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Biosurfactant from endophytic *Bacillus cereus*: Optimization, characterization and cytotoxicity study

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ABSTRACT

Aim: The increased importance of biosurfactant in the recent past is mainly due to their applications in various industries ranging from petroleum to pharmaceuticals. Their biodegradability and environmental compatibility with low toxicity makes it even more interesting. Microbial production of biosurfactant is found to be a viable option as they are diverse, eco-friendly, facilitate large scale production, able to perform under extreme conditions etc. One class of microbes that is endophytes are known to show great potential in producing different varieties of medically and industrially significant biological compounds. The present study focuses on the screening and production of biosurfactant from endophytic bacteria.
Methodology and results: Of all the isolates tested, one endophyte identified as *Bacillus cereus* HM998898 was found to produce maximum biosurfactant. Statistical method Plackett burman was used to optimize the media for the maximum production and the ideal composition was found to be KNO_3 (1 g/L), Gingley oil (2 mL), K_2HPO_4 (2.5 g/L), KH_2PO_4 (0.75 g/L), $MgSO_4 \cdot 5H_2O$ (0.5 g/L), $FeSO_4.7H_2O$ (0.005 g/L) and NaCl (0.025 g/L). The extracted biosurfactant was characterized and was identified to be glycolipid. This was further tested for biocompatibility against Fibroblast (3T3) cells and was evaluated for their anti tumor activity against Hep2 cells.
Conclusion, significance and impact of study: The biosurfactant produced was found to induce toxicity to cancer cells at appreciable levels while they remained non-toxic to normal cells supporting the possible applications of biosurfactant in medical field.

Keywords: Endophytic bacteria, *Bacillus cereus*, biosurfactant, Plackett-Burman design, cytotoxicity

INTRODUCTION

The search for novel compounds for their use in medicine and industry is an evergreen task for the research community. The main features expected in these compounds are their compatibility with a living system, less toxicity, biodegradability, ease of production and manipulation. Biosurfactants gained interest in the recent past owing to its potential applications in medicine and environment.

Biosurfactants are the surface-active amphiphilic molecules obtained from a variety of microbes. They contain hydrophilic and hydrophobic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively. Chemically they are glycolipids, glycoproteins and lipopeptides that are expected to display varied properties and physiological functions (Singh and Cameotra, 2004; Banat *et al.*, 2010).

They are known to play a key role in remediation due to their efficacy as dispersion agents and display less toxicity and high biodegradability (de Cássia *et al.*, 2014). They are also found to be effective at extreme temperatures and pH (Singh *et al.*, 2007). They also possess certain additional advantages over synthetic surfactants by being diverse in their structure and biodegradable thereby making them an effective option for various commercial applications in the petroleum, biomedical, pharmaceutical and food processing industries (Cooper and Zajic, 1980; Desai and Banat, 1997).

Of late, researchers stated that biosurfactants are important for the growth and survival of microbes. For instance, *Bacillus subtilis* requires biosurfactant for the formation of fruiting body. Likewise, biofilm formation in *Pseudomonas aeruginosa* needs biosurfactant. These studies suggest the role of biosurfactant in the survival of microbes.

Therefore, exploring different microorganisms to produce biosurfactants is a continuous process.

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Especially large-scale production using new microorganisms, novel sources as raw materials is investigated to minimize the cost of production and increase the efficacy (Jorge *et al.*, 2013). Endophytes are one such collection of microbes which are known to produce several bioactive compounds with potential medical and industrial applications. To the best of our knowledge, very few reports are available on the production of biosurfactants using endophytes. Hence, the present investigation is carried out to use endophytic bacteria to produce biosurfactants and optimize the media for enhancing the production using the Plackett Burman statistical design. The compatibility of the biosurfactant is also checked by performing toxicity studies on cell lines.

MATERIALS AND METHODS

Screening of endophytic bacteria to produce biosurfactant

The endophytic bacteria that were isolated and studied by us earlier (Sunkar and Nachiyar, 2012a) were screened for the production of biosurfactants by growing these bacteria (1×10^{-8} CFU/mL) in Mineral salt media composed of KH_2PO_4 (1.4 g/L), K_2HPO_4 (2.7 g/L), $MgSO_4{\cdot}7H_2O$ (0.6 g/L), $FeSO_4{\cdot}7H_2O$ (0.01 g/L), NaCl (0.05 g/L), $CaCl_2$ (0.02 g/L), nitrogen source (1 g/L), carbon source (2%) for 5 days. The culture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant is collected and screened to produce biosurfactants using the following tests.

Emulsification test

The bacteria were grown in Mineral salt medium for 5 days at 37 °C. The reaction mixture contained 2 mL of oil (cooked oil) and 2 mL of culture supernatant obtained after centrifugation and vortexed for 1 min. The mixture was incubated for 24 h after which the emulsification index is calculated by measuring the height of the emulsified layer (Cooper and Goldenberg, 1987). The formula used for calculating emulsification index is given below:

$$E_{24}=h_{emulsion} / h_{total} \times 100\ \%$$

Oil spreading Assay

Oil spreading assay was conducted according to the procedure given by Morikawa *et al.* (2000). The microlitre of oil was added to the surface of 40 mL of distilled water in a petri dish to form a thin oil layer. Then, 10 µL of culture supernatant were gently placed on the centre of the oil layer. The presence of biosurfactant would displace the oil and a clear zone would form. The diameter of the clearing zone on the oil surface would be visualized under visible light and measured after 30 sec, which correlates to the surfactant activity, also known as oil displacement activity.

