Changes in cellular fatty acid composition induced by phenol and catechol in *Pseudomonas vesicularis* and *Pseudomonas stutzeri*

Agnieszka Mrozik[1], Sylwia Łabużek[1], Zofia Piotrowska-Seget[2]
[1]Department of Biochemistry,
[2]Department of Microbiology,
University of Silesia, Katowice, Poland

Changes in cellular fatty acid composition induced by phenol and catechol in *Pseudomonas vesicularis* and *Pseudomonas stutzeri*

Summary

The changes in cellular fatty acid profiles, determined by gas chromatography, of *Pseudomonas vesicularis* and *Pseudomonas stutzeri* growing in a modified minimal medium with catechol or phenol are presented in this paper. *P. vesicularis* increased its ratio of saturated/unsaturated fatty acids from 1.92 to 4.05 and 5.72 when grown on glucose, phenol and catechol, respectively. In the case of *P. stutzeri,* the ratio changed from 2.56 to 8.28 and 4.65 under the same growth conditions. The increase in the abundance of saturated and cyclopropane fatty acids and forming new *iso* and *anteiso* fatty acids during growth of tested strains in the presence of catechol and phenol are suggested as a possible mechanism to tolerate tested aromatic compounds.

Key words:
catechol, phenol, fatty acids, *Pseudomonas vesicularis*, *Pseudomonas stutzeri.*

Address for correspondence

Agnieszka Mrozik,
Department
of Biochemistry,
University of Silesia,
Jagiellońska 28,
40-032 Katowice, Poland.

biotechnologia

1 (64) 244–252 2004

1. Introduction

Aromatic compounds such as phenol, catechol, benzene and toluene are common pollutants found in water, soil and sediments. The fate of these substances is of great environmental concern due to their toxic and mutagenic properties (1,2).

Aromatic solvents at low concentrations disturb many biological processes occurring in bacterial cell, especially respiration, growth, ions and nutrients transport and at saturating concentrations they may even cause lysis of the cells (3,4). The loss of essential ions is probably a crucial event in the toxic action of many phenolic compounds (5). It has been shown that the main cellular site in which such compounds are accumulated is the bacterial membrane. Accumulation of aromatic solvents in the membrane affects the physico-chemical properties of the lipid bilayer and, consequently, its biological functioning. These lipophilic compounds induce perturbations of lipid ordering, membrane permeability and fluidity (6-8). Bacteria that are able to survive in the presence of aromatic compounds exhibit a wide range of fast, adaptive responses (9). The main adaptative reactions are alterations in the composition in the membrane, particularly in fatty acid composition, phospholipid headgroups and in the protein content (10-12). In response to toxic compounds, microorganisms modify their lipid composition by *de novo* synthesis of fatty acids, isomerization of *cis* to *trans* unsaturated fatty acids and form cyclopropane and branched fatty acids (13-16). These tolerance responses effectively reduce the accumulation of toxic compounds in the membrane. They are called "homeoviscous adaptation" and have been described for several gram-negative bacteria, especially from the genus *Pseudomonas* (5,9,16-18).

The aim of this study was to determine the changes in the cellular fatty acids of *Pseudomonas vesicularis* and *Pseudomonas stutzeri* during growth in the presence of catechol and phenol.

2. Materials and methods

2.1. Microorganisms and culture conditions

P. vesicularis was isolated from mixed populations of activated sludge microorganisms adapted to the degradation of phenol. It was identified on the basis of the cellular fatty acid profile by gas chromatography using Hewlett-Packard Microbial Identification System (MIS) (19). *P. stutzeri* was obtained from Polish Collection of Microorganisms of Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wrocław. The culture were grown in a modified minimal medium containing: 3.78 g of $Na_2HPO_4 \times 12H_2O$; 0.5 g of KH_2PO_4; 5.0 g of NH_4Cl; 0.2 g of $MgSO_4 \times 7H_2O$ and 0.1 g of yeast extract in 1000 ml of deionized water (20). Single aromatic substrate, catechol or phenol, was added at a concentration of 4 mM. The final pH of the medium was 7.2-7.3. The cells were also grown on Kojima et al. (20) medium supplemented with 5.5 mM (1 g l^{-1}) glucose for comparison of fatty acid profiles in the bacteria grown in the presence and absence of aromatic substrates. The liquid cultures were grown in 500 ml flask on rotary shaker at 30°C. The sam-

ples of the cultures were withdrawn periodically for the analysis of cell density (OD) and substrate concentrations.

