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BIOCHEMICAL CHARACTERIZATION OF ANTI-MICROBIAL ACTIVITY OF GLYCOLIPIDS PRODUCED BY *RHODOCOCCUS ERYTHROPOLIS*

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Abstract

Bacteria exhibiting antimicrobial activity were isolated from contaminated sites in Riyadh area, Saudi Arabia. It was charaterized and identified as *Rhodococcus erythropolis*. The biosurfactants produced by *Rhodococcus erythropolis* were partially purified and characterized by HPLC and GC-MS. The influence of antimicrobial activities were obtained by using agar diffusion method against some Gram positive and Gram negative pathogenic bacteria involved (*Escherichia coli*, *Pseudomonas aerouginosa*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Proteus spp*., *Salmonella* spp. *Corynebacterium* spp., *Streptococcus pneumoniae* and *Staphylococcus aureus*); and two pathogenic fungi viz., *Aspergillus niger* and *Aspergillus flavus*. These surface active agents exhibited high inhibitory activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Aspergillus flavus* among the tested ones. The results clearly indicated that the antibacterial and antifungal activity of the used glycolipids vary with the species of the organisms used. The microbes examined by scanning electronic microscope (SEM), were totally deformed and exhibited severe destruction. Abnormal cell division was observed at high frequencies among cells that tried to divide in the presence of the *Rhodococcus erythropolis* glycolipids . Many cells were enlarged, elongated, empty hosts, or fragmented, consistent with the extremely low viability. Thus, the study ascertains the value of the use of these glycolipids which could be of considerable interest to the development of new anti-microbial materials for medical applications as water purification plants, dental surgery equipments and pharmaceutical purposes. The potential activity of these microorganisms to produce useful antimicrobial compounds is great and must be better explored.

Introduction

Microbial contamination by microorganisms is very serious problem in many applications as medical devices, water purification plants and hospital and dental surgery equipments etc. Production of antimicrobial compounds seems to be a general phenomenon for most bacteria (Olanrewaju, 2007). An admirable array of microbial defense systems is produced, including broad-spectrum classical antibiotics, metabolic by-products such as organic acids and lytic agents as lysozyme (Adeskan *et al*., 2008). In addition, several types of protein exotoxins and bacteriocins, which are biologically active peptide moieties with bactericidal mode of action were described by Riley *et al*., (2002).

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Antibiotics have been used in many fields including agriculture, veterinary and pharmaceutical industry. Bacteria have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic ,and enzymes like cellulase and xylanase used in waste treatment (Oskay *et al*., 2004).

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Biosurfactants produced by certain microorganisms are surface-active metabolites produced by microorganisms when grown on water miscible or oily substrates: they either remain adherent to microbial cell surfaces or are secreted in the culture broth (Abouseouda *et al*., 2008). They possess the characteristic property of reducing the surface and interfacial tensions using the same mechanisms as chemical surfactants (Singh *et al*., 2007). Microbial surfactants constitute a diverse group of surface-active molecules and are known to occur in a variety of chemical structures, such as glycolipids, lipopeptides and lipoproteins, fatty acids, neutral lipids, phospholipids and polymeric and particulate structures (Desai & Banat, 1997). The features that make them commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability and hence, greater environmental compatibility, better foaming properties (useful in mineral processing) and stable activity at extremes of pH, salinity and temperature (Chen *et al*., 2007).

The search for new antimicrobial agents is a field of utmost importance. The prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide (Singer *et al*., 2003). Antimicrobial agents are known to be polymeric biocides. These biocides agents have been engineered to mimic antimicrobial peptides which are used by the immune systems of living things to kill bacteria (Emily *et al*., 2010).

On the other hand, Infectious diseases are the leading cause of death world-wide. Antibiotic resistance has become a global concern (Westh *et al*., 2004). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens (Bandow *et al*., 2003). These antimicrobial agents may enhance the efficiency and selectivity of currently used antimicrobial agents, while decreasing associated environmental hazards because antimicrobial agents are generally nonvolatile and chemically stable (Martins *et al*., 2001). The ideal antimicrobial agent would be totally specific for microbe as well as devoid toxicity for the host. For such theoretic agent the obvious site of the attack would be some structure unique to the microbe not found on the host. This makes this material a prime candidate for use in areas of medicine as a means to fight infection, in the food industry to prevent bacterial contamination, and in water sanitation to inhibit the growth of microorganisms in drinking water (Kenaway *et al*., 2007).

The aim of this work was to investigate the antimicrobial activity of bacterial isolate from certain contaminated sites in Riyadh area in Saudi Arabia.

Materials and Methods

Microorganisms and culture conditions: The microorganisms used in this study were isolated from contaminated soil in Riyadh, Kingdom of Saudi Arabia. The bacteria were grown aerobically in a mineral salt medium (Márquez-rocha & Vanessa, 2001), containing 0.5% Na $HPO_4(12H_2O)$, 0.1% KH_2PO_4, 0.05% $(NH_4)_2\ SO_4$, $MgSO_4.(7H_2O)$, (pH 7.0) in Erlenmeyer flasks with a working volume of 1 liter. The enrichment of these strains was carried out using Luria Bertani (LB) media. Furthermore, 1 mM eicosan was used a sole of carbon and energy source. Strain identification was carried out using the sequence of the gene encoding 16S rRNA according to the methods of Rochelle *et al*., (1995).

