FATTY ACID COMPOSITION AND THE GROWTH SUBSTRATE OF FREE AND IMMOBILIZED CELLS OF FLAVOBACTERIUM CHLOROPHENOLICA.

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ABSTRACT

The fatty acid profiles of free and immobilized cells of Flavobacterium chlorophenolica were studied when these cells were grown on phenol, 4-chlorophenol or p-nitrophenol as a sole source for carbon and energy in comparison with cells grown on glucose. The total fatty acid content increased as a result of the immobilization process especially when 4—chlorophenol was applied, which resulted in increasing the fatty acid content by1.35 fold compared to that of the free cells (16.67 %). Moreover, the 4-chlorophenol showed an increase in the relative % of the unsaturated and the branched-chain fatty acid contents for the free cells (10.49 and 20.18%) and for the immobilized cells (11.31 and 29%) compared with the glucose grown cells. The major saturated fatty acid for both the free and the immobilized cells was the palmitic acid (16:0) which was followed by the pentadecanoic acid (15:0), their relative % ranged from 45.31 to 56.48% and from 0.0 to 15.42% of the total fatty acids, respectively. In addition, the palmitoleic acid (16:1) showed a great relative % compared to the oleic acid (18:1) for both free and immobilized cells with a relative content ranging from 7.49% to 8.06% and from 7.49% to 10.02%, respectively. Moreover, the most presented branched-chain fatty acid when these aromatic compounds were utilized by the free and the immobilized cells of F. chlorophenolica was iso 17:0 fatty acid with a relative % average of 6.73 and 10.82, respectively.

INTRODUCTION

Carbon source utilization studies can distinguish between microbial communities in different habitats on the basis of substrate utilization or metabolic pathways. Fatty acid profiling is used to describe microbial strains or communities or to differentiate among environmental samples by their fatty acid fingerprints (Kenndy, 1994).

However, the recent molecular biological studies for the identification of the bacterial species in comparison to the fatty acid methyl ester analysis method showed that, the assemblage of organisms present in an environmental sample can contain thousands of distinct bio-types of bacteria. They are also very time consuming due to the construction and screening of clonal libraries (Chandler *et al.,* 1997; Gray and Herwig, 1996). Moreover, many investigators proved that there are strong correlation between LH-PCR results and fatty acid methyl esters (FAME) results used for the identification of the bacterial isolates (Ritchie *et al.,* 2000).

Meanwhile, the aromatic hydrocarbons are found to be toxic to different bacterial species due to their high partition into the cell membrane (Sikkema *et al.,* 1994 & 1995). In addition, the change in the degree of saturation of the bacterial fatty acids is a well-known reaction in the presence of such membrane active compounds (Diefenbach *et al.,* 1992 and Keweloh *et al.,* 1991). In addition, the effect of the aromatic compounds on the fatty acid composition of different bacterial species, particularly, those

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which are able to utilize high concentrations of such aromatic compounds has not been studied intensively (Tsitko *et al.,* 1999), specially under the immobilization condition.

Moreover, it is well known that environmental or physiological factors or culturing conditions affect the fatty acid composition of bacteria (Rose, 1989 and Scherer *et al.,* 2003). However, the fatty acid analysis of bacteria is usually performed using free cells grown on glucose as a sole carbon with little attention to the effect of other carbon sources and other culturing conditions like immobilization of these bacterial cells. Therefore, the objective of this study was to investigate the response of the fatty acid profiles of the free and immobilized cells of *Flavobacterium chlorophenolica* when grown on different aromatic compounds (phenol, 4-chlorophenol and p-nitrophenol) as a sole carbon and energy source, in comparison with glucose as standard growth substrate

MATERIAL AND METHODS
Isolation and identification

The isolation process was carried out from a sediment sample of El-Max Gulf, Alexandria to obtain a bacterial species able to utilize relatively high concentrations of different aromatic compounds as a sole carbon and energy source. 15g sediment sample was spread in 15cm petri-dish and enriched for three weeks with phenol, 4-chlorophenol or p-nitrophenol at a concentration of (200mg/l). The identification of this bacterial isolate was carried out using the fatty acid methyl ester (FAME) analysis and identified as *Flavobacterium chlorophenolica* with a kind aid of the Microchek Inc. Microbial lab., Vermont, USA.

Culturing medium

The isolated *F. chlorophenolica* was cultivated in 1 liter flasks with 200 ml of mineral medium composed in (g/l) of: 0.5 $MgSO_4$, 1.0 K_2HPO_4, 8.0 Na CL, 1.0 $(NH_4)_2SO_4$ and supplemented with phenol,

4-chlorophenol or p-nitrophenol (200mg/l) as a sole carbon and energy source. The cultures were inoculated with 5ml of bacterial suspension (OD $\simeq$ 1.0) and incubated under shaking conditions (180rpm) at 28°C to early stationary phase.

