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Keywords: *Thermomyces lanuginosus*, lipase, solid-state fermentation, hull-less pumpkin oil cake, flax oil cake, hemp oil cake

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Article

Lipase Production by Solid-State Cultivation of *Thermomyces Lanuginosus* on By-Products from Cold-Pressing Oil Production

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Abstract: This study shows that by-products obtained after cold-pressing oil production (flax oil cake, hemp oil cake, hull-less pumpkin oil cake) could be used as substrates for the sustainable and cost-effective production of lipase when cultivating *Thermomyces lanuginosus* under solid-state conditions ($T = 45\text{ }^{\circ}\text{C}$, $t = 9$ days). Lipase showed optimum activity at $T = 40\text{ }^{\circ}\text{C}$. The produced lipase extract was purified 17.03-folds with a recovery of 1% after gel chromatography. Three different batch experiments were performed in order to test the possibility of using the lipase in biodiesel production. Experiments were performed with a commercial, unpurified enzyme, and partially purified lipase with sunflower oil and methanol as substrates in a batch reactor at $40\text{ }^{\circ}\text{C}$. During the experiments, the operational stability of the enzyme was studied. The obtained results clearly showed that produced crude and purified lipase can be used for biodiesel production, but the process needs some additional optimization. As for operation stability, it was noticed that the commercial enzyme was deactivated after 30 h, while produced crude enzyme remained 8.25% of its activity after 368 h.

Keywords: flax oil cake; hemp oil cake; hull-less pumpkin oil cake; solid-state fermentation; lipase; *Thermomyces lanuginosus*

1. Introduction

In recent years the need for the development of sustainable industrial processes based on green chemistry has led to the application of solid-state fermentation, a fermentation approach where microorganisms are cultivated on complex biomaterials, mostly industrial wastes or by-products. In this regard, solid-state fermentations are greatly investigated for the purpose of lipase production. Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) are comprised of a large group of enzymes that differ in substrate specificity and regioselectivity. They can catalyze reactions of hydrolysis, esterification, and transesterification, and exhibit the catalytic activity under mild process conditions, which has an impact on the reduction of energy consumption [1,2]. In the terms of regioselectivity, lipases can be divided into three groups: (a) Sn-1,3-specific: Hydrolyze ester bonds at position sn-1 and sn-3; (b) Sn-2-specific: Hydrolyze ester bond at position sn-2.; (c) non-specific: Hydrolyze ester bonds at any positions. Non-specific lipases are widely used for biodiesel transesterification for complete hydrolysis of the triglyceride. The lipase from *Thermomyces lanuginosus* is an Sn-1,3-specific lipase [3].

Lipases are mostly produced from filamentous fungi, but basically, they can be produced from any organism that has fat-degrading enzymes. Lipase production belongs to one of the dominated

industrial enzyme productions [4]. Lipases can be used in the production of fine chemicals, detergents, in paper manufacture, feed and food industries, leather industry, medicine, and especially in biodiesel production [5]. The big economic aspect for applying lipases in industrial processes is its need to recover and recycle, whereas significant researches are dedicated to the investigations of immobilization techniques. The reason why a lot of attention is given to the investigation of lipase application in biodiesel production is the fact that in industrial biodiesel production a huge amount of chemicals and energy are used and a great number of soaps are formed, along with a considerable amount of wastewater. Therefore, this process cannot be categorized as *green*. Industrial biodiesel plants usually operate with base and/or acid-catalyzed formation of fatty acid alkyl esters by transesterification of oils of the corresponding fatty acids with short chain alcohol. The advantages of enzymatic methods are low reaction energy requirements, easy separation of glycerol, and the complete conversion of the free fatty acids presented in feedstock into alkyl esters. Disadvantages are possible enzyme inhibition by methanol and high costs of the enzyme.

Many papers on lipase production using solid-state cultivation of fungi on different low-cost media derived from agroindustry residues have been published thus far, although no data of its successful commercialization on a large scale are available. With the purpose of lipase production, *Aspergillus niger* was cultivated on a variety of agricultural wastes [6–9], *Rhizopus oryzae* on sugarcane bagasse [10], *Aspergillus* species on olive pomace [11,12], *Trichoderma* strains on oil-palm fruit-bunch medium [13], *Penicillium camemberti* KCCM 11268 on wheat bran [14], *Schizophyllum commune* ISTL04 on *Leucaena leucocephala* seeds [15], etc.

