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LIPOLYTIC ACTIVITY OF BIOCATALYSTS OBTAINED AFTER IMMOBILISATION OF FUNGAL BIOMASS OF *RHIZOPUS* GENUS IN SIRAN

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ABSTRACT

Siran was used as a ceramic carrier for the immobilisation of *Rhizopus microsporus* and *Rhizopus nigricans* fungi. Siran was cross-linked with glutaraldehyde or silanised to improve the capacity and stability of biomass immobilisation. The conditions of the obtained enzymatic preparations (immobilised and free) maintained in cooled (-20°C) acetone were determined.
The immobilised biocatalysts and especially those obtained after biomass immobilisation on the glutaraldehyde-modified Siran, exhibited stable lipolytic activity for 60 days at 4°C and may be applied in 6-7 cycles of periodic lipid hydrolysis.
The Siran immobilised biocatalysts were substrate-specific and exhibited lipolytic activity in 10°C - 55°C and pH 4.5 - 10.5. They may be used for hydrolysis of animal waste fats, e.g. poultry or pork fat.

Key words: immobilisation, Siran, *Rhizopus microsporus*, *Rhizopus nigricans*, activity of lipases, fat hydrolysis

INTRODUCTION

Immobilisation of microbial cells on carriers enhances the activity of their intracellular enzymes e.g. lipases, proteases. In addition, such enzymes retain the substrate specificity and enzymatic activity for a longer time and the immobilised biocatalysts are easier to handle in continuous processes.

The most crucial stages in the process of obtaining immobilised biocatalysts include: the selection of strains with specified substrate activity, the selection of immobilisation technique and the carrier properties, e.g. porosity, charge etc.

In the immobilisation of microbial biomasses, porous and microporous carriers are frequently used. Their advantages include great porosity (up to 50% of carrier volume), mechanical stability, potentially high endurance of the carrier to immobilised biomass, and a short diffusion distance between the outer and the inner surface of the carrier.

Previous studies have indicated the possibility of microbiological modification of animal waste fats, e.g. beef tallow or poultry fat [2,3]. Fractions of mono-, diacylglycerols and fractions of free fatty acids useful in chemical, cosmetic, and pharmaceutical industry can be obtained in this way. The biocatalysts obtained after Siran immobilisation of *Rhizopus* fungi biomass with lipolytic activity were used to improve the effects of animal waste fat biodegradation.

The measurements included:

- the characteristics of Siran properties (QVF Engineering), i.e. determination of specific gravity, capacity of fungal biomass adsorption and its improvement,
- the determination of the optimal dose of the carrier for the culture of fungi with reference to adsorption capacity and activity of the biocatalysts obtained,
- defining the conditions of obtaining immobilised biocatalysts and characteristics of their properties,
- the application of the immobilised biocatalysts obtained in the reaction of lipid hydrolysis in a two-phase system (a comparative analysis of the lipolytic activity in relation to the temperature and pH),
- an evaluation of the stability of the biocatalysts lipolytic activity during storage and determination of the number of applicable cycles,
- the application of the biocatalysts obtained for the hydrolysis of animal waste fats.

MATERIALS AND METHODS

Fat substrates. The following fat was used in the experiment: poultry waste fat from the Poultry Processing Plant "Indykpol S.A." (Olsztyn, Poland), pork waste fat collected from the waste water of the Meat Processing Plant in Olsztyn (Poland), rapeseed oil (Z.P.T. "Boilmar"), sun flower oil (Kruszwica-Cereol), and soybean oil (Z.P.T. "Olvit").

Carrier. Siran A and B (QVF Engineering).

Microorganisms.

- *Rhizopus microsporus* from the strain collection of the Institute of Microbiology and Viral Diseases of the Ukrainian Academy of Sciences, Kiev, Ukraine;
- *Rhizopus nigricans* from the strain collection of the Institute of Biotechnology of Agricultural and Food Industry, Warsaw, Poland.

Pure cultures of fungi were cultivated on agar-wort slants at 30°C for 4 days and used immediately or after 1-month storage at 4°C.

Culture medium and preparation of inoculum. The inoculum was prepared using YPG culture medium with the following composition: glucose - 2% (w/v), yeast extract - 2% (w/v), and peptone - 1% (w/v). The inoculum of fungi was prepared in 100ml of medium (sterilised in 121°C/20 min) in 500ml conical flasks by washing the fungal biomasses from slants with physiological solution.
The fungal inoculum were prepared in a shake culture in a G-25 shaker (New Brunswick) with 200rev/min at 30°C for 24h.

Conditions of fungal biomass multiplication and immobilisation. The fungi cultures were performed in 200ml of YPG culture medium enriched with 2% (v/v) of sun flower oil *(Rhizopus nigricans*) or with 2% (v/v) of soybean oil *(Rhizopus microsporus*). The following doses of Siran were added to the cultures: 5, 10, 15, and 20 % w/v. A shaking culture was performed at 30°C for 72h, 200rev/min. In parallel, a fungal culture without a carrier addition was also performed.

