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New Biocatalyst with Multiple Enzymatic Activities for Treatment of Complex Food Wastewaters

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Summary

The cells of filamentous fungus *R. oryzae* entrapped in the polyvinyl alcohol cryogel are capable of producing various extracellular hydrolytic enzymes (proteases, amylases, lipases) and are used for the treatment of complex wastewaters of food industry. Five types of media simulating the wastewater of various food enterprises were treated under batch conditions for 600 h. Fats containing mostly residues of unsaturated fatty acids, as well as casein, glucose, sucrose, starch, soybean flour and various salts were the main components of the treated wastewaters. The immobilized cells concurrently possessed lipolytic, amylolytic and proteolytic activities. The level of each enzymatic activity depended on the wastewater content. The physiological state of immobilized cells was monitored by bioluminescent method. The intracellular adenosine triphosphate (ATP) concentration determined in the granules with immobilized cells was high enough and almost constant for all the period of biocatalyst application confirming thereby the active metabolic state of the cells. The study of mechanical strength of biocatalyst granules allowed revealing the differences in the values of modulus of biocatalyst elasticity at the beginning and at the end of its use for the wastewater treatment. The decrease in chemical oxygen demand of the tested media after their processing by immobilized biocatalyst was 68–79 % for one working cycle.

Key words: immobilized fungus cells, polyvinyl alcohol cryogel, wastewater treatment

Introduction

The treatment of food industry wastewater represents a great problem. The volumes of wastewaters usually obtained per each tonne of product in various fields of food industry are as follows (in m^3/t): 1.7 in milk-processing, 2 in cheese making, 15 in meat processing, 7 in brewing and 8 in fish processing (*1*). The main components of such wastewaters are proteins (up to 0.5 %), fats (up to 3.8 %), carbohydrates (up to 1.2 %) and salts (totally up to 10 %) (*2–5*). In fact, they are present in various ratios. *Rhizopus* sp. is capable of concurrent secretion of different hydrolytic enzymes (lipases, amylases, proteases, *etc.*) and the composition of the extracellular polyenzymatic complexes usually varies depending on the content of nutrient medium (*6–9*).

The approach enabling improvement of the treatment of food industry wastewaters is based on the use of fungus cells and wastewaters as media for cultivation of the microorganisms producing hydrolytic enzymes (*10*). On the one hand, this approach allows the hydroly-

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sis of the components of complex media, and on the other hand, it supplies the microorganism with products of hydrolysis as nutrition sources, necessary to maintain its viability.

It is known that immobilization of filamentous fungi notably decreases the negative influence of metabolites and some wastewater components on the mycelium (*11,12*). As a result of that, a prolonged effective exploitation of immobilized biocatalyst can be realized. The reuse of immobilized cells in the same process allows to overcome a problem of utilization of accumulated biomass, which arises with the use of free cells. According to the literature data, polyvinyl alcohol (PVA) is the best carrier for the immobilization of cells used for the treatment of various wastewaters (*13–15*).

Earlier we showed the successful treatment of wastewaters of fat-and-oil industry by a biocatalyst prepared using *Rhizopus oryzae* cells immobilized in PVA cryogel (*16*). The biocatalyst hydrolyzed 92–98 % fats and provided up to 70 % decrease in chemical oxygen demand (COD) of wastewaters for one working cycle. The purpose of the work is to investigate the bioremediation of five various models of complex food industry wastewaters. It was necessary to estimate the state of the biocatalyst, the duration of its possible use and an efficiency of its exploitation for such wastewater treatment.

Materials and Methods

Cultivation and immobilization of fungus cells

Fungus spores of *Rhizopus oryzae* F-814 were accumulated on potato dextrose medium containing (in g/L): glucose 20, $MgSO_4$ 0.2, $CaCO_3$ 0.2, potato 200, and agar 20. Granules of immobilized biocatalyst were obtained by entrapment of fungus cells in the PVA cryogel according to the previously patented procedure (*16*). PVA (99–100 % hydrolyzed, M_r=86 000) was purchased from Acros Organics (Belgium).

The batch cultivation of immobilized cells was carried out in Erlenmeyer flasks with 60 mL of each type of model wastewater on a shaker Lab-Therm (Adolf Kühner, Switzerland) under aerobic conditions with constant agitation at 180 rpm and 28 °C. The media were refreshed after each working cycle (on average 30 h).

The wastewater treatment with the immobilized biocatalyst was carried out under batch conditions in the bioreactor Biostat (1 L; B. Braun Melsungen AG, Germany) at pH=6.0 and 28 °C. The content of model wastewaters is presented in Table 1. All media were prepared using 100 mM potassium-phosphate buffer with 1.7 g/L of NH_4Cl and 0.25 g/L of $FeCl_3 \cdot 6H_2O$. Starch and glucose were employed as carbohydrate sources, and the sum of their concentrations was represented as the concentration of carbohydrates. Each medium was replaced by a fresh one each working cycle.