Detection of biosurfactants by methylene blue method for active substance (MBAS): Assay was carried out useing the method described by Lide (1991); Jones & Esposito (2000) with some modifications. 1 ml of eicocane mineral salt medium culture was vigorously shaken for 30s with 0.003% methylene blue, and then an equal amount of chloroform was added to the sample. The mixture was left for 20 min to extract the methylene blue anionic surfactant ion pair into chloroform layer. At this point, it is

necessary to note that all blue dye has migrated into the chloroform layer. The tube was centrifuged at 3,000 rpm for 5 min. After the extraction with chloroform, the absorbance of each sample was measured at 625 nm against a reference of pure grade chloroform.

Detection of biosurfactant activity: Samples of the culture media were centrifuged at 8,000 rpm for 20 min. The emulsifying activity and stability of the culture supernatant was measured by adding 0.5 ml of the supernatant and 0.5 ml of eicocane to 4.0 ml of distilled water. The tube was vortexed for 10s, held stationary for 1 min, and then visually examined for turbidity of a stable emulsion. Emulsification power was measured by vortexing equal volumes of the centrifuged culture with eicosane for 1 min and determining the percentage of volume occupied by the emulsion. The mixture was allowed to settle for 24 h and the height of the emulsion was measured as described according to Moussa *et al*., (2006).

Detection and quantification of the biosurfactants: Ten ml of the culture supernatants (pH 6.5) were concentrated by the addition of $ZnCl_2$ to a final concentration of 75 mM. The precipitated material was dissolved in 10 ml Sodium phosphate buffer (pH 6.5) and extracted twice with equal volumes of diethylether. The pooled organic phases were evaporated to dryness and the pellets dissolved in 100 ml of methanol. The concentrated culture supernatants were spotted on paper filter discs and then put onto agar plates containing 5% sheep blood. The blood agar plates were incubated at room temperature for 2 days. In addition, blue agar plates containing 0.2 mg ml^{-1} cetyltrimethylammonium bromide (CTAB) and 5 mg ml^{-1} methylene blue were used to detect extracellular biosurfactants production according to the method described by Ron & Rosenberg (2002) Biosurfactants were observed by the formation of halos around the colonies.

Purification and identification of the biosurfactants: Partial purification of the biosurfactants was carried out by centrifuging 200 ml liquid culture at 10,000 rpm for 10 min., at 4°C. The floating materials resulting from the treatment of the supernatant by 40% Ammonium sulphate were collected by centrifugation and then dissolved in a small fraction of water. Chilled acetone was added to the solution to remove the protein and acetone-insoluble materials. This step was critical in the purification procedure because the biosurfactant was soluble in acetone at high concentration. The rest of eicosane that may have remained in the acetone fraction was removed by extracting it three times with hexane (Kim *et al*., 2000). For sugar fatty acid esters (SFAE) separation, the mixture was subjected to extraction with warm hexane (50°C) and filtration. This step resulted in fatty acids in the organic phase and sugar esters, enzymes and sugars onto the filter (Yan, 2001).

Hydrophilic moiety determination: Determination of the Hydrophilic moiety was carried out according to dinitrosalicyclic assay (DNSA) that depends mainly on reducing ends of carbohydrates (Jeffries *et al*., 1998). Reagent composition was 1% of 3,5-dinitrosalicylic acid (DNSA), 30% of Sodium potassium tartrate and 0.4 M NaOH. Equal volumes of the sample and the previously mentioned reagent were mixed and heated in a boiling water bath for 10 min. After rapid cooling to RT and diluting with 10 volumes of water, the absorbance at 570 nm was measured. Sucrose was used as a reference for the calibration curve. For further identification of the sugar moiety, the analysis was carried out according to the method described by Yan (2001). The analyses were performed using Hewlett & Packard Gas Chromatograph (HP 5890 with automatic injector HP

7673A and integrator HP 3396A). The derivatisation process was carried out using N,O-bis-(trimethylsilyl) acetamid (BSA).

Hydrophobic moiety determination: Methyl esters of fatty acids were prepared as previously described by Morrison & Smith (1964). Equal amounts from the sample and 1M of methanolic NaOH were mixed well in a glass vial. The mixture was incubated in a water bath at 60°C for 20 min., in order to cleave off fatty acids. Equal amount of BF_3/methanol was then added after cooling in ice for 1 min in order to create methyl esters. After incubation at 60°C for 10 min, 200 µl of saturated NaCl was added and mixed well. The sample was transferred to a 1.5 ml Eppendorf vial, then 300 µl hexane was added and finally centrifuged at 13,000 rpm for 10min. The hexane layer was separated. The extract was dried over anhydrous Na_2SO_4 before analysis with GC-MS. The supernatant was transferred to a fresh vial. The water layer was extracted for second time and the hexane layer was combined with the previous extract, then subjected to GC-MS. The System parameters: inlet temperature was 250°C and detection temperatures was 280°C, a temperature program with an initial oven temperature 160°C for 1 min, final temperature 240°C and heating rate 10°C/min to 240°C with Helium as carrier gas at $p = 0.37$ atm.

