Biological Utilisation of Fatty Waste – Initial Laboratory Examination

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Summary

This article presents the process of biodegradation of aliphatic substances in oily bleaching soil. For the experiment, oily bleaching soil from the NZPT (a fat-processing plant) from the town of Brzeg was used. Its characteristic features were a high aliphatic substance content and low (3.8-4.5) pH. By means of experiment three composites of bacteria and yeast were prepared with the use of field and library strains. In a 44-day process, the mixture of field and library strains caused the aliphatic substance content in the oily bleaching soil to drop by 69-71%. The application of bacterial and yeast strains proved efficient for the aliphatic low-pH waste.

Key words:
biodegradation, oily bleaching soil, aliphatic substances, autochtonous (field) microflora.

1. Introduction

In the recent years, research has been carried out on industrial waste degradation with the use of microorganisms (1,2,15). It usually concerns waste of pharmaceutical, chemical, petrochemical and food industries (2,7,8,16). In the process of degradation, enzymatic capability of selected microorganisms is used to break multimolecular organic compounds down to simple, environment friendly substances. The microorganisms used for biodegradation are usually obtained by selection and initial adaptation (4).

A serious problem for the environmental protection is food industry waste, especially by-products of fat processing. The waste is

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loaded with water-insoluble substances, such as fats, fatty acids, aldehydes and ketones, which form suspension or emulsion.

It is particularly difficult to cultivate oily bleaching soil (OBS) – waste created in the process of refining vegetable oil. It consists mainly of bentonite, aliphatic substances and vegetable dye (6,10,11).

Vegetable oil content in the OBS ranges from 3 to 35%, depending on filtration and storage methods. The oil found in the OBS is usually rape oil. The OBS is found in the form of loose, oily mass, or after placing it in sedimentation basins, it may form oily silt, impermeable to water and air. This feature of the OBS blocks its degradation processes in sedimentation basins and slows them on its surface. Oily bleaching soil stored in sedimentation basins pollutes the atmosphere and becomes a potential source of contamination of ground waters and soil.

Considering the large scale of the problem it seems useful to work out a method of microbial utilisation of aliphatic substances contained in the OBS.

This paper presents microorganism selection methods suggested for biological utilisation, as well as the results of biodegradation of the oily bleaching soil fatty waste.

2. Research Material and Methods

2.1. Oily bleaching soil (OBS)

The OBS was obtained from the Fat Processing Factory in Brzeg, where it had been stored in four non-isolated 300-400 m^2 sedimentation basins, 3.5-4.5 metres deep. Averaged samples were prepared for the experiment: from sedimentation basin no. 2 surface material was mixed with material gathered from the depths of ca. 1, 2, 3 and 4.5 m, and from sedimentation basin no. 3 superficial OBS was mixed with OBS taken from the depths of ca. 1, 2, 3 and 3.5 m.

2.2. Microbial composites used for the research

Three microbial composites – A, B and C – were used for the examination.

A. Composite A was prepared with bacterial and yeast strains taken from the culture maintained by the Department of Microbiology and Biotechnology of the Opole University. The strains were selected according to their lipolytical activity, especially the ability to hydrolyse fats and oxidise fatty acids (3,5,12,16,19,26). Then the strains were cultured on nutritious agar at 30°C for 48 hrs. Next, they were sowed onto nutrients for culturing lipolytic microorganisms, i.e. the nutrients containing Tween 80 and tributirine. This was done by the surface method. The incubation was conducted at 30°C and its duration was 72 hrs. Every 24 hrs visual assessment of the changes in the nutrient area around the sowed strains was conducted and then compared to sterile nutrients. On the nutrient containing Tween 80 the fat-degrading colonies were surrounded by a turbid zone, colonies on the nutrient containing tributirine were surrounded by a transparent zone. The area of both zones was propor-

