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Full paper

Biodiesel fuel production by *Aspergillus niger* whole-cell biocatalyst in optimized medium

Nailya R. Almyasheva [a], Maria I. Shuktueva [a, b], Daria A. Petrova [a, c], Dmitry S. Kopitsyn [a], Mikhail S. Kotelev [a], Vladimir A. Vinokurov [a], Andrei A. Novikov [a, *]

[a] Gubkin University, 65-1, Leninsky Prospect, Moscow, 119991, Russia
[b] Gause Institute of New Antibiotics, 11, Bolshaya Pirogovskaya, Moscow, 119867, Russia
[c] Moscow State University, 1, Leninskie gory, Moscow, 119991, Russia

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ABSTRACT

Methanolysis of sunflower oil catalyzed by immobilized *Aspergillus niger* mycelium was studied in a packed-bed reactor. The optimal cultivation parameters for *A. niger* were determined using full factorial and steepest ascent experimental designs. Sunflower oil, yeast extract and soybean meal were selected as the best carbon and nitrogen sources and were used in the subsequent experiments. Intracellular lipase activity and cell mass concentration were respectively 3.2 and 2.4 times greater and cultivation period decreased by 24 h compared with the initial medium. The optimum values of these most significant parameters were as follows: sunflower oil (13.2 g/L), yeast extract (6.2 g/L), soybean meal (7.4 g/L) and incubation period (72 h) at 30 °C. With *A. niger* biocatalyst grown in optimized conditions, the biodiesel fuel yield reached 23.1% after sixth pass of recycled reaction mixture through the reactor.

1. Introduction

Biodiesel fuel is typically a mixture of fatty acid methyl esters (FAME). The most usual method for biodiesel production is alkaline transesterification of renewable lipid sources, such as rapeseed, sunflower, palm and soybean oils. Substitution of chemical catalysts by lipolytic enzymes will involve the cheap low-grade raw materials and waste oils in a process (Robles-Medina et al. 2009). Considerable attention has been devoted recently to investigation of enzymatic biodiesel production processes. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triglycerides (TAG), via diacylglycerols (DAG) and monoacylglycerols (MAG), to produce glycerol and free fatty acids (FFA), as well as reactions like esterification, transesterification and aminolysis (Gog et al. 2012). Broad substrate specificity and high stability in non-aqueous media place lipases among the most used biocatalysts (Hasan et al. 2006). They are applied in agrochemical, food, cosmetic, pharmaceutical, textile and other industries (Sharma et al. 2001).

Good producers of lipases are fungi of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Candida* and bacteria of the genera *Bacillus*, *Pseudomonas* and *Burkholderia* (Gupta et al. 2004; Singh and Mukhopadhyay 2012). Various methods of enzymes immobilization on solid supports have been developed in order to improve enzyme activity and stability under chemical reaction conditions and to facilitate separation of the biocatalysts from the reaction mixture (Zhang et al. 2012). Currently, there are many commercially available biocatalysts which can be used to produce biodiesel. For example, Lipozyme TL IM – a lipase from *Thermomyces lanuginosa*, immobilized on silica gel, has been found efficient and gave a FAME yield of 98% from palm oil after 12 h (Khor et al. 2010). Use of Novozym 435, which is a lipase from *Candida antarctica*, immobilized on macroporous poly(methyl methacrylate), gave FAME with a yield of 92% from soybean oil after 48 h (Go et al. 2013). However, industrial use of these biocatalysts in biodiesel production is limited by both the high cost and the rapid deactivation under transesterification conditions. As a result of the ethanolysis of soybean oil, Novozym 435 retains 84% of its initial activity after 9 cycles in a batch reactor, while Lipozyme TL IM has only about 10% of its initial activity left after just the first cycle (Hernández-Martín and Otero 2008).

An alternative to expensive catalysis by purified lipases is whole-cell biocatalysis – the approach in which the enzyme-producing organisms are used directly as biocatalysts. In this

* Corresponding author.
E-mail address: gubkin.biotech@gmail.com (A.A. Novikov).