Drop collapse method

Drop collapse test was performed to screen the biosurfactant production (Jain *et al.*, 1991). The bacterial strains were inoculated in mineral salt medium with 0.1% cooked oil and incubated for 48 h. Two microlitre of oil was applied to the well regions delimited on the covers of 96-well microplates and these were left to equilibrate for 24 h. The 48 h culture was centrifuged at 12,000 × g for 15 min at 25 °C to remove the cells. Five microlitre of the supernatant was transferred to the oil-coated well regions and drop size was observed after 1 min with the help of a magnifying glass. The result was considered to be positive when the diameter of the drop was increased by 1 mm from that which was produced by distilled water which was taken as the negative control.

Blood haemolysis test

The fresh bacterial culture was streaked on blood agar plates and incubated for 48 to 72 h at 37 °C. The plates were then observed and the presence of clear zone around the colonies indicates the presence of biosurfactant producing organisms (Mulligan *et al.*, 1989).

Optimizing the carbon and nitrogen source

Biosurfactant production is significantly influenced by nutrients provided to the organism. Hence in the present investigation, various carbon sources (2 mL) namely cooked oil, Refined oil, Gingley oil, Amla oil, Coconut oil, Olive oil and nitrogen sources namely KNO_3, urea, NH_4Cl, NH_4NO_3 were used for evaluation of the most appropriate carbon and nitrogen sources for the production of biosurfactants.

Growth kinetics and biosurfactant production

Growth kinetics and corresponding production of the biosurfactant was followed for a period of 10 days. The endophytic *Bacillus cereus* was inoculated in mineral salt media and a day wise analysis of growth and biosurfactant production was estimated based on emulsification index.

Media optimization for the production of biosurfactant using Plackett Burman design

The effectiveness of an industrial fermentation is decisively dependant on the medium composition as this critically has an effect on the yield, concentration of the product and volumetric productivity. Conventional methods of optimising the media involve the changing of one constituent at a time and identifying the best media component. This method is laborious and time consuming when there are many factors to be tested. Plackett Burman design is a statistical method that is used to evaluate the most significant media components for biosurfactant production. An 8-Run Plackett Burman design was applied to reflect the relative importance of

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various fermentation factors involved in the production of biosurfactant. For each variable a high (+) and low (-) levels were tested. The variables used for the design are provided in Table 1.

Table 1: Variables affecting biosurfactant production and their high and low levels.

Variables	Component	High(g/L)	Low(g/L)
A	KNO_3	1.0	0.5
B	Oil	2.0	1.0
C	K_2HPO_4	2.5	1.25
D	KH_2PO_4	1.5	0.75
E	$MgSO_4 \cdot 5H_2O$	0.5	0.25
F	$FeSO_4 \cdot 7H_2O$	0.1	0.005
G	NaCl	0.05	0.025

Extraction and partial purification of biosurfactant

The produced biosurfactant was extracted by acid precipitation method. The cell free supernatant was subjected to an acid precipitation as described by Mawgoud *et al*. (2008). Briefly, the pH of the supernatant was adjusted to 2.0 with 4 M HCl and left overnight at 4 °C. Afterwards, the precipitate was collected by centrifugation (10,000×*g*, 10 min, 4 °C.) and washed twice with acidified water (pH 2.0). The precipitated crude biosurfactant is dissolved in 40 mL of demineralized water and the pH was adjusted to 7.0 using 1 M NaOH. This is followed by dialysis against demineralized water at 4 °C dialysis membrane (cut-off 6000‑8000 Da) for 48 h for purification. Finally, the purified biosurfactant was freeze dried and stored at −20 °C for further use.

Characterization of biosurfactant

The nature of biosurfactant was initially identified by the standard tests commonly employed namely Phenol-Sulphuric Acid Test, Biuret Test, CTAB (cetyl trimethylammonium bromide) /Methylene Blue agar plate test and Rhamnose test and was further characterized by instrumental analyses.

Fourier Transform Infra-red (FTIR) analysis

The major functional groups of the exopolysaccharide were identified using FTIR spectrophotometer. About 0.5 mg of dried sample was ground with 150 mg of KBr crystals and was made into a pellet using a hydraulic press. The pellets were subjected to FTIR analysis using PerkinElmer spectroscope.

HPLC analysis

Free sugars were determined by a High-Performance Liquid Chromatography (HPLC). A stainless-steel column of 0.05 m long and 4.6 mm in internal diameter followed along with a stain-less steel column 0.15 m long and 4.6 mm in internal diameter, both packed with amino propyl silyl silica gel for chromatography (3 µm) maintained at 38 °C was used. Mobile phase at a flow rate of 1.0 mL/min was prepared by dissolving 0.253 g of sodium dihydrogen phosphate in 220 mL of water and 780 mL of acetonitrile. Refractometer maintained at a constant temperature is used as detector (Agilent Bio HPLC Column Selection Guide). Identification of sugars was done by comparing the relative retention times of sample peaks with that of standards.1.00 mg of the test Sample was mixed with 2 mL of water and 2.5 mL of acetonitrile with gentle heating. 0.5 mL of water was added to the sample to form the test solution. Reference solution was prepared with glucose and fructose in 20 mL of water and 25 mL of acetonitrile with gentle heating and 5 mL of water.