Acetone (-20°C) was added in the amount of 1:1 (v/v) to the fungal culture with the carrier. After 2 hours, the carrier was separated from the remaining liquid and rinsed three times with cooled acetone. A biocatalyst obtained this way was dried at room temperature and stored at 4°C. A similar procedure was applied to the fungi control culture (without Siran addition).

Variants of obtaining the immobilised biocatalysts:

A. The immobilisation of fungal biomass was performed according to the above-described method using a 10%-addition of native Siran.
B. Application of Siran modified with glutaraldehyde. Siran was fixed in a 2.5% (v/w) water solution of glutaraldehyde and mixed in a magnetic stirrer for 2 hours at 4°C. Next, the carrier was separated, washed three times with distilled water and added (10%) to the culture of fungi. The immobilisation procedure as in variant A.
C. Application of Siran after silanisation and modification with glutaraldehyde. Siran was kept for 30 in the HCl/MeOH solution (1:1), and then rinsed with distilled water. Neutralised Siran was kept for 30 min in concentrated H_2SO_4 and rinsed with distilled water till pH 7.0 was achieved. Next, the carrier was dried at room temperature. The dried Siran was kept for 12h in a 2.5% solution of APTS (3-aminopropyltrimethoxysilane) in anhydrous toluene, and rinsed with 99% ethanol and water. After silanisation, the carrier was immersed in a 2.5% water solution of glutaraldehyde and prepared as in variant B.

Determination of the lipolytic activity of biocatalysts with the method of pH-static titration in emulsion system.

The composition of reaction mixture:

- 5% (w/v) of rapeseed oil
- 2% (w/v) of arabic gum
- 20 mM of $CaCl_2$
- 10 mM of NaCl.

The reaction mixture was homogenised for 3 x 5 min in an Ultraturrax T25 homogeniser (IKA). The examined sample (0.5 g of biocatalyst or 0.5 g of biomass) was added to 30ml of emulsion. The reaction was carried out at 37°C for 10 min, pH 8.

One unit of lipolytic activity (LAU) is an equivalent of the number of μmoles of fatty acids liberated in 1 min under assay conditions by lipases present in 1g of the biocatalyst.

The effect of conditions on the biocatalysts lipolytic activity determined in the emulsion system. The reaction mixture was prepared, with the composition as given above. To 30ml of emulsion, 1g of the biocatalyst was added to perform the process of hydrolysis. The lipolytic activity was determined with the method of pH-static titration.

1. *The effect of pH on the lipolytic activity of the biocatalyst.* The reaction of lipid hydrolysis was carried out at a constant temperature of 37°C and pH of 2.5, 3.5, 4.5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9.5, and 10.5.
2. *The effect of temperature on the lipolytic activity of the biocatalyst.* The reaction of lipid hydrolysis was carried out at a constant emulsion pH 7.0 at temperatures of: 10°C, 20°C, 25°C, 30°C, 37°C, 45°C, 55°C, and 65°C.

The evaluation of the stability of biocatalyst lipolytic activity during storage. The biocatalysts were stored at 4°C. During storage, their lipolytic activity was determined with: rapeseed oil, poultry fat, and pork fat. The assays of the lipolytic activity were performed with the method of pH-static titration after 7, 14, 21, 30, and 60 days.

The evaluation of the multiplication factor of biocatalyst application. The hydrolysis of poultry waste fat was carried out with the biocatalysts obtained in a two-phase system under the following conditions: pH 7.0, temp. 30°C, 24 h. When the degree of hydrolysis corresponded to a 50%-loss of their initial activity, the biocatalyst was separated, rinsed with distilled water, and re-used in subsequent cycles.

RESULTS

The suitability of Siran was determined as a carrier for the immobilisation of the biomass from the fungi of *Rhizopus* genus capable of synthesising intracellular acylhydrolases. Of the two available types of the carrier, differing in terms of structure and specific gravity, the best capacity of adsorption was obtained with Siran B (Table 1)

Table 1. The characteristics of Siran (OVF Engineering)

Parameters	Siran A	Siran B
Beads size (mm)	approx. 1-2	approx. 3-5
Porosity (µm)	300	300
Specific gravity (g/cm3)	0.463	0.388
Capacity of biomass adsorption (mg d.m./g dry carrier) (the highest obtained)	38.76	67.23

It was found that the carrier dose added to the fungal culture determined the capacity of biomass adsorption (Table 2). Considering the results of other studies in that area, as well as the economic and technological aspects, a 10%-addition of the carrier to the fungal culture was applied in further investigations.

