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INFLUENCE OF THE CULTURE CONDITIONS ON LIPOLYTIC ACTIVITY OF *Bacillus cereus* AND *Bacillus mycoides*

WPŁYW WARUNKÓW ŚRODOWISKA NA AKTYWNOŚĆ LIPOLITYCZNĄ SZCZEPÓW *Bacillus cereus* I *Bacillus mycoides*

Abstract: The aim of the research was the evaluation of lipolytic activity of *B. cereus* and *B. mycoides* strains, in reference to carbon source, pH and the temperature. In the research, two strains of *Bacillus cereus* and *Bacillus mycoides* each, isolated from the soil and water, were applied. The sources of carbon in culture media were fatty substrates: tributyrin, Tween 40, Tween 60, Tween 80 and glucose. The lipolytic activity was measured by means of titration at pH ranging from 5 to 8 and the temperature ranging from 30 °C to 60 °C. The results were presented in the units of lipolytic activity [U cm^{-3}]. In the conducted research, the amount of liberated lipolytic activity depended on the type of fatty substrate in the medium, pH and the temperature. The strains under study showed the lowest activity at pH 5 and 6, and the highest at pH 7 and 8. In these conditions, most of the strains showed the lipolytic activity, even in case of the lack of fatty substrate in the medium. The highest amount of lipolytic activity was liberated at pH 8 in the medium with Tween 40, and the highest results (0.88 [U cm^{-3}]) were noted for the soil strain *B. cereus*. When analysing the influence of the temperature on the lipolytic activity, it was stated that the highest amount of lipolytic activity was noted at 30 and 40 °C, and the lowest at 50 and 60 °C. The best results were obtained for most of the strains at 30 °C, in medium with Tween 40, and the most active was the soil strain of *B. mycoides* (0.88 [U cm^{-3}]). The exception is *B. cereus*, as it liberated 1.38 [U cm^{-3}], in the medium with glucose. Taking into account all analysed sources of carbon and parameters, it seems that the most active were *B. mycoides* strains.

Keywords: *Bacillus cereus, Bacillus mycoides*, lipases, tributyrin, Tween

Lipases are an important group of biotechnologically valuable enzymes. They are defined as hydrolases of glycerol esters EC 3.1.1.3, and are the enzymes of high catalytical potential. They are produced by plants, animals and microorganisms, of which the last group remains in the centre of attention. Many kinds of bacteria possess

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the ability to produce them, among others bacteria of *Bacillus* kind [1, 2]. A common interest in bacterial lipases is connected with their role as biocatalysts in many biochemical processes. They have diverse applications in a wide variety of industries ranging from detergent, oleochemical, organic synthesis, dairy, fat and oil modification to pharmaceutical. They have many applications for stereospecific hydrolysis and synthesis of a wide variety of technologically valuable esters [1, 2].

The interest in microbial lipase production has risen in the last decades, because of its large potential in environmental protection as wastewater treatment as well as decomposition and removal of oil substances. Effective breakdown of solids and the clearing and prevention of fat blockage or filming in waste systems are important in many industrial operations. Bacterial lipases are also involved in solution of such environmental problems as the breakdown of fats in domestic sewage and anaerobic digesters [3].

As shown by data in literature [2, 4–6], they are varied in terms of their enzymatic activity, which depends on the species of microbes and the culturing conditions (eg pH of the growth medium, temperature, source of nitrogen and presence of lipids in the medium). Therefore, screening of microorganisms with lipolytic activities could facilitate the discovery of novel lipases.

The aim of undertaken research was the evaluation of lipolytic activity of *Bacillus cereus* and *Bacillus mycoides*, isolated form the natural environment, in reference to carbon source, pH and the temperature.

Materials and methods

The object of the study were 4 *Bacillus* strains:

– 2 *Bacillus cereus* strains marked as: A96 and G10, isolated from soil and water, respectively,

– 2 *Bacillus mycoides* strains marked as: A134 and G3, isolated from soil and water, respectively.

