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PRODUCTION AND OPTIMIZATION OF EXOCELLULAR LIPASES PRODUCED BY *Proteus mirabilis*

PRODUKCJA I OPTYMALIZACJA ZEWNĄTRZKOMÓRKOWYCH LIPAZ *Proteus mirabilis*

Abstract: Lipases are produced by plants, animals and microorganisms, but only those produced by the microorganisms are important to industry due to their various enzymatic properties and substrate specificity. Despite the fact, that such enzymes are synthesized by many species of microorganisms, new environmental strains able to synthesize exocellular lipases are searched for. The aim of conducted research was to assess the lipolytic activity of *Proteus mirabilis* depending on pH and temperature. The activity was analysed in the pH range between 4-9 and temperatures 5-60ºC. The lipolytic activity was assessed by means of titration and the results were presented as the amount of released µmoles of fatty acids. The analysis of pH influence showed that the highest amount of µmoles of fatty acids was released by the strain under study at pH 4 (264.75 µmol/cm^3). Synthesized lipases were most active at 35-45ºC and the amount of released µmoles of fatty acids ranged between 264.75 and 116.25 µmol/cm^3. Lipases produced by *P. mirabilis* due to their biological activity showed in the wide range of pH and temperature may be an alternative to lipolytic products currently applied in the industry.

Keywords: *Proteus mirabilis,* lipolytic activity, culturing conditions

Introduction

The global industrial enzymes market is projected to reach USD 6.30 billion by 2022 in terms of value [1]. In the forthcoming years we shall witness a significant increase in enzymes production due to their expanding application as detergents, pharmaceuticals, food and beverages. Lipases constitute third group, after proteases and carbohydrases, in terms of their total sale number [1]. Lipases catalyze the breakdown of ester bonds between glycerol and fatty acids in different lipids. The enzymes are responsible for the breakdown of triglycerides which provide energetic substances for living organisms. The reaction catalyzed by lipases is carried out between the lipid and aquatic phase, which dissolves the enzyme. The products of hydrolysis are fatty acids, diacylglycerols, monoacylglycerols and glycerol. Due to their versatile catalytic properties, lipases have wide range of industrial applications including detergent, textile, and dairy industries, oil processing, production of surfactants and synthesis of chiral pharmaceuticals [2-6].

Many different lipases have been identified in and isolated from bacteria belonging to the following species: *Acitenobacter*, *Pseudomonas*, *Bacillus*, *Geobacillus*, *Burkholderia*, *Serratia* and *Staphylococcus.* Bacterial lipases are exocellular enzymes of molecular weight 19-75 kDa [6], isolated to the growing medium in the late phase of logarithmic growth and produced in the process of submerged fermentation [2]. At present, bacterial lipases belonging to these from Family I, Subfamily I.1 and I.2 are perhaps studied most closely. Lipases from Subfamily I.1 and I.2 are nearly exclusively found in *Pseudomonas* and *Proteus* species [4, 7]. The process of their synthesis and secretion is influenced by many

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environmental factors, such as: sources of carbon and nitrate, ions, the presence of polysaccharides which are not metabolized by bacteria, temperature and pH. Therefore, the latest trend in lipase research is the development of novel and improved lipases.

The aim of the research was an assessment of different environmental factors such as: temperature, pH, incubation time on the lipolytic activity of the new strain *Proteus mirabilis* KMN16.

Materials and method

Isolation and identification

Proteus mirabilis strain KMN16 was isolated from meat waste contaminated soil. The isolation process was performed on tributyrin agar plates incubated at 30°C. Colonies showing clear zones around them were picked out and transferred to nutrition agar. The Identification of the isolate was performed with the rapid miniaturised system API ID 32GN and API 20E (bioMérieux, France), following the suppliers instructions. Complementary tests used for the final identification of the isolate were as follows: macroscopic and microscopic morphologies and biochemical analysis following Bergey's manual of determinative bacteriology.

Lipase production

The ability of *P. mirabilis* KMN16 to produce lipase was tested in tributyrin medium containing [%]: tributyrin oil 1.0, pepton 1.0, NaCl 0.5, $CaCl_2$ 0.01. The pH was maintained at 7. The strain was suspended in 5 cm^3 of sterile water and used as the inoculum for the pre culture to obtain an initial cell density to adjust the turbidity of 0.5 McFarland standard. The suspension of bacterial culture was incubated in 100 cm^3 of liquid medium on a rotary shaker at 100 rpm and incubated at 30°C. After 7 and 14 days of incubation, the culture was centrifuged at 10,000 rpm for 15 min at 4°C and the cell free culture supernatant was used as the source of exocellular lipases.

