Polish Journal of Environmental Studies Vol. 13, No. 6 (2004), 729-735

Original Research

Catabolic Activity of *Bacillus* and *Desulfotomaculum* Bacteria in Media Containing Rape-Seed Oil Methyl Esters (RMe)

M. Waligórska[1], K. Seifert[1], F. Domka[2]*, A. Korzeniowski[2]

[1] Adam Mickiewicz University, Faculty of Chemistry, Grunwaldzka 6, 60-780 Poznań, Poland
[2] University of Economics, Faculty of Material Science, Al.Niepodległości 10, 60-967 Poznań, Poland

Received: 31 October 2003
Accepted: 13 May 2004

Abstract

Catabolic activity of *Bacillus licheniformis* and *Desulfotomaculum ruminis* bacteria in media containing rape-seed oil methyl esters (RMe) has been studied. The degree of the esters' biodegradation was assessed on the basis of the kinetic parameters of the process of microbiological reduction of nitrates or sulphates, taking place with the involvement of RMe as electron donors.

It has been shown that RMe in a wide range of concentrations are effective sources of carbon and energy for *Bacillus licheniformis* bacteria. At the esters concentration of 0.3% in the medium, the conversion of nitrates reached 100%, COD was reduced by 80% (biodegradation of RMe) and biomass increases three times. *Desulfotomaculum ruminis* bacteria in the media with RMe probably needs a long period of adaptation to this carbon source in the medium.

Keywords: rape-seed oil methyl esters, denitrification, *Bacillus licheniformis*, sulfate reduction, *Desulfotomaculum ruminis*

Introduction

Esters of fatty acids present in plant oils belong to biodegradable materials [1, 2]. This group also includes rape-seed oil methyl esters (RMe), used by themselves or in mixtures with diesel oil, in engines fuelled by this kind of fuel. The esters mix very well with diesel oil although their increasing contribution causes deterioration of ecological properties of such a mixture. For example, at concentration 10% of RMe in diesel oil increases the mixture sensitivity to bacteriological contamination [3, 4] causing changes in its physicochemical properties. The use of rape-seed oil methyl esters in biofuel can create potential threads to the environment by contaminating soil and surface waters with products of RMe decomposition such as methanol - known to be toxic to micro-organisms. It should be remembered that not all materials believed to be biodegradable are. Unfortunately, there is a lack of a generally accepted method for classification of materials according to their biodegradation ability. Biodegradability cannot be assessed on the basis of visual observations or physical properties of the samples in different conditions of micro-organism activity. The reliable assessment of material biodegradability should be based not only on the observations in the period of incubation of micro-organisms in the medium with the material tested but also on the basis of a study of the process of conversion and its kinetic parameters. Moreover, so far no recommendations have been presented by the Committee appointed by ASTM to propose a procedure for determination of the degree of biodegradability of materials [5]. Therefore, the problems related to reliable assessment of the biodegradability of commercial products often being a mixture of a few chemical compounds, are still unresolved. Unfortu-

*Corresponding author

Table 1. Composition of the medium used in the process of denitrification.

Macroelements		Microelements	
compound	concentration [g/dm^3]	compound	concentration [mg/dm^3]
$CaCl_2$	1.0	$MnSO_4xH_2O$	3.47
NH_4Cl	0.25	H_3BO_3	0.86
$Fe(NO_3)_3x9H_2O$	0.43	$CuSO_4x5H_2O$	1.2
$Na_2HPO_4x12H_2O$	2.5	$Ni(NO_3)_2x6H_2O$	0.24
KNO_3	10.0	$Co(NO_3)_2x6H_2O$	0.28
$MgSO_4x7H_2O$	0.5	$Zn(NO_3)_2x6H_2O$	0.1
sodium lactate	10.06	$(NH_4)_6MoO_{24}x4H_2O$	0.18
		$NaHSeO_3$	0.0088

nately, the problem also concerns RMe recently promoted as an alternative fuel.

Our paper presents preliminary results of a study of the degree of RMe biodegradability carried out in batch culture, on the basis of measurements of COD changes and kinetic parameters of denitrification or desulfurication taking place with the involvement of *Bacillus licheniformis* or *Desulfotomaculum ruminis* bacteria.