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case, intracellular enzymes expressed on the cell wall or on the membrane are used instead of extracellular enzymes (Jin et al. 2009). A conversion of cottonseed oil of more than 85% was achieved after 60 h of methanolysis with bacteria *Pseudomonas* sp. immobilized with sodium alginate (Ali et al. 2011). Yeasts *Rhodotorula mucilaginosa* immobilized on sugarcane bagasse have been used as biocatalyst for the production of FAME from marine microalga *Chlorella salina* oil. The maximum yield was found to be 85% under optimized conditions, and the whole-cell biocatalyst showed good stability in 10 cycles without significant loss of activity (Surendhiran et al. 2014). Nevertheless, the most used whole-cell biocatalyst preparation technique is immobilization of mycelial fungi, such as *Rhizopus oryzae,* using porous biomass support particles, because it does not require chemical additives or cell preproduction (Adachi et al. 2011; Ribeiro et al. 2011). Optimization of enzymatic transesterification conditions (water content, substrate molar ratio, use of cosolvents) allows obtaining a high yield of FAME – more than 92% (Ban et al. 2001; Li et al. 2007; Sun et al. 2010; Yoshida et al. 2012).

The aim of the present work is to develop the biocatalyst for biodiesel production based on immobilized *A. niger* DSM 823. To enhance biomass and intracellular lipases production, we optimized growth medium using mathematically designed experiments.

2. Materials and methods

2.1. Chemicals

Tributyrin, glycerol and malt extract were purchased from Sigma Aldrich (St. Louis, MO, USA), phosphoric acid, potassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Carl Roth (Karlsruhe, Germany), gum arabic was purchased from Rushim (Moscow, Russia), peptone, dextrose and yeast extract were purchased from Helicon (Moscow, Russia). Sunflower oil and soybean meal were of food grade.

2.2. Microorganism and cultivation condition

The filamentous fungus *Aspergillus niger* DSM 823 was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig). *Aspergillus niger* stock culture was maintained at 4 °C on potato-dextrose agar plates and subcultured at monthly intervals. Submerged cultivation of *A. niger* has been carried out on a rotary shaker at 220 rpm and 30 °C in 750 mL Erlenmeyer flask, containing 200 mL of nutrient medium. Inoculum was prepared by transferring a loopful of spores from agar plates to the medium. The basal medium consisted of 15.0 g dextrose and 10.0 g peptone in 1 L tap water.

2.3. Intracellular lipase activity assay

Mycelium of *A. niger* was filtered, washed with 0.1 M phosphate buffer (pH 6.5) and freeze-dried. Lipase activity was determined by GC using tributyrin as substrate. Substrate solution was prepared by mixing tributyrin emulsion (10% w/w) and phosphate buffer with a ratio of 1:1 (v/v). Dried mycelium (10 mg) was added to 900 μL of substrate solution and the mixture was incubated at 28°C for 24 h. Enzymatic hydrolysis was stopped by addition of 50 μL of phosphoric acid (85%). Reaction mixture was centrifuged at 10,000 *g* for 10 min and the butyric acid concentration was assayed in supernatant by gas chromatography. One unit of lipolytic activity was defined as 1 mmol of butyric acid that is produced by the catalytic action of the 1 mg of mycelium per h under the conditions described above.

2.4. Selection of medium components

Submerged cultivation of *A. niger* in 15 nutrient media was carried out in order to evaluate the ability of various carbon and nitrogen sources to provide active growth and intracellular lipases biosynthesis. These 15 media were formed by combining five carbon sources and three nitrogen sources pairwise. Dextrose, sunflower oil, glycerol, molasses and malt extract were used as carbon sources in concentration of 10 g/L, peptone, soybean meal and yeast extract - as nitrogen sources in concentration of 5 g/L. The cultivation time was 96 h. Biomass yield was estimated as mass of freeze-dried mycelium weighed on GR-300 analytical balances (A&D Company, Tokyo, Japan) produced per 1 L of medium.

2.5. Experimental design and statistical analysis

The composition of nutrient medium for *A. niger* was optimized using mathematically designed experiments (Maksimov 1980). The effects of three factors, viz. sunflower oil (g/L), soybean meal (g/L) and yeast extract (g/L) were evaluated by a 2^3 full factorial design. Each parameter had three levels: low (−1), middle (0) and high (+1), as shown in Table 1. Submerged cultivation of *A. niger* was carried out for 96 h. The total lipolytic activity per 1 mL of culture medium was taken as the dependent variable or response (Y). The regression equation, which includes all interaction terms, was used to calculate the predicted response:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_{ij} \beta_{ij} x_i x_j + \sum_{ijk} \beta_{ijk} x_i x_j x_k$$

where Y represents the predicted response (U/mL culture medium); β_0 is the model constant; β_i are linear effects; β_{ij} and β_{ijk} are interaction effects; x_i, x_j и x_k are coded levels of variables X_i, X_j и X_k.