Test organisms: Cultures of Gram negative *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus* spp., *Salmonella* spp., and Gram positive *Bacillus cereus*, *Streptococcus pneumonia* and *Staphylococcus aureus* and two pathogenic fungi *Aspergillus niger* and *Aspergillus flavus* were used in the study. The source of microbes was obtained from the College of Science, Botany and Microbiology Department, KSU.

Anti-fungal activity: The fungi were subcultured onto Czapek Dox agar (Oxoid). Czapek's Dox agar medium was prepared using 30.0 g of sucrose, 3.0 g of $NaNO_3$, 0.5 g of MgSO4, 0.1 g of KCl , 0.01 g of FeSO4, 1.0 g of K_2HPO, and 13.0 g agar in 1.0 L distilled water in a flask shaken vigorously in an automatic shaker for 20 to 30 minutes. The medium pH was adjusted to 7.3. The media was autoclaved at 121°C for 15 min., then poured in Petri dishes. The plates were incubated at room temperature at 25°C for 7 days. Growth was assessed every day and the result expressed in mm of colony diameter. *Rhodococcus erythropolis glycolopids* like discs was placed on agar without fungi as negative control. The antifungal assay plates were incubated at 37°C for 0, 2, 5, 10, 25, 30, 60, 120, 180 and 240 mins. The diameters of the inhibition zones were measured in mm.

Anti-bacterial activity: Inhibitory test was carried out as described by Rasadah *et al*., (1988) with some modifications: biosurfactants were tested by the disc diffusion method (Anon., 1996) then put on the surface of the Petri dishes after the fungal growth. The inhibition zones were estimated after 0, 2, 5, 10, 25, 30, 60, 120,180 and 240 mins.

Preparation of bacterial inoculums: The Gram negative *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus* spp., *Salmonella* spp., and Gram positive *Bacillus cereus*, *Streptococcus pneumonia* and *Staphylococcus aureus* were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 5 min, pellet was suspended in distilled water and the cell density was standardized spectrophotometrically (A_{570} nm). The test microorganisms were seeded into respective medium by spread plate method 100 µl (10^8 cells/ml) with the 24h cultures of bacteria growth in nutrient broth. *Rhodococcus erythropolis* glycolopids like

discs (5 mm in diameter) was placed on test organism-seeded plates. *Rhodococcus erythropolis* glycolopids like discs was placed on agar without bacteria as negative control. The antibacterial assay plates were incubated at 37°C for 4h. The diameters of the inhibition zones were measured in mm.

Scanning electron micrograph of the used bacteria and fungi: Fungal cells were prepared for scanning electron microscopy (SEM) (Amray Model 1820 Scanning Electron Microscope, UK) King Faisal Specialist Hospital and Research Center, Saudi Arabia according to the initial fixation and dehydration steps previously published (Moore, *et al*., 1992). After the antifungal and antibacterial tests, the shape of the cells was examined. The cells were fixed at 24°C for 60 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), dehydrated with a serial concentration of ethanol, and then dried on a critical point dryer (HCP-2; Hitachi Co.). The dried cell samples were coated with gold, and examined using a scanning electron microscope (S-4100; Hitachi Co.). For transmission electron microscopy, dehydrated cells were embedded in a medium type LR white resin (Sigma Chemical Co., St. Louis, Mo.), which was polymerized at 60°C for 24 h. Specimens were then viewed with the scanning electron microscope.

Results and Discussion

Based on the 16S rRNA gene sequences, comparative sequences analysis, physiological characteristics and the morphology of the cells group suggested that the isolate belongs to *Rhodococcus* with highest identity to *Rhodococcus erythropolis.* Results obtained from the disc method in the present study relieved that these glycolipids exhibited potential antibacterial activity against *Escherichia coli and Pseudomonas aeruginosa* and antifungal activity against *Aspergillus niger* and *Aspergillus flavus* among all the tested microbes (Figs. 1 and 2). When tested by the disc diffusion method, *Rhodococcus erythropolis* glycolopids showed significant activity against *Pseudomonas aeruginosa* after 300 mins (4 mm) followed by *Escherichia coli* after 420 mins (3.5mm), then *Aspergillus niger* after 240 mins (3mm) and *Aspergillus flavus* after 360 mins (3 mm).

The biosurfactants are an amphiphilic agent with both lipophilic and hydrophilic structural moieties in its molecule. These amphiphilic agents are widely used for industrial, agricultural, food, cosmetic and pharmaceutical applications. Most of these compounds are chemically synthesized and potentially cause environmental and toxical problems (Schramm *et al*., 2003).The glycolipids were hydrolyzed to sugar and fatty acids by alkaline hydrolysis. For characterization of the hydrophobic moiety, a methylation reaction of the fatty acids obtained from the alkaline hydrolysis of the glycolipid was performed. The resulting fatty acid methyl esters were analyzed by GC-MS. The organism produced large quantities of glycolipids. However the composition of fatty acids in these glycolipids was different from other known glycolipids (Fig. 3). The fatty acids in the glycolipids were eicosanoate and tetraeicosanoate. Dinitrosalicyclic assay (DNSA) was used to detect the sugar moieties of these biosurfactants. The sugar moiety was analyzed according to the method described in Lebensmittel Bedarfsgegenstände und Futtermittelgesetzbuch, Germany (LMBG). The results showed that the *Rhodococcus erythropolis*, biosurfactants consisted of two different sugars, rhamnose and glucose with retention times of 13.6 and 24.6 mins, respectively (Fig. 4).

