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Production, Characterization and Evaluation of Potential Applications of Bioemulsifier Produced by an Endophytic Actinomycete EARN5 isolated from *Andrographis paniculata*

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A number of natural, synthetic, plant and animal derived emulsifiers are already been used at commercial level but some of the disadvantages and limitations with these available emulsifiers have forced researchers to search for suitable and feasible alternatives. Emulsifiers of microbial origin can solve the problem to a large extent and therefore there is a need to explore their possibilities on regular basis. In present investigation, the endophytic actinomycetes have been used to explore their potential of producing bioemulsifier is an important attempt in this regard. The isolation of endophytic actinomycetes were made from the tissues of different parts of medicinal plants which were obtained from the medicinal plant garden of Panjab University, Chandigarh, India. It resulted in isolation of a total of seven actinomycetes isolates. All of which were successfully isolated in to pure form and screened out for their potential to produce the bioemulsifier in maltose yeast extracts broth (MYEB). Isolate no. EARN5 which was identified as the species of *Actinomycetes* was found to exhibit the maximum emulsification activity with different oils and hydrocarbons used. There after the studies pertaining to optimization of various parameters to enhance the yield of the test bioemulsifier was also performed. The optimization of physical parameters revealed the maltose yeast extract as the best production medium, 7 days as the most suitable incubation period, 29±1°C as the optimum temperature, pH of 7 and 130 rpm as the optimum agitation speed. The biochemical parameters revealed the cellulose and yeast extract as the best sources of carbon and nitrogen respectively. The test bioemulsifier was also evaluated for the type of emulsion it formed and its stability. Besides, it was also characterized for various physical (solubility, SEM, critical micellar concentration and surface tension), molecular (FTIR and elemental analysis) and chemical properties (protein, carbohydrates and lipid contents). The test bioemulsifier was also evaluated for its efficiency to remove the oil from contaminated water and cream from full cream milk. The results obtained on the studies found to possess both the properties in the test bioemulsifier.

Keywords: Bioemulsifier, Biosurfactants, Emulsion, Endophytes, Actinomycetes, Emulsification Activity, Emulsification Index, CMC

An emulsion is a blend or mixture of two immiscible liquids, with droplets of one phase (dispersed phase) distributed in the other phase (continuous phase). In general these are unstable systems in which the dispersed phase droplets tend to

agglomerate or coalescence and separate out. The boundary between the two phases is called the interface. Emulsifiers or bioemulsifiers contain both hydrophilic and lipophilic parts. The hydrophilic head is easy to hydrate (water soluble) and the lipophilic tail is impossible or very difficult to hydrate (oil soluble). Emulsifiers concentrate at the interface between oil and water to reduce the surface or interfacial tension, thereby making the emulsion more stable. In many industries emulsifiers are referred to as 'surfactants' or biosurfactants which is an abbreviation of surface active agents varying in chemical properties and molecular size (Chanlarfah, 2003). While the low molecular weight surfactants are often glycolipids, the high molecular weight surfactants are either polyanionic containing covalently-linked hydrophobic side chains or complexes containing both polysaccharides and proteins.

Microorganisms produce bioemulsifiers or biosurfactants to mediate solubilization of hydrophobic compounds in their environment to be able to utilize them as substrates (Margesin and Schinner, 2001), however, this fact may not be always true. Few microbes produce bioemulsifiers or biosurfactants on water soluble substrates (Gunther *et al.,* 2005). Due to their interesting properties such as lower toxicity, higher biodegradability, higher foaming capacity and higher activity at extreme temperatures, pH levels and salinity, bioemulsifiers have been increasingly attracting the attention of the scientific community as promising candidates for the replacement of a number of synthetic emulsifiers (Banat *et al*., 2000). They can be produced by a wide diversity of bacteria, actinomycetes (Kokare *et al*., 2007), yeasts and fungi. Generally, polymeric bioemulsifiers are released in the extracellular medium of the fermentation broth (Boyle and Reade 1983). However, they can also be attached to the cell surface (Dikit *et al*., 2010). Bioemulsifier production by actinomycetes has been reported in very few cases. A glycolipids based emulsifier has been obtained from *Rhodococcus erythropolis* and *R. aurantiacus*. While surface active lipids based bioemulsifier obtained from *Nocardia erythropolis* (Kretschmer *et al*., 1981). Although several fungi, archaea and bacteria have been reported, but the work on endophytic actinomycetes, still needs sincere attention. So far, only few studies have been undertaken to assess the incidence of endophytic actinomycetes. Endophytic actinomycetes have attracted attention in the search for novel bioactive natural compounds that can be used as new drugs replacing those against which pathogenic strains have rapidly acquired resistance. Endophytic actinomycetes have been isolated from stem and root interior of many plants, such as snakevine, tomato, banana, wheat, medicinal plants etc. (Castillo *et al*., 2002). The association of actinomycetes with plants found to confer many advantages such as the production of anti-microbials, extracellular enzymes, phytohormones and siderophores. They also help in phosphate solubilization and plant protection against abiotic and biotic stresses (Bailey *et al*., 2006). In the literature search, there were no reports on bioemulsifier production from endophytic actinomycetes. Keeping in view the scarcity of work in this regard, the present investigation was undertaken to isolate endophytic actinomycetes of selected medicinal plants and to explore their potential to produce bioemulsifier(s) that could have commercial importance.

Materials and Methods

Collection of plant samples and isolation of endophytic actinomycetes

In order to isolate the endophytic actinomycetes, parts of different medicinal plants including leaves, roots and stems were collected from the medicinal plants garden of Panjab University, Chandigarh, India. The isolation of endophytic actinomycetes from the leaves, roots and stems of the plant was made as per method of Arunachalam *et al., (*2010). In this method healthy samples were washed by running tap water for 1-2 minutes to remove the soil

particles completely. The resultant were subjected to a five-step surface sterilization procedure in which 4 to 10-min wash in 5%NaOCl, followed by 10-min wash in 2.5% $Na_2S_2O_3$, a 5-min wash in 75% ethanol, 2 min wash in sterile water, and a final rinse in 10% $NaHCO_3$ for 10 min to disrupt the plant tissues and inhibit the fungal growth was done. A control was also maintained in other plate to observe the appearance of probable epiphytes by spreading the final washed solution onto modified Con's agar (K_2HPO_4:0.05gms, $MgSO_4.7H_2O$:0.2gms, dextrose:1gms, gelatin:1.5gms, agar-agar:20gms, distilled water:1000ml, pH:7) (Ravikumar *et al*., 2011). Incubated the plates at 28±1°C and observed for the appearance of any epiphyte to validate the surface sterilization protocol. After the sterility check, the surface sterilized tissues were crushed in sterile pestle and mortar and crushed materials were spread on the plates containing modified Con's agar medium. Plating was done in duplicates and all the plates were incubated at 28±1°C. Observations were made daily until the growth appeared. The pure culture of all the isolates of actinomycetes were maintained and preserved on actinomycetes isolation agar (AIA) medium (Himedia).

