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INVESTIGATION OF THE FAT FRACTION ENZYMATIC HYDROLYSIS OF THE WASTE FROM PRODUCTION OF HYDROGENATED FAT BY THE LIPASE *RHIZOPUS JAPONICUS*

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Abstract. In the article the conditions of enzymatic hydrolysis of fat fraction of waste from production of hydrogenated fat by the lipase Rhizopus japonicus are considered, namely, the influence of pH of the medium (pH-optimum, pH-stability) and temperature (thermal optimum, thermal stability). The scope of applications of lipases in various branches of the national economy, including for utilization of numerous fatty waste and by-products of oil and fat industry, is disclosed. The main reasons of biotechnological potential of microbial lipases are considered. Objects of research were the lipase *Rhizopus japonicus* and waste from the demetallization stage of the hydrogenated fat production. Detected, that the optimum pH value for *Rhizopus japonicus* lipase is 7.0, reducing the pH of the medium from the optimum to pH 6.0 is accompanied by a decrease in activity by 30%, and an increase from 7.0 to 9.0 – decrease by 20%. The maximum activity of the enzyme is observed in the region of physiological values of the temperature. It has been established that the lipase optimal temperature is 40°C. The results of the *Rhizopus japonicus* lipase stability study showed that incubation of the enzyme at pH 2.5 resulted in a complete loss of lipolytic activity after 30 minutes, and at alkaline pH, the enzyme was more stable. Incubation of lipase *Rhizopus japonicus* for 30 min at pH 9.0 leads to loss of lipolytic activity by 25% of the maximum, and total loss of activity occurs after 2.5 h. The study of pH-stability of *Rhizopus japonicus* lipase at an optimal pH of 7 showed that after 60 min of incubation, the enzyme lost 15% of the lipolitical activity, and after 60 min – 50%. Complete loss of Rhizopus japonicus lipase activity at pH 7.0 takes place after 150 minutes of incubation. The results of the study of thermal stability of lipase showed that at a temperature of 40°C and 60°C, the lipase activity remained rather stable for 50 minutes and completely lost after 150 minutes of incubation. At 80°C and 100°C, lipase activity was lost after 40 minutes and 50 minutes of incubation, respectively. The results of the study indicate the prospect of enzymatic hydrolysis of fat fraction of waste by Rhizopus japonicus lipase. The results obtained should be used to improve the processing technology of waste oil and fat industry food and processing industries.

Key words: ecological biotechnology, oilseed fat industry, waste, lipase *Rhizopus japonicus*, enzymatic hydrolysis, stability.

ДОСЛІДЖЕННЯ ФЕРМЕНТОЛІЗУ ЖИРОВОЇ ФРАКЦІЇ ВІДХОДІВ ВИРОБНИЦТВА САЛОМАСУ ЛІПАЗОЮ *RHIZOPUS JAPONICUS*

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Анотація. У статті розглянуто умови ферментолізу жирової фракції відходів виробництва саломасу ліпазою *Rhizopus japonicus*, а саме, вплив pH середовища (pH-оптимум, pH-стабільність) та температури (термооптимум, термостабільність). Розкрито область застосування ліпаз в різних галузях народного господарства, в тому числі для утилізації численних жирових відходів та побічних продуктів оліє-жирової промисловості. Розглянуто біотехнологічний потенціал мікробних ліпаз. Об'єктами дослідження обрано ліпазу *Rhizopus japonicus* і відходи зі стадії деметалізації при виробництві саломасу. Виявлено, що для ліпази *Rhizopus japonicus* оптимальне значення pH середовища складає 7,0 од. pH, зниження pH середовища від оптимального до pH 6,0 супроводжується зменшенням активності на 30%, а підвищення з 7,0 до 9,0 од. pH – зменшенням на 20%. Максимальна активність ферменту спостерігається в області фізіологічних значень температури. Встановлено, що термооптимум ліпази має місце за температури 40°C. Результати дослідження pH-стабільності ліпази *Rhizopus japonicus* показали, що інкубація ферменту при pH 2,5 приводить до повної втрати ліполітичної активності через 30 хвилин, а при лужних значеннях pH фермент є більш стабільним. Інкубація ліпази *Rhizopus japonicus* протягом 30 хв при pH 9,0 приводить до втрати ліполітичної активності на 25% від максимальної, а повна втрата активності наступає через 2,5 год. Вивчення pH-стабільності ліпази *Rhizopus japonicus* за оптимального значення pH 7 показало, що через 60 хв інкубації фермент втрачає 15% ліполітичної активності, а через 60 хв – 50%. Повна втрата активності ліпази *Rhizopus japonicus* при pH 7,0 має місце через 150 хв інкубації. Результати дослідження термостабільності ліпази показали, що за температури 40°C та 60°C активність ліпази залишалась доволі стабільною протягом 50 хв і повністю втрачалась через 150 хв інкубації. За температури 80°C та 100°C активність ліпази втрачалась через 40 хв та 50 хв інкубації відповідно. Результати дослідження свідчать про перспективність ферментативного гідролізу жирової фракції відходів ліпазою