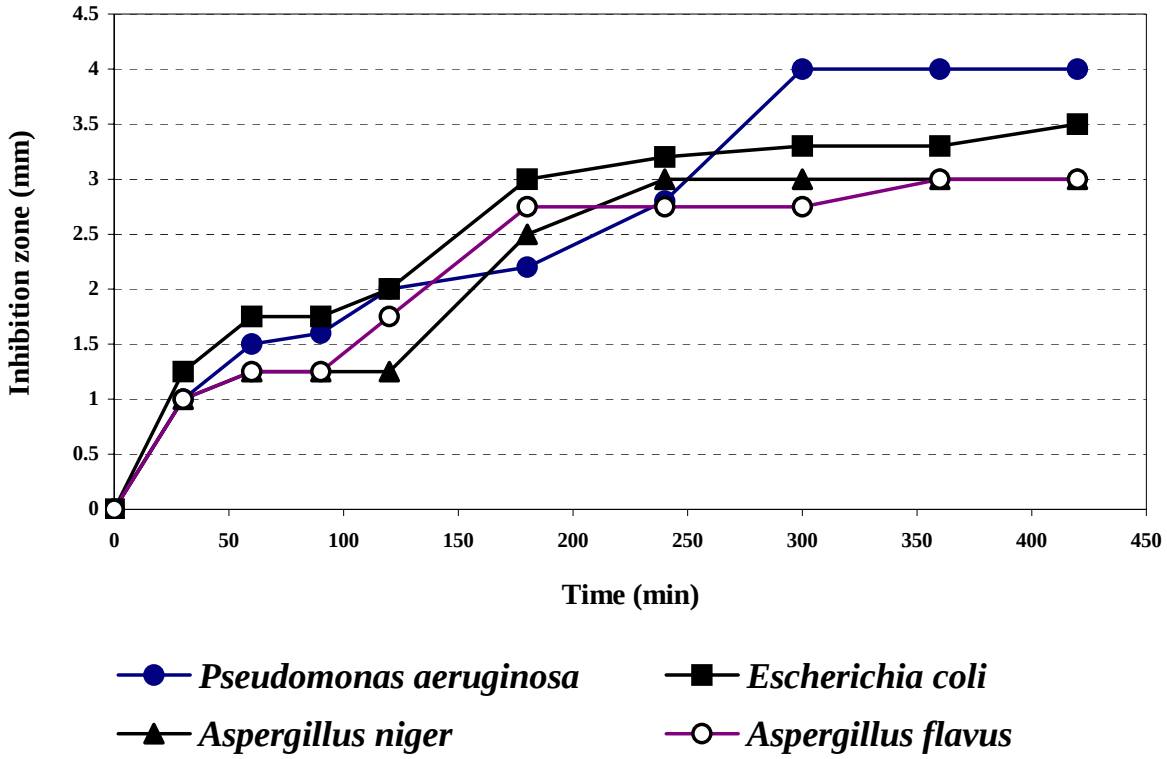

Fig. 1. Inhibition zone (mm) of *Rhodococcus erythropolis* glycolipids against against two pathogenic Fungi: *Aspergillus niger* and *Aspergillus flavous* and two pathogenic bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*.