Table 2. The effect of dose and type of Siran added to fungi culture on the capacity of biomass adsorption and activity of the biocatalysts obtained

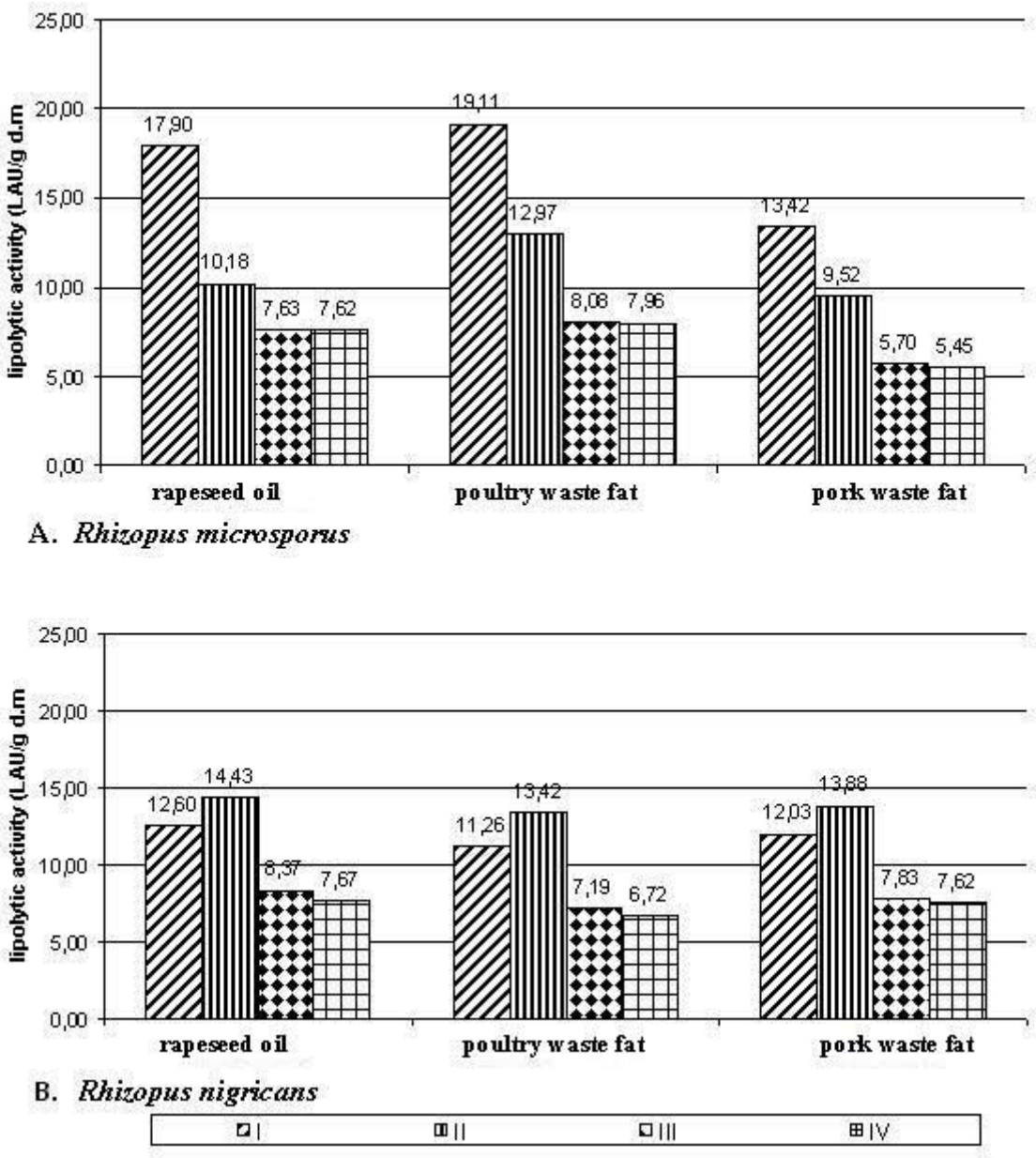
Dose of carrier (% w/v)	Lipolytic activity of the biocatalyst* (LAU/g carrier)				Capacity of biomass adsorption (mg d.m./g dry carrier)			
	Siran A		Siran B		Siran A		Siran B	
	Rhizopus microsporus	*Rhizopus nigricans*	*Rhizopus microsporus*	*Rhizopus nigricans*	*Rhizopus microsporus*	*Rhizopus nigricans*	*Rhizopus microsporus*	*Rhizopus nigricans*
5	4.25	3.03	6.50	4.34	11.76	17.66	44.01	41.41
10	4.55	3.59	7.92	4.68	21.16	35.66	55.84	47.55
15	3.83	3.12	4.55	3.87	31.76	37.06	62.77	50.03
20	3.53	3.07	4.07	3.28	32.16	38.76	67.23	52.16

*lipolytic activity of the biocatalyst determined in the emulsion system with the use of rapeesed oil

So as to improve the biochemical properties of the biocatalysts obtained in the initial stage of the research, Siran was modified by means of cross-linking with glutaraldehyde or silanisation.

