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ORIGINAL RESEARCH PAPER

KINETIC STUDIES ON BIODEGRADATION OF LIPIDS FROM OLIVE OIL MILL WASTEWATERS WITH FREE AND IMMOBILIZED *Bacillus sp.* CELLS

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Abstract: The studies on the biodegradation of lipids from olive oil mill wastewater with free and immobilized *Bacillus sp.* cells indicated that the maximum specific rate of the process is reached at pH = 8. The use of immobilized cells allows to increasing the number of biodegradation process cycles, but reduces the rate of the process. In this case, the process rate depends on the biocatalysts size and cells concentration inside them. Thus, at bacterial cells concentration of 9 g d.w./100 mL biocatalyst, the apparent specific rate varied from 4.65 to 1.46×10^{-2} h^{-1} by increasing the biocatalyst particles diameter from 3 to 4.2 mm.
The cumulated influences of the particles size and cells concentration have been included in a mathematical model for the apparent specific rate of lipids biodegradation. The model offers a good concordance with the experimental data, the average deviation being of ± 7.38%.

Keywords: *Bacillus sp., immobilized cells, kinetics, lipids, wastewater*

INTRODUCTION

Lipids are organic biomolecules produced by microbial, vegetal and animal cells [1]. These compounds are insoluble in water, but soluble in non-polar solvents. Among the complex lipids, a particular class includes the triglycerides, namely fats and oils.
According to the Global Industry Analysts Report, the worldwide production of vegetable oils was 130 millions tonnes in 2010, being estimated to 144 millions for 2011 and 169 millions tonnes for 2015 [2]. This evolution is in relation with the population and, implicitly, consumption increase, as well as with the diversification of the vegetable oils utilization from food to chemical synthesis.
The oil producers are important sources of wastewaters, the characteristics of pollutants depending on the vegetable raw materials and used technologies. The olive oil represents about 3% from the worldwide production of vegetable oils, most of this quantity being consumed in Europe. The wastewaters resulted from olive oil mills are important pollutants, due to their high organic content (lipids 0.2-1%, carboxylic acids 0.5-1.5%, sugars 1-8%, polyphenols and pectins 1-1.5%, tannins, polyalcohols, etc.) [3, 4]. Most of the problems associated with the pollution generated by olive oil mills wastewaters are direct related to the polyphenols, because these coumpounds affect drastically the activity of the environment microorganisms [5, 6].
For these wastewaters treatment several physical and chemical methods have been proposed and applied (decantation, concentration by evaporation, filtration and ultrafiltration, reverse osmosis, flotation, adsorption, oxidation and photo-oxidation, etc.) [7-15]. Recently, some biological methods have been tested [4, 5, 16-24]. The biological treatment is based on aerobic or non-aerobic processes using free or immobilized bacteria (*Burkholderia cepacia, Phormidium sp.*, *Oscillatoria sp., Chroococcus sp., Enterobacter aerogenes, Mucor racenosus*), yeasts (*Candida oleophila, Candida tropicalis*, *Yarrowia lipolytica*), fungus (*Aspergillus niger, Phanerochaete crysosporium, Lentinus edodes, Pleurotus ostreatus, Funalia trogii, Geotrichum candidum, Mucor rouxii, Absidia coerulea, Penicillium restrictum, Penicillium verucosum*) and *lipases*. In these systems, the triglycerides are bioconverted to long chain fatty acids, which are finally oxidated to acetate or propionate [25, 26].
The use of free or immobilized microorganisms or enzymes is rather expensive. In the same time, the use of active sludge induces the appearance of flotation, its intensity becoming important at high oils content [27-29]. This phenomenon is due to the adsorption of lipids in sludge and reduces the efficiency of biological treatment.
For these reasons, our studies are focused on the analysis of the performances of the biological treatments of olive oil mill wastewaters using an anaerobic system containing free and immobilized bacteria. In this paper, the results of the kinetic studies on the lipids bioconversion and the influence of the main factors on process rate are presented.

EXPERIMENTAL

The experiments have been carried out in 100 mL (80 mL working volume) small anaerobic bioreactors, each containing 60 mL olive oil - water emulsion (the oil concentration was 10 mL/L emulsion). The bioreactors have been placed on a rotary shaker at 150 rpm and incubated at 40°C.

