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A Study of Antibacterial Activity of Fatty Acids Extracted from *Pseudomonas* sp. LP1 by using Chromatography technique

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ABSTRACT

A bacterium that was capable of metabolizing C1- compounds was isolated and identified in Moscow State Academy of Fine Chemical Technology (Russia), as Pseudomonas sp. LP1 based on detailed taxonomic studies. This organism is a new strain of obligate methylotrophic bacteria, which was cultured on the methanol-containing medium (1%) as its sole carbon source. This study included culture of Pseudomonas sp. LP1 in M9 modified medium. The lipids were extracted from biomass of bacteria by organic solvents where a mixture of fatty acid methyl esters (FAMEs) was obtained. Antibacterial activity of total fatty acids was tested against some standard strains of both gram positive and gram negative bacteria by using wells in agar plates. Fatty acids mixture were found to have antibacterial effects. Thin-layer chromatography was used to tentatively identified the mixture components of fatty acids. A gas-liquid chromatographic system referred that Palmitic (16:0), Palmitoleic (16:1) and Oleic (18:1) acids were present.

Introduction:

Some microorganisms have a number of unique enzymes that enable them to generate energy from methanol and synthesize all cell constituents required for their growth and reproduction using one-carbon source. They are able to synthesize a variety of carbon compounds from methanol as an alternative to chemical processes (1).

Methylotrophic bacteria are a diverse group of organisms that possess a great number of specialized enzymes that enable them to grow on reduced carbon substrates without carbon – carbon bonds and use these as energy as well as a carbon source. (2). Two major groups of methylotrophs can be distinguished, depending on the range of utilizable substrates. Obligate methylotrophs can utilize only one carbon compounds including methanol as sole sources of carbon and energy by way of the ribulose monophosphate pathway(2), whereas facultative methylotrophs are able to grow on a variety of other organic multicarbon compounds.

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These microorganisms have a wide applications in biotechnology and biochemistry (3, 4). The potential of these bacteria for the development of economically competitive bioprocesses based on methanol as an alternative carbon source, bringing together biological, technical and economic considerations (2, 5) so that they can be used commercially to produce fine and bulk chemicals (2, 6). All of these factors led to increased interest in the methylotrophic bacteria in the last years.
Lipids are one of these important biological compounds that can be obtained by these bacteria. Fatty acids are the major component of lipids, and the physical, chemical, and physiological properties of a lipid class depend primarily on its fatty acid composition. The purpose of this paper was identification of fatty acids produced by *Pseudomonas* sp. LP1 using thin layer chromatography (TLC) and gas liquid chromatography (GLC) methods then study the inhibitory effect on some bacterial strains.

Materials and Methods:

Materials:

Fatty acid methyl esters used as standards were purchased from Sigma. All other chemicals were from CHIMMED Company (Russia). Experiments were

performed in Moscow State Academy of Fine Chemical Technology (Russian Federation).

Microorganisms:

The bacteria used in the antibacterial test were: gram-positive (*Bacillus pumilus* ATCC 14884 and *Staphylococcus aureus* ATCC 8625) and gram-negative (*Escherichia coli* ATCC 10541 *Pseudomonas aeruginosa* ATCC 15422).

Biomass preparation of bacteria:

Bacteria under study were cultivated in modified M9 medium (7) at fixed laboratory conditions (28°C and pH 7.0) for 48 hours and by using batch culture. Cell biomass was separated by centrifugation at 2000 $\times g_{av}$ for 10min.

Lipid extraction:

Total lipid was extracted from bacterial cells according to the method described by Gómez-Brandón *et. al*. (8) with some modifications. A weight of (3.8gm) of bacterial cells were dispensed into a separating funnel and (60ml) chloroform:methanol (1:2) were added. This solution was shaken for 2 min and allowed to stand for 1h. Chloroform (20ml) was added, followed by distilled water (20ml). The chloroform layer that separated was transferred into a flask and the residue was re-extracted twice as before. The extracts were combined and the solvent was concentrated by blowing nitrogen gas.