A detailed review on lipase from *T. lanuginosus* is given by Fernandez-Laufente [16]. *T. lanuginosus* lipase is a single chain protein comprised of 269 amino acids. Its molecular weight is 31,700 g/mol with an isoelectric point at pH 4.4. It tends to form bimolecular aggregates, confronting their open active centers, where each monomer can have a different activity, stability, and selectivity. *T. lanuginosus* lipase is thus far used in a different area, such as modification and production of new oils and fats, and biodiesel production. It has also been applied in organic chemistry and environmental protection [16].

In this paper, the utilization of the by-products from the vegetable oil production (oil cake) as a cheap substrate and carrier for the growth of *T. lanuginosus* with the aim to produce lipase is presented. *T. lanuginosus* was chosen as the working microorganism, since it is thermophilic fungus, and enzymes from thermophiles are more heat stable than those obtained from mesophiles [17]. Oil cakes are chosen to be substrates since they have a certain amount of oils in their composition [18]. An efficiency of crude lipase extract, partially purified lipase, and commercial lipase from *T. lanuginosus* was evaluated in experiments of transesterification of sunflower oil and methanol. This is, to the best of our knowledge, the first time when *T. lanuginosus* was used for lipase production when cultivated on hull-less pumpkin (*C. pepo* L.), flax (*L. usitatissimum* L.), and hemp (*C. sativa* L.) oil cakes.

2. Materials and Methods

2.1. Substrates

Hull-less pumpkin (*C. pepo* L.), flax (*L. usitatissimum* L.) and hemp (*C. sativa* L.) oil cakes were obtained from the local company (SME Lazić, Vraneševci, Croatia). The oil cakes used in all the experiments were from the same batch of oil production and were frozen at $-20\text{ }^{\circ}\text{C}$ prior to using. The initial moisture of the oil cakes delivered to the laboratory was 6.93%, 7.08%, and 7.91%, for hull-less pumpkin, hemp, and flax oil cake, respectively.

2.2. Microorganism and Commercial Enzyme

Thermomyces lanuginosus Tsiklinsky (ATCC $\text{\textcircled{R}}76323^{\text{TM}}$) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The fungus was grown on PDA (Potato Dextrose Agar) agar plates at $45\text{ }^{\circ}\text{C}$ for 7 days and stored at $4\text{ }^{\circ}\text{C}$. Spore suspension of the inoculum was prepared by mixing 5 mycelial discs (6 mm in diameter) with 10 cm^3 of water.

Lipase from *T. lanuginosus* (Lipolase 100L) was purchased from Sigma-Aldrich Handels GmbH, Vienna, Austria.

2.3. Enzyme Production

2.3.1. Solid-State Fermentation

Solid-state fermentations were performed in glass jars of 670 cm³. The height of the substrate layer in each jar was 6.5 cm. In order to obtain 60% of the substrate moisture, 50 g of the substrate was mixed with 52 cm³, 53 cm³, and 49 cm³ of distilled water, for flex, hemp, and hull-less pumpkin oil cake, respectively. Prior to inoculation, jars with substrates were autoclaved (121 °C/20 min) and cooled overnight. The substrate in each jar was inoculated (5 mycelial discs, 6 mm in diameter suspended in 10 cm³ of water, homogenized and incubated for 9 days at 45 °C without agitation in an incubator with the air fan set on 20% (KB 115, BINDER GmbH, Germany). Weight loss was monitored by weighing samples before and after biological treatment. For each substrate, 27 jars were inoculated. Every day, 3 jars were withdrawn and samples were taken for measuring lipase activity. To obtain crude enzymatic extract 1.0 g of the sample was extracted by 5 cm³ of phosphate buffer (0.1 M, pH 7) at 4 °C for 30 min and then centrifuged for 10 min at 15,000 g at 4 °C (Multifuge 3 L-R Centrifuge, Heraeus, Germany). Each extraction was done in duplicate. Lipase activity was measured from liquid extracts.