Analytical methods

The concentration of biomass and the levels of extracellular hydrolytic (lipolytic, amylolytic and proteolytic) activities were controlled throughout the process of biocatalyst exploitation. The specific activity was related to 1 g of dry biomass. The amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate (tributyrin, Vekton, Russia) for 1 min at 40 °C and pH=6.5 was accepted as a unit of lipolytic activity (*6*). The amylase activity was determined by colorimetric method with the use of standard reagent (Ekoservis, Russia). One unit of amylolytic activity was defined as the amount of enzyme catalyzing the production of 1 mg of dextrin per minute as the result of enzymatic hydrolysis of 1 % starch at 30 °C and pH=4.7.

Concentrations of D-glucose were assayed by the enzymatic methods using the glucose oxidase-peroxidase kit (Impakt, Russia). One unit of glucoamylolytic activity was defined as the amount of enzyme that catalyzed the production of 1 mg of glucose per minute as the result of enzymatic hydrolysis of 1 % starch solution at 30 °C and pH=4.7. The protease activity was determined according to the standard method using Folin's reagent (*17*). The protein concentration in all samples was controlled using the Bradford method. To control starch concentration, the absorption of starch-iodine complex was registered on an Agilent 8453 UV-Visible spectroscopy system (Germany) at 580 nm.

The concentrations of lipids were determined by gravimetric method (*18*). COD was detected using bichromatometric method (*19*) on a COD Reactor (Hach, USA). The concentration of immobilized cell biomass was controlled using bioluminescent method of determination of intracellular adenosine triphosphate (ATP) concentration. The latter parameter was analyzed in immobilized fungi by bioluminescent method with luciferine-luciferase reagent (Lumtek, Russia). To extract ATP from the free fungus cells, an aliquot (0.1 mL) of cell suspension in culture medium was centrifuged (Beckman J2-21 centrifuge, USA) at 5000 rpm for 10 min, then biomass precipitate was weighed and treated with 1 mL of dimethylsulphoxide (DMSO) for 5 min. ATP was extracted from the immobilized cells by the treatment of

Table 1. The content of model wastewaters used for immobilized biocatalyst treatment

No.	Model media of food productions	γ/(g/L)				
		lipids	carbohydrates	proteins	$MgCl_2 \cdot 6H_2O$	$CaSO_4 \cdot H_2O$
1	Starch processing	5.0	50.0	20.0	0.9	0.1
2	Meat processing	10.0	35.0	1.0	6.0	4.3
3	Wastewater from abattoirs	8.0	30.0	20.0	1.3	1.0
4	Mixed dairy processing	21.0	0.1	0.5	5.1	3.2
5	Soybean processing factory	0.5	0.1	0.9	0.5	0.5

weighed granules (up to 300 mg) with 1 mL of DMSO for 5 min. The cell extracts (50 μL) were added to the microcuvettes with aliquots of luciferase reagent (50 μL), and the bioluminescence intensity was measured on a microluminometer 3560 (New Horizons Diagnostic, USA). The precise ATP concentrations in the tested samples were calculated using calibration plot, graphed in accordance with a known approach (*20*) and determining the correlation between the ATP concentration and dry cell biomass.

The modulus of elasticity (on compression) of the biocatalyst containing immobilized spores and mycelium was determined using standard methods (*21*).

Results and Discussion

An opportunity of effective and long-term use of the biocatalyst prepared using *R. oryzae* cells immobilized in PVA cryogel for the biological treatment of model wastewaters of food industries containing various complex mixtures of proteins, fats and carbohydrates was investigated. The study of kinetics of immobilized biomass accumulation in all tried model wastewaters allowed revealing the growth of immobilized fungus cells for the first 100 h of cultivation (Fig. 1). It was shown that the growth intensity of immobilized cells strongly depended on the composition of model wastewaters, and the lower protein concentration in the medium provided lower levels of immobilized biomass accumulation. It is necessary to note that there was no mycelium outlet from the biocatalyst granules throughout the period of biocatalyst exploitation.

