International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-2 (2015) pp. 65-81**
http://www.ijcmas.com

Original Research Article

Isolation, Characterization and Screening of Bioemulsifier Producing Micro-Organisms from Soil Sample (Kaas Pathar, Satara, India) and its Application

Chaitrali Atre, Tanushree Bhandari, Madhuri Kothawade and Shilpa Mujumdar*

Department of Microbiology, Modern College of Arts, Science and Commerce, Shivajinagar, Pune-05, India
**Corresponding author*

A B S T R A C T

The study on bioemulsifier aims to screen bacterial strains isolated from rhizopspheric soil from Kaas Pathar (Satara, India) for bioemulsifier production. The strains isolated from soil sample collected from Kaas Pathar (Satara, India) were characterized. 10 bacterial strains were isolated out of which 7 bacterial strains are Gram positive and remaining 3 bacterial strains are Gram negative. The 3 Gram negative strains (Kp1, Kp2 and Kp3) were further characterized based on capsule staining, motility and biochemical tests. The Gram Negative bacterial strains were used in screening for bioemulsifier production using emulsification assay and emulsification index which was carried out by using Groundnut oil, Soyabean oil, Petrol, Kerosene and Machine oil as substrates. All the three strains (Kp1, Kp2 and Kp3) after 72h showed maximum value for emulsification assay which was about 4.0 Eu/ml and emulsification index was about 40% for Kp1 while 44% for Kp2 and Kp3. The partially purified bioemulsifier was used further for the studies such as anti-microbial activity, antibiotic activity, anti-fungal activity and anti-diabetic activity.

Keywords

Bioemulsifier, Antibiotic activity, Antifungal activity and Anti Diabetic activity

Introduction

Bioemulsifiers are amphipathic polymers while biosurfactants are surface active chemicals produced by large number of bacteria, yeast and fungi (Letizia Fracchia *et al.*, 2012). Due to their unique structures, physicochemical properties, rheological properties, surface active and chemical composition bioemulsifiers find applicability in different fields (Letizia Fracchia *et al.*, 2012). Bioemulsifiers are widely used in food industry, agriculture industry, pharmaceuticals industry, petro chemistry, paper and pulp industry (Mujumdar, 2002). The development of this line of research is of immense importance mainly due to the present concern regarding the protections of the environment which is getting amplified day after day that is resulting into natural calamities that ultimately results into deterioration of human health and the environmental factors that is generating adverse effects. They are known to increase bioavailability of carbon source (Banat *et al.*, 2008). Depending upon their chemical structure bioemulsifiers has been classified as high molecular weight

(BE) and low molecular weight (BS) compounds. Bioemulsifiers are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex of these mixtures. Biosurfactants are glycolipids or lipopeptides in which rhamnolipids, trehalolipids and sophorolipids compounds predominate (Rosenberg *et al.,* 2002).

Many microorganisms such as *E.coli, Acinetobacter junni, Aeromonas caviae, Pseudomonas fluorescens, Klebsiella pneumonia*, *Bacillus* sp synthesize an extracellular polymeric substances called as bioemulsifier (Cameron *et al.,* 1988). Most of the bioemulsifier produced by the microorganisms are consider to be the secondary metabolites but some may play a vital role in the survival of the microorganisms either by facilitating nutrient transport, microbe-host interaction or it might act as biocide agents. Bioemulsifier are high molecular weight polymeric, ampiphilic surface active molecules such as Biodispersan, Liposan, Alsan, Mannoprotein etc that are involved in the stabilizing oil in water by forming stable emulsion or the emulsion of the two immiscible liquids may be formed by binding water insoluble substrates together (Ron et al., 2002). This property is particularly common amongst microorganisms that degrade water soluble hydrophobic compounds such as petroleum. Bioemulsifiers are applied in relatively low concentration and are functionally effective at extreme pH and temperature or other extreme environmental condition (Rosenberg *et al.,* 1995; Letizia Fracchia *et al.,* 2012).

The role of this molecules includes increasing the surface area and bioavailability of the hydrophobic water insoluble substrates, heavy metals binding, bacterial pathogenesis, quorum sensing and biofilm productions (Banat *et al.,* 2008).

Materials and Methods

Soil samples were collected from Kass Pathar, Satara, India. The soil sample were suspended in the sterile phosphate buffer saline, 10 fold serial dilution of the soil sample was carried out from 10^{-1} to 10^{-10} and each dilution were spread plated on sterile Luria agar (LA) plates and on sterile *Acinetobacter* minimal media (AMM) and the plates were incubate at 37^{0}C for 24h. After 24h isolated colonies were further streaked onto sterile LA plates and sterile AMM plates for purification.

Characterization of the isolates

All the isolated bacterial strains were characterized based on Gram Character. Out of 10 isolates, 7 bacterial isolates were Gram positive and remaining 3 bacterial isolates were Gram negative. The 3 Gram negative strains (Kp1, Kp2 and Kp3) were selected further for characterization up to genus level based on catalase test, oxidase test, oxidative fermentative test, nitrate reductase test, gelatinase test, triple sugar ion test and sugar tests (manitol, xylose, fructose, raffinose, rhamnose sugar) (Mujumdar, 2002).

Screening for bioemulsifier production

Gram negative strains (3) were further screened for bioemulsifier production. Luria Bertani broth was used for the production of bioemulsifier. Luria Bertani broth was selected as it showed the maximum bioemulsifier production as compared to other medium such as AMM. Bioemulsifier production was carried out in 100 mL LB broth in a 500 mL conical flask at room temperature (28°C) with aeration at 100 rpm. Inoculum (Kp1, Kp2 and Kp3) was

grown separately in the same medium and the culture was inoculated into the sterile LB broth. Strains were tested for bioemulsifier production by using different oil substrates such as groundnut oil, soya bean oil, mustard oil, sesame oil, machine oil, petrol and kerosene as hydrocarbon source (analytical grade).

Bioemulsifier production was observed after every 24h by employing emulsification assay and emulsification index for 72 h that was detected by measuring absorbance at 400nm (Mujumdar, 2002; Kokare *et al.,* 2007).

Emulsification index

The 24hgrowth of three strains (Kp1, Kp2 and Kp3) was used for the emulsification assay. The cells were separated by centrifugation at 10,000 rpm for 15min at 30^0C. Three ml of the cell free culture broth was mixed with 0.5 ml of test oil, vortexed for 2 min and incubated at 30^0C for 1h for the phase separation.

