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Immobilization of *Enterobacter aerogenes* E13 strain culture medium, distinguished by its lipase activity, in the ethylcellulose microcapsules

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Lipases – enzymes with hydrolytic activity widely applied in many fields of the industry, used to hydrolyze certain substrates and synthesize organic compounds. One of the main methods of enzyme stabilization is that of their immobilization using various carriers. For economic purposes, microorganism culture media with catalytic activity can be immobilized by skipping the purification.

The way to immobilize lipase producing bacterium *Enterobacter aerogenes* E13 strain culture medium in ethylcellulose microcapsules by microcapsulation has been applied in this work. The influence of the microcapsulation process conditions towards the specific lipasic activity and immobilization activity of immobilized preparation has been examined. The properties of immobilized preparation have been determined: pH and temperature optimums, stability and physical indexes. The rapeseed oil has been hydrolyzed with this preparation and peresterified with methanol.

Key words: lipase, *Enterobacter aerogenes*, microcapsulation, hydrolysis, peresterification

INTRODUCTION

Vegetable and animal fats make up not a small part of agricultural, meat reproduction and food industry waste. Also, they are present in the household and communal slurry. Environmentally friendly biotechnological waste degradation and recycling methods are applied more and more while solving technological waste management problems. In the fields of lipid biotechnology, the most versatile catalysts are lipases with a variety of industrial applications [1]. The increase in the stability of lipases and other enzymes is usually reached by immobilizing them on various carriers. This way, an enzyme not only becomes more resistant to the environmental conditions, but it is also convenient to reuse [2–5]. Also, it is advantageous to use not only immobilized enzymes, but also immobilized enzyme-cell complexes. By immobilizing enzymes and the cells producing them together, the preparation not only can gain special qualities, but also the expensive purification stage of the cultural medium can be discarded [6]. The preparations of complexically immobilized enzymes and cells have lots of advantages in prices, efficiency, stability and polyfunctionality in comparison with both, immobilized enzymes and free cells [7]. Microcapsulation technologies are most fitting for the immobilization of cell and enzyme complexes. During microcapsulation, no chemical bonds are made between the substance being immobilized and the carrier; substances can be immobilized complexically, the obtained preparations have a big surface area and quite good resistance to the environmental factors [3, 4, 7]. The microcapsule membrane controls the passage of molecules into the microsphere – big objects enclosed in membrane are held up in it and small molecules of substrate, product or others substances of small molecular mass can freely diffuse through the synthetic membrane [3, 6].

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The immobilization of *Enterobacter aerogenes* E13 strain culture medium distinguished by its lipase activity, in the ethylcellulose microcapsules is being discussed in this work. The preparations with lipasic activity can be used for the treatment of oil waste [8–9]. By using immobilized lipasic preparations it's possible not only to degrade oil waste, but also to recycle it into bio-fuel [10, 11], bio-oil and other compounds [12, 13].

The goal of this work is to create the bacterial preparation with lipasic activity through the immobilization of *Enterobacter aerogenes* E13 strain cultural medium by means of microcapsulation, also to evaluate the properties of the immobilized preparation.

MATERIALS

Enterobacter aerogenes E13 strain cultural sample and methyl oleate from JSC "Biocentras", ethyl cellulose (EC), polyvinyl alcohol, paranitrophenylbutyrate (p-NPB) and hexane from Sigma Aldrich, CCl_4, NaOH and Izopropanol from Lachema, NaH_2PO_4 and Na_2HPO_4 from Fluka, methanol from Chempur, acetic acid, acetone and metallic iodine from Penta, aluminum oxide TLC plates from Merck, rapeseed oil from JSC "Tikras kelias".

METHODS

Spectrophotometric assessment of lipase activity
Add 2.35 ml of heated to 30 °C universal buffer (UB) (pH 8), 100 µl of culture medium solution, 25 µl of 10 mM p-NPB solution in isopropanol into a 3 ml cuvette and maintaining the 30 °C temperature for 180 seconds observe the absorption change at 410 nm. Lipolitic activity is calculated with the formula:

$$\mathrm{La} = \frac{\Delta A/\mathrm{min.} \times \mathrm{M} \times \mathrm{D} \times \varepsilon}{t \times m},$$

ΔA/min. – absorption change at 400 nm per minute;
M – reaction mixture mass, g;
m – test preparation mass, g;
ε – p-nitrophenol extinction coefficient, 18 300 M–1;
D – dilution of the initial sample.

