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ORIGINAL ARTICLE

BIOSURFACTANT PRODUCTION USING PINEAPPLE JUICE AS MEDIUM BY PSEUDOMONAS FLUORESCENS ISOLATED FROM MANGROVE FOREST SOIL.

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Abstract:

In the present investigation, Pineapple juice has been tested for its suitability for biosurfactant production using Pseudomonas fluorescens MFS03 isolated from the crude oil enriched mangrove soil. The strain recorded maximum biosurfactant production (9.43 g/l) when pineapple juice was used as a medium. Biosurfactant production was confirmed by conventional screening methods including hemolytic activity, drop collapsing test, oil displacement method, CTAB, lipase activity and emulsification index. The isolate shows lowest surface tension of 25.4 mN/m, an interfacial tension of 0.98 mN/m and a CMC of 34 mg/l. The active compound was extracted with ethyl acetate. The extract was fractionated by TLC to isolate the pure compound. Based on the TLC, chromatogram the biosurfactant was identified as glycolipid type. FT-IR spectrum revealed that the important adsorption bands at 3466.24, 2926.45, 1743.47, 1407.30 and 1162.26 cm-1 indicates the chemical structure of rhamnolipid. The results demonstrated that the Pineapple juice can be a suitable medium for the biosurfactant production, which can improve the process economical.

KEY WORDS:

Pseudomonas fluorescens, Biosurfactant, Pineapple juice, Rhamnolipid, Mangrove forest soil.

INTRODUCTION:

Microbial compounds that exhibit pronounced surface and emulsifying activities are classified as biosurfactants. Microbial biosurfactants are a structurally diverse group of amphipathic surface-active molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases that have different degrees of polarity and hydrogen bonding (Desai and Banat., 1997; Mulligan, 2005). Most microbial surfactants are complex molecules, comprising of different structures that include lipopeptides, glycolipids, polysaccharide-protein complexes, fatty acids and phospholipids (Neto et al., 2008). As compared to their oil-based (synthetic) counterparts, biosurfactants are biodegradable, environmentally benign, stable under extreme conditions, and they can be produced in situ from inexpensive renewable substrates (Benincasa, et al., 2010). Therefore, biosurfactants are gaining increasing interest as eco-friendly alternatives to synthetic surfactants (Banat et al., 2000).

Besides their wide potential applications in bioremediation and environmental protection, biosurfactants constitute an important class of industrial chemicals with useful potential applications in almost every sector in industry (Ashis and Das, 2010). Several biosurfactants have been isolated and characterized. However, to date biosurfactants are still not able to compete with chemically synthesized

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surfactants. This is due to high production costs, poor strain productivity, and the use of expensive substrates (Cameotra and Makkar, 1998).

To overcome this problem, in recent years, there has been an unprecedented increasing interest in the more efficient utilization of agro-industrial residues because its application provides an alternative way to reduce the production cost and help to solve many environmental hazards.

The main problem related to use of alternative substrates as culture medium is to find a waste with the right balance of nutrients that permits cell growth and product accumulation (Makkar and Cameotra, 1999). A variety of cheap raw materials including, plant-derived oils (Oliveira et al, 2009), oil wastes (Thavasi, et al., 2008b), starchy substances (Fox and Bala, 2000) cashew apple juice (Parthasarathi and Sivakumaar, 2009) and agriculture residues (Moldes, et al., 2007) have been reported to support biosurfactant production.

The aim of this present study is to isolate and characterize biosurfactant-producing microorganisms from the mangrove forest soil and to develop a cost-effective biosurfactant production using pineapple juice as medium. According to the literature evidenced, this the first investigation on production of biosurfactant using pineapple juice as a substrate.

MATERIALS AND METHODS:

Isolation of heterotrophic bacteria from mangrove soil

One hundred gram of freshly collected soil samples from mangrove forest soil, Pichavaram, Tamilnadu, India (latitude 11°20' to 11°30' North and longitudes 79°45' to 79°55' East) were enriched with 10 ml of crude oil (obtained from Indian Oil Corporation, Chennai, Tamilnadu, India), incubated at room temperature (28 ± 2°C) for 30 days (Jobson, et al., 1972). After a month period of incubation the soils were analyzed for microbial population using standard microbiological procedures.