Fatty acid analysis:
Extraction process

The cells were harvested ($\simeq$ 50 mg wet cells) by centrifugation and washed twice with sterile saline solution (0.8% NaCl). The lipid content was extracted by a chloroform /methanol mixture (2:1) using a soxhlet for about 6h at 80º C.

Saponification of the lipid extract

The saponification process for the obtained dried lipid residues were carried out for 2h at 80°C using 5ml of absolute ethanol containing 0.2ml of 30% aqueous KOH and the acidification process occurred by using 2ml of 1.5N HCl. The resulting fatty acids were extracted five times using n-hexane, then a complete drying of the used solvent was achieved.

Methylation of the fatty acids

This process was carried out according to Bligh and Dyers (1959), the fatty acids were heated for 2h at 80-90ºC in presence of 10 ml of a freshly prepared mixture solution composed of dry methanol, benzene and concentrated H_2SO_4 (8.6,1.0, 0.4ml, respectively). After cooling, 15 ml of bi-distilled water were added, then the methyl esters were extracted five times using 5ml portions of n-hexane. The resulted extract was dried and concentrated using anhydrous $(Na)_2SO_4$ and a genital stream of nitrogen gas(0.5 L/min).

Estimation of fatty acid methyl esters (FAME)

The FAME were analyzed by Microchek Inc. Microbial lab. Vermont, USA, using gas chromatography (GC)-mass spectrometry with an HP 6890A gas chromatograph equipped with an HP 5972A mass selective detector (Hewlett Packard Co., Porlo Alto, California) and an HP Ultra 2 cross-linked 5% phenyl-methyl silicon capillary column

(25m by 0.2mm 0.33μm). The oven temperature was programmed with injection and 1-min hold at 80°C, followed by an increase to 160°C at 60°C min⁻¹, a hold at 160°C for 28 min., and an increase at 5°C min⁻¹ to 230°C. Individual FAME was identified by comparing their mass spectra with standard kits (Ana-labs, North Haven, Conn.). The fatty acid content of the cells was calculated as the average of three independent cultivations.

Immobilization process :

Free fatty acid Na-Alginate (medium viscosity-sigma) was prepared by extraction with chloroform-methane mixture using soxhlet as mentioned before. 2g of the prepared alginate was used for entrapping the cells of *F. chlorophenolica* according to Tanaka, *et al.*(1979). Where 20% (V/V) beads were transferred to the culture mineral medium containing phenol, 4-chlorophenol or p-nitrophenol (200 mg/l). These cultures were inoculated and incubated as described for the free cells.

After incubation the beads were washed twice using sterile saline solution (0.8% NaCl) then dissolved using phosphate buffer (1M, pH 7), after complete dissolution (∼1.0 h) centrifugation and extraction processes were carried out as described for the free cells.

RESULTS

The data of the fatty acid composition of the free cells of *Flavobacterium chlorophenolica* due to the utilization 200mg/l of phenol, 4-chlorophenol or p-nitophenol as sole carbon source is presented in Table (1). It was showed that, the relative % of the total fatty acid contents increased by 1.46, 2.97 and 2.02 fold, respectively, as a result of using these aromatic compounds in comparison with glucose (5.62%).

In addition, the contents of the unsaturated and the branched-chain fatty acids were also increased as a result of utilizing these substrates .The maximum relative % was obtained with the use of 4-chlorophenol (10.49% and 20.18%, respectively). On the other hand, the total saturated fatty acid content illustrated in Figure (1-A) showed slight decrease with the use of p-nitrophenol, while phenol and 4-chlorophenol resulted in decreasing the saturated fatty acid content (6.9-7.5 %, respectively) compared to glucose (73.98% of the total fatty acid).

The immobilized cells of *F. chlorophenolica* using fatty acid-free alginate gel material resulted in an increase of fatty acid content compared to the free cells. This reflected the great effect of the substrate usage, where they showed fatty acid content ranging from 1.16 to1.35 fold more than the corresponding substrate using the free cells (Table-2). Moreover, the saturated fatty acid content decreased from 65.11%, using the glucose substrate, to 59.6% and 58.11% when 4-chlorophenol and p-nitrophenol were applied, respectively. On the other hand, phenol showed an increase in the saturated fatty acid content (68.86%) and a decrease in the unsaturated fatty acid content (9.75%).