tional to the lipolytic activity of the strain. Fourteen bacterial and yeast strains were tested. The assessment of their lipolytic activity is shown in Table 1. The analysis of the growth of selected strains proved that after three days of incubation the most active in lipase production were *Pseudomonas areuginosa, Pseudomonas fluorescens, Serratia marcescens* as well as *Bacillus subtilis* and *Bacillus macerans.* Also *Staphylococcus aureus, Azotobacter* sp., and *Streptococcus* sp. were characterised by good lipolytic properties. Among the selected yeast, the *Candida lipolytica* strain proved to be the best in fat degradation. Finally, after the elimination of potentially pathogenic species, 7 strains of bacteria and yeast were chosen for composite A. They included: *Pseudomonas fluorescens, Pseudomonas fragi, Candida lipolytica, Bacillus subtilis, Bacillus macerans, Streptococcus* sp. and *Azotobacter* sp. The selected strains were individually cultured in liquid broth with the addition of 1% glucose. They were incubated for 48 hrs. After the culturing period 20 ml of each strain were gathered to create composite A. It contained $9x10^7$ bacteria and yeast cells per 1 ml of the nutrient.

Table 1

The assessment of the efficiency of the library strains in the lipase production

The name of strain	Lipase secretion					
	Tween 80			Tributirine		
	after 24 hrs	after 48 hrs	after 72 hrs	after 24 hrs	after 48 hrs	after 72 hrs
Pseudomonas fluorescens	–	+	++	+	+	++
Pseudomonas fragi	+	++	+++	+	+++	+++
Candida lypolitica	+	++	++	–	+	++
Candida tropicalis	–	–	+	–	+	++
Serratia marcescens	+++	+++	+++	++	+++	+++
Micrococus luteus	–	–	–	–	–	+
Staphylococus aureus	+	++	+++	–	+	++
Proteus vulgaris	+	+	+	–	–	–
Bacillus subtilis	+	++	++	+	++	+++
Azotobacter sp.	++	++	++	+	++	++
Bacillus coagulans	+	+	++	+	++	++
Bacillus macerans	+	++	++	+	++	+++
Streptococus sp.	+	+	++	+	++	++
Pseudomonas areuginosa	+++	+++	+++	+++	+++	+++

– no fat degradation, + weak fat degradation, + + good fat degradation, + + + very good fat degradation.

B. Composite B was composed of strains isolated from averaged OBS samples. 10 g of the OBS were added to 90 ml of physiological salt. Next, the suspension was shaken for 60 min at room temperature and then filtred through filtre-paper. The microorganisms obtained from the filtrate were further culured in mineral nutrient containing 0,1% emulsified

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vegetable oil. Incubation was conducted for 15 days at room temperature. In the filtrate containing OBS mainly *Bacillus subtilis, Pseudomonas* sp., *Nitrosomonas* sp. and *Pseudomonas denitrificans* were found. After the culturing period 300 ml of the culture was sampled to create composite B. It contained $1x10^7$ bacteria cells per 1 ml of nutrient.

C. Composite C was made up of library strains used for composite A and strains isolated from the OBS samples (composite B). After culturing, equal quantities of these strains were mixed until the capacity of 300 ml was obtained. Composite C contained $5x10^7$ bacteria and yeast cells per 1 ml of the nutrient.

2.3. Nutrients used for the microbial cultures

To culture the microorganisms the following nutrients were applied:
A. Mineral nutrient:

$(NH_4)_2SO_4$	– 2 g
K_2HPO_4	– 3 g
KH_2PO_4	– 2 g
$MgSO_4 \times 7H_20$	– 0.5 g
Distilled water	– 1000 ml

1 g/1000 ml of rape oil was added as the source of carbon.

B. Nutrient A with 100 g of tributitine, solidified with 2% agar.

C. Nutrient A with 10% Tween 80 added, solidified with 2% agar.

D. Nutritious agar.

E. Yeast – Pepton – Glucose agar.

F. Broth with 1% glucose added.

2.4. The examination design and the usage of microbial composites

100 ml of mineral nutrient (A) without oil was placed in each of the 24 flasks used for the examination, each having the capacity of 300 ml. 30 g of the OBS from sedimentation basin no. 2 were added to 12 flasks, 4 of which were inoculated with 5 ml of composite A, 4 with composite B and the remaining 4 with composite C. 12 flasks containing OBS from sedimentation basin no. 3 were prepared in a similar manner. Additionally, 2 check sets (4 flasks for sedimentation basin no. 2 and 4 for basin no. 3) were prepared. Their composition was analogous but they were not inoculated with the microbial cultures. The culture was maintained for 44 days at room temperature in aerified sets.

