Original Article

MOLECULAR CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF BIOSURFACTANT PRODUCING ISOLATE STREPTOMYCES CELLULOSAE VAR. PD66

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Abstract

In the present study, the actinomycetes isolate named as PD66 (Streptomyces cellulosae var.) was screened and identified based on 16S rRNA nucleotide sequence and phylogenetic evolutionary relationship with other strains by National Collection of Industrial Microorganisms (NCIM), Pune. Biosurfactant production by the isolate PD66 was carried out using Kim's medium with 3% olive oil as a carbon source and incubated at 27ºC for 7 days on rotary shaker at 150rpm. After 7 days, the contents of the flask were centrifuged and collected the cell free supernatant for performing the preliminary tests like oil displacement, para film-m test for confirmation of biosurfactant production, these two tests were given positive results for biosurfactant production obtained from the selected isolate PD66. Lipase activity was also checked and confirmed the extra cellular lipase enzyme release during the growth of isolate PD66 using tributyrine agar medium. Surface tension and emulsification index were also measured for evaluation of biosurfactant present in cell free supernatant broth using SLS (Sodium lauryl sulphate) as a standard. Determination of extracellular antibacterial activity of biosurfactant samples was determined for every 24hrs upto 10days using cell free supernatant by agar diffusion method. Selected isolate PD66 has showed antibacterial activity against E.coil and Staphylococcus aureus. After extraction of cell free supernatant with ethylacetate, the resultant crude extract was tested for identification of bioactive metabolite by TLC method and Bioautography technique.

Keywords: Biosurfactant, 16S rRNA sequence, phylogenetic tree analysis, antibacterial activity, bioautography technique.

1. INTRODUCTION

Biosurfactants are the important compounds obtained from both chemical and biological sources, they plays vital role in the recent trends to improve the soil fertilization by reducing hazardous hydrocarbon and contaminated oils present in soil [1]. Antibiotics produced during biosurfactant production are having very much pharmaceutical, medical and therapeutic attention in the treatment of several diseases like antibacterial, antifungal, antiviral infections and a very powerful tool for treating several types of cancers, pulmonary diseases, and anti-tubercles diseases etc.

Recent studies suggested that biosurfactants are also a good active molecules used in the prevention of sars covid 19 infections by binding to the hydrophobic nature of viral lipid membrane present in the covid 19 virus and also used in formulations of many types of sanitizers, hand washes and most recently suggested to use as masks coated with single layer biosurfactant, so that they prevent infectious diseases coming from outside environment to our body [2]. One of the medical importance of biosurfactant is in replacement therapy in new born babies who are not having natural surfactants from lungs at their birth [3].

Actinomycetes are filamentous gram positive bacteria looks like fungi. They are produced from both marine and soil samples. Marine actinomycetes are the good bioactive metabolite producers [4]. Nearly 80% of antimicrobial compounds are reported from actinomyctes. Streptomyces sp. are the goldmines for the production of antibiotics. They produce potent bioactive metabolites like antibiotics, therapeutically useful enzymes, industrially important lipids etc [5]. Streptomyces produced antimicrobial compounds act effectively against plant infections caused by insects and several types of harmful bacteria and fungal strains present in the soil, they may affect the crop yield during the cultivation and growth [6]. In the present study actinomycete isolate PD66 identified as Streptomyces cellulose var. from actinomycetes. It was isolated from the plastic waste dumping yard soil samples, Visakhapatnam and screened for its biosurfactant production and antibacterial activity.