These processes include the conversion of nitrates to free nitrogen or sulphates to hydrogen sulphide, using organic carbon as a source of electrons [6]. The processes are used practically for efficient waste purification [7] and are characterized by the highest yield in the conditions of appropriate supply of nitrates or sulphates and carbon substrate. The type of carbon source significantly affects the efficiency and dynamics of conversion and thus permits assessment of the biodegradability of a material studied on the basis of kinetic parameters of substrate elimination in the period of bacteria incubation.

Materials and Methods

The Process of Denitrification

Pure culture of *Bacillus licheniformis* bacteria grown in a lactate medium (Table 1) was adapted for 45 days in a medium containing rape-seed oil methyl esters (RMe). The process was conducted in bottles of 120 ml in capacity at 22±1°C, and at pH 8. The medium was inoculated with 4% vol. of the culture collected after 24 hours of growth. The samples were mixed by a magnetic stirrer. The adaptation was performed by gradual reduction of the amount of sodium lactate in the medium and replacing it by RMe. The adaptation was enhanced when the contribution of the inoculum increased to 20% vol.

Except for sodium lactate, the reagents used were analytical grade produced by POCh-Gliwice. Sodium lactate was purchased from Fluka.

Rape-seed oil methyl esters, whose characterization is given in Table 2, were produced at the Poznań Institute of Agricultural Equipment.

Table 2. Physical and chemical properties of methyl esters of fatty acids of rape-seed oil (RMe).

Physical and chemical properties of RMe	
density at 20°C [g/dm^3]	0.881
cold filter blocking point [°C]	- 12
kinematics viscosity at 20°C [mm^2/s]	7.73
sulphur content [mg/kg]	18

Kinetic study was conducted in bottles of 600 ml in capacity, filled with 500 ml of the medium containing RMe as the only source of carbon and energy, in the concentrations from 0.075 to 1.2% vol.

The course of the process carried out at 22°C, and at pH 8 was followed on the basis of time changes in the concentration of nitrates and nitrites.

The concentration of nitrates was measured spectrophotometrically in the reaction with brucine and concentrated sulphuric acid [8].

The concentration of nitrites was determined by the Griess-Ilosvay method by measuring absorption of the dye complex between nitrites with sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride at the wavelength of λ=520 nm [9].

Biomass was measured as a protein [g/dm^3] by the spectrophotometrical Lowry method at λ=750 nm [10].

COD_{total} (without biomass separation) before and after the process was measured by the use of dichromate [11]. The same method was used for determination of COD of non-inoculated medium and of post-reaction mixture where the later was filtrated through a Millipore (0.22 μm) filter. It was done in order to determinate COD of unused RMe and metabolites formed in the process. However, it turned out that the excess of the studied esters was adsorbed on the surface of the filters (the studies on adsorption of RMe on Millipore filters showed that total amount of RMe in the medium

adsorbed on the filter) so the COD describes only the metabolites dissolved in water.

The observed growth yield (Y_{obs}) was calculated from the following equation:

$$\frac{Y_{obs}}{1-Y_{obs}} = \frac{biomass\ produced}{\Delta COD_{total}}\ [\text{g COD/g COD}] \quad (1)$$

where the decrease of COD_{total} (ΔCOD) was expressed as the difference between COD before and after reaction without biomass separation [12]. The concentration of biomass was expressed in COD units through multiplication by a factor 1.48 [13].

Because the observed growth yield (Y_{obs}) depends on the ratio of the initial concentration of the substrate to the initial concentration of biomass (S_o/X_o), the observed biomass increase yield can be described by the Yu Liu equation [14] in the form:

$$\frac{1}{Y_{obs}} = \frac{1}{Y'_{obs}} + \frac{1}{(Y_w)_{min}} \frac{S_O/X_O}{S_O/X_O + K_{S/X}} \quad (2)$$

where:

Y_{obs} - the growth yield observed [g protein (COD)/g COD]

Y'_{obs} - the observed growth yield in a substrate limited-culture

$(Y_w)_{min}$ - minimal energy spilling-related growth yield [mg biomass(COD)/mg COD)

S_o - the initial concentration of the organic substrate

X_o - the initial biomass concentration

$K_{s/x}$ - the S_o/X_o ratio-related saturation constant [mg COD/mg biomass(COD)].

