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Production and Characterization of Biosurfactant from *Bacillus subtilis* YB7

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Abstract: Biosurfactants (BS) are of recent interest due to their unique properties including structural diversity, higher biodegradability, lower toxicity and exhibit stable activity at extreme conditions including pH, temperature and ionic strength. They are found to have applications in medical and environmental fields. Some of the BS are potential antimicrobial agents and are suitable alternatives to synthetic antibiotics. In this perspective, a surfactant producing microorganism has been screened and isolated from a polymer dump site, which was characterized as *Bacillus subtilis* YB7. This microorganism was capable of producing 0.5 g L^{-1} of crude extract (dry weight) in the mineral salt medium. The FTIR and LC/MS analysis of the crude extract showed the presence of similar functional groups and six isoforms, respectively as that of the commercially available Sigma surfactin. However, the proportions of the peaks vary from sigma surfactin. Molecular mass of the major peak in the crude extract is 1023 $(M+H)^+$ which is equivalent to C_{14} surfactin analog, whereas it is 1035 (C_{15} surfactin) in case of sigma surfactin. The crude extract reduced the surface tension of water from 72 to 30 mN m^{-1} at the CMC of 40 μM. This particular strain produced unique type of cycliclipopeptide analogs.

Key words: Surfactin, *Bacillus subtilis* YB7, drop collapse test, oil spreading test, cyclic lipopeptide, wilhelmy plate method

INTRODUCTION

Bisurfactants (BS) or microbial surface active compounds are amphiphiles with polar and non polar moieties that tend to preferentially partition at interfaces and thus reduce the interfacial/surface tension (Desai and Banat, 1997). Growing interest in the field of BS can be attributed to their lower critical micelle concentration; toxicity and higher biodegradability compared with their chemical counterparts (Van-Hamme *et al*., 2006; Ron and Rosenberg, 2001; Neu, 1996; Rosrnberg and Ron, 1999). These molecules can be synthesized by microorganisms by using renewable resources including oil and food industrial wastes (Maneerat, 2005). Hence, they are called as green alternatives. There are five main classes of BS, namely lipopeptides; glycolipids; fatty acids including neutral lipids and phospholipids; polymeric and particulate (Neu, 1996; Rosrnberg and Ron, 1999). Among these, the first two classes show wide range of applications in food, cosmetics and pharmaceutical industries (Cameotra and Makkar, 2004; Rodrigues *et al*., 2006; Singh and Cameotra, 2004; Singh *et al*., 2007). They are used in removing soil contaminants including heavy metals, oils and other toxic pollutants (Van-Hamme *et al*., 2006; Mulligan, 2005; Neu, 1996; Singh *et al*., 2007). Some of the BS are potential antimicrobial agents and are suitable alternatives to synthetic antibiotics (Cameotra and Makkar, 2004; Rodrigues *et al*., 2006; Singh and Cameotra, 2004).

Except for a few, the physiological roles of BS in general are not yet completely characterized. BS are essential for the motility (swarming and gliding) of the microorganisms. For example, surfactin production and flagellar biosynthesis are crucial for swarming motility in *B. subtilis* (Van-Hamme *et al*., 2006; Kearns and Losick, 2003). Similarly, *Serratia marcescens* depends on serrawettin for surface locomotion and access to water repelling surfaces (Van-Hamme *et al*., 2006; Matsuyama and Nakagawa, 1996) 3-(3-Hydroxyalkanoyloxy). This author kit is designed to alkanoic acid (HAA), the intermediate in the rhamnolipid biosynthesis pathway, plays an essential role in swarming motility (Van-Hamme *et al*., 2006; Deziel *et al*., 2003). Bisurfactants (BS) regulate the attachment and detachment of the microorganisms to or from solid substrates (Van-Hamme *et al*., 2006; Ron and Rosenberg, 2001; Neu, 1996). Rhamnolipids are essential to maintain the architecture of the biofilm (Van-Hamme *et al*., 2006; Neu, 1996) and are considered as one of the virulence factors in *Pseudomonas* sp. (Van-Hamme *et al*., 2006;

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Ron and Rosenberg, 2001; Neu, 1996). Rhamnolipids, mannosylerythritol lipid (Arutchelvi *et al*., 2008) and surfactin show antimicrobial properties. Thus they confer a competitive advantage to the organism during colonization and cell-cell competition (Van-Hamme *et al*., 2006; Ron and Rosenberg, 2001). BS enhance the accessibility of hydrocarbon substrates by forming small emulsion droplets and increase the surface area of the insoluble substrates (Van-Hamme *et al*., 2006; Ron and Rosenberg, 2001; Neu, 1996).