The inoculum was produced in the basal medium consisted of 1.0 g · dm^{-3} yeast extract, K_2HPO_4 3.0 g · dm^{-3}, KH_2PO_4 2.0 g · dm^{-3}, $(NH_4)_2SO_4$ 2.0 g · dm^{-3} and $MgSO_4 \cdot 7H_2O$ 0.5 g · dm^{-3}. The sources of carbon in culture media were the following fatty substrates: tributyrin, Tween 40, Tween 60, Tween 80 and glucose. The cultures were maintained in Erlenmeyer flasks of 250 cm^3 capacity containing 50 cm^3 of respective growth medium with an inoculum of density equal to E = 2, obtained from the 48-hour culture on a nutrient broth. Incubation was conducted on a rotary shaker for 2 days at 30 ^oC.

Samples were collected after 2 days of culturing and centrifugated for 20 min at 4000 rpm. The extracellular lipolytic activity was marked in the obtained supernatant by means of titration towards the same substrates as the ones added to the growth media (the proper treatment). In the control treatment the supernatant was replaced with water. Lipolytic activity was estimated at pH ranging from 5 to 8, and at temperature ranging from 30 to 60 ^oC. The amount of liberated fatty acids was determined by titration with 0.05 M NaOH solution against 2 % phenolophthalein as an indicator, and calculated as

a subtraction between the proper treatment and the control treatment results. The results were presented in the units of lipolytic activity. The unit was expressed as the amount of μmoles of 0.05 M NaOH required to neutralize fatty acids liberated by the lipases contained in 1 cm^3 of post-culture liquid within 1 minute. The lipolytic activity was expressed in the unit U cm^{-3}.

Results and discussion

In the presented paper, 4 bacterial strains of *Bacillus* kind were screened for their ability to synthesize lipolytic enzymes on culture media containing different source of carbon, at pH ranging from 5 to 8 and the temperature ranging from 30 to 60 °C.

In conducted tests, lipolytic activity depended on the carbon source in the medium, pH and the temperature, while individual *B. cereus* and *B. mycoides* strains showed varied activity in exocellular lipases production.

For the soil strain *B. cereus* A96*,* a reverse correlation between their lipolytic activity and the temperature has been observed (Fig. 1) and it has been stated that the temperature growth caused a decrease in lipolytic activity U. The highest values were obtained at 30 °C in the presence of glucose as carbon source – 1.38 U cm^{-3}, and the lowest at 60 °C in the medium of Tween 80 and glucose – 0.13 U cm^{-3}. The highest, 10-fold, decrease in the lipolytic activity, has been recorded on the medium with glucose, while Tween 40 seemed to be the most stable and favourable source of the fatty substrate for *B. cereus* A96 (Fig. 1).

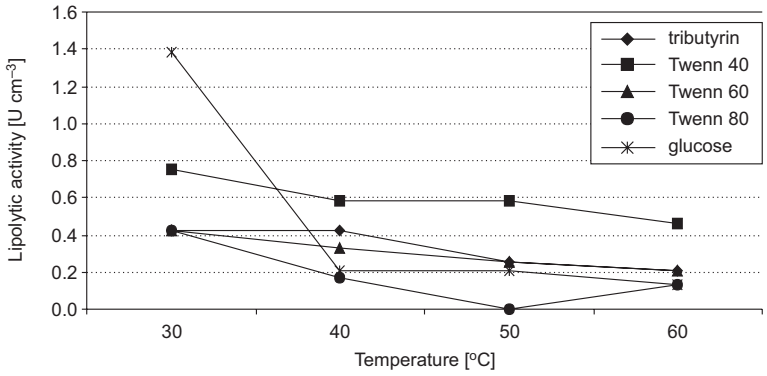

Fig. 1. Influence of temperature on the lipolytic activity of *Bacillus cereus* A96

In the presented paper, there has been no clear relationship found between the lipolytic activity and the temperature in case of *B. cereus* G10 strain isolated from water (Fig. 2). However, the highest activity has been recorded at 30 °C and the lowest at 60 °C, regardless of the carbon source. The most favourable source of the fatty substrate was Tween 40, where the highest values of the activity have been noted, regardless of the temperature. The highest value of 0.71 U cm^{-3} was noted at 30 °C. The least favourable source of carbon was Tween 80 and glucose, as irrespective of the

