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# LIPOLYTIC ACTIVITY OF *Bacillus* sp. ISOLATED FROM THE NATURAL ENVIRONMENT

## AKTYWNOŚĆ LIPOLITYCZNA *Bacillus* sp. WYIZOLOWANYCH Z ŚRODOWISKA NATURALNEGO

**Abstract:** In the study 10 bacterial strains of *Bacillus* kind were screened (*B. cereus*, *B. firmus*, *B. mycoides*, *B. pumilus* i *B. subtilis*) for their ability to synthesize lipases on the media containing the following fatty substrates as carbon sources: tributyrin and Tween 40, 60, 80. The growth media were incubated for 15 days and the results were recorded after 2, 4, 8 and 15 days. The results were presented as the amount of liberated μmoles of fatty acids [μmol] and the units of lipolytic activity U/cm$^3$. Tested bacterial strains displayed different levels of activity of extracellular lipases depending on the type of the fatty substrate source and the time of culturing. For most of the tested strains the maximum production of lipases took place on the 15$^{th}$ day of culturing. The most effective growth medium in the process of enzymes biosynthesis was the medium with tributyrin, and both *B. cereus A96* and *G10* strains distinguished from the others by the highest activity liberating on average 97.5 and 69.375 μmoles of fatty acids. The value of lipolytic activity was increasing during the experiment from the value of 0.958 to 1.375 U/cm$^3$ for *A96* strain and from 0.625 to 3.125 U/cm$^3$ for *G10* strain. Taking into account all tested growth media, the lowest lipolytic activity was displayed by *B. pumilus Tw3* strain.

**Keywords:** *Bacillus* sp., lipases, tributyrin, Tween

Lipases, defined as hydrolases of glicerol esters EC 3.1.1.3, are the enzymes of high catalytical potential. They catalyse the hydrolysis and trans-esteryfication of triacylglycerols, enantioselective synthesis, and hydrolysis of a variety of esters. They are produced by plants, animals and microorganisms, of which the last group remains in the centre of attention. Many bacteria have the ability to produce them but the most important in the process are the species: *Pseudomonas, Staphylococcus* and *Bacillus* [1–3].

Bacterial lipases are the extracellular water-soluble enzymes, produced in the late phase of logarithmic growth. All known bacterial lipases belong to the group of α/β – hydrolases of 3-dimentional structure. Lipases are characterized by their unique ability to catalyse reactions at the interface of a lipid phase and the aqueous phase [1, 4].

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A wide interest in bacterial lipases is linked to their role as biocatalysts in many biochemical processes. They are used, among others to produce detergents, food, paper, pharmaceuticals and in the environmental protection. However, according to data from literature [1, 2] the microorganisms are varied in terms of their enzymatic activity, which depends on the species of the microbes and the culturing conditions (*eg* pH of the growth medium, temperature, source of nitrogen and presence of lipids in the medium). Therefore, bearing in mind immense application abilities of microbiological lipases, there has been research done in order to find new strains, able to synthesize significant amounts of highly active enzymes.

The aim of presented research was the evaluation of the ability to synthesize extracellular lipases by selected *Bacillus* sp. strains isolated from the natural environment, depending on the source of fatty substrate and incubation time.

## Materials and methods

The objects of the study were the following 10 bacteria strains:
– 5 *Bacillus pumilus* strains marked as: *M1, Tw3, A115, G4, G8*,
– *Bacillus subtilis G2*,
– 2 *Bacillus cereus* strains marked as: *A96* and *G10*,
– *Bacillus mycoides G3*,
– *Bacillus firmus Tw4*.

The source of fatty substrates in the growth media were 10 % solutions of the following: tributyrin, Tween 40, Tween 60 and Tween 80. The cultures were maintained in Erlenmeyer flasks of 250 $cm^3$ capacity containing 50 $cm^3$ of the respective growth medium and placed on a rotary shaker for 15 days at 30 $^oC$. The cultures were introduced with an inoculum of density equal to E = 2 (standardized with the use of a spectrophotometer) obtained from the 48-hour culture on a nutrient broth.

Samples for the analysis were collected after 2, 4, 8 and 15 days of culturing and centrifuged for 20 minutes at 4000 rpm. In the obtained supernatant the extracellular lipolytic activity was marked by means of titration towards the same substrates previously added to the growth medium (the proper treatment). In the control treatment the supernatant was replaced with water. The amount of liberated fatty acids was estimated 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 as the amount of liberated μmoles of fatty acids [μmol] and 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

In the presented paper 10 bacterial strains of *Bacillus* kind were screened, within 15 days, for their ability to synthesize lipolytic enzymes on the growth media containing

different sources of fatty substrates. The obtained results proved the variety among tested *Bacillus* strains in terms of extracellular lipases production according to the source of fatty substrate in the growth medium and incubation time.