The variable X_i was coded as x_i according to the equation:

$$x_i = \frac{X_i - X_{i(0)}}{\Delta X_i}$$

where x_i is coded value of the variable X_i; $X_{i(0)}$ is the value of X_i at the middle level and ΔX_i is variation step length.

The effect of every factor was determined by Student's t-test, the statistical significance of a model was evaluated by Fisher's test. Based on the results obtained from full factorial experiment, optimization of medium components by steepest ascent design was carried out.

2.6. Immobilization

The carrier used for *A. niger* immobilization were 7-mm cubes of polyurethane foam with particle voidage more than 97% and pore size of 100–500 μm. The immobilization of mycelium within polyurethane foam was spontaneously achieved during submerged cultivation in optimized media. Cultivation of *A. niger* has been carried out on a rotary shaker at 220 rpm and 30 °C in a 750 mL Erlenmeyer flask, containing 200 mL of nutrient medium and 1 g of

Table 1
Codes and actual levels of independent variables for full factorial experimental design.

Variables	Symbols	Coded levels			Variation step length, λ
		−1	0	+1	
Yeast extract (g/L)	X_1	2.5	5.0	7.5	2.5
Soybean meal (g/L)	X_2	2.5	5.0	7.5	2.5
Sunflower oil (g/L)	X_3	10.0	15.0	20.0	5.0

carrier. Optimized culture medium consisted of 13.2 g sunflower oil, 7.4 g soybean meal, 6.2 g yeast extract in 1 L tap water. The cultivation time was 72 h. Immobilized mycelium was filtered, washed with 0.1 M phosphate buffer (pH 6.5) and freeze-dried.

2.7. Methanolysis

Methanolysis of sunflower oil was carried out in a packed-bed reactor (PBR), which is a glass column (10 mm in internal diameter and 300 mm in height), filled with 4 g of dried biocatalyst. The reaction mixture was supplied into the top of PBR with space velocity of 0.2 h^{-1}. The reaction mixture contained 18.3 g of sunflower oil, appropriate amount of methanol and 15% (v/v) of distilled water. One batch cycle consists of six passes with residence time of 8 h. Methanol was added into the system in four steps (1 molar equivalent of methanol at each of the first, second, third and fourth passes). The reaction mixture was emulsified by sonication (400 W; 20 kHz; Digital Sonifier S-450D, Branson, Danbury, CT, USA) for 10 min prior to reaction. Methanolysis was performed at 28 °C. Samples of effluent from PBR were centrifuged at 10,000 *g* for 10 min and the upper oil layer was analyzed by HPLC.

2.8. Analysis

Butyric acid content in lipolytic assay was quantified using a Crystal-5000.2 gas chromatograph (Chromatec, Yoshkar-Ola, Russia), equipped with a flame-ionization detector and a Chromosorb-102 glass column (3 m × 3 mm). The column was held at 215 °C. The total analysis time was 13 min.

HPLC analysis of the transesterification reaction mixtures was performed with Agilent 1260 Infinity series chromatograph (Santa Clara, CA, USA). Components of the reaction mixtures (TAG, DAG, MAG, FFA, and FAME) were separated on a commercially packed Luna C18 HPLC column (25 cm × 4.6 mm i.d. × 4 μm particle size, Phenomenex, Torrance, CA, USA) and then detected by evaporative light scattering detector (ELSD, G4260B, Agilent). Individual peaks were identified by comparing their retention times with those of pure TAG and FAME in standard mixtures and with those of TAG in common vegetable oils of known composition. The temperature of the ELSD was kept at 50 °C and the flow rate of the nebulizing gas N_2 at 10 L/min. The mobile phase was a mixture of a solvent A (methanol) and a solvent B (a mixture of 2-propanol–hexane, 5:4 v/v) at a gradient composition descending from 100% A to 50% A and 50% B in 22 min. The injection volumes of 10 μL and the flow rate of 1 mL/min were used in all experiments. The column temperature was held constant at 40 °C. All samples were dissolved in solvent B. The total analysis time was 30 min.