2. MATERIALS AND METHODS

2.1 Screening of biosurfactant producing actinomycete isolate:

Selected isolate of PD66 was screened for biosurfactant production using Kim's medium composed of NaNO3 1g/L, KH_2PO_4 0.1g/L, $MgSO_4$ 0.1g/L, CaCO3 0.1g/L, Yeast extract 0.2g/L, Dist. water 1000ml, Olive oil 30ml/L was used as sole source of carbon at pH 6.0±0.2 and incubated at 27ºC for 7 days at 150rpm on orbital shaking incubator [7]. After 7 days of incubation the biosurfactant flasks were removed from the orbital shaker and centrifuged at 4000 rpm for 20 min at 4ºC, after centrifugation collected the cell free supernatant and conduct the preliminary identification tests for biosurfactant production obtained from selected isolate PD66.

2.2 Preliminary identification tests for biosurfactant production:

2.2.1 Oil displacement test

Oil displacement method is a preliminary test for identification of biosurfactant production and it is very easy and take less time to perform[8]. 40ml of distilled water was poured in to petri plate (90mm) and then add 15µl of oil on the surface of water to form thin layer. A 10microlitre of cell free broth was added on the oil layer, immediately the oil was displaced and the displaced zone was measured and this was compared with SLS as standard and un inoculated kim's media as negative control.

2.2.2 Parafilm-M test

Parafilm-M test is used to detect the whether the biosurfactant is hydrophobic and hydrophilic. This test was performed by using 1% bromothymol blue mixed with 10µl of Cell free supernatant containing biosurfactant and 10µl of cell free supernatant was placed on the surface of hydrophobic surface containing Parafilm-M along with positive control SLS and negative controls water and un inoculated kim's media[9]. The diameter of drop was observed and measured after 1minute, if the shape of the drop is collapsed it indicates the presence of biosurfactant production.

2.2.3 Lipase activity

1%Tributyrine agar medium was used for the screening of lipase activity produced by the selected isolates. This medium containing 1% tributyrine, 5g/l of peptone, 3gm/l of yeast extract, 20gm/l of agar and 1L distilled water and adjusted pH at 7.5±0.2.The selected isolates were streaked on the surface of the tributyrine plates and then incubated 27ºC for 7days [10]. The clear zone around the growth of selected isolate PD66 was observed after 7 days indicates the extracellular lipase enzyme production.

2.2.4 Surface tension

Surface tension of the biosurfactant was determined with the help of stalgmometer [11]. A 5ml of selected isolate PD66 was added to 250ml Erlenmeyer flask containing 45ml of kim's production medium at pH 6±0.2 and incubated for 7 days at 27ºC on rotary shaker at 150 rpm. After 7 days of incubation the cultured broth was centrifuged at 4000rpm for 20min at 4°C and cell free clear supernatant was collected by further filtration from the biomass. The obtained cell free supernatant was used for determination of surface tension of biosurfactant and Standard SLS was used as a standard and water as a negative control. Calculate the surface tension of three samples by the formulae.

Surface tension of sample = $\rho 2n1/\rho 1n2 \times$surface tension water

$\rho 1$=density of distilled water

$\rho 2$=density of sample

n1=no of drops of water

n2=no of drops of samples

2.2.5 Emulsification index:

Emulsification index is an efficient method for evaluation of biosurfactant, it is easy to perform and also to get clear observation for calculating emulsification index results [12]. For evaluation of emulsification index, the cell free supernatant was used to perform this test. 6 ml of kerosene was added to the test tube and to this added 4 ml of cell free supernatant of biosurfactant and vortexed for 5 min. and then leave it for 24hrs. Standard SLS and un inoculated kim's medium were used as positive control and negative controls respectively for this test and then observed emulsification index results for samples and compared. If the biosurfactnt was present in the cell free supernatant it forms a white emulsion with the kerosene layer and emulsification index was calculated by using the below given formulae. If biosurfactant was not produced two separated layers can be seen.