2.2. Determination of catechol, phenol and cell density

The concentration of catechol was measured every two hours using spectrophotometry method proposed by Evans (21). Similarly, every two hours, phenol concentration was determined by Lurie and Rybnikova method (22). The cell density was monitored spectrophotometrically by measuring the absorbance at the wavelength of 600 nm.

2.3. MIDI-FAME analysis

For extraction of cellular fatty acids, cells at mid-exponential phase growing with 4 mM of aromatic substrate or 5.5 mM of glucose were harvested by centrifugation (8000 g) at 4°C for 30 min. The cell pellets were washed twice with 0.85% NaCl to remove residue culture medium. To decrease the humidity of bacterial cell, pellets were left for 2h at room temperature. Next, 55 mg of bacterial biomass were transferred in duplicate to reaction tubes (Pyrex) and 1 ml of first reagent (150 g NaOH in 1 litre of 50% methanol) for saponification was added. The samples were incubated for 30 min at 100°C in water bath. To methylate liberated fatty acids, 2 ml of reagent II (6N HCl in aqueous methanol) was added to each tube and incubated again for 10 min at 80°C in water bath. The fatty acid methyl esters (FAMEs) were extracted from the aqueous phase by addition of 1.15 ml of reagent III (hexane/methyl tert-butyl ether, 1:1, v/v) to each tube. Then, the samples were rotated end-over-end for 10 min. After removing aqueous (lower) phase, 3 ml of 1.2% NaOH in H_2O were added and the tubes were rotated again for 5 min (23). Finally, the organic (upper) phase containing FAME was transferred to a gas chromatography vial (Hewlett-Packard). The fatty acids were analysed by gas chromatography (Hewlett-Packard 6890, USA) using capillary column Ultra 2-HP (cross- linked 5% phenyl-methyl silicone 25 m, 0.22 mm ID, thickness 0.33 μm) and hydrogen as a carrier gas. FAME were detected by a flame ionisation detector (FID) and identified by MIS (Microbial Identification System) software, using the aerobe method and TSBA library version 3.9 (MIDI, USA).

The fatty acids were designed by the number of carbon atoms followed by a colon, the number of double bonds, and then by a position of the first double bond from the methyl (ω) end of the molecule. The prefixes *c* or *t* indicate *cis* or *trans* configuration of the double bond, *cy* – cyclopropane fatty acids, Me – the position of the methyl group from the acid end, and -OH indicates the position of the hydroxyl group from the acid end of the molecule. The branched fatty acids are designed as *iso* and *anteiso* if the methyl branch is one or two carbon from the ω end of acyl chain.

3. Results and discussion

Each strain of the bacteria used was individually tested for its ability to degrade catechol and phenol in a single substrate system. It was found that both strains are able to degrade 4 mM (376 mg l^{-1}) of phenol and 4 mM (440 mg l^{-1}) of catechol used as a sole carbon and energy source. Both strains utilized 4 mM of catechol fast within 6-8 h of incubation, but they degraded phenol slowly. *P. vesicularis* metabolized 4 mM of phenol within 15 h, and *P. stutzeri* within a 24 h cycle of observation. The cell growth and substrate removal profiles are illustrated in Figures 1-4.

