

Selection of fat-degrading microorganisms for the treatment of lipid-contaminated environment

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To develop effective microbial agents applicable to complex technology for grease wastes utilization, a total of 124 microorganisms were screened for their ability to degrade lipidic compounds. The screening yielded five strains (UP2, F2, E13, Kl1 and N3) showing lipolytic activity and rapidly degrading sunflower and olive oil, tallow and lard. Among them, strains E13 and N3 were found to have the highest lipase activity and the more intensive rates of the degradation of saturated (palmitic and stearic) and unsaturated (oleic and linoleic) fatty acids and triglycerides containing these fatty acids. The strains were obtained from the culture collection of JSC “Biocentras” and identified as *Enterobacter aerogenes* E13 and *Arthrobacter* sp. N3. When a mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 was grown in a mineral medium containing 0.5% of sunflower oil, the hydrolysis products were diglycerides, monoglycerides and free fatty acids. Moreover, the mixed culture was consuming these hydrolysis products during cultivation at 30 °C. Investigation of lard biodegradation in black soil showed that a mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 degraded lard about 7 times more intensively than indigenous soil microorganisms (after six weeks of degradation in black soil, the concentration of lard was reduced by 87.5 and 8.0%, respectively). Therefore, the mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 may be used for an effective grease waste reduction in a complex cleaning technology.

Key words: fat degradation, *Enterobacter*, *Arthrobacter*, lipase

Abbreviations: FOGs, fats, oils and greases; COD, chemical oxygen demand; TLC, thin layer chromatography; LCFA, long chain fatty acid

INTRODUCTION

Fats, oils and greases (FOGs) are released into the environment with wastewater derived from the food processing industry, restaurants and kitchens or by accidental spills of oils. If not treated, they provoke an environmental effect similar to that of petroleum oil due to common physical properties. In natural environments, FOGs result in coating of animals and plants with oil, reduction of oxygen transfer rate and a high chemical oxygen demand (COD) in wastewater [1]. On the other hand, accumulation of FOGs in wastewater collection and handling systems leads to clog-

ging of drainpipes, appearance of unpleasant odour and corrosion of sewer pipes [2].

The main constituents of FOGs are animal fats and vegetable oils. They also comprise a combination of glycerol and free fatty acids whenever hydrolysis has taken place [3]. When FOGs are spread in ecosystems, they may be used by indigenous lipid-degrading microorganism as an additional source of carbon and energy.

Many microorganisms capable of degrading FOGs have been isolated from soil and water samples. For example, studies carried by Ruiz et al. [4] have shown that nearly half of 724 strains isolated from soil rich in organic matter degraded olive oil and tributyrin, and the most active strain belonged to the genus *Bacillus*. Other genera of

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bacteria, such as *Pseudomonas*, *Burkholderia*, *Acinetobacter*, *Escherichia*, and fungi (*Candida*, *Rhodotorula*, *Yarrowia*) are known to have the ability to degrade contaminants as well as *Bacillus* [5–11]. However, the natural microbial degradation of FOGs is slow due to their low lipolytic activity. Furthermore, the biodegradation of lipids is often limited by the antimicrobial effect of hydrolysis products such as long-chain fatty acids [2, 3].

Selection of microorganisms with a high degradation activity for FOGs and their application for the removal of pollutants is one of the ways to enhance biodegradation. There are reports and patents on the use of active lipid-degrading microorganisms, particularly mixed cultures, developed in laboratory for the biological removal of FOGs [5, 11–15]. The aerobic degradation of FOGs in wastewater by active microbial cultures is a common and one of the most efficient practices of biological treatment. However, biodegradation is rather slow and insufficient when wastewater is highly polluted by FOGs. Another alternative method to deal with a high fat content in wastewater is mechanical separation of fatty wastes from aqueous systems and their *ex situ* biodegradation in soil by composting or other biological treatment [15]. Moreover, *ex situ* biodegradation can be applicable for microbial destruction of food residuals rich in oil and grease.

Recently, increasing attention has been focused on the hydrocarbon-degrading microorganisms that have lipolytic activity [16–19]. For instance, Domenico et al. [18] have investigated diesel oil degrading bacteria isolated from Antarctic seawater and found that 90% of the isolates had grown on both diesel oil and Tween 80. As noticed by Margesin et al. [16], the ability of microorganisms to degrade hydrocarbons and lipids is related to the fact that similar enzymes are involved in both degradation processes. In general, the initial step of hydrocarbons oxidation gives primary alcohols further converted to the corresponding fatty acids which are metabolized through the β -oxidation pathway, just as fatty acids derived from triglycerides after the hydrolytic action of lipolytic enzymes [17, 20]. Therefore, application of microbial cultures with a high hydrolytic activity toward fats and petroleum hydrocarbons could improve the biological treatment of the environment, particularly of wastewater polluted by FOGs and mineral oil.