E (24) = (Height of emulsion formation)/(Total height of solution)×100

2.3 Morphology of isolate PD 66:

Actinomycetes has shown the substantial morphological distinction among the Gram positive bacteria. Actinomycetes formed branched filamentous hyphae, which seems like fungi and also shown rod shaped cell structures like Gram-positive bacteria [13]. Determination of the morphology of selected isolate PD66, 0.05ml of spore suspension was added to 10 ml of sterile starch casein agar medium and poured into petri dish and spread the medium like a thin film and the plate was incubated at 27ºC for 7 days in cooling incubator. After 7 days of incubation observe the growth of actinomycetes colonies on the surface of the medium and the plate was focused under the trinocular microscope (LAMOMED CX R3 USA) at 10X and 45X magnification, images were recorded by the camera and then examined the spores shape and arrangement of spore structures of selected actinomycetes isolate PD66.

2.4 Time course of antibacterial activity:

For this assay, submerged fermentation was employed for determination of anti-bacterial agents release during the biosurfactant production. The 5 ml of selected isolate PD66 culture was added to 45 ml of kim's medium containing olive oil as carbon source and incubated at 27ºC for 10 days at 28ºC on rotary shaker at 150rpm. Every 24hrs of time period 5ml of sample was collected from production flask, this process was carried out from 1 to 10 days. After collection of 10 days of samples, they were centrifuged at 4000rpm for 20minuts at 4ºC. Then the cell free supernatant was separated from the bio mass and this cell free supernatant was used for determination of antibacterial activity by agar well diffusion method against Gram negative and Gram positive bacteria(E.coli, and Staphylococcus aureus) [14]. Nutrient agar was prepared and sterilized at 121ºC for 20min at 15lb pressure. A fresh 24hr culture of bacterial suspensions of both bacteria were serially diluted and checked the OD at 600nm to adjust that 0.6-0.7 and they were added individually to the bacterial culture media and then they were poured into petri plates separately for the both bacteria and allowed for solidification for 10min[15]. Then 10 cups were made for samples (collected for 10 days) in each petri plate with the help of cork borer. 10 samples of each 50µl of cell free supernatant were added into cups and then petri plates were incubated at 32ºC for 24 hrs in bacteriological incubator. After 24hrs of their incubation period, both plates were observed and the clear inhibition zones around the cups indicate the antibiotics production during the biosurfactant fermentation.

2.5 Identification of 16S rRNA sequence and phylogenetic analysis:

The identified 16S rRNA sequence was deposited in the Gen bank of NCBI and this was used to BLASTN search for identification of the closely related sequences with the percentage similarity and then the construction of phylogenetic tree was done by neighbour joining method using MEGA (Molecular Evolutionary Genetics Analysis) version 6.0software [16].

2.6 Production and extraction of biosurfactant:

After the all primary screening tests were give positive results for biosurfactant production using kim's medium containing olive oil as a carbon source, then started biosurfactant production process was carried out by submerged fermentation. 5ml of inoculum culture was inoculated into 45ml of production medium in 250ml Erlenmeyer flask and incubated at 27ºC for 7 days on rotary shaker at 150rpm [17]. After 7 days of incubation, the production flask was removed from the shaker and centrifuged at 4000rpm at 4ºC for 20minuts, and then separate the cells from the medium by filtration and collected the cell free supernatant. The collected sample was used for extraction process for getting crude biosurfactant. Extraction of biosurfactant was done using cell free supernatant. The crude broth was extracted with ethyl acetate (1:1 ratio) using separating funnel at room temperature and carefully collected the ethyl acetate organic layer from aqueous layer and this process was repeated three times[18]. The

organic layer was subjected to evaporation by using rotary evaporator to remove the ethyl acetate and collected the honey colour crude extract into the sterile screw cap tubes and stored in refrigerator until they will use for further analysis.