3. Results and discussions

Effect of culture conditions on production of extracellular lipases by *A. niger* is well investigated and purified enzymes have been studied for different industrial applications in food and detergent industries, as well as in fatty acids esters synthesis (Saisubramanian et al. 2006; Edwinoliver et al. 2010; Romero et al. 2012). The aim of this study was to determine the optimal fermentation parameters for enhanced *A. niger* whole-cell lipase yield.

Fig. 1 shows time courses of biomass and intracellular lipase accumulation on glucose-peptone basal medium at optimal growth temperature (30 °C). Intracellular lipase activity increased along with the cell growth and reaches maximum (0.58 U/mg mycelium) after 96 h of fermentation. Further incubation led to decrease in lipase activity, as previously described in the literature for strain *A. niger* MTTC 2594 (Edwinoliver et al. 2010). As can be seen at

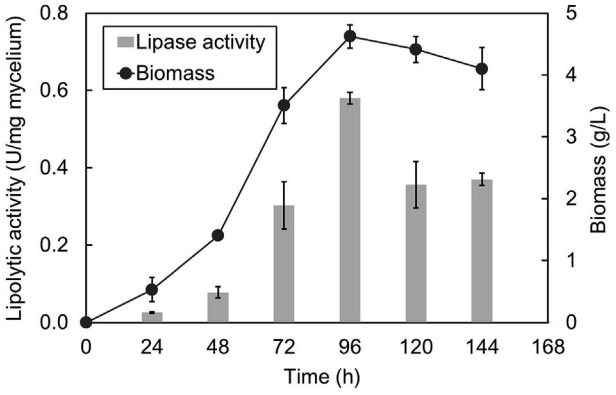

Fig. 1. Intracellular lipase production and mycelial growth during submerged cultivation by *Aspergillus niger* DSM 823 in glucose-peptone basal medium at 30 °C.

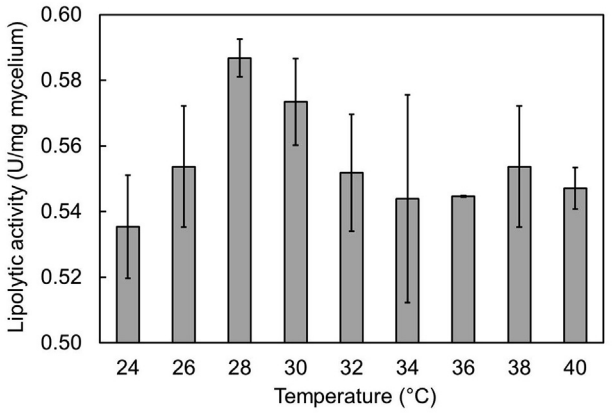

Fig. 2. Effect of reaction temperature on the activity of intracellular lipase from *Aspergillus niger* DSM 823 towards tributyrin during incubation at 28°C for 24 h.

Fig. 2, intracellular lipases from *A. niger* were stable over a wide temperature range (24–40°C) with optimum at 28°C. Therefore, *A. niger* was cultivated for 96 h and lipase activity was measured at 28°C in all subsequent experiments.

In order to obtain an effective whole-cell biocatalyst, it is necessary to select the carbon and nitrogen sources for the maximum lipase activity and biomass yield. The production of lipases by many fungi species was significantly enhanced with addition of oils to medium (Cihangir and Sarikaya 2004; Athalye et al. 2013). For example, activity of lipases produced by *Rhizopus oryzae* ATCC 34612 on the medium containing cottonseed oil was two times higher than the activity of lipases produced on medium with glucose. However, the combination of glucose and oil significantly increased the cell mass concentration compared to medium with single carbon source (Athalye et al. 2013). Glycerol was found to have positive effects on lipase production by *Rhizopus delemar* (Haas and Bailey 1993). A high yield of lipase from *Aspergillus* sp. was observed when yeast extract and peptone were used as nitrogen sources (Cihangir and Sarikaya 2004). The addition of soybean meal to the production medium increased both the lipase activity and cell growth of *Penicillium simplicissimum* (Vargas et al. 2008).