In the experiments, free and immobilized mixture of *Bacillus sp.* has been used (*Bacillus subtilis, Bacillus megaterium, Bacillus licheniformis, Bacillus ortoliquefaciens* in equal ratios). The concentration of free bacteria was of 1 g d.w./100 mL medium.
The immobilization has been carried out by bacterial cells inclusion into the alginate matrix, respecting the method given in literature [30]. In this purpose, 3.1 - 14.4 g d.w. bacterial mixture was mixed with 100 mL of 5% aqueous solution of sodium alginate. The biocatalysts particles have been obtained by dripping this suspension at constant pressure through a capillary into a solution of 0.2% $CaCl_2$. Capillaries with three different diameters have been used and the obtained particles of immobilized cells had the following diameters: 3.0, 3.6 and 4.2 mm. In all cases, the volumetric fraction of the immobilized cells into the medium was 0.10.
The fermentation end has been considered when either the olive oil was completely consumed or its concentration remained constant for 12 h. Any mechanical damage of the biocatalyst due to the shear forces was recorded during the experiments.
The process evolution has been analyzed by means of the variation of total lipids, using the spectrophotometric method with triolein [31].

RESULTS AND DISCUSSION

Olive oil biodegradation by free cells of *Bacillus sp.*

The variation of total lipids concentration from wastewater during the biodegradation process with free *Bacillus sp.* cells is plotted in Figure 1. The pH has not been controlled, its value decreasing continuously from 8.5 to 5.3, due to the fatty acids accumulation in the medium. As it can be seen from Figure 1, without pH adjustment at a given level, the process duration is 50 - 52 h, the lipids amount decreasing for about 4.9 times from the initial value.
For emphasizing the influence of pH, the lipids biodegradation process has been carried out in a similar manner, by maintaining this parameter at the following prescribed values: 5, 6, 7, 8. In this purpose, the emulsion has been prepared by mixing olive oil with citrate buffer solution with the desired pH-value. In this case, the variations of total lipids concentration during the process for each pH-value indicate that the optimum pH-domain is neutral to low alkaline one, the most important reduction of total lipids concentration being recorded for pH = 7 (for 19.8 times) and pH = 8 (for 25.7 times), respectively (Figure 2). Moreover, at these pH-values the duration of the biodegradation process was minimum (48 h at pH = 7 and 44 h at pH = 8).
The process efficiency is significantly affected in the acidic domain. Therefore, at pH values of 5 and 6, the initial concentration of lipids has been reduced only for 2 and 2.9 times, respectively, the process duration increasing to 64 and 60 h.
These results are in concordance with those previously reported in literature, which indicated that the lipids consumption rate is maximal in the low alkaline domain of pH [22, 23].

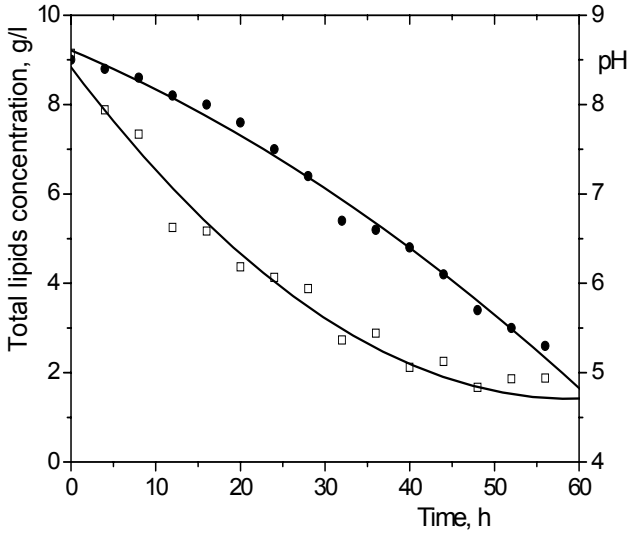

Figure 1. *Variation of total lipids concentration and pH during the lipids biodegradation with free cells of* ***Bacillus sp.*** *(□ - total lipids concentration, ● - pH)*