Rhizopus japonicus. Отримані результати доцільно використовувати для удосконалення технології переробки відходів оліє-жирової галузі харчової та переробної промисловостей.

Ключові слова: екологічна біотехнологія, оліє-жирова промисловість, відходи, ліпаза *Rhizopus japonicus*, ферментоліз, стабільність.

Introduction. Formulation of the problem

Fat and products of hydrolysis of fats are valuable raw materials for food and processing, as well as other industries such as rubber, paint, cosmetic and others. The search for biotechnological methods of hydrolysis of the fat fraction of the waste oils of the oil and fat industry is an urgent task [1-5]. To effectively carry out the process of enzymatic transformation, it is necessary not only to have an active substance but also to know on what conditions its maximum enzymatic activity is manifested and what factors and how affect the enzymes.

Analysis of recent research and publications

Every year in Ukraine, in the process of production of oil and fat products at various stages, numerous fatty waste and by-products are formed. According to expert estimates, the volume of these wastes in general in Ukraine is 30 million tons, and the average level of their industrial processing barely exceeds 30% of the mass produced. The process of hydration of vegetable oils in oil and fat production is accompanied by the formation of large-scale waste, the main of which is the spent sorbent from the stage of extra bleaching and spent catalyst, the fat content of which can reach 50%. The main method of utilization of these wastes is burial at solid waste landfills. As a rule, for 1 ton of commodity product (hydrogenated fat) 1.4–2.0 kg of spent sorbent and 0.4–0.5 kg of spent catalyst are formed [6,7].

Biotechnological processes of processing are based on natural processes, which practically have no side effects, are productive and safe for biota and components of the environment.

Microbial lipases form an important group of biotechnologically valuable enzymes [8]. The reasons for the enormous biotechnological potential of microbial lipases are the fact that they:

− function better at low temperatures;
− Can be used as a substitute for aggressive chemical compounds, which reduce environmental hazard, prevent contamination, spare the use of corrosion-resistant equipment;
− highly specific, therefore eliminating the formation of adverse undesirable products and the need for complex technological processes;
− possible modification through immobilization, which contributes to their multiple use;
− Can be used for treatment and disposal of waste containing harmful compounds;
− They can decompose naturally with destructors, so all the chemical components of enzymes are included in the natural ecological cycle [8].

The individual characteristics of the enzyme in many respects depend on the characteristics of the producer and can vary significantly even in microorganisms of one kind, which is associated with species, and sometimes with strain differences. Many researchers pay close attention to the influence of pH and temperature on the stability of lipase. Moreover, these two factors have an important application value and should be taken into account. For each enzyme there are optimal values of these quantities, in which it exhibits maximum activity. The optimal pH and temperature values for lipases from different sources vary widely enough. The pH-optimum for lipases from different sources varies from 4.0 to 9.0. However, the optimum for most lipases is in the pH range from 6.0 to 8.0. Some authors noted changes in the optimum pH of the enzyme with increasing degree of purification [9-12]. The optimal temperature values for most lipases vary in the range of 30–40°C. Some of the representatives are heat-resistant and operate at sufficiently high temperatures – 55–70°C. Moreover, some lipase representatives are stable and active at -20°C, and lipase rye retains its activity even after 12 hours of deep cooling to -45°C [9-12]. Similar data are available for lipase of microbial origin [13,14].

An important property possessed by enzymes is their substrate specificity. High specificity of enzymes in relation to substrates opens great prospects for their practical use [15].