Current world wide production of surfactants is around 12.5 M tones per year, worth approximately US$28 billion, growing at the rate of about 5,00,000 t year^{-1} (Edser, 2006). They have application in study, perfume, fragrance, food and pulp industries, metal processing and environmental remediation (Cameotra and Makkar, 2004; Rodrigues *et al*., 2006; Singh and Cameotra, 2004; Singh *et al*., 2007). It is expected that biosurfactants may take up a part of this market share also.

In this study, a biosurfactant producing microorganism was isolated and the biosurfactant was partially characterized as cycliclipopeptide using FTIR and LC/MS analysis. This crude extract was capable of reducing the surface tension of water from 72-30 mN m^{-1}. Eventhough, it showed similarity in functional groups and number of cyclic lipopeptide isoforms, the amount of each isoform is different from the well characterized commercially available Sigma surfactin. The major isoform of the crude extract is C_{14} surfactin. The isolated microorganism was characterized as *Bacillus subtilis* using 16S rDNA.

MATERIALS AND METHODS

All the chemicals used for the following experiments were purchased from Sigma, Himedia and SRL. Soil samples were collected from plastic dump site located at Pallikaranai, Chennai, India.

Isolation of biosurfactants producing microorganisms: In order to isolate biosurfactant producing microorganisms, soil from plastic dump site was taken. One milliliter of the soil suspension was inoculated in the Minimal Medium (MM) (Cacciari *et al*., 1993) (solution A (g L^{-1}) contains KH_2PO_4-3; Na_2HPO_4-6; NH_4Cl-2; NaCl-5 and solution B (g L^{-1}) contains glucose-0.5; $MgSO_4.7H_2O$- 0.1; pH 7.0±0.2) and mineral salt medium (MSM) (contains (g L^{-1}) $NaNO_3$-2.5; K_2HPO_4-1.0; KH_2PO_4-0.5; $MgSO_4$-0.5; KCl-0.1; $FeSO_4$-0.01; $CaCl_2$-0.01; Glucose-0.5; pH-6.8-7.0) (Nair *et al*., 2007). In this medium sterilized polypropylene or hexadecane was added along with the glucose. After 10 days of inoculation, spread plating of serially diluted culture suspension was done on nutrient agar plates. Isolated single colonies were inoculated into the 3 mL of MM/MSM and incubated in the temperature control shaker at 37°C and 180 rpm. The culture supernatant was collected from the above cultures after 24 h and checked for the production of biosurfactants by using drop collapse method and oil spreading method (Youssef *et al*., 2004). The microorganisms that showed positive results with drop collapse and oil spreading were further taken for the large scale production and characterisation.

Characterisation of the isolated biosurfactant producing microorganisms: A bacterium was isolated from the above experiments was named as YB7. This was further characterized based upon the 16S rDNA analysis. Genomic DNA was isolated from the pure culture and 16S rDNA fragment was amplified using consensus primers and high fidelity PCR polymerase. The PCR product was cloned and plasmid DNA was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homolog for the microbe.

Isolation of biosurfactants from the microorganisms: YB7 was inoculated in 1 L of MM and MSM, respectively. Crude extract was isolated from the culture supernatant as described by Copper *et al*. (1981).

FTIR analysis: A Jasco N4200 spectrometer at a resolution of 2 cm^{-1}, in the frequency range of 4000-400 cm^{-1}, calibrated with polystyrene standards was used for the identification of functional groups and type of bonds present in the crude extract.

HPLC analysis: The crude extract was separated using reverse phase HPLC (Agilent) with C18 (5 µm pore size; Purospher column) column and 80% acetonitrile with 0.1% trifluoroacetic acid as the mobile phase. Flow rate was kept at 1 mL min^{-1}. UV detector was used.

ESI-MS analysis: The lipopeptide was analyzed by mass spectrometry in positive electrospray mode. Applied ion spray voltage and ion source temperature were 5000 V and 450°C, respectively.