2.5. The assessment of the effects of bioutilisation

The effect of the utilising action of the microorganisms was assessed microbiologically and chemically on the 14^{th}, 24^{th}, 34^{th} and 44^{th} day of the experiment.

2.5.1. The microbiological assessment embraced:

A. The determination of the total number of bacteria and yeast by the tabular method according to norm PN-75C-04615/03 (17).

B. The identification of bacteria and yeast by the microscopic, macroscopic and biochemical methods using the mini-API analyser.

2.5.2. The chemical assessment embraced:

A. The determination of aliphatic substance content according to norm PN-76R-640753 (18).

B. The gravimetric determination of the total organic substance content after the intitial extraction with chloroform.

C. The determination of the acidity level after the dissolution of fat in an organic solvent and after the titration of the obtained solution with the potassium hydroxide solution.

D. The measurment of the pH level with the CP-315 pH-metre cooperating with the silver-chloride electrode of the EsAgP-301W type.

3. Results and Discussion

3.1. The assessment of the biodegradation of organic compounds by selected microorganisms

Figure 1 presents the reduction of the total organic substance content obtained during the experiment.

The data from Figure 1 show that in all the examination sets the organic substance content was reduced. The highest reduction level was observed in the two sets containing composite C. In the set containing OBS from sedimentation basin no. 2 the reduction was 52.9% and in the set containing the OBS from sedimentation basin no. 3 it was 59%. In the sets with composite B containing the bacterial strains isolated from the OBS the reduction of the organic substance was 52.3% for the set with the OBS from sedimentation basin no. 2, and 48.9% for the set containing the OBS from sedimentation basin no. 3. Considerably smaller reduction took place in the sets containing composite A with the li-

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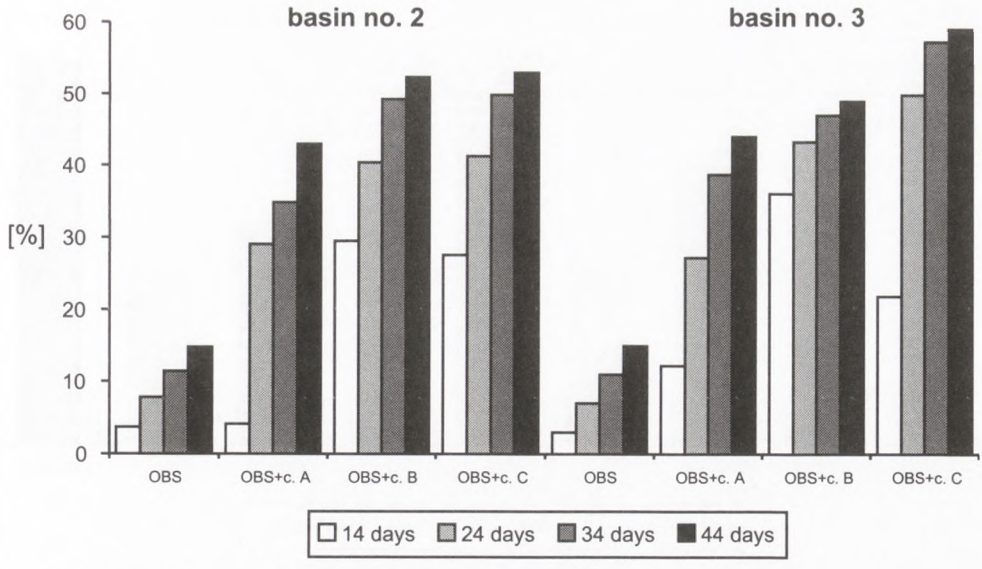

-c. A – composite A; -c. B – composite B; -c. C – composite C

Fig. 1. The organic substance reduction in the OBS in the consecutive days of biodegradation.

brary strains. The organic substance content dropped by 42.9% for the set containing the OBS from sedimentation basin no. 2. For the set containing the OBS from set no. 3 the organic substance content dropped by 44%. In the check set, on the other hand, the organic substance content reduction was 14% for the set containing the OBS from basin no. 2, and 15% for the one containing the OBS from basin no. 3.