Aqueous phase was removed carefully and absorbance was recorded at 400 nm. Sterile LB broth was used as blank. Emulsification assay was expressed in terms of emulsification activity per ml (EU/ml) (Patil and Chopade, 2001; Mujumdar, 2002).

Emulsification index

The 24hgrown culture of the three strains (Kp1, Kp2 and Kp3) was used for the emulsification assay. The cells were separated by centrifugation at 10,000 rpm for 15min at 30^0C. Two ml of the cell free culture broth was mixed with 2.0 ml of the test oil, vortexed for 2min and incubated at 30^0C for 24h. After 24h the emulsification index was measured and percent emulsification index was calculated (Maneerat *et al.,* 2008; Letizia Fracchia *et al.,* 2012).

Effect of growth kinetics on the bioemulsifier production

To check the effect of growth kinetics on bioemulsifier production, LB broth was inoculated with three strains (Kp1, Kp2 and Kp3) separately, emulsification index and emulsification assay was carried out after every 24h for a period of 7 days at 400nm (Patil and Chopade 2001; Maneerat et al., 2008; Doshi *et al.,* 2010).

Effect of environmental factors on bioemulsifier production

Bioemulsifier production of3 selected strains (Kp1, Kp2 and Kp3) were tested for different physico-chemical conditions**.**

Effect of temperature

To observe the effect of different temperature on bioemulsifier production, LB broth was inoculated with three strains (Kp1, Kp2 and Kp3)separately and incubated at different temperature ranges such as 28□C, 30□C and 37□C. Bioemulsifier production was measured after every 24h by emulsification assay at 400 nm (Patil and Chopade, 2001, Maneerat et al., 2008; Maniyar *et al.*, 2011).

Effect of pH

The effect of pH on bioemulsifier production was studied, LB broth was adjusted to different pH ranges such as 4, 6, 7 and 9 and was inoculated with Kp1, Kp2 and Kp3 strains separately. Bioemulsifier production was measured after every 24h by emulsification assay at 400 nm (Patil and Chopade, 2001; Maneerat *et al.,* 2008; Maniyar *et al.*, 2011).

Effect of aeration

Effect of aeration on production of bioemulsifier was detected by incubating LB inoculated withKp1, Kp2 and Kp3 strains separately at different aeration conditions such as 50, 75, and 100 rpm. Bioemulsifier production was measured after every 24h by emulsification assay at 400 nm (Patil and Chopade, 2001; Maneerat *et al.,* 2008; Doshi *et al.,* 2010; Maniyar *et al.,* 2011).

Effect of salt concentration

Experiments on the effect of salt concentration were carried out by using varied concentrations of NaCl such as 2 %, 3 %, 4%and 5% w/v. Bioemulsifier production and activity were measured after every 24h by emulsification assay at 400 nm (Patil and Chopade, 2001; Maneerat et al., 2008; Doshi *et al.,* 2010; Maniyar *et al.,* 2011).

Effect of different oils and hydrocarbons on bioemulsifier activity

The emulsification assay was performed after centrifugation with different oils such as soyabean oil, groundnut oil, machine oil and hydrocarbons such as petrol and kerosene at 1% (v/v) concentration. Emulsification assay was carried out and emulsification was measured at 400 nm (Patil and Chopade, 2001; Mujumdar, 2002; Bashetti *et al.,* 2012).

Acetone extraction and partial purification

The selected strains (Kp1, Kp2 and Kp3) were subjected for partial purification as it showed good bioemulsifier activity. Partial purification was carried out by taking 1000ml of 72h grown Luria Broth Culture at 28^0C, 30^0C and at 37^0C respectively and centrifuged at 10,000 rpm for 20 min at 4^0C of temperature. After centrifugation, three volume of chilled acetone was added in the cell free broth, mixed thoroughly and it was incubated at 4^0C for 24h. After 24h the mixture was centrifuged at 10,000rpm for 20 min at 10^0C and the brown precipitate was collected. This brown colour precipitate was dissolved in maximum 3ml of chilled sterile distilled water and dialyzed extensively against sterile distilled water at room temperature for 48^0C. Distilled water was changed after 12h. The dialysate was than frozen at -20^0C and lyophilized (Thermo, model: Savant Modulyod freeze dryer, USA) (Kaplan *et al.,* 1987; Doshi *et al.,* 2010; Maniyar *et al.,* 2011).

Chemical analysis of partially purified bioemulsifier

The partially purified bioemulsifier was chemically analysed. Reducing sugars were estimated by using DNSA method with glucose as a standard. Quantification of the lipids was done by method given by Reddy *et al.,* (1983). Protein content was measured by Follin-Lowry method and bovine serum albumin was used as standard (BSA) (Kaplan *et al.,* 1987; Doshi *et al.,* 2010; Maniyar *et al.,* 2011; Bashetti *et al.,* 2012).

Cleaning property of partially purified bioemulsifier

The bioemulsifier obtained from, Kp1, Kp2 and Kp3 was subjected for cleaning activity to check the cleaning property of bioemulsifier, 10 µg of purified powder of bioemulsifier was dissolved in 5ml of distilled water. Clean glass tubes were taken and about 7ml glycerol, groundnut oil, machine oil and soyabean oil were added separately. Then the tubes were inverted and oils were removed in such a way that the portion of the oil should stick to the walls of

the tubes. After this, 1ml of bioemulsifier solution (10µg/ml) was added drop by drop, very slowly in each of the tubes by holding the tube in the horizontal position, and the inner surface of each glass tube was observed carefully for cleansing property (Karanth *et al.,* 1999; Mujumdar, 2002).

Determination of surface tension of partially purified bioemulsifier

To detect the surface tension, partially purified bioemulsifier powder of all the three strains was mixed with the water. Surface tension of bioemulsifiers was measured by standard spinning drop method using optical tensiometer (Dynamic contact angle surface tensiometer Dataphysics, (DCAT 11), Germany) (Kokare *et al.,* 2006).

Antimicrobial activity of partially purified bioemulsifier

Antimicrobial activity of the partially purified bioemulsifier after lyophilisation was tested against a variety of human pathogens by using well diffusion method. The microbial strains used were *Pseudomonas, Acinetobacter, Bacillius, Proteus, E. coli, Staphylococcus, Salmonella typhi, and Klebsiella pneumoniae.* The LB plates were then incubated at 30^0C for 24h. After 24 h the plates were observed for zone of inhibition (Maneerat *et al.,* 2008, Ahmed *et al.,* 2013).