Surface tension measurement: Surface tension of the crude extract solution was measured at 25°C using the surface tensiometer (NIMA technology model DST9005) which works based upon the principle of wilhelmy plate method. Crude extract was dissolved in alkaline water (pH 9.0) and prepared in 0 to 280 µM concentration range. The surface tension versus concentration curve was used to determine Critical Micelle Concentration (CMC).

RESULTS AND DISCUSSION

Isolation and characterisation of biosurfactants producing microorganisms: Gram staining of YB7 showed that it is a gram positive and rod shaped microorganism. Based upon the 16S rDNA sequence homology and phylogenetic analysis, YB7 was detected as *Bacillus subtilis* and the nearest homolog was found to be *Bacillus axarquiensis* which is known to produce biosurfactants. Phylogenetic tree is shown in Fig. 1.

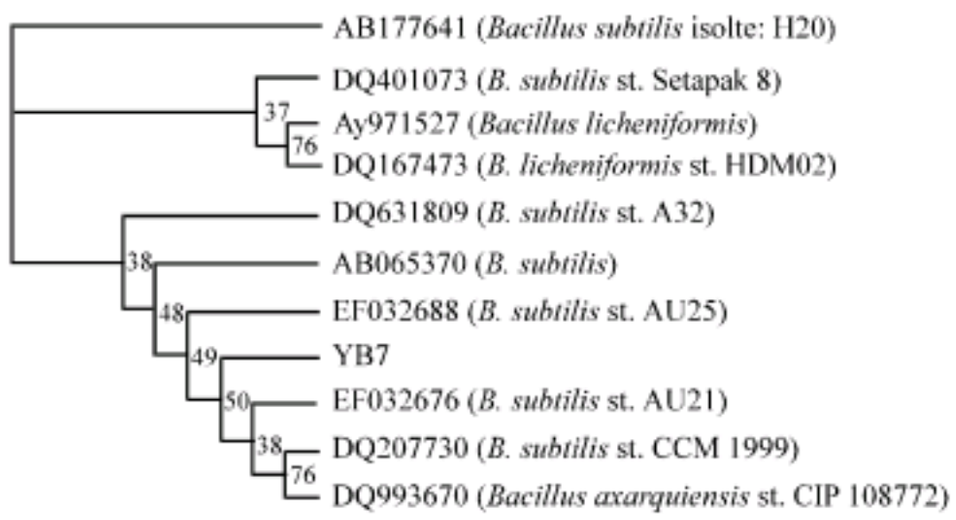

Fig. 1: Phylogenetic tree

Isolation and characterisation of biosurfactants: We have used two different medium (MM and MSM) for the production of biosurfactant by *Bacillus subtilis*YB7. YB7 produced 0.072 g L^{-1} of crude extract in MM whereas 0.5 g L^{-1} in MSM.

FTIR analysis of the crude extract from YB7: From Fig. 2, we can conclude that the crude biosurfactant from YB7 is a cyclic lipopeptide since the spectrum of the commercially available surfactin and isolated crude extract is superimposable and showed the presence of lactone ring, C-N bond, Amide II bond, aliphatic chain and -OH/-NH groups. These results confirm that it is a cyclic lipopeptide.