Lipases are widely used in the processing of fats and oils, detergents and degreasers in the food industry; in the production of glycerol, fatty acids, mono- and diglycerides; synthesis of fine chemicals and pharmaceuticals; for utilization of oil and fat industry waste, sewage treatment and sewage communications; in medicine, as therapeutic agents; light industry; agriculture; the production of detergents, paper, cosmetics and biodiesel. Lipase can be used to accelerate the degradation of fatty waste and synthetic plastics [15-17].

Active producers of lipases are bacteria of genera *Pseudomonas, Bacillus, Rhizopus, Aspergillus, Geotrichum, actinomycetes Streptomyces, Thermoactinomyces, Candida* yeast (Yarrowia). From the results of previous studies [18], it is evident that the greatest activity in relation to hydrogenated fat was shown by lipase Rhizopus japonicus. Dosage of the enzyme Rhizopus japonicus depends on the raw material and process, the enzyme activity is 50,000–5,058,593.8 units/g, the optimum action is 25–45°C, the optimum pH 4.0–8.0 [18].

The purpose of the work is to select the conditions for productive enzymatic hydrolysis fatty fraction of waste formed during the hydrogenation of vegetable oils by means of the lipase of the microbial origin of *Rhizopus japonicus*.

The task of the work is to determine the pH optimum, thermal optimum, pH stability and thermal stability of lipase *Rhizopus japonicus* in relation to hydrogenated fat.

Research materials and methods

The objects of the study were lipase *Rhizopus japonicus* produced by Enterprise Enzim (Ladyzhin,

Vinnitsa region, Ukraine), which shows the greatest activity in relation to hydrogenated fat [18], which substantiates its use for hydrolysis of the latter (PJSC «Vinnytsia OZHK»).

In previous studies [18], the effectiveness of the lipase *Rhizopus japonicus* use for hydrogenated fat hydrolysis was shown. The hydrogenated fat production process is shown in the figure 1.
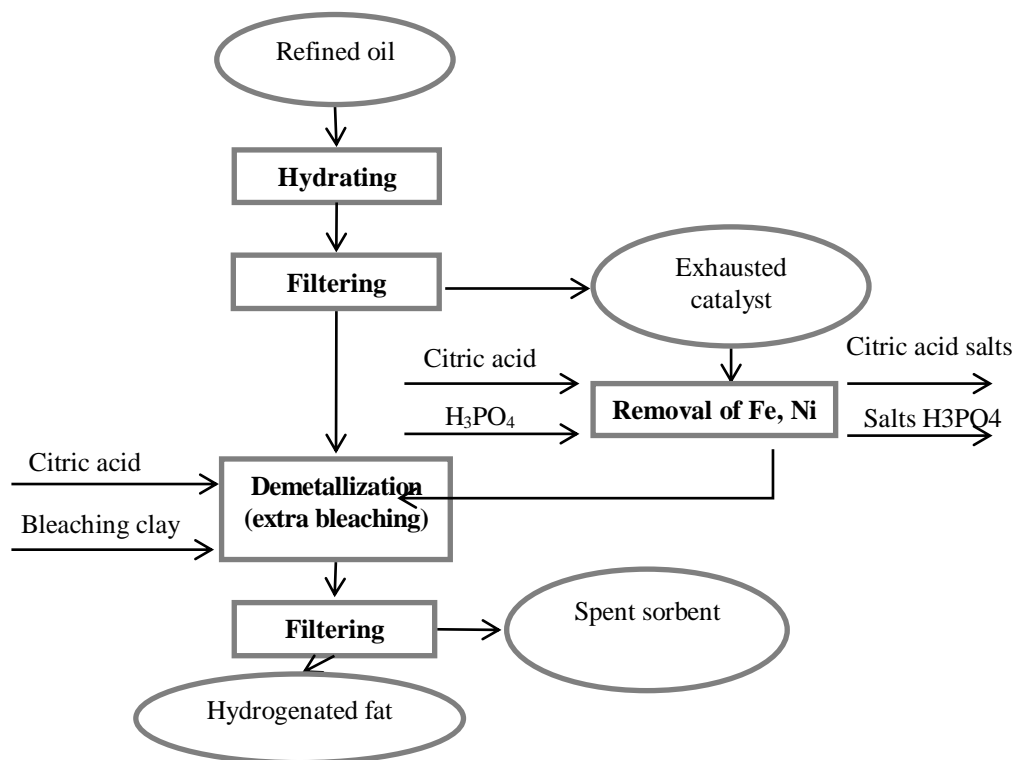

Fig. 1. Technological scheme of the production of hydrogenated fat

To provide the characteristics of the enzyme as a substrate, hydrogenated fat was used, which is the main component of its production waste.