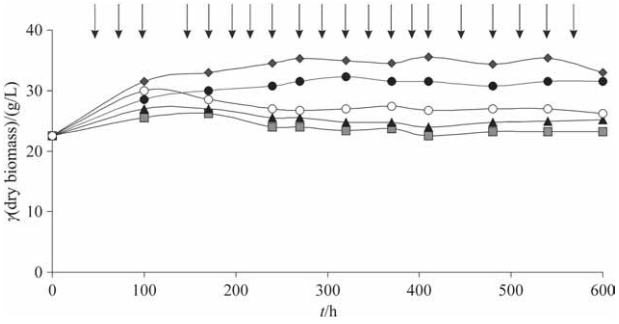

Fig. 1. Biomass of immobilized cells during the long-term treatment of model food industry wastewaters: ◆ – 1, ■ – 2, ▲ – 3, ● – 4, ○ – 5 (samples were numbered according to the data from Table 1). The replacement of each nutrition medium by fresh one is marked by the arrows

The extracellular enzymatic activities determined in the culture media depended on the nutrition source for immobilized cells mostly present in the media (Fig. 2). Therefore, in medium no. 4, containing the maximum concentration of fats (Table 1), the highest lipolytic activity was established. The top level of glucoamylolytic activity was shown in medium no. 1. Obviously, it was caused by the highest starch concentration (50 g/L) in that model wastewater. Along with that, the presence of all four controlled enzymatic activities of hydrolytic enzymes secreted by the immobilized cells was discovered in all tested media.

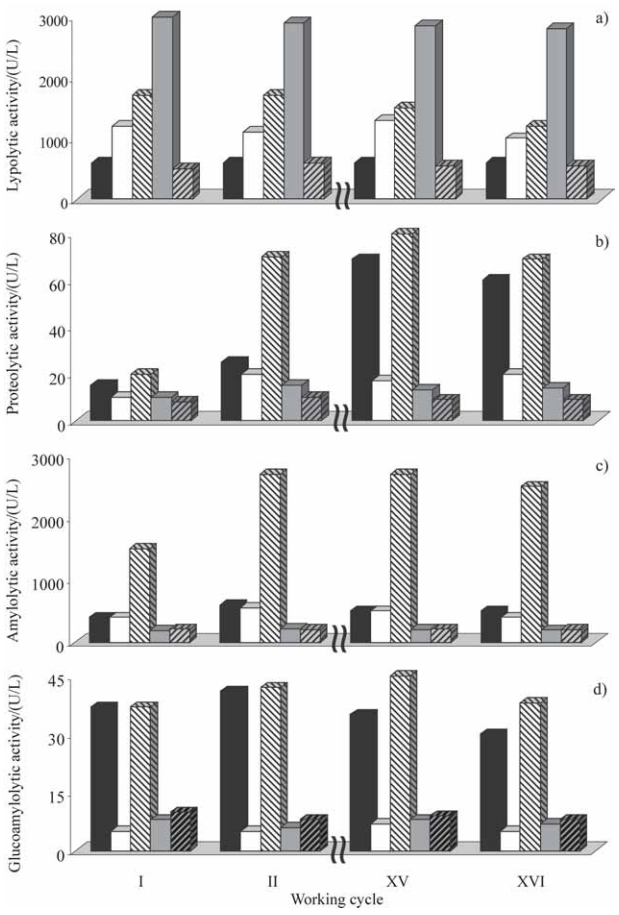

Fig. 2. Hydrolytic activity of enzymes: (a) lipase, (b) protease, (c) amylase and (d) glucoamylase, determined in the wastewaters during the biocatalyst exploitation (numeration of wastewater samples corresponds to Table 1: ■ – no. 1, □ – no. 2, ▨ – no. 3, ▩ – no. 4, ▨ – no. 5)

Analysis of energetic status of immobilized mycelium localized inside the biocatalyst granules showed that cells possessed a high level of intracellular ATP concentration throughout the biocatalyst exploitation (Table 2). The values of modulus of elasticity determined for the granules with immobilized spores and with the mycelium taken at different stages of its use for the treatment of model wastewaters allowed to establish the essential influence of the medium content on the mechanical characteristics of the granules (Table 2). Thus, the inflexibility of granules after their long-term use in the wastewater no. 1 was 3 times higher than in the medium no. 4, initially containing notably higher fat concentration. In spite of the differences in the mechanical strength of the granules, all values of the modulus were above 10 kPa and therefore acceptable for long-term exploitation of immobilized biocatalysts.

Changes in the pH of model wastewaters were observed during their treatment with the biocatalyst. Independently from the initial content of media there was a slow decrease in pH from 5.9 or 7.5 down to 4.2 or 5.7 in each working cycle (Table 3). The COD level and residual concentrations of proteins, carbohydrates and fats were controlled at the end of each working cycle (Table 3). A decrease of 90–100 % in the concentrations of the

Table 2. ATP concentrations (nmol/g dry biomass) in the granules of biocatalyst based on immobilized cells and modulus of elasticity (E/kPa) of biocatalyst after its exploitation for various working cycles*