The efficiency of immobilization process
The efficiency of lipase immobilization process was determined according to article [14] as follows:

$$\eta = \left[\frac{E_0 V_0 - E_1 V_1}{E_0 V_0} \right] \times 100\,(\%),$$

where E_0 – lipase activity in the solution before immobilization, E_1 – lipase activity in the solution after immobilization, V_0 *and* V_1 – volumes of cultural sample before and after immobilization, respectively.

Microcapsulation technique
The immobilization of cultural medium is based on physico-chemical methods; microcapsules are created with mixing of the liquid phases of polymer solvent and cultural medium. In this case, the ethyl cellulose was used as a polymer, carbon tetrachloride as solvent, immobilizing object was *Enterobacter aerogenes* E13 bacteria culture medium.

Ethyl cellulose was dissolved in carbon tetrachloride. Cultural medium was added into a conical flask with the previous solution. The flask was stoppered and inserted into the spreader. During the mixing time, micro-drops in the polymer-solvent phase created the polymeric shell around the drops of cultural medium. The third phase with microcapsules has been observed at the phase boundary. After mixing, the suspension from the flask has been poured into the tall vial and a phase rich with microcapsules has been collected with pipette. In order to remove the remaining solvent, the phase with microcapsules has been flushed with water several times, then poured in a thin layer on to the glass surface and left in an oven at 30 °C until it became dry.

The influence of immobilization conditions on the specific lipasic activity of immobilized preparation
Immobilization was repeated using different concentrations of ethyl cellulose solutions in carbon tetrachloride, different ETCM and cultural sample phase volumes; different pH and concentration of cultural samples have been used. The impact of mixing speed and immobilization conditions on the resulting lipolitic activity of immobilized product has been studied.

Microscopy
Optical microscope was used to verify whether the structure of the obtained product is microcapsules.

The study of immobilized lipase spontaneous inactivation during the storage
Bacterial preparations obtained after each immobilization were numbered and stored in open glass vials at the temperature of 4 °C. Every 1–2 weeks, the specific lipolitic activity of the microcapsulated preparation was determined by spectrophotometric method.

Temperature and pH optimum
Temperature and pH optimum of free and immobilized lipase were tested by determining the activity at different temperatures and universal buffer pH rates.

Hydrolysis of rapeseed oil by using microcapsulated *Enterobacter aerogenes* E13 strain cultural medium
Immobilized preparation (activity 19527 U/g) was powdered and put into the thermostatable column. The emulsion of rapeseed oil and 0.5 M Sorensen buffer (1 : 1) with PVA (2%

of the mixture medium) used as an emulsifier was being filled through the top of the column by a peristaltic pump (20 rpm). At the bottom of the column, the reaction products and unused substrate were collected into the glass with emulsion, which was being filled into the column again.

Esterification of rapeseed oil with methanol

Esterification reaction was carried out by taking 5 g of rapeseed oil that was added to 0.5 ml of 0.5 M Sorensen phosphatic buffer (pH 8) and 0.5 g of microcapsulated cultural sample. Mixture was thermostated at 40 °C. Every 1.5 hours, for 3 times, 0.33 g of the methanol-hexane (1 : 1) was added to the mixture. The sample was taken less than 1.5 hours after the last filing of methanol-hexane. The sample was studied by thin layer chromatography.

Determination of the hydrolysis and esterification reactions by thin-layer chromatography

Hexane : ether : acetic acid (70 : 30 : 1) mixture has been used as a carrier. 0.1 ml of reaction mixture was dissolved in 0.9 ml of hexane : ether solvent (1 : 1). 2 ml of this solution was put on a silica plate; a mix of rapeseed oil and methyl oleate was used as control. The plate was placed in a chamber with a carrier. When the carrier front reached the upper limit, the plate was removed, dried and placed in a painting chamber with crystalline iodine, which develops the chromatogram.