In general, the immobilized cells of *F. chlorophenolica* demonstrate greater relative % of the total unsaturated and the branched-chain fatty acid contents 11.31% and 29% respectively, when 4-chlorophenol was applied, compared with that of glucose (10.28% and 18.06%, respectively).

However, it was observed that the major saturated fatty acid obtained was palmitic acid (16:0), its relative % ranged from 50.29% to 56.27% for the free cells and from 45.31% to 56.48% for the immobilized cells. This was followed by the Pentadecanoic acid (15:0) which resulted in a relative content ranging from 11.82% to 15.42% for the free cells and from 0.0 to 13.44% for the immobilized cells of *F. chlorophenolica*. Moreover, the unsaturated fatty acid, palmitoleic acid (16:1), was found in high relative content compared to the oleic acid (18:1), ranging from 7.49% to 8.06% for the free cells and from 7.49% to 10.02% for the immobilized cells (Tables, 1 & 2).

The data illustrated in Fig. (1) indicated that the fatty acid composition of *F. chlorophenolica* under the utilization of these four growth substrates (glucose, phenol, 4-chlorophenol and p-nitrophenol), can be divided into three common groups. The major group was the saturated fatty acids occupying ~ 70 and 63% of the total fatty acid content of the free and the immobilized cells, respectively. This group was followed by the unsaturated fatty acids which occupied ~7.49 – 13.06% and 9.75- 11.31% of the total fatty acid content of both types of cells, respectively. While the third group was the branched-chain fatty acids (~15.57- 20.18% and 18.06- 29%, respectively). The most presented individual of this fatty acid group was the iso 17:0 fatty acid, its relative % ranged from 5.26 to 8.12% (for the free cells) and from 5.26 to 15.02% (for the immobilized cells). Moreover, it was observed that, the trend of each fatty acid group obtained by *F. chlorophenolica* was not greatly affected by the immobilization process, except in the case of glucose where the saturated fatty acid group decreased from 73.98% to 65.11% as a result of the immobilization (Fig., 1- A & B).

Table 1: Fatty acid composition of the free cells of *F. chlorophenolica* grown on mineral medium with different carbon sources [@].

Fatty acid(s)	Relative % of fatty acid(s)			
	Glucose	**Phenol**	**4-Chlorophenol**	**p-Nitrophenol**
14:0	2.29	4.02	3.12	5.21
15:0	15.42	12.68	11.82	14.77
16:0	56.27	52.16	53.46	50.29
Sum of saturated	73.98	68.86	68.4	70.27
16:1(*9c*)	7.49	7.59	8.06	7.95
18:1(*9c*)	ND	2.16	2.43	5.11
Sum of unsaturated	7.49	9.75	10.49	13.06
15:0 ante-iso	2.13	3.02	5.46	3.86
16:0 iso	5.82	4.68	4.56	5.28
17:0 iso	5.26	8.12	7.12	6.43
17:0 ante-iso	2.46	ND	3.04	ND
Sum of branched-chain	15.67	15.82	20.18	15.57
Total content*	5.62	8.2	16.67	11.57

[@]Each substrate concentration was 200mg/l. * mg fatty acid/100mg wet cell extracted.
ND: not detected

Table 2: Fatty acid composition of the immobilized cells of *F. chlorophenolica* grown on mineral medium with different carbon sources [@].

Fatty acid(s)	Relative % of fatty acid(s)			
	Glucose	Phenol	4-Chlorophenol	p-Nitrophenol
14:0	ND	4.02	3.12	ND
15:0	13.44	12.68	ND	12.8
16:0	51.67	52.16	56.48	45.31
Sum of saturated	65.11	68.86	59.6	58.11
16:1(*9c*)	7.49	7.59	8.23	10.02
18:1(*9c*)	2.79	2.16	3.08	ND
Sum of unsaturated	10.28	9.75	11.31	10.02
15:0 ante-iso	4.56	3.6	1.28	3.86
16:0 iso	8.24	5.02	6.12	7.53
17:0 iso	5.26	12.53	15.02	10.46
17:0 ante-iso	ND	< 0.5	6.58	ND
Sum of branched-chain	18.06	21.15	29	21.85
Total content*	6.54	10.23	22.43	15.21

[@]Each substrate concentration was 200mg/l. * mg fatty acid/ 100mg wet cell extracted.
 ND: not detected

DISCUSSION

The occurrence and abundance of microbial fatty acids have been used by many investigators for the identification of microorganisms in microbial communities (Schutter and Dick 2000; Noble *et al.,* 2000). Also they can be used as indicators of substrate usage (Abraham *et al.,* 1998; Boggs *et al.,* 1998). Moreover, they can be used in distinguishing virulence among certain bacterial species (Inglis *et al.,* 2003).