3.2. The assessment of the aliphatic substance biodegradation by selected microorganisms

In parallel to the decreasing organic compound content, the aliphatic substance content was also reduced. The results are shown in Figure 2.

The greatest reduction of the aliphatic substance content was observed in all the sets during the first 24 hrs of the experiment. The greatest reduction was ascertained in the sets containing composite C. In the set containing the OBS from sedimentation basin no. 2 the reduction reached 69.4% and in the set containing the OBS from basin no. 3–71.6%. In the sets containing composite A with the library strains, the aliphatic substance content dropped by 55.1% in the case of the OBS from sedimentation basin no. 2 and 43.2% in the case of the OBS from sedimentation basin no. 3. The reduction of the aliphatic substance content was the smallest in the sets containing composite B with the bacteria strains isolated from the OBS. The aliphatic substance content dropped in them by 34.7% and 31.6% in the case of the OBS from sedimentation basins no. 2 and 3 respectively.

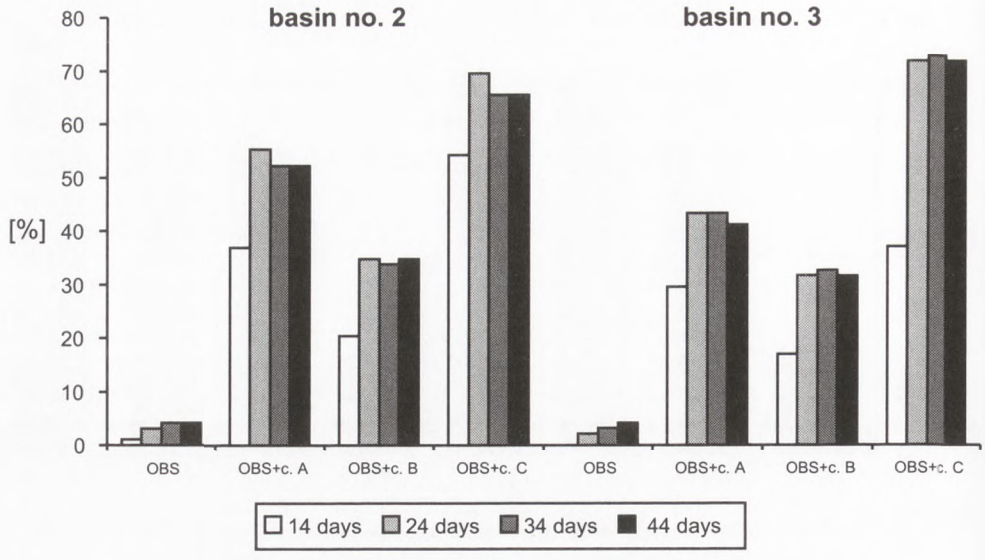

Fig. 2. The aliphatic substance reduction in the OBS in the consecutive days of biodegradation.

3.3. The number of microorganisms during the aliphatic substance biodegradation process

The number of microorganisms in the examined sets were determined by the total number of bacteria in 1 cm^3 of the culture. The results are presented in Tables 2 and 3.

The present data prove that in all the sets the total number of bacteria and yeast dropped during the first 14 days of the process. This might have been caused by the low pH (3.8-4.5) which remained such throughout the experiment and possibly hindered the growth of the microorganisms. The change in the pH in the examination sets is presented in Table 4. Such low pH was caused by the degradation of vegetable oil to fatty acids, which increased the acidity of the environment. The changes in the acidity levels in particular examination sets are presented in Figure 3. Although the increase of the acidity level did not cause the microorganism count to drop, it did not have a considerable influence on the biodegradation, which was the most intensive in the first fourteen days of the process (Figure 1 and 2). In the subsequent days of the experiment the acidity level in both the sedmentation basins remained on a level higher than in the OBS sample without the composite (Figure 3).

