 14, and 21, a sample of each soil slurry was serially diluted, plated on R2A agar (Himedia), and incubated for 1 week. After incubation, plates were enumerated, and morphologically different bacteria were selected for qualitative biosurfactant screening. Isolated colonies were inoculated into 5 ml mineral salts medium (MSM) containing 2% glucose as the sole carbon and energy source. The MSM was a mixture of solution A and solution B. Solution A contained (per liter) 2.5 g of $NaNO_3$, 0.4 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NaCl, 1.0 g of KCl, 0.05 g

of $CaCl_2 \cdot 2H_2O$, and 10 ml of concentrated phosphoric acid (85%). This solution was adjusted to pH 7.2 with KOH pellets. Solution B contained (per liter) 0.5 g of $FeSO_4 \cdot 7H_2O$, 1.5 g of $ZnSO_4.7H_2O$, 1.5 g of $MnSO_4 \cdot H_2O$, 0.3 g of K_3BO_3, 0.15 g of $CuSO_4 \cdot 5H_2O$, and 0.1 g of $Na_2MoO_4 \cdot 2H_2O$. One milliliter of solution B was added to 1,000 ml of solution A to form the MSM. The broth cultures were incubated with shaking (200 rpm) for 7 to 9 days at 23°C. The cell free supernatants were then tested for the presence of surfactant by using the qualitative drop collapse method.

Qualitative drop collapse tests were performed in the polystyrene lid of a 96 microwell 12.7 by 8.5 cm plate. The lids have 96 circular wells (internal diameter, 8 mm). A thin coat of 20W -40 oil was applied to each well. The coated wells were equilibrated for 24 hours at 23ºC and then 5µl of each supernatant was delivered into the center of each well. If the drop remained beaded after 2 minutes, the result was scored as negative. If the drop spread and collapsed the result was scored as positive for the presence of biosurfactant. All the positive isolates in drop collapse test were further subjected to secondary qualitative screening for biosurfactant production by beta-haemolytic activity.

Qualitative screening for biosurfactant producing isolates by beta-haemolytic activity on blood agar plates:

Since the beta-hemolytic activity is indicative of surface activity, qualitative screening for biosurfactant producing isolates was carried out using the Blood agar plates. The strains subjected to drop collapse test were subsequently inoculated on blood agar plates and were incubated at 37°C. After 24 hours the presence of hemolytic zone was observed (Youssef *et al*., 2004; Rodriguez *et al*., 2006).

Quantitative measurement of surface activity:

All the isolates that tested positive in the drop collapse test were then tested quantitatively for biosurfactant production with the drop weight method described by Sabesan *et al*., in 2002. The isolates were grown in 5ml of mineral salt medium amended with 2% glucose. Cell suspensions were centrifuged at 5000 rpm for 15 minutes and the cell free supernatant was poured into a burette. The bottom of the burette consists of a rubber tube attached with glass tube of 3 mm diameter. An empty pre-weighed beaker was placed under the burette and the supernatant was released slowly drop by drop. 50 drops were poured in to the beaker and it was weighed to determine the weight of 50 drops.

The mass of one drop was calculated by using the formula

$$\text{Mass of one drop (M)} = \frac{\text{Beaker + Sample weight – Beaker Weight}}{\text{Number of drops}}$$

Then the surface tension of the supernatant was calculated by using the formula

$$\text{Surface tension (T)} = \frac{Mg}{\pi r} \times 10^{-3} \times nm^{-1}$$

Where

M = Mass of one drop
g = Gravity
r = Radius of glass tube

Surface activity of each isolate was calculated by the following formula:

Surface activity = Surface tension of uninoculated medium – surface tension of supernatant.

Genus level identification of all the biosurfactant producing isolates:

The biosurfactant producing isolates determined by quantitative studies were identified up to genus level by studying phenotypic characters like gram staining, motility and biochemical characteristics like oxidase, catalase, IMVIC, urease, nitrate reduction and sugar fermentation tests. The methods described by Cappuccino (1999) was followed for all the procedures. All these results were compared with Bergey's Manual of Determinative Bacteriology to determine the genus (Holt *et al.*, 1994).