When comparing the effect of the carrier preparation variant and procedures of fungal biomass immobilisation onto Siran on the lipolytic activity of the biocatalysts, it was found that the most advantageous results were obtained in variant B, i.e. with glutaraldehyde-modified Siran (Figure 1). Depending on the substrate used, the lipolytic activity ranged from 9.52 LAU/g d.m. to 12.97 LAU/g d.m. of biocatalyst from *Rhizopus microsporus* and from 13.42 LAU/g d.m. to 14.43 LAU/g d.m. of biocatalyst from *Rhizopus nigricans* (Figure 1).

Fig. 1. The effect of substrate and biocatalyst preparation variant on the lipolytic activity.
Biocatalysts:
I – obtained after biomass maintenance with acetone (control sample)
II – obtained with a native carrier (variant A)
III – obtained with a glutaraldehyde-modified Siran (variant B)
IV – obtained with a silanised Siran (variant C)



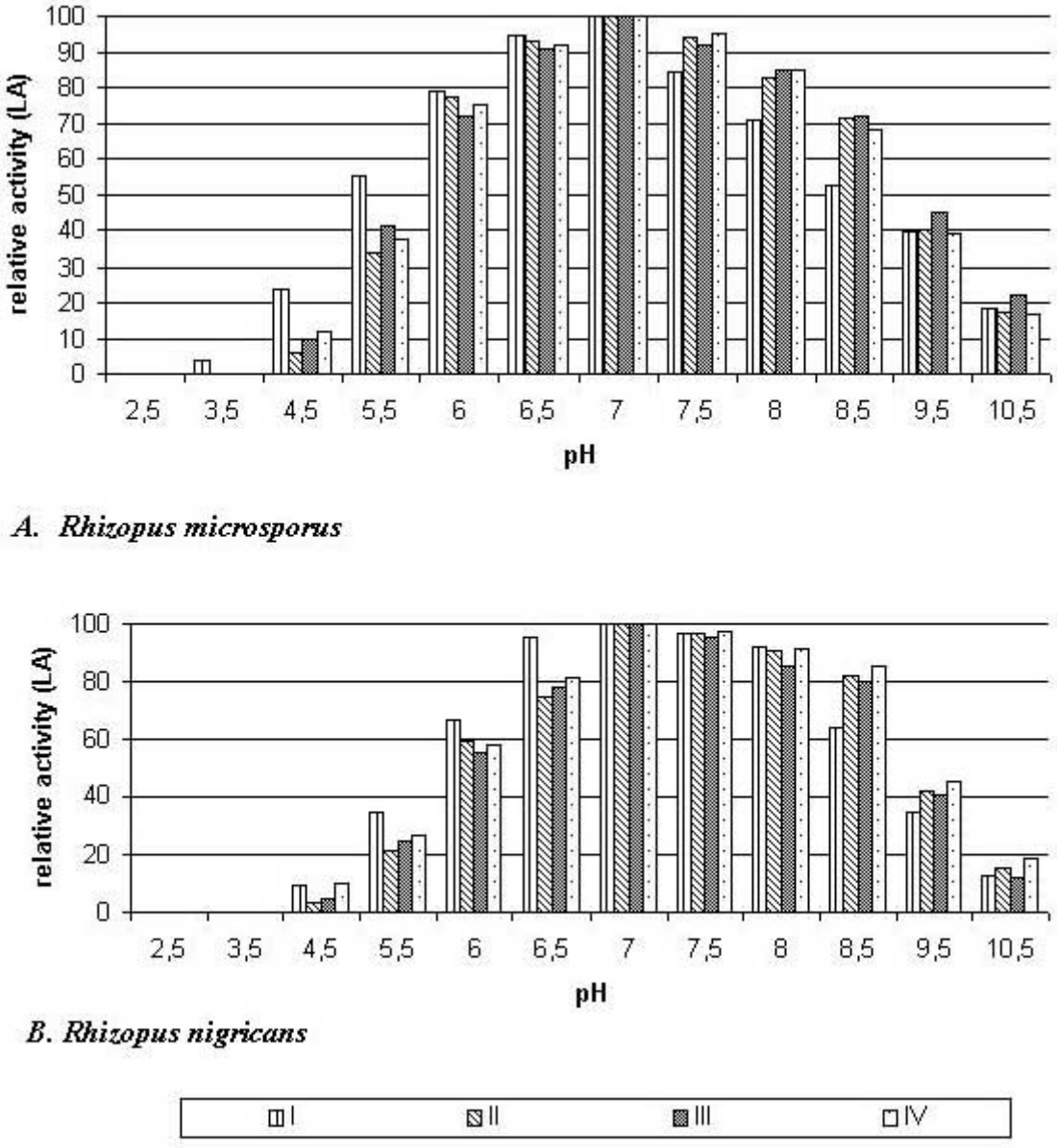
* Lipolytic activity in LAU/g d.m. of biocatalyst determined in the emulsion system with the addition of fatty substrate.