In previous works, we have studied hydrocarbon degrading bacteria and their application for cleaning the environment from petroleum contamination. We have found that hydrocarbon-degrading bacteria belonging to the genus *Arthrobacter* degraded a mixture of fuel oil and diesel fuel more rapidly when grown in a complex nutrient medium containing sunflower oil or oleic acid [21, 22]. So, these results lead to the presumption that our hydrocarbon-degrading bacteria might be applicable for removing not only hydrocarbon but also FOGs.

The aim of this study was to select lipidic compounds degrading microorganisms and to develop a microbial composition applicable to a complex technology for grease waste utilization. In particular, microorganisms isolated from an environment contaminated with fats and bacteria applicable for removal of hydrocarbons were tested for their ability to degrade animal fats, vegetable oils, triglycerides and fatty acids and for lipase activity. Furthermore, the effect of a mixture of lipid-degrading microorganisms on removing fats from aqueous and soil environments was investigated.

MATERIALS AND METHODS

A total of 124 microbial cultures obtained from the culture collection of JSC “Biocentras” and isolated from the environment polluted with various lipids were examined.

Nutrient agar (Oxoid, Basingstoke, UK) was used for plating microbial strains, and nutrient broth (Oxoid, Basingstoke, UK) was used for the subculture and preculture of the strains. The enrichment medium composed of 1 g/l sunflower oil, 0.5 g/l KH_2PO_4 , 0.5 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.1 g/l MgSO_4 , 0.1 g/l CaCl_2 and 1 g/l of yeast was used to enrich lipid-degrading microorganisms. Tween-peptone agar containing 10.0 g/l peptone, 5.0 g/l NaCl, 0.1 g/l CaCl_2 , 5 ml/l Tween 80 and 18 g/l agar was used for the screening of lipid-degrading microbes [23]. To investigate the ability of the strains to degrade fatty substrates, a mineral medium was used. The mineral medium had the following composition: 0.5 g/l KH_2PO_4 , 0.5 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.1 g/l MgSO_4 and 0.1 g/l CaCl_2 (pH 6.8).

For flask cultures, 750 ml Erlenmeyer flasks containing 100 ml of nutrient broth, enrichment or the mineral medium and 10% of submerged cultures grown in nutrient broth were applied for inoculation.

For isolation of lipid-degrading microorganisms, a suspension of 1% (w/v) soil or wastewater samples was incubated in an enrichment medium at 30 °C and 200 rpm for 72 h. After the incubation, microbial cultures were plated on nutrient agar for single colony isolation. Subsequently, strains isolated and obtained from culture collection were plated on Tween-peptone agar and incubated at 30 °C for 72 h for screening of lipid-degrading microbes. Strains that showed an opaque halo around the colonies were selected for further experiments.

The ability of strains to degrade fatty substrates was investigated in a mineral medium supplemented with 0.1% of vegetable oil (olive and sunflower), animal fats (lard and tallow), triglycerides (tripalmitin, tristearin, triolein and trilanolein) and fatty acids (oleic, linoleic, palmitic and stearic). Inoculated and blank flasks were incubated at 30 °C, 200 rpm for 24 h. The degradation intensity of the fats was calculated by comparing the content of residual

fats in experimental and blank flasks. All experiments were carried out in triplicate.

The best fat degraders were tested for their capability of degrading fats in a mixed culture. For the analysis of their ability to degrade fats in an aqueous environment, cultivation was carried out under sterile conditions in 2000 ml flasks with 500 ml of mineral medium supplemented with 0.5% of sunflower oil on a rotary shaker at 30 °C and 200 rpm. During the cultivation, samples were taken at appropriate time intervals for the analysis of cell growth, oil degradation and lipase activity. Fat biodegradation by a mixed bacterial culture in soil was investigated under non-sterile conditions in plastic boxes filled with 100 g of black soil artificially polluted with 2.0% of lard. Lard-contaminated soil in experimental and blank (non-inoculated) boxes was incubated at 30 °C, soil was periodically mixed, and moisture was kept at 15–20%.