Fatty acid methyl esters preparation:

The FAMEs was prepared as follows method by (9) with slight modifications. The lipid sample (100mg) was refluxed with a solution of potassium hydroxide (1M) in 95% ethanol (2ml) for 1 hour alternatively, reaction at room temperature overnight was equally effective. The solution was cooled, water (5ml) was added and the mixture was extracted thoroughly with hexane-diethyl ether (1:1, v/v; 3 x 5ml). The solvent extract was washed with water, dried over anhydrous sodium sulfate and the non-saponifiable materials were recovered on removal of the solvent in a rotary evaporator. The water washings were added to the aqueous layer, which was acidified with hydrochloric acid (6M) and extracted with diethyl ether-hexane (1:1, v/v; 3 x 5ml). The Fatty acid methyl esters recovered after washing the extract with water, drying it over anhydrous sodium sulfate and removing the solvent by evaporation. fatty acids were divided into two parts, first part for testing the inhibitory effect and the second part, for analysis of fatty acids using gas-liquid chromatography.

Antibacterial activity:

The modified agar well diffusion method of Perez *et al.* (10) was employed. The tested bacteria were cultured in nutrient broth at 37°C for 18 hours. The plates were allowed to solidify and 10^5 CFU/ml of bacterial cultures were spread to agar. A well was prepared in the plate with the help of a cork-borer (6 mm). 50µl (0.5mg/ml) of the fatty acids was introduced into the well. The plates were incubated overnight at 37°C. Bacterial growth was determined by measuring the diameter of zone of inhibition. For each bacterial strain, controls were maintained where pure solvents (5% DMSO) **(**11) were used instead of the fatty acids mixture.

Analysis of fatty acids:

The fatty acid composition of *Pseudomonas* sp. LP1 was determined by converting the lipids to fatty acids methyl esters (FAMEs) and then analyzing by:
Thin layer chromatography (TLC): Reverse-phase TLC was performed on RP-18F-254 (Merck 0.25mm×200 mm×200 mm) using acetonitrile: acetic acid (1:1, v/v).
Gas-liquid chromatography (GLC): Fatty acid methyl esters were analyzed by gas-liquid chromatography method (GLC) using a HP 6850 gas chromatograph equipped with a 40 m x 0.25 mm WCOT capillary column. The temperature was maintained at 175ºC for 3 min, then was increased to 195ºC, where it was maintained for a further 17 min. Hydrogen was used as a carrier gas at a flow-rate of 3 ml/min. Each fatty acids methyl ester in extract identifying retention times with those of known standard FAME (Lipid Standard, Sigma).

Results and Discussion:

Analysis and separation of fatty acid methyl esters typically are achieved by a variety of chromatography systems, such as thin-layer chromatography (TLC) and

gas-liquid chromatography (GLC). In this study, chromatographic techniques performed were effective analytical systems, which allow together valuable information about the lipid structure that extracted from *Pseudomonas* sp. LP1. It could be said that these bacteria gave considerable yield of lipids that to be a good source of some fatty acids.

Separation of saturated fatty acid methyl esters may be enhanced by employing Reverse-phase TLC where separation by degree of unsaturation and carbon number (table 1). On Reverse-phase TLC, fatty acid methyl esters interact with a polar solvent on a non polar stationary phase in a manner that allows more rapid migration of molecules with greater polarity and lower mass. Fatty acid methyl esters of our bacteria showed three spots corresponding to Oleic, Palmitic and Palmitoleic acid methyl esters, respectively (see Figure 1). This analysis showed different R_f values with the acetonitrile: acetic acid solvent systems (table 1).

The fatty acid composition was then determined by gas-liquid chromatography. GLC analysis of fatty acids is performed following their conversion to apolar, methyl esters (12). The GLC analysis confirmed the data that was obtained from the TLC analysis of the mixture as revealed higher amount of unsaturated fatty acids than saturated fatty acids (table 2).