2.3.2. Enzyme Purification

Enzyme lipase was partially purified according to the method described by Singh et al. [15]. Briefly, 1 mmol dm⁻³ of phenylmethylsulfonyl fluoride (PMSF) was added into the crude enzyme extract in order to prevent enzyme degradation during the purification process. After that, lipase was precipitated in the supernatant by the addition of ammonium sulphate (70% saturation concentration) with continuous stirring at 4 °C. The precipitate collected after 20 min of centrifugation (21,255 g) and 4 °C (Universal 320R, Hettich Zentrifugen, Tuttlingen, Germany) contained the majority of lipase existing in the sample. The precipitate was then dissolved in sodium phosphate buffer (4.5 cm³, pH 8) that contained 1 mmol dm⁻³ PMSF and dialyzed for 24 h with three changes against the same buffer. The concentrated lipase was then filtered (Filter Chromafil® AO-20/3; 0.45 µm, Macherey-Nagel GmbH, Dueren, Germany) in order to remove larger molecules that could potentially clog the gel filtration column. Further purification was performed on the gel filtration column Superdex™ 200 (GE Healthcare Life Sciences, Barcelona, Spain) (1.2 cm × 20 cm). The column was pre-equilibrated with 50 mmol dm⁻³ sodium phosphate buffer containing 0.15 mol dm⁻³ NaCl (pH 8). The mobile phase was the same buffer and the flow rate was 0.5 cm³ min⁻¹. Fractions of 0.5 cm³ were collected at the bottom of the column and analyzed for lipase activity and protein concentration using the Bradford method. All purification steps were carried out at 4 °C. Fractions containing active lipase were pooled and stored at -20 °C.

2.4. Analytical Methods

2.4.1. Measurements of Lipase Activity

The reaction mixture contained a buffer-substrate solution (1 mmol dm⁻³ pNP-palmitate), Tris-HCl buffer (pH 8.0, 50 mmol dm⁻³), gum arabic (1 g dm⁻³) in the final volume of 3.9 cm³. The mixture was pre-incubated at 40 °C for 5 min, and the reaction started by the addition of 0.1 cm³ of crude enzyme extract. After 5 min of incubation at 40 °C, the reaction was stopped by the addition of 1.5 cm³ of Marmur solution. Absorbance was measured spectrophotometrically (ThermoSpectronic, Helios, Cambridge, UK) at 410 nm after centrifugation (Z 326 K, Hermle Labortechnik GmbH, Wehingen, Germany) at 15,000 g for 10 min. The amount of released pN-phenol was calculated using a molar extinction coefficient of 0.29866 dm³ mmol⁻¹ cm⁻¹. One unit (U) was defined as the amount of lipase that degrades 1 µmol of pNP-palmitate in 1 minute.

2.4.2. Measurement of Fatty Acid Methyl Esters (FAME)

The FAME concentration was determined by the method of Budžaki et al. [19]. Briefly, samples were collected during the process, diluted 10x in hexane, and filtrated (hydrophobic PTFE Syringe filters, pores: 0.45 μm , diameter: 25 mm) in order to remove the enzyme. The samples were analyzed by gas chromatograph (Shimadzu GC-2014, Kyoto, Japan) equipped with a flame ionization detector (FID) and Zebron ZB-wax GC capillary column (Torrance, CA, USA, length 30 m, I.D. 0.53mm and film thickness 1.00 μm). The method consisted of maintaining the temperature at 180 $^{\circ}\text{C}$ for 1 min and then heating up to 230 $^{\circ}\text{C}$ at a rate of 5 $^{\circ}\text{C min}^{-1}$. The total determination time was 20 min using helium as a carrier gas at 1.97 $\text{cm}^3 \text{min}^{-1}$. Peaks identification was carried out using the standard FAME mix GLC-10 that was also used for calibration curves preparation. The retention times for the corresponding fatty acid esters were 7.494 min for palmitic, 10.192 min for stearic, 10.545 min for oleic, 11.257 min for linoleic, and 12.336 min for linolenic acids. All the measurements were performed in triplicate and confidence interval was calculated taking in consideration standard deviation, number of measurements, and confidence range. In the 95% confidence range, the results showed no statistical difference.

2.4.3. Enzyme Characterization

In order to find the optimal temperature, the activity of the lipase was measured at a temperature range from 30 $^{\circ}\text{C}$ to 50 $^{\circ}\text{C}$ in 0.1 mol dm^{-3} phosphate buffer, pH 7.5. Each measurement was carried out twice. To determine the storage stability of the enzyme, the crude enzyme extract was stored at (a) $T = 25^{\circ}\text{C}$, (b) $T = 4^{\circ}\text{C}$, and (c) $T = -20^{\circ}\text{C}$. For experiments (a) and (b), the activity was measured every day over 5 days, while for experiment (c) it was measured at 7 days intervals during a 3 months period. In order to avoid the influence of thawing on enzyme activity, the samples were stored separately, 12 samples in total. The activity was measured by standard assay method described above.