2.7 Bioautography technique:

Bioautography technique is the one of the efficient and major test for identification of antibiotics present in the crude extracts. For this test the obtained crude extract was used to perform bioautography method [19]. Two bacteria of Gram negative and Gram positive (E.coli, Staphylococcus aureus) were used for this technique, nutrient agar media for bacteria growth was prepared and sterilized and the bacteria suspensions were prepared and inoculated 25µl suspension to 25ml of nutrient agar media and then allowed for solidification for 10minuts. A small amount of crude extract was placed on the bottom of TLC plate (which is previously coated with silica gel) with the help of capillary tube and then run this TLC plate in 25% ethyl acetate and hexane as a mobile phase. Then the TLC plates were removed from the chromatography chamber and allowed for air dry and then each TLC plate was placed on the surface of respective solidified medias against both bacteria, after that two petri plates were kept in refrigerator for 1hr for diffusion of antibiotics from TLC plates to solidified medias. After 1hr the TLC plates were removed carefully from the surface of cultured agar media with the help of sterile forceps and then incubated the plates in bacteriological incubator at 32ºC for 24hrs. After incubation period, the plates were observed and inhibition zones were recorded [20].

3. RESULTS:

The selected isolate PD66 was sub cultured and maintained on starch casein agar medium, the maintained isolate was showed in fig:1. Kim's medium containing 3% olive oil as carbon source for screening of biosurfcant showed white milky emulsion in the production flask and no emulsion formed in un inoculated kim's control flask, the results were showed in fig:2 (A, B and C). The production of biosurfactant was evaluated by preliminary tests of oil displacement test ,it showed displaced zone on the surface of the water indicated the presence of biosurfactant and it showed 7.5cm displaced zone and 9.5cm, there is no displaced one for control un inoculated kim's, the results were represented in fig:3 The cell free supernatant was showed hydrophilicity nature by parafilm-m test indicated the biosurfactant production. The drop of biosurfactant was collapsed on the surface of the parafilm-m and this was compared with standard SLS showed expanded drop size and negative controls water and un inoculated broth showed no collapse of drops and they remain constant and showed dome shape on surface of parafilm-m has showed in fig:4. In case of lipase activity, a clear zone around the growth of the isolate PD66 was observed in tributyrine agar plate indicated that the selected isolate have the ability to produce extracellular lipase enzyme during their growth. The obtained clear zone result was given in fig: 5.

Surface tension of the cell free supernatant have showed the values for PD66 was 31.62 dynes/cm , standard SLS has shown 23.14 dynes/cm and water was 53.35 dynes/cm. all these results were showed in fig:6 (D). The selected isolate PD 66 was formed white colour emulsion with the kerosene layer and showed 50.02 %, and standard SLS was 80.02% emulsification index whereas there was no emulsion formed in case of control (un inoculated broth). The emulsification results were represented in fig: 6 (A, B, C and D).

3.1 Morphology of isolate PD66:

The morphological characters of the selected strain of PD66 showed purple colour for gram- staining like Gram-positive bacteria and observed open spirals and hairy like long hyphal structures with spore chain formation and branched filaments like fungi under trinocular microscope at 4000X magnification and the morphological image was showed in fig: 7.

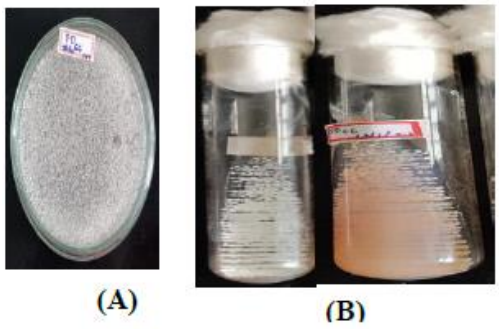

Fig:1 Selected isolate of PD 66 A) pour plate of PD66 and B) maintained PD66 culture in a slant