The parameters of the equation were determined by the graphical method.

The values of Y'_{obs} were determined directly from the relation between $1/Y_{obs}$ and S_o/X_o, for $S_o/X_o = 0$. The other parameters were determined having converted eq. 3 into the linear form:

$$\frac{1}{\frac{1}{Y_{obs}} - \frac{1}{Y'_{obs}}} = (Y_w)_{min} K_{S/X} \frac{1}{S_O/X_O} + (Y_w)_{min} \quad (3)$$

in which $(Y_w)_{min}$ is the intercept corresponding to $S_o/X_o = 0$, whereas $(Y_w)_{min} * K_{s/x}$ is the slope of the line.

Desulfurication

Pure culture of the *Desulfotomaculum ruminis* bacteria was grown in a Starkey medium composed of sodium lactate and macro and microelements (Table 3). The medium was inoculated with a 4% inoculum. The process was conducted at 37°C and at pH 7.

The kinetic study was carried out for different concentrations of sodium lactate and RMe. The concentration of sodium lactate was chosen so that the C/S ratio was 1.8 or 0.9. In these media the process of desulfurication was limited by the concentration of organic carbon. The tested RMe was introduced into the media in the amounts from 0.74 to 58.3 % vol., and then the media were inoculated with 20% vol. of the inoculum. Parallel experiments were conducted in the medium containing sodium lactate of the same C/S ratio and without RMe (reference samples).

The process was controlled by measurement of concentration of liberated hydrogen sulphide, which was blown by nitrogen from the reactors to absorption bulbs containing cadmium acetate. The concentration of sulphides was determined by the iodometric method [15]. The same method was used to determine the content of sulphides in the sediment after the process. The total concentration of sulphides formed in the process of desulfurication was the sum of the sulphides determined in the solution and in the sediment.

Results and Discussion

Denitrification

Our experiments testing the effect of rape-seed oil methyl esters (RMe) on the process of denitrification in a medium containing sodium lactate (C/N = 2.4) have shown that the esters in the concentrations from 9 to 33.3% are not toxic towards the bacteria *Bacillus licheniformis*. In all experiments total reduction of nitrates and nitrites to free nitrogen was observed, similarly as in the reference samples without RMe.

Table 3. Chemical composition of a standard Starkey medium.

Macroelements		Microelements	
compound	concentration [g/dm^3]	compound	concentration [mg/dm^3]
$CaCl_2x6H_2O$	0.25	$MnSO_4x4H_2O$	0.06
NH_4Cl	1.0	H_3BO_3	0.017
Mohr salt	0.50	$CuSO_4x5H_2O$	0.24
K_2HPO_4	5.0	$Ni(NO_3)_2x6H_2O$	0.003
Na_2SO_4	2.43	$Co(NO_3)_2x6H_2O$	0.1
$MgSO_4x7H_2O$	2.0	$Zn(NO_3)_2x6H_2O$	0.02
sodium lactate	10.06	$(NH_4)_6MoO_{24}x4H_2O$	0.02
		$NaHSeO_3$	2 x 10^{-8}

Table 4. Parameters of denitrification taking place with the use of the *Bacillus licheniformis* bacteria in the medium containing RMe as the only source of carbon and energy.