Taxonomic identification of the potent biosurfactant producing strain CS14 using 16s r DNA studies:

Taxonomic identification of the best biosurfactant producing strain CS14 was performed using 16s rDNA studies (Heuer *et al.,* 1997; Mishra and Doble, 2008). Universal eubacterial 16s rDNA PCR primers, 27f- forward primer and 1492r reverse primer were synthesized (Xu *et al.*, 2003; Bento *et al.,* 2005). The bacterial pellet obtained after centrifugation of the overnight culture grown in Luria Bertani Broth medium were boiled at 98°C for 10 minutes in the boiling water bath and kept on ice immediately, which was then used as DNA template 2 µl of forward and reverse primers were added along with 25 µl of 2x PCR Master Mix (Bangalore Genie, India) and made up to 50 µl with

PCR grade water. A Program of 94° C for 1 minute for denaturing, 42° C for 50 seconds for annealing and 72° C for 1 minute for extension were used Eppendorf PCR Mastercycler up to 25 cycles. The confirmations of the amplicons were done by running the samples in 1% agarose gel along with DNA ladder marker. The sample was sequenced at Ocimum Biosolutions Ltd., Hyderabad, Andhra Pradesh, India and was submitted to GenBank of National Centre for Biotechnology Information and accession numbers was obtained.

Analysis of the sequenced bases with the help of NCBI-BLAST:

The partial 16s rRNA gene sequence in FASTA format was used for similarity search against non-redundant (nr) database by Basic Local Alignment Search Tool (blastn) at National Centre for Biotechnology Information server (Altschul *et al*., 1997).

Phylogenetic tree construction:

Phylogenetic tree was constructed using 16s rRNA gene sequence obtained, 8 highly homologous sequences identified by blastn result and one unrelated sequence was also selected as out group. Totally, 10 16s rRNA sequence including outgroup were selected for phylogenetic tree construction. Similarity matrix was prepared using Dnadist program in PHYLIP analysis package (Jiang *et al*., 2009) using jukes cantor corrections. Phylogenetic tree was constructed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software package version 5 (Tamura *et al*., 2011).

RESULTS AND DISCUSSION

Collection of contaminated soil samples:

The soil samples were collected from different places in and around Kanchipuram using the procedure described by Bodour *et al*., in 2003. The results are shown in **Table 1.**

Enumeration of soil microbial counts:

The average Standard Plate Count was 2.81 x 10^8 CFU/g of contaminated soil samples (**Table 2**).

Qualitative screening for biosurfactant producing isolates by drop collapse test:

Totally 3662 colonies were obtained from 3, 7, 14 and 21 days retrieval of all 14 contaminated soil samples over a period of 3 months from which 212 morphologically different colonies were tested for biosurfactant production employing 96 well microplate lid and 22 positive strains were identified. All the 22 positive strains were further assessed for beta-haemolytic activity on blood agar plates as secondary confirmation and secretary nature of biosurfacants (Table 2).

Qualitative screening for biosurfactant producing isolates by beta-haemolytic activity on blood agar plates:

Since the beta-hemolytic activity is indicative of surface activity, qualitative screening for biosurfactant producing isolates was carried out using the Blood agar plates. The 22 positive strains from drop collapse test were subsequently inoculated on blood agar plates and were incubated at 37°C. Totally, 15 positive strains showed good beta-haemolytic activity on blood agar plates. The surface active isolates obtained in this study were designated as Isolate CS1 – CS15.