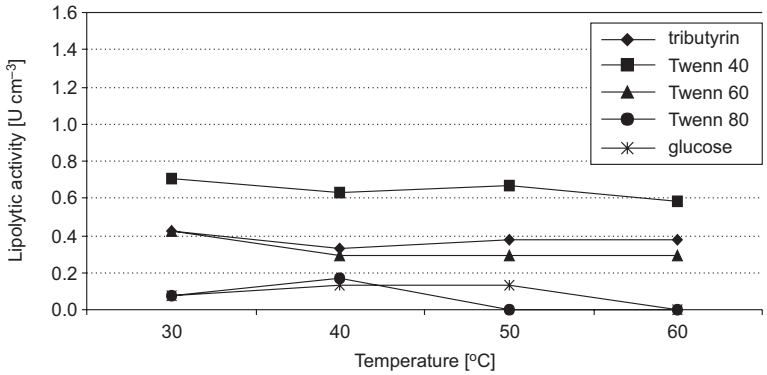

Fig. 2. Influence of temperature on the lipolytic activity of *Bacillus cereus* G10

temperature, obtained results were the lowest. Also, the activity of exocellular hydrolases has not been noticed at 50 °C and 60 °C in case of Tween 80, and at 60 °C in case of medium containing glucose (Fig. 2).

Growing temperature did not promote exocellular production of lipases in case of *B. mycoides* A134 and G3 strains (Fig. 3 and 4).

The exception has been noted for *B. mycoides* A134 strain on the medium with glucose, where the lipolytic activity grows from the initial value of 0.83 U cm^{-3} at 30 °C to 1.46 U cm^{-3} at 40 °C, to reach the value of 0.46 U cm^{-3} at the temperature 60 °C (Fig. 3).

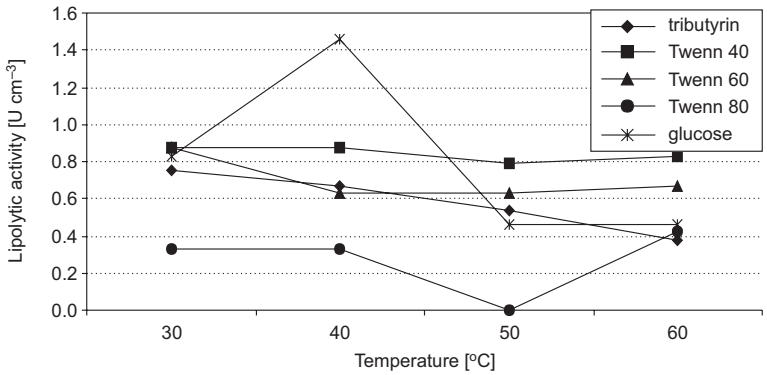

Fig. 3. Influence of temperature on the lipolytic activity of *Bacillus mycoides* A134

The most favourable medium for *B. mycoides* A134 strain, among media containing fatty substrate as the source of carbon, is medium with Tween 40. Recorded values of the lipolytic activity were the highest at all temperatures, when compared with other fatty substrates. The lipolytic activity at 30 °C amounted 0.88 U cm^{-3} and decreased slightly to the value of 0.83 U cm^{-3} at 60 °C. The least favourable fatty substrate was Tween 80, as at 30 °C measured activity amounted only 0.33 U cm^{-3}, and at 50 °C the strain did not show exocellular activity of hydrolases (Fig. 3).