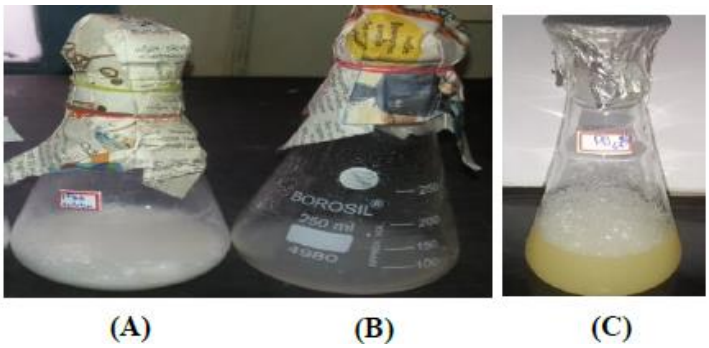

Fig: 2 Production flask of biosurfactant A) biosurfactant flask and B) control flask (un inoculated flask) C) cell free supernatant contained biosurfactant

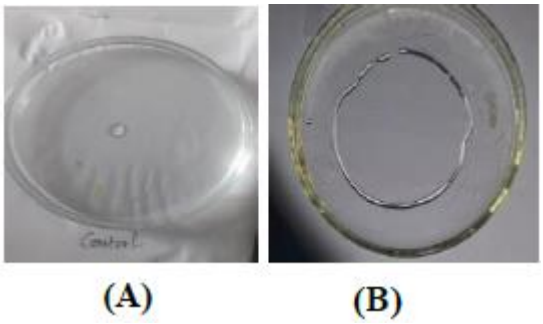

Fig: 3 Oil displacement test showed positive results of displaced zone A) Control and B) Biosurfactant of selected isolate PD66 showed displaced zone

Fig: 4 Parafilm-M test showed a hydrophilicity nature of biosurfactant against negative controls

Fig: 5 Lipase activity has showed clear zone around the growth of PD 66 isolate

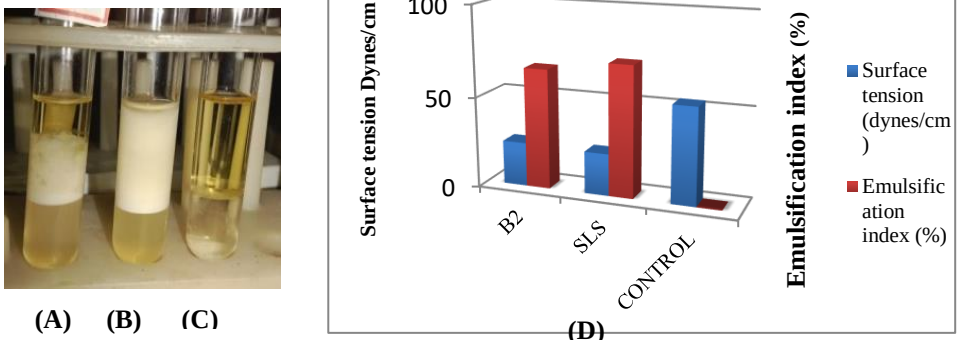

Fig: 6 Emulsification index A) PD66 B) SLS, C) Control (un inoculated kim's medium) and D) Both results regarding reduced surface tension and increased % emulsification index

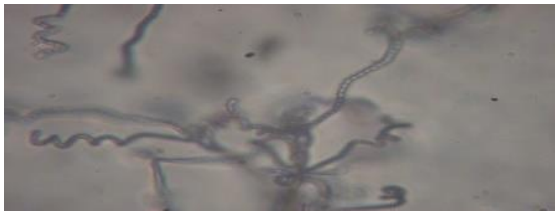

Fig: 7 Morphology of PD66 under 4000X magnification

Time course of antibacterial activity:

The selected isolate PD66 was showed significant results for production of antibacterial activity for both Gram negative and Gram positive bacteria. By performing every day assay, the antibacterial were produced from 1st day to 10 day with increased zone diameters. The selected bacteria strains showed a inhibition zone diameter around the cups, for bacterial strains E.coli showed for 1st day 24 mm zone diameter and which is gradually increasing to every 24hr, at 10th day it was showed increased zone diameter of 52mm.whereas in Staphylococcus aureus 1st day it was showed 22 mm and at 10th day 56 mm. both the Gram positive and Gram negative bacteria showed high amount of inhibition zone diameters. It indicated the selected isolate PD66 had an antibacterial activity against both Gram negative and Gram positive bacteria. Both the bacteria were showed high rice of inhibition zones at 10th day. The inhibition zone diameters showed in fig: 8 (A, B, and C and the measured inhibition zone diameters were mentioned in table: 1.