Cell count was determined by plating serial dilutions of samples on nutrient agar plates and incubating at 30 °C for 24 h.

After biodegradation, fat substrates were extracted from experimental and blank flasks with tetrachlormethane. The content of fat substrates was determined by IR-spectrophotometry at 2930 cm^{-1} [24].

To determine sunflower oil degradation products, thin layer chromatography (TLC) was carried out. The residual oil and the degradation products were extracted from the culture liquid with hexane. TLC was performed using glass silica gel plates (Fluka), with a mobile phase of hexane, diethyl ether and acetic acid (70 : 28 : 2, v/v/v). The spots of oil and hydrolysis products were visualized by saturated iodine steam.

Lipase activity was measured by the spectrophotometric method using p-nitrophenyl palmitate as a substrate [25] as follows. An appropriately diluted submerged culture (0.2 ml) was added to 1.8 ml of the substrate solution prepared by mixing one part of solution A (40 mg of p-nitrophenol dissolved in 12 ml of 2-propanol) with 19 parts of solution B (50 mM Sorensen's phosphate buffer pH = 8.0 with 0.2% sodium deoxycholate and 0.1% gum arabic). The

reaction mixture was incubated at 40 °C for 3–5 min, and the substrate hydrolysis was determined by measuring the increase in absorbance at 400 nm. One unit of lipase activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per minute under the assay conditions described.

Calculations were performed with Origin software. Experimental results were expressed as the arithmetic mean \pm standard deviation.

RESULTS AND DISCUSSION

The lipid-degrading capability of 52 petroleum hydrocarbons and greases degrading microbial cultures obtained from the culture collection of JSC "Biocentras" and 72 strains isolated from wastewater and solid wastes contaminated by FOGs were initially tested on Tween-80 peptone agar. Twenty-six strains which formed opaque zones around microbial colonies, indicating lipolytic activity, were selected. Subsequently, the fat degradation ability of the selected strains was investigated in a mineral medium supplemented with 0.1% of animal fats and vegetable oils. Five strains (UP2, N3, KL1, E13 and F2) were found to degrade all test fats at the highest rate. During 24 h of cultivation, the strains degraded 24.9–42.3% of tallow, 25.9–45.7% of lard, 38.4–56.6% of sunflower oil and 40.3–58.9% of olive oil. The strains were selected for further investigations.

When the environment is being contaminated by fats for a prolonged period, indigenous microorganisms can break them down and induce accumulation of long-chain fatty acids (LCFA), including palmitic, stearic and oleic acids, which form 75–90% of those present [2]. There are only a few studies concerning the aerobic degradation of fatty acids; the more common practice is anaerobic digestion [2, 3, 14]. In addition, LCFA have been reported to inhibit the activity of various microorganisms [3]. Therefore, it was interesting to investigate the ability of the selected strains to degrade saturated (palmitic and stearic) and unsaturated (oleic and linoleic) fatty acids and triglycerides containing

Table. Effect of microbial strain on degradation of fatty acids and triglycerides

Substrate	Degradation (%) by strain:				
	E13	F2	UP2	N3	KI1
Oleic acid	95.0 \pm 1.55	78.3 \pm 2.33	90.0 \pm 5.22	95.2 \pm 2.72	65.5 \pm 4.21
Linoleic acid	94.3 \pm 1.27	95.7 \pm 2.03	95.7 \pm 1.37	92.3 \pm 3.74	87.1 \pm 5.19
Stearic acid	28.5 \pm 1.05	16.5 \pm 1.56	17.5 \pm 1.02	29.3 \pm 1.47	11.3 \pm 1.05
Palmitic acid	31.2 \pm 2.50	12.7 \pm 2.20	19.0 \pm 0.58	30.1 \pm 2.26	17.0 \pm 0.95
Triolein	87.8 \pm 4.00	51.0 \pm 2.50	59.3 \pm 2.93	65.9 \pm 3.14	35.2 \pm 1.73
Trilinolein	95.5 \pm 3.92	93.0 \pm 2.93	67.0 \pm 3.47	98.3 \pm 1.15	59.5 \pm 6.44
Tristearin	41.9 \pm 2.55	6.0 \pm 0.22	13.0 \pm 1.15	40.3 \pm 3.10	10.3 \pm 0.86
Tripalmitin	23.0 \pm 1.47	0	8.4 \pm 0.72	15.9 \pm 0.99	2.0 \pm 0.4

these acids under aerobic conditions. As is seen in Table, all test strains degraded unsaturated fatty acids and the corresponding triglycerides more intensively than saturated. For example, unsaturated and saturated fatty acids were biodegraded 65.5–95.7 and 11.3–31.2%, respectively, and the corresponding triglycerides were degraded 35.2–98.3 and 2.0–41.9%, respectively. Strains E13 and N3 showed the highest rates of degradation of the test fatty acids and triglycerides after 24 h of cultivation.