Qualitative assay

Lipolytic activity of *P. mirabilis* KMN16 was determined by the diffusion plate method in tributyrin agar medium containing tributyrin oil 1%. To perform the assay, the holes of 5 mm diameter were bored in agar and 200 mm^3 of supernatant was introduced to each. The media were incubated at different temperatures ranging from 5 to 60ºC for 7 days. Lipolytic activity of tested strain was determined by measuring the diameter of hydrolytic zones around each hole.

Assay of lipase activity

The lipase activity was assessed by means of titrimetric analysis and determined with tributyrin emulsion as a substrate. The reaction mixture contained 1 cm^3 of the enzymatic suspension, 4 cm^3 of respective buffer and 5 cm^3 of tributyrin emulsion. In the control treatment the supernatant was replaced with sterile water. The mixture was incubated for 30 minutes and the reaction was terminated by adding 10 cm^3 of acetone-ethanol (1:1) mixture. The amount of liberated fatty acids was determined by titration with 0.05 M

NaOH solution against 2% phenolphthalein as an indicator, and calculated as a subtraction between the proper treatment and the control treatment results. The result was presented as the amount of liberated μmoles of fatty acids. One lipase unit (U) was defined as the amount of enzyme which liberated 1 μmol of fatty acids per minute.

Effect of pH and temperature on lipase activity

For determination of optimal temperature of the activity, the supernatant containing the enzyme was incubated at different temperatures ranging from 5 to 60°C, at pH 7.0 (0.05 M phosphate buffer). The optimal pH was determined at 35°C in buffer solutions of pH values from 4 to 9 using different buffers (0.05 M citrate buffer pH 4-6; 0.05 M phosphate buffer pH 7-8; 0.05 M Tris-HCl buffer pH 9). Enzymatic activity was measured according to a standard titrimetric protocol.

Results and discussion

The sample of meat waste contaminated soil showed high bacterial count. The colony described as KMN16 showed maximum zone of clearance when plated on tributyrin agar. Morphological and biochemical studies were carried out on KMN16 isolate. The analysis clearly demonstrated that strain KMN16 belongs to the genus *Proteus* and exhibited maximum biochemical similarity with *P. mirabilis* (99% of biochemical similarity).

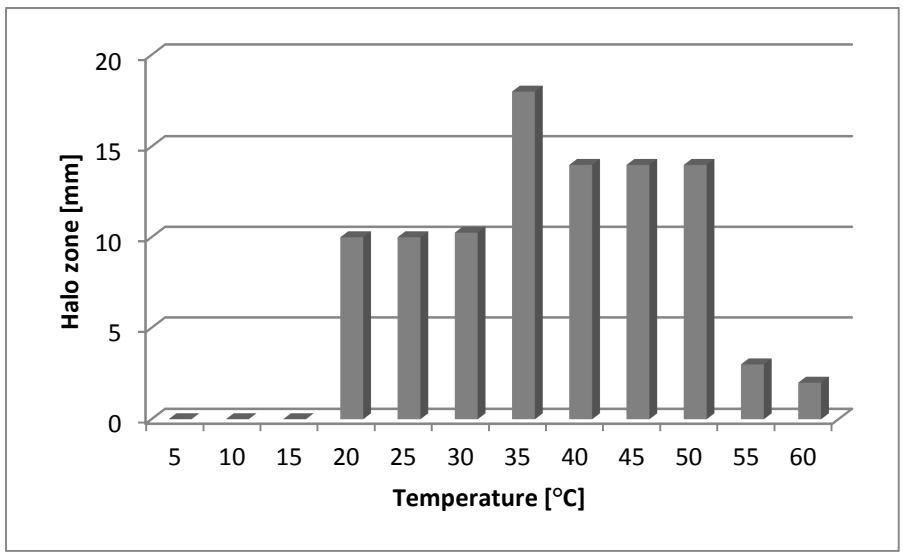

Fig. 1. Lipase activity on tributyrin agar plate after 7 days

P. mirabilis KMN16 was tested in terms of its ability to synthesize exocellular lipases in different environmental conditions concerning time, temperature and pH of the culturing medium. The first phase of the research consisted of screening tests on the medium with tributyrin incubated at temperature ranging from 5 to 60°C (Fig. 1). The ability to produce

lipases by strain KMN16 was assessed according to the hydrolytic zones. They were observed only after 7 days of incubation around the holes containing supernatant. The biggest hydrolytic zone of 18 mm was obtained at 35°C. Slightly smaller zones were noted at temperatures ranging from 20 to 30°C (ca. 10 mm) and between 40 and 50°C (14 mm). At the lowest and the highest temperatures amounting 5-15°C and 55-60°C respectively the strain KMN16 did not produce lipases or synthesized them at a low amount. The differences between obtained hydrolytic zones were statistically insignificant in this case (Fig. 1).