SCREENING FOR BIOSURFACTANT PRODUCTION

Haemolytic activity

Isolated strains were screened on blood agar plates containing 5% (v/v) human blood and incubated at room temperature for 24 hr. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carillo, et al., 1996). The diameter of the zone of clearance is a qualitative method used as an indicator of biosurfactant production.

Drop collapsing test

Two microliters of mineral oil was added to each well of a 96 well microtitre plate lid. The lid was equilibrated for 1 h at room temperature and then 5µl of the culture supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Youssef et al., 2004).

Oil displacement test

Fifteen micro litre of weathered crude oil placed on the surface of distilled water (40 µl) in a petridish (150 mm in diameter). Then 10 µl of the culture supernatant was gently dropped on the centre of the oil film. The diameter and area of clear zone were measured and calculated after 30 seconds as described by (Morikawa et al., 1993).

Cetyl Trimethyl Ammonium Bromide (CTAB) plate assay procedure

Biosurfactant producers were detected by blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg/ml) and methylene blue (5 µg/ml). Shallow wells were cut on the agar plate surface using the gel puncher. Ten µl of the inoculum was added into each well. The plates were incubated for 48 h at 34°C. In control plates only medium without inoculum was added in the wells. Biosurfactants were observed by the formation of dark blue colour colonies against the light blue colour agar background (Siegmund and Wagner, 1997).

LIPASE ACTIVITY:

The isolates that produce lipase were screened using tributyrin agar plates. Tributyrin (1%) was added to minimal salt agar medium. The pH of the medium was adjusted to 7.3–7.4 using 0.1 N NaOH. A loopful of inoculum was streaked on to the tributyrin agar plates. The plates were incubated at 34 °C for 48 h. After incubation, the plates were examined for the formation of clear zone around the colonies.

Identification of biosurfactant producer

The cultural, morphological and biochemical characters of the isolate was studied and the species level is identified by following Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1974).

PREPARATION OF PINEAPPLE JUICE MEDIUM:

100ml of Mineral salt medium (MSM) broth was sterilized in an autoclave at 121o C for 15 min. The clarified pineapple juice with various concentrations i.e. 2, 4, 6 and 8 percent was added to the sterilized MSM broth. Same way PAJ was used as such without inorganic mineral salts to prove the effect of PAJ on the biosurfactant production. For the comparison, defined medium (MSM with 2% glucose) was included in this study. The sterilized MSM broth was inoculated with 5ml of the isolate MFS03 and incubated at room temperature over an orbital rotary shaker set at 129rpm min-1 for 3 days.

Extraction of Biosurfactant

The culture broth was centrifuged at 10,000 × g for 30 min to discard the cells. The cell-free supernatant was acidified with 2 N HCl to attain pH 2 and extracted with ethyl acetate (1:1 ratio, 5 times). The extracted fractions were combined and evaporated to dryness under reduced pressure in a rotary vacuum evaporator (Rotavapor R-205; Buchi, Bern, Switzerland). Biosurfactant from the extract were purified on a silica gel (100-200mesh, 30×2 cm) column washed with chloroform and then eluted with 3% methanol in chloroform to remove traces of contaminants.

CHEMICAL CHARACTERIZATION OF BIOSURFACTANT

Surface activity:

Surface tension was measured with a du Nouy Tensiometer (Kruss Digital-tensiometer 10, Hamburg, Germany) at room temperature (28 ± 2°C). Twenty ml volume of each cell free culture broth was placed into a clean 50 ml glass beaker and placed onto the tensiometer platform. Cell free culture broth was equilibrated for 15 min in a small weighing dish prior to the surface tension determination. A platinum wire ring submerged into the solution was then slowly pulled through the liquid–air interface, to measure the surface tension (mN/m). Surface tension measurement values were recorded and expressed as mN/m. Between each measurement, the platinum wire ring was rinsed three times with water, followed by acetone and was allowed to dry. The surface tension value shown is the average of three replicates from the same culture. Control consisted of a sterile culture medium plus an inoculum, at initial at 28 ± 2°C. Distilled water and isopropanol were used as standards (duNouy, 1919; McInerney et al., 1990).