In general, liquid lipidic compounds (vegetable oils, unsaturated fatty acids and triglycerides containing these fatty acids) whose melting point is lower than the experimental temperature were more preferred by the examined microbes. In case of solid lipidic compounds, when added to the mineral medium, they formed irregular clumps which were less available for microbial and enzymatic attack. Therefore, a complete degradation of solid lipids by microbial cultures needs more time. These results are similar to those reported by other authors [1, 9] who have found the biodegradation rate to depend on the physical state of lipids.

At the initial stage of FOGs biodegradation microbes usually secrete extracellular lipases which hydrolyze the ester bond of glycerides into glycerol and free fatty acids [7]. The low lipolytic activity of microbial cultures can result in slow processes of the biological removal of FOGs. Therefore, to further analyse lipid degradation by the selected five strains, the time course of lipase production was investigated (Fig. 1). The results indicated that lipase

activity of all microbial cultures increased during cultivation. Strain E13 showed the highest lipase activity not only after 8 h of cultivation, but also after 48 h (0.32 ± 0.0015 and 0.45 ± 0.022 U/ml, respectively). If to compare the lipase activity of strains E13 and N3, the activity of strain N3 was lower in the beginning of the cultivation, while after 48 h it was close (0.41 ± 0.019 U/ml) to the activity of strain E13.

Strains E13 and N3, degrading lipidic compounds intensively and being the most active lipolytic strains, were chosen for further studies. The strains were obtained from the culture collection of JSC “Biocentras”. They had been previously identified using the criteria of Bergey’s Manual of Determinative Bacteriology [26] as *Enterobacter aerogenes* E13 and *Arthrobacter* sp. N3. It is worth mentioning that in our previous works we selected *Arthrobacter* sp. N3 strain as an active hydrocarbon degrader which is used for cleaning the environment from petroleum pollutants [21, 22].

To assess the strains’ application for the bioremediation of lipid-contaminated environments, a mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 was grown in both aqueous (mineral medium containing 0.5% of sunflower oil) and solid (black soil with 2.0% of lard) systems. The mixed culture of the selected strains was chosen for the experiment based on our previous studies [21] and reports of other researchers [9, 13], indicating that mixed microbial cultures have a higher biodegradation potential than single strains.