In case of *B. mycoides* G3 strain the most favourable was the medium with Tween 40, similarly to *B. mycoides* A134 (Fig. 4). The highest values of the lipolytic activity have been obtained in the temperature ranging between 30 and 60 °C and amounted 0.83–0.67 U cm^{-3}. Similar changes, to the aforementioned, have been noted in reference to the medium with glucose, but recorded values of the lipolytic activity have been significantly lower. At the temperature of 30 °C the lipolytic activity amounted 0.58 U cm^{-3}, grew up to the value of 0.71 U cm^{-3} at 40 °C and reached the value of 0.33 U cm^{-3} at the highest temperature. The lowest difference in the lipolytic activity, at respective temperatures, have been obtained for the media with the following fatty substrates: tributyrin, Tween 40 and Tween 60 (Fig. 4).

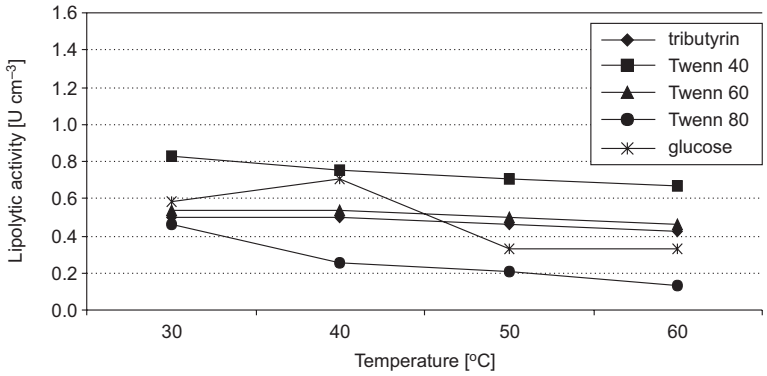

Fig. 4. Influence of temperature on the lipolytic activity of *Bacillus mycoides* G3

Presented paper considers also the influence of pH on the lipolytic activity of tested *B. cereus* and *B. mycoides* strains, with reference to the source of carbon in the culture medium. Some correlation has been observed in most cases, regardless to the strain tested and the culture medium, namely pH increase in the range between 5–8 promotes the increase in the lipolytic activity (Figs. 5–8).

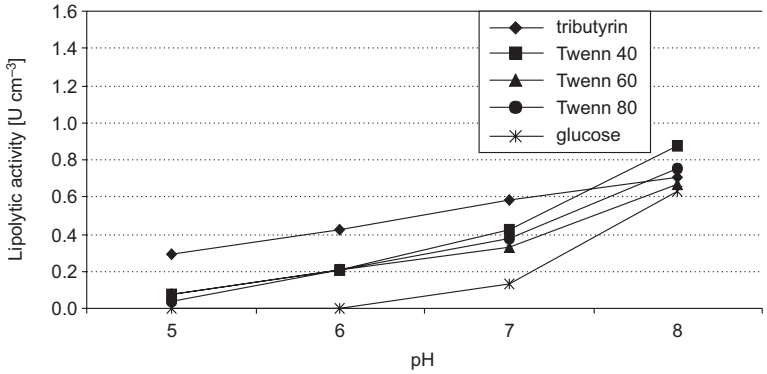

Fig. 5. Influence of pH on the lipolytic activity of *Bacillus cereus* A96

The most favourable culture media for the soil strain *B. cereus* A96 are those containing tributyrin and Tween 40, as the source of the fatty substrate. The gradual growth of the lipolytic activity from the initial amount of 0.29 U cm^{-3} at pH 5, up to the amount of 0.71 U cm^{-3} at pH 8 was observed in case of tributyrin. The lipolytic activity on Tween 40 was initially lower, when compared with tributyrin, and finally obtained the highest recorded value for the strain under study, equal to 0.88 U cm^{-3} at pH 8.

B. cereus A96 did not show the lipolytic activity at pH between 5–6 on the culture medium with glucose. It was recorded only at pH 7 and amounted 0.13 U cm^{-3}, growing up to the value of 0.63 U cm^{-3} at pH 8 (Fig. 5).

In case of *B. cereus* G10 strain, the most effective source of carbon in the process of exocellular lipases biosynthesis, were also fatty substrates under study (Fig. 6), for which recorded values of the lipolytic activity did not differ significantly at pH range between 5–7. The differences were observed at pH 8, where the highest value of 0.71 U cm^{-3} was obtained in the medium with Tween 40, and the lowest value of 0.38 U cm^{-3} in the medium with Tween 60.