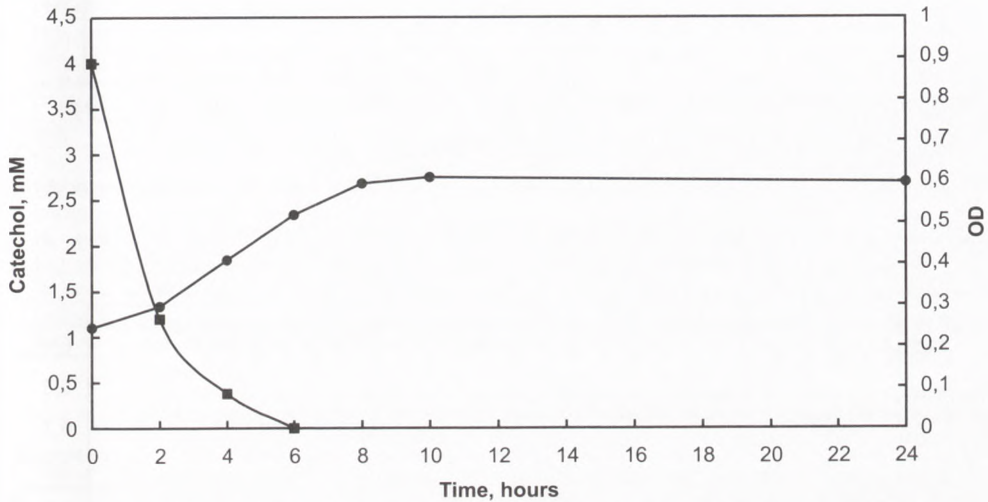

Fig. 1. Biodegradation 4 mM of catechol (–■–) and growth rate (–●–) of *Pseudomonas vesicularis*.

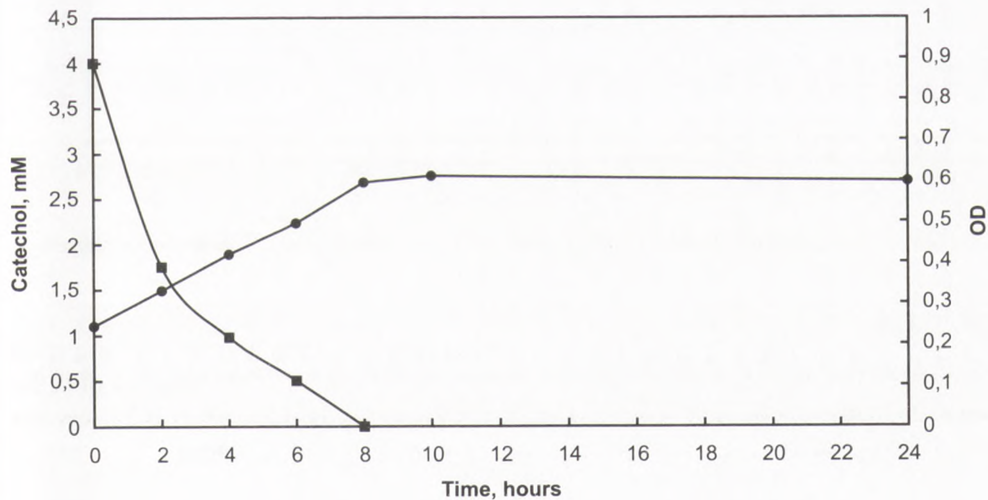

Fig. 2. Biodegradation 4 mM of catechol (–■–) and growth rate (–●–) of *Pseudomonas stutzeri*.

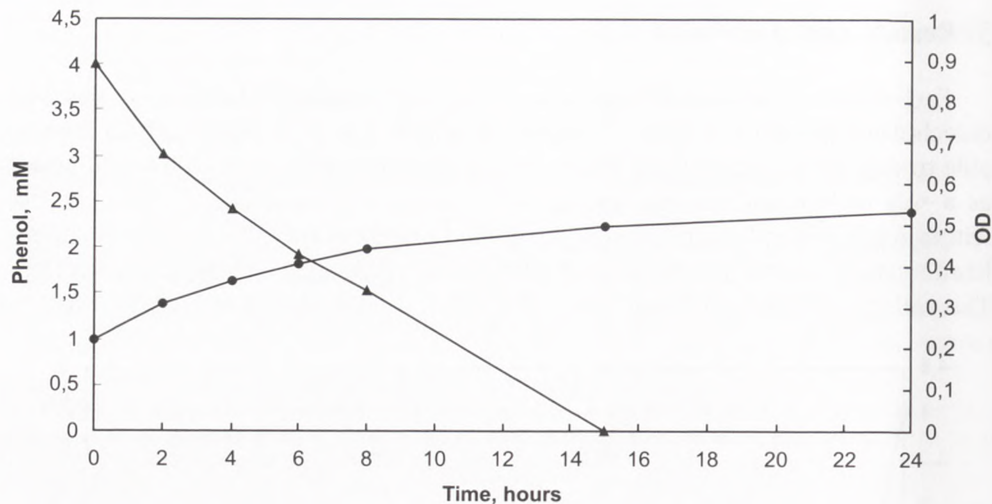

Fig. 3. Biodegradation 4 mM of phenol (–▲–) and growth rate (–●–) of *Pseudomonas vesicularis.*