2.4.4. Biodiesel Production

Biodiesel production from edible sunflower oil (Oil refinery Čepin, Croatia) purchased at a nearby market, using lipase as a biocatalyst was performed in a laboratory batch reactor under following reaction conditions: V (reactor) = 400 cm^3 , V (reaction mixture) = 200 cm^3 , $T = 40^{\circ}\text{C}$, $n = 300 \text{rpm}$, 0.1 mol dm^{-3} phosphate buffer pH 7.5, $t = 368 \text{h}$. The reaction mixture was comprised of sunflower oil and methanol in a ratio of 1:3.4 and the reaction started by the addition of enzyme dissolved in 0.1 mol dm^{-3} phosphate buffer pH 7.5 into the reaction mixture. Water/buffer (8% (w/w)) was added to ensure water/lipid interface formation since it is necessary for lipase activation [20]. Three different experiments were performed, the first one by using commercial enzyme ($V.A. = 344,204 \text{U cm}^{-3}$), the second by using unpurified enzyme obtained after solid-state fermentation using hemp oil cake as a substrate ($V.A. = 60 \text{U cm}^{-3}$) and third one, by using partially purified lipase ($V.A. = 1020 \text{U cm}^{-3}$). Samples were collected during the process and analyzed using gas chromatography.

During the process, the samples were also withdrawn for the purpose of analyzing enzyme activity in order to investigate enzyme operational stability.

2.4.5. Data Processing

Enzyme operational stability decay rate constant (k_d) was described by first-order kinetics (Equation (1)).

$$\frac{d(\text{relative activity})}{dt} = -k_d \cdot \text{relative activity} \quad (1)$$

It was estimated by non-linear regression analysis from experimental data. The least-squares method implemented in the Scientist®3.0 software package (Micromath®, Saint Louis, MO, USA) was used.

3. Results

3.1. Lipase Production During Solid-State Cultivation of *T. Lanuginosus* on Oil Cakes

Oil cakes were chosen to be substrates for *T. lanuginosus* solid-state cultivation based on our previous work, where it was shown that the oil cakes were comprised of a high ether extract content that indicated a high lipid content. Ether extract content in hemp seed oil cake was 12.3% db, in hull-less pumpkin seed oil cake it was 36.2% db, while in flax seed oil cake it was 21.4% db. Additionally, they were also comprised of high protein content; 38.3% db (hull-less pumpkin oil cake), 32.4% db (flax seed oil cake), and 24.8% db (hemp seed oil cake) [18]. Chemical composition of oil cakes indicated that they were good substrates for the use of lipase production during solid-state cultivation of *T. lanuginosus*, based on the following assumptions: (a) No need to add an extra source of oils; (b) no need to add additional carbon source in the form of simple sugars for the microorganisms growth [21]; (c) low content of phenolic compounds known as inhibitors of fungal growth.

When cultivating lipase-producing fungi on agro-industrial waste it is common to add additional carbon sources (glucose, maltose, lactose or fructose) [11]. Based on previous results [18], only hull-less pumpkin oil cake is comprised of all 3 investigated sugars (glucose, fructose, and sucrose), while no sugars were detected in the flax oil cake. Hemp oil cake contained sucrose. *T. lanuginosus* started to grow after one day of cultivation, and mycelial penetration was visible by eye. After 9 days, all jars were fully covered with mycelia, but higher activities were obtained when *T. lanuginosus* was cultivated on the hemp oil cake (Figure 1). Carbon catabolic repression of lipase by glucose is known for some fungi, such as *R. miehei*, *A. niger*, *R. delemar*, *F. oxysporum*, etc. [21]. The results of this study also indicated possible lipase inhibiting effect of glucose or fructose since the lowest lipase activities were obtained when *T. lanuginosus* was cultivated on hull-less pumpkin oil cake.