Similarly to the soil strain, the lipolytic activity was not recorded if the medium did not contain the fatty substrate and glucose was the source of carbon. Such case was observed at pH between 5–6. At pH 7 and 8 measured values of the activity amounted 0.17 and 0.46 U cm^{-3} respectively (Fig. 6).

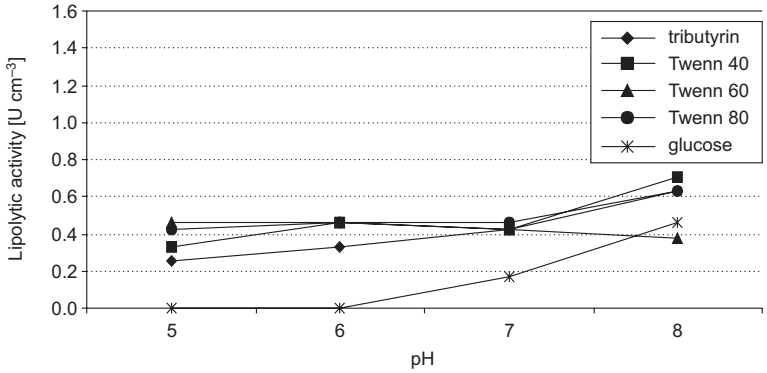

Fig. 6. Influence of pH on the lipolytic activity of *Bacillus cereus* G10

In own research, when analysing the influence of pH on the lipolytic activity, it has been noted that bacterial strains *B. mycoides* A134 and G3 preferred different sources of fatty substrates in comparison with *B. cereus* strains. The highest values of the lipolytic activity have been obtained on the culture media with addition of Tween 80, Tween 60 and Tween 40 (Fig. 7 and 8).

B. mycoides A134 strain revealed the highest lipolytic activity at pH 5–7 on the medium with Tween 80, and the recorded values ranged between 0.21–0.54 U cm^{-3}. However, in the presence of Tween 40 lipolytic activity of *B. mycoides* A134 was the highest and amounted 0.75 U cm^{-3}.

The strain did not show the ability to produce exocellular lipases at pH between 5–7, with no fatty substrate in the culture medium. Only at pH 8, the activity was noted at the level of 0.75 U cm^{-3} in case of the medium with glucose and it was one of the highest values obtained for the strain (Fig. 7).

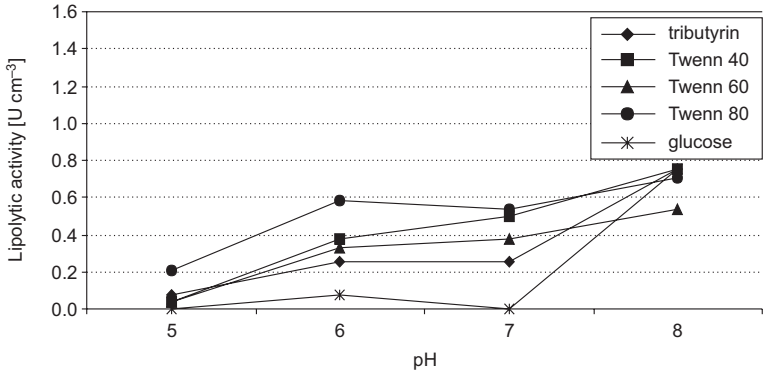

Fig. 7. Influence of pH on the lipolytic activity of *Bacillus mycoides* A134

The last strain under study – *B. mycoides* G3*,* preferred Tween 60 and Tween 80 as the sources of the fatty substrate (Fig. 8). The value of lipolytic activity on Tween 60, was growing from the amount of 0.54 U cm^{-3} at pH 5 to 1.0 U cm^{-3} at pH 8, which was the highest value recorded in the experiment. In Tween 80 case, the initial value of lipolytic activity at pH 5 and 6 was slightly higher and amounted 0.63 U cm^{-3} and 0.75 U cm^{-3}, respectively. At pH 8, it amounted 0.92 U cm^{-3} and was a bit lower when compared to the amounts obtained on the medium with Tween 60. It seems, that *B. mycoides* G3 strain, as the only one among all tested, uses Tween 60 as the most effective source of the fatty substrate in the process of extracellular lipases biosynthesis.