From the data obtained in this study the immobilization process increased the total fatty acid content in a range of about 1.16-1.35 fold compared with the free cells of *F. chlorophenolica* when grown on different growth substrates (glucose, phenol, 4-chlorophenol and p-nitrophenol) as a sole carbon and energy source. In addition, the use of 4-chlorophenol resulted in increasing the relative % of palmitoleic acid (16:1 *9c*) and (iso and ante-iso17:0) branched-chain fatty acids by both free or immobilized cells of *F. chlorophenolica* compared with glucose. These results are in a partial agreement with that of Tsitko *et al.* (1999) who found that, the use of 4-chlorophenol as a sole carbon source for growing *Rhodococcus opacus* resulted in increasing the content of *trans*-hexadecenoic acid (16:1 *9c*) compared with cells grown on fructose.
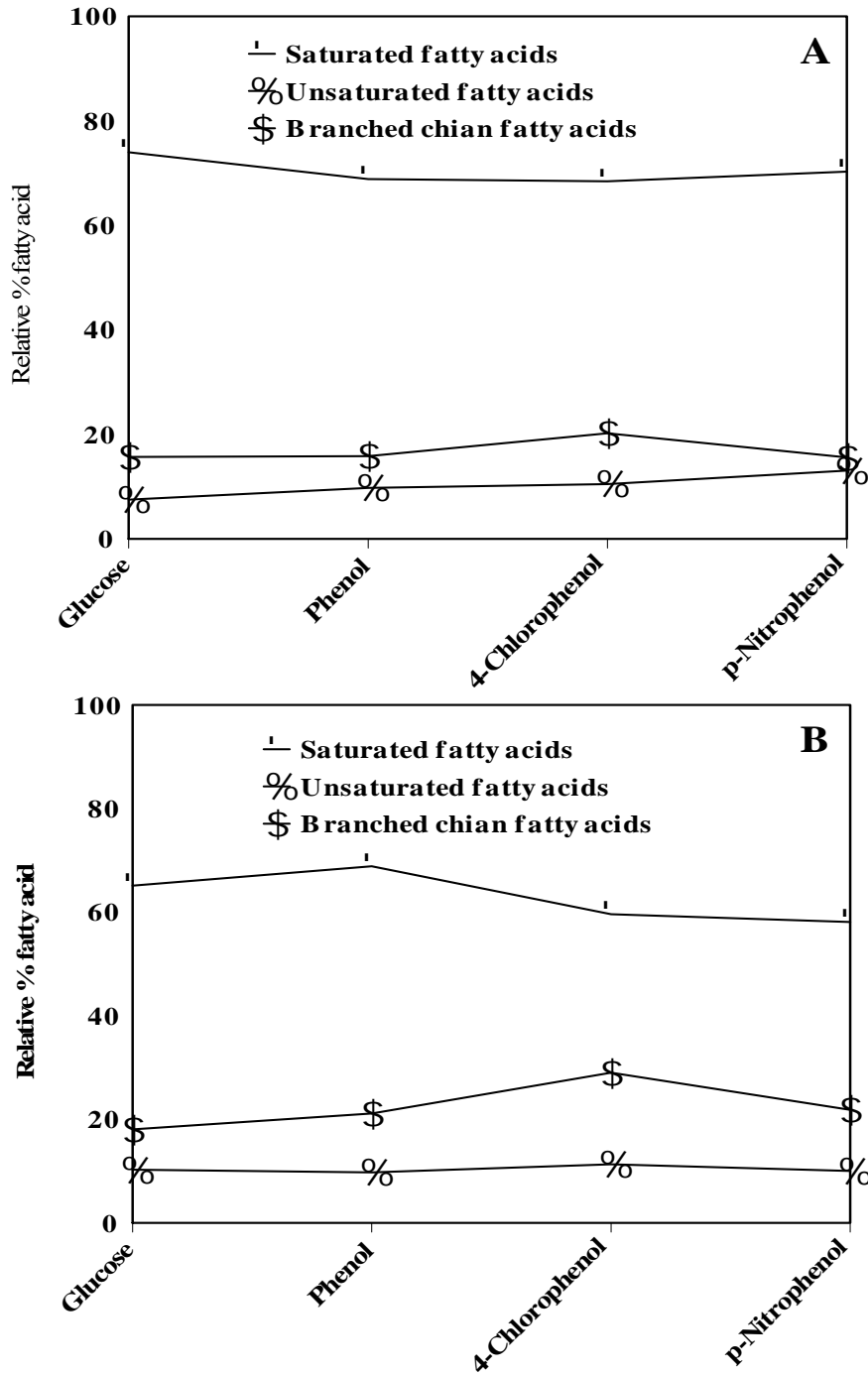
Fig. 1: Effect of different carbon sources on the fatty acid composition of the free cells (A) and the immobilized cells (B) of *Flavobacterium chlorophenolica*.