RESULTS AND DISCUSSION

Micro capsules with lipasic activity were obtained after *Enterobacter aerogenes* E13 cultural medium entrapment in ethylcellulose matrix (Fig. 1). The highest specific activity was achieved using about 20 g/l EC-TCM solution (Fig. 2), when cultural sample and the EC-TCM solution volume ratio was 2 : 1 (Fig. 3). Higher speed mixing in the microcapsulation process lead to a higher specific lipasic activity of immobilized preparation (Fig. 4). Maximum activity was achieved by maintaining 250 rpm and mixing for 1–1.5 hours (Fig. 5). Lipasic activity of immobilized prepa-

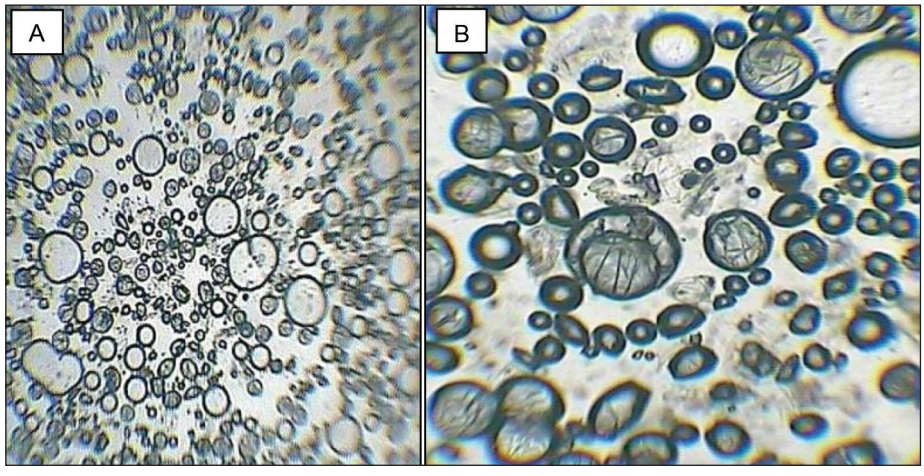

Fig. 1. Ethyl cellulose microcapsules with *Enterobacter aerogenes* E13 cultural sample: A – enlarged 10x, B – enlarged 100x

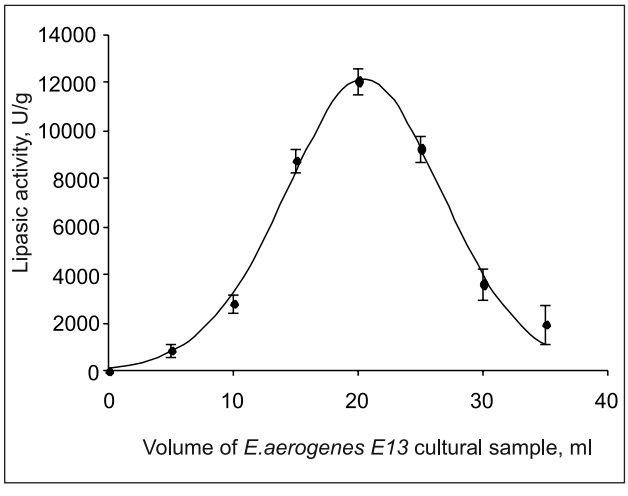

Fig. 2. The dependence of immobilized preparation lipasic activity on the concentration of EC used for immobilization in EC-TCM solution

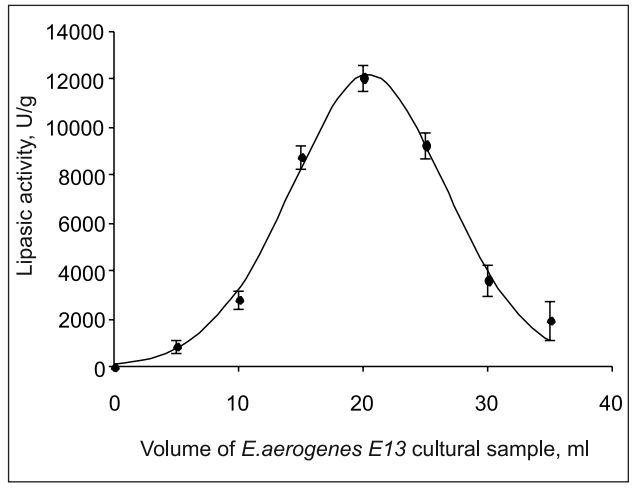

Fig. 3. The dependence of immobilized preparation lipasic activity on the volume (ml) of cultural medium used for immobilization