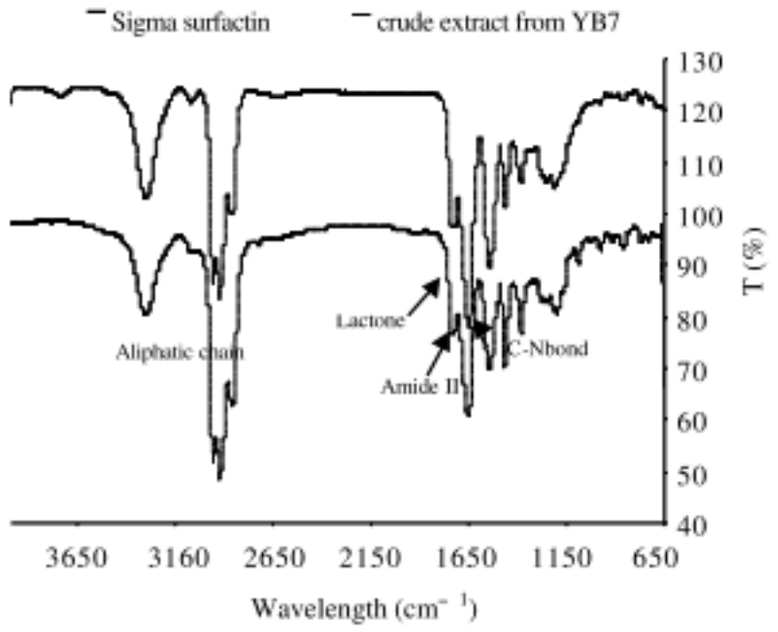

Fig. 2: Comparison of FTIR of sigma surfactin and crude extract of YB7

Reverse phase HPLC analysis of crude extract: Figure 3 shows the HPLC chromatogram of commercially available surfactin (purchased from sigma) and the isolated crude extract (Fig. 4) was similar. Commercially available and isolated cyclic lipopeptide showed six peaks with almost same retention time except the area under 3 peaks which are indicated by the black arrows. This shows that the polarity of the isoforms of crude extract is similar but the amount of the isoforms is different from that of the commercially available surfactin.

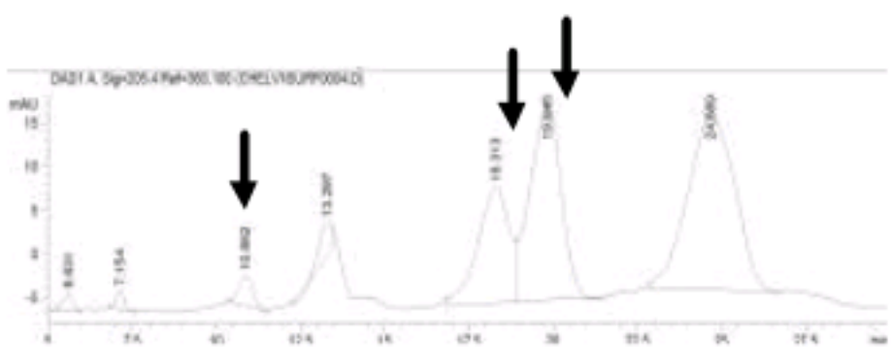

Fig. 3: HPLC chromatogram of sigma surfactin

ESI-MS analysis of surfactin: Figure 5 shows the molecular masses corresponding to each peak in HPLC chromatogram. The masses are equivalent to the masses of C_{12}-, C_{13}- and C_{14}-surfactin analogs. However, C_{15}-surfactin analog is absent in crude extract which is reported as a major isoforms in commercially available surfactin. C_{12}-surfactin surfctin analog is present which is not there with sigma surfactin. The report on the presence of C_{12} is very few (Kowall *et al*., 1998; Yakimov *et al*., 1996; Oka *et al*., 1993).

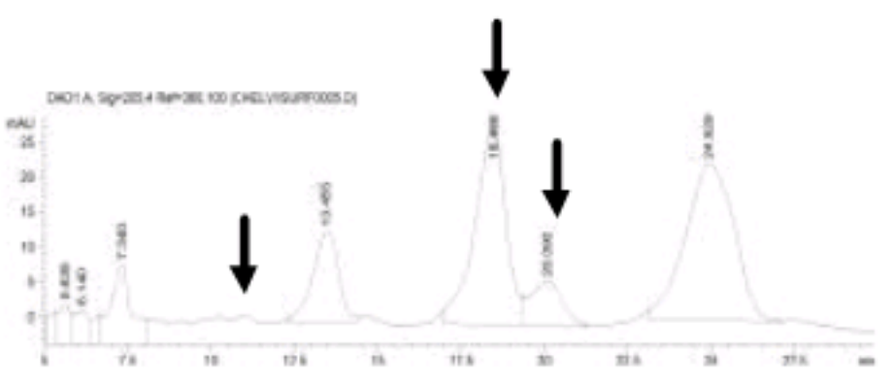

Fig. 4: HPLC chromatogram of crude extract

Surface tension analysis: Figure 6 shows the variation of surface tension versus concentration of the crude extract. The Critical Micelle Concentration (CMC) value was estimated to be around 40 μM, while the minimum surface tension value was 30 mN m^{-1}, which was almost similar to (27 mN m^{-1} for sigma surfactin) (Cooper *et al*., 1981; Kowall *et al*., 1998; Yakimov *et al*., 1996) providing