The lipolytic activity was determined by the titrimetric method of Ota and Yamada [18], which is based on the calculation of the amount of fatty acids formed during the hydrolysis of the substrate. As a substrate, 40% emulsion of hydrogenated fat was used, which was stabilized with polyvinyl alcohol. An activity unit was taken up by the amount of enzyme that liberates 1 micromole of acid from 40% saline suspension at 37°C for 1 hour. In a test tube containing 8–15 mg of the sample, 1 cm³ of water, 0.1 cm³ of a 0.1% solution of lipase, 0.8 cm³ of phosphate buffer (pH of the medium corresponded to the pH-optimum of lipase) and thermostat for 5 minutes at 37°C Then, 1.0 cm³ of a 40% suspension of hydrogenated fat with polyvinyl alcohol was added, thermostated under the same conditions. Exactly after 1 h of incubation, the reaction was interrupted by the addition of 5 cm³ of 96% ethanol. In the control sample, the emulsion of hydrogenated fat was added after the addition of

ethanol. In the control and experimental samples, three drops of phenolphthalein solution were added and titrated with 0.05 N sodium hydroxide solution to a light pink color. The level of lipase activity was estimated in terms of the difference in the amount of alkali that went for the titration of the experimental and control samples with the hydrolysis of 40% emulsion of hydrogenated fat.

The activity of lipase was calculated according to the formula:

$$LA = \frac{(A - A_1)}{B} * 100 \qquad (1)$$

where A – is the amount of 0.05 M NAOH that went for the titration of the prototype, cm³;

A_1 – is the amount of 0.05 M NAOH that went for the titration of the control sample, cm³;

B – the amount of enzyme in the reaction mixture, mg;

100 – coefficient for conversion into a micromolar;

LA – lipolytic activity.

The pH-optimum lipase was determined in such a way that buffer solution with different pH values of the medium in the range of 2.5–12.0 was added to the enzyme samples of equal activity and the enzymatic activity was determined. Determination of pH optimum of the enzyme preparation was carried out at 37°C using the following buffer solutions: glucine-NaCl-HCl, 0.1 mol/dm³ (pH 2–3.5); Acetate buffer solution, 0.1 mol/dm³ (pH 3.5–5.5); phosphate buffer solution, 0.1 mol/dm³.

The thermo optimum of the enzyme was determined by studying for the same enzyme activity at a temperature of 20 to 80°C in the corresponding buffer, which corresponds to the pH optimum of this enzyme.

To determine the pH-stability, the enzyme samples that were identical in activity were incubated at different pH values of 2.5–12.0 for 0–360 min, then the pH of the solution was adjusted to an optimum value and the enzyme activity was determined. The thermal stability of the lipase was studied in such a way that the activity level of the enzyme sample in the appropriate buffer solution, which corresponds to pH optimum, was incubated at 20, 37, 45 and 60°C for 0 to 360 min, then the temperature was adjusted to 37°C and the activity of the enzyme was determined.

Results of the research and their discussion

In order to optimize conditions for enzymatic hydrolysis of hydrogenated fat with lipase, the study of physical and chemical properties of lipase had been carried out.

The catalytic activity of enzymes is strongly influenced by the pH of the medium, which is carried out by ionizing the individual components of the enzymatic reaction. Enzymes are active only at a certain pH range and in most cases, for each enzyme, there is a certain optimum pH. The presence of such an optimum can have several reasons: 1) the influence of pH on the reaction rate (under saturation with the substrate); 2) the influence of pH on the affinity of the enzyme to the substrate; 3) the effect of pH on the stability of the enzyme, which may be irreversibly inactivated by pH values different from the optimal. These factors, as a rule, operate in combination with each other. The action of these factors can be

distinguished experimentally. Changes in the state of ionization of the enzyme when changing the pH are due to the existence of a series of different ionic forms, and the distribution of the entire amount of the enzyme between these ionic forms depends on the pH and the ionization constants of different groups. Since catalytic activity is usually only one of these forms, the form of pH-dependence has the form of a bell. Changes in the composition of the enzyme when changing pH are described by the scheme:

$$EH^+ \xrightarrow{K_1} EH \xrightarrow{K_2} E^-$$
$$H^+ \qquad H^+$$
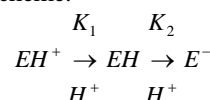
where EH⁺, EH, E⁻ – «acid», «neutral» and «alkaline» form of the enzyme, respectively; K_1 i K_2 – are the corresponding dissociation constants [13,19].