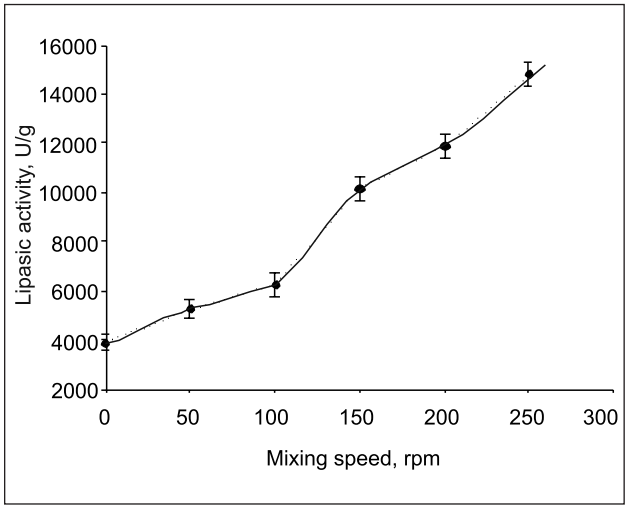

Fig. 4. The dependence of microcapsulated cultural medium on the mixing speed during immobilization

ration also depends on the pH value of the used cultural sample. When pH of the cultural medium is lower than 8 before immobilization, the specific lipasic activity of immobilized preparation significantly starts to fall. At higher pH (starting with 9), phase boundary basically disappears after immobilization and the homogenic mass of immobilization components is obtained. When pH of immobilization conditions is getting higher, the specific activity of the microcapsulated preparation also increases. When pH reaches 8, the specific activity of the preparation becomes stable and maintains an approximately maximal value. The dependence of specific lipasic activity of the obtained bacterial preparation on pH is expressed graphically in Fig. 6.

Temperature and pH optimum

The effect of native and immobilized cultural medium samples at different pH values of the reaction mix showed that immobilized preparation better works at the wider range

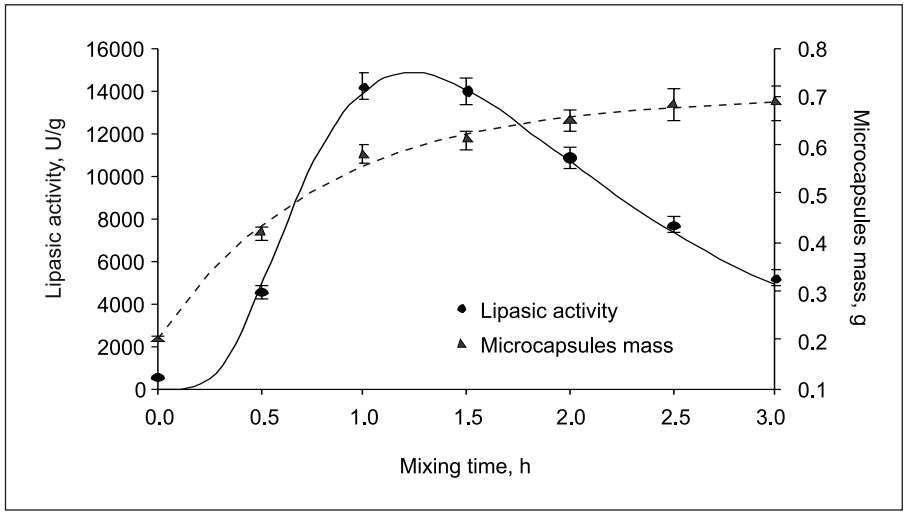

Fig. 5. The dependence of lipasic activity of the immobilized enzyme on the duration of microcapsule formation