Screening of endophytic actinomycetes for bioemulsifying activity

The screening of endophytic actinomycetes for bioemulsifying activity was done as per method of Kokare *et al*. (2007) by growing the successfully isolated cultures separately in maltose yeast extract broth (MYE) (maltose :10gms, glucose :10gms, yeast extract: 5 gms and NaCl 5gms and distilled water: 1000ml) under shaking at 120 rpm and pH7 for seven days. Cells were separated after respective incubation period by centrifugation at 10,000rpm for 15 min at 30±0.5°C. Cell free supernatant (3ml) was mixed with 0.5 ml oil (almond, olive, mustard, sunflower, groundnut, sesame) and hydrocarbon (kerosene, xylene and petrol) by vortexing vigorously for 2 min and incubated at 30°C for 1h for phase separation. Aqueous phase was removed carefully and its absorbance was recorded at 400nm. The blank was prepared separately by replacing the cell free supernatant with sterile MYE broth. An absorbance of 0.010 units at 400nm multiplied by dilution factor, if any, was considered as one unit of emulsification activity per ml (EU/ml) (Patil *et al*., 2001).
The isolate giving the maximum emulsification activity was selected for further studies regarding bioemulsifier production.

Identification of the screened out endophytic actinomycete

The pure isolate of the selected endophytic actinomycete was identified to its genus level by performing biochemical (Glucose, Arabinose, Mannitol, Sucrose, Rhamnose, Maltose, Hugh leifson (HL), Catalase, Nitrate reduction, Triple sugar iron agar, Indole, Methyl red, Voges prausker Citrate, Lysine, Ornithine, Arginine, Urease and PPA), cultural (color, size, aerial mass color, pigments, texture, surface, shape, margin of the colony) and microscopic (hyphal details, spore shape, branching pattern etc.) studies of taxonomic importance following as per Waksman (1959).

Optimization of cultural parameters for enhancing bioemulsification activity

The optimization studies for enhancing the bioemulsification activity was done in a series of experiments by changing one variable at a time approach and keeping the other factors fixed at a specific set of conditions (production medium: maltose yeast extract broth; incubation period: 7 days; temperature: 30±0.5°C; pH: 7 and agitation: 120 rpm).All the experiments were performed in triplicates and at the end of each experiment emulsification activities were determined by calculating Emulsification index (EI). For this, the Oil/hydrocarbon (as mentioned before) was added to culture broth (1:1 v/v), vortexed for 2 min and allowed to stand for one hour. Control was prepared by

replacing the culture broth with sterile broth. EI was calculated by the height of emulsion measured by taking the layer formed in between aqueous and oil/hydrocarbon layer divided by total length of the liquid (Ellaiah *et al*., 2002) and multiplied by 100 which give rise to the EA and expressed in EA% and mean standard deviation (SD) were calculated.

Optimization of physical parameters

This included the optimization studies for the best medium, optimum time course that is incubation period of bioemulsifier assay, temperature, pH, and agitation. Optimization for selecting the best production medium was done by carrying the fermentation separately in different media i.e. Maltose yeast extract broth (MYEB)-Maltose:10gms, Glucose: 10gms, Yeast extract:5gms, NaCl:5gms and Distilled water:1000ml); Glycerol yeast extract broth (GYEB)-Yeast extract: 2 gms, Glycerol:10ml, Distilled water: 1000ml); Luria broth(LB)-Casein enzymic hydrolysate: 10 gms, Yeast extract :5gms,NaCl: 5 gms and Distilled water 1000ml); Tryptone broth (TYEB)-Tryptone:10gms, Yeast extract:5gms, NaCl:5gms, Distilled water:1000ml and nutrient broth (NB)- Peptic digest of animal tissue: 5gms, NaCl: 5gms, Beef extract: 1.5 gms, Yeastextract:1.5gms and Distilled water:1000ml). For incubation period the isolate was grown at different incubation periods ranging from 3 to 9 days. The optimum value of pH was standardized by taking different values of pH i.e., 5, 6, 7, 8 and 9. The pH values were adjusted by 1N HCl or 1N NaOH. Temperatures taken for optimization were 27, 29, 31, 33, 35, 37 and 40±1°C. While the agitation speed used for optimization were 120, 130, 140, 150, 160 and 170 rpm.

Optimization of biochemical parameters

The biochemical parameters taken for optimization were different sources of carbon and nitrogen. A total of nine different carbon sources (xylose, dextrose, lactose, mannitol, fructose, maltose, sucrose, starch and cellulose) and seven different nitrogen sources (ammonium nitrate ($NH_4NO_{3)}$, ammonium chloride (NH_4Cl), ammonium sulphate ($NH_4SO_{3)}$, peptone, yeast extract, sodium nitrate ($NaNO_3$), and potassium nitrate (KNO_3) were used.

Extraction and partial purification of bioemulsifier

The bioemulsifier produced in the optimized medium by the test organism was extracted from the cell free broth by solvent extraction method of Maneerat and Dikit, (2007). In this method, the test bioemulsifier in the supernatant was extracted by mixing with an equal volumes of chloroform: methanol in the ratio of 1:1 and mixed in separatory funnel, shaken vigorously and allowed to stand for 30 min. The aqueous layer formed at the bottom of the separatory funnel was removed and the white layer of bioemulsifier just above the aqueous layer was collected in a glass petridish and left in oven at (40-45°C) till dryness for evaporation of solvents, then the bioemulsifier was collected by scrubbing and preserved in a clean screw capped glass vial as partially purified dried powder.

Determination of emulsification potential of test bioemulsifier with different oils and hydrocarbons

Emulsification potential of the test bioemulsifier was determined as per modified method of Cameron *et al*., (1988). For this 1ml of each test oils (almond, mustard, olive, and groundnut, sunflower and sesame oil)and hydrocarbons (kerosene, petrol and xylene) was added separately to 1ml of 1.5 %(w/v) of the test bioemulsifier and vortexed for 3min. The emulsification activity (EA%) was determined after 1hour whereas the emulsification index (EA%) representing the emulsion stability was determined after 24, 48, 72 and 96 hours The EA% and $\%EA_{24\text{-}96}$were calculated by dividing the measured height of emulsion layer by the total height of the reaction mixture.