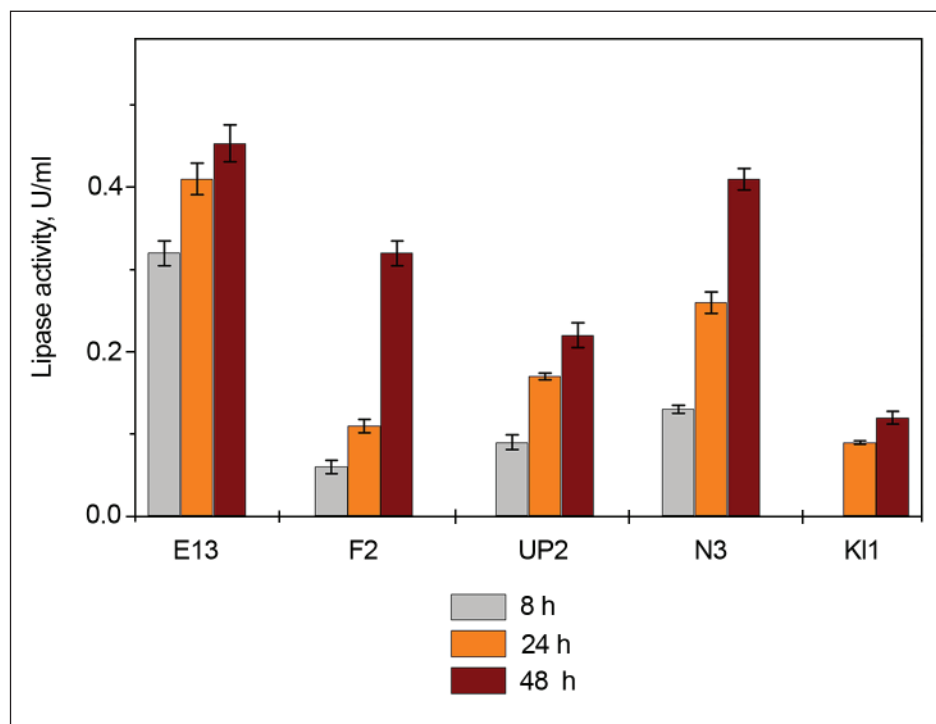


Fig. 1. Comparison of lipase activity of fat-degrading microorganisms

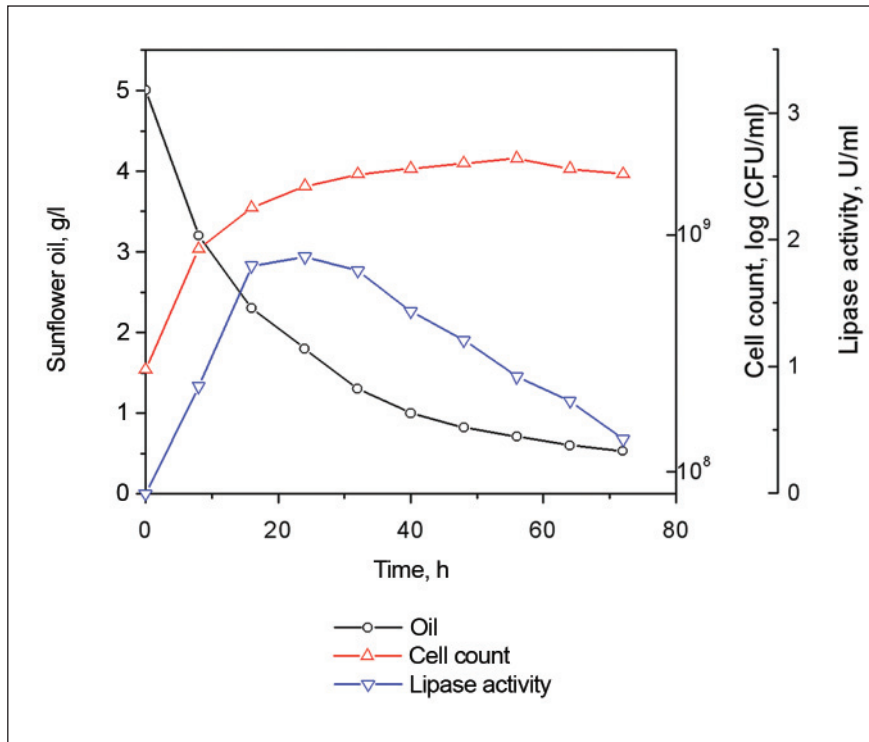


Fig. 2. Dynamics of cell number, sunflower oil concentration and lipase activity during cultivation of mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 in mineral medium with sunflower oil

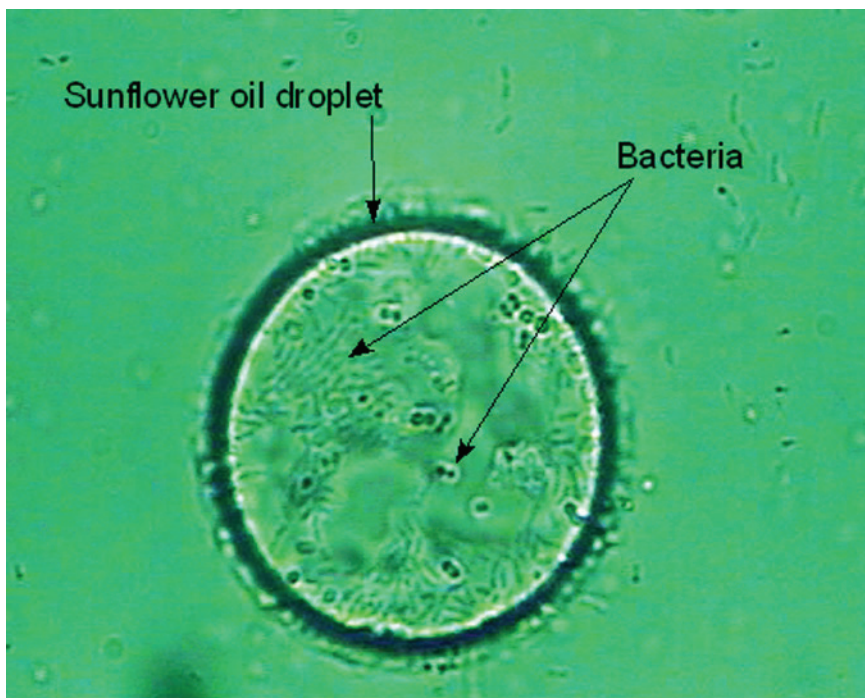


Fig. 3. Interaction between bacteria and sunflower oil in mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 grown in mineral medium supplemented with 0.5% of sunflower oil