The acidity level was gradually decreasing, which along with the above mentioned data indicates that the process of aliphatic substances biodegradation did take place. The presence of the *Candida lipolytica* strain in composite A and C allowed for an efficient biodegradation of the aliphatic substances in the low pH of the OBS. The research conducted by Rymowicz et al. (19,20) confirms that the yeast of the *Candida* kind is necessary for the biode-

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gradation of vegetable fats at low pH. In the quoted research it was discovered that the amount of yeast grew comparatively rapidly at pH 3.5 and 5.5 however, the biomass efficiency was lower in the former case.

Table 2

Quantitative microbiological examination in the consecutive days of biodegradation

Examination	Sedimentation basin	Total quantity of bacteria (units/cm^3)				
		The duration of biodegradation (in days)				
		1	14	24	34	44
OBS	2	0.4×10^3	0.6×10^3	0.4×10^3	0.1×10^3	0.1×10^3
OBS+c. A[1]	2	150.0×10^3	11.7×10^3	120.0×10^3	197.4×10^3	238.0×10^3
OBS+c. B	2	17.0×10^3	6.7×10^3	10.0×10^3	4.0×10^3	34.0×10^3
OBS+c. C	2	70.0×10^3	46.7×10^3	77.0×10^3	60.0×10^3	177.7×10^3
OBS	3	0.8×10^3	0.1×10^3	0.6×10^3	1.2×10^3	0.9×10^3
OBS+c. A	3	150.0×10^3	5.5×10^3	3.6×10^3	7.3×10^3	272.7×10^3
1	2	3	4	5	6	7
OBS+c. B	3	17.0×10^3	5.1×10^3	11.0×10^3	11.3×10^3	40.1×10^3
OBS+c. C	3	70.0×10^3	15.1×10^3	7.6×10^3	10.6×10^3	176.7×10^3

[1] – c. A – composite A

Table 3

Quantitative microbiological examination in the consecutive days of biodegradation

Examination set	Sedimentation basin	Total quantity of yeast (units/cm^3)				
		The duration of biodegradation (in days)				
		1	14	24	34	44
OBS	2	0.4×10^3	1.4×10^3	1.2×10^3	0.2×10^3	0.4×10^3
OBS+c. A	2	150.0×10^3	63.3×10^3	3.7×10^3	10.8×10^3	172.8×10^3
OBS+c. C	2	70.0×10^3	63.3×10^3	6.7×10^3	11.3×10^3	175.8×10^3
OBS	3	0.8×10^3	2.0×10^3	0.7×10^3	0.4×10^3	0.1×10^3
OBS+c. A	3	150.0×10^3	10.9×10^3	10.9×10^3	10.3×10^3	228.0×10^3
OBS+c. C	3	70.0×10^3	24.0×10^3	10.2×10^3	10.1×10^3	72.3×10^3

Table 4

pH during biodegradation

Examination set	Sedimentation basin	pH in the sets				
		The duration of biodegradation (in days)				
		1	14	24	34	44
OBS	2	4.2	4.3	4.3	4.2	4.2
OBS+c. A	2	4.2	4.3	4.1	4.1	4.0
OBS+c. B	2	4.3	4.5	4.1	4.0	4.0
OBS+c. C	2	4.3	4.5	4.5	4.4	4.1
OBS	3	4.1	4.2	4.2	4.1	4.1
OBS+c. A	3	4.1	4.1	4.2	4.0	3.9
OBS+c. B	3	4.2	4.5	4.1	4.1	4.0
OBS+c. C	3	4.2	4.2	4.1	3.9	3.8