Table 1: Collection of contaminated soil samples

S. No	Soil Sample name	Location	Contamination	Geographical coordinates
01	SS-1	Petrol bunk	Petrol and Diesel	12° 50’ 47.00”N; 79° 41’ 44.62”E
02	SS-2	Welding shop	Metal and waste oil	12° 50’ 11.07”N; 79° 42’ 21.66”E
03	SS-3	Ration shop	Kerosene	12° 49’ 05.48”N; 79° 41’ 32.63”E
04	SS-4	Mechanic shop	Motor oil	12° 50’ 11.24”N; 79° 42’ 22.46”E
05	SS-5	Motor cycle Shed	Motor oil	12° 50’ 09.50”N; 79° 42’ 22.11”E
06	SS-6	Welding shop	Metal and waste oil	12° 49’ 05.68”N; 79° 41’ 36.72”E
07	SS-7	Old bus shed	Motor oil,	12° 51’ 25.34”N; 79° 31’ 47.80”E
08	SS-8	Petrol bunk	Petrol and Diesel	12° 49’ 58.54”N; 79° 42’ 13.38”E
09	SS-9	Mechanic shop	Motor oil	12° 49’ 17.19”N; 79° 41’ 53.78”E
10	SS-10	Generator engine room	Motor oil, Diesel	12° 51’ 31.48”N; 79° 43’ 52.71”E
11	SS-11	Bus shed	Motor oil, Diesel	12° 50’ 04.57”N; 79° 42’ 18.01”E
12	SS-12	Mechanic shop	Motor oil	12° 50’ 20.40”N; 79° 42’ 32.70”E
13	SS-13	Petrol bunk	Petrol and Diesel	12° 49’ 03.34”N; 79° 41’ 34.45”E
14	SS-14	CPCL, Chennai	Crude oil	13° 09’ 47.45”N; 80° 16’ 46.37”E

Table 2: Enumeration of soil microbial count and Qualitative screening for biosurfactant producing isolates

Sample No.	Location	SPC x 10^8 CFU/ml	No. of colonies in R2A agar			No. of Positives in Qualitative Screening
			Days of incubation in water	Total No. of colonies	Colonies selected for screening	
SS-1	Petrol bunk	0.033	3	154	7	0
			7	21	5	1
			14	23	5	1
			21	9	3	0
SS-2	Welding shop	0.047	3	92	5	0
			7	18	4	0
			14	16	5	0
			21	11	2	0
SS-3	Ration shop	0.050	3	32	4	0
			7	7	2	0
			14	17	3	0
			21	9	3	1
SS-4	Mechanic shop	6.43	3	134	5	2
			7	56	3	0
			14	24	3	0
			21	14	2	0
SS-5	Motor cycle Shed	7.13	3	198	6	0
			7	112	7	0
			14	73	3	0
			21	34	2	0
SS-6	Welding shop	0.045	3	114	4	0
			7	13	2	0
			14	8	2	0
			21	14	3	1
SS-7	Old bus shed	3.14	3	157	6	2
			7	76	4	1
			14	39	3	0
			21	18	3	0
SS-8	Petrol bunk	3.06	3	158	4	0
			7	89	6	2
			14	56	4	0
			21	28	2	0
SS-9	Mechanic shop	0.80	3	134	5	0
			7	80	3	0
			14	65	2	0
			21	27	3	0
SS-10	Generator engine room	1.10	3	112	3	0
			7	71	3	0
			14	48	2	0
			21	14	3	0
SS-11	Bus shed	4.3	3	158	6	0
			7	83	5	0
			14	34	3	1
			21	13	3	0
SS-12	Mechanic shop	0.93	3	149	7	0
			7	93	4	0
			14	48	5	1
			21	19	4	1
SS-13	Petrolbunk	7.93	3	198	5	0
			7	87	3	0
			14	46	3	0
			21	17	2	0
SS-14	Sludge sample	4.34	3	156	6	1
			7	86	4	0
			14	74	3	0
			21	20	3	0
Total number of colonies				**3662**	**212**	**15**

Quantitative measurement of surface activity of biosurfactant producing isolates using drop weight method:

All the isolates that tested positive in both drop collapse test as well as by beta-haemolytic activity assay were then tested quantitatively for biosurfactant production with the drop weight method. The results are shown in **Table3.** The Isolate CS14 showed maximum surface activity of 51.38 x 10^{-3} nm^{-1}. It was able to reduce the surface tension of mineral salt media from 88.8 x 10^{-3} nm^{-1} to 37.42 x 10^{-3} nm^{-1}. As observed by Mulligan, in 2004 and Gnanamani *et al*., in 2010, a good biosurfactant must reduce the surface tension of the water from 72 to 35 mN/m and should show a surface activity of at least 37 mN/m. Isolate CS14 showed a surface activity of 51.38 mN/m and can be categorized as very good biosurfactant producer.

