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Optimization and Characterization of Lipopeptide Biosurfactant

KEYWORDS: Bacillus spp., whey, mineral salt medium and surfactin

*** Dr.Priya Iyer**
PG and Research Department, Department Of Biotechnology, Women's Christian College, College Road,Chennai-600 006. *Corresponding author

P.Sandhya
PG and Research Department, Department Of Biotechnology, Women's Christian College, College Road,Chennai-600 006.

ABSTRACT *Lipopeptides are the bioactive peptides and some constituents of these compounds are surfactin, fengycin, iturins, mycosubtilins and bacillomycins. Among these lipopeptides, surfactin is produced by Bacillus subtilis that has strong anti-microbial,anti-viral and anti-larvicidal activities. In the present study, Bacillus spp. was chosen for lipopeptide production and the growth medium used was whey + mineral salt media. The optical density of the growth medium was checked for 72 and 96 hours and the best results were observed in 72 hour culture; thus chosen for lipopeptide production.The biomass and the crude obtained were estimated to be 21.72 g/L and 5.0 g/L respectively. The crude obtained was characterized for its surfactant molecule using MALDI-MS and FTIR. MALDI-MS showed the highest peak of 1029.8 at the intensity of 69.4 indicating the presence of surfactin molecule. FTIR also proved the presence of surfactin*

Introduction:

Biosurfactants are amphiphilic compounds produced by bacteria, fungi and yeast. They belong to various classes including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipo-polysaccharides. The properties/applications of biosurfactants includes excellent detergency, emulsification, foaming, dispersing traits, wetting, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovering (oil) which allows biosurfactants an ability to replace some of the most versatile process chemicals. In addition biosurfactants are promising natural surfactants that offer several advantages over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability (**R.Thavasi et.al**).Optimization takes place under laboratory conditions before up scaling for mass production. Although there is considerable information available on laboratory-scale fermentation of Bacillus, published literature has declined as processes became commercially more significant. (**Sharp et al., 1989**).A great deal of research has been carried outon these cyclic lipopeptides and their structures have been fully characterized. The ability toisolate, purify and characterize these structures is extremely important, providing detailedinformation with regard to different cultivation condition and biological activities. Similarmethods can be used for both lipopeptides and biopolymers especially when attempting todetermine their amino acid sequences. The experimental techniques used to isolate, purify andanalyses these biosurfactant are widely varied from simple colorimetric assays giving anapproximate indication of the type of compounds present to the more complex mass spectrometric techniques that provide information on molecular mass and structural features. Massspectrometry provides essential information in the identification of these structures usingsophisticated MS/MS experiments and software technologies(**T. J. P. Smyth et.al 2010**).The bacillus spp. was isolated and screened for its surfactant activity in the previous study. Thus in the present study, the growth medium and culture conditions are optimized. The obtained product was characterized using MALDI-MS and FTIR.

Materials and methods:

Inocula:

The Bacillus isolate was selected for the biosurfactant production. Thus the fresh culture of the isolate was inoculated into 10mL nutrient broth and allowed for overnight culture with shaking at 30°C set for 150 rpm.

Optimization of Growth media:

The growth media used for the lipopeptide production is whey and mineral salt media g/dL(Milk Whey-50ml; Disodium Hydrogen Phosphate-1.5; Glucose-0.30;Ammonium Sulphate-0.4; Dipotassium Hydrogen Phosphate - 0.10; Magnesium Sulphate -0.04; Mangnese Sulphate- 0.010; Calcium Chloride - 0.16; deionised Water - 50ml at PH-7.2+ 2 at 35°C) in the ratio of 1:1. The medium was autoclaved at 121°C under 15 lbs pressure for 15mins. The sterilized medium was cooled and inoculated with 1mL of the seed inocula. This inoculated medium was incubated in Orbital shaker at 30°C for 96 hrs set at 150rpm. After 24 hrs 10mL of the culture was taken and centrifuged at 10,000rpm for 20min. Optical density of the supernatant was read at 620nm for detection and quantification of the biosurfactant production. This procedure was done for 48, 72 and 96 hours. The OD values were recorded for each hour in Table 2.

Mass Production:

The growth medium was optimized using the OD values obtained in Table 2 and the Bacillus was subjected to mass production up to 1 litre. Whey + mineral salt medium (1L) was inoculated with 10mL of the seed inoculum and incubated in Orbital shaker at 30°C for 72 hrs set at 150rpm.