Anti-fungal activity of partially purified bioemulsifier

The anti-fungal activity of the partially purified bioemulsifier (Kp1, Kp2 and Kp3) was tested against *Candida* species and *Aspergillus* species. For anti-fungal activity disc diffusion method was performed on sterile Potato Dextrose agar plates. The PDA plates were incubated at room temperature for 24 h and the plates were observed for zone of inhibition (Sawal *et al.,* 2003, Ademe *et al.,* 2013).

Antibiotics and metals resistances of partially purified bioemulsifier

The partially purified Bioemulsifier of Kp1, Kp2 and Kp3 were determined for resistances pattern against 6 antibiotics such as Doxycycline, Cephalosporin, Cifixime, Sulfamethoxazol and trimethoprim (S+T), Neomycine and Amikacin and simultaneously the resistances pattern was checked against metals such as nickel, iron, sulphur, copper and ammonium by spot inoculation method. The two fold serial dilution was carried on Muller-Hinton agar plates. The plates were incubated at 28□C, 30□C and 37□C for 24h (Maneerat *et al.,* 2008).

Plasmid isolations of Kp1, Kp2 and Kp3

To check the presence of plasmid, plasmid isolation for all three strains Kp1, Kp2 and Kp3 was carried out by method of Kado and Lui. 1% Agarose gel electrophoresis in Fast buffer (Sodium hydroxide and boric acid) was carried out. Gel was stained with eithidium bromide. Plasmid bands were observed under Transilluminator (Superfit, R1350116, India) (Kokare et al., 2007).

Anti-diabetic activity of partially purified bioemulsifier

In vitro inhibition of porcine α – amylase (ex porcine pancreases SRL) was carried out by DNSA method using starch as a substrate. 50µg/ml (O.D. of the Porcine enzyme was adjusted at 280nm at 0.4) of porcine pancreatic α – amylase was dissolved in phosphate buffer with pH7. 1 mg/ml of the porcine α – amylase was

incubated with 1 mg/ml test bioemulsifier powder (Kp1, Kp2 and Kp3) at 37^{0}C for 10 min in dark. 10 µM/ml stock solution of starch was used as substrate. After 10 min of incubation,0.1 ml of the test sample was taken to which 0.9 ml of starch solution was added later followed by incubation in dark for 10min at 37^{0}C. Reducing sugar was estimated by using DNSA assay at 540nm and the enzyme units were expressed as micro molar per minute. For control a blank was taken consisting of distilled water and DNSA while the other control that is ES blank (consists of starch as substrate and porcine α – amylase as enzyme). Percent inhibition of α – amylase was calculated (Ghosh *et al.,* 2011).

Results and Discussion

Isolation and characterization

From the morphological and biochemical analysis (Table 1) and by comparing the results with Bregey's Manual of bacteriology [9th edition], it may be concluded that the Kp1and Kp3 may belong to Genus *Flavobacterium* sp, Kp2 strain may belong to genus *Acidomonas* sp.

Effect of growth kinetics on bioemulsifier activity

The three strains Kp1, Kp2 and Kp3 showed increase in bioemulsifier activity upto 72h. After 72h the bioemulsifier activity declined (Fig.2).

Effect of physico-chemical properties on bioemulsifier activity

Kp1 showed maximum bioemulsifier activity at a temperature of 30□C, Kp2 at 28°C and Kp3 at 37°C (Fig.3). All the three strains showed maximum bioemulsifier activity at pH 7 (Fig. 4), NaCl concentration of 2% (w/v) (Fig. 5) and aeration rate of 100 rpm (Fig. 6). Thus these physico-chemical conditions were found to be optimum for bioemulsifier production.

Effect of different oils and hydrocarbons on bioemulsifier activity

Substrates such as petrol, kerosene, machine oil, groundnut oil and soybean oil were used to check the effect of substrate on emulsification. Out of five substrates groundnut oil and soybean oil were found to be the best substrate and were efficiently used by all the isolates as a source of carbon. The value of emulsification assay for these substrates was as high as 4 EU/ml (Fig. 7).

Chemical analysis of partially purified bioemulsifier

The bioemulsifier produced by Kp1 is light brown in colour, hygroscopic in nature, completely soluble in cold water with yield of 0.22g/L, having reducing sugar of 58%, protein content of 28% and lipid content of 14%. The bioemulsifier produced by Kp2 is dark brown in colour, gummy in nature and completely soluble in cold water with yield 0.19g/L, having reducing sugars of 57%, protein content 32%, and lipid content of 11%.

The bioemulsifier produced by Kp3 is light brown in colour, gummy in nature and completely soluble in cold water with yield 0.11g/L having53% of reducing sugar, protein content of 30%, and 17% lipid (Table 2). The results indicate that all three bioemulsifiers are proteoglycan in nature.

Cleansing property of partially purified bioemulsifier

Cleansing activity of bioemulsifier was tested using glycerol, groundnut oil and soya bean oil. Kp1, Kp2 and Kp3 could efficiently remove glycerol but could not clean groundnut oil and soya bean oil.

Surface tension of partially purified bioemulsifier

Kp1 showed reduction in surface tension of water upto 64.83 dynes/cm, Kp2 showed reduction in surface tension of water up to 66.25 dynes/cm and Kp3 showed reduction in surface tension of water up to 68.08 dynes/cm respectively (Table 3). These results indicate that the bioemulsifier produced by Kp1, Kp2 and Kp3 has less ability to reduce surface tension hence it is bioemulsifier and not a biosurfactant.

Antimicrobial activity of partially purified bioemulsifier

Antimicrobial activity of bioemulsifier produced by Kp1, Kp2 and Kp3 was checked against *Pseudomonas, Acinetobacter, Bacillus, Proteus, E. coli, Staphylococcus, Salmonella typhi, and Klebsiella pneumoniae* by well diffusion method. The bioemulsifier produced by Kp1, Kp2 and Kp3 strains showed a zone of exhibition for *Acinetobacter sp* and *Pseudomonas sp* while all other test organisms were not inhibited by the bioemulsifier produced by all the three strains.

Antifungal activity of partially purified bioemulsifier

Antifungal activity of bioemulsifier produced by Kp1, Kp2 and Kp3 was checked against *Candida sp* and *Aspergillus sp* by well diffusion method. Bioemulsifier of Kp3 strain showed zone of inhibition with 6mm in diameter and that of Kp2 strain showed zone of inhibition with 2mm in diameter against *Aspergillus sp.* However the bioemulsifier of Kp1 showed a zone of exhibition for *Candida sp* with 4mm in diameter.