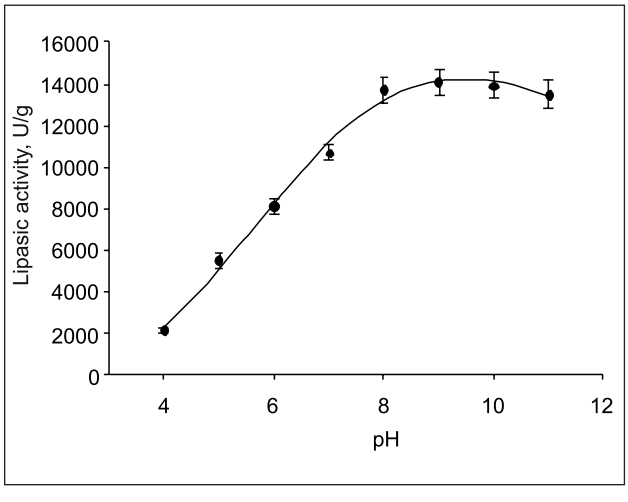

Fig. 6. The influence of the immobilized mix pH on lipasic activity of the obtained preparation during microcapsulation

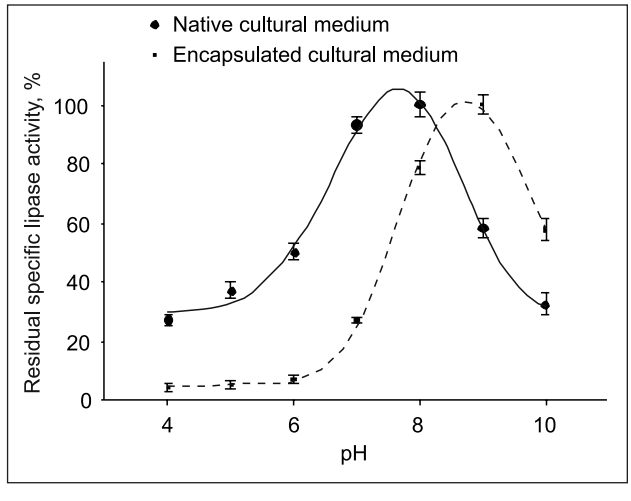

Fig. 7. The effect of reaction mixture pH on the activity of native and immobilized lipase samples

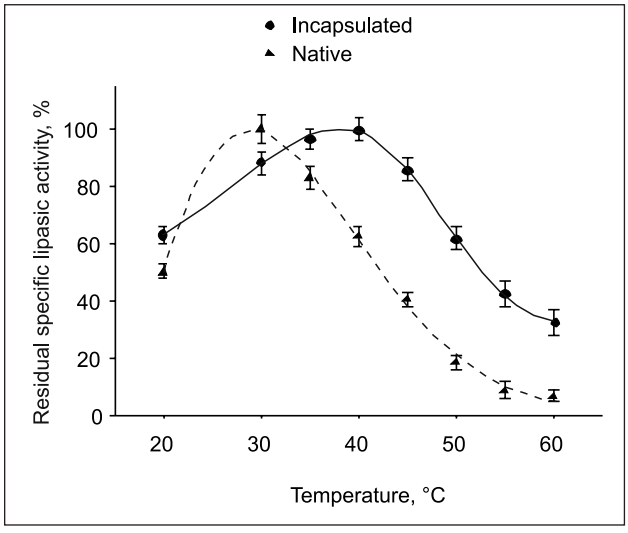

Fig. 8. The effect of reaction temperature on the activity of native and immobilized lipase samples

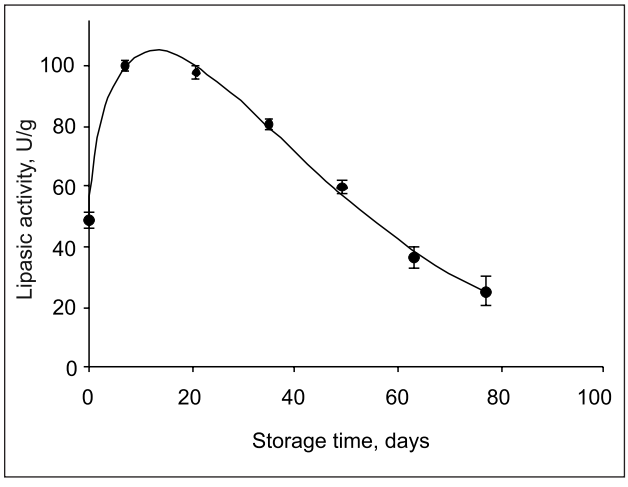

Fig. 9. The storage stability of microcapsulated lipase from *Enterobacter aerogenes* E13

of pH than the native cultural medium. The maximal value of activity in research conditions has been reached at lower pH values in comparison with the pH optimum of native cultural medium (Fig. 7).