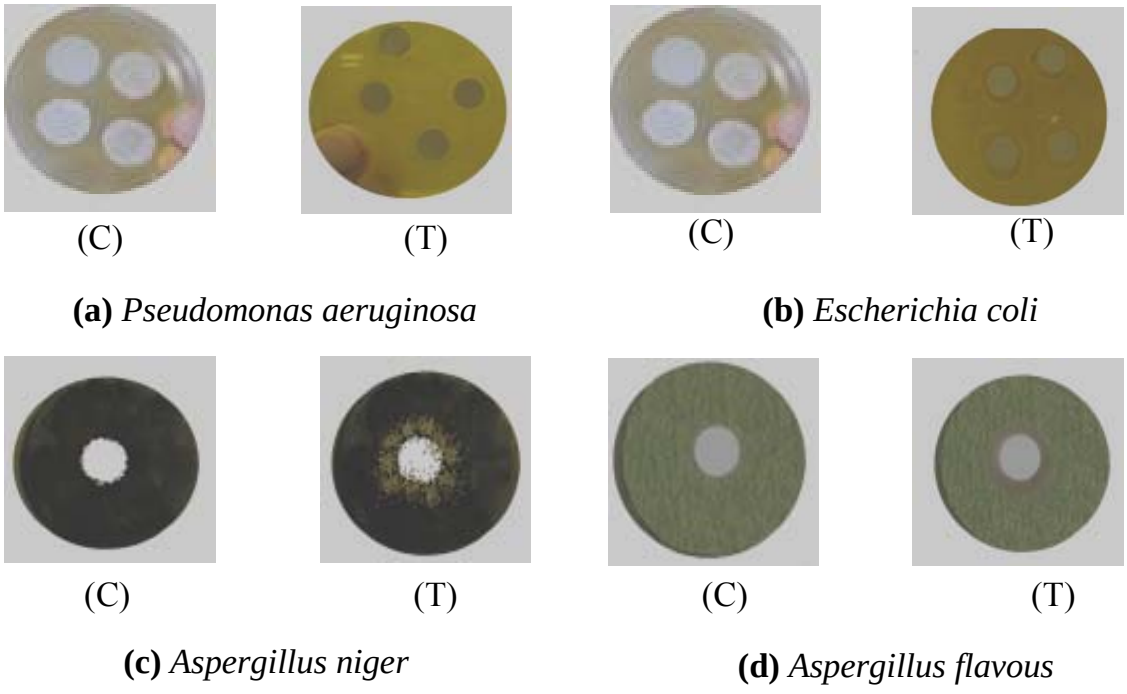

Fig. 2. Inhibition Zones of *Rhodococcus erythropolis* glycolipids against two pathogenic Fungi: *Aspergillus niger* and *Aspergillus flavous* and two pathogenic bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*. C: control & T: treated.

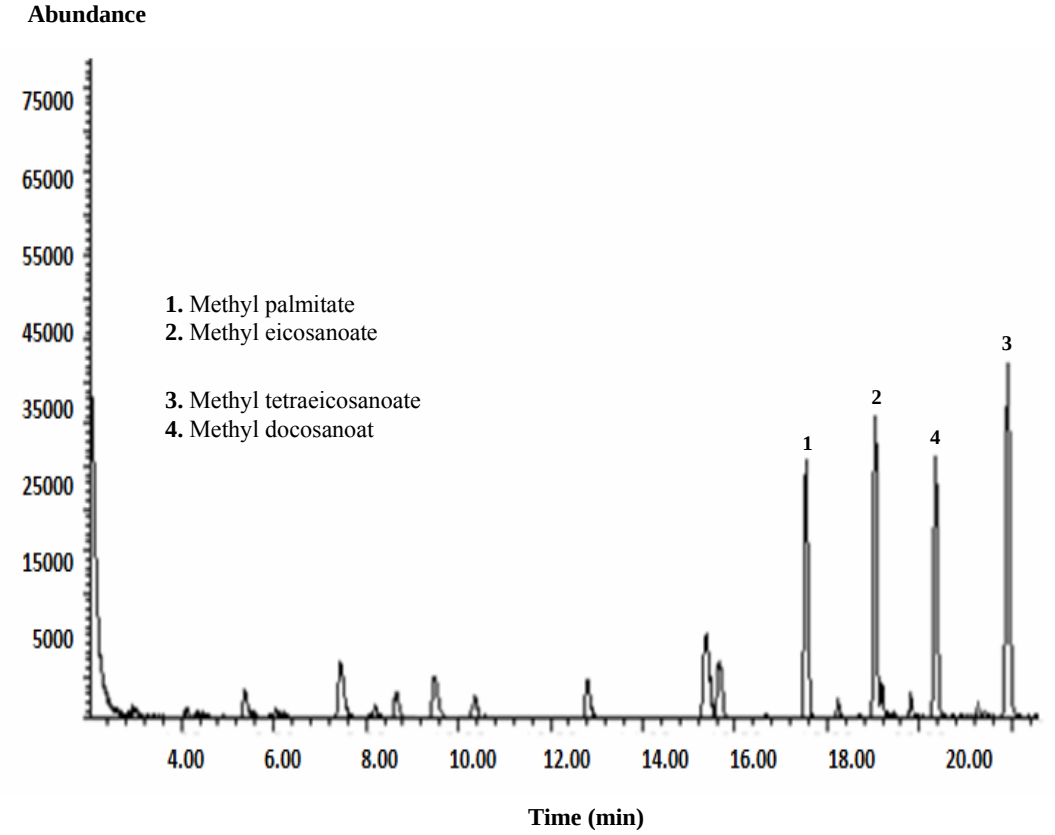

Fig. 3. GC profile of fatty acids methyl ester from the glycolipids produced by *Rhodococcus erythropolis*.