In the presented research the most effective source of fatty substrate in the process of extracellular lipases biosynthesis was tributyrin (Fig. 1). After 15 days of culturing the highest amount of fatty acids was liberated by *B. cereus G10* strain, on average 97.5 µmoles. The value of lipolytic activity for this strain was increasing from 0.625 U/cm$^3$ on the second day to the value of 3.125 U/cm$^3$ on the 15$^{th}$ day of culturing. Similar activity was displayed by the other strain under study *B. cereus A96*, however its lipolytic activity was considerably lower and fluctuated during the test from 0.958 to 1.375 U/cm$^3$ (Fig. 2). But for the strain *B. mycoides G3,* which belongs to the same group, the correlation was different. The lipolytic activity was decreasing from 1.0 to 0.167 U/cm$^3$. The lowest amount of fatty acids was liberated by *B. pumilus Tw3,* on average 13.125 µmoles (Fig. 1). Tributyrin was not a favourable environment for the extracellular lipases production, as with time the lipolytic activity was decreasing from the value of 0.333 to 0.208 U/cm$^3$ (Fig. 2). The remaining strains of *B. pumilus* were

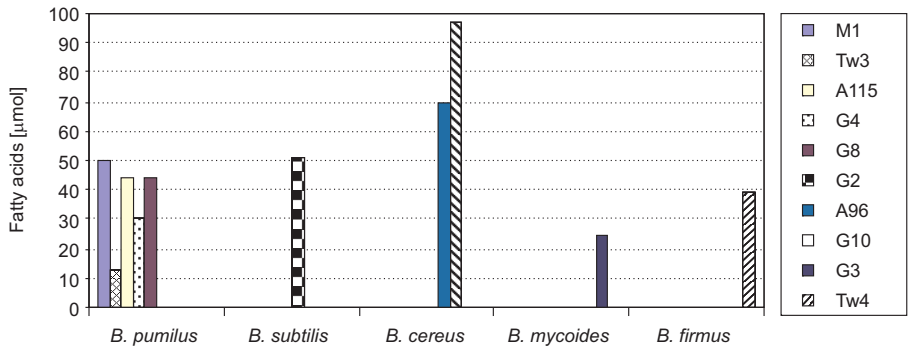


Fig. 1. The influence of tributyrin on extracellular lipases production by selected *Bacillus* strain

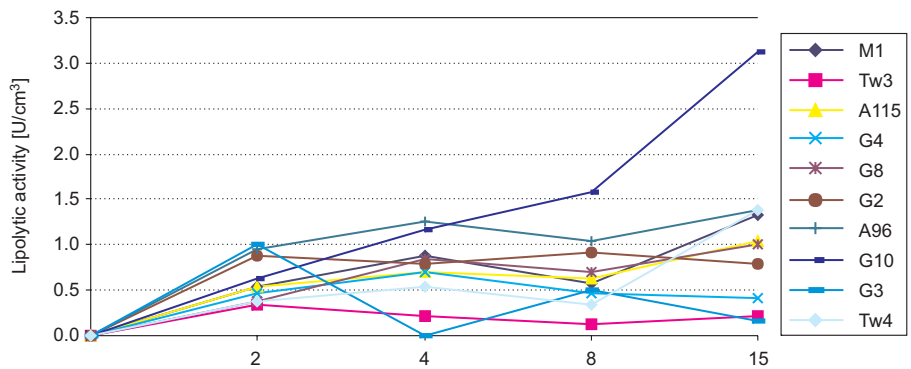


Fig. 2. The influence of the culturing time on extracellular lipases production in the presence of tributyrin on selected *Bacillus* strains

liberating much more of fatty acids, reaching the following values: on average 30.625 μmoles for the *G4* strain, 43.75 μmoles for the *A115* and *G8* strains and 50 μmoles for the *M1* strain (Fig. 1). The lipolytic activity was increasing of 0.5–0.7 unit for most of the mentioned strains during the experiment. Only in case of *G4* strain the activity was decreasing slightly from 0.458 to 0416 U/cm$^3$ (Fig. 2).

*B. subtilis* strain *G2* belonging to the same group as *B. pumilus*, liberated on average 50.625 μmoles of fatty acids and its lipolytic activity was decreasing from 0.875 to 0.792 U/cm$^3$ (Fig. 1, 2).