The optimal working temperature of native cultural medium was 35 °C; upon the increase or lowering of the temperature by 10 degrees, the lipasic activity clearly decreased (Fig. 8). The highest activity of cultural medium immobilized in ethyl cellulose microcapsules is reached at 40 °C. In comparison with native cultural medium, the immobilized preparation is more stable in a wider temperature range.

Study of the spontaneous inactivation of immobilized lipase during the storage

As shown in Fig. 9, in a week after the creation of preparations, their lipasic activities increased more than twice and

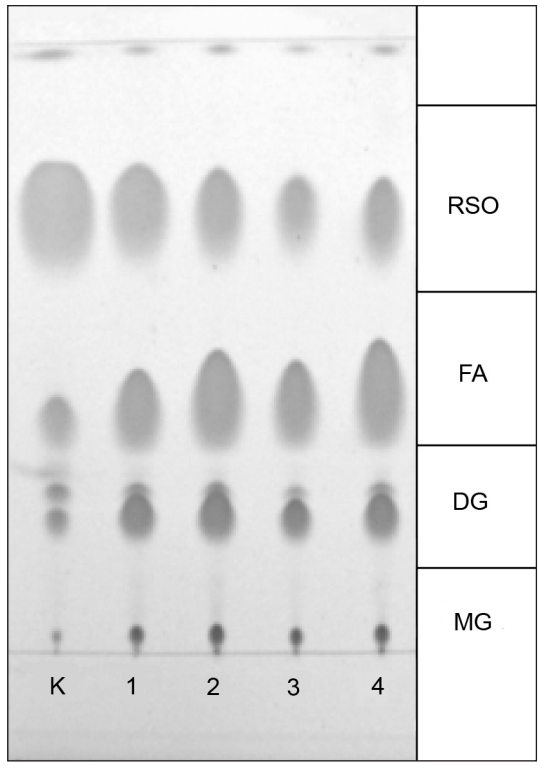

Fig. 10. The results of thin layer chromatography of the rapeseed oil hydrolyzed with microcapsulated lipase from *E. aerogenes* E13. K – rapeseed oil, 1 to 4 – the time of hydrolysis (in hours). RSO – rapeseed oil, FA – fatty acids, DG – diglycerides, MG – monoglycerides

then started gradually to decline; in 56 days after the creation of preparations the activities reached a starting point and in twenty more days decreased by half. The increase of lipasic activity during the first week can be explained by the drying of the unstoppered preparation at 0 °C, thus decreasing in mass. Due to a decrease in mass, after measuring a gram of preparation, there might have been more immobilized biologically active agents than a week before.

Hydrolysis of rapeseed oil by using microcapsulated *Enterobacter aerogenes* E13 strain cultural fluid

It can be seen from a thin layer chromatogram shown in Fig. 10 that the amount of rapeseed oil (RSO) in the reaction sample decreases during the reaction; the blots of free fatty acids (FA), diglycerides (DG) and monoglycerides (MG) are getting bigger and bolder. Judging from the sizes of the blots, in four hours the amount of rapeseed oil decreased and the amount of free fatty acids increased almost twice in comparison with the control point. From this, we can gather that hydrolysis reaction is proceeding properly when using the obtained MC preparation.

The microcapsule film partially melted after the reaction. After drying the remaining preparation, the specific activity was 10 463 U/g – almost twice less in comparison with the starting 19 527 U/g.