Antibiotic and metal resistance of Kp1, Kp2 and kp3

All the three isolates showed resistance to antibiotics Doxycycline, Cephalosporin, Cifixime (zifi), Neomycine and Amikacin. While Kp1was found susceptible to Sulfamethoxazol and trimethoprim (S+T) antibiotic. While Kp2 and Kp3 were found to be resistance to Sulfamethoxazol and trimethoprim (S+T) antibiotic (Fig 8). All three isolates showed resistance towards all six metals.

Plasmid isolation of Kp1, Kp2 and Kp3

Isolation of plasmid was carried out by using Kado and Lui method for all the three strains. A high molecular weight plasmid was seen in case of all the three strains Kp1, Kp2 and Kp3.

Antidiabetic activity of partially purified bioemulsifier

The Kp1 bioemulsifier inhibited porcine pancreatic α-amylase by 86%, the Kp2 bioemulsifier inhibited porcine pancreatic α-amylase by 78.1% and Kp3 bioemulsifier inhibited porcine pancreatic α-amylase by 88.6%. All the isolates could efficiently degrade amylase (Table 4 and Fig. 8a-8c). From the results we can say that the bioemulsifier may have an anti-diabetic properties which can be formulated with medicine and can be used for in the field of pharmacology we need to do further studies in this area.

The present study on bioemulsifiers has demonstrated appreciable bioemulsification activity by strains isolated from rhizopspheric soil sample taken from Kaas Pathar (Satara, India). The isolated strains Kp1, Kp2 and Kp3 based on their biochemical and morphological analysis

were determined as *Acidomonas* and *Flavobacterium species*. Since both the species is found in soil it is possible that the isolated strains may belong to *Acidomonas and Flavobacterium species* but further conformation about the genus should be done through 16s rRNA sequencing and MALDI. Maximum bioemulsifier production occurred at 30□C for Kp1, 28□C for Kp2 and 37□C for Kp3. The optimum temperature of 28□ C, 30□ C and 37□C for bioemulsifier production can be due to the habitat of the organisms. The result is similar to that of bioemulsifier produced by *Streptomyces sp* S22 isolated from garden soil which showed maximum bioemulsifier production at room temperature (Doshi *et al.,* 2010), the bioemulsifier produced by *Streptomyces sp* S1 isolated from marine sediments showed maximum emulsification activity at 28□C (Kokare *et al.,* 2007). A pH of 7 was found to be optimum for bioemulsifier production for all three strains (Kp1, Kp2 and Kp3) this is due to the pH of soil from which the bacterial strains were isolated. In general pH of soil is always neutral to alkaline hence these strains showed similar results in case of pH. This result is similar to that of bioemulsifier produced by *Streptomyces sp* S1 isolated from marine sediment which showed maximum production of bioemulsifier at pH 7 where bioemulsifier activity was reduced in both acidic and basic pH (Kokare *et al.,* 2007). A contrary results was observed to that of bioemulsifier produced by *Streptomyces sp* S22 which showed maximum emulsification activity at pH 6 (Doshi *et al.,* 2010; Maniyar *et al.,* 2011).The test strains showed increase in bioemulsifier production as the concentration of NaCl increases, a salt concentration of 2% (w/v) was optimum for bioemulsifier production. This result is similar to that of bioemulsifier produced by *Actinopolyspora* sp A18 isolated from garden soil which showed maximum emulsification activity at 2% (w/v) of NaCl (Doshi *et al.,* 2010; Maniyar *et al.,* 2011). Aeration rate of 100rpm was found to be optimum for bioemulsifier production for Kp1, Kp2 and Kp3.The report for bioemulsifier production by *Actinopolyspora* A18 shows that aeration rate of 250 rpm is optimum for bioemulsifier production (Doshi *et al.,* 2010, Maniyar *et al.,* 2011, Bashetti *et al.,* 2012).

The bioemulsifier production takes place by degrading hydrocarbons and utilizing them as a source of nutrient in a nutrient limited condition. Groundnut oil, soyabean oil were excellently emulsified by all strains (Kp1, Kp2 and Kp3) indicating that they served as a good substrate for emulsification and a carbon source. As compared to hydrocarbons effect of oils on bioemulsifier production is more significant which indicates that efficiency of bioemulsifier to degrade oil was more than the hydrocarbons (Kokare *et al.,* 2007). However emulsification of petrol, kerosene, machine oil was poor which suggests that the strains are unable to grow in presence of hydrocarbons. This report is contrary to that of bioemulsifier produced by *Streptomyces* sp SS20 isolated from contaminated soil, the bioemulsifier of which can efficiently degrade both hydrocarbons and oils (Hayder *et al.,* 2014). The emulsifier was also soluble in water thus it can be easily used in formulation of pesticides, pharmacy, food and medicine.

The strains Kp1, Kp2 and Kp3 failed to reduce the surface tension of water which indicates they must be bioemulsifier and not biosurfactants. This result is against Emulsan, a bioemulsifier produced by *A.calcoaceticus* RAG-1 that showed decrease in surface tension of water by 10 dynes/cm (Gutnick and Shabtai 1987).In

case of rhamnolipids produced by *Pseudomonas* sp isolated from contaminated soil showed a reduction in surface tension up to 26 dynes/cm suggesting it as biosurfactants (Viramontes *et al.*, 2010).

The bioemulsifier can act a very effective cleansing agent. However Kp1, Kp2 and Kp3 did not showed any cleansing activity against the oils and hydrocarbons used.

Chemical analysis of the bioemulsifier produced by Kp1, Kp2 and Kp3 indicates that it is proteoglycan in nature. The result is similar to that of bioemulsifier from *Actinopolyspora* sp A18 which is also a proteoglycan in nature (Doshi *et al.*, 2010). The bioemulsifier sample produced by all the three strains consists of significant amount of lipid fraction.

A zone of exhibition was seen for *Acinetobacte*r, *Pseudomonas* when bioemulsifier produced by Kp1, Kp2 and Kp3 was used in antimicrobial assay. The reason for zone of exhibition is not known but it might be concluded that it may be due to the presence of certain component that supports the growth of organism for which it warrants further investigation. All three isolates failed to inhibit *S. typhi*, *E. coli, Staphylococcus, Salmonella typhi, Bacillus and Proteus.* The same result was observed in case of bioemulsifier Serrrawettins produced by *Serratia* species that failed to show inhibition activity against *E.coli, Staphylococcus, Bacillus* and *Proteus* (Ahmed *et al.*, 2013). However the Kp2bioemulsifier showed a zone of inhibition for *Klebsiella.* This result is against the bioemulsifier of *Bacillus licheniformis* strain 104 which showed a distinct antimicrobial activity against the same pathogens (Gomma *et al.*, 2013).