Determination of emulsion type

The type of emulsion formed by the test bioemulsifier was determined as per drop dispersion method of Rieger (1986). In this method a Whatman filter paper no. 1 was used to determine the emulsion type. If the emulsion is water-in-oil (w/o) type, the droplet of emulsion remains as a droplet on the filter paper. While in case, the emulsion is oil-in-water (o/w) type, it disperses rapidly on the filter paper.

Determination of temperature, pH and NaCl stability of emulsion

The temperature, pH and NaCl stability of the emulsion formed by the test bioemulsifier was determined as per the method of Dixit *et al*. (2010). For this 1% (w/v) of the test bioemulsifier was used. For determination of temperature stability, the test bioemulsifier was dissolved in distilled water and the emulsion thus formed was exposed to different temperatures (20, 40, 60, 80 and 100) °C. While the pH stability of the emulsion formed by the test bioemulsifier was determined by incubating the emulsions at different pH like 2, 4, 6, 8 and 10 for 30 min at 30±0.5°C. The salt stability was determined at different concentrations of NaCl (i.e. 1, 2, 3, 4 and 5%). The stabilities of these parameters were also compared with the standard emulsifier, gum Arabic.

Characterization of test bioemulsifier

The bioemulsifier produced by the test actinomycete isolate was characterized for various parameters including solubility of the bioemulsifier which was determined in various organic solvents (ethanol, methanol, chloroform, petroleum ether and acetone, water). The solubility of the partially purified bioemulsifier was determined by immersing the 2mg of the bioemulsifier in different solvents and compared with the standard emulsifier, gum Arabic. The solubility was observed after 24 hours and result was described as '+' if it was completely soluble, '*' and '-' for partially soluble and insoluble respectively. The surface tension property of the test bioemulsifier was measured using a tensiometer at room temperature. The concentration at which micelles began to form was represented as the critical micelle concentration (CMC). At the CMC, sudden changes in surface tension, electrical conductivity and detergency were observed. The CMC was automatically determined by measuring the surface tension of the bioemulsifier in distilled water till the constant value was obtained. Scanning electron microscopy of the test bioemulsifier was also done to determine various surface related properties. While the FTIR analysis was performed to detect various functional groups present in the chemical structure of the test bioemulsifier. The elemental analysis was performed to determine the presence of various organic elements in the test bioemulsifier. Besides, the test bioemulsifier was also analyzed for chemical composition including the protein, carbohydrates and lipid contents as per methods of Lowry *et al.,* (1951), Dubois *et al*., (1956) and Frings and Dunn (1970) respectively.

Evaluation of test bioemulsifier to remove heavy oil from contaminated water and cream from full cream milk

The ability of the test bioemulsifier to remove the heavy oil from oil contaminated water and cream from full cream milk was determined as per method of Joaad, (2002) in this method a sample of river water (100 ml) was contaminated by adding 20 ml of heavy oil (xylene) and mixed vigorously for two hours to insure a good mixing of oil in water. Then, 25 ml of distilled water containing (5 mg/ml) of the test bioemulsifier was mixed with equal volume (25 ml) of the contaminated water, shaken for 15 min, the excess and floating oil was removed. The effect of addition of the bioemulsifier on the clarity, colour and smell of contaminated water were observed. The river water and heavy oil were used as control. The ability of the test bioemulsifier to remove the cream from full cream milk was also determined. For this, a five ml of full fat milk was mixed with 25

mg of test bioemulsifier, shaken vigorously and separation of the cream layer was observed. The full cream milk was taken as control.

Results and Discussion

Isolation of endophytic actinomycete

The isolation of different endophytic actinomycetes were made from the plant samples collected from the medicinal plant garden of Panjab University, Chandigarh, India. The collected plant materials were taken to the laboratory and processed for isolation of endophytic actinomycetes. Processing of different medicinal plant samples of *Catharanthus roseus (Vinca rosea*/Sadabahar*), Ocimum sanctum (*Tulsi*), Andrographis paniculata (*Kalmegh*) and Azadirchta indica* (Neem) resulted in isolation of seven different endophytic actinomycetes (Table 1). A control (efficacy of surface sterilization) plate was also maintained by spreading the final distilled wash water (after surface sterilization) on modified Con's medium. Absence of microbial growth on control plates indicated the effectiveness of surface sterilization. All the seven endophytic cultures obtained were isolated successfully into pure form and maintained on AIA medium.

Table 1: List of seven endophytic actinomycetes cultures isolated from different parts of themedicinal plants.

S. No.	Isolates number	Host plants	Plant parts
1	EARN1	*Catharanthus roseus (Vinca rosea)*	Root
2	EARN2	*Catharanthus roseus (Vinca rosea)*	Stem
3	EARN3	*Catharanthus roseus (Vinca rosea)*	Leaf
4	EARN4	*Ocimum sanctum (*Tulsi*)*	Leaf
5	EARN5	*Andrographis paniculata (*Kalmegh*)*	Leaf
6	EARN6	*Andrographis paniculata (*Kalmegh*)*	Leaf
7	EARN7	*Azadirchta indica* (Neem)	Leaf

Table 2: Emulsification activities of seven isolates of endophytic actinomycetes with different oils and hydrocarbons.

S. No.	IsolateNo.	Almond (EU/ml)	Mustard (EU/ml)	Olive (EU/ml)	Sunflower (EU/ml)	Groundnut (EU/ml)	Sesame (EU/ml)	Petrol (EU/ml)	Kerosene (EU/ml)	Xylene (EU/ml)
1	EARN1	70 ±0.01	40.9 ±0.03	76.5 ±0.01	135.9 ±0.01	96.7 ±0.01	89.2 ±0.01	100.7 ±0.01	91.7 ±0.02	88.2 ±0.01
2	EARN2	95.9 ±0.02	132. 6±0.01	94.8 ±0.03	86.5 ±0.02	83.8 ±0.03	77 ±0.02	77.3 ±0.04	82.2 ±0.02	78.8 ±0.01
3	EARN3	78 ±0.01	48.4 ±0.02	170.8 ±0.02	130.6 ±0.01	94.1 ±0.02	57 ±0.03	118.3 ±0.01	145. 7±0.03	76.8 ±0.01
4	EARN4	92.7 ±0.03	98.8 ±0.03	128.2 ±0.01	100.4 ±0.01	141 ±0.01	45.8 ±0.03	143 ±0.02	140.4 ±0.01	77 ±0.01
5	**EARN5**	**106.2 ±0.02**	**144.4 ±0.02**	**180 ±0.01**	**133 ±0.02**	**155.5 ±0.01**	**56.7** ±0.01	**149 ±0.01**	**147.2 ±0.01**	**87** ±0.01
6	EARN6	100.2 ±0.01	49.7 ±0.04	79.8 ±0.02	88.3 ±0.02	70.4 ±0.02	67.5 ±0.01	64.9 ±0.03	121.6 ±0.03	63 ±0.01
7	EARN7	60 ±0.01	35.1 ±0.03	65.8 ±0.01	78.2 ±0.01	71.4 ±0.01	45.8 ±0.01	84.4 ±0.04	83.6 ±0.01	65 ±0.01