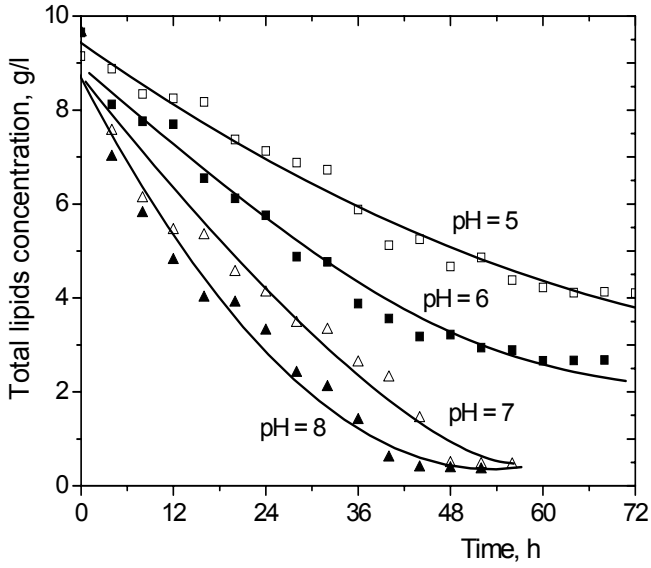

Figure 2. *Variation of total lipids concentration during the biodegradation process with free cells of* ***Bacillus sp.*** *at constant pH-value*

For kinetic analysis of the lipids biodegradation, the model proposed by Pavlostathis and Giraldo-Gomez has been taken into consideration [32]:

$$-\frac{dC_{TL}}{dt} = k_d \cdot C_{TL} \qquad (1)$$

The solution of equation (1) represents the expression of a straight line:

$$\ln \frac{C_{TL0}}{C_{TL}} = k_d \cdot t \qquad (2)$$

The corresponding straight lines for the considered pH-values are plotted in Figure 3. Consequently, from the slopes of the straight lines it is possible to determine the values of the specific rate of biodegradation, k_d, at different pH-values (Table 1).

Table 1. *Values of specific rate of lipids biodegradation process with free cells of Bacillus sp.*

pH value	variable	5	6	7	8
$\boldsymbol{k_d \times 10^2}$, h^{-1}	3.81	1.58	3.04	6.27	7.03

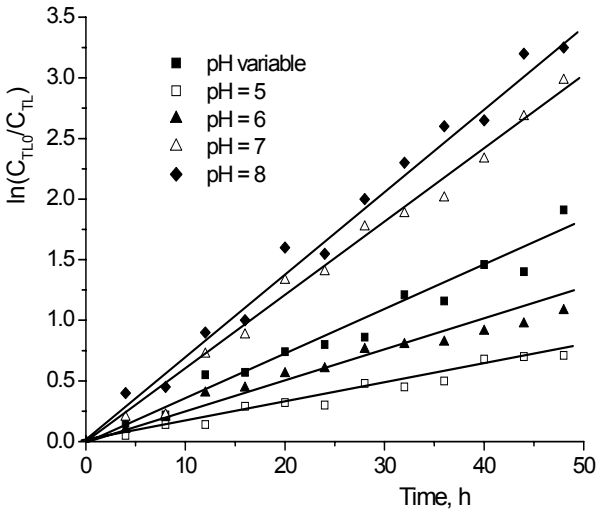

Figure 3. *Graphical representation of straight lines given by equation (2)*

As it was discussed above, the increase of pH from 5 to 8 exhibits a positive effect on the biochemical process rate, the value of specific biodegradation rate being accelerated for 4.5 times (Figure 4).

Olive oil biodegradation by immobilized cells of *Bacillus sp.*

The results presented in Figures 5 - 7 suggest that the immobilized bacterial cells can be used for many biodegradation cycles. But, although the cells concentration in the medium was similar to that for system containing free bacterial cells, the duration of the lipids biodegradation process was higher, especially due to the supplementary resistance induced by the internal diffusion of lipids inside the biocatalyst particle.
The biocatalyst activity, respectively the possible number of biodegradation cycles, depends mainly on the size of immobilized cells particles. Thus, for the smallest particles (diameter of 3 mm), six biodegradation cycles can be carried out, but the process performance is significantly reduced over the first two cycles (Figure 5).