concentration MEFARO [%v/v]	process duration [h]	final concentration $N\text{-}NO_3$ [g/dm^3]	final concentration $N\text{-}NO_2$ [g/dm^3]	degree of reduction $N\text{-}NO_3$ toN_2 [%]	biomass increase [g/dm^3]	decrease of COD_{total} [g O_2/dm^3]	COD a) [g O_2/dm^3]	decrease of COD b) [%]	Y_{obs} [mg protein (COD)/ mg COD]
0.075	68	0.740	0.289	26.5	0.238	1.36	0.43	79	0.206
0.15	68	0.451	0.374	41.1	0.363	2.13	0.66	81	0.201
0.3	115	0	0.005	99.6	0.660	4.06	1.46	79	0.194
0.6	115	0	0.004	99.7	0.776	5.0	1.62	-	0.187
1.2	76	0	0.003	99.9	0.722	5.2	1.68	-	0.170

a) COD measured in the post-reaction mixture after filtering off the biomass through Millipore filters, b) COD determined as a ratio of the difference between COD_o and COD a) to COD_o

In kinetic studies the bacteria were used after adaptation to the medium containing RMe, which was the only source of carbon and energy. The process is illustrated in Figs. 1, 2 and in Table 4.

In the media containing 0.075% and 0.15% vol. of RMe the process was inhibited after 68 hours. The concentration of $N\text{-}NO_3$ after the process was finished was 0.74 and 0.451 g/dm^3, respectively, while the concentration of $N\text{-}NO_2^-$ was 0.289 and 0.374 g/dm^3. Biomass growth was 0.238 and 0.363 g/dm^3 and the corresponding decrease of COD_{total} was 1.36 and 2.13 g/dm^3. In these two media the presence of RMe at the concentrations used limited the process of denitrification, so its inhibition was related to the exhaustion of organic carbon. An increase of RMe concentration to 0.3% vol. caused practically total reduction of nitrates and nitrites. Our studies have shown (data not included) that RMe at the concentration of 0.3% vol. was sufficient for denitrification of 1.43 $N\text{-}NO_3^-$ g/dm^3. Thus, RMe, at 0.3% vol. concentration, was almost completely consumed (initial concentration of $N\text{-}NO_3^-$ in the medium was 1.4 g/dm^3). The reduction of nitrates and nitrites was accompanied by a significant growth of biomass, equal 0.66 g/dm^3 and a decrease of COD_{total} equal 4.06 g O_2/dm^3 (Table 4, Fig.1).

A similar degree of nitrate conversion was obtained in the media in which the RMe concentration was 0.6 and 1.2 % vol. The amount of biomass produced increased to 0.789 g/dm^3, and to 0.7722 g/dm^3 respectively. This process was accompanied by a decrease in the level of COD by 5.0 gO_2/dm^3 for 0.6% vol. and 5.2 gO_2/dm^3 for 1.2% vol. of RMe.

Analysis of COD of post-reaction mixture after filtration through Millipore filters (which represents only the COD of the metabolites – see Materials and Methods) shows that COD increased from 0.43 g O_2/dm^3 to 1.46 g O_2/dm^3 when the concentration of the esters changed from 0.075 to 0.3% vol.. A further increase of RMe concentration caused an insignificant increase of COD (from 1.46 to 1.68 g O_2/dm^3). The decrease of COD (%) in the media containing from 0.075 to 0.3% vol. RMe is 79, 81, 78% and is very close to the values characterizing the process of denitrification (Table 4).

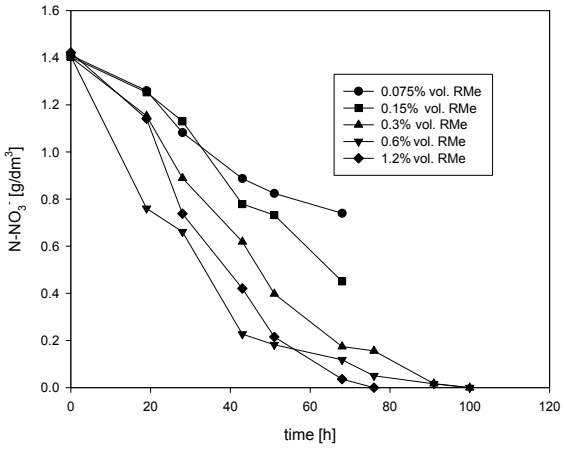

Fig. 1. The effect of the content of RMe on the process of denitrification carried out with the use of the *Bacillus licheniformis* bacteria (pH = 8, 22°C).