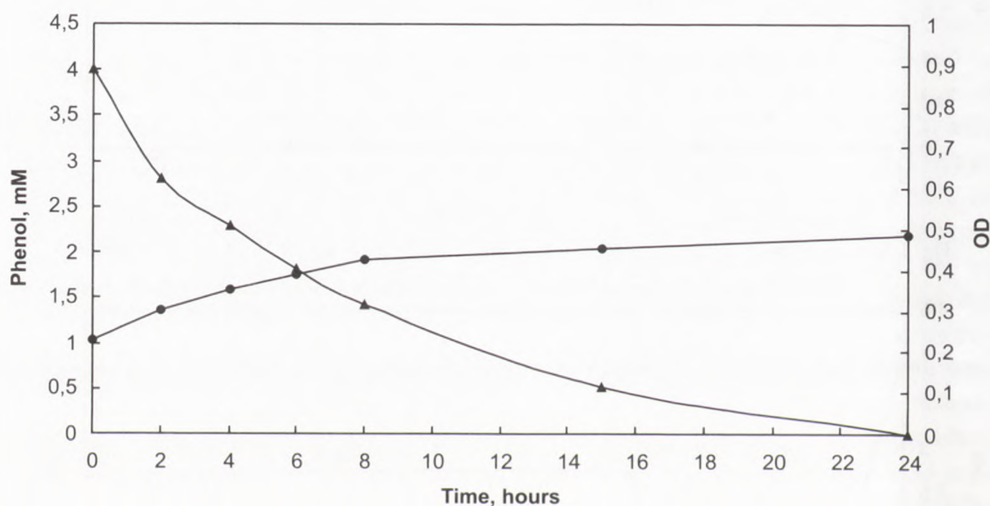

Fig. 4. Biodegradation 4 mM of phenol (–▲–) and growth rate (–●–) of *Pseudomonas stutzeri.*

The fatty acid profiles of both catechol-grown and phenol-grown strains were compared to each other and to glucose-grown cells to identify the changes of cellular fatty acid composition caused by aromatic compounds. Tables 1 and 2 show the total fatty acid compositional changes in *P. vesicularis* and *P. stutzeri*.

Table 1

Percentages of total fatty acids from *P. vesicularis* grown in the presence of glucose or catechol or phenol

Fatty acid	(%) of total fatty acids		
	glucose	catechol	phenol
Saturated			
10:0	0.42	0.25	0.66
110:0 3OH	2.09	0.00	0.00
12:0	13.18	8.79	7.58
12:0 2OH	0.37	4.90	4.84
14:0	1.30	1.86	1.53
15:0	0.27	0.00	0.00
15:0 *iso*	0.00	0.75	0.64
15:0 *anteiso*	0.00	0.00	0.41
16:0	29.93	44.22	42.72
16:0 *iso*	0.00	0.00	0.66
17:0 *cy*	5.59	17.85	13.26
18:0	0.98	1.35	1.19
19:0	0.37	2.85	1.70
19:0 *cy* ω8*c*	11.24	1.94	4.63
Unsaturated			
16:1 ω7*c*	8.98	5.49	6.51
18:1 ω8*c*	0.53	2.33	2.31
18:1 ω7*c*/ω9*t*/ω12*t*	24.75	7.00	10.87
Unknown	0.00	0.42	0.49
Total	100	100	100

Table 2

Percentages of total fatty acids from *P. stutzeri* grown in the presence of glucose or catechol or phenol

Fatty acid	(%) of tatal fatty acids		
	glucose	catechol	phenol
Saturated			
10:0	0.37	0.00	0.39
12:0	3.94	7.69	5.54
12:0 2OH	5.70	4.54	0.00
14:0	1.46	1.92	1.66
15:0	0.42	0.00	0.00
15:0 *iso*	0.00	0.83	0.88
15:0 *anteiso*	0.00	0.50	0.54
16:0	45.86	44.85	40.16
17:0	0.40	0.00	0.00
17:0 *cy*	10.99	15.05	27.71
18:0	0.97	1.65	1.47
19:0	0.98	1.88	0.00
19:0 *cy* ω8*c*	0.56	3.40	10.87
Unsaturated			
18:1 ω9*c*	1.56	2.57	0.72
20:1 ω9*t*	0.00	0.17	0.00
18:1 ω7*c*/ω9*t*/ω12*t*	6.90	5.86	6.08
Other			
16:1 ω7*c*/15:0 *iso* 2OH	19.45	9.09	3.98
Unknown	0.44	0.00	0.00
Total	100	100	100