The results obtained in screening tests proved that lipolytic activity of *P. mirabilis* KMN16 was noted after at least 7 days of culturing. Therefore, the amount of liberated µmoles of fatty acids was measured after 7 and 14 days and the most optimal conditions for culturing was 35°C and pH 7.

The analysis of lipolytic activity of *P. mirabilis* KMN16 shows that the highest amount of fatty acids was liberated under the most optimal conditions after 7 days of incubation (Figs. 2 and 3). Strain KMN16 synthesized the highest amount of µmoles of fatty acids at 35°C and pH 4 which amounted over 260 µmoles. As the pH was getting higher, the amount of fatty acids was decreasing rapidly to the amount of 107.25 µmoles at pH 5 and gained constant value of 58 µmoles in the pH range of 8-9. After 14 days of incubation, no influence of pH was noticed in the process of lipases synthesis. Analyzed range of pH showed that the amount of liberated µmoles of fatty acids remained at a constant level and mounted between 49-47 µmoles (Fig. 2). The highest amount of liberated fatty acids at pH 7 optimal for *P. mirabilis* KMN16 was noted after 7 days of incubation at 35°C and amounted over 94 µmoles. Further rise of temperature caused reduction in the amount of µmoles of fatty acids. However, in the temperature range between 40 and 45°C these differences were statistically insignificant and liberated µmoles of fatty acids amounted 90. Regardless of the incubation time, no ability to synthesize fatty acids was noted under psychrophilic conditions - below 15°C (Fig. 3).

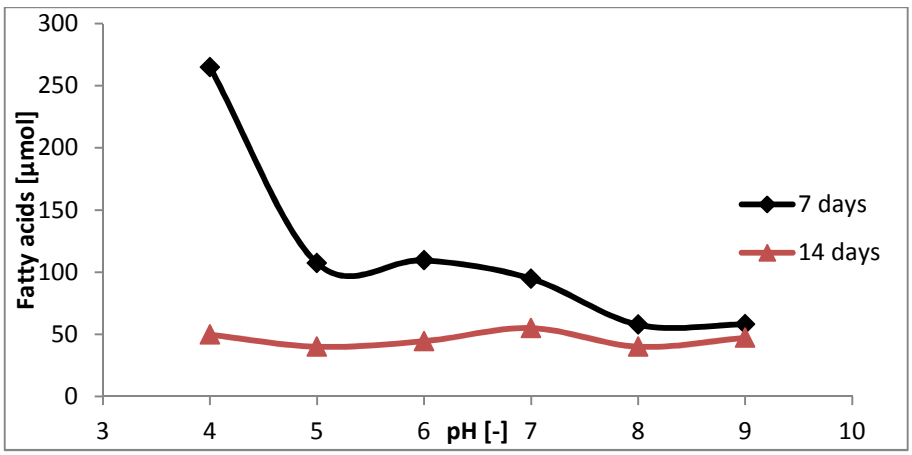

Fig. 2. Effect of different pH on lipase production by *P. mirabilis* KMN16

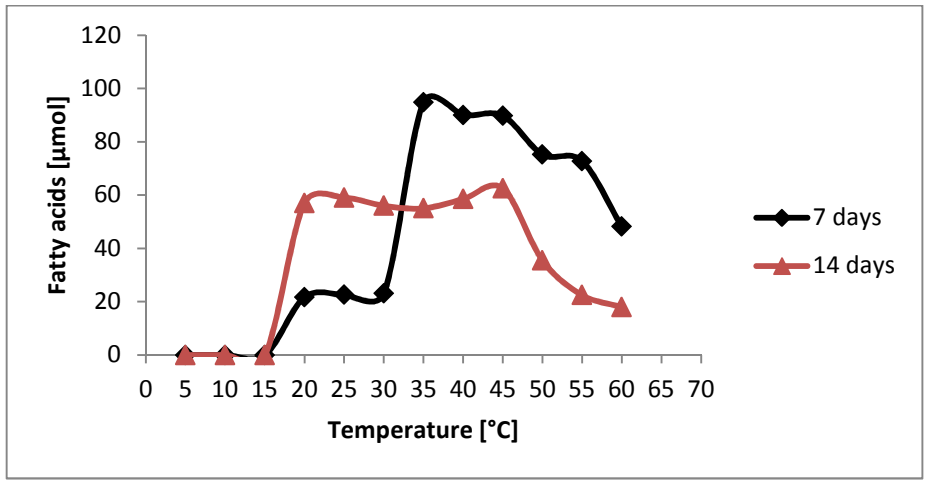

Fig. 3. Effect of different temperature on lipase production by *P. mirabilis* KMN16