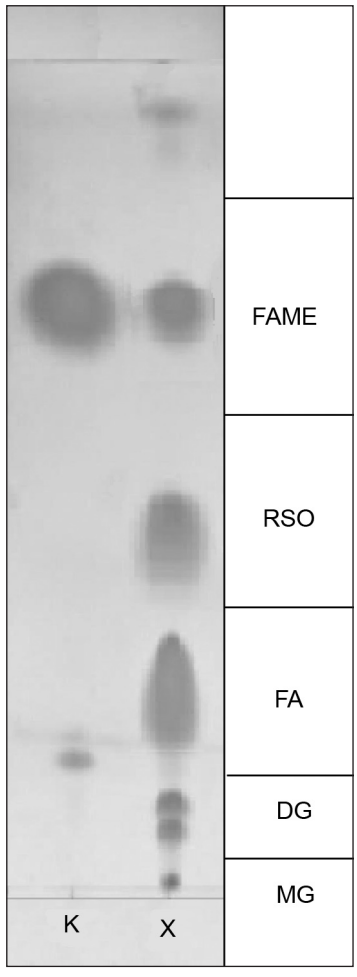

Fig. 11. TLC of rapeseed oil peresterification with methanol using encapsulated lipase. K – Methyl oleate, X – sample after peresterification process, FAME – Fatty acids methyl ester, RSO – rapeseed oil, FA – fatty acids, DG – diglycerides, MG – monoglycerides

Esterification of rapeseed oil with methanol

During the peresterification of rapeseed oil with methanol as during hydrolysis microcapsule preparation, it partially disintegrates and gains the consistency of sticky yellowish gel. As seen in the thin layer chromatogram pictured in Fig. 11, the bacterial microcapsule preparation obtained during this work catalysis the reaction of rapeseed peresterification with methyl alcohol.

CONCLUSION

During this research, *Enterobacter aerogenes* E13 cultural sample was immobilized in ethyl cellulose microcapsules. The obtained preparation can be used for the hydrolysis and peresterification reactions. However, ethyl cellulose carrier is not resistant to low-polarity liquids, like fatty acids or some esters, thus there is no advantage in reusage of immobilized preparation. On the other hand, *Enterobacter aerogenes* E13 cultural sample immobilization in ethyl cellulose microcapsules provides *better* storage stability. Higher microcapsulated lipase temperature optimum and lower pH optimum in comparison to non-immobilized cultural sample in some cases can be beneficial. This type bacterial-lipasic preparation can be used for the technologies for the purification of water from oil waste, as a filter filling and as a component for lipid sorbents. Due to cells also being immobilized into microcapsules, at relatively small waste concentrations, the immobilized complex should not only hydrolyze fats to fatty acids and glycerol, but also degrade them into the smaller products of *E. aerogenes* E13 cell metabolism.

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LIPAZINIU AKTYVUMU PASIŽYMINČIO *ENTEROBACTER AEROGENES E13* KULTŪRINIO SKYSČIO IMOBILIZAVIMAS ETILCELIULIOZĖS MIKROKAPSULĖSE

Santrauka

Lipazės – hidroliziniu aktyvumu pasižymintys fermentai, plačiai taikomi daugelyje pramonės sričių tam tikrų substratų hidrolizei bei organinių junginių sintezei. Vienas pagrindinių fermentų stabilizavimo būdų – jų imobilizavimas naudojant įvairius nešiklius. Ekonominiais sumetimais atsisakant gryninimo stadijos, gali būti imobilizuojami kataliziniu aktyvumu pasižymintys mikroorganizmų kultūriniai skysčiai.

Darbo metu taikytas mikrokapsuliavimo būdas lipazę produkuojančio *Enterobacter aerogenes E13* bakterijų kamieno kultūrinam skysčiui imobilizuoti etilceliuliozės mikrokapsulėse. Ištirtas mikrokapsuliavimo proceso sąlygų poveikis imobilizuoto preparato specifiniam lipaziniam aktyvumui bei imobilizacijos efektyvumui. Nustatytos imobilizuoto preparato savybės: pH ir temperatūrų optimumai, stabilumas, fizikiniai rodikliai. Preparatu buvo hidrolizuotas ir metanoliu peresterintas rapsų aliejus.

Raktažodžiai: lipazė, *Enterobacter aerogenes*, mikrokapsuliavimas, hidrolizė, peresterifikacija