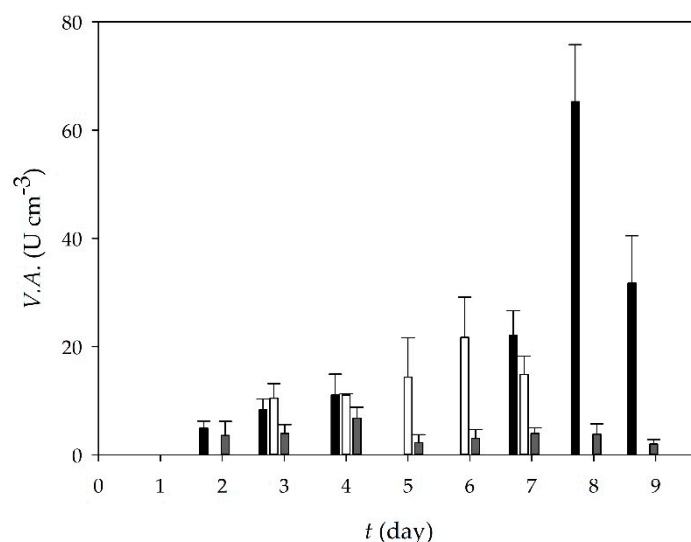


Figure 1. Lipase activities during *T. lanuginosus* cultivation on different oil cakes (■ hemp oil cake, □ flax oil cake, and ▒ hull-less pumpkin oil cake).

The results of lipase activities are presented as an average value of the measurements from 3 jars and standard deviation (Figure 1).

The substrate value, from an economic point of view, should be evaluated through the substrate conversion process to the desired product regardless of its low price. Roughly, factors that determine whether the biomass conversion process into a product is feasible or not, are the operational costs and the market price of the product obtained, since the capital investments are relatively low [22]. Castilho et al. [22] presented a detailed cost analysis of lipase production by solid-state fermentation on babassu cake enriched with olive oil in bench-scale (100 m³ of lipase concentrate per year).

Obtained results showed the feasibility of lipase production by a solid-state fermentation process with agro-industrial waste as a substrate. For a lipase annual capacity of 100 m³, the payback time was 1.5 years due to 47% lower production cost than the product selling price.

3.2. Enzyme Purification

Partial purification of lipase produced in solid state fermentation from *T. lanuginosus* lead to a 17.03 fold increase in a specific activity, with a recovery of 1% achieved after gel chromatography. Similar results were also obtained when lipase was purified after isolation from different strains. For lipase produced from *Bacillus stearothermophilus*, purification resulted in a 9.53 fold increase in specific activity and 2.49% recovery yield [23]. When an extracellular lipase from *Microbacterium* sp. was purified, a total yield of 20.8% and a 2.1-fold increase in specific activity was achieved [24]. In addition, when lipase produced by *Streptomyces bambergiensis* OC 25-4 strain was partially purified with ammonium sulphate precipitation, dialysis, and gel filtration chromatography, a 2.73-fold increase in a specific activity with 0.53% recovery was achieved [25]. For lipase produced from *T. lanuginosus*, purification resulted in a 10-fold increase in specific activity of the enzyme with 25% of recovery [26]. All mention examples coupled with the one presented in this research clearly indicate that additional optimization of the purification process is necessary in order to achieve overall higher lipase activity.

3.3. Enzyme Characterization

The activity of purified lipase was measured in a temperature range from 30 to 50 °C in phosphate buffer at pH 7.5 in order to establish the influence of temperature on the reaction rate in the assay conditions described in paragraph 2.4.1. As evident from Figure 2, the optimal temperature was 40 °C.

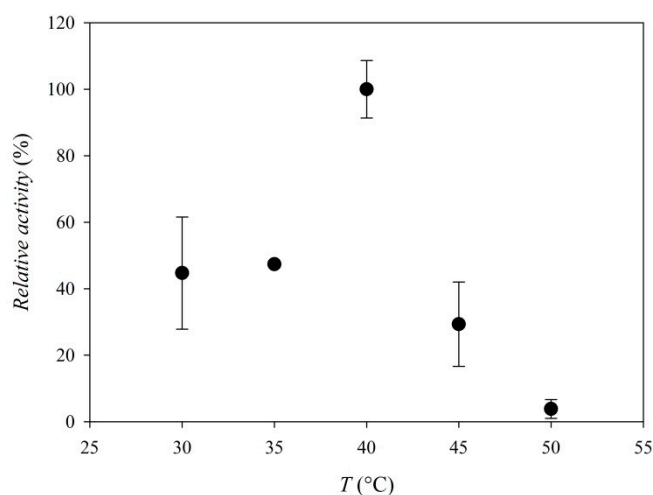


Figure 2. Dependence of lipase activity on temperature.