Isolation of Biosurfactant:

The 72 hr culture was chosen for the production of the biosurfactant based on the trial studies. The extraction procedure is a combination of acid precipitation and solvent extraction **(Vater et.al; 2002)**.The culture broth was centrifuged in a cooling centrifuge at 10,000rpm for 20mins at 4°C to remove the cells.The supernatant collected was acidified by the addition of concentrated HCl to pH 2.0and allowed for overnight precipitation at 4°C. The pellet was extracted from the supernatant using ethyl

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acetate solvent in a separating funnel.Supernatant and the solvent were shaken vigorously in the separating funnel and allowed for the precipitation of the biosurfactant. The biosurfactant molecules get separated from the supernatant and they were collected separately from the supernatant. The solvent containing supernatant was discarded.The collected precipitate was heated to 40°C for the evaporation of the solvent and the dried mass was resuspended in deionized water. This suspension was stored at 4°C and used for the further studies.

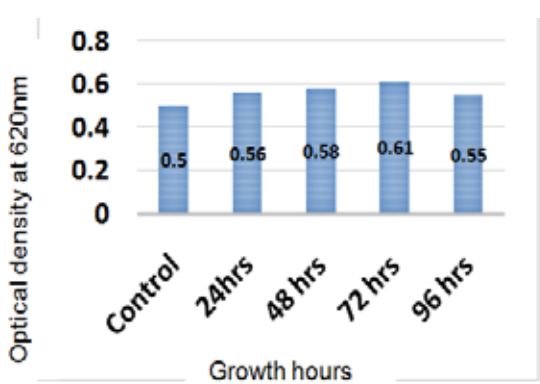
Estimation of Biomass:
The biomass of the pellet and the crude precipitate was estimated by calculating the dry weight and the results were recorded.

Detection of the Surface Active Compounds:
Matrix Assisted Laser Desorption Ionistion-Mass Spectroscopy (MALDI-MS) Analysis:
Molecular weight of the crude biosurfactant molecule was determined using MALDI-MS from Sophisticted Analytical Instrument Facility (SAIF), Indian Institute of Technology,Chennai and the results were recorded in Table 3.

Fourier Transform Infra-Red Analysis:
Fourier transform infrared spectroscopy (FTIR) was used to determine the functional groups and the chemical bondspresent in the biologically active fraction of the biosurfactantand thus determine its chemical nature.The spectralmeasurementswere carried out in the absorbance mode.Thespectra were normally acquired with the use of 4 cm^{-1} resolutionyielding IR traces over the range of 400–4000 cm^{-1}. The IR spectra of each sample were the averageof 32 data scanning over the entire range of wave numbers. The analysis was done in Crystal Growth Department, Anna University,Chennai and the results were recorded.

Results and Discussion:
Optimization of Growth media:
Table 1: OPTICAL DENSITY OF THE WHEY +MSM CULTURE FOR DIFFERENT HOURS(trial)

Growth Hours	Optical density
control	0.50
24hrs	0.56
48 hrs	0.58
72 hrs	0.61
96 hrs	0.55



Graph 1:OPTICAL DENSITY OF THE WHEY +MSM CULTURE FOR DIFFERENT HOURS(trial)

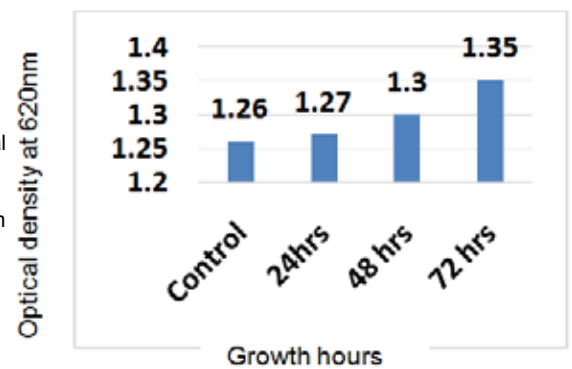
From the trial studies shown in graph 1, it was confirmed that 72 hrs culture produced the maximum OD and hence the growth medium was optimized and the culture was mass cultured to 1litre for the lipopeptide production.

Mass Culture of Bacillus:
Whey+mineral salt media was optimized by the above study and the mass production to 1 litre was done for lipopeptide production.