Antifungal activity using *Candida* and *Aspergillus* suggested that bioemulsifier produced by Kp2 and Kp3 strain inhibited *Aspergillus sp*. This results is similar to that of bioemulsifier Serrrawettins produced by *Serratia sp* that showed zone of inhibition against *Aspergillus sp* (Ahmed *et al.*, 2013). The antifungal activity of Kp2 and Kp3 indicate that the bioemulsifier can acts on lipid content in the fungal cell, which in turn results into the formation of spores in the fungal cell wall that inhibit the fungus can be used in antifungal agents. However the bioemulsifier produced by Kp1 showed zone of exhibition against *Candida sp* which suggest that there is a component present in the bioemulsifier that enhances the growth in order to find the component further investigation is needed.

Table.1 Results for biochemical tests of Kp1, Kp2 andKp3

Strain	Oxidase	Catalase	Sucrose	Lactose	Raffinose	Fructose	TSI	OF test	Nitrate Reductase test
Kp1	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Kp2	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Kp3	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Table.2 Properties and chemical analysis of partially purified bioemulsifier produced by Kp1, Kp2 and Kp3

Characteristics	Kp1	Kp2	Kp3
Colour and texture	Light brown, hygroscopic	Dark brown, gummy powder	Light brown, gummy powder
Yield	0.22g/L	0.19g/L	0.11g/L
Solubility	Completely soluble in cold water	Completely soluble in cold water	Completely soluble in cold water
Reducing sugar	58%	57%	53%
Protein	28%	32%	30%
Lipid	14%	11%	17%

Table.3 Surface tension of partially purified bioemulsifier produced by Kp1, Kp2 and Kp3

Strains	Surface Tension of Water (dynes/cm)	Reduction in surface tension (dynes/cm)
Kp1	72.11	64.83
Kp2	72.11	66.25
Kp3	72.11	68.08

Table.4 Anti-diabetic activity of bioemulsifier produced by Kp1, Kp2 and Kp3 on porcine α-pancreatic amylase

Strain	Percent inhibition (%)
Kp1	86%
KP2	78.1%
KP3	88.6%

Figure.2 Effect of growth kinetics on bioemulsifier activity and production by Kp1, Kp2 and Kp3

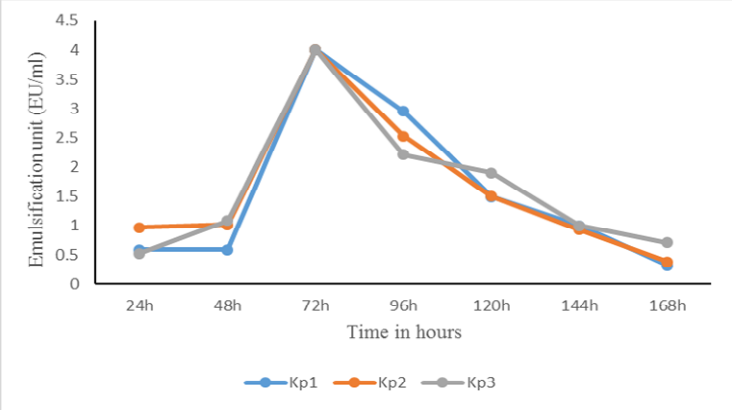


Figure.3 Effect of temperature on bioemulsifier activity and production by Kp1, Kp2 and Kp3

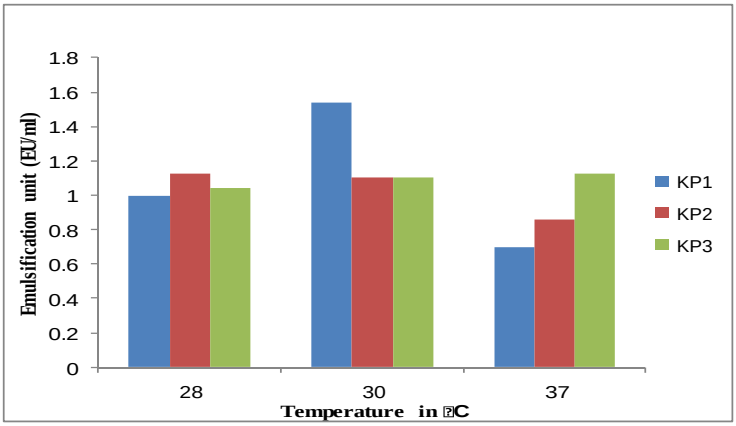


Figure.4 Effect of pH on bioemulsifier activity and production by Kp1, Kp2 and Kp3

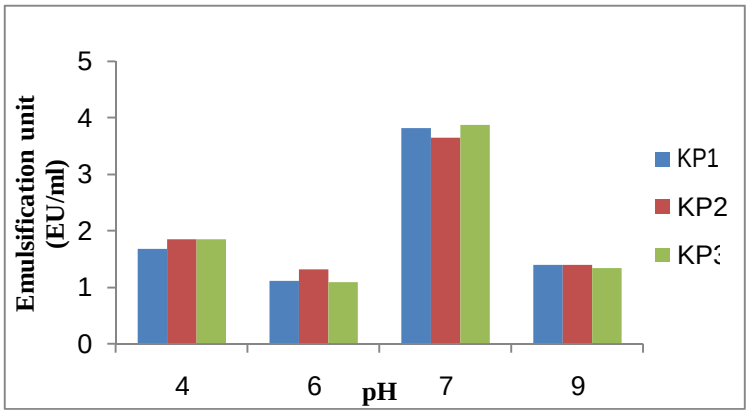


Figure.5 Effect of salt concentration on bioemulsifier activity and production by Kp1, Kp2 and Kp3

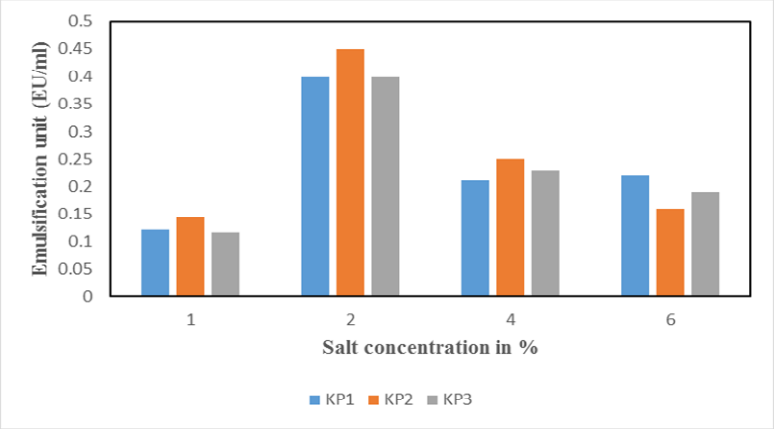


Figure.6 Effect of aeration on bioemulsifier activity and production by Kp1, Kp2 and Kp3