GC-MS analysis

Twenty milligrams of the EPS were hydrolysed with 2 mL of 2 M Tri Fluoroacetic Acid (TFA) at 100 °C for 2 h. After hydrolysis, methanol was added into the dry sample and evaporated. The hydrolysate was then subjected to GC–MS analysis (GCMS-QP2010Ultra, Shimadzu) for the determination of the monosaccharide composition (Vidyalakshmi *et al.*, 2016).

Cytotoxicity using MTT assay

The cytotoxic effect of the biosurfactant was assessed against the 3T3 Fibroblast cell lines and Hep2 cell by MTT assay (Sunkar *et al.*, 2017). Varying concentrations of the biosurfactants were used (1.0, 0.5, 0.25, 0.13, 0.06, 0.03 and 0.02 mg/mL) and the % cytotoxicity was calculated using the formula:

$$\% \text{ Cytotoxicity} = Abs_{Control} - Abs_{Test} / Abs_{Control} \times 100$$

Anti-microbial activity

Antimicrobial activity was carried out using agar well diffusion assay (Sunkar and Nachiyar, 2012b). The test organisms used were *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Enterobacter* sp., *Serratia* sp. and *E. coli*. The activity was noted in terms of the zones of inhibition obtained.

RESULTS AND DISCUSSION

The prime reasons for the spreading interest in biosurfactants are their environmental-friendly nature, biodegradability (Mohan *et al.*, 2006) and low toxicity (Flasz *et al*., 1998). Their unique structures provide new properties that classical surfactants may lack. The sources of biosurfactants are diverse from terrestrial to marine organisms (Das *et al.*, 2010) and the quest for novel organisms to produce biosurfactants remains an incessant process. In this line, the present study tries to investigate the ability of endophytic bacteria to produce biosurfactant.

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Table 2: Screening results of seven strains for the production of biosurfactant.

Endophytic bacterial isolates	Emulsification Index (%)		Oil Spreading	Drop Collapse	Haemolysis
CBGP	52.38	++	-	+	-
CBGL	25.00	+	-	+	-
ADA	60.15	+++	+	+	+
GX1	11.29	+	-	-	-
GX2	15.6	+	-	-	-
GX3	-	-	-	-	-
GX4	17.9	+	-	-	-
GX5	20.7	+	-	-	-
SYZ 4	18.6	+	-	-	-
P4B	54.54	++	+	+	-
P3B1	53.12	++	-	+	-
P1A	50.00	++	-	+	-
P2A	21.5	+	-	-	-
P2B	15.9	+	-	-	-
WT	22.5	+	-	-	-
ARA	24.6	+	-	-	-

(For Emulsification index "+" = 10-30 % EI; "++" = 30-55 % EI ; "+++"= > 55% EI; For the other tests "-" indicates negative result, "+" indicates positive result)

Screening of endophytic bacteria to produce biosurfactant

Endophytic bacteria that were isolated earlier (Sunkar and Nachiyar, 2012a) were used in the current study where they were subjected to standard screening tests to produce biosurfactant namely oil spreading test, drop collapse test and emulsification index and the results are provided in the following table (Table 2).

The table clearly indicates that certain bacteria have the ability of emulsifying oil. From the results obtained, it is very much evident that the endophytic bacterial isolates ADA and P4B1 were producing noticeable amounts of biosurfactant and the isolate ADA which is showing the maximum emulsification index (60.15%) is selected for further studies. The isolate ADA was identified as *Bacillus cereus* HM998898.1 and described elsewhere (Sunkar and Nachiyar, 2012b). Several bacteria displayed promise in producing biosurfactants. Strains like *Bacillus subtilis* ICA56 (Franc *et al.*, *2*015) with bioremediation potential, *Bacillus methylotrophicus* USTBa (Chandankere *et al*., 2013) isolated from petroleum reservoir which was later used for the degradation of crude oil, *Bacillus subtilis* group (PTCC 1696) which was isolated from an Iranian oil field, have been investigated which displayed potential stability under different temperatures and pH (Ghojavand e*t al*., 2008).

Optimizing the carbon and nitrogen source

The production of biosurfactant was found to be dependent on the composition of the medium. It is well known that the growth of cells and amassing of metabolic products were powerfully influenced by the composition of the medium which constitutes carbon sources, nitrogen sources, salinity and other factors which favors the high yield of metabolites (Muthusamy *et al*., 2008). The endophytic bacterium *Bacillus cereues* was cultured in MS media with varying carbon sources and nitrogen sources and the results are provided in table 3.