EU/ml: emulsification activity per ml. Values are expressed as mean SD of triplicate observations

Screening for bioemulsification activity

All the successfully isolated seven endophytic isolates of actinomycetes were screened out for presence of emulsification activity. The result obtained revealed that all the isolates were capable of producing bioemulsifier and showed the significant emulsification activity with all the oils and hydrocarbons used. However, the isolate No EARN5 was found to be the most efficient producer of bioemulsifier having the maximum emulsification activity with olive oil (vegetable oil) and petrol (hydrocarbon)as 180±0.01 and 149±0.01 EU/ml respectively (Table 2; Fig. 1).

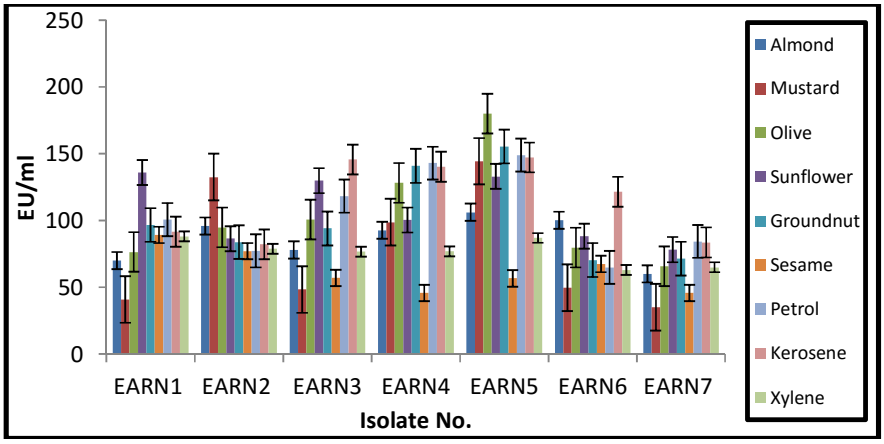

Fig. 1: Emulsification activities of 7 isolates of endophytic actinomycetes with different oils andhydrocarbons.*EU/ml: emulsification activity per ml. Values are expressed as mean SD of triplicate observations.*

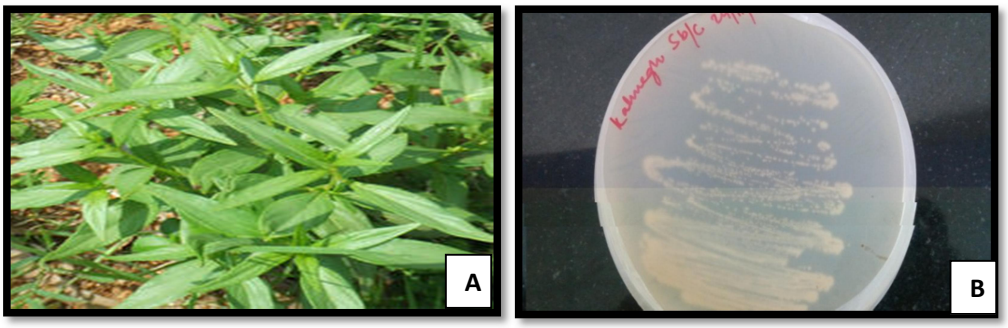

Fig. 2.i) A): Host plant (*Andrographis paniculata*); B): Colony of pure endophytic actinomycete (*Actniomyces* sp.) culture on modified Con's agar medium.

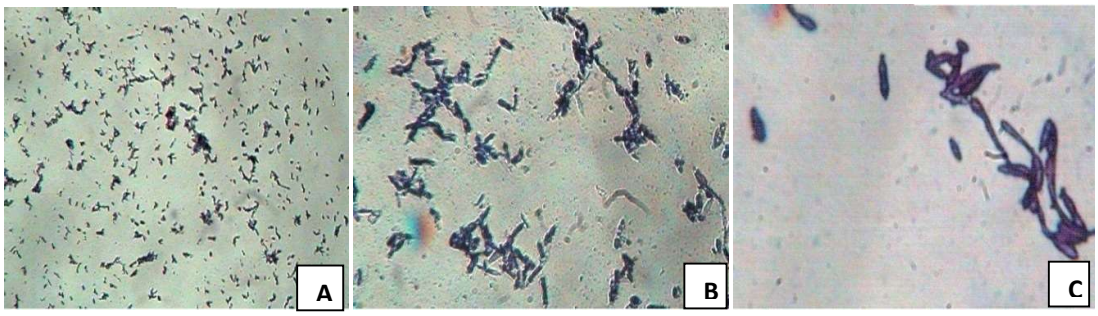

Fig. 2.ii) A: Microscopic details of the endophytic actinomycetes: A) at 10X; B) at 45X; C) at 100X.

Table 3: Cultural, microscopic and biochemical characteristics of the selected endophytic culture of *Actinomyces sp.* EARN5.

S.No.	Identification parameters	Isolate No. EARN5
Cultural characteristics		
1	Colony color and shape	White, Round
2	Colony diameter	2-3mm
3	Presence of spores	Present
4	Spore surface aerial spore mass color	White
5	Pigments	
	a. Reverse side pigment	Negative
	b. Melanoid pigment	Negative
	c. Diffusible pigment	Negative
Microscopic characteristics		
1.	Gram stain	Positive
2.	Spore	Present
3.	Hyphae	Pseudohyphae
4.	Length of Hyphae	Short
5.	Arrangement	Cluster
6.	Width	1-2mm
7.	Filament	Present
Biochemical Characteristics		
1	Growth range	5-9pH
2	Sugars	
	a. xylose	Positive
	b. Glucose	Acid
	c. Arabinose	Positive
	d. Mannitol	Negative
	e. Sucrose	Positive
	f. Rhamnose	Negative
	g. Maltose	Positive
3	Hugh leifson(HL)	Positive
4	Catalase	Negative
5	Nitrate reduction	Positive
6	Triple sugar iron agar	K/A
7	Indole	Negative
8	Methyl red	Negative
9	Vogesprausker	Negative
10	Citrate	Negative
11	Lysine	Positive
12	Ornithine	Positive
13	Arginine	Negative
14	Urease	Negative
15	PPA	Negative
16	Motility	Non motile

Identification of selected endophytic actinomycete

The isolate of actinomycete EARN5, which was screened out as the best bioemulsifier producing isolate on the basis of its maximum emulsification activity was tentatively found to belong to genus *Actinomyces* as per studies made on its specific cultural, microscopic and biochemical characteristics (Fig. 2i & 2ii; Table 3).