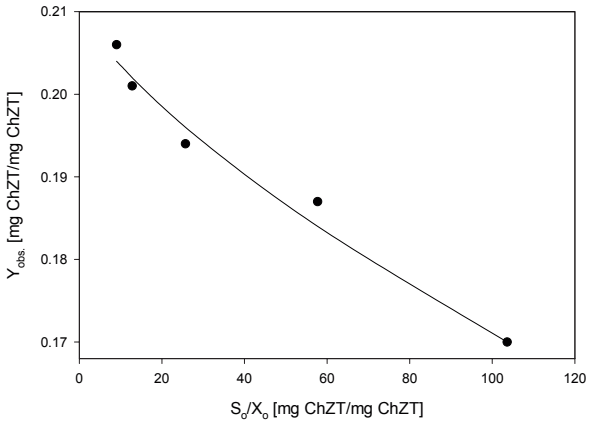

Fig. 2. The yield of biomass increase ($Y_{obs.}$) and the ratio of the initial substrate concentration (S_o) to the initial biomass concentration (Y_o), i.e. (S_o/X_o).

Table 5. The effect of the content of RMe on the process of desulfurication taking place with the use of *Desulfotomaculum ruminis* bacteria in media of different C/S ratio, at 37°C.

Content of RMe [% v/v]	Lactate medium (C/S = 1.8)	Lactate medium (C/S = 0.9)
	concentration of sulphides [g/dm^3]	concentration of sulphides [g/dm^3]
0 (reference sample)	0.63	0.44
0.74	-	0.44
1.24	-	0.43
4.8	-	0.43
9.1	0.61	0.42
16.7	0.59	-
33.3	0.61	0.34
41.2	0.60	-
50	0.47	0.25
58.3	-	0.06

On the basis of the biomass produced and COD_{total} values the coefficient of the yield Y_{obs} was determined from eq. (1), which describes the efficiency of the use of the organic substrate in the process studied and thus the degree of RMe biodegradability. This coefficient is 0.206 in the medium containing 0.075% vol. RMe and decreases to 0.170 when the esters concentration increases to 1.2% vol., so with increasing ratio of S_o/X_o. This is related to the different way of using the organic substrate as a source of carbon and energy in the media containing an excess of the esters. The organic substrates used by microorganisms are converted into intracellular metabolites and energy (ATP) used for maintenance, biomass production and product formation. According to Pirt's theory [16] there is some minimum rate of substrate assimilation above which fast biomass growth occurs. This minimum rate of the substrate assimilation corresponds to the rate of the endogenic processes. This theory effectiveley describes the growth of microorganisms in the substrate-limited media. However, it does not give correct description of the phenomena taking place in the phase of growth in substrate-sufficient cultures. In such experiments the consumption of the substrate is much greater than in the substrate-limited cultures. A quantitative description of the phenomena given by Yu Liu [14] assumes that the total amount of the substrate used by the micro-organisms is a sum of the substrate needed for maintenance, growth of biomass and used in different futile processes involving energy spilling taking place as a result of the mismatch between the anabolic and catabolic processes [17]. The model proposed by Yu Liu (eq. 2) relating the growth yield Y_{obs} with the ratio S_o/X_o well describes the experimental data (Fig.2). Therefore, we can assume that the excessive use of the substrate at high S_o/X_o ratios is related to the energy spilling and futile processes.

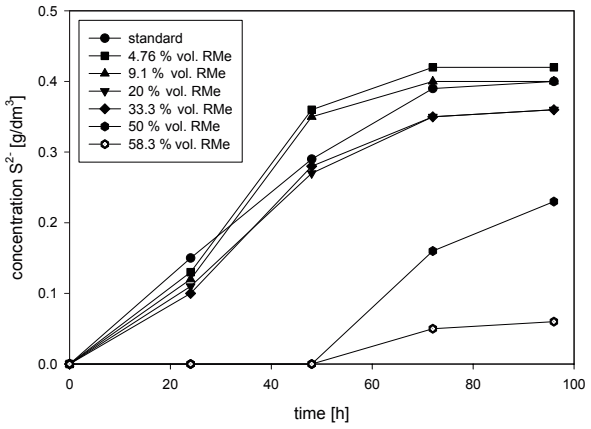

Fig. 3. The effect of RMe concentration on the kinetics of desulfurication taking place with the use of the *Desulfotomaculum ruminis* bacteria, (C/S = 0.9, 37°C).