Among the carbon sources, it was found that significant production was noticed when gingley oil was used (EI 75%) followed by cooked oil (EI 63.63%). Likewise, all nitrogen sources used were supporting the production on biosurfactant but the most favorable was found to be KNO_3 (EI 70%) followed by urea (EI 50%). Hence for further studies gingley oil and KNO_3 were used as carbon and nitrogen sources.

Though certain reports suggested that olive oil (Abouseoud *et al.*, 2007), glucose (Aparna *et al.*, 2012) were considered to be the ideal carbon source, the results in the present study showed gingley oil to be the best carbon source for the biosurfactant production. Abouseoud *et al.* (2007) reported that olive oil was found to be best suitable for the production of biosurfactant using *Pseudomonas fluorescens*. Certain strains of bacteria like *Virgibacillus salaries* showed significant production of biosurfactant using fried oil thereby making it a cost-effective option (Elazzazy *et al*., 2015). Coconut oil was also found to be a suitable carbon source for the production of biosurfactant with *Pseudomonas aeruginosa* F23 (Patil *et al*., 2014). These reports indicate the diverse ability of the microorganisms to utilize different kinds of carbon sources for the production of biosurfactant.

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Table 3: Optimization of carbon and nitrogen source.

Emulsification index		Result	EI %
Carbon source	Cooked refined oil	+++	63.63
	Uncooked refined oil	-	Nil
	Olive oil	-	Nil
	Gingley oil	+++	75.00
	Coconut oil	++	50.00
	AmLa oil	-	Nil
Nitrogen source	Urea	++	50
	KNO_3	+++	70
	NH_4Cl	++	45
	NH_4NO_3	++	35

The present study reports that KNO_3 was ideal for the maximum production of biosurfactant which goes well in accordance with the reports of Saikia *et al*. (2012) and Patil *et al*. (2014) which stated that potassium nitrate was found suitable to produce biosurfactant. Another strain *Bacillus subtilis* MTCC 2423 was found to produce maximum amount of biosurfactant with potassium nitrate compared to other nitrogen sources (Makkar and Cameotra, 1998). The result is further supported by a study conducted by Lan e*t al*. (2015) who reported that nitrate nitrogen was more effective than amino nitrogen for biosurfactant production.

Growth kinetics and biosurfactant production

Experiments have been conducted to estimate the production of biosurfactant in relation with the growth of the organism periodically using emulsification index as parameter (Figure 1).

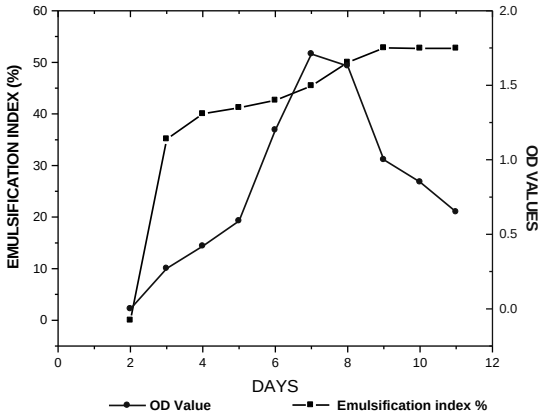

Figure 1: Growth kinetics and biosurfactant production.

The Figure 1 clearly illustrates that time positively influenced the production of the biosurfactant. There was an increase in the OD values and a simultaneous increase in the biosurfactant indicating the relation between the growth and biosurfactant production. The production of biosurfactant was estimated day wise and it was observed that there is an increase in the biosurfactant production with time. The production measured in terms of EI showed a sudden increase in the first two days after which the increase was found to be gradual and steady. This trend was followed for a period of 8 days (EI 52%) after which the values were found to be stable without much variation. This can be directly correlated to the growth of the bacteria as there was an increase in the OD values till 7 days followed by a downtrend indicating the decline phase of the bacterial growth. Correspondence of these two parameters clearly indicates that the production of biosurfactant was consistent with the growth of the bacteria. Once the decline phase started (day 7), the production of biosurfactant seemed to remain the same without much variation.

The production of biosurfactant was observed chiefly during the exponential phase of growth which implies that this could be a product of primary metabolism complementing with the biomass development suggesting growth associated kinetics (Abouseoud *et al*., 2007; Persson *et al*., 1988). Similar trend was observed by the studies conducted by Ruwaida *et al*. (1991) for *Rhodococcus*, strain ST-5 and Tabatabaee *et al*. (2005) where in the production was in correlation with the growth of the bacteria. This feature also proposes that biosurfactant can be well produced under chemostat conditions or by immobilized cells (Klein and Wagner, 1987). This kind of production was also supported by studies carried out by Abbasi *et al*. (2012) who reported that *Pseudomonas aeruginosa* was producing maximum rhamnolipid after 10 days after which there was a decrease in the production.

Design of media using Plackett Burman model

Microbial growth rate depends on chemical and physical parameters such as medium components, pH, aeration and agitation rate. The microbial cells producing primary and metabolites are influenced by medium components that include carbon, nitrogen and other inorganic salts (Cutchins *et al.*, 1952). Hence searching for the ideal constituents and their optimization for enhanced production involves several factors and is considered to be a tedious task.