Such changes can occur in the native ferment, in the enzyme-substrate complex or substrate. It is shown that pH influences the rate of enzymatic reaction in three ways: the action on the magnitude of the rate of enzymatic reaction (Vmax), the formation of the enzyme-substrate complex and the stability of the enzyme molecule [18,20].

Lipazes contain different groups that are capable of ionizing, but their catalytic activity is detected in a narrow range of pH. In this regard, one can predict that only one of the ionic forms of the enzyme (active center) is catalytically active. In this case, the distribution of the entire amount of the enzyme between these ionic forms depends on the pH and ionization constants (pK) of individual groups located in the active center.

For any enzyme, there is an optimal pH and temperature for which it exhibits maximum activity. For lipase Rhizopus japonicus, the optimum pH value of the medium is 7.0 units pH, and reducing the pH of the medium from the optimum to pH 6.0 is accompanied by a decrease in activity by 30%, and an increase from 7.0 to 9.0 units. pH – a decrease of 20%, which allows us to conclude that lipase is sufficiently sensitive to change in pH (Fig. 2).

The optimum temperature for most lipases varies in the range of 37–43°C, the pH-optimum of *Rhizopus japonicus* lipase was determined at 40°C (Fig. 3).
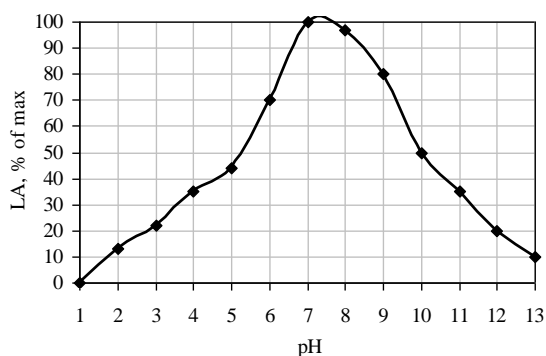

Fig. 2. pH- optimum lipase *Rhizopus japonicus*
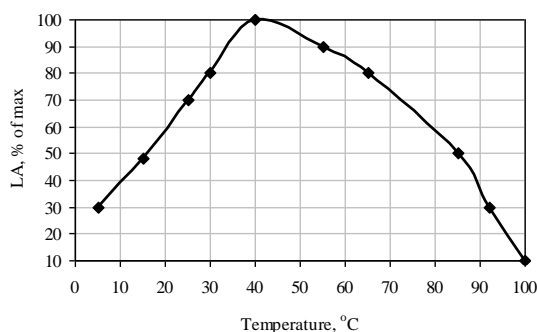

Fig. 3. Thermo-optimum lipase *Rhizopus japonicus*

It was established that the inactivation of enzymes under the influence of pH and temperature is the result of previous ionization processes, which deeply affect electrostatic interactions in the enzyme molecule.

Due to the fact that the lipolysis reaction is heterogeneous, that is, it proceeds on the surface of the phase separation of oil and water, the course of its flow can be altered by action directly on the enzyme or its sorption on the interphase surface, as well as on the orientation of the substrate molecules and the diffusion of lipolysis products.

The effect of temperature on the rate of enzymatic reactions may be due to the effect of various factors. The temperature affects the stability of the enzyme, the decay rate of the enzyme-substrate complex, the affinity of the enzyme to the substrate, etc. [18].

At carrying out the enzymatic reaction two different factors defining the influence of temperature act simultaneously, on one hand- increase of initial speed, on the other hand – causing the denaturation of enzyme under the influence of temperature, which causes continuous decrease of concentration of active enzyme. The optimum temperature depends on the correlation between the influence of temperature on the speed of the enzymatic reaction and its effect on the enzyme denaturation rate [18,20].

The position of the thermooptimum of the enzymatic activity of the preparation with Rhizopus japonicus was determined at an optimum value of pH activity (7.0) and a temperature of 10–100°C.