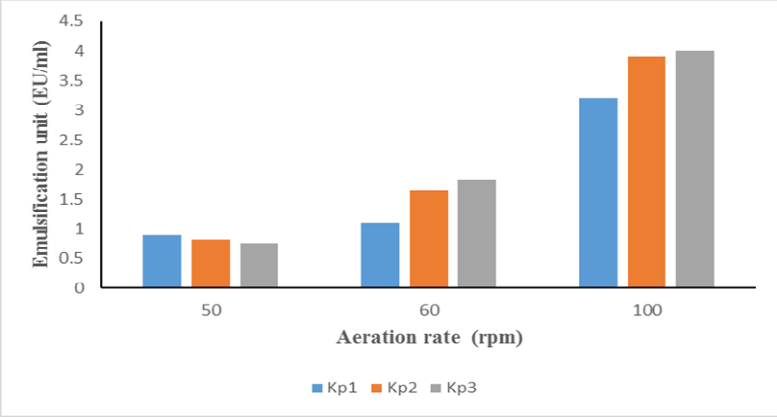


Figure.7 Effect of substrates on bioemulsifier activity and production by Kp1, Kp2 and Kp3

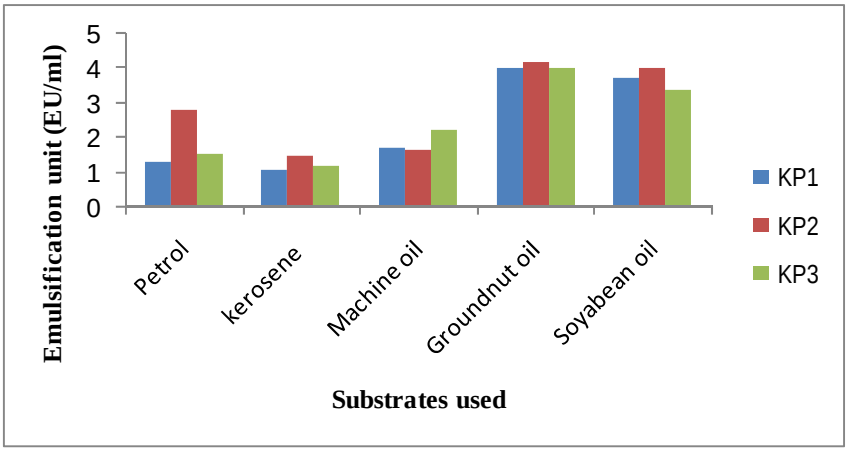


Figure.8a Anti diabetic activity of Kp1 bioemulsifier

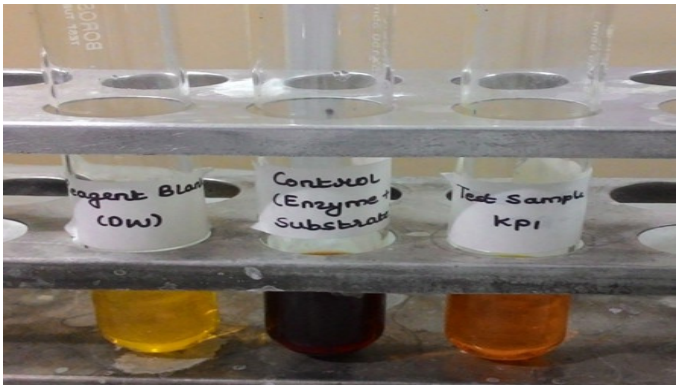


Figure.8b Anti diabetic activity of Kp2 bioemulsifier

Figure.8c Anti diabetic activity of Kp3 bioemulsifier

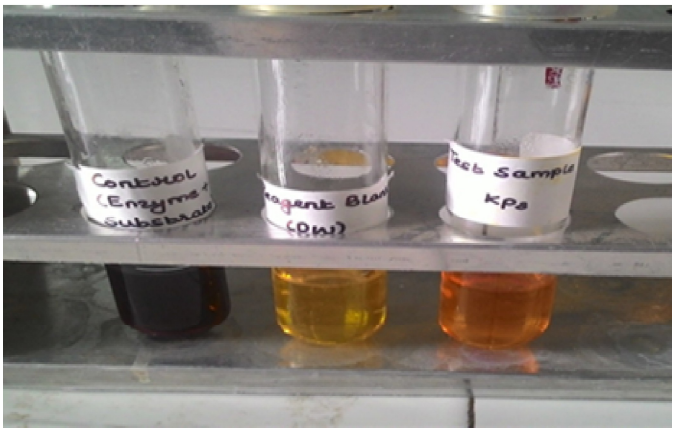
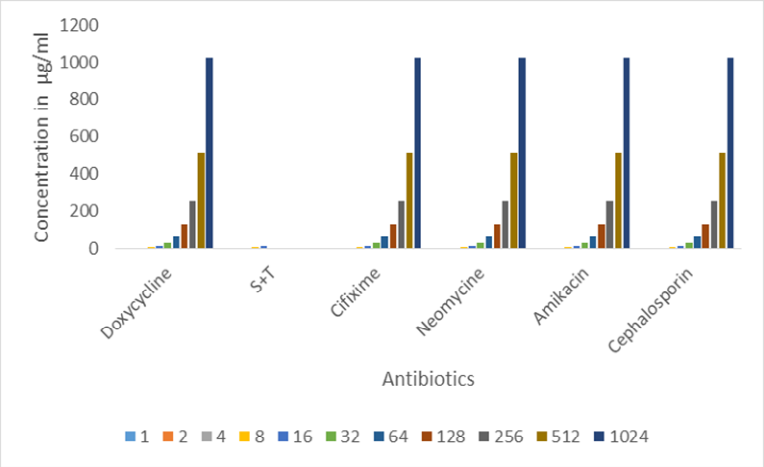


Figure.9 Effect of antibiotics on Kp1, Kp2 and Kp3



Note: (S+T): Sulfamethoxazol and trimethoprim

The antibiotic resistance pattern of three isolates was checked against antibiotics Doxycycline, Cephalosporin, Cifixime (zifi), Neomycine, Sulfamethoxazol and trimethoprim (S+T) and Amikacin. The strains Kp1, Kp2 and Kp3 out of 6 antibiotics showed resistance to 5 antibiotics which suggests that the bioemulsifiers have good antibiotic resistance pattern . The existing drugs can be formulated with these bioemulsifier products. Also all three (Kp1, Kp2 and Kp3) isolates showed resistance towards all six metals at a concentration which indicates that it can be used in bioremediation of heavy metals. The results are similar to that of bioemulsifier produced by *Streptomyces sp* VITDDK3 isolated from saltpan soil that also showed a significant heavy metal resistance (Laxmipathy *et al.*, 2010).