Optimization of physical and biochemical parameters for bio -emulsifier production

The optimization of various physical parameters (media, incubation period, temperature, pH and agitation speed) revealed the maltose yeast extract broth (MYEB) as the best production medium giving the maximum emulsification activity 42.5±0.01EA% (Fig. 3).While a period of 7 days was found to be best giving the maximum emulsification activity 46.5±0.01EA% (Fig. 4). Regarding rest of the parameters, the temperature of 29±1°C was found to be optimum with the maximum emulsification activity of 47.4 ±0.01 EA% (Fig. 5).

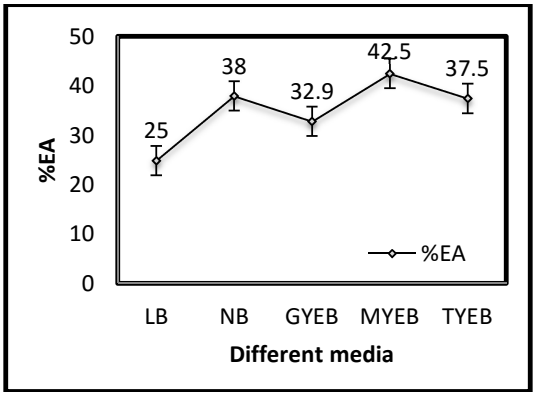

Fig. 3: Effect of various production media on the bioemulsifier production by endophytic isolate of *Actinomyces sp.* EARN5. *Culture conditions: Incubation period: 7 days; Temperature: 29±1°C; pH: 7; Agitation speed; 120rpm Carbon source: Maltose; Nitrogen source: Yeast extract. Values are mean SD of triplicate experiments.*

While, the pH of 7 was found to support the maximum emulsification activity (48.21±0.02 EA%). However, it decreased steeply with the increase in pH from 7-9 (Fig. 6). Similar results were observed by Patil&Chopade, (2001) while working on bioemulsifier production by *Actinopolyspora juni* SC14 which showed the maximum bioemulsifier activity at slightly alkaline pH (7.2). Regarding agitation speed, the fermentation carried out at 130rpm gave the maximum emulsification activity (Fig.7).The result obtained on biochemical parameters revealed the cellulose and yeast extract as the best sources of carbon and nitrogen giving the maximum emulsification activity 55.6±0.1 and 56.2±0.1EA% respectively (Fig. 8 & 9).

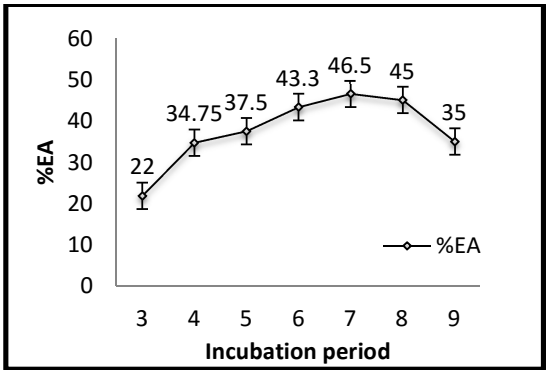

Fig. 4: Effect of different incubation days on the bioemulsifier production by endophytic isolate of *Actinomyces sp.* EARN5. *Culture conditions: Medium: Maltose yeast extract broth; Temperature: 30±1°C: pH: 7; Agitation speed: 120rpm; Carbon source : Maltose; Nitrogen source : Yeast extract. Values are mean SD of triplicate experiments.*

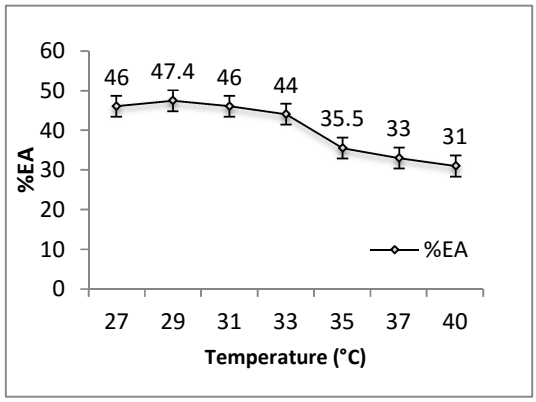

Fig. 5: Effect of different incubation temperatures on the bioemulsifier production by endophytic isolate *Actinomyces sp* EARN5. *Culture conditions: Medium: Maltose yeast extract broth; Incubation period: 7 days; pH: 7; Agitation speed: 120rpm; Carbon source: Maltose; Nitrogen source : Yeast extract. Values are mean SD of triplicates experiments*

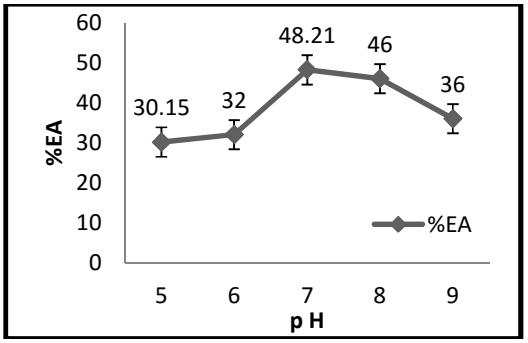

Fig. 6: Effect of different pH on the bioemulsifier production by endophytic isolate of *Actinomyces sp*. EARN5**.** *Culture conditions : Medium: Maltose yeast extract broth; Incubation period: 7 days; Temperature: 29±1°C; Agitation speed: 120rpm; Carbon source: Maltose; Nitrogen source : Yeast extract. Values are mean SD of triplicates experiments.*

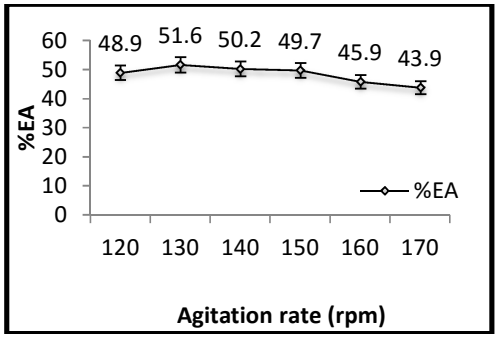

Fig. 7: Effect of different agitation rate on the bioemulsifier production by endophytic isolate of *Actinomyces sp.* EARN5**.***Culture conditions: Medium: cellulose yeast extract broth; Incubation period: 7 days; Temperature: 29±1°C; pH: 7 Agitation speed: 130rpm; Carbon: Cellulose. Values are mean SD of triplicates experiments.*

Extraction and partial purification of the crude bioemulsifier

The bioemulsifier produced by *Actinomyces sp* EARN5 was extracted by using chloroform and methanol in the ratio of 1:1. This resulted in extraction of white to cream colour, finely granulous, powder which was used further for characterization purpose.