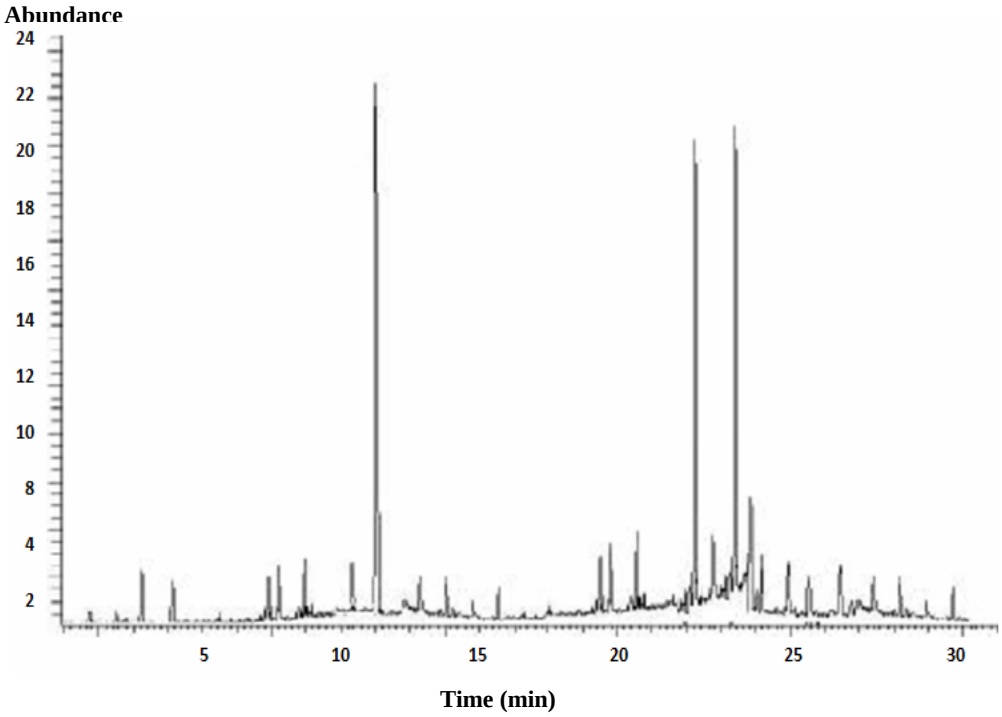

Fig. 4. Sugars in biosurfactants produced by *Rhodococcus erythropolis*.

On the other hands, the bacterial cells examined using SEM, were totally deformed and exhibited severe destruction. The surfaces of the bacterial cells were damaged and had become rough and swollen, but unlysed. In contrast, it was found that intact cells had a smooth surface with overall intact morphology (Figs. 5, 6, 7, 8). It was observed that the structure of the cell wall surface layer was wrinkled, and round pores were partially deformed, indicating that the cytoplasmic structures were flushed out of the cells. Abnormal cell division was observed at high frequencies among cells that tried to divide in the presence of the *Rhodococcus erythropolis glycolopids* . Many cells were enlarged, elongated, empty ghosts, or fragmented, consistent with the extremely low viability. The effect was investigated and compared with the control cells. In fact, certain antimicrobial agents inhibit bacterial growth by binding to the outside of the bacteria, permeabilizing the outer membrane and disrupting the cytoplasmic membrane. It was found that the efficacy of the agents increased against Gram-ve bacteria and acts in a similar fashion to other cationic biocides (Rawlinson *et al*., 2010)

For fungi, the effect of the *Rhodococcus erythropolis* glycolopids caused pronounced deformation and visible shrinkage in fungal strains studied. It was observed that the cell shrinkage and significantly reduced, but the most important result was a considerable reduction of wrinkling and deformation of the fungal cells. The fungal cells were observed in damaged pellets and somewhat resembled pseudohyphae that were highly irregular and aborted.

In fact, biosurfactants can be described as a class of with antimicrobial agents that have the ability to inhibit the growth of microorganisms as bacteria, fungi or any other micro-organisms. These biosurfactants have been engineered which are used by the immune systems of living things to kill bacteria and fungi. They are active against microorganisms by interaction with the cellular membrane and aimed to kill microorganisms (Rivardo *et al*., 2009).

Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites (Das *et al*., 2009); thus, prior adhesion of biosurfactants to solid surfaces might constitute a new and effective means of combating colonization by pathogenic microorganisms (Rivardo *et al*., 2009). Pre-coating vinyl urethral catheters by running the surfactin solution through them before inoculation with media resulted in a decrease in the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enterica*, *E. coli* and *Proteus mirabilis* (Ajesh and Sreejith, 2009). Furthermore, Rodrigues *et al*., (2004) demonstrated that biosurfactants greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation.

The results clearly indicate that the antibacterial and antifungal activity of the used glycolipids produced by *Rhodococcus erythropolis* vary with the species of the organism used. Thus, this study ascertains the value of the used of these glycolipids which could be of considerable interest to the development of new anti-microbial materials for medical applications as water purification plants hospital, dental surgery equipments and pharmaceutical purposes.