Maximum intracellular lipase activity of *A. niger* (1.12 U/mg mycelium) was observed when the medium was supplemented with yeast extract and sunflower oil, however, cell mass concentration did not exceed 2.5 g/L (Figs. 3 and 4). In contrast, cell mass concentration was higher when soybean meal was used as nitrogen

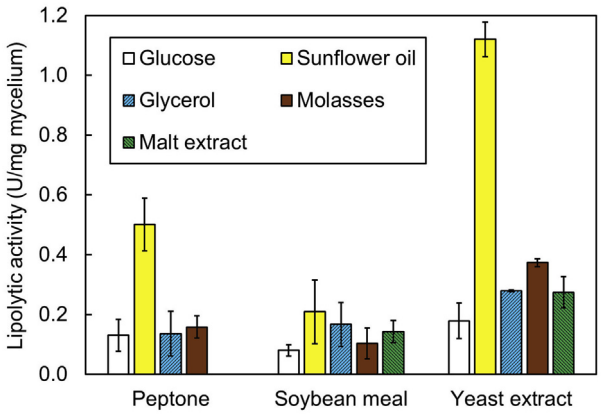

Fig. 3. Effect of carbon and nitrogen sources on intracellular lipase production by *Aspergillus niger* DSM 823 during 96-h cultivation at 30 °C.

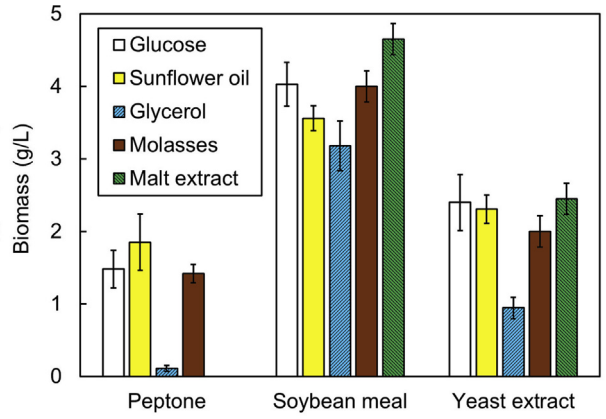

Fig. 4. Effect of carbon and nitrogen sources on mycelial growth by *Aspergillus niger* DSM 823 during 96-h cultivation at 30 °C.

source (3.2–4.7 g/L), but minimum lipase activity was detected. Thus, medium consisting of 5.0 g yeast extract, 5.0 g soybean meal and 15.0 g sunflower oil in 1 L tap water was chosen for the further optimization.

From the results of two-level full factorial experiment (Table 2) a regression equation was obtained, which can be expressed as follows after elimination of non-significant interaction coefficients by Student's t-test:

$$Y = 5,06 + 0,84x_1 + 1,72x_2 - 0,60x_3 - 0,21x_1x_2x_3$$

It indicated that all three factors – concentrations of sunflower oil, yeast extract and soybean meal, and their interaction were significant at the 95% confidence level. The model suggested that higher concentrations of yeast extract and soybean meal increase total lipase activity, but higher concentration of sunflower oil acts in the opposite way. The statistical significance of model equation was evaluated by Fisher's test, which revealed that this regression is statistically significant.

Based on a model equation, the steepest ascent design was performed in order to optimize the composition of nutrient medium for *A. niger* increasing or decreasing the value of a variables according to its regression coefficients (Table 3). The path of the steepest ascent started from the center of the full factorial design. Maximum intracellular lipase activity (1.31 U/mg mycelium) and cell mass concentrations (11.12 g/L) were obtained on medium consisting of 6.2 g yeast extract, 7.4 g soybean meal and 13.2 g sunflower oil in 1 L tap water.

Optimized medium provided uniform pellet growth of *A. niger* without sporulation during submerged cultivation up to 96 h. The study of growth dynamics of *A. niger* (Fig. 5) showed that cell mass reached maximum (11.8 g/L) after 60 h of cultivation compared to 96 h in the case of basal glucose-peptone medium (Fig. 1). Maximum intracellular lipase activity of 1.87 U/mg mycelium was obtained after 72 h of submerged cultivation, this being three times higher than the maximum lipase activity of mycelium grown in basal medium. It is worth noting that both biomass and lipase activity became considerably high after only 48 h, thus optimization

Table 2
The matrix of the full factorial experiment, and the corresponding experimental data.