The initial experiments to determine the ideal carbon and nitrogen source revealed that gingley oil and KNO_3 were best suited for the production of biosurfactants. In spite of identifying the carbon and nitrogen source, the ideal composition of the nutrients required for the maximum production of biosurfactant. This can be determined by using one variable at a time approach. Since this is time consuming, Plackett Burman statistical design is preferred to determine the optimal concentrations of the components for the enhanced production. It facilitates the examination of upto N-1 variables with N experiments on the assumption that

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there exist no interactions between the components. The study employed 7 variables in 8 trials to evaluate the influence of the media components on the biosurfactant production. Table 4 provides the results obtained after the experiment in terms of EI and the weight of the biosurfactant for each trial. Trial 1 gave the maximum production of biosurfactant which constituted KNO_3 (1 g/L), Gingley oil (2 mL), K_2HPO_4 (2.5 g/L), KH_2PO_4 (0.75 g/L), $MgSO_4 \cdot 5H_2O$ (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.00 5g/L) and NaCl (0.025 g/L). The effect of the components is based on the difference between the average of H and L responses for each variable (Equation 1). The effect of an independent variable on the response is the difference between the average response for the four experiments at H and the average value for four experiments at L and given in Equation 2.

$$\boldsymbol{\Sigma A\,(H) - \Sigma A\,(L)} \quad \text{Equation 1}$$

$$\boldsymbol{A = \Sigma AH/4 - \Sigma AL/4} \quad \text{Equation 2}$$

$$2(\Sigma A\,(H) - \Sigma A\,(L))/8 \quad \text{Equation 3}$$

The experimental error can be calculated by averaging the mean squares of the dummy effects of E and G. The final stage is to identify the factors which are showing large effects. In the above experiment, this was done using an F-test which is calculated by using the formula:

Factor mean square / error mean square

The regression coefficient, F value and P value of the factors were calculated for biosurfactant production using statistical design. The response was analyzed by analysis of variance (ANOVA) a statistical parameter that provides the significant of the model employed. Table 5 gives the details of the trails with varying concentrations along with the response in terms of biomass produced.

On the basis of statistical tests results obtained (Table 5), A (KNO_3), B (Oil) and C (K_2HPO_4) was found to be the most significant components influencing the production of biosurfactant. The importance of the variables influencing the production of the biosurfactant and the results of Plackett- burman are viewed in a better way by Pareto chart (Figure 2).

Table 4: Plackett Burman experimental design and the corresponding response.

Trials	A	B	C	D	E	F	G	Response	
								EI (%)	Weight of the biosurfactant (g/L)
1	H	H	H	L	H	L	L	60.00	11.32
2	L	H	H	H	L	H	L	52.00	0.15
3	L	L	H	H	H	L	H	50.00	2.27
4	H	L	L	H	H	H	L	55.26	8.62
5	L	H	L	L	H	H	H	58.97	0.24
6	H	L	H	L	L	H	H	53.55	1.00
7	H	H	L	H	L	L	H	54.05	9.69
8	L	L	L	L	L	L	L	52.45	8.49

Table 5: Statistical analysis of effects of medium constituents on biosurfactant production as per Plackett burman design.

	A KNO_3	B Oil	C K_2HPO_4	D KH_2PO_4	E $MgSO_4 \cdot 5H_2O$	F $FeSO_4 \cdot 7H_2O$	G NaCl
∑H	4.18	4.25	3.03	4.19	3.12	3.63	3.96
∑L	3.04	2.97	4.19	3.03	4.1	3.59	3.26
D	1.14	1.28	-1.16	1.16	-0.98	0.04	0.7
MS	1.2996	1.6384	1.3456	1.3456	0.9604	0.0016	0.49
MSE				0.0601			
F Test	2.7019	3.4062	2.7975	2.7975	1.9967	0.0033	1.0187
P value	0.0117	0.0029	0.0131	0.0141	0.0635	1.0	0.4329

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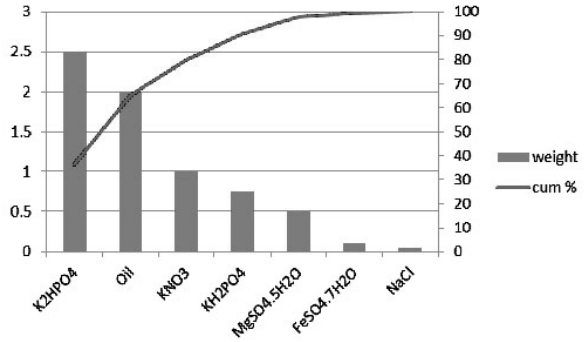

Figure 2: Pareto-Plot for Plackett-Burman parameter estimates for seven medium components.

It elucidates the order of significance of variables that effect the biosurfactant production. The significant factors were found to be KH_2PO_4, oil source and KNO_3. These factors can be later used for media optimization using response surface methodology in future studies.

Extraction and characterization of biosurfactant

The biosurfactant produced by the endophytic bacterium was extracted (white precipitate), dried and used for further studies. The different types of biosurfactants are glycolipids, lipopeptides and phospholipids and are identified using the standard tests namely Phenol-H_2SO_4 test, Biuret test, and phosphate test which are performed with the cell-free supernatant of the endophyte *Bacillus cereus*.