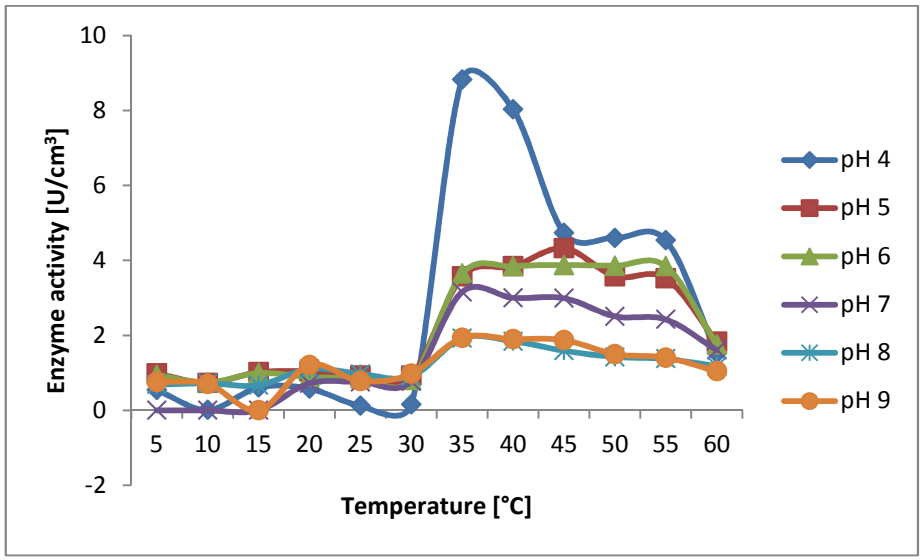

Fig. 4. Lipolytic activity of *P. mirabilis* KMN16 after 7 days incubation

In Figures 4 and 5 lipolytic activity of *P. mirabilis* KMN16 is shown in the units of lipolytic activity in the full range of analyzed both temperatures and pH. After 7 days of incubation, regardless of the pH value, in the temperature range between 5-30°C no lipolytic activity or the lowest activity was noted. The significant increase in the activity was noted at the temperature over 30°C. The strain showed the highest activity at pH 4 and temperatures between 35-40°C (8.83 U/cm^3). Under test conditions at pH between 5 and 9 and at temperature range from 35 to 55°C the enzyme was stable. The lipolytic activity

measured at various pH levels (pH 5-9) was statistically insignificant (Fig. 4). After 14 days of incubation, no enzymatic activity or only low value of it was noted at the temperatures between 5 and 15°C. The results obtained in this case were ambiguous, as regardless of the pH, increased enzymatic activity was observed in the temperature range of 25 and 45°C. The highest lipolytic activity amounting 3.45 U/cm^3 was noted again at pH 4 at the lowest temperature amounting 25°C (Fig. 5).