Temperature is an important limiting factor for the storage of enzymes. In order to investigate long-time storage, 3 different temperatures were chosen at low temperatures below 20 °C and to investigate 5-days crude enzyme storage at 4 °C and 25 °C for the purpose of enzyme manipulation during laboratory experiments. The results of the measurements of crude extract enzyme storage stability at 4 °C and 25 °C during the time are presented in Figure 3.

After 5 days, it was shown that the crude enzyme is stable at 25 °C. However, after 5 days, 89.3% of the activity retained when the enzyme was stored at 4 °C. No loss in activity was gained during enzyme storage at −20 °C.

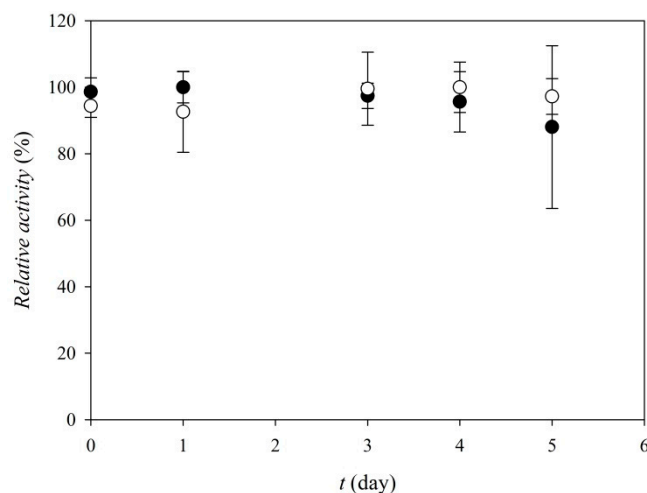


Figure 3. Enzyme stability during storage at different temperatures: (○) $T = 25\text{ }^{\circ}\text{C}$, (●) $T = 4\text{ }^{\circ}\text{C}$.

3.4. Biodiesel Production

After the lipase was purified, 3 different experiments were performed, the first one by using a commercial enzyme, the second by using an unpurified enzyme, and the third one, by using partially purified lipase.

As mentioned in the introduction, lipases can catalyze reactions of hydrolysis, esterification, and transesterification [1,2]. Since the excess of water in the process favors hydrolyses [27], the process was performed at a water content of 8%(w/w) that minimized hydrolyses [28]. In esterification and transesterification reactions, excess methanol is usually used to shift the reaction towards FAME formation [29]. Another advantage of methanol excess is the reduction of reaction mixture viscosity that leads to minimization of diffusion limitations and enhancement of reaction rate [28]. All the reactions were performed in an oil/methanol ratio of 1:3.4.

Dynamic change of fatty acids methyl esters (FAME) content (w/w) is presented in Figure 4.

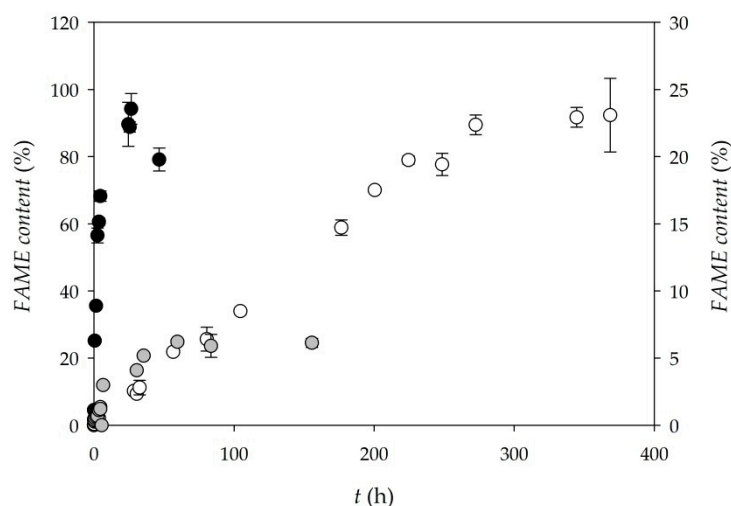


Figure 4. Fatty acids methyl esters (FAME) content when the reaction is performed by using (●) commercial, (○) unpurified and (●) purified enzyme (○ and ● are presented on the secondary axis).