A comparative analysis of the lipolytic activity of the biocatalysts obtained indicates a relatively high activity of the preparations from free, acetone-maintained fungal biomass. It fluctuated from 13.42 LAU/g d.m. to 19.11 LAU/g d.m. for the preparations from *Rhizopus microsporus* and from 11.26 LAU/g d.m. to 12.60 LAU/g d.m. of biocatalysts for the preparations from *Rhizopus nigricans*.

The obtained biocatalysts, both free as well as those immobilised onto Siran, demonstrated substrate specificity. It was shown by the differences in their lipolytic activity depending on the substrate used, i.e. universal oil, poultry waste fat or pork waste fat (Figure 1).

The lipolytic activity of the biocatalysts obtained depends on the acidity of the reaction mixture (Figure 2). The most desirable lipolytic activity was obtained at the acidity of the reaction mixture of pH 7.0.

Fig. 2. The lipolytic activity of the biocatalysts in relation to pH

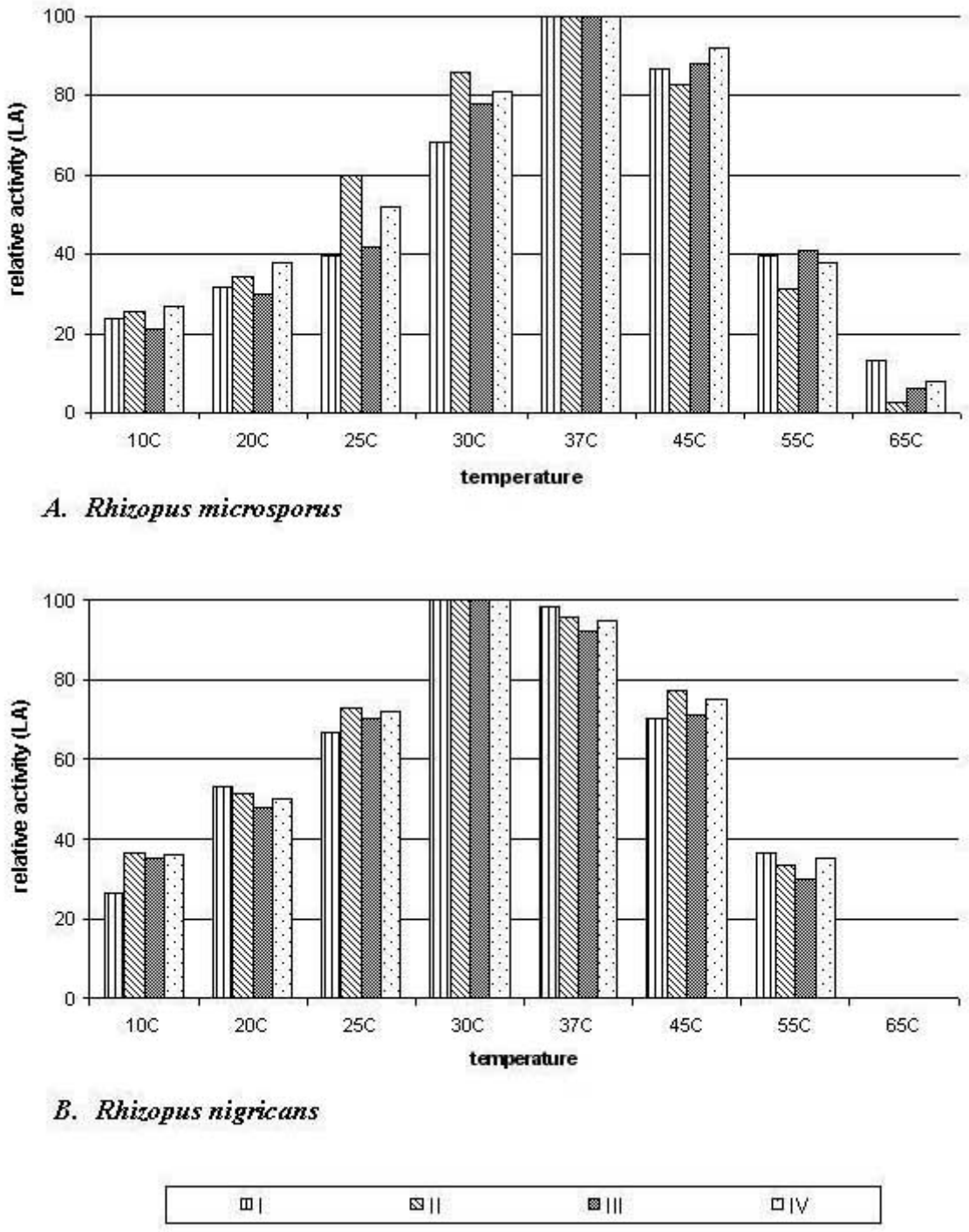


Explanations as in fig. 1.