Positive results were obtained for phenol-H_2SO_4 test and CTAB test indicating the presence of sugars and glycolipids. Further characterization of the biosurfactant was carried out by FTIR and GC MS analysis.

FTIR analysis

The possible functional groups present in the sample were identified by FTIR analysis and results are provided in Figure 3.

The IR spectra showed broad peaks were obtained at 3394 cm^{-1} corresponds to the O–H stretching vibrations. The peak at 2854 cm^{-1} that was obtained in both the spectra corresponds to C-H stretching vibrations of aliphatic groups. Other small peak noticed at 2288 cm^{-1} implies a –C≡C– stretch. Two peaks in the range 1746 to 1641 cm^{-1} correspond to the C=O stretching of ester groups. The peaks obtained in the range 1200 - 1460 cm^{-1} indicate the presence of C-O-C, C-C, and C-H groups usually present in the carbohydrates. Weak bands at 3010 and 1746 cm^{-1} indicate the presence of Rhamnolipid (Zhao *et al.*, 2013). The peaks usually found in between 1300 to 1150 cm^{-1} indicate the presence of CH wag of alkyl halides and the smaller peaks obtained from 725 to 524 cm^{-1} corresponds to alkyl groups. The result obtained in the present investigation suggests the presence of lipids in the biosurfactant. Another study where in *Pseudomonas aeruginosa* M14808 was used for biosurfactant synthesis revealed similar groups in FTIR spectra. Also, *Streptomyces* sp. MAB36 displayed the ability to produce a glycolipid biosurfactant that was identified by the peaks obtained in the FTIR spectra (Manivasagan *et al*., 2014). Further a report by Park *et al*. (1998) also specified similar peaks that confirmed the presence of glycolipids in the biosurfactant produced by *P. aeruginosa* YPJ-80 using chromatographic and spectroscopic techniques. The peaks obtained in the present investigation are in agreement with the previous studies and points out that the possible chemical structures may be identical to glycolipids and rhamnolipids which are made up of rhamnose rings and long hydrocarbon chains (Pornsunthorntawee *et al.*, 2008).

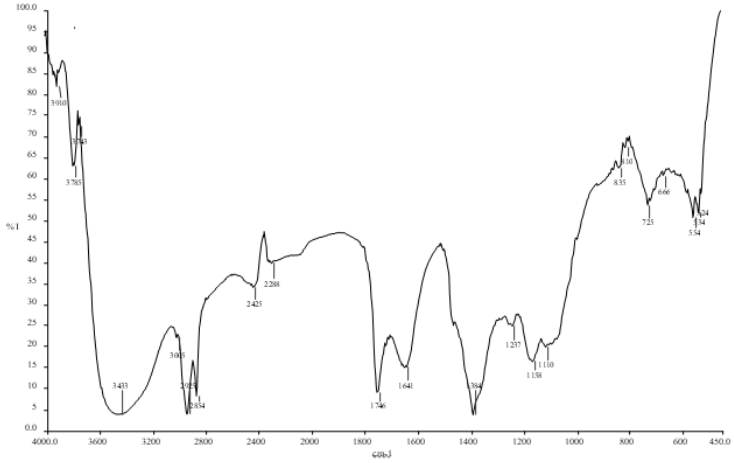

Figure 3: FTIR spectrum of the biosurfactant.

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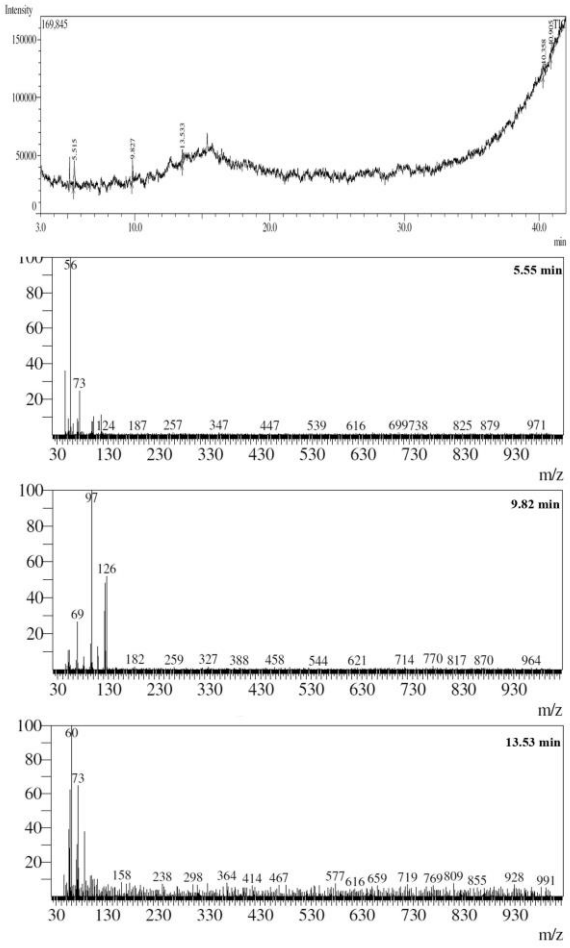

Figure 4: GC MS analysis of the biosurfactant.