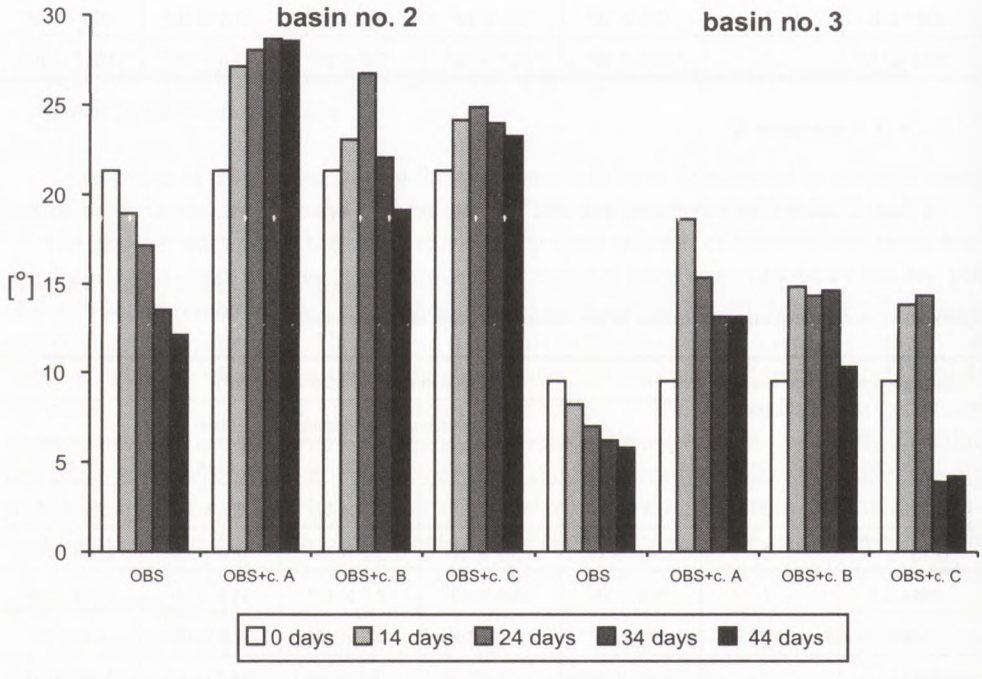

Fig. 3. Changes in the acidity level in the consecutive days of biodegradation.

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After the period of adaptation of the microorganisms to the unfavourable conditions, their number was growing irregularly but gradually in all the cultures. The largest growth of bacteria cells as compared to their number in the first day of the experiment was observed in the set with composite C. In the sets containing the OBS from sedimentation basin no. 3 with composite C, the number of bacteria on the 44^{th} day of the experiment increased from 70×10^3 units/cm^3 to ca. 177×10^3 units/cm^3. In the case of the applied composites A and B, the growth in the number of bacteria was smaller and amounted from 17×10^3 units/cm^3 to ca. $35\text{-}40 \times 10^3$ units/cm^3, and from 150×10^3 units/cm^3 to ca. $240\text{-}270 \times 10^3$ units/cm^3 for sedimentation basins no. 2 and 3 respectively (Figure 2). As the culturing process progressed, it was observed that the bacilli of the *Bacillus* kind began to dominate over other identified bacteria. This could have been caused by their acidity resistance (3). In the case of yeast, after an initial drop in its quantity the growth began only after the 34^{th} day of the experiment. On the 44^{th} day of incubation its quantity was comparable to that of bacteria (Figure 3). Similarly to bacteria, also in the case of yeast the most rapid growth of its cells was observed in the sets with composite C. As regards composite A, it was only for the OBS from basin no. 3 that a larger growth was noted.

4. Results and Conclusions

1. After 44 days of the experiment the applied composites caused the aliphatic substance content to drop by 30% and the organic substance by 40%.

2. It was composite C, which was a mixture of library and field strains, that helped to achieve the best results in the reduction of organic compounds (53-59%) and aliphatic substance (69-71%).

3. To utilise low pH aliphatic waste it is necessary to use a mixture of bacterial and yeast strains.

4. The results of the experiment indicate the possibility of utilising the aliphatic compounds with the use of adequately selected microbial strains.

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