The polar column (WCOT) was used in the separation of FAMEs, and according to (13), the saturated components are eluted before the unsaturated fatty acids with the same chain-length (fig. 2). Results of experiments show in table 1 and 2, the presence of two unsaturated fatty acids (Palmitoleic acid, C16:1 and Oleic acid, C18:1) versus one saturated fatty acid (palmitic acid, C16:0).

Present study is in accordance with the study (14) that showed the presence of three fatty acids from the lipids extract of the methylotrophic bacterium (strain Kr3). Doronina and co-workers (15) showed the same results when studying a novel methylotrophic bacterium *Methylophaga alcalica* sp. nov.. In contrast, other studies showed that a novel species of methylotrophic bacteria *Methylobacillus arboreus* sp. nov., and *Methylophaga aminisulfidivorans* sp. nov. (16, 17) respectively, together produce only (C16:0) and (C16:1).

The FAMEs extract possessed antibacterial activity against a total of 4 microorganisms (2 gram-positive and 2 gram-negative bacteria). The results of the test demonstrated that the Dimethylsulfoxide (5%) did not appear any activity against the tested bacteria, and this result in accordance with the study of Ramachandran *et. al.* (18). The mean zone of inhibition of the FAMEs, assayed against the tested organisms ranged between 11 and 26 mm (Table 3). The results of the present study demonstrated that these fatty acid methyl esters were more effective against Gram-positive bacteria than the Gram-negative bacteria, these results are compatible with recent results obtained by Huang *et. al.* (19).

Previous study (20) reported that the exact process of the antibacterial activity of fatty acids is not clear, but the main target of it action is the cell membrane, where fatty acids disrupt the electron transport chain and oxidative phosphorylation. Besides interfering with cellular energy production, fatty acid action may also result from the inhibition of enzyme activity, impairment of nutrient uptake, generation of peroxidation and auto-oxidation degradation products or direct lysis of bacterial cells.

Conclusions:

Microorganisms are the main drivers of the biological mechanisms involved in these processes, thus their characterization in terms of their FAME profiles would clearly improve the understanding and optimization of such processes.

This study was mainly focused on the lipids (fatty acids) of *Pseudomonas* sp. LP1. Palmitic acid, Palmitoleic and Oleic were identified in the lipid composition of *Pseudomonas* sp. LP1, thus the study of the fatty acids composition of *Pseudomonas* sp. LP1 is worthwhile. The antibacterial screening of the fatty acids exhibited strong antibacterial activity against both gram-positive and gram-negative tested organisms. The potential for commercial or biomedical exploitation of antibacterial fatty acids from methylotrophic bacteria, requires more scientific attention of these types of bacteria.

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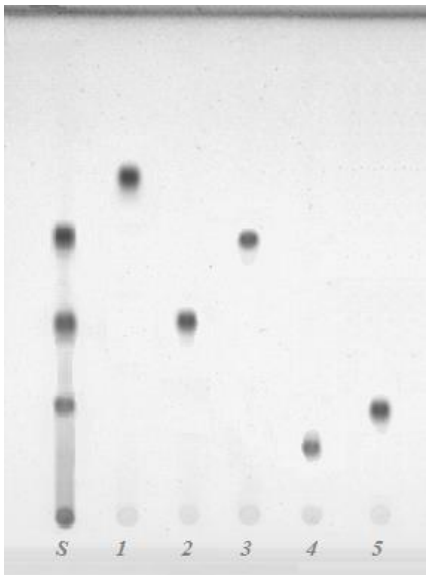

Figure 1. Separation of FAMEs from *Pseudomonas* sp. LP1 on reversed-phase thin layer chromatography. Lane S: fatty acids (as methyl esters) extracted from bacteria; **Lane 1**: methyl myristate; **lane 2**: methyl palmitate; **Lane 3**: methyl palmitoleate; **Lane 4**: methyl stearate; **Lane 5**: methyl oleate, as standard.

Table 1. TLC analysis of total fatty acids (as methyl esters) of *Pseudomonas* sp. LP1.