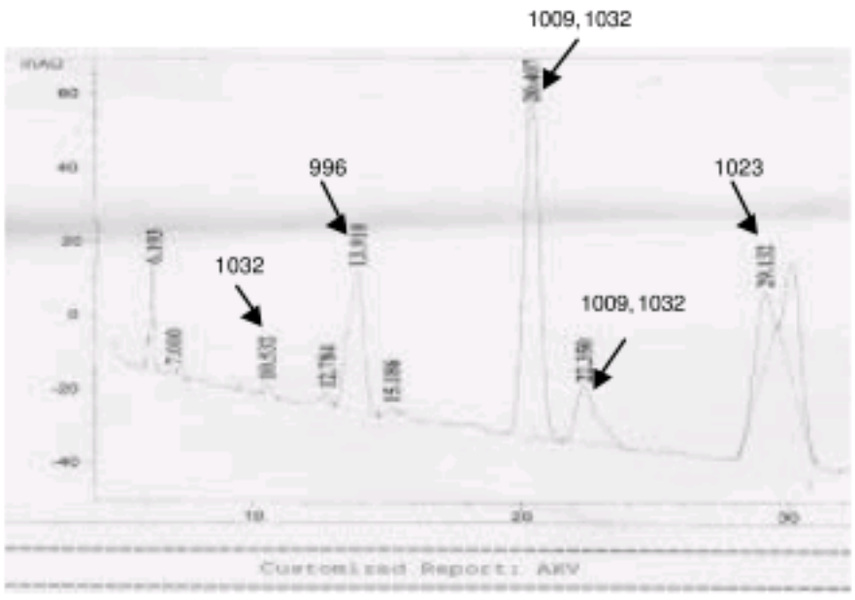

Fig. 5: LC/ESI MS analysis of crude extract

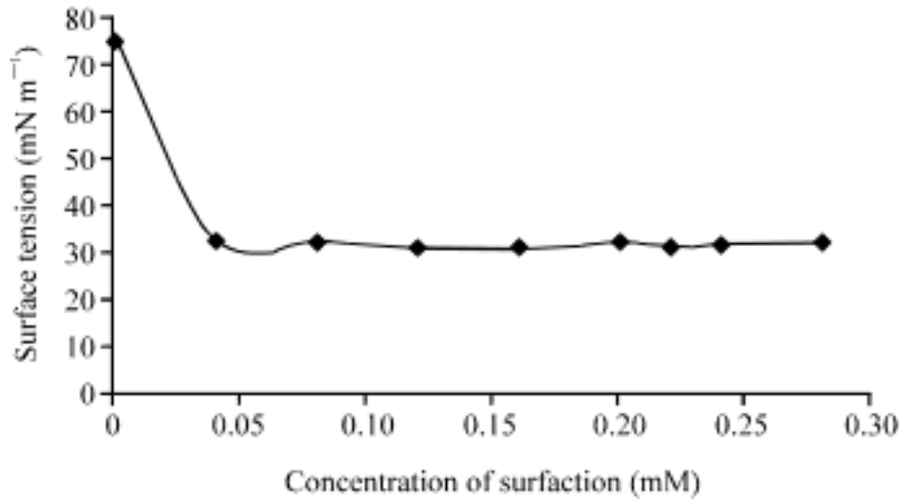

Fig. 6: Surface tension versus concentration of crude extract plot

further evidence to validate that these lipopeptides were surfactin analogs with minor variations in the molecular structure.

CONCLUSION

A Biosurfactant producing microorganism was isolated from plastic dump site and characterized as *Bacillus subtilis* YB7. This microorganism produced 0.5 g L^{-1} of biosurfactant (crude extract dry weight) in mineral salt medium. Crude extract of biosurfactant was isolated from the medium by acid precipitation and characterized as cyclic lipopeptide from FTIR analysis. This showed the presence of aliphatic chain and cyclic ester group. LC/MS analysis of the crude extract showed the molecular mass equivalent to C_{14}-surfactin analog which is a major component in the crude extract. This particular strain of *Baillus subtilis* YB7 did not produce C_{15}-surfactin and at the same time produced a rare C_{12} surfactin analog. This reduced the surface tension of water from 72 to 30 mN m^{-1}. All these differences confirm the uniqueness of this particular strain and the isolated crude extract which is partially characterized as cyclic lipopeptide. Amino acid sequencing and determination of the fatty acid chain length are crucial in order to eliminate the ambiguity in molecular identification which is under progress.

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