Emulsification activity

The emulsifying activity of the biosurfactant was determined by using the cell free culture broth (Cooper and Goldenberg, 1987). The assay was carried out by adding hexane, heptanes, soybean oil and diesel oil and then vortex for 1 min individually. The emulsification was determined by measuring the height of emulsion layer after 24 h.

Emulsion index E24 (%) were determined by

$$E_{24}(\%) = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

Bacterial adherence to hydrocarbons (BATH)

BATH assay was carried out as described by (Rosenberg et al., 1980), Pseudomonas fluorescens MFS03 cell was harvested by centrifuging at 10,000 rpm for 15 min and washed twice with 4 mL PUM Buffer (pH 7.1) containing 16.9 g of K2HPO4, 7.3 g of KH2PO4, 18 g of urea and 0.2 g of MgSO4.7H2O, again resuspended in same buffer. The initial density of cell suspension measured spectrophotometrically at 400 nm. Bacterial cell suspension (8ml) was then mixed with (2ml of hexadecane incubated at room temperature (28 ± 2°C) for 10 minutes and vortexed for 2 minutes, the mixture left undisturbed for the phase separation for 15 min, the bottom layer was recovered and the OD 400 was measured. Adherence percent to hexadecane was measured before and after the addition of hexadecane and multiple by 100.

$$\text{Hydrophobicity index (\%)} = 100\left(\frac{1 - \text{ODa}}{\text{ODc}}\right)$$

ODc - initial density of the cell suspension
(ODa) - Cell density remained in the aqueous phase after 15 minutes

ANALYTICAL METHOD

To identify the chemical nature of the compound, the residue was applied in preparative silica gel TLC plates. Chromatograms were developed with 96% ethanol:water (7:3) for amino acids; chloroform:acetic acid:water (60:30:10) for sugars and chloroform:methanol:water (65:25:4) for lipids. Fractions were isolated and eluted with corresponding buffer and subjected to quantification of macromolecules such as protein, carbohydrate and lipid. Protein was estimated using the method of Lowry et al. (1951) , using bovine serum albumin as standard. Carbohydrate with phenol–sulphuric acid method by Chaplin and Kennedy, (1994), with glucose as standard and lipid was estimated for free fatty acid using the method of Sadasivam and Manickam, (2004) with cholesterol as standard.

FTIR SPECTRAL ANALYSIS OF BIOSURFACTANT

The FT-IR spectra was recorded in a Thermo Niocolet, AVATAR 330 FT-IR system, Madison WI 53711-4495, in the spectral region of 4000-400 cm-1 using potassium bromide (KBr) solid cells. The analysis was done in the Department of Chemistry, Annamalai University, India. The spectra were recorded and analyzed using the standard methods described by the previous authors (Yin, et al., 2008; Pornsunthorntawee et al., 2009).

ANTIMICROBIAL ACTIVITY OF SURFACE ACTIVE COMPOUND

The extracted compound as well as the culture supernatant of Pseudomonas fluorescens MFS03was tested for antimicrobial activity using well diffusion method and area of the halo was calculated (Cappuccino and Sherman, 1999), extracted active compounds were tested against human pathogens such as Candida albicans, E. coli, Proteus mirabilis, Klebsiella pneumonia, Haemolytic streptococcus. Muller Hilton agar plates were prepared and swabbed with pathogens well was made with a steel cork borer (1 cm dia) and 50µl of each of extracted compound was added in wells, incubated at 30°C for 24 h after incubation, the clear halo was measured and the area of inhibition in mm2 was calculated.

RESULT AND DISCUSSION:

Isolation and identification of biosurfactant producing bacteria

Totally five strains were isolated from the crude oil enriched mangrove forest soil. Among these strain Pseudomonas fluorescens MFS03 shows higher activity, for the further studies Pseudomonas fluorescens MFS03 was selected and studied in detailed for the biosurfactant production. The cultural, morphological and biochemical characters of the isolate was studied and the species level is identified by following Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1974).