A significant percent reduction of porcine pancreatic α –amylase by Kp1, Kp2 and Kp3 bioemulsifier indicates that the bioemulsifier may have anti-diabetic activity. The present study focuses on the inhibition activity of only one enzyme i.e. α – amylase by DNSA which is a preliminary test only for screening the anti-diabetic properties, although inhibition of glycosidase should checked further in order to confirm the anti-diabetic properties followed by the testing on animal models and pancreatic cell lines induced with diabetics. The future studies are must in order to make this statement credible.

From the present study it can be concluded that the strains Kp1, Kp2 and Kp3 isolated from soil sample efficiently produce bioemulsifier. The bioemulsifier produced showed anti-diabetic properties anti-fungal activity thus suggesting their applications in food, pharmaceutical and cosmetics industries.

Acknowledgement

We would like to express our sincere gratitude to Dr. R. Zunjarrao, Principal, Modern College for his support. We would like to take this opportunity to thank Dr. P. K. Dhakephalkar, Scientist F, Agharkar research institute, Pune for providing the facility of Lyophilisation. We would also like to express our thanks to Dr. Mrs Surekha Satpute for providing us with the facilities of Optical Tensiometer to determine the surface tension.

References

Berna Elya, Amarila Malik, Purwa Indah Septimahanani and Bianca Loranza.2012. Antidiabetic activity test by inhibition of α- glucosidase and phytochemical screening from the most active fractions of Buni stem barks and leaves. Parma Teach.4: 1667-1671.

C. R. Kokare, S. S. Kadam, K. R. Mahadik and B. A. Chopade.2007. Studies on bioemulsifier production from marine *Streptomyces* sp S1. Indian Journal of Biotechnology.6: 78-84.

Cirigliano. M. C and G. M. Cameron.1984.Isolation of bioemulsifier from *Candida lipolytica*. Appl Environ Microbiol.48:747-750.

Cooper. D. G and C .R. MacDonald.1981.Enhanced production of surfactins from Bacillus subtilis by continuous product removal and metal cation additions. Appl Environ Microbiol.42:408-412.

D. R. Cameron, D. G. Cooper and R. J. Neufeld.1988. The mannoprotein of *Saccharomyces cerevisiae* is an effective bioemulsifier. Appl Environ Microbiol. 54(6): 1420.

Das. K and A. K. Mukharjee .2005. Characterization of biochemical properties and biological activites of biosurfcatants produced by *Pseudomonas aeruginosa* mucoid and mucoid strains. Appl Microbiol Biotchnol.69:192-199

Desai. A. J, Patel. K. M and J. D. Desai.1988.Emulsifier production by *Pseudomonas* fluorescence during the growth on hydrocarbon. Curr Sci.57:500-501

Doshi Dhawal, Javed .P Maniyar, Smita S Bhuyan and Shilpa Mujumdar. 2010. Studies on bioemulsifier production by *Actinopolyspora* sp.A18 isolated from garden soil. Indian Journal of Microbiology.9:391-396

Mujumdar. 2002. Studies on the isolation, distribution, bio typing, characterization, production of antibiotics, bioemulsifier and plasmid Pup1125 encoded indole acetic acid production and its role in plant growth promotion by *Acinetobater species* from rhizosphere of wheat.

Eliora Z Ron and Eugene Rosenberg.2001. Natural role of biosurfactants. Environmental Microbiology.3 (4): 239-246.

Eliora Z Ron and Eugene Rosenberg.2002. Biosurfactants and oil bioremediation. Elsevier Science Biotechnology.13: 249–252.

Eman Zakaria Gomma and Abeer Ahmed Rushdy.2014. Antimicrobial activity of biosurfactants produced by *Bacillus licheniformis* strain M104 grown on whey. Journal of applied microbiology. DOI: 10 1007/s 13213-013-0733-7.

Entissar Faroun Ahmed and Shatha Salman Hassan. 2003. Antimicrobial activity of a bioemulsifier produced by *Serratia marcescens* S10. 16 (1): 147-155.

Fardos M. Bokhari.2009. Antifungal activity of some medicinal plants used in Jeddah. Saudi Arabla. Mycopathy.7 (1): 51-57.

Fukuoka. T, Morita and M. Konishi.2007.Characterization of new glycolipids biosurfactant triacetylated mannosylerythritol lipids produced by *Pseudozyma*. Biotachnol Lett.29:1111-1118

J. R. Patil and B. A. Chopade. 2001. Studies on bioemulsier production by *Acinetobacter* strains isolated from healthy human skin. Journal of Applied Microbiology. 3: 290-298.

Juan Carlos Camacho Chab, Jean Guezennec, Manuel Jesus Chan Bacab, Elvira Rios-Leal, Corinne Sinquin, Raquel Muniz Salazar, Susana del C. De la Rosa-Garcia, Manuela Reyes Estebanez and Benjamín Otto Ortega Morales. 2013. Emulsifying Activity and Stability of a Non-Toxic Bioemulsifier Synthesized

by *Microbacterium* sp, MC3B-10. Int. Journal of Molecular biology Sci.14: 18959-18972.

Kaplan, N. Z. Zosim and E. Rosenberg. 1987. Reconstitution of emulsifying activity of *A.calcoaceticus* BD4 emulsan by using pure polysaccharide and protein. Appl. Environ. Microbial.53:440-444.

Kulnaree Phetrong, Aran H. Kittikun and Suppasil Maneerat. 2008. Production and characterization of bioemulsifier from a marine bacterium *Acinetobacter calcoaceticus* subsp. *anitratus* SM7. Songklanakarin J. Sci. Technol.30 (3): 297-305.