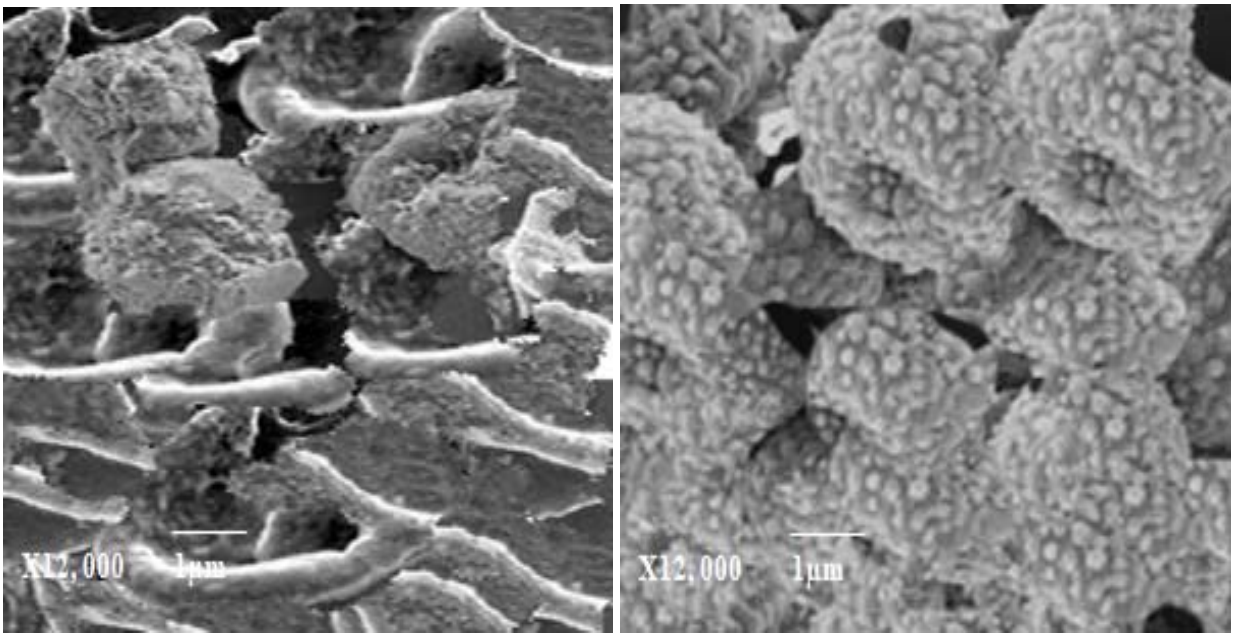

Fig. 5. Scanning electron micrograph of antifungal effects of the used *Rhodococcus erythropolis* glycolipids on *Aspergillus niger***.** Intact fungus (right) and affected fungus (left).

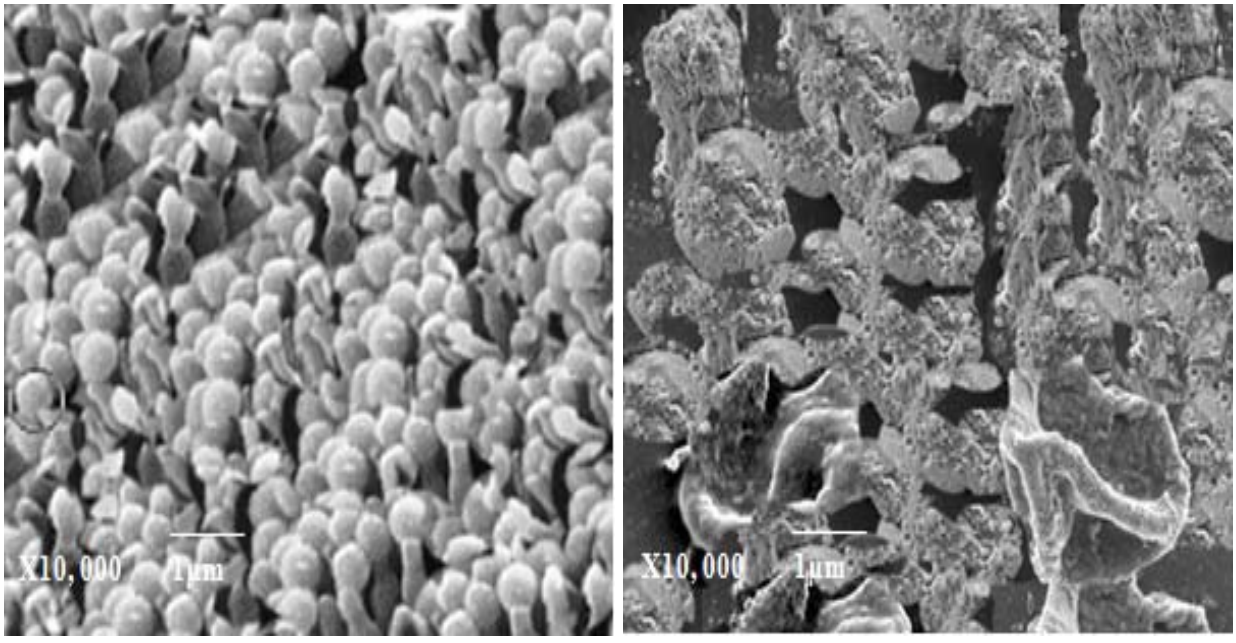

Fig. 6. Scanning electron micrograph of antifungal effects of the used *Rhodococcus erythropolis* glycolipids on *Aspergillus flavous*. Intact fungus (right) and affected fungus (left).