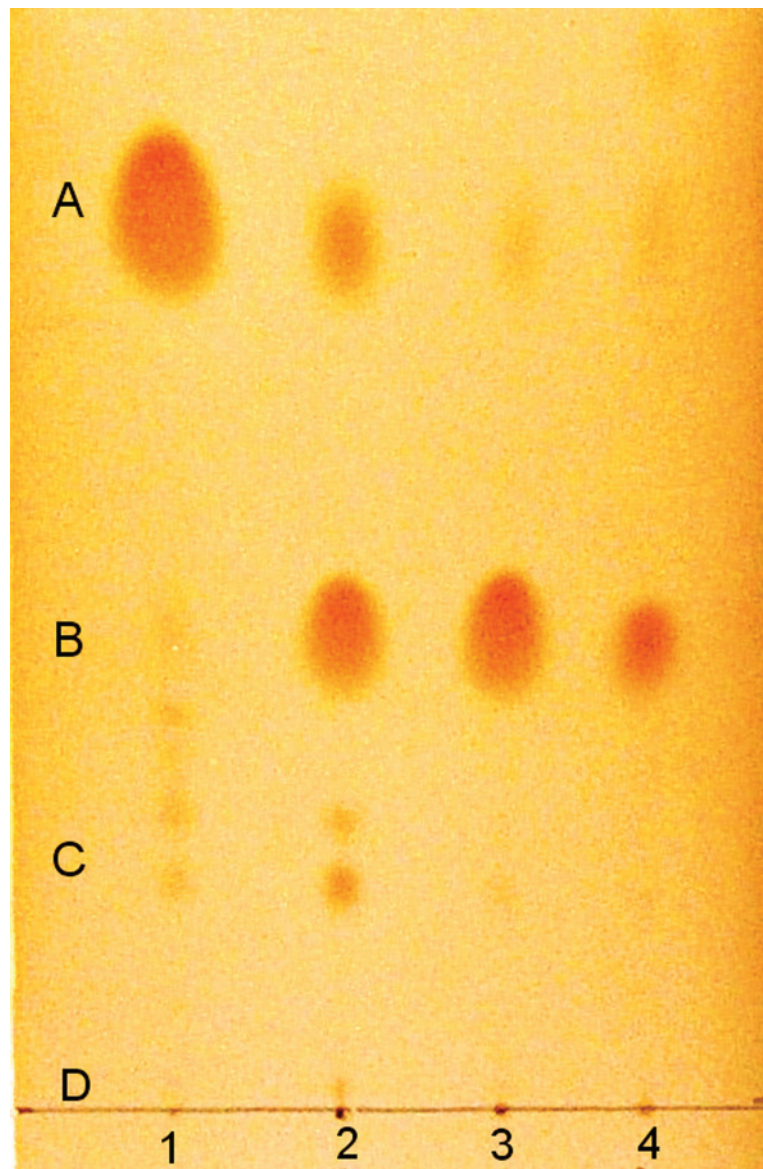


Fig. 4. Thin-layer chromatogram of products obtained after sunflower oil biodegradation by a mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3. Line 1 – 0 h; 2 – 24 h; 3 – 48 h; 4 – 72 h; A – triglycerides, B – fatty acids, C – diglycerides, D – monoglycerides

With a mixed culture cultivated in a mineral medium supplemented with sunflower oil under sterile conditions (Fig. 2), the most intensive reduction of the substrate was observed after 8 h of cultivation. Then biodegradation gradually slowed down; however, after 72 h of degradation by the mixed culture, the concentration of sunflower oil decreased from the initial value of 5.0 to 0.52 g/l (90% of oil was degraded). Similarly to sunflower degradation rate, the most intensive increase in bacterial cell number (about 3 times) was detected during the first 8 hours of cultivation. The maximum cell count ($2.1 \cdot 10^9$ CFU/ml) was reached at 56 h, and later the microbial population slightly decreased. In addition, the mixed culture of

strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 dispersed sunflower oil in the mineral medium by producing fine oil droplets and subsequently colonised them (Fig. 3). The dispersion of sunflower oil may be related to the biosurfactants produced by the strains under study. According to the report of Matsumiya et al. [5], emulsification with biosurfactants increases the interaction between microbial enzymes and lipids and thus enhances lipid degradation ability. As shown in Fig. 2, lipase activity rapidly increased during 16 h of the mixed culture cultivation and was more than 3 times higher compared with the activity of the strains grown separately in the nutrient medium without fats for 48 h (Fig. 1). The maximum lipase activity