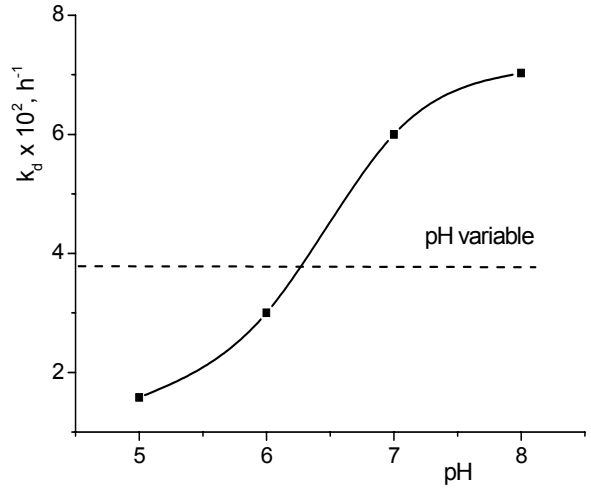

Figure 4. *Influence of pH on the specific rate of lipids biodegradation by free cells of* ***Bacillus sp.***

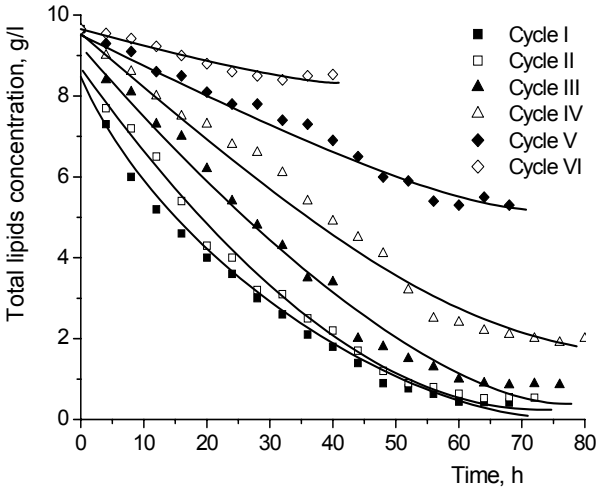

Figure 5. *Variation of total lipids concentration during the biodegradation process with immobilized cells of* ***Bacillus sp.*** *for biocatalyst particles diameter of 3 mm (pH = 8)*

The initial amount of lipids has been reduced for 22.3 times during the first biodegradation cycle, and for 18.7 times during the second one, the both cycles duration being of 60 - 64 h. Excepting the higher duration, these results are similar to those recorded for free *Bacillus sp.* cells.

Satisfactory results have been recorded also for the third cycle with the smallest biocatalyst particles, the reduction ratio of the lipids concentration being of 11.6. But, the process duration increased to 72 h. Starting with the cycle IV, both the rate of the lipids biodegradation and the biocatalysts activity are significantly diminished. Thus, from cycle IV to VI, the initial amount of total lipids was reduced for 4.1 to 1.3 times,

simultaneously with the decreasing of the process duration from 60 to 28 h. The shorter duration does not indicate an increase of the biodegradation rate, because it represents the time needed to reaching a constant level of lipids concentration in medium. This constant level of lipids concentration becomes closer to the initial one by increasing the number of cycles. The diminution of the biocatalyst activity can be attributed to the clogging of the alginate particles due to the lipids accumulation inside them, which hinders the substrate and products diffusion from and, respectively, to the external medium.

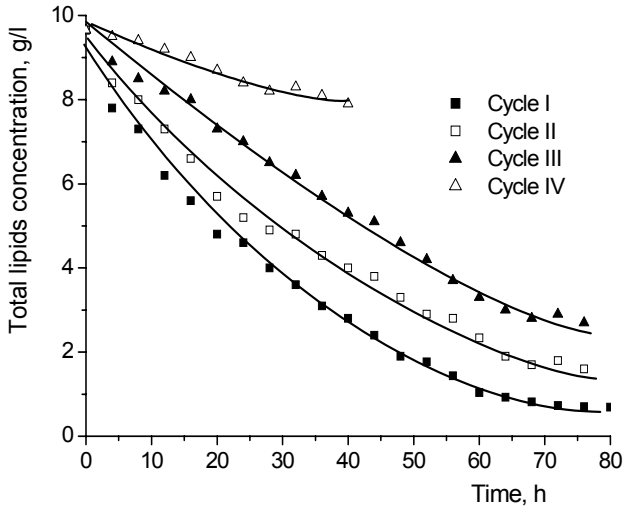

Figure 6. *Variation of total lipids concentration during the biodegradation process with immobilized cells of* ***Bacillus sp.*** *for biocatalyst particles diameter of 3.6 mm (pH = 8)*