The last strain under study, *B. firmus Tw4,* produced on average 39.375 μmoles of fatty acids and its activity increased of 1 unit within 15 days, from the initial value of 0.375 to 1.375 U/cm$^3$ (Fig. 1, 2).

Generally, the highest values of extracellular lipases activity in the presence of tributyrin were recorded on the 15$^{th}$ day of the experiment. The only exception were the strains: *Tw3* and *G3*, for which the maximum values were obtained on the 2$^{nd}$ day, and the strains *G4* and *G2* with the highest values on the 4$^{th}$ and 8$^{th}$ day respectively (Fig. 2).

In the presented study the lipolytic activity was also assessed towards long-chain synthetic lipids: Tween 40, 60, 80. The most favourable growth medium for the extracellular lipases production was the medium with the ddition of Tween 40. The highest amount of fatty acids on this medium was produced by *B. subtilis G2* – on average 34.375 μmoles (Fig. 3). Also high mean values of μmoles of fatty acids were obtained with the strains of *B. pumilus: G4* and *G8*, and recorded values were 30 and 31.25 μmoles respectively. The lowest amount of fatty acids was liberated by the strains *B. firmus Tw4* (on average 16.25 μmoles) and *B. pumilus Tw3*, on average 14.37 μmoles (Fig. 3).

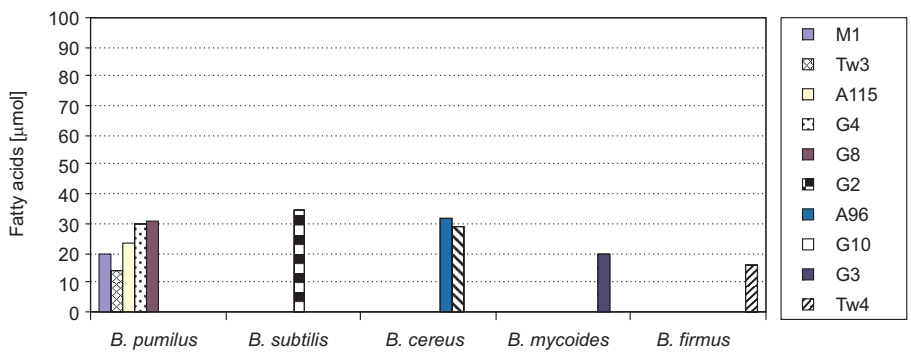


Fig. 3. The influence of Tween 40 on extracellular lipases production by selected *Bacillus* strains

It is difficult however to find the correlation between the time of culturing and lipolytic activity of tested strains on Tween 40 (Fig. 4). In case of the following strains: *B. cereus A96*, *B. subtilis G2* and *B. mycoides G3* the maximum values were obtained on the 2$^{nd}$ day and was decreasing on the following days. The highest lipolytic activity was noted for *B. cereus A96* strain and amounted 0.833 U/cm$^3$ which corresponded with the highest amount of μmoles of fatty acids obtained on the medium with the addition of Tween 40.

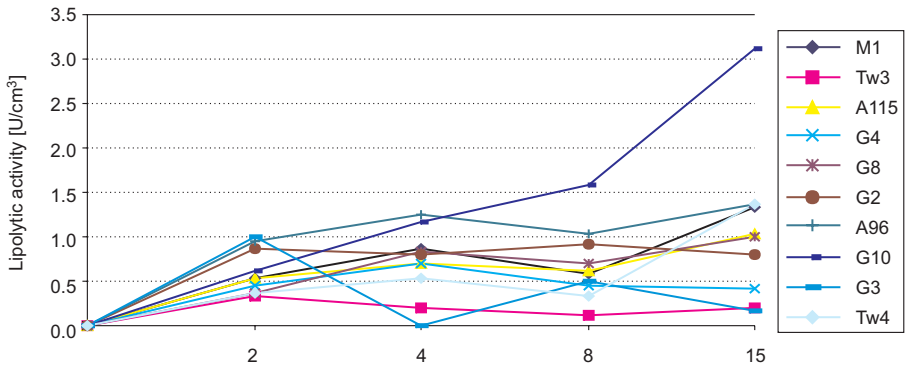


Fig. 4. The influence of the culturing time on extracellular lipases production in the presence of Tween 40 on selected *Bacillus* strains

While the maximum amounts of lipolytic activity for *B. cereus G10* were obtained on the 4th day, for the strains *B. pumilus Tw3, A115* and *G8* they were recorded on the 8th day, and for the strains *B. pumilus M1*, *B. firmus Tw4* and *B. pumilus G4* on the last day of the experiment (Fig. 4).