Spots	S FAMEs extracted from bacteria		Standards 1 C14:0	2 C16:0	3 C16:1	4 C18:0	5 C18:1
R_f Values	C18:1	0.24	0.69	0.40	0.57	0.15	0.24
	C16:0	0.40					
	C16:1	0.57					

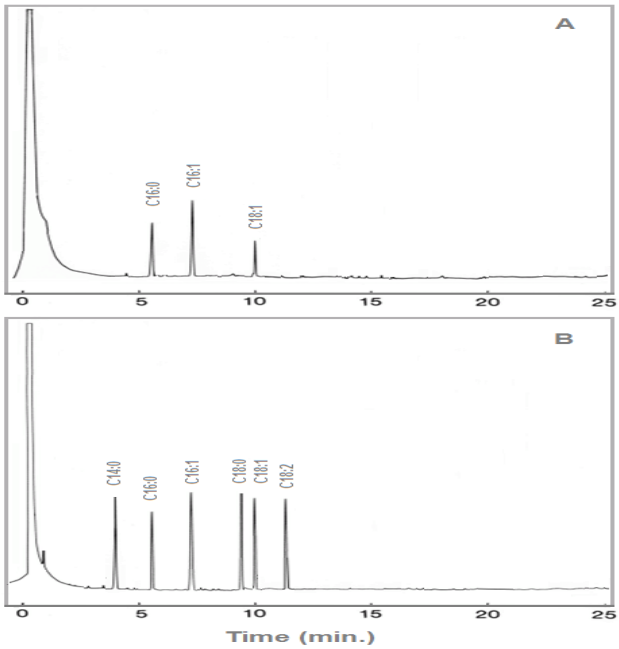

Figure 2. Separation of fatty acid methyl esters of *Pseudomonas* sp. LP1 by GC. (A): Fatty acid methyl esters extracted from bacteria**. (B)**: Standard FAMEs.

Table 2. Fatty acid composition of *Pseudomonas* sp. LP1

Peak No.	Retention time (min.)	Fatty acid	No. of carbon atom
1	5.6	Palmitic acid	C16:0
2	7.5	Palmitoleic acid	C16:1
3	10.0	Oleic acid	C18:1

Table 3. Antibacterial activity of Fatty acid methyl esters of *Pseudomonas* sp. LP1.

Microorganisms	Mean diameter of growth of inhibition zones (mm)	
	Fatty acids (as methyl esters)	Control [a]
Bacillus pumilus ATCC 14884	19	-
Staphylococcus aureus ATCC 8625	26	-
Escherichia coli ATCC 10541	11	-
Pseudomonas aeruginosa ATCC 15422	15	-

[a] The effect of solvent (5% DMSO) alone.

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دراسة الفعالية المضادة للبكتريا للأحماض الدهنية المستخلصة من *Pseudomonas* sp. LP1 باستخدام تقنية الكروماتوغرافيا

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الخلاصة:

عزلت إحدى أنواع البكتيريا القادرة على التمثيل الغذائي للمركبات الحاوية على ذرة كربون واحده وشخصت في اكاديمية موسكو الحكومية لتكنولوجيا الكيمياء الدقيقة في روسيا الاتحادية باسم Pseudomonas sp. LP1. هذا الكائن هو سلاله جديدة من البكتيريا المتغذية على الميثانول (1%) كمصدر وحيد للكربون. تناولت الدراسة إكثار البكتيريا في الوسط M9 المحَور. استخلصت الدهون من الكتلة الحية للبكتيريا بواسطة المذيبات العضوية ومن ثم تم الحصول على أسترات المثيل للأحماض الدهنية. اختبرت فعالية الأحماض الدهنية المضادة للبكتيريا ضد بعض سلالات البكتيريا الموجبة والسالبة لملون كرام القياسية وباستخدام طريقة الانتشار في وسط الأكار ووجد أن مزيج الأحماض الدهنية المستخلصة من Pseudomonas sp. LP1 له تأثير مضاد للبكتريا. تم فصل الأحماض الدهنية المستخلصة وتشخيصها بواسطة كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا الغاز وثبت وجود كل من حامض البالمتيك, حامض البالميتوليك وحامض الاوليك.