The biocatalysts obtained were also found to demonstrate lipolytic activity across a wide range of temperatures, namely at 10°C - 65°C in the case of the preparation from *Rhizopus microsporus* and at 10°C - 55°C for the preparation from *Rhizopus nigricans.*

The highest lipolytic activity was reported for the preparations from *Rhizopus microsporus* and *Rhizopus nigricans* at 37°C and 30°C, respectively (Figure 3).

Fig. 3. The lipolytic activity of the biocatalysts depending on the temperature

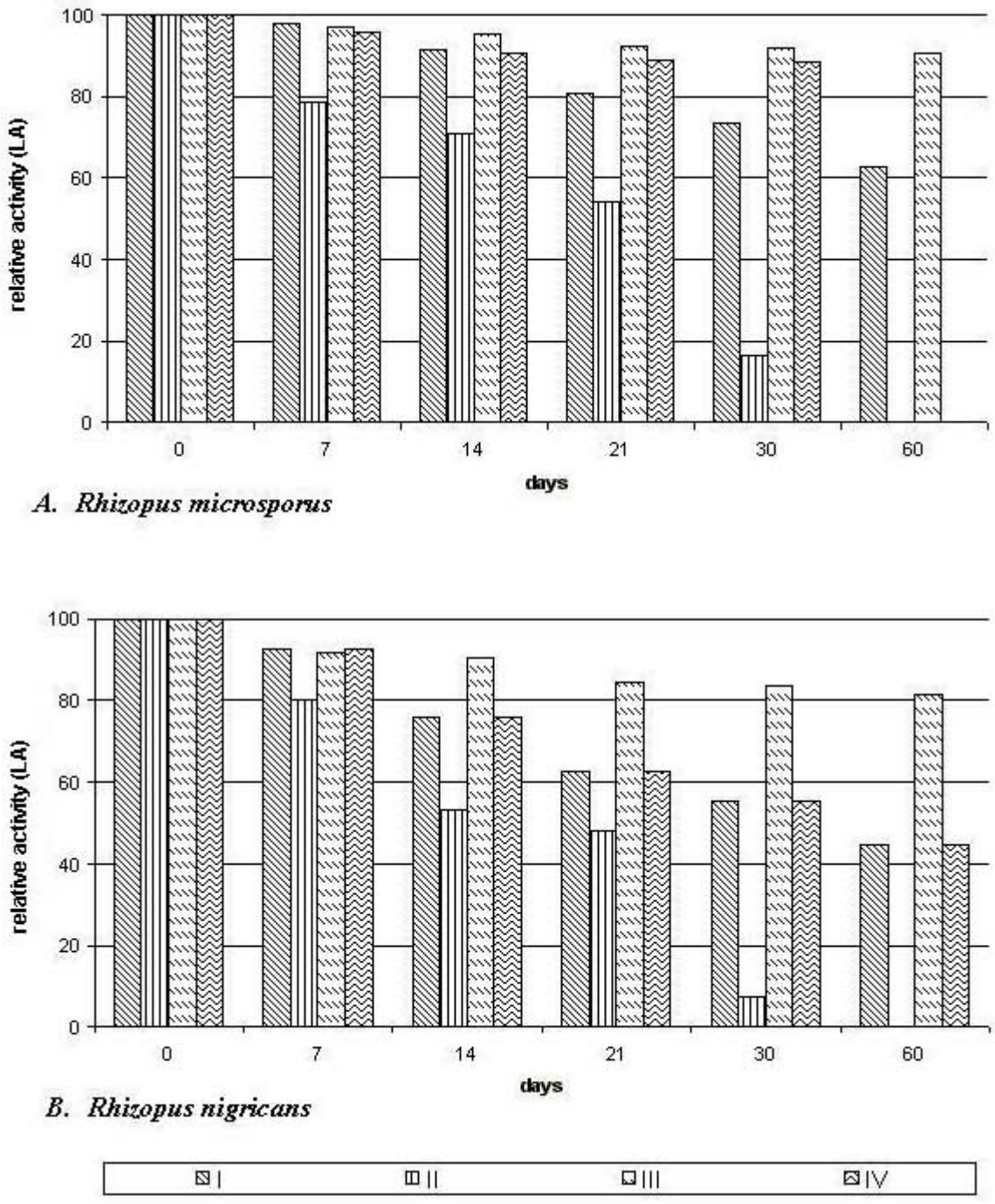


Explanations as in fig. 1.

It should be emphasised that the immobilisation procedures applied had little impact on the thermostability of the lipolytic activity of the biocatalysts obtained. Similar observations were made with reference to the susceptibility of the biocatalysts obtained to the changes in the reaction mixture acidity.