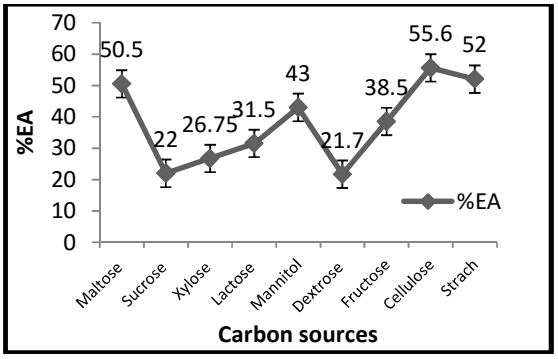

Fig. 8: Effect of different carbon sources on the bioemulsifier production by endophytic isolate pf *Actinomyces sp*. EARN5.*Culture conditions: Incubation period: 7 days; Temperature: 29±1°C; pH: 7; Agitation speed: 130rpm; Nitrogen source : Yeast extract. Values are mean SD of triplicates experiments.*

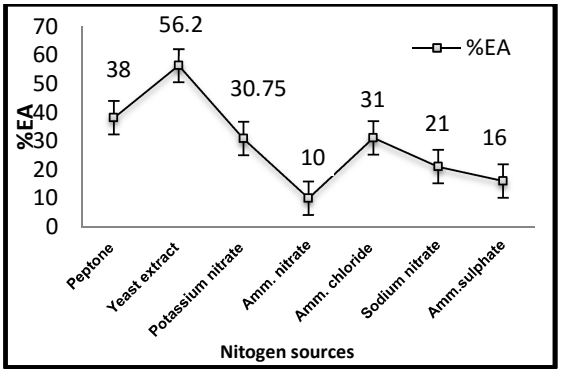

Fig. 9: Effect of different nitrogen sources on the bioemulsifier production by endophytic isolate *Actinomyces sp*. EARN5.*Culture conditions: Medium: Maltose yeast extract broth; Incubation period: 7 days; Temperature: 29±1°C; pH: 7; Carbon source: Maltose; Nitrogen source : Yeast extract. Values are mean SD of triplicates experiments.*

Emulsification potential of test bioemulsifier with different oils

The result obtained revealed the emulsification activity of the test bioemulsifier was quite fair with all the oils and hydrocarbons. However, it was found to be maximum (54.5%) with olive oil and remained stable for 72 hours which was the maximum as compared to the stability with other oils and hydrocarbons (Fig. 10).

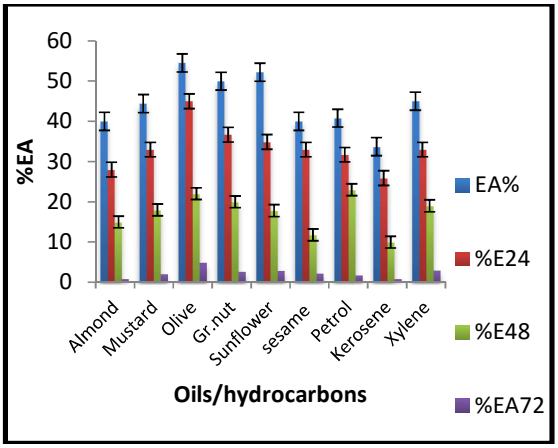

Fig.10: Emulsification activity and stability of the test bioemulsifier with different oils and hydrocarbons. *Values are expressed as mean SD of triplicate experiments*

Determination of Emulsion Type

Type of emulsion formed by the test bioemulsifier was determined as per drop dispersion method. The result obtained revealed the emulsion formed by the test bioemulsifier with olive oil was water in oil type (w/o) type. The test emulsion did not disperse on the filter paper confirmed it as water –in –oil emulsion (Fig. 11).

Fig. 11 : Drop dispersion test showing the test bioemulsifier was water in oil type as the test bioemulsifier remained in drop form at the end of the test.

Determination of temperature, pH and NaCl stability of the test bioemulsifier

The temperature stability of the emulsion formed by the test bioemulsifier found maximum (40±02) EA% between 60 and 80°C. (Fig.12). Thus, it was concluded that the stability of the emulsion was much affected by the temperature due to hydrolytic action at higher temperatures. Shafeeq *et al.*, (1989), also reported the maximum emulsification activity between 60 and 70°C while working on bioemulsifier production by *Pseudomonas sp.*

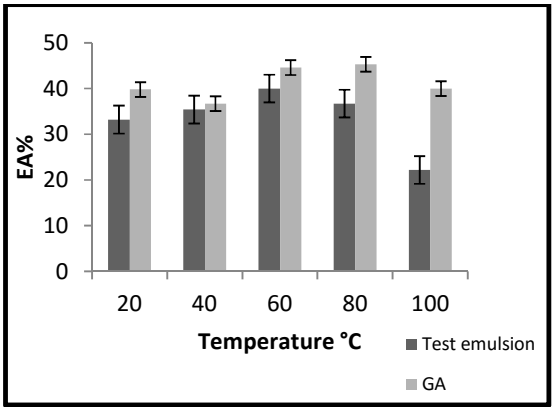

Fig. 12: Effect of different temperatures on the stability of emulsion formed by the test bioemulsifier. *Values are mean SD of triplicate experiments.*

Regarding pH, the emulsion was found stable between pH 2 and 6 (Fig. 13) and remained most stable at pH 6 with constant emulsifying activity of 42±0.01EA% up to 24hrs.