When the biotransformation process was performed with commercial lipase, obtained results corresponded to the previously published results [19]. High FAME content of $94.25 \pm 4.55\%$ was observed after 26 h. Budžaki et al. [19] suggested that for higher FAME content higher oil to methanol

ratio should be considered. In the literature [30], the ratio (usually from 3.5 to 12) depends on several factors like choice of feedstock, the molecular weight of alcohol, and microbial source of enzyme.

On the other hand, too much excess can have several negative effects. The first one is possible emulsification of glycerol that could lead to a reduction of FAME yield because recombination of FAME and glycerol could occur [31]. The second one is deactivation of enzyme since short chains of methanol can cause structural changes of lipase [32].

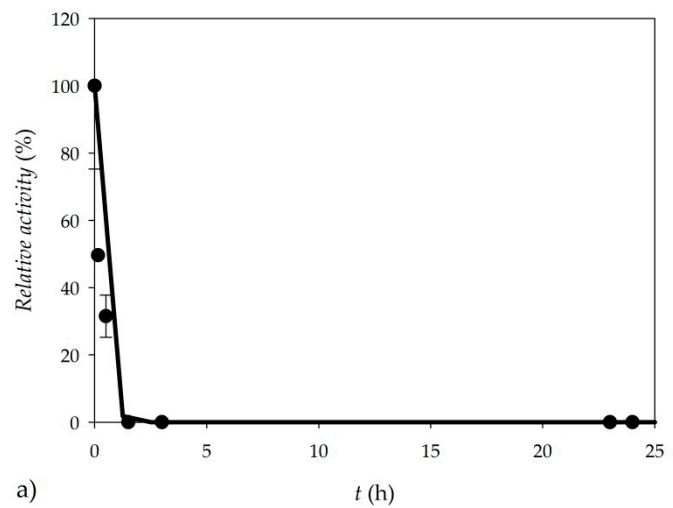
When the reaction was performed with the unpurified enzyme, 5-fold lower FAME content was noticed for 6-fold longer reaction time. The reason for this was a significantly lower enzyme volume activity of produced, unpurified enzyme. On the other hand, results clearly indicated that lipase produced using by-products of oil production could be used as a catalyst for biodiesel production. To make the process more economical (higher FAME content in shorter reaction time), additional lipase production process optimization is necessary.

As a final step, the third reaction was performed with purified enzyme. Since the volume activity was only 17.03 higher in comparison to the one of the unpurified enzyme, for the first 60 h of the reaction, slightly higher FAME content was observed. On the other hand, the reaction was stopped after no change in FAME content was noticed. The reason for this is the loss of enzyme activity.

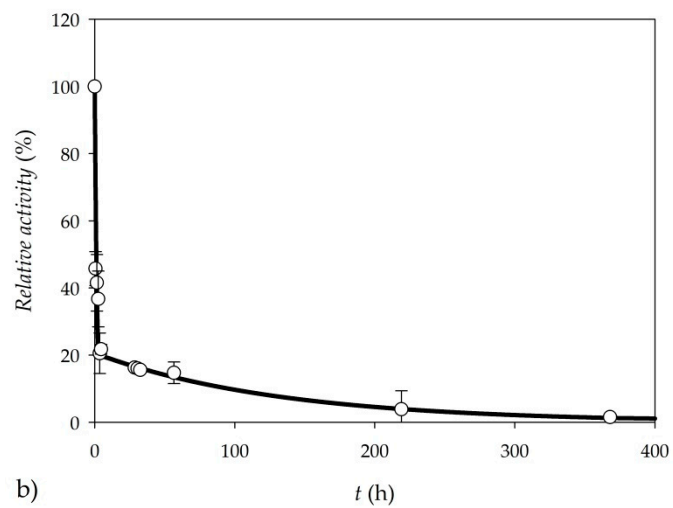
The results of enzyme operational stability decay compared with the commercial enzyme are presented in Figure 5. The results of lipase activities were presented as relative values.