Genus level identification and characterization of all the biosurfactant producing isolates:

All the biosurfactant producing isolates CS1 – CS15 were identified up to genus level by studying phenotypic characters like gram staining, motility and biochemical characteristics like oxidase, catalase, IMVIC, urease, nitrate reduction and sugar fermentation tests. These results suggested that the Isolates CS1-CS15 belong to the genus of *Providencia sp., Pseudomonas sp., Providencia sp., Bacillus sp., Pseudomonas sp., Bacillus sp., Bacillus sp., Pseudomonas sp., Micrococcus sp., Bacillus sp., Pseudomonas sp., Pseudomonas sp., Rhodococcus sp., Bacillus sp.* and *Aeromonas sp.,* respectively.

Mukherjee *et al*., in 2006, observed that most of the biosurfactant research related to production trials has been mainly confined to microorganisms, such as *Pseudomonas sp., Bacillus sp.* and *Candida sp*. It may be due to the fact that these species produce biosurfactants in high amounts and get selected in quantitative screening procedures. In the present study also *Bacillus sp.* CS14 was selected based on the high surface activity of 51.38 10^{-3} nm^{-1}, during the quantitative screening.

Taxonomic identification of the potent biosurfactant producing strain CS14 using 16s r DNA studies:

Approximately 1.5 kb DNA fragment was amplified (**Figure 1**) and was sequenced. The sequence was submitted to GenBank of National Centre for Biotechnology Information and accession number HQ902895 was obtained.

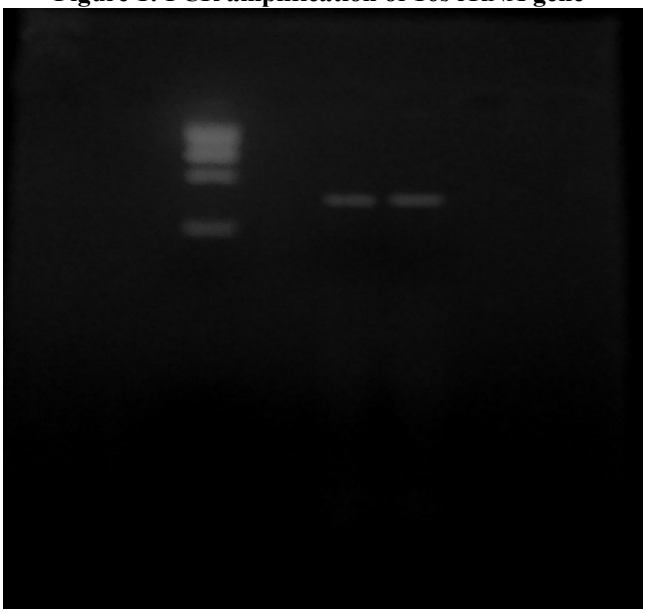
Phylogenetic tree construction:

For the 16s rRNA gene sequence, 8 highly homologous sequences were identified by blastn results and were downloaded and one unrelated sequence was also selected as out group and phylogenetic tree was constructed (**Figure 2**). The phylogenetic tree revealed that the best biosurfactant producer Isolate CS14 is *Bacillus subtilis*.

Table 3: Quantitative measurement of surface activity of biosurfactant producing isolates using drop weight method and selection best of biosurfactant producer.