Table 2: OPTICAL DENSITY OF THE WHEY +MSM CULTURE FOR DIFFERENT HOURS

Growth Hours	Optical density
Control	1.26
24hrs	1.27
48 hrs	1.30
72 hrs	1.35

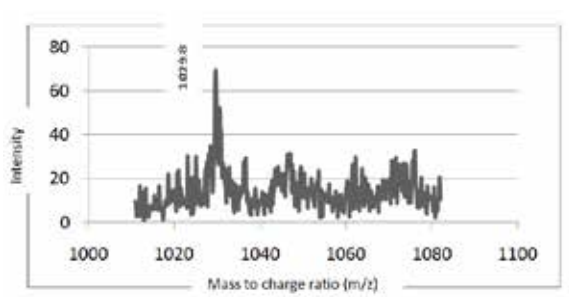
Graph 2: OPTICAL DENSITY OF THE WHEY +MSM CULTURE FOR DIFFERENT HOURS



Optical density at 620nm
Growth hours

Estimation of Biomass:
Graph 2 exhibits the highest OD value for 72 hour culture and the value decreased after 72 hours. The biomass of the 72 hour culture was estimated to be 21.72g/L and the crude biosurfactant amount was 5.0g/L using whey as the carbon source. The extraction process was done with ethyl acetate according to the method of recovery described by **Jitendra D.Desai**. Similar results were observed in the method followed by Paulo Andre Vincente. The maximum concentrations of biomass and crude biosurfactant obtained were 7.8 g/L and 2.0 g/L using glucose as the carbon source.

Detection of the Surface Active Compounds:
Matrix Assisted Laser Desorption Ionization-Mass Spectroscopy (MALDI-MS) Analysis:
Graph3: MOLECULAR WEIGHT OF DIFFERENT COMPOUNDS AND THEIR INTENSITIES



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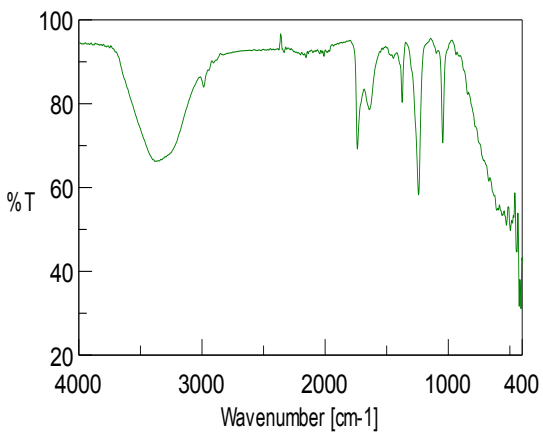
The results obtained from MALDI showed high peaks at m/z values between1020-1040. The mass peaks obtained were1018.5, 1020.5,1027.5&1027.9. The highest peak was obtained for 1029.8 having the intensity of 69.4 which were similar to the peaks for surfactin and the results were interpreted in Graph 3. Similar results were observed by **A.M. Manonmaniet.al.** MALDI-TOF spectrum study of CMM showed well-resolved groups of peaks at m/z values between 1000 and 1080. The mass peaks obtained were at m/z 1030.6, 1046.7, 1044.6, 1052.6, 1058.7, 1060.7, 1066.7 and 1074.8.The purified mosquito pupicidal metabolite from *B. subtilissubsp. subtilis* was earlier identified as a cyclic lipopeptide, surfactin by IR, NMR and MALDI-TOF analysis. This study also showed that the CMM when subjected to MALDI-TOF analysis exhibited peaks indicative of surfactin. The group of peaks could be attributed to the isoform ensembles of surfactins produced by the mosquitocidal strain. It was clear that the cyclic lipopeptide surfactin was the main component produced by the strain isolated.

FTIR Analysis:



The lipopeptide nature of the biosurfactant was further confirmed by the IR spectra of the compound. A broad absorbance with wave numbers ranging approximately from 3500 cm^{-1} to 3200 cm^{-1} having its maxima at 3300 cm^{-1} was seen. Absorbance in this region is caused as a result of C–H stretching vibrations and N–H stretching vibrations andis a characteristic of carbon-containing compounds with amino groups. An absorbance in this region also signifies the presence of intramolecular hydrogen bonding. The peak with highest absorbance in the spectrum wasobserved at 1700 cm^{-1}. Absorbance in this region signifies the presence of peptide group in the molecule. Peaks at 1000 and 1200 $cm^{-1}$are probably because of C–O–C vibrations in esters.**P.Das et.al** has obtained a peak with highest absorbance in the spectrum at 1656 cm^{-1}. Absorbance in this region signifiesthe presence of peptide group in the molecule.The lipopeptide biosurfactant, surfactin (Sigma) also yielded a similar IRabsorption pattern and absorbed approximately at the same wave number positions. This type of FTIR spectra is characteristicof lipopeptides, e.g. other lipopeptide biosurfactants like lichenysin reported in literature have alsoyielded similar IR absorption spectra **(Lin et al. 1994; Yakimov et al. 1995).**

CONCLUSION:

From the results inferred from MALDI-MS and FTIR, the crude sample was found to contain surfactin as the surface active compound. Thus the crude molecule was further analyzed for its antimicrobial and anti-larvicidal properties in the future studies.

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