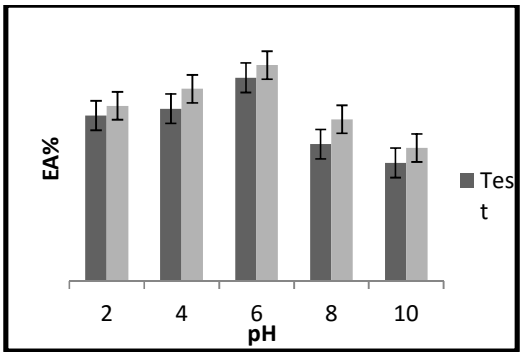

Fig. 13: Effect of different pH values on the stability of emulsion formed by the test bioemulsifier. *Values are mean SD of triplicate experiments*

Effect of NaCl stability carried out on 1% (w/v) of the test emulsion revealed the 3% concentration (w/v) of NaCl as the

optimum and gave the maximum emulsification activity of (45±0.02) EA % and this was in range of emulsification activity of the standard emulsifier, the gum Arabic, which was showing the emulsification activity between 2-4% of NaCl concentration (Fig. 14).A decrease in emulsification activity was observed with further increase in concentration of NaCl. Maniyar *et al.*, (2012) also reported the similar results, while working on production of bioemulsifer by *Rhodococus ruber* Z25 and *Streptomyces sp*. S1 they observed bioemulsifier stability at NaCl concentration of 2.5 and 3% respectively.

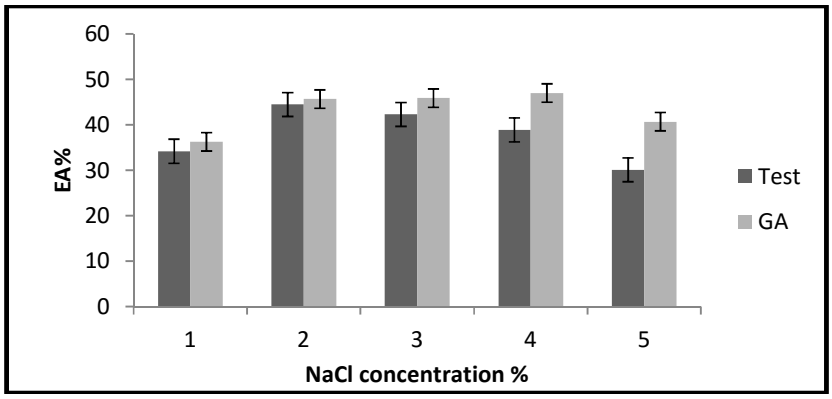

Fig. 14: Effect of different concentrations of NaCl on the emulsion formed by the test bioemulsifier.

Characterization of test bioemulsifier

The test bioemulsifier was also characterized for important parameters including the solubility, which was evaluated by dissolving it in various organic solvents and it was found partially soluble in water, completely soluble in chloroform: methanol in the ratio of 1:1 and insoluble in acetone, benzene, ethanol (Table 4). It can be concluded from the results obtained that the test bioemulsifier was very much comparable with Gum Arabic, the standard, in terms of solubility.

Table 4: Solubility with different organic solvents

S.NO.	Solvent	Standard Gum Arabic	Test bioemulsifier
1	Water	**+**	*****
2	Ethyl alcohol	*****	*****
3	Chloroform: methanol	*****	**+**
4	Acetone	+	**-**
5	Benzene	**-**	**-**
6	Petroleum ether	**-**	**-**

*+: Completely soluble; *: Partially soluble; -: Insoluble*

Determination of surface tension and critical micelle formation, which is the concentration of the bioemulsifier at which the formation of micelles take place. And at this or more concentration no further decrease in surface tension occurs. It is evident from the results obtained that the surface tension of the test bioemulsifier was 45.6 Dynes/cm and the CMC was 450mg/L and corresponds to the surface tension value of 65.5 Dynes/cm. From the present study it can be concluded that the

bioemulsifier produced by *Actinomyces Sp.* EARN5 reached saturation condition at CMC which leads to change in arrangement of hydrophophic moieties toward the medium and hydrophilic moieties towards liquid solution resulted in stability of surface tension at this concentration. The test bioemulsifier was also analyzed under SEM for its various surface properties and the result obtained revealed that the particles of the test bioemulsifier were irregular in shape, rough and contained pores which were responsible for its characteristic solubility and water holding capacity (Fig. 15).

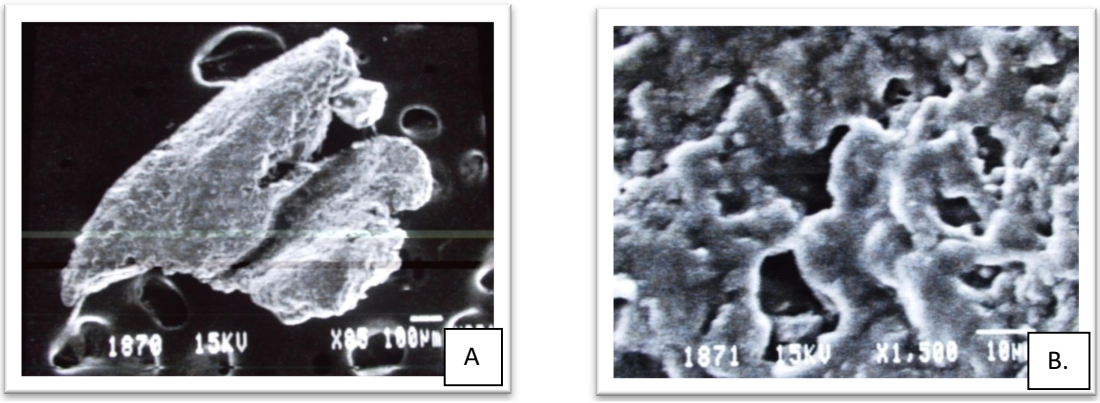

Fig. 15: SEM photomicrograph of the test bioemulsifier showing irregular, porous and rough surface of the particles: A) at 85 and B) at 1500X.

The studies pertaining to FTIR spectroscopy of the test bioemulsifier showed the presence of the different functional groups like OH, CH, CC , CN, NO, carboxylate and C-halides at 3397.12 cm^{-1}, 2923.17, 2852.23 cm^{-1}, 1654.16 cm^{-1}, 1033.14, 1142.11 cm^{-1}, 1550.24 cm^{-1}, 1076.14, 1109.11 cm-1, 637.22 , 704.30 and 758.35 cm^{-1} respectively (Fig. 16).