The application of Tween 60 as a fatty substrate in the growth medium did not promote the extracellular lipases biosynthesis. The values of µmoles of liberated fatty acids were the lowest for this medium in case of all the bacterial strains when compared with other fatty substrates. In the presence of Tween 60, the amount of liberated fatty acids did not exceed 30 µmoles (Fig. 5). The lowest mean values of fatty acids were noted for *B. pumilus Tw3* and amounted 5 µmoles, and the highest 29.375 µmoles were noted for *B. pumilus G4*.

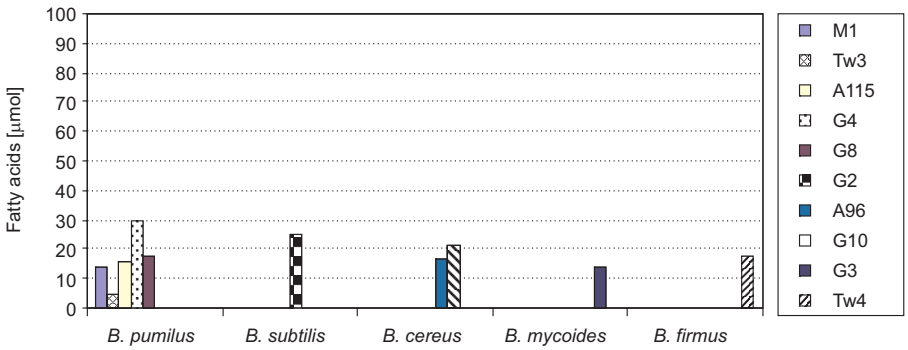


Fig. 5. The influence of Tween 60 on the production of extracellular lipases by selected *Bacillus* strains

In Tween 60 environment, the lipolytic activity was increasing with the time of the research (Fig. 6). Most of tested strains obtained the maximum activity on the 15th day of culturing. The highest activity of 0.75 U/cm$^3$ was measured for *B. pumilus G4* strain, slightly lower value of 0.625 U/cm$^3$ was recorded for *B. cereus G10* strain and 0.5 U/cm$^3$ value characterized the following strains: *B. pumilus G8*, *B. subtilis G2* and

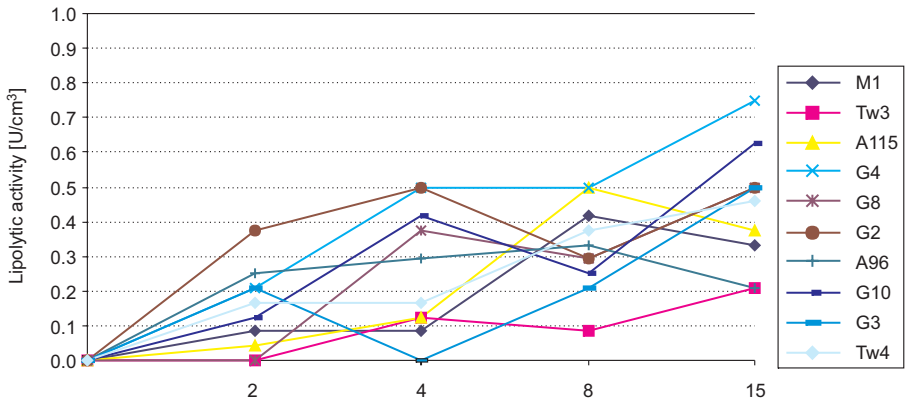


Fig. 6. The influence of the culturing time on extracellular lipases production in the presence of Tween 60 on selected *Bacillus* strains

*B. mycoides G3*. Only 2 strains obtained the maximum value of lipolytic activity on the 8th day of the experiment and they were *B. pumilus M1* and *A115*.

It's worth noticing that 2 of the strains under study, namely *B. pumilus Tw3* and *G8* liberated extracellular lipases only on the 4th day of the research. It confirms the thesis that a fatty substrate in the growth medium induces the biosynthesis of lipolytic enzymes.

Tween 80 – the last tested synthetic source of fatty substrate was a favourable source for extracellular lipases production. During the experiment, the most vigorous strain was *B. subtilis G2* liberating on average 33.125 µmoles of fatty acids similarly to the amounts liberated on Tween 40. For the rest of the bacterial strains the amount of liberated fatty acids fluctuated around 20 µmoles. The lowest results were recorded for the strains *B. pumilus A115* and *B. mycoides G3*, obtaining 15.0 and 15.625 µmoles respectively (Fig. 7).