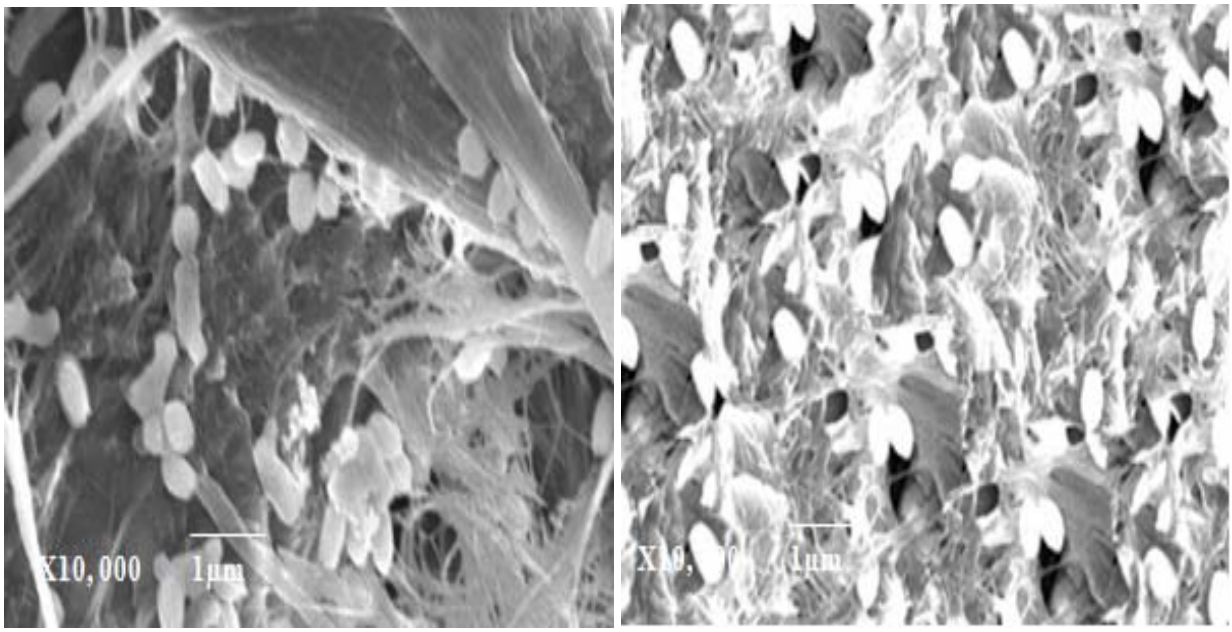

Fig. 7. Scanning electron micrograph of antibacterial effects of the used *Rhodococcus erythropolis* glycolopids on Intact bacterial cells (right) and the affected ones (left).

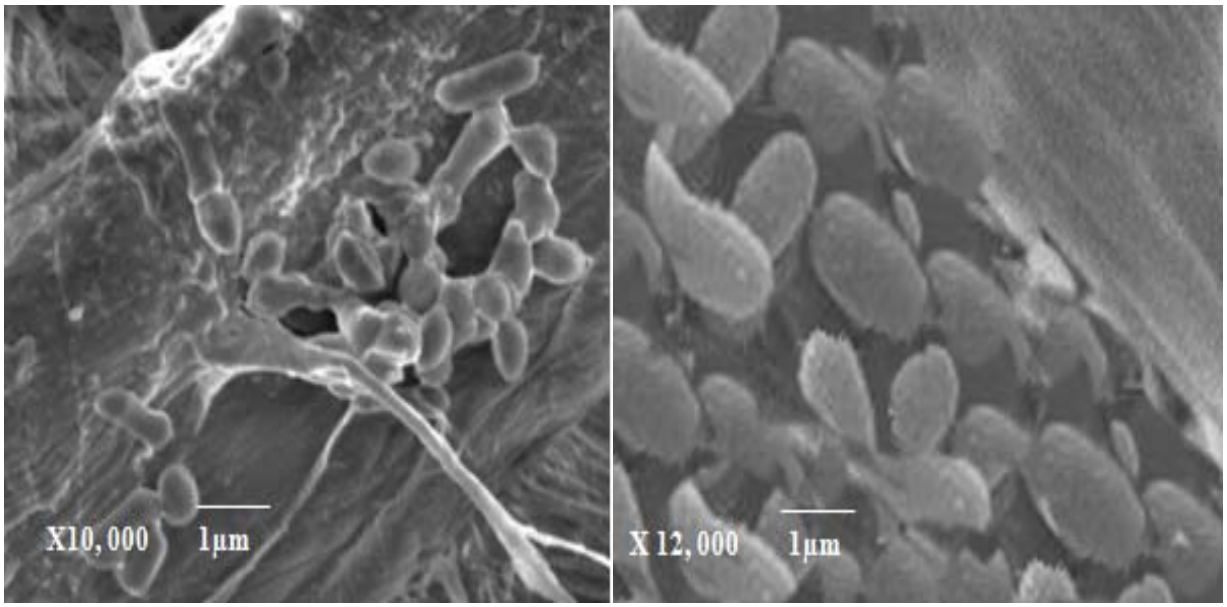

Fig. 8. Scanning electron micrograph of antibacterial effects of the used *Rhodococcus erythropolis* glycolopids on *Pseudomonas aeruginosa*. Intact bacterial cells (right) and the affected ones (left).

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