However, variations obtained in the fatty acid profiles indicated that the substrate usage has great effect on the fatty acid composition of *F. chlorophenolica.* This result was clearly discussed by Velicer (1999) and Mackenzie *et al.*(2002), who showed the selectivity and the adaptation of the bacteria toward the substrate which may affect the metabolic pathways. Moreover, on a comparison of the differences of the obtained fatty acid profiles they showed un-uniform pattern even under the same conditions of extraction and methylation. These variations were also found by many investigators, which indicated that the fatty acid composition may be influenced by other factors (Abraham *et al.,* 1998; Nichols *et al.*, 2002).

The fatty acid profiles of *F. chlorophenolica* obtained in this study indicated the predominance of the palmitic acid (16:0) in comparison to the pentadecanoic acid (15:0), and the presence of palmitoleic acid (16:1) and the oleic acid (18:1). These results were in agreement with many investigators, Basile *et al.* (1995) who found the mass spectra of gram-negative microorganisms were characterized by the presence of the palmitoleic and the oleic acids and with the abundance of the palmitic acid. In addition, Bertone, *et al.* (1996) studied the fatty acid methyl ester composition of a total of 71 marine strains which were characterized by the predominance of 16:0, (16:1 *cis* and *trans)*,15:0 iso ,and (18:1 *cis* and *trans*) fatty acids. On the other hand, Kankaanpää *et al.*(2004) mentioned the most abundant bacterial fatty acids identified were oleic (18:1 *9c*), vaccenic (18:1 *11c*) and di-hydrosterculic acids especially when bacteria were cultivated in presence of such fatty acids.

However, Tsitko *et al.* (1999) observed that aromatic compounds enhance the formation of the fatty acids in the bacterial cells to be more adaptive in degrading such alternative carbon sources in nature. They also mentioned that these fatty acids may play an important role in the protection of the cells

against the toxic effect of such aromatic compounds (Heipieper *et al.*,1992). Moreover, the increase in the content of the branched-chain fatty acids obtained in this study (due to the use of aromatic carbon sources) was explained by the same investigators, since they mentioned that the branched-chain fatty acids may participate in the adaptation of *Rhodococcus opacus* to these aromatic hydrocarbons. Also Nichols *et al.* (2002) noticed that there was a high degree of variation in the percentage of the branched–chain fatty acids of *Listeria monocytogenes* as an adaptive pathway when these cells were grown under difficult physiological conditions.

In general, the entrapment of *F. chlorophenolica* in fatty acid-free alginate gel material exhibited less fatty acid content compared with the use of normal alginate gel material, where the fatty acid content was decreased with about 2.4 fold (data not shown). These results are in agreement with that of Scherer *et al.* (2003), who observed the growth of *Helicobacter pylori* on fatty acid-free agar which showed less fatty acid content compared with the use of the blood agar containing fatty acids. On the other hand, they observed no appreciable differences between the fatty acid profiles of the laboratory-adapted strains and the freshly isolated strains of *H. pylori* (strains ATCC 43504, ATCC 1932 and ATCC 700392). However, these results are not in agreement with those obtained in the present study and some previous studies. Where, the fatty acid profiles of *F. chlorophenolica* and other bacterial species showed great variations due to the culturing and extraction conditions (Schutter and Dick, 2000).

From the present study it may be concluded that, the free and the immobilized cells of *Flavobacterium chlorophenolica* were able to utilize phenol, 4-chlorophenol and p-nitrophenol as a sole carbon and energy source. Moreover, this utilization resulted in increasing the fatty acid content of the free

and the immobilized cells of this bacterium, especially when 4-chlorophenol was applied which increased both unsaturated and the branched-chain fatty acid contents compared to the glucose grown cells. These results may be explained according to the results obtained by Chanama and Crawford (1997) and Lee and Xum (1997). Where they showed that the cells of *F. chlorophenolica* are more adaptive to the chlorophenolic compounds since they are characterized by having *pcpA* gene which is essential for such utilization.

To our knowledge it is the first documented work studying the effect of the immobilization process using fatty acid-free alginate gel material, on the fatty acid composition of *Flavobacterium chlorophenolica* with the utilization of different aromatic compounds.

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