The above conclusion is supported by the effect induced by increasing the biocatalysts particles size. Contrary to the alcoholic or succinic fermentation with immobilized yeast and bacterial cells [33, 34], the increase of the particles diameter leads to the significant decrease of the lipids biodegradation rate. According to Figures 6 and 7, the number of cycles corresponding to the lipids consumption decreases to 4 for biocatalyst particles with 3.6 mm diameter, and to 3 for the particles with 4.2 mm diameter. Moreover, it can be observed the evident differentiation between the curves plotted for each cycle (from the first to the last considered cycle, the initial concentration of total lipids has been reduced for 14 to 1.1 times for the intermediary particles, respectively for 2.7 to 1.1 times for the bigger ones).

These results can be suggestively underlined by plotting the dependence between the average rate of lipids biodegradation and the size of immobilized cells particles. The average rate of process is defined by the following relationship:

$$\bar{r}_d = \frac{C_{TL0} - C_{TL}}{t}, \text{ g·L}^{-1}\text{·h}^{-1} \quad (3)$$

Thus, Figure 8 indicates that the average rate decreases by increasing the particles diameter. In this case, because the biodegradation process occurs in the diffusional

regime, the real value of the specific rate cannot be established. But, the kinetics of the process can be quantitatively described by means of the apparent specific rate of the lipids biodegradation, which depends on the biocatalyst particles diameter and cells concentration inside the particles. For calculating the value of the apparent specific rate, the modified equation (1) could be used:

$$-\frac{dC_{TL}}{dt} = k_{dC}{}' \cdot C_{TL} \tag{4}$$

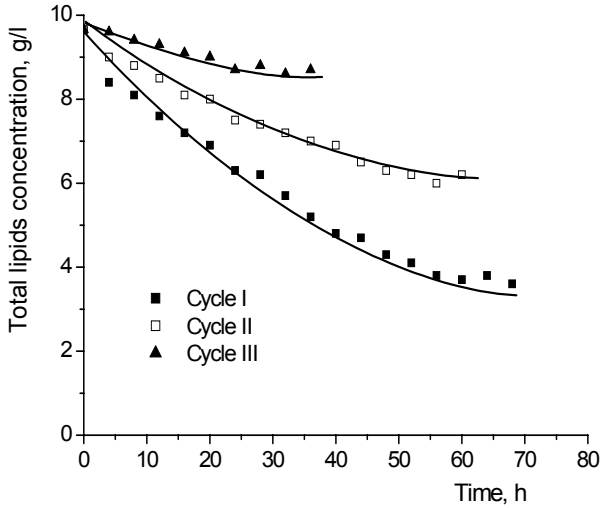

Figure 7. *Variation of total lipids concentration during the biodegradation process with immobilized cells of* ***Bacillus sp.*** *for biocatalyst particles diameter of 4.2 mm (pH = 8)*

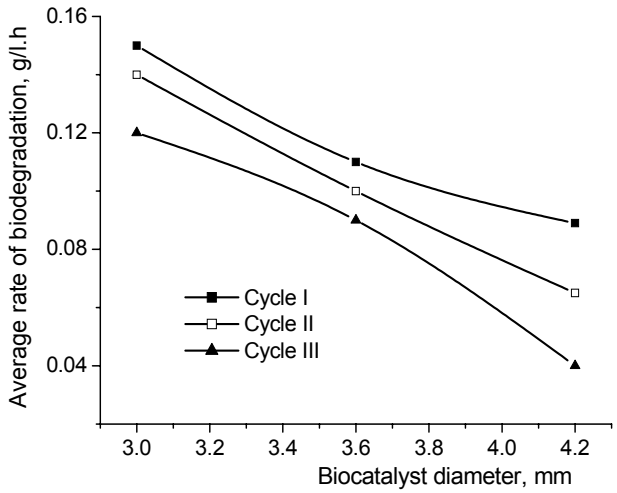

Figure 8. *Influence of biocatalyst particles size on average rate of lipids biodegradation with immobilized cells of* ***Bacillus sp.***

Using the previous algorithm and considering only the first cycle of biodegradation for each particles size, the straight lines given in Figure 9 have been obtained. The values of the apparent specific rate have been determined from these straight lines slopes and are indicated in Table 2. These values have not taken into consideration the influence of the cells concentration.