GC-MS analysis

The GC-MS analysis of the ethyl acetate extract of the biosurfactant indicated the presence of 9-Hexadecenoic acid, methyl ester (Z) and 1-Eicosanol with the retention time 14.95 and 15.85 respectively. This result goes well in accordance with the findings of Sharma *et al*. (2015) who reported the presence of long chain fatty acids, mainly C-16 long fatty acids in the biosurfactant produced by *Enterococcus faecium*. Many studies revealed that Hexadecanoic acid was a main fatty acid in glycolipids that were studied earlier. A study by Saravanakumari and Mani (2010) reported the presence of octadecanoic acid with a sugar moiety in the biosurfactant produced from *L lactis*. Another report demonstrated that Rhamnolipids are extensively isolated glycolipids that were composed of β-hydroxydecanoic acid molecules as branched fatty acids (Desai and Banat, 1997). Some reports also suggested the presence of Palmitic acid and stearic acid in cell bound biosurfactant produced by *L. pentosus* (Vecino *et al*., 2014).

The GC MS spectra (Figure 4) gave a peak at 5.5 min corresponding to 4 oxopentanoic acid, a breakdown product of hexose. A peak at 9.82 min indicates the presence of 5-Hydroxymethylfurfural, which is the dehydration product of fructose. The presence of glucose can be identified from the peak at 13.53 min.

HPLC analysis (Figure 5) further confirmed the presence of glucose and fructose with the RT value of 20.87 min and 28.12 min respectively. HPLC also indicated the presence of rhamnose at 33.45 min. The presence of sugars along with fatty acids was earlier reported by Saravanakumari and Mani (2010) wherein they isolated a biosurfactant which was a xylolipid. Rhamnose was also reported to be a component of biosurfactant as demonstrated by Lan (2015). The presence of fatty acids and sugars identified by GC MS and HPLC clearly indicate the glycolipid nature of the biosurfactant.

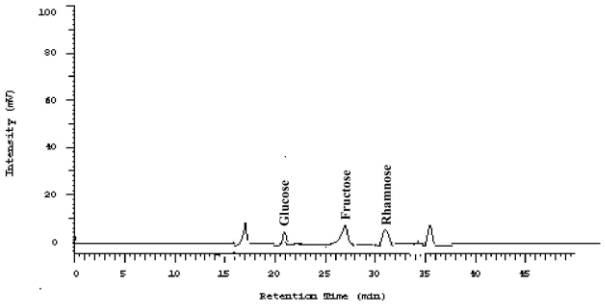

Figure 5: HPLC chromatogram of biosurfactant.

Cytotoxicity studies

The discovery and adaptability of new molecules in the medical field requires their irritant potential evaluation which at present is carried out using various cell lines. Moreover, the commercial applications of biosurfactants in the medical field have increased during the past decade. Their antibacterial, antifungal and antiviral activities make them relevant molecules for applications in combating many diseases and as therapeutic agents. In view of the potential offered by the biosurfactant in medical field, a study was carried out to assess the compatibility of biosurfactant with cells. The toxicity of the biosurfactant was checked against normal Fibroblast cells 3T3 and Hep2 cancer cells (Figure 6).

The toxicity of the biosurfactant was checked on normal cells (Fibroblast cells 3T3), for 4 h, 24 h and 48 h and the results are provided in Figure 6a. As concentration increased the toxicity slowly increased to around 50%. Prolonged exposure with even with higher concentrations also showed 50% toxicity. The results clearly indicate that the biosurfactant produced was compatible as 50% toxicity was obtained only after exposing for 24 h, where the IC_{50} value is found to be 0.27 mg/mL while there was not even 50% toxicity after 4 h of exposure at lower concentrations. It was noticed that even after 48 h of exposure, significant cytotoxicity was not noticed as the IC_{50} value was 0.25 mg/mL thus making it compatible with cells and thereby proposing its

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use in medical field. The results obtained in the present investigation goes in accordance with the results of Jadhav *et al*. (2013) which demonstrated the non-toxic nature of biosurfactant produced by a bacterium *Oceanobacillus* sp. BRI 10 even at high concentrations. Another report by Diaz *et al.* (2013) clearly suggested lower levels of cytotoxicity of biosurfactant against fibroblast cells than SDS, a chemical surfactant.