SCREENING OF BIOSURFACTANT PRODUCTION

Haemolytic activity

Haemolytic activity of Pseudomonas fluorescens MFS03 showed a clear zone diameter of 9 mm around the colony. Haemolytic activity is the widely used method to screen biosurfactant production. Literatures evidenced that biosurfactant productions of new isolates was preliminary screened by haemolytic activity. In the present study, a significant correlation was established between the haemolytic activity and surfactant production. Blood agar lysis has been used to quantify surfactin (Moran et al., 2002) and rhamnolipids (Johnson et al., 1980) and has been used to screen for biosurfactant production by new isolates (Yonebayashi et al., 2000).

Drop collapsing and oil spreading test

For the confirmation of surface activity, the drop collapsing and oil displacement method was performed. In drop collapsing test Pseudomonas fluorescens MFS03 with flat drop was observed, it shows positive results. The drop collapse technique depends on the principle that a drop of liquid containing a biosurfactant will collapse and spread completely over the surface of oil (Bodour et al.,1998). In oil spreading test Pseudomonas fluorescens MFS03 showed a positive result with a zone diameter of 3.2 cm (Table-1). It was studied by the method described by (Morikawa, et al., 2000). Based on this observation, it was confirmed that Pseudomonas fluorescens MFS03 was a biosurfactant producing bacteria.

CTAB and Lipase activity:

The formation of dark blue colour zone on blue agar plates (Fig) confirmed the production of biosurfactant by Pseudomonas fluorescens MFS03 it also shows positive results for the lipase activity. Lin et al. (1998) assayed the biosurfactant producing ability, to form clear halos in methylene blue / cetyltrimethylammonium bromide (CTAB) plate and N-cethylpyridinium chloride- methylene blue agar plate. According to Kokare et al. (2007), lipase acts on water – oil surfaces and therefore it was suggested that the lipase production is one of the characteristic of biosurfactant/ bioemulsifier producers.

CHEMICAL CHARACTERIZATION OF BIOSURFACTANT:

Activity of Biosurfactant:

In this study, for the defined medium the carbon source is replaced by pineapple juice medium at 2, 4, 6 and 8% concentration and the biosurfactant production was studied. The study revealed that pineapple juice medium recorded maximum biosurfactant production for the isolate MFS03. The maximum biosurfactant was achieved with MFS03 (9.43g/l) (Fig-1) in pineapple juice as such incorporated medium. However, the yield was found to be inferior, when pineapple juice was used at 2, 4 and 6 percent concentration in the defined medium. The emulsification activity of the biosurfactant derived from pineapple juice medium was found to be superior to defined medium and defined medium supplemented with pineapple juice at various concentrations (Fig-1).The pineapple juice medium supported maximum dry cell biomass for MFS03 (7.42 g/l) (Fig-2). The surface tension reduction was maximum in pineapple juice medium for the isolate MFS16 (25.42 mN/m) (Fig-2), an interfacial tension of 0.98mN/m and a CMC of 34 mg/l respectively. Surfactin, one of the most effective surfactant known so far, showed a surface tension of 25.0mN/m, an interfacial tension <1.0mN/m and a CMC of 25 mg/L (Cooper et al., 1981).Thus the study indicated that pine apple juice may be used for biosurfactant production. Makkar and Cameotra (1997) reported that biosurfactant was produced using two strains of B. Subtilis using molasses as a substrate. Rocha et al., 2007 reported cashew apple juice (CAJ) as a complex medium for Pseudomonas aeruginosa growth and production of biosurfactant.

ANALYTICAL METHOD:

Thin layer chromatography:

The active compound was enriched by the step-by-step liquid-liquid partitioning in chloroform methanol mixture and silica gel adsorption chromatography. TLC of the ethyl acetate extract showed a single spot in the chromatogram of lipid, carbohydrate and protein with Rf values 0.84, 0.73 and 0.80 respectively. From the TLC fractions the macromolecules was estimated and it found to be as follows:

56.2% lipid, 42.8% carbohydrate and 0.39 % protein. From the estimation and TLC analysis, the compound was partially characterized as glycolipid.