When analysing the influence of pH on the lipolytic activity of *B. mycoides* G3 strain, it has been noted, that it was the only active strain even if the medium did not

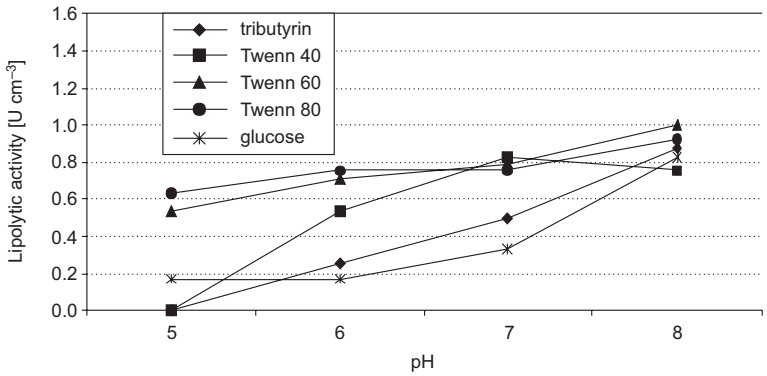

Fig. 8. Influence of pH on the lipolytic activity of *Bacillus mycoides* G3

contain fatty substrate. The value of its lipolytic activity in the presence of glucose increased from the value of 0.17 U cm^{-3} at pH 5 to 0.83 U cm^{-3} at pH 8 (Fig. 8).

Lipase production is influenced by temperature, pH and medium composition. The process of lipases biosynthesis conducted by strains *B. cereus* and *B. mycoides* was the most intensive at the temperature ranging between 30–40 ^{o}C. Hasan et al [7], Shaoxin et al [8] and Alkan et al [9] have also found similar results in case of *B.* sp. FH5, *B. cereus* C71 and B. *coagulans*. For *B. cereus* C71 [8] the lipase was active at temperature ranging between 30–45 ^{o}C with its most intensive activity at 33 ^{o}C. Whereas, for *B. coagulans* [9] and *B.* sp. FH5 [7], maximum activity have been observed at 37 ^{o}C.

The protein nature of enzyme under study means that pH will affect the ionization state of the amino acids which determines the primary and secondary structure of the enzyme and hence, controls its overall activity [10]. The optimum activity of lipase was observed at pH between 7.0–8.0. Isolates of *B.* sp. have been found to produce lipolytic enzymes under alkaline conditions [9]. Lipase from *B. subtilis* and *B. licheniformis* have been of particular interest because they exhibit optimal activity and stability at extreme alkaline pH values greater than 9.5 [11]. These enzymes, however are thermolabile. Another lipase produced by *B.* sp. RSJ-1 has shown maximum activity at pH between 8.0–9.0 [10]. These results are in contrast to those of lipase from the *B. cereus* and *B. mycoides* under study, which are thermotolerant but display maximum activity at moderate alkaline pH between 7.0–8.0. Hasan et al [7] and Alkan et al [9] found similar results from *B.* sp. FH5 and *B. coagulans*, respectively.

Lipases were defined as the enzymes hydrolyzing long-chain acyglycerols (≥ 10 carbon atoms). However, it is known that most of lipases are also active on short-chain fatty acid esterase [8]. In own research, the biosynthesis of exocellular lipases in most cases, was more effective in medium with fatty substrate than with the addition of glucose. This may prove, that the synthesis of these enzymes is induced by the lipids. In case of fatty substrates, the highest values of lipolytic activity have been noted mostly in the presence of Tween 40 (C10) as the source of carbon, and noted values of lipolytic activity were higher in case of *B. mycoides* strains. Similarly, lipase from *B. cereus* C71 [8] showed higher activity toward substrate with C12 than C16 and C18. These results are in contrast to the ones obtained for the lipase from *B.* sp. FH5 tested by Hasan et al [7]. In the present study it has been found that maximum lipase levels were obtained when Tween 80 (C18) was used as a source of lipid which served both as a carbon source and an inducer for a lipase production. A low lipase level in a medium with glucose has also been reported by Hasan et al [7].