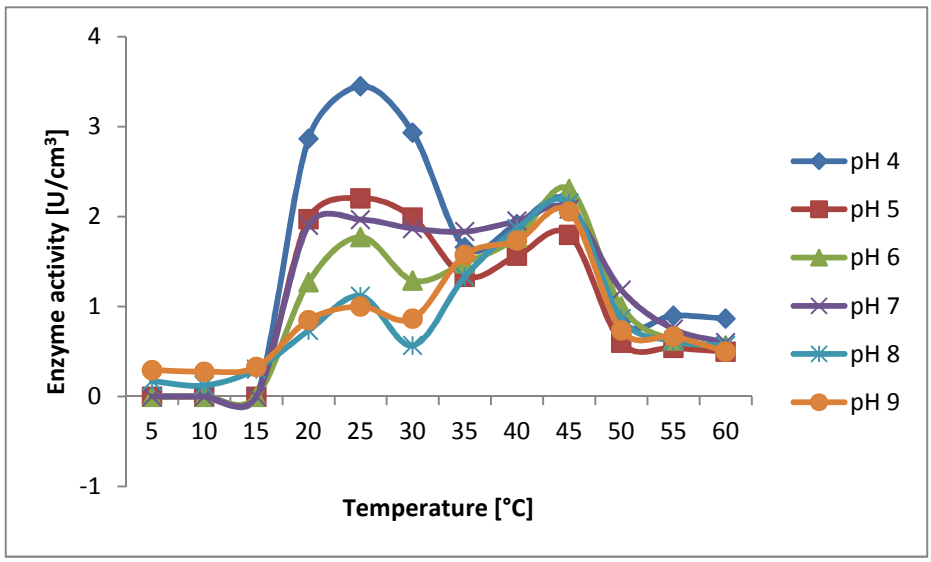

Fig. 5. Lipolytic activity of *P. mirabilis* KMN16 after 14 days incubation

On the basis of obtained results it may be concluded that optimal conditions for the strain *P. mirabilis* KMN16 to synthesize lipases is pH amounting 4 and the temperature range between 35-45°C. However, in an alkaline and thermophilic environment, strain under study KMN16 showed low lipolytic activity regardless of the incubation time.

Conclusions

The effect of pH on the lipolytic activity was determined in six different buffers covering the range of pH 4 to 9. In conducted research, the enzyme was most active at pH 4. In general, bacterial lipases are stable in a wide range of pH from 4 to 11 [9]. However, the optimum pH value will vary greatly from one enzyme to another. For example the optimum value of pH for pancreatic lipase amounts 8, for gastric lipases it ranges between 4-5 and for lipases used in the process of biodiesel production pH amounts 4.7 [10]. However, the majority of other research papers prove that the optimal pH value in the process of bacterial lipases production is neutral or alkaline. The research papers on lipases produced by strain *P. fluorescens* SIK W1 show that it prefers an acidic optimum of pH 4.8. Whereas, in the research of Bradoo et al. [11] the strains *B. stearothermophilus* SB-1 and *B. licheniformis* SB-3 retained their lipolytic activity at pH 3.0.

Bacterial lipases generally have temperature optima in the range of 30-60°C, which was proved by the authors' own research. According to Korman and Bowie [12] and Korman et al. [13] bacterial lipases from Subfamily I.1 and I.2 catalyze the hydrolysis of triacylglycerol at the temperature range between 25-45°C and are used extensively as biocatalysts. As the authors show, the lipase from *P. mirabilis* belongs to the *Proteus*/psychrophilic family of lipase Subfamily I.1 and is a promising catalyst for biodiesel production because it can tolerate high amounts of water in the reaction. In conducted research, the enzyme was most active at 35°C, which allows to include lipases produced by strain KMN16 in Subfamily I.1. An application of new psychrophilic lipase produced by *P. mirabilis* KMN16 in an industrial process of biodiesel production enables to reduce the costs as it decreases the temperature of the process and thus reduce the demand for energy.

The results obtained in this study show that the stability of *P. mirabilis* KMN16 lipase at pH 4 and at temperature 35°C have proved it to be a potential psychrophilic and acidic lipase and a candidate for industrial applications such as biodiesel production. Further studies are needed to enhance lipase production in strain KMN16.

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PRODUKCJA I OPTYMALIZACJA ZEWNĄTRZKOMÓRKOWYCH LIPAZ *Proteus mirabilis*

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Abstrakt: Lipazy są wytwarzane przez rośliny, zwierzęta i mikroorganizmy, ale tylko te pochodzenia mikrobiologicznego są ważne dla przemysłu ze względu na zróżnicowane właściwości enzymatyczne i specyficzność substratową. Mimo że enzymy te są syntetyzowane przez wiele gatunków mikroorganizmów, to nadal poszukuje się nowych, środowiskowych szczepów zdolnych do syntezy zewnątrzkomórkowych lipaz. Celem przeprowadzonych badań była ocena aktywności lipolitycznej *Proteus mirabilis* w zależności od pH oraz temperatury. Aktywność lipolityczną analizowano w zakresie pH 4-9 oraz w temperaturach 5-60ºC. Do oznaczenia aktywności lipolitycznej posłużono się metodą miareczkową, a wyniki podano jako ilość uwolnionych µmoli kwasów tłuszczowych. Analizując wpływ pH, stwierdzono, iż najwięcej µmoli kwasów tłuszczowych badany szczep uwolnił przy pH 4 (264,75 µmol/cm^3). Lipazy syntetyzowane przez badany szczep wykazywały największą aktywność, gdy reakcję prowadzono w zakresie temperatur 35-45ºC. Ilość uwolnionych wówczas µmoli kwasów tłuszczowych mieściła się w przedziale od 264,75 do 116,25 µmol/cm^3. Lipazy *P. mirabilis* ze względu na aktywność biologiczną w szerokim zakresie pH oraz temperatury mogą stać się alternatywą dla obecnych na rynku preparatów lipolitycznych stosowanych w przemyśle.

Słowa kluczowe: *Proteus mirabilis*, aktywność lipolityczna, warunki hodowli