Table: 1 Antibacterial activity of the selected isolate PD66 (inhibition zones diameters in milli meter (mm)

Number of days	1	2	3	4	5	6	7	8	9	10
E.coli (MTCC 1687)	24	26	30	32	34	38	40	44	48	52
Staphylococcus aureus (731)	22	24	32	34	36	40	44	50	52	56

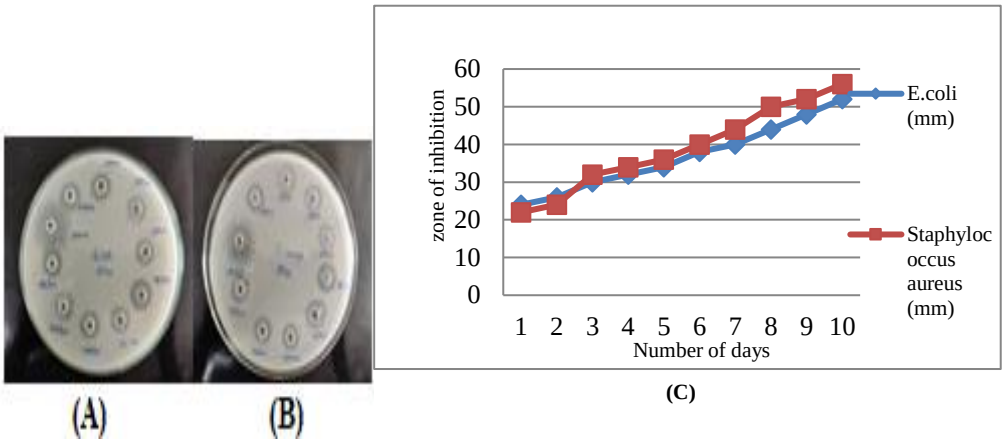

Fig: 8 Antibacterial activity of PD 66 showed inhibition zones around the cups at different time intervals ,the results showed in A) E.coli B) Staphylococcus aureus and C) increased zones of inhibition with increased time period

3.2 Identification of 16S rRNA sequence and phylogenetic tree analysis:

The selected isolate was identified as Streptomyces cellulosae var. done by NCIM, Pune. The 16S rRNA sequence was analysed and sequence was submitted to genbank and the provided the accession number for the sequence is ON150857.1 and the results of phylogenetic tree analysis of selected strain Streptomyces cellulose var. PD 66 was showed in fig: 9.