Actually, the variation in enzyme production at different temperatures or pH values and medium composition resulted from bacterial strains specificity.

Conclusions

The research proved significant diversity of lipolytic activity of *B. cereus* and *B. mycoides* strains, towards the source of carbon, pH and the temperature analysed in the experiment. Based on the obtained results following conclusions were drawn:

1. The highest amount of lipolytic activity were liberated by the strains at 30 ^{o}C and pH equal to 8. Under these conditions the most favourable medium was with the addition of Tween 40, as the source of fatty substrate.

2. Strains under study were active even if there was no fatty substrate in the growth medium.

3. The most active were *B. mycoides* strains. Additionally, because of its wide substrate specificity, the enzyme can be used not only for short-chain fatty acids (contained by tributyrin) but also for medium-chain fatty acids (contained in Tween 40), which will greatly broaden its environmental applications.

4. Individual strains of *B. cereus* and *B. mycoides* showed diversity in their lipolytic activity, which was influenced by the environment from which they were isolated.

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WPŁYW WARUNKÓW ŚRODOWISKA NA AKTYWNOŚĆ LIPOLITYCZNĄ SZCZEPÓW *Bacillus cereus* I *Bacillus mycoides*

Samodzielna Katedra Biotechnologii i Biologii Molekularnej
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Abstrakt: Celem podjętych badań była ocena aktywności lipolitycznej szczepów *B. cereus* oraz *B. mycoides* w zależności od źródła węgla, pH oraz temperatury. Do badań wykorzystano 2 szczepy *Bacillus cereus* oraz 2 szczepy *Bacillus mycoides* wyizolowane z gleby i wody. Źródłem węgla w pożywkach były substraty tłuszczowe: tributyryna, Tween 40, Tween 60, Tween 80 oraz glukoza. Aktywność lipolityczną oznaczono w zakresie pH od 5 do 8 oraz temperaturach od 30 do 60^{o}C metodą miareczkową, a wyniki podano w jed-

nostkach aktywności enzymatycznej [U cm^{-3}]. W przeprowadzonym doświadczeniu aktywność lipolityczna uzależniona była od rodzaju substancji tłuszczowej zawartej w podłożu, pH oraz temperatury. I tak, badane szczepy *B. cereus* oraz *B. mycoides* wykazywały najmniejsza aktywność przy pH 5 oraz 6. Natomiast największą aktywność stwierdzono przy pH 7 i 8. W tych warunkach większość szczepów wykazywała aktywność lipolityczną nawet przy braku substratu tłuszczowego w podłożu. Największe wartości aktywności lipolitycznej uzyskano przy pH 8 na podłożu z dodatkiem Tween 40, a największe wartości (0,88 U cm^{-3}) uzyskano dla glebowego szczepu *B. cereus*. Analizując wpływ temperatury na aktywność lipolityczną stwierdzono, iż najwyższą aktywność odnotowano w temperaturze 30 i 40 ^{o}C, a najmniejszą w 50 i 60 ^{o}C. Największe wartości, dla większości szczepów, uzyskano w temperaturze 30 ^{o}C na podłożu z dodatkiem Tween 40, gdzie najbardziej aktywnym okazał się glebowy *B. mycoides* (0,88 U cm^{-3}). Wyjątek stanowi glebowy *B. cereus*, dla którego wartość aktywności lipolitycznej na podłożu z dodatkiem glukozy wynosiła aż 1,38 U cm^{-3}. Uwzględniając wszystkie analizowane źródła węgla i parametry, najaktywniejszymi były szczepy *B. mycoides.*

Słowa kluczowe: *Bacillus cereus*, *Bacillus mycoides*, lipazy, tributyryna, Tween