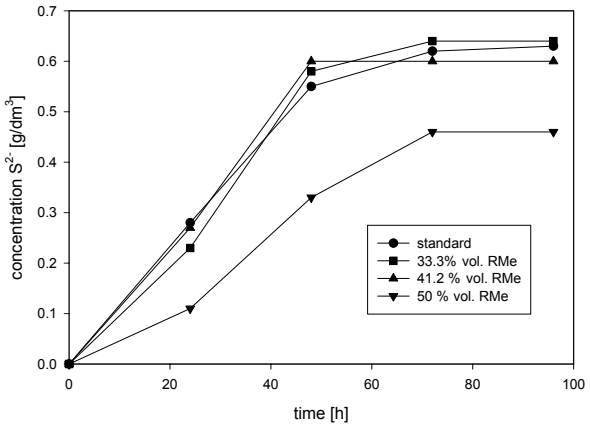

Fig. 4. The effect of RMe concentration on the kinetics of desulfurication taking place with the use of the *Desulfotomaculum ruminis* bacteria (C/S = 1.8, 37°C).

Desulfurication

The process of desulfurication was conducted with the use of the *Desulfotomaculum ruminis* bacteria adapted to the media with RMe. The experiment was carried out in a medium whose C/S ratios are 0.9 or 1.8.

The results given in Table 5 and in Fig. 3 show that the concentrations of sulphides formed after 72 hours of the process in the media containing sodium lactate (C/S=0.9) and esters in the amount up to 33.3% are close to the concentration of sulphides produced in reference samples. This means that RMe does not inhibit the growth of sulphide reducing bacteria (SRB). The increase of the esters concentration above 33.3% significantly extends the induction period needed for adaptation of the bacteria to the medium and lowers the concentration of sulphides. For 50% vol. of RMe in the medium it was 0.25 g S^{-2}/dm^3, while in the reference sample it was 0.45 g S^{-2}/dm^3.

In the medium richer in organic substrate (C/S=1.8) an addition of the esters in 41.2% vol. does not affect sulphide formation (Fig. 4). An increase in the esters concentration up to 50% vol. causes the reduction of sulphides producing up to 0.47 g S^{2-}/dm^3 (see Fig. 4). A comparison of the desulfurication process in the media with C/S=0.9 and 1.8 shows that the inhibition is more significant in the medium which contains less sodium lactate.

The results of this study indicate that rape-seed oil methyl esters (RMe) are easily biodegradable by the bacteria *Bacillus licheniformis*. In this process RMe can be used as the only source of carbon and energy in concentrations in which the process is limited by the substrate (0.075 and 0.15% vol.) as well as in an excess (from 0.3 to 1.2% vol.).

With the RMe concentration changing from 0.075% to 0.3% vol., the degree of nitrate and nitrite conversion increases from 25.6% to 100%, the amount of biomass increases from 0.238 to 0.66 g/dm^3 and COD decrease changes from 1.36 to 4.06 gO_2/dm^3. A further rise in the initial concentration of the esters up to 0.6% results in a further small increase in biomass and COD decrease.

In the systems studied the value of COD of the post-reaction mixture after filtering off the biomass through Millipore (0.22 μm) filter varies from 0.43 to 1.46 gO_2 /dm^3, when concentration RMe changes from 0,075 to 0.3%, which gives the COD loss of an order of 80%.

The ratio of biomass produced to the amount of consumed organic carbon (Y_{obs}) decreases with increasing initial concentration of RMe in the medium (Fig.2). This fact confirms the hypothesis put forward by Zang [17] and Yu Liu [14], saying that in the media with the energy source exceeding the catabolic needs of the bacteria cells, the production of energy can be faster than its use by the cells. In this situation the microorganisms are not capable of regulating the rate of catabolism exactly to needs of anabolism and, consequently, the efficiency of coupling these processes is lowered by energy spilling and futile cycle. Therefore, much more substrate must be oxidized to provide the energy needed for growth of the bacteria [14].