FT-IR spectrum analysis

FT-IR analysis of the biosurfactant showed that, the most important adsorption bands located at 3466.24 (OH bond, typical polysaccharides), 2926.45 and 2856.23(CH band: CH2-CH3, hydrocarbon chains), 1743.47 and1601.26 cm-1(for C=O, C=O ester bond), 1407.30 cm-1(C-N amide groups). The C-O stretching bands at 1162.26-1232.88 cm-1 confirm the presence of bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings and 846.93,652.05(for the CH2 groups) (Fig.6). Therefore, it can be concluded that the biosurfactant produced by Pseudomonas fluorescensMFS03 is a rhamnolipid. (Fig. 3). Carrera et al., (1993) reported the FT-IR spectrum of cyclic lipopeptides like surfactin produced by B. Subtilis and lincheysin produced by B. Licheniformis as the most effective biosurfactant. In this study, the FT-IR spectral analysis of biosurfactant produced from Pseudomonas fluorescens MFS03 was further corroborates with the findings of (Rodrigues et al., 2006 Pornsunthorntawee et al., 2008).

Antimicrobial activity of surface active compound

The extract of Pseudomonas fluorescens MFS03 and the supernatant showed a wide activity against the pathogenic culture, the results are show in Fig. 4. In the extract E. coli and Proteus mirabilis showed a higher activity followed by Candida albicana and Klebsiella pneumonia. In comparison with the extract and supernatant, the extract showed a wide activity. So this biosurfactant production strain has a wide antimicrobial activity. According to Tsuge et al. (1996) lipopeptide surfactants are potent antibiotics mainly the surfactin, sterptofactin, gramicidin produced by the microorganism had the wide antimicrobial activity (Peypoux et al., 1999; Richter et al., 1998; Krauss and Chan, 1983) compared to the glycolipid producing strain. A glycolipid surfactant from the Candida antartica has demonstrated antimicrobial activity against gram positive bacteria. Therefore the future use of these biosurfactant as broad spectrum of antibiotics is a promising result.

CONCLUSION:

In conclusion, the present study is an attempt to find economically cheaper sources for the large scale production of microbial biosurfactants. The Pseudomonas fluorescens MFS03 used in this study were able to produce Rhamnolipid in pineapple juice as medium proved to be a suitable substrate for production; results obtained in biosurfactant production with Pineapple juice suggested the possibility of industrial production of biosurfactants using economically cheaper sources. Satisfactory emulsification activity of the biosurfactant against different hydrocarbons indicated its diverse applicability against different hydrocarbon pollution. It is well known phenomenon that the amount and type of a raw material contribute considerably to the production cost in most of biotechnological processes, so the above medium can serve as low-cost medium for biosurfactant production thus can make the process economical.

Tables and figures:

Tests	Results
Haemolytic activity	9 mm
Oil spreading test	3.2 cm
Drop collapsing test	+
CTAB assay	8.6 mm
Lipase activity	+

Table. 1 Screening results of *Pseudomonas fluorescens* MFS03

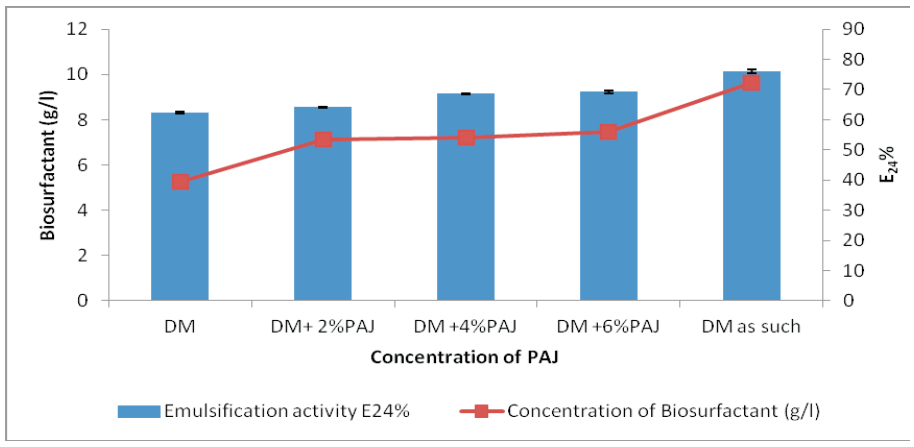

Fig.1. Effect of pine apple juice on production of biosurfactant and emulsification activity (E_{24}%) by *Pseudomonas fluorescence* MFS03