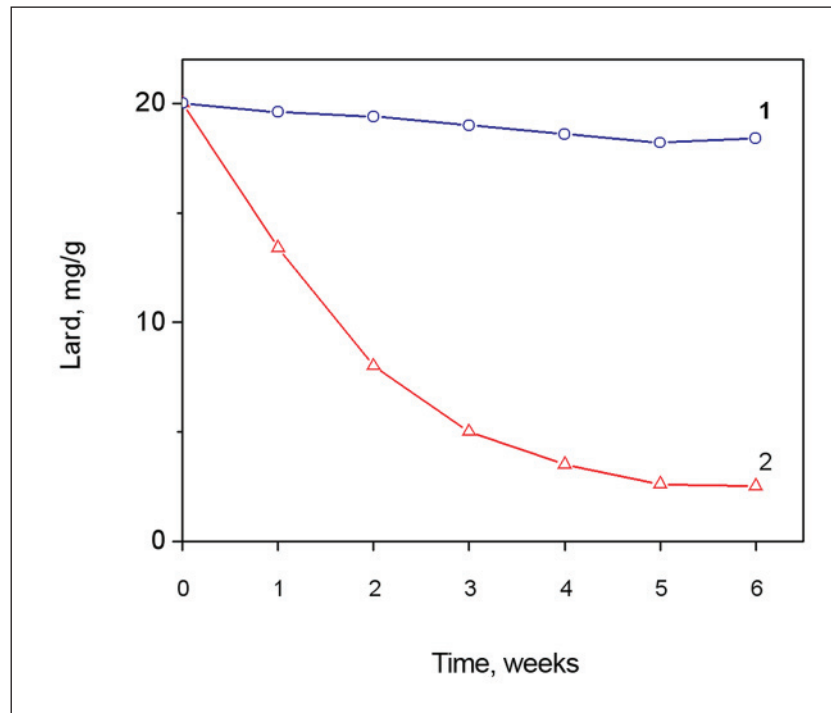


Fig. 5. Degradation of lard in black soil with a mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3. 1 – indigenous microbes (control), 2 – mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3

(1.87 U/ml) was detected after 24 h, and then it continuously decreased. It is obvious that the high lipase production during the first 24 h of cultivation resulted in an intensive hydrolysis of sunflower oil (Fig. 4). After 24 h of cultivation, sunflower biodegradation products were free fatty acids, monoglycerides and diglycerides. Later, the glycerides were hydrolysed to fatty acids which were also metabolized by the bacterial cultures.

Overall, in the final phase of the investigation, the decrease in the number of bacteria as well as in biodegradation rate and lipase activity may be explained by the substrate depletion, the inhibitory effect of the metabolites and a remarkable pH decline. The medium pH decreased even to a value of 3.8 after 72 h of cultivation. A similar correlation between the acidification of the medium and lipid degradation rate was reported in the literature [10]. The authors suggested that changing the medium buffering capacity and pH control would be required for an effective lipid degradation by bacteria.

Fat degrading microorganisms sometimes lose their ability to degrade fats in open treatment systems due to the effects of indigenous microorganisms [5]. For the practical application of the bacterial mixture to FOG-contaminated environment treatment, lard biodegradation by a bacterial mixture was investigated under non-sterile conditions; the results are presented in Fig. 5. As is seen

from the lard degradation profile, in the first two weeks of the investigation the reduction of lard concentration was most intensive and had an almost linear character. During this period, a mixed culture of *E. aerogenes* E13 and *Arthrobacter* sp. N3 strains utilized about 60.0% of the lard added. Later, the rate of lard degradation in soil gradually got slower, similarly to the sunflower oil degradation by a mixed culture in an aqueous medium; however, after six weeks the concentration of lard in samples treated with the mixed culture was reduced to 2.5 mg/g, i. e. 87.5% of the lard was degraded. In comparison, indigenous soil microorganisms degraded only 8.0% of lard during the same period. Therefore, treatment with a mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 enhanced lard removal from soil about seven times.