The results indicate that the concentration of 0.3% vol. RMe is the optimum in a medium containing 1.4g $N\text{-}NO_3/dm^3$. In these conditions the process of denitrification is complete, the biomass production is not too high (0.66 g/dm^3), and the balance of COD indicates that the esters have degraded almost 100%.

As has been mentioned in [18] the process of denitrification in a medium containing diesel oil is much slower and requires a much higher volume ratio of the oil to the mineral medium. It is supposed that an addition of RMe to diesel oil will increase the growth of microorganisms and thus also the rate of biodegradation of organic substrates. It has been shown that of rape-seed oil methyl esters in the concentration up to 33.3% does not inhibit the process of desulfurication but RMe was not used as the source of carbon and energy. Maybe esters could be used by the *Desulfotomaculum ruminis* bacteria after longer adaptation to the medium.

Acknowledgements

Joanna Hyża and Agata Waśko students of the University of Economics have taken part in the experimental work.

References

1. CULSHAW F.A., The Potential of Biodiesel from Oilseed Rape. Proceedings of I. Mech. E., Journal of Power and Energy, **207**, **1993.**
2. PODNIAŁO A., Fuel, Oil and Lubricant in ecological exploitation. W.N.T., Warsaw, pp. 108, **1993** (in Polish).
3. ŁUKASIK Z., Normalisation in quality requirements for methyl esters of fatty acids in rape oil used as a fuel., EKODIESEL'96 Meeting III, Warsaw **1996** (in Polish).
4. POITRAT E., Trials Programme on Engines with Rope Methyl Ester (RME) in France. Fuels 1st International Colloquium. Technische Akademie Esslingen **1997**.
5. SWIFT G., Biodegradation test and the need for standardisation, in Biodegradable Plastics Socity. International Symposium on Biodegradable Polymers, Tokyo, October 29-31, **1990**.
6. BATH H., TREBST A., Biology of Inorganic Nitrage and Sulfur, Berlin Heidelberg New York , p. 116, **1981**.
7. DOMKA F., JUSZCZAK A., Between Biology and Chemistry. W.N.UAM, Poznan, **2000** (in Polish).
8. Physico-chemical Methods of Investigation of Water and Sewage (Collective works in Polish) Warsaw, p.131, **1976**.
9. MARCZENKO Z., Colorimetric Determination of Elements, PWN, Warsaw, **1979**.
10. LOWRY O.H., ROSENBROUGHT N.J., FAAR A.L., RANDALL R.J., Protein measurement with pholin-phenol reagent. J.Biol.Chem., **193**, 265, **1951**.
11. Standerds Methods for the examination of Water and Wastewater, 18th edit., 5220, **1992**.
12. COPP J.B., DOLD P.L., Comparing Sludge Production under aerobic and Anoxic Conditions, Wat.Sci.Tech., **38**, (1), 285, **1998**.

13. BAKER P.S., DOLD P.L., COD and Nitrogen Mass Balances in Activated Sludge Systems., Wat.Res., **29**, (2), 633, **199**5.
14. YU LIU, Bioenegetic Interpretation on the S_o/X_o Ratio in Substrate – Sufficient Batch Culture, Wat.Res., **30**, (11), 2766, **1996**.
15. WILLIAMS W.I., Determination of Anions, PWN, Warsaw, **1985** (in Polish).
16. PIRT S.J., The Maintenance energy of bacteria in growing cultures., Proc.R.Soc.Lond.B **163**, 224, **1965**.
17. ZENG A.P., DECKWER W.D., A kinetic model for substrate and energy consumption of microbial growth under substrate – sufficient conditions. Biotechnol.Prog., **11**, 71, **1995**.
18. WALIGÓRSKA M., DOMKA F., KOPRAS I., IDZIAK W., The Effect of Petroleum Products on the Process of Denitrification., Pol.J.Environ.Stud., **7**, (1), 39, **1998**.