Letizia Fracchia, Massimo Cavallo, Maria Giovanna Martinotti and Ibrahim M. Banat. 2012. Biosurfactants and bioemulsifiers biomedical and related applications -present status and future potentials. Biomedical Science Engineering and Technology. 20: 227-230.

M. C. Cirigliano and G. M. Carman. 1985. Isolation of bioemulsifier from *Candida lipolytica*. Appl Environ Microbiol. 50(4):846.

Maniyar. P. Javed, Dhawal Doshi, Smita. S. Buyan and Shilpa Mujumdar. 2010. Bioemulsifier production by *Streptomyces sp.* 22 isolated from garden soil. Indian Journal of experimental biology.49:293-297

Md. Afjalus siraj, Md. Reaz, Emrul Hossain, Malik Salahuddin.2013. Assay of Antidiabetic activity of *Hemidesmusindicus* by gut perfusion and six segment methods on long Evans rats. Pharmacology online.3: 81-87.

Navon-Venezia S, Zosim Z and A Gotttieb.1995.Alasan, a new bioemulsifier from *Acinetobacte*r. Appl. Environ. Microbiol. 61(9):3240.

Noordam. W. G and D. B. Janssen.2002.Rhamnollipids surfactants stimulates uptake of hydrophobic compounds by. *Pseudomonas aeruginosa*. Appl Environ Microbiol.60:4502-4508.

Omer Erturk. 2006. Antibcaterial and antifungal activity of ethanolic extracts from eleven spice plants. Cellular and molecular biology.3: 275-279.

P. F. F. Amaral, J. M. Silva, M. Lehocky, A. M. V. Barros Timmons, M. A. Z. Coelho, I. M. Marrucho, J. A. P. Coutinho. 2006. Production and characterization of a bioemulsifier from *Yarrowia lipolytica*. Process Biochemistry.41:1894–1898.

Ramarathnan. R. B and YChen.2007.Molecular and biochemical detection of fengycin and bacillomycin D-producing *Bacillus* sp antagonistic to fungal pathogens of canola and wheat. Can J Micobiol.53: 901-911

Reddy. P. G, Singh. H. D, Pathak M. G, Bhagat S. D and J. N. Baruah.1983.Isolation and functional characterization on hydrocarbon emulsifying and solubilizing factors produced by *Pseudomonas* species. Biotechnol and Bioengineering.25:387-401.

S Navon Venezia, Z. Zosim, Gottlieb, R. Legmann, S. Carmeli, E. Z Ron and E. Rosenberg. 1995. Alasan, a new bioemulsifier from *Acinetobacter*. Appl. Environ. Microbiol. 61(9): 3240-3245.

S. M. M. Dastgheib, M. A. Amoozegar, E. Elahi, S. Asad, I. M. Banat. 2008. Bioemulsifier production by a halothermophilic *Bacillus* strain with potential applications in microbially oil recovery. BiotechmLeu. 30:263-270.

Sabina Viramontes Ramos, Martha Cristina Portillo. 2010. Selection of biosurfactant/ bioemulsifier producing bacteria from hydrocarbon contaminated soil. Brazilian Journal of Microbiology 41: 668-675.

Satpute K. Surekha, Arun G. Banpurkar, P. K. Dhakephalkar, I. M. Banat and B. A. Chopade. 2010. Methods for investigating biosurfactants and bioemulsifier: a review. Critical reviews in biotechnology. 1-18.

Schippers C, Genssner K and T. Muller.2000.Microbial degradation of phenanrhrene by addition of sophorolopids mixture.Biotechnol.83:189-198.

Soon Huat Tiong, Chung Yeng Looi, Hazrina Hazni, Aditya Arya, Mohammadjavad Paydar, Won Fen Wong, Shiau-Chuen Cheah, Mohd Rais Mustafa and Khalijah Awang. 2013. Antidiabetic and antioxidant properties of alkaloids from *Catharanthus roseus*. Molecules.18: 9770-9784.

Sougata Ghosh, Mehul Ahire, Sumersing Patil, Amit Jabgunde, Meenakshi Dusane. 2012. Antidiabetic Activity of Gnidia glauca and Dioscorea bulbifera: Potent Amylase and Glucosidase Inhibitors. Hindawi Publishing Corporation Evidence-Based Complementary and Alternative Medicine. doi:10.1155/2012/929051.

Sung Heun, Chung Yeng Looi, Hazrina Hazni, Aditya Arya. 2011. *Myroides marinus* sp. Nov, a member of the family *Flavobacteriaceae* isolated from seawater. International Journal of Systematic and Evolutionary Microbiology: 61. 938–941.

Suppasil Maneerat and Paweena Dixit.2005. Bile acid are new products of marine bacterium, *Myroides sp* strain SM1. Appl Microbial Biotechnol.2: 67-98.

Suppasil Maneerat1 and Paweena Dikit.2007. Characterization of cell-associated bioemulsifier from *Myroides* sp. SM1, a marine bacterium. J. Sci. Technol. 29(3): 769-779.

Tabatabaee, M. Mazaheri Assadi, A. Noohi, V A Sajadian. 2005. Isolation of biosurfactant producing bacteria from oil reservoirs. Iranian J Env Health Sci Eng. 2: 6-12.

Tanaka Y, Takashi T and U Kazuhike.1997.Method of producing IturinA and antifungal agent for profound mosis. Biotechnol Ad.57:234-235.

Toren A, Segal G and EZ Ron.2001.Emulsifiying activities of purified alasan proteins from *Acinetobacter radioresistens* KA5.Appl Environ Microbiol.67:1102-1106

Tullack P, Hill A and JFT Spencer.1967. A new type of macro cyclic lactone from *Torulopsis apicola*. J chem soc chemcommun.9:584-586

Van Dyke MI, Lee H and JT Trevors.1997.Application of microbial surfactants. Biotech Adv .2:241-252.

Wang X, Gong L and S Liang.2005.Algicidal activity of rhamnolipid biosurfactant produced by Pseudomonas aeruginosa. Harmful algae.4:433-443.

Xuansheng Hu. 2013. Antidiabetic activities of aqueous extract from *Actinidia kolomikta* root against α-glucosidase. Journal of Pharmacognosy and Phytochemistry.2 (4):43-57.

Yakimov. M. M, Fredrickson H. L and K. N. Timmis.1996.Effect of heterogeneity of hydrophobic moieties on surface activity of Lichenysin A lipopeptide biosurfactant from *Bacillus licheniformis* BAS 50.Biotechnol Appl Biochem.23:13-18.

Zosim Z, D.L. and E Rosenberg. 2011.Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. Int.J. Mol.Sci.12:165