Run	Variables			Lipase activity (U/mg mycelium)	Cell mass (g/L)	Total lipase activity (U/mL culture medium)	
	X_1	X_2	X_3			Experimental	Predicted
1	−1	−1	−1	1.03 ± 0.03	4.18 ± 0.17	4.30 ± 0.14	4.30
2	+1	−1	−1	1.10 ± 0.09	4.58 ± 0.35	5.04 ± 0.21	5.04
3	−1	+1	−1	0.65 ± 0.08	8.51 ± 0.84	5.56 ± 0.43	5.39
4	+1	+1	−1	0.82 ± 0.04	9.40 ± 0.25	7.72 ± 0.59	7.89
5	−1	−1	+1	0.84 ± 0.06	1.26 ± 0.21	1.05 ± 0.25	1.22
6	+1	−1	+1	0.66 ± 0.08	4.55 ± 0.48	2.95 ± 0.03	2.78
7	−1	+1	+1	0.56 ± 0.03	10.80 ± 0.88	6.08 ± 0.34	6.08
8	+1	+1	+1	0.71 ± 0.07	10.89 ± 0.87	7.76 ± 0.19	7.76

Table 3
Design and results of steepest ascent experiment.

Run	X_1	X_2	X_3	Lipase activity (U/mg mycelium)	Biomass (g/L)	Total lipase activity (U/mL culture medium)
0	5.0	5.0	15.0	0.81 ± 0.19	7.66 ± 0.61	6.21 ± 0.94
1	5.4	5.8	14.4	0.89 ± 0.08	10.02 ± 0.48	9.04 ± 0.47
2	5.8	6.6	13.8	0.94 ± 0.00	11.02 ± 0.31	10.31 ± 0.29
3	6.2	7.4	13.2	1.31 ± 0.11	11.12 ± 0.43	14.53 ± 0.76
4	6.6	8.2	12.6	1.17 ± 0.07	10.92 ± 0.22	12.76 ± 1.12
5	7.0	9.0	12.0	1.10 ± 0.13	9.45 ± 0.92	10.36 ± 0.35

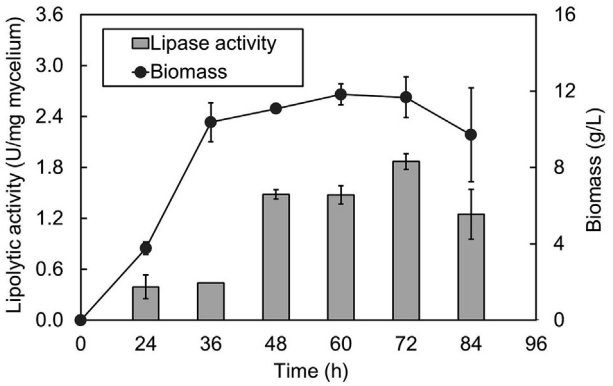

Fig. 5. Intracellular lipase production and mycelial growth during submerged cultivation by *Aspergillus niger* DSM 823 in optimized medium at 30 °C.

of nutrient media made it possible to shorten the cultivation period twice while getting higher lipase activity.

Aspergillus niger mycelium was immobilized within polyurethane foam (2.18 ± 0.21 g of dry cells/g carrier) during submerged cultivation in optimized media for 72 h. The immobilized mycelium was observed under SEM, and it was found intensely covering the polyurethane matrix, as one can see at Fig. 6. The obtained biocatalyst was tested for FAME production from sunflower oil in PBR system. As shown at Fig. 7A, biodiesel yield increased with time and reached 23.1% (w/w) at the sixth pass. Since *A. niger* lipase is characterized by 1,3-regiospecificity toward a triglyceride, an increase in MAG content was observed at the beginning stages of the reaction. After the third pass of the reaction mixture through the PBR, the remarkable reduction in MAG content was observed, which could be explained by the acyl migration from the *sn*-2 position to the *sn*-1 or *sn*-3 position (Macrae 1983), with the consequent