Catechol and phenol in the concentration of 4 mM in comparison with control sample (bacteria grown only on 5.5 mM glucose) influenced the cellular fatty acid composition in both strains. The total level of saturated fatty acids in *P. vesicularis* increased from 65.74% in control to 79.82% and 84.76% in the presence of phenol and catechol, respectively. These changes were expressed as the saturated/unsaturated ratio and showed an increasing tendency from 1.92 in control to 4.05 and 5.72 in the samples with phenol and catechol, respectively. The increase in the saturated/unsaturated ratio was also observed in the case of *P. stutzeri*. However, in phenol treatment, this ratio was twice as high (8.28) as in *P. vesicularis* Gutierrez *et al.* (24) obtained similar results studying the changes in whole cell-derived fatty acids induced by benzene in *Rhodococcus* sp. 33. The correlation between an increase in a degree of saturation of membrane fatty acids and an increased tolerance towards the toxic compounds in phenol-degrading strain *Pseudomonas putida* P8 was observed by Heipieper et al. (17). Our results confirm the thesis that fatty acid acyl chains of the phosphilipids in the cell membrane come closer together and increase the rigidity of the membrane contributing to a role in tolerating the catechol and phenol (4). The increasing degree of membrane lipid saturation appeared to be one of the major adaptive mechanisms of bacteria cells to the presence of many aromatic compounds (15,24,25).

In *P. vesicularis,* six fatty acids dominated the fatty acid profile in glucose treatment, four saturated and two unsaturated. These were 12:0, 16:0, 17:0 *cy*, 19:0 *cy* ω8*c*, 16:1ω7*c* and 18:1 ω7*c*/ω9*t*/ω12*t*. The same fatty acids dominated in this strain after catechol and phenol treatment except of 19:0 *cy* ω8*c*. The highest increase in abundance of single fatty acid was observed in the case of 16:0 and 17:0 *cy*. The distribution of 16:0 increased from 29.93 (control) to 42.72% (phenol) and 44.22% (catechol), whereas the abundance of 17:0 *cy* increased from 5.59 (control) to 13.26% (phenol) and 17.85% (catechol). On the other hand, the abundance of 16:1ω7*c* and 18:1 ω7*c*/ω9*t*/ω12*t* markedly decreased (tab. 1).

The whole cell fatty acid profile of *P. stutzeri* was slightly different while compared with *P. vesicularis* in the presence of glucose in culture medium. The abundance of 16:0 fatty acid was 30% higher than in *P. vesicularis* whereas the distribution of this fatty acid was at the similar level after catechol and phenol treatment. Apart from 16:0, the following fatty acids dominated in the control sample 12:0 2OH, 17:0 *cy*, 16:1ω7*c* and 18:1 ω7*c*/ω9*t*/ω12*t*. Catechol and phenol caused the markedly decrease in the abundance of two cyclopropane fatty acids (17:0 *cy* and 19:0 *cy* ω8*c*). Simultaneously, the decrease in the abundance of dominated unsaturated fatty acids was observed (tab. 2).

It was found that the abundance of branched and cyclic fatty acid in tested strains was higher in the presence of catechol and phenol than in bacteria grown on glucose. Similarly, Kim et al. (26) observed the increase of percentage of 17:0 *cy* isolated from *Ralstonia eutropha* H850 grown on biphenyl. Russel and Fukunaga (27) observed that these fatty acids increase the fluidity of the cytoplasmatic membrane as

they retain the ability to slide each other, as they cannot form crystalline structure. Cyclopropane fatty acids confer fluidity upon the cell membrane and assist in the tolerance towards aromatic compounds and other environmental factors (10,28). The properties of cyclopropane fatty acids imply that topologically novel protein--lipid interactions occur in the biosynthesis of these fatty acids (29).

It is interestingly that catechol and phenol in both strains induced the formation of new types of fatty acids which were absent in a control sample. These were mainly branched fatty acids such as 15:0 *iso*, 15:0 *anteiso*, 16:0 *iso* and unsaturated fatty acid 20:1 ω9*t* (in *P. stutzeri*).