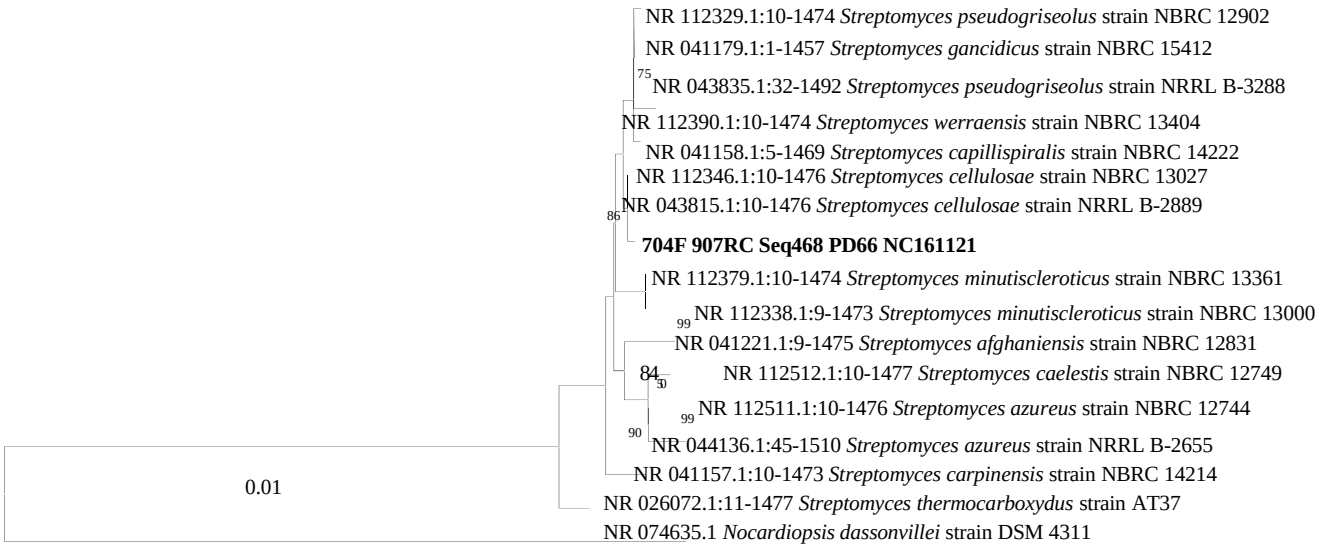

Fig: 9 Phylogenetic tree analysis of Strain Streptomyces cellulose var. PD 66

3.3 Production and extraction of biosurfactant:

After several types of organic solvents used for extraction of biosurfactant, Ethyl acetate was selected for this process by taking the high amount of recovery of biosurfactant compared with the other solvents like Hexane, Chloroform and methanol

respectively. Extraction was done by using cell free supernatant of selected strain of PD66 (Streptomyces cellulose var.) and ethyl acetate at 1:1 ratio this was showed in fig: 10. We got a crude honey colour extract and this is subjected to TLC with 25% ethyl acetate and hexane as a mobile phase and analysed the different compounds by subjected to iodine chamber, due to the high vapour pressure released by iodine chamber, the TLC plate was saturated in the chamber and turn to light brown colour and after a few seconds detected the some unsaturated and aromatic compounds appeared like dark brown spots. Then the compounds spots were analysed by spraying methanol-H_2SO_4 reagent and then kept the TLC plate at 110ºC for 30minuts, noted the different compounds Rf values.

Fig: 10 Extraction of biosurfactant

3.4 Bioautography technique:

Determined the antibacterial compounds released from biosurfactant production by using bioautography technique against both Gram positive and Gram negative bacteria. After 24hrs of incubation, the plates were removed from the incubator and observed the results. Both bacteria were given inhibiton zones around the antibiotic diffusion area on the surface of media, and the Gram positive bacteria showed 12mm diameter and 15mm for Gram negative bacteria were recorded and the inhibition zones of both petri plates were showed in fig: 11. This indicated that the obtained partially purified biosurfactant produced antibacterial compounds during the production.

Fig: 11 Antibacterial activity of PD 66 showed inhibition zone around the diffused antibiotic area on bioautography plate

4. CONCLUSION

In this era, biosurfactants have producing beneficial results for all the fields like pharmaceutical, cosmetic industries, medicine, agriculture and bioremediation of soils & contaminated water areas etc. Biosurfactants have lead the way considerable attentiveness for present and future application. In this study, biosurfactants producing isolate obtained from soil sample and it produced a considerable amounts of antibacterial metabolites. The antibacterial activity of the biosurfactant, which in turn to focus on the useful applications for treatment of several diseases , in the field of agriculture for soil remediation and confiscation of several types of infections caused by the microorganisms present in the soil. So many researches are reported as Streptomyces sp. are the potential producers for release of high amount of antibiotics during biosurfactant production by submerged fermentation process.

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