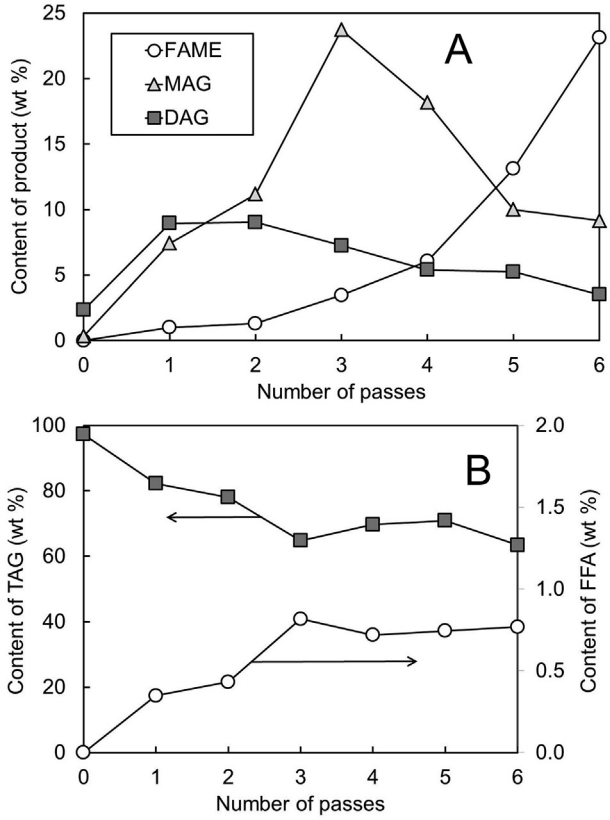

Fig. 7. Biodiesel production (A) and TAG utilization (B) from sunflower oil with *Aspergillus niger* DSM 823 whole-cell biocatalyst in PBR system.

transesterification to FAME. Acyl migration hypothesis is supported by the less prominent decrease in TAG (Fig. 7B) and DAG

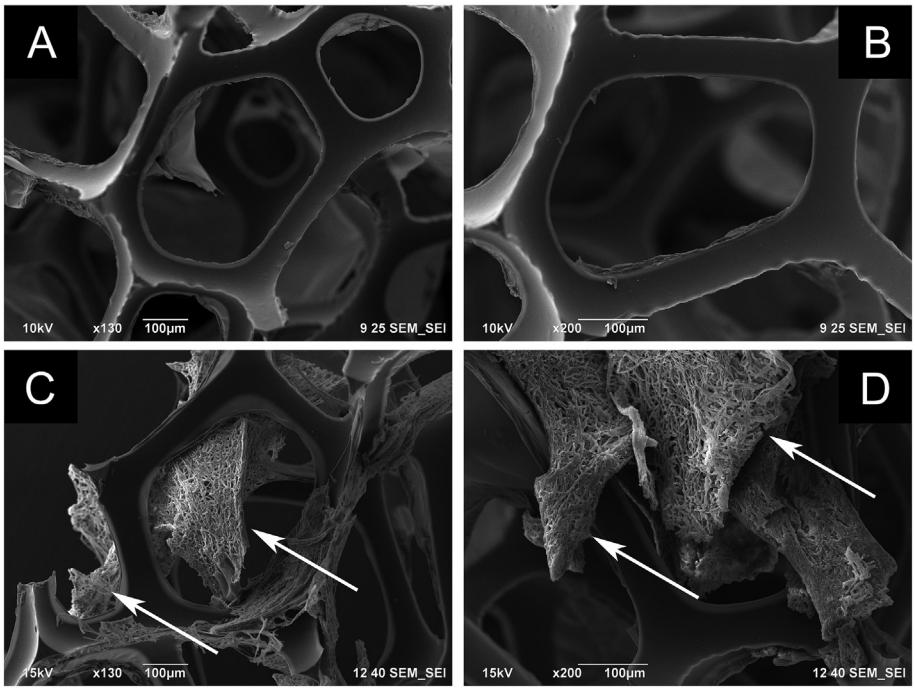

Fig. 6. SEM micrographs of polyurethane foam (A,B), and *Aspergillus niger* DSM 823 mycelium (indicated with white arrows) immobilized within polyurethane foam (C,D). *Bars:* 100 µm.

(Fig. 7A) content. FFA content in the reaction mixture did not exceed 1% (w/w) (Fig. 7B).

4. Conclusion

Optimization of growth medium leads to improvements in biomass yield and lipase activity of *A. niger* mycelium. Grown in optimized medium, immobilized *A. niger* mycelium is able to catalyze the biodiesel production for a prolonged period. Observed dynamics of MAG in the reaction mixture assumes acyl migration and thus promises high yield of FAME in transesterification catalyzed by living mycelium.

Disclosure

The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of the Russian Federation where they were performed.

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