Many processes in modern environmental biotechnology, particularly biotransformations and bioremediation, are inhibited by the effects of aromatic solvents on bacterial cells. Microorganisms which are able to offer a successful physiological/biochemical adaptation are better suited to colonize aromatic compounds' polluted sites. The understanding of all adaptative mechanisms occurring in bacterial cells as a response to this compounds allow to use solvent-tolerant microorganisms in cleaning-up contaminated sites (30,31).

Literature

1. Bouwer E. J., Zehnder A. J. B., (1993), Trends Biotechnol., 11, 360-367.
2. Willams P. A, Sayer J. R., (1994), Biodegradation, 5, 195-217.
3. Sikkema J., Weber F. J., Heipieper H. J., de Bont J. A. M., (1994), Biocatalysis, 10, 113-122.
4. Sikkema J., de Bont J. A. M., Poolman B., (1995), Microbiol. Rev., 59, 201-222.
5. Diefenbach R., Heipieper H. J., Keweloh H., (1992), Appl. Microbiol. Biotechnol., 38, 382-387.
6. Yap L. F., Lee Y. K., Poh C. L., (1999), Appl. Microbiol. Biotechnol., 51, 833-840.
7. Beney L., Gervais P., (2001), Appl. Microbiol. Biotechnol., 57, 34-42.
8. Tsitko I. V., Zaitsev G. M., Lobanok A. G., Salkinoja-Salonen M. S., (1999), Appl. Environ. Microbiol., 65, 853-855.
9. Ramos-Gonzales M. I., Godoy P., Alaminos M., Ben-Bassat A., Ramos J. L., (2001), Appl. Environ. Microbiol., 67, 4338-4341.
10. Sajbidor J., (1997), Crit. Rev. Biotechnol., 17, 87-103.
11. Fang J., Barcelona M. J., Alvarez P. J. J., (2000), Appl. Microbiol. Biotechnol., 54, 382-389.
12. Denich T. J., Beaudette L. A., Lee H., Trevors J. T., (2003), J. Microbiol. Meth., 52, 149-182.
13. Weber F. J., Isken S., de Bont J. A. M., (1994), Microbiology, 140, 2013-2017.
14. Heipieper H. J., Keweloh H., Rehm H. J., (1991), Appl. Environ. Microbiol., 57, 1213-1217.
15. Heipieper H. J., Weber F. J., Sikkema J., Keweloh H., de Bont J. A. M., (1994), Trends Biotechnol., 12, 409-415.
16. Heipieper H. J., Loffeld B., Keweloh H., de Bont J. A. M., (1995), Chemosphere, 30, 1041-1051.
17. Heipieper H. J., Diefenbach R., Keweloh H., (1992), Appl. Environ. Microbiol., 58, 1847-1852.
18. Kobayashi H., Takami H., Hirayama H., Kobata K., Usami R., Horikoshi K., (1999), J. Bacteriol., 181, 4493-4498.
19. Mrozik A., Łabużek S., (2002), Acta Microbiol. Polon., 51, 367-378.
20. Kojima Y., Itada N., Hayaishi O., (1961), J. Biol. Chem., 236, 2223-2231.
21. Evans W. C., (1974), J. Biochem., 41, 373-380.
22. Lurie J. I., Rybnikova, (1968), *Goschimizdat*, Moskwa.
23. Sasser M., (1990), *MIDI Technical Note* 101. Microbial ID, Inc., Newark, DE, USA.
24. Guttierez J. A., Nichols P., Couperwhite I., (1999), FEMS Microbiol. Lett., 176, 213-218.

25. Keweloh H., Diefenbach R., Rehm H. J., (1991), Arch. Microbiol., 157, 49-53.
26. Kim I. S., Lee H., Trevors J. T., (2001), FEMS Microbiol. Lett., 200, 17-24.
27. Russel N. J., Fukunaga N., (1990), FEMS Microbiol. Rev., 75, 171-182.
28. Grogan D. W., Cronan J. E., (1986), J. Bacteriol., 166, 872-877.
29. Grogan D. W., Cronan J. E., (1997), Microbiol. Mol. Biol. Rev., 61, 429-441.
30. Daugulis A. J., (2001), Trends Biotechnol., 19, 457-462.
31. Whiteley A. S., Wiles S., Lilley A. K., Philp J., Bailey M. J., (2001), J. Microbiol. Meth., 44, 79-88.