The results of enzyme operational stability decay (Equation (1)) compared with the commercial enzyme expressed as relative activities indicated that it can be seen that the commercial and purified enzyme activity decreased faster than the activity of the crude enzyme extract. Enzyme operational stability decay rate was calculated to be: $k_{d\text{commercial}} = 3.20 \pm 0.49 \text{ h}^{-1}$, $k_{d\text{unpurified}} = 0.63 \pm 0.21 \text{ h}^{-1}$ for the first 2.5 h and $0.007 \pm 0.0005 \text{ h}^{-1}$ until the end of the experiment and $k_{d\text{purified}} = 0.95 \pm 0.21 \text{ h}^{-1}$. The enzyme operational stability decay would be apparent from the concentration vs. time dependence if a lower enzyme concentration was used, which was the case when the crude enzyme extract was used.

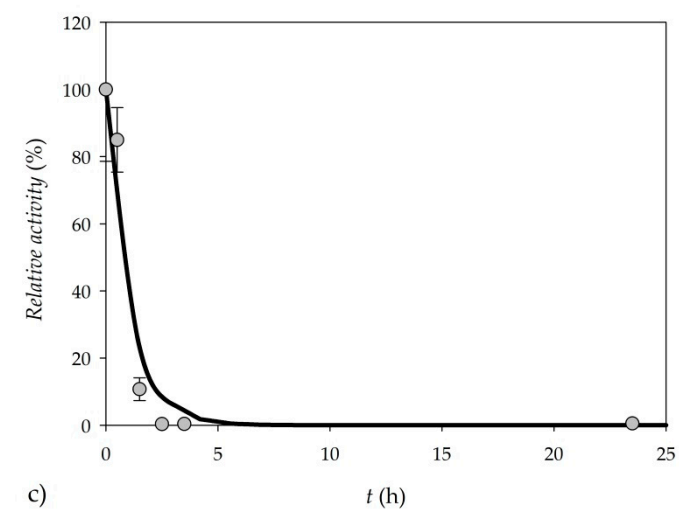
According to the literature, there can be several reasons for the decrease of lipase activity. Some of them are mechanical forces caused by mixing, presence of low chain alcohols, the formation of glycerol, and water content [33,34]. According to Toress et al. [35], the loss of enzyme activity over time is a result of thermal degradation and alcohol inhibition. Lipases are mostly deactivated by methanol since the degree of degradation is inversely proportional to the number of carbon atoms in the alcohol [36]. Kamal et al. (2013) [37] claimed that methanol has a high tendency to strip off tightly bounded water from the enzyme and can reach deeper into the enzyme active site. As a consequence, the lipase structure was changed and the activity was decreased. According to Robles et al. [33], little or no deactivation has been noticed when a methanol to oil molar ratio below 3 was used. On the other hand, Maceiras et al. [38] claimed that low amounts of methanol deactivate the free enzyme, whereas higher concentrations of methanol were necessary to deactivate the immobilized enzyme. Salis et al. [39] found that *C. antarctica* lipase B was progressively inactivated by methanol in amounts above 1/2 molar equivalent. In the work of Šalić et al. (2018) [40] commercial lipase from *T. lanuginosus* was completely deactivated by methanol after 25 h using higher amounts of methanol. However, the purified enzyme in this study followed the same deactivation trend despite the fact that a lower oil to methanol ratio was used. On the other hand, crude extract remained active for a longer period of time, which in the end had an effect on overall FAME content. Based on the obtained results and the fact that methanol as a substrate has a significant effect (substrate inhibition) on the process, and in order to mitigate this effect, the process should be conducted as a fed-batch. Other possible solutions were the use of other alcohol as an alternate to methanol or the use of organic solvents as well as to use immobilized enzyme.



a)



b)



c)

Figure 5. Investigation of enzyme stability in an oil/methanol reaction mixture ($V_{\text{reactor}} = 400 \text{ cm}^3$, $n = 300 \text{ rpm}$, $T = 40 \text{ }^\circ\text{C}$, $t = 368 \text{ h}$) for (a) commercial, (b) unpurified, and (c) purified enzyme (— mathematical model).

4. Conclusions and Future Work

In conclusion, in the cold press oil industry, remarkable amounts of by-products arise after oil production. Its utilization is very important regarding economical gain. In this paper, a simple method for the lipase production by the application of three different oil cakes as the substrate for *T. lanuginosus* solid-state cultivation was suggested. In the term of highest lipase production, hemp oil cake was shown to be the best substrate. Further optimization study for higher lipase activities and scale-up of the process in tray bioreactor, and lipase application in the biodiesel production in fed-batch experiments are foreseen.

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