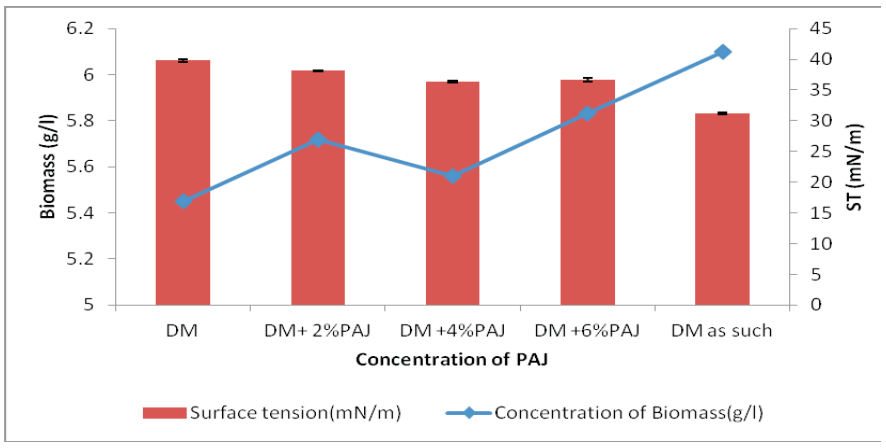

Fig.2. Effect of pineapple juice on biomass production and the surface activity of the biosurfactant produced by *Pseudomonas fluorescens* MFS03

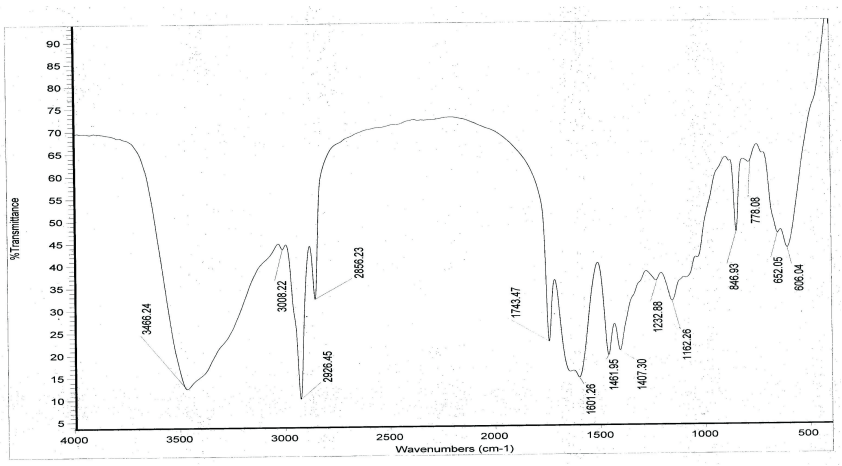

Fig. 3 . Fourier transform infrared (FTIR spectra) of rhamnolipid produced by *Pseudomonas fluorescens* MFS03.

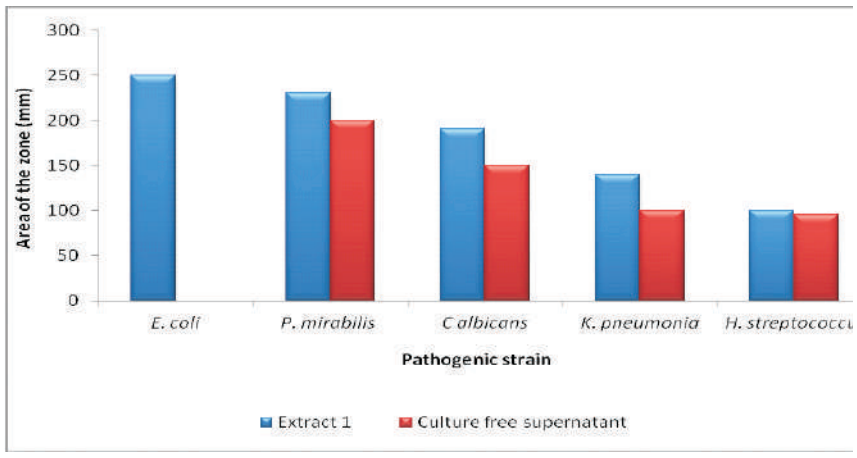

Fig.4. Antimicrobial activity of culture free supernatant and extract of the *Pseudomonas fluorescens* MFS03.

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