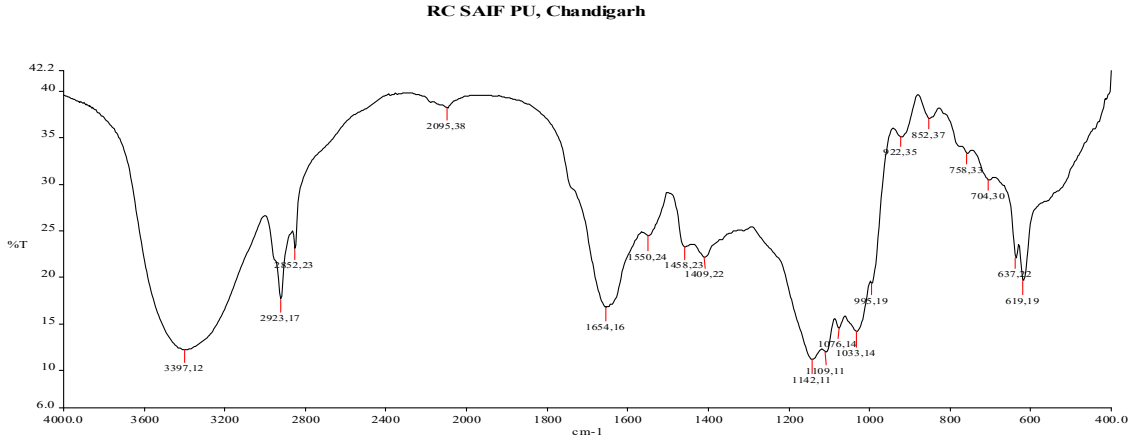

Fig. 16: FTIR spectrum of the test bioemulsifier produced by an endophytic isolate of *Actinomycetes sp.*EARN5

The elemental analysis revealed the presence of Nitrogen (2.464%), Carbon (35.151%) and Hydrogen (5.494%) in the test bioemulsifier (Fig.17). While the chemical analysis revealed the 8.6% protein, 9.06% carbohydrate and 76.9% lipid content in the test bioemulsifier.

Retention Time (min)	Component Name	Element %
0.817	Nitrogen	2.464
1.250	Carbon	35.151
3.042	Hydrogen	5.494
		43.110

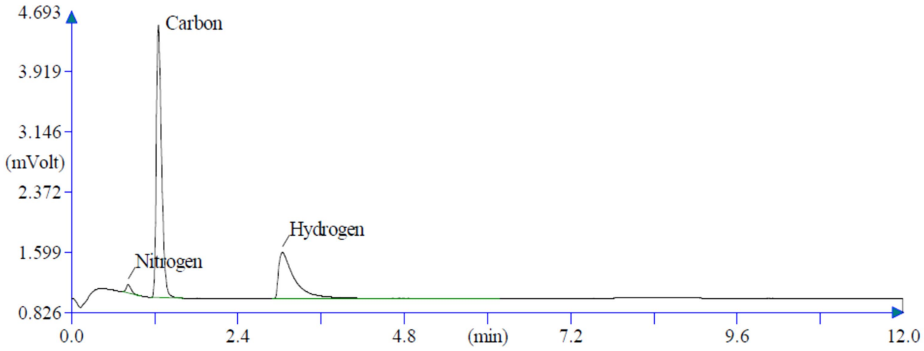

Fig.17: Carbon, Hydrogen, and Nitrogen (CHN) analysis of the test bioemulsifier.

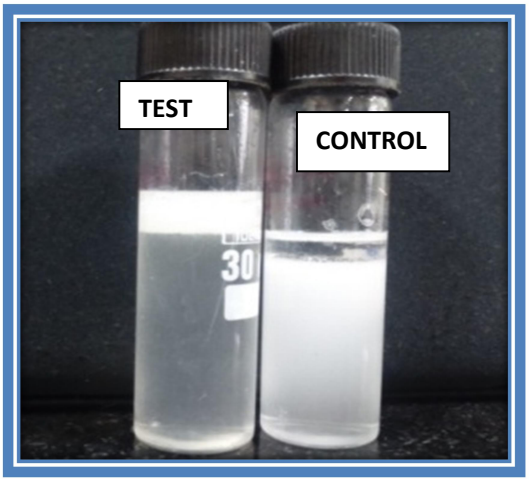

Fig.18: The test bioemulsifier of endophytic *Actinomyces sp* EARN5 removing oil from oil contaminated water. *TEST: After adding the bioemulsifier; CONTROL: Before adding the bioemulsifier.*

Evaluation of test bioemulsifier for removing oil from oil contaminated water and cream from full cream milk

The potential of test bioemulsifier for removing oil from contaminated water and cream from full cream milk was also studied and the result obtained revealed the bioemulsifier was quite efficient in removing oil from contaminated water which was evident from the turbidity and oily smells of the oil contaminated water which decreased or nearly disappeared when mixed with the test bioemulsifier (Fig. 18). And the cream content also got separated from the full cream milk on mixing with the test bioemulsifier (Fig. 19).

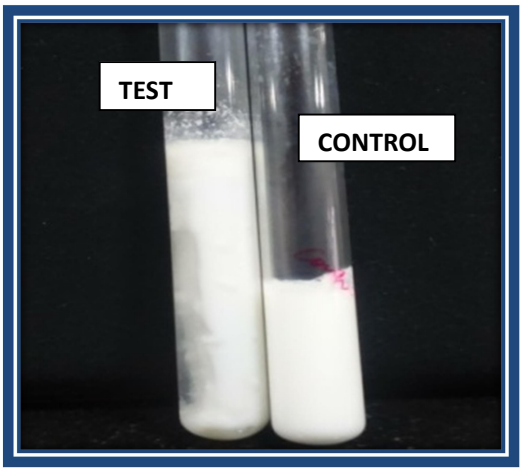

Fig. 19: The test bioemulsifier of endophytic *Actinomyces sp.* EARN5 removing the cream from full cream milk. *TEST: After adding the bioemulsifier; CONTROL: Before adding the bioemulsifier.*

Conclusion

Present study explored the production of bioemulsifier by an endophytic actinomycete, (*Actinomyces* EARN5) isolated from *Andrographis paniculata.* The bioemulsifier produced was able to remove oil from contaminated water and cream from full cream milk. Thus, adding the new perspective to endophytic actinomycetes, the research on which is very scarce according to available literatures. Microbial derived polymeric biosurfactants /bioemusifiers have many interesting functional properties such as the ability to reduce the surface and interfacial tension, emulsification and de-emulsification capacities, the foaming potency, dispersing capacity, the solubilization and mobilization abilities etc. These permitted their multifaceted applications in environmental, pharmaceutical, industrial and agricultural sectors. Owing to these fascinating properties and wide spectrum applications there is need to screen more isolates of actinomycetes especially from newer biotopes and to evaluate their properties which may lead to the development of novel value added products of microbial origin.

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