S.No	Isolate	Surface tension x 10^{-3} nm^{-1}	Surface activity x 10^{-3} nm^{-1}
1	Uninoculated MSM	88.8	0 (Control)
2	CS1	49.11	39.69
3	CS2	58.4	30.40
4	CS3	66.29	11.04
5	CS4	50.20	38.60
6	CS5	79.52	09.28
7	CS6	86.53	02.27
8	CS7	55.24	19.67
9	CS8	86.5	02.30
10	CS9	79.52	09.28
11	CS10	86.5	02.30
12	CS11	67.8	21.00
13	CS12	45.97	15.76
14	CS13	56.98	22.24
15	**CS14**	**37.42**	**51.38**
16	CS15	42.32	46.48

Figure 1: PCR amplification of 16s rRNA gene



Lane 3: 1kb DNA Ladder marker, Lane 5,6: PCR amplified product ~1.5 kb

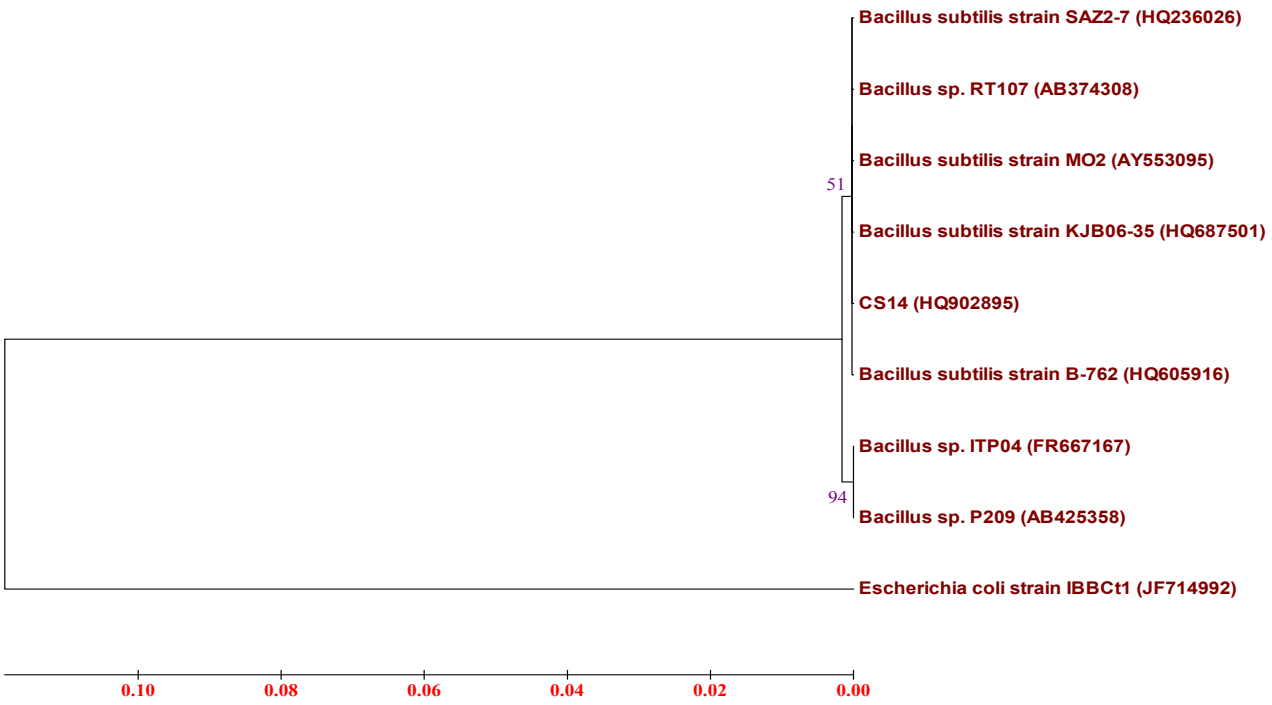


Figure2: Phylogenetic relationships of biosurfactant producing bacterial isolate CS14 derived from 16S rRNA gene sequence homology

CONCLUSION

The present study is the first preliminary assessment on the presence of biosurfactant producing bacterial population in Kanchipuram contaminated soils. A potent biosurfactant-producing bacterial isolates *Bacillus subtilis* CS14 with a surface activity of **51.38 10^{-3} nm^{-1}** was isolated and chosen as research target for biosurfactant production, to be described in a further work.

ACKNOWLEDGEMENT

One of the authors, B.Ramesh sincerely thanks Dr.K.R.Venkatesan, Principal, Sri Sankara Arts and Science College for all the support provided to conduct this research work.

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