Model media no.**	1st working cycle		3rd working cycle		10th working cycle		16th working cycle	
	ATP	E	ATP	E	ATP	E	ATP	E
1	467±5	60.3±2.3	459±9	69.2±6.4	481±8	77.1±6.1	476±5	79.6±3.9
2	456±4	59.8±3.3	438±7	56.2±3.4	447±7	44.4±5.5	432±6	37.7±7.4
3	483±8	58.7±6.3	401±5	54.3±5.5	423±4	50.1±6.6	415±10	46.3±4.5
4	489±9	56.4±6.5	433±12	30.1±5.0	430±10	29.9±8.9	422±11	26.0±4.2
5	447±7	58.5±9.5	462±13	62.8±9.2	481±11	77.1±6.1	456±7	75.2±5.1

*The initial value of the modulus of elasticity of all granules at the beginning of biocatalyst exploitation was the same and equal to (55.6±3.1) kPa
**Numeration of wastewaters corresponds to Table 1

Table 3. The COD level and the concentrations of the main components of the model wastewaters at the beginning and at the end of biocatalyst working cycle

Model media no.*	Before treatment		After treatment		γ(end of each working cycle)/(g/L)		
	γ(COD)/(g/L)	pH	γ(COD)/(g/L)	pH	lipids	carbohydrates	proteins
1	9.9±0.07	6.5	2.5±0.04	4.3	0.6±0.02	4.0±0.05	1.6±0.03
2	6.8±0.06	7.5	1.5±0.02	5.7	0.8±0.03	1.8±0.04	0
3	7.6±0.07	6.0	1.8±0.03	5.1	0.5±0.01	1.8±0.03	1.4±0.02
4	8.2±0.08	5.9	2.7±0.06	4.5	1.7±0.03	0	0
5	3.3±0.05	6.3	0.7±0.01	4.2	0	0	0

main controlled medium components was constantly observed, and also 60–68 % decrease in COD was revealed independently from the type of the tried wastewater.

Analysis of various biological systems used for food wastewater treatment by immobilized cells of microorganisms showed that there are a number of general approaches to the solution of the problem. The first one is aimed at the removal of oil-containing ingredients from the wastewaters, since fats are among the most difficult-to-degrade components of food wastes. The effectiveness of the treatment mainly depends on the type of microorganism used in the process. For example, the application of bacterial strains *Rhodobacter sphaeroides* NR-3 and *R. sphaeroides* S entrapped in calcium-alginate and agar gels, respectively, guaranteed the 3.0- to 3.5-fold decrease in the concentration of cooking oil in sewage wastes for 6 days under anaerobic conditions (*22*). The reduction of 39.4 % of the initial level of COD was registered after biological treatment of wastes by immobilized cells that was conducted for 39 days. The efficiency of the removal of lipid components, demonstrated in our work with application of immobilized cells of filamentous fungi under aerobic conditions, was much higher compared to the discussed results, since the aerobic conditions allow intensifying the biodegradation of organic matter and the fungus cells possessed higher lipolytic activity as compared to photosynthetic bacteria.

It is known that some microorganisms (especially immobilized filamentous fungi), which are used for the effective treatment of complex food wastewaters (*23,24*), should be supplied with special nutritional additives to provide a significant decrease in COD (85–100 %). Contrary to that, the *R. oryzae* cells used in our work in immobilized form did not manifest the same needs and could degrade the organic matter in the presence of a narrow set of salts regularly present in the wastewaters (Table 1).

The character of the cell carrier is another important factor influencing the efficiency of the application of immobilized cells for the food wastewater treatment (*25*). The low porosity of carrier structure sometimes demands an introduction of emulsifiers (anionic detergents) to the oil-containing wastes (*26*) or their multiple dilutions before the treatment (*27*). Such necessity was not revealed when the macroporous PVA cryogel was applied as immobilizing carrier for the fungus cells in this work.

Conclusion

The long-term use (at least for 600 h) of the biocatalyst developed using *R. oryzae* cells immobilized in the PVA cryogel for the treatment of complex wastewaters containing proteins (up to 20 g/L), fats (up to 21 g/L), carbohydrates (up to 50 g/L) and salts (up to 10.3 g/L) was demonstrated. The constant level of intracellular ATP concentrations of immobilized cells testifies to their high metabolic activity throughout the operation time of the biocatalyst. Application of biocatalyst enabled 2.5- to 3.0-fold decrease in COD within one working cycle. It should be noted that fats and oils present in the food industry wastewaters usually inhibit the metabolism of

activated sludge widely used for the biological treatment of food industry wastes, since the hydrophobic film that covers its surface notably impairs mass transfer conditions and thereby shortens the half-life of activated sludge functioning (*26*,*28*). The pretreatment of wastewaters with the biocatalyst should allow to essentially reduce the concentrations of substances with negative effect or completely eliminate them, and therefore the efficiency of biological treatment of wastewaters could be improved in general, since the application of immobilized biocatalyst aims at the increase in efficacy and duration of activated sludge operation.

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