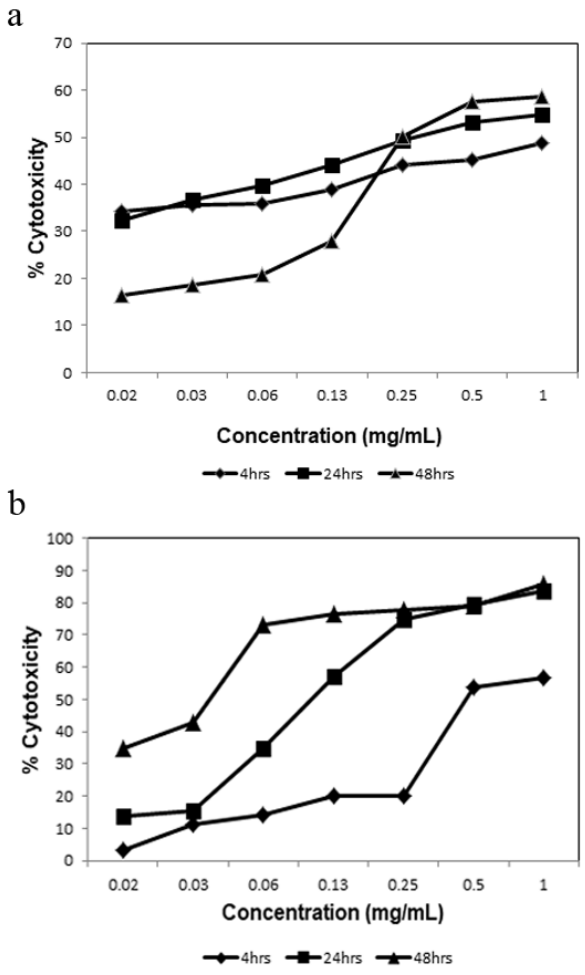

Figure 6: Cytotoxicity of the biosurfactant against: a) Fibroblast 3T3 cells, b) Hep2 cells.

Simultaneously, anticancer activity of the biosurfactant was also evaluated against Hep2 cell lines using varying concentrations at different time intervals and the results are provided in Figure 6b. From the results obtained, it was observed that there was time and dose dependent cytotoxicity against Hep2 cell lines. After 4 h, it was noted that a concentration of 0.465 mg/mL was required to bring about 50% toxicity. It was found that 0.10 mg/mL was needed to induce cytotoxicity when exposed for 24 h. But only 0.03 mg/mL of the biosurfactant was found to induce 50% toxicity when exposed for 48 h. These results clearly indicate the potential of biosurfactant as an anticancer agent as 75% cytotoxicity was brought about with the mentioned concentrations. The IC 50 values were calculated for 4 h (0.465 mg/mL), 24 h (0.1 mg/mL) and 48 h (0.03 mg/mL). This study therefore demonstrates the potential of biosurfactant as a probable anticancer agent as this was able to bring about appreciable level of apoptosis in Hep2 cells.

Certain reports state that these compounds disrupt cell membrane that leads to cell lysis by increasing the membrane permeability that eventually led to the leakage of metabolites. The modification in the membrane structure alters its function significantly and disturbs the entire biological process (Heerklotz and Seelig, 2006; Lee *et al*., 2012). Of late evidences are available on the ability of biosurfactant to induce apoptosis. A study by Zhao *et al.*, (2000) reported that glycolipids played a crucial role in arresting the growth of tumor cells. This was identified by the accumulation of B 16 cells in sub-G0/G1 phase which is an indication of apoptosis which was observed by the condensation of chromatin and DNA fragmentation in mouse melanoma B 16 cells. Another glycolipid produced from *Sphingobacterium detergens* was found to be effective against $CaCO_2$ human colorectal cancer cells (Diaz *et al*., 2013). Likewise, a surfactin made of lipopeptide was shown to induce apoptosis in MCF 7 breast cancer cells (Cao *et al.*, 2010). The results obtained in the present investigation advocate the capability of biosurfactant in bringing about apoptosis and hence can be considered as a promising candidate in cancer management.

Anti-microbial activity

The anti-microbial activity was carried out using six different bacterial strains as test organisms. They were *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Enterobacter* sp., *Serratia* sp. and *E. coli*. The isolated biosurfactant did not show noteworthy zones of inhibition but noticeable zones were developed against *Staphylococcus* sp. and *Serratia* sp. Though there are reports on the efficacy of biosurfactant as antimicrobial agents (Gudina *et al.*, 2010; Ghribi *et al.*, 2011), our results reveal that the biosurfactant is not very effective against the pathogenic bacteria (data not shown).

CONCLUSION

The potential of endophytes in the production of biosurfactant has been explored in the present investigation. Endophytic bacteria were screened for the production of biosurfactant and it was observed that the endophyte *Bacillus cereus* showed positive results for all the screening tests employed. Media was optimized for the production of biosurfactant using various carbon and nitrogen sources and it was found that gingley oil and KNO_3 were the best sources respectively. Plackett Burman design was employed to optimize the media using 7 variables and 8 trials and the first trial with the composition KNO_3 (1 g/L), Gingley oil (2 mL), K_2HPO_4 (2.5 g/L), KH_2PO_4 (0.75 g/L), $MgSO_4 \cdot 5H_2O$ (0.5 g/L),

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$FeSO_4 \cdot 7H_2O$ (0.005g/L) and NaCl (0.025g/L) gave the maximum emulsification index (60%) with maximum biosurfactant production (11.32 g). The biosurfactant was found to be a glycolipid based on the tests and instrumental analysis carried out. The biosurfactant produced was studied for its toxicity against normal Fibroblast 3T3 cell lines and Hep2 cell lines. The biosurfactant didn't show much of toxicity against the normal cell lines but showed significant toxicity against Hep2 cell lines making them potential candidate for various medical purposes. This study puts endophytes in the list of biosurfactant producers and the increased production using Plackett burman model further promotes a large-scale production.

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