Our experimental results indicate that the selected mixed culture of strains *E. aerogenes* E13 and *Arthrobacter* sp. N3 actively degraded vegetable and animal fats in both aqueous and solid systems. So, bacterial strains can be regarded as effective biological agents applicable to cleaning the environment contaminated with FOGs. Moreover, a mixed bacterial culture can be used for the treatment of environments simultaneously contaminated by lipids and petroleum hydrocarbons, since *Arthrobacter* sp. N3 strain is also known as an active petroleum hydrocarbon degrading bacterium [22].

CONCLUSIONS

1. A total of 124 microorganisms were screened for the ability to degrade lipidic compounds. Strains UP2, F2, E13, K11 and N3 were found to degrade sunflower and olive oil, lard and tallow most intensively.

2. Strains UP2, F2, E13, K11 and N3 were studied for lipase activity and for the capability of degrading saturated (palmitic and stearic) and unsaturated (oleic and linoleic) fatty acids and triglycerides formed from the acids. Strains E13 and N3 showed the highest lipase activity and degradation rates. The strains were identified as *Enterobacter aerogenes* E13 and *Arthrobacter* sp. N3.

3. Sunflower oil degradation by a mixed culture of *E. aerogenes* E13 and *Arthrobacter* sp. N3 strains was investigated in a mineral medium supplemented with 0.5% of oil. The results of the investigation showed that sunflower oil degradation products were free fatty acids, monoglycerides and diglycerides which were consumed during the cultivation.

4. The results of lard degradation in black soil by a mixed culture of *E. aerogenes* E13 and *Arthrobacter* sp. N3 strains indicated that the mixed bacterial culture enhanced lard biodegradation about 7 times in comparison with indigenous soil microorganisms.

5. A mixed culture of *E. aerogenes* E13 and *Arthrobacter* sp. N3 strains may be used for an effective reduction of FOGs in a complex cleaning technology.

ACKNOWLEDGEMENTS

This research is related to the Eureka project E!3726 supported by the Agency for International Science and Technology Development Programmes in Lithuania.

Received 11 August 2009
Accepted 3 November 2009

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RIEBALUS SKAIDANČIŲ MIKROORGANIZMŲ, TINKANČIŲ APLINKAI VALYTI NUO RIEBALŲ, ATRANKA

Santrauka

Siekiant surasti mikroorganizmus, kuriuos būtų galima pritaikyti kompleksinėje riebalinių atliekų utilizavimo technologijoje, buvo patikrintas 124 kamienų gebėjimas skaidyti riebalinius substratus. Nustatyta, kad UP2, F2, E13, K11 ir N3 kamienai greičiausiai skaidė saulėgrąžų ir alyvuogių aliejų, kiaulienos taukus bei jautienos lajų, o efektyviausi buvo E13 ir N3 kamienai, kurie pasižymėjo aukščiausiu lipazės aktyvumu bei intensyviausiai skaidė sočiąsias (palmitino ir stearino) ir nesočiąsias (oleino ir linolo) riebalų rūgštis bei iš jų sudarytus trigliceridus. Šie kamienai buvo gauti iš UAB „Biocentras“

mikroorganizmų kolekcijos ir yra identifikuoti kaip *Enterobacter aerogenes* E13 ir *Arthrobacter* sp. N3. Mišrią *E. aerogenes* E13 ir *Arthrobacter* sp. N3 kamienų kultūrą auginant mineralinėje terpėje su 0,5 % aliejaus nustatyti šie aliejaus hidrolizės produktai: riebalų rūgštys, monogliceridai ir digliceridai. Be to, pastebėta, kad bakterijos šiuos hidrolizės produktus naudojo augimui. Tyrimo rezultatai, gauti kiaulienos taukus juodžemyje skaidant su mišria *E. aerogenes* E13 ir *Arthrobacter* sp. N3 kamienų kultūra, rodo, kad ši mišri bakterijų kultūra riebalus skaidė apie 7 kartus intensyviau nei vietiniai dirvožemio mikroorganizmai (kiaulienos taukų koncentracija dirvožemyje per 6 savaites sumažėjo atitinkamai 87,5 ir 8,0 %). Taigi mišrią *E. aerogenes* E13 ir *Arthrobacter* sp. N3 kamienų kultūrą galima taikyti kompleksinėje riebalinių atliekų šalinimo technologijoje.

Raktažodžiai: *Enterobacter*, *Arthrobacter*, lipazė, riebalų skaidymas