A beneficial effect of the conditions applied to the obtaining of the immobilised biocatalysts, especially with the use of glutaraldehyde-modified Siran, was reported when determining their lipolytic activity stability during 60 days of storage at 4°C. Still, the losses in the lipolytic activity reached as little as 10% for the biocatalysts from *Rhizopus microsporus* and ca. 18% for those from *Rhizopus nigricans* (Figure 4).

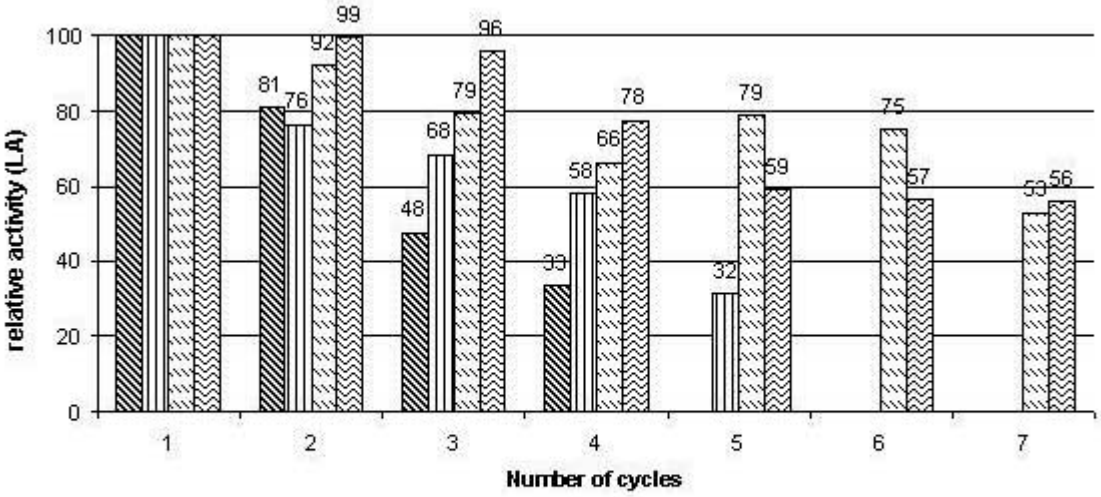
Fig. 4. The effect of the biocatalyst preparation variant on the losses in lipolytic activity during storage at 4°C



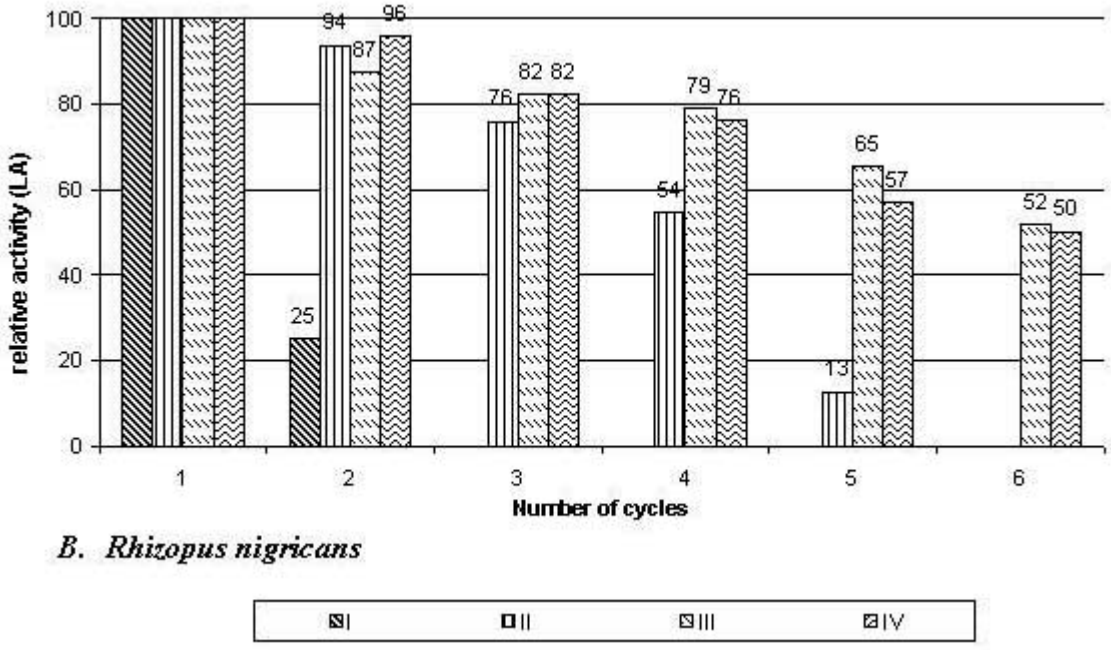
Explanations as in fig. 1.