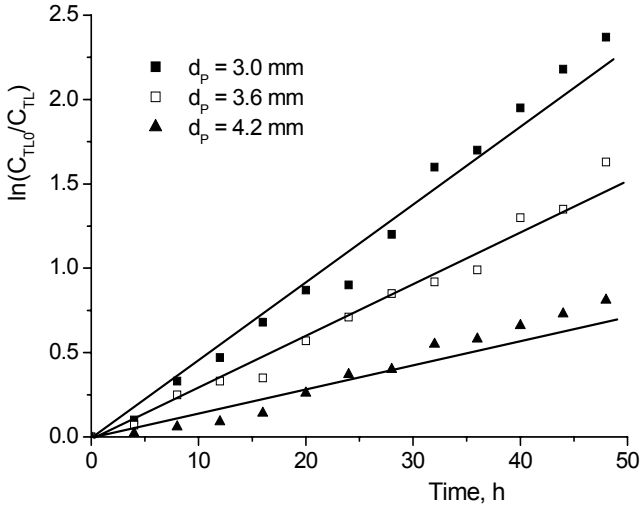

Figure 9. *Graphical representation of straight lines given by equation (2) considering the immobilized* ***Bacillus sp.*** *cells*

Table 2. *Values of apparent specific rate of biodegradation process, k_d'*

d_P, mm	3.0	3.6	4.2
$k_d^{'} \times 10^2$, h^{-1}	4.65	3.12	1.46

The magnitude of the effect of biocatalyst particles size on the biodegradation rate can be described by the variation of the ratio between the apparent specific rate and specific rate obtained for free cells with the particles diameter (Figure 10). The graphical dependence suggested the following polynomial correlation between the two parameters:

$$k_d^{'} = k_d \cdot \left(1 + 5{,}93 \cdot 10^{-2} \cdot d_P - 5{,}54 \cdot 10^{-2} \cdot d_P^2\right) \quad (5)$$

For quantifying the influence of cells concentration on apparent specific rate, the straight lines corresponding to the smallest biocatalysts have been plotted at different *Bacillus sp.* concentration inside the alginate particles (Figure 11).

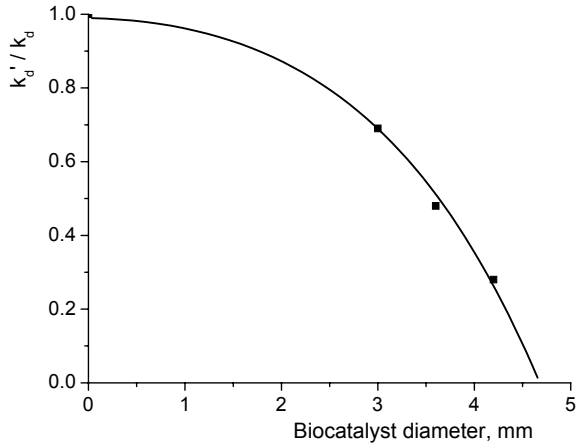

Figure 10. *Influence of biocatalyst particles size on ratio k_d'/k_d*

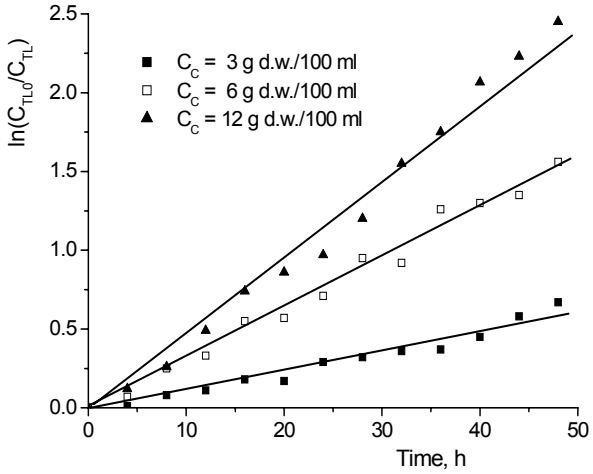

Figure 11. *Graphical representation of straight lines given by equation (2) considering the immobilized* ***Bacillus sp.*** *cells and various cells concentration (biocatalyst diameter of 3 mm)*

The values of apparent specific rate of lipids biodegradation indicate its significant amplification for cells concentration up to 9 g d.w./100 mL biocatalyst (Table 3).