From the experimental data, the study shows (Fig. 3) that the thermo-optimum of lipase is 40°C. Lipase is stable in the range of 15–85°C, keeping 50% of the maximum activity. With an increase in temperature, activity is sharply reduced.

Thus, the maximum activity of the enzyme is observed in the region of physiological values of the temperature range with its rapid decrease with a further rise in temperature due to the thermal denaturation of the native protein molecule, the change in the nature of the microenvironment of the native enzyme.

Results on the study of lipase pH-stability (Figure 4) indicate that lipase is least stable at pH 2.5. Under conditions of incubation at pH 2.5, lipolytic activity is lost after 30 minutes. When the enzyme is incubated at pH 9.0, the lipolytic activity is completely lost after 2.5 hours.

Thus, the enzyme does not change its activity within one hour of its incubation at an optimum pH value with its subsequent loss, which may be due to denaturation changes in the protein molecule.

As can be seen from the results of the study (Fig. 5), at 100°C, the lipolytic activity of native lipase after 20 minutes its incubation under these conditions decreased to 2% of the original. The highest lipase stability was observed at 40°C – lipase activity remained stable for 1.5 h. and completely lost after 3 h of incubation.
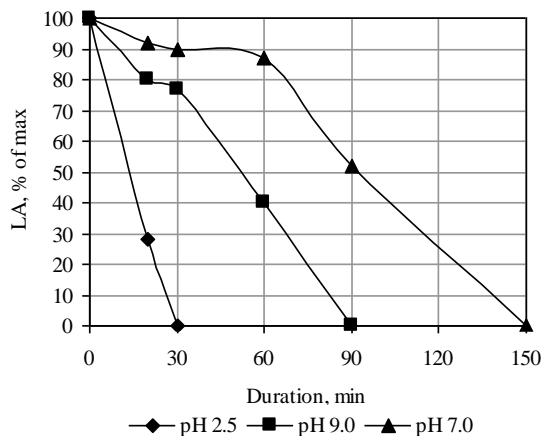

Fig. 4. pH- stability of the lipase Rhizopus japonicus (at 40 ° C)
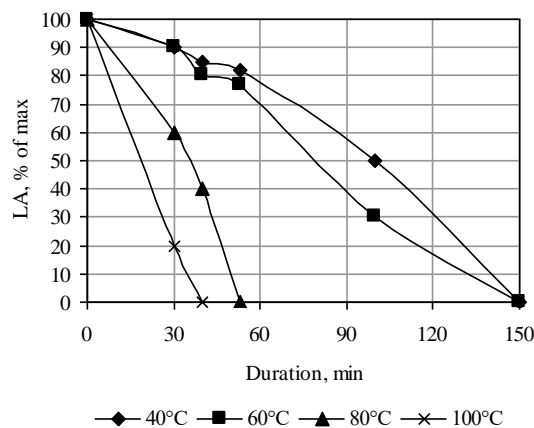

Fig. 5. Thermal stability of the lipase Rhizopus japonicus (pH 7,0)

Conclusion

The experimental results obtained from the study indicate the prospect of fermentolysis by the lipase Rhizopus japonicas of fat fraction of waste generated during the hydrogenation of vegetable oils. The conditions for productive hydrolysis of waste are: the weight ratio of lipase: substrate 1:50 [18], the medium temperature of 40°C, the pH of the reaction mixture 7.0, the reduction of pH from optimal to pH 6.0 is accompanied by a decrease in activity by 30%, and an increase from 7.0 to 9.0 units. pH – a decrease of 20%,

which allows us to conclude that lipase is sufficiently sensitive to change in pH.

It has been established that lipase is stable in the range of 15–85°C keeping 50% of its maximum activity. With an increase in temperature, activity is sharply reduced. At 100 °C, the lipolytic activity of native lipase after 20 minutes of its incubation under these conditions decreased to 2% of the original. The highest lipase stability was observed at 40°C – lipase activity remained stable for 1.5 h. and was completely lost after 3 hours of incubation. Thus, the maximum activity of the enzyme is observed in the region of physiological values of

temperature with its rapid decrease with a further increase in temperature due to the thermal denaturation of the native protein molecule, the change in the nature of microenvironment of the native enzyme.

The results of research should be used for improvement of the technology of processing of waste of the oil and fat branch of the food and processing industry.

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