An important qualitative parameter of the immobilised biocatalysts is the multiplication factor of their application in cyclic hydrolysis of poultry waste fat. It was found that after the immobilisation of the investigated fungi biomass on glutaraldehyde-modified Siran or on silanised Siran, the obtained biocatalysts lost ca. 50% of their initial activity after 7 and 6 cycles of the poultry waste fat hydrolysis, for the preparations from *Rhizopus microsporus* and *Rhizopus nigricans*, respectively (Figure 5). These results may be found to be satisfactory. They form a sound basis for continued research into determination of the conditions of continuous hydrolysis of lipids with the use of immobilised preparations of lipases synthesised by *Rhizopus microsporus* or *Rhizopus nigricans*.

Fig. 5. The multiplication factor of biocatalyst reuse in relation to preparation variant



A. Rhizopus microsporus



B. Rhizopus nigricans

Explanations as in fig. 1.
*Biocatalysts were used in the hydrolysis of poultry waste fat.

DISCUSSION

In preparation of immobilised biocatalysts, immobilisation is applied more often to enzymes than to microbial biomasses, mainly of those synthesising extracellular enzymes [4,6,7,8].

Strains synthesising intracellular lipase are found to dominate among known fungi. They include *Rhizopus microsporus* and *Rhizopus nigricans* used in the presented research. They produce lipases with desirable biotechnological properties such as stable lipolytic activity across a wide range of pH and temperatures. These

properties predispose their application in the biodegradation of waste lipids, e.g. in wastewaters of the meat fat and the gastronomic industries.
In searching for carriers suitable for obtaining immobilised biocatalysts with lipolytic activity, attention was paid to Siran, due to its advantageous physicochemical properties, including resistance to changes across a wide range of temperatures and acidity levels, as well as the capability of regeneration and multiple applications in the immobilisation of biomasses.

It is worth emphasising that Siran was successfully applied in the immobilisation of *Teredinobacter turnire* biomass in periodical biosynthesis of extracellular proteases [5]. The physicochemical properties, e.g. porosity, specific gravity, of Siran used in the above- mentioned paper were similar to the corresponding properties of the carrier used in the presented research. It should be emphasised that Beshay and Moreira [5] used the obtained preparation in 7 cycles without any considerable loss in the immobilised biomass capability to produce proteases.

In contrast to the immobilisation procedures for microbial biomasses synthesising extracellular enzymes, in the search for immobilised biocatalysts from *Rhizopus microsporus* or *Rhizopus nigricans* (the producers of intracellular enzymes) biomass, attention should be paid to: the yield and stability of biomass adsorption, its disintegration after immobilisation, and maintenance of the enzymatic preparations obtained. The suggested method for obtaining biocatalysts with lipolytic activity seems to be simple and economical, compared to the immobilisation procedure for enzymes beforehand isolated from the disintegrated biomass.

In the evaluation of the quality of Siran as a carrier, it was found that its capacity for immobilisation of biomass may be up-graded by cross-linking with glutaraldehyde or silanisation.

The biocatalysts obtained after immobilisation of fungal biomass onto modified Siran were successfully applied in a few cycles of poultry waste fat hydrolysis. Due to the troublesome and expensive procedure of silanisation, the modification of Siran with glutaraldehyde seems to be more beneficial and sufficient. It should be emphasised, however, that silanisation of glass and ceramic carriers (similar to Siran) often considerably improve the capability of covalent binding of microbial cells. An example may be the results of research by Shriver-Lake et al. [9], where immobilisation of *E. coli* cells was carried out on silanised glass beads.

For practical reasons, the relatively advantageous properties of the enzymatic preparations obtained after maintenance of *Rhizopus microsporus* or *Rhizopus nigricans* biomass with cooled (-20°C) acetone may be interesting. This procedure may be acknowledged as the specific immobilisation of enzymatic proteins onto the structures remaining after dehydration of filamentous fungi with acetone.

Satisfactory results were also obtained in previous studies into obtaining and applying intracellular lipase preparations from *Rhizomucor miehei* and *Yarrowia lipolytica* maintained with acetone [1]. The results of the studies performed may form the basis for obtaining immobilised preparations from *Rhizopus microsporus* or *Rhizopus nigricans* and their application in the process of the continuous hydrolysis of animal waste fats.

CONCLUSIONS

The research performed showed that Siran, a ceramic carrier, is suitable for the immobilisation of biomass from the fungi of *Rhizopus* genus producing intracellular acylhydrolases.

To obtain preparations with a stable lipolytic activity, predisposed to multiple uses in the process of lipid hydrolysis, modification of Siran properties (e.g. cross-linking of its porous structure with glutaraldehyde) is advisable.

The obtained immobilised biocatalysts with lipolytic activity demonstrate the substrate specificity, which was identified in the hydrolysis of rapeseed oil, poultry waste fat and pork waste fat. The immobilised lipase preparations from the fungi examined demonstrate activity in 10°C - 55°C and pH 4.5 - 10.5. They may be applied in the biodegradation of waste lipids disposed in the wastewaters of the meat, fat or gastronomic industries.

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