Table 3. *Values of apparent specific rate of biodegradation process, k_{dC}'*

C_C, g d.w./100 mL	3.0	6.0	9.0	12.0
$k_{dC}' \times 10^2$, h^{-1}	1.41	3.30	4.65	5.02

By means of these results, the correlation between the ratio of the apparent and real specific rates and the concentration of *Bacillus sp.* cells was plotted in Figure 12 and suggests the following linear dependence:

$$k_{dC}' = 0{,}109 \cdot C_C \cdot k_d' \quad (6)$$

In these circumstances, the cumulated influences of the biocatalyst particles diameter and cells concentration can be included in the particular kinetic model for the apparent specific rate of lipids biodegradation by immobilized *Bacillus sp.* cells:

$$k_{dC}' = \left(0{,}109 + 6{,}46 \cdot 10^{-3} \cdot d_P - 6{,}04 \cdot 10^{-3} \cdot d_P^2\right) \cdot C_C \cdot k_d \quad (7)$$

As it can be seen from Table 4, equation (7) offers a good concordance with the experimental data and is valid also for the higher sizes of biocatalyst particles.
These experiments have been carried out also at rotation speeds of 180 and 200 rpm, but any important differences have been observed, this indicating the limiting role of the internal diffusion in the particles. Higher rotation speed could lead to the mechanical disruption of the biocatalysts.

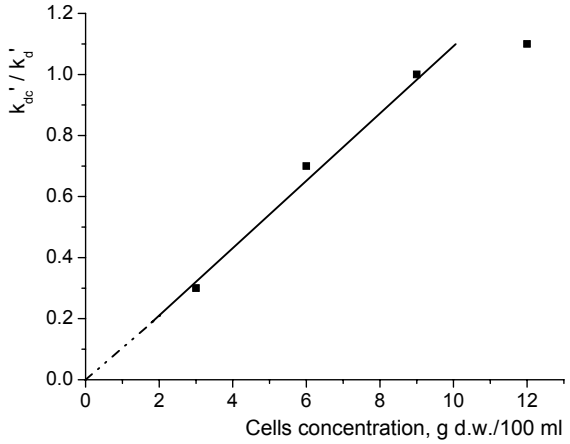

Figure 12. *Influence of bacillus sp. cells concentration on ratio k_{dC}'/k_d*

Table 4. *Comparison between the experimental and calculated values of apparent specific rate of lipids biodegradation, k_{dC}'*

d_P, mm	C_C, g d.w./100 mL	$k_{dC}'_{exp.} \times 10^2$, h^{-1}	$k_{dC}'_{calc.} \times 10^2$, h^{-1}	**Average deviation**, %
3.0	3	1.41	1.12	± 7.38
3.0	6	3.30	3.24	
3.0	9	4.65	4.86	
3.6	9	3.12	3.41	
4.2	9	1.46	1.47	

CONCLUSIONS

The studies on the biodegradation of lipids from olive oil mill wastewater with free and immobilized *Bacillus sp.* cells indicated that the optimum pH-value is 8, the process duration varying from 44 h to over 60 h.

The use of immobilized cells allows to increasing the number of biodegradation process cycles, depending on the biocatalyst particles size. But, the rate of the process is significantly decreased by cells immobilization, due to the supplementary step of lipids internal diffusion and to the clogging of the alginate particles.
The influences of the particles size and cells concentration on process rate have been included in a mathematical model for the apparent specific rate of lipids biodegradation. The model offers a good concordance with the experimental data, the average deviation being of ± 7.38%.

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NOTATIONS

C_C - cells concentration inside the alginate particle, g d.w./100 mL
C_{TL} - total lipids concentration in wastewater, g/L
C_{TL0} - initial total lipids concentration in wastewater, g/L
d_P - diameter of biocatalyst particle, mm
k_d - specific rate of lipids biodegradation process, h^{-1}
k_d' - apparent specific rate of lipids biodegradation process considering only the biocatalyst particles size influence, h^{-1}
k_{dC}' - apparent specific rate of lipids biodegradation process considering both the biocatalyst particles size and cells concentration influences, h^{-1}
t - time, h

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