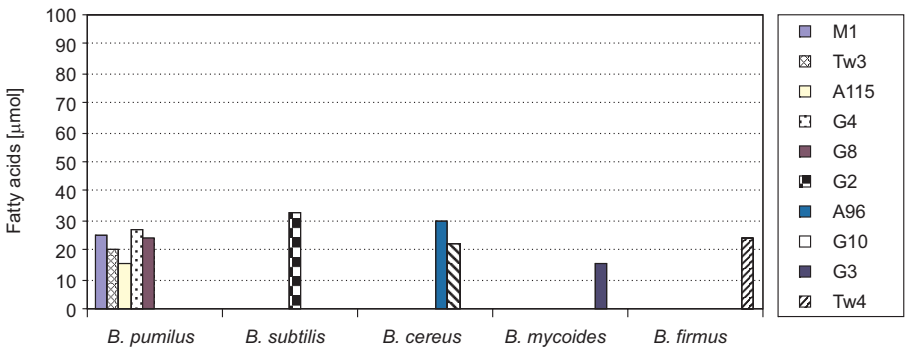


Fig. 7. The influence of Tween 80 on the production of extracellular lipases by selected *Bacillus* strains

Tween 80, analogous with Tween 40, revealed no clear correlation between incubation time, lipolytic activity, the species or the bacterial strains (Fig. 8). The lipolytic activity was decreasing during the experiment in case of the strains *B. cereus*

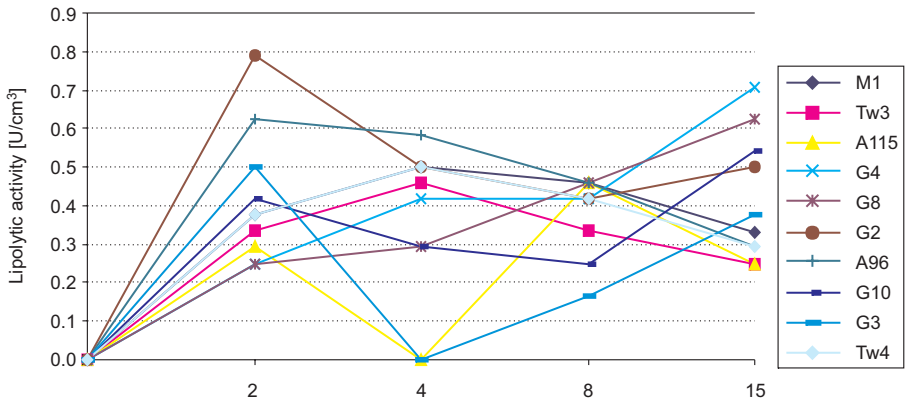


Fig. 8. The influence of the culturing time on extracellular lipases production in the presence of Tween 80 on selected *Bacillus* strains

*A96*, *B. subtilis G2* and *B. mycoides G3* (Fig. 8), similarly to the growth medium with the addition of Tween 40. For the rest of the strains the activity was increasing, to obtain its peak on the 4$^{th}$ day of culturing for the strains *B. pumilus M1* and *Tw3*, on the 8$^{th}$ day of culturing for the strains *B. pumilus A115* and *B. firmus Tw4* and finally on the 15$^{th}$ day of culturing for the strains *B. pumilus G4* and *G8*, *B. cereus G10.* The values obtained on the 15$^{th}$ day were among the highest recorded values and amounted 0.708 U/cm$^3$ for the *B. pumilus G4* strain, slightly lower 0.625 and 0.542 U/cm$^3$ for the strains *B. pumilus G8* and *B. cereus G10* respectively (Fig. 8).

Generally, the highest values of lipolytic activity on the analyzed growth media were obtained on the 8$^{th}$ and 15$^{th}$ day of incubation, which may suggest that the substrate was not used up completely. But it is still difficult to explain the activity drop on the following days of culturing. One possible reason [5–9] for the activity decrease is said to be the substrates exhaustion and growing pH of the environment. The activity drop could also be caused by the inhibitory interaction of forming products. Therefore, the study is to be continued towards setting the optimal culturing conditions, which may play an important role in the process of fatty wastes managing and the environmental protection as well.

## Summary and conclusion

Conducted research proved significant diversity between particular *Bacillus* strains in terms of their lipolytic activity, when different sources of fatty acids were considered and enabled to state that:

1. Biosynthesis of lipases catalysed by *Bacillus* strains was the most intense on the medium with trybutyrin, and the least intense on the medium with the addition of Tween 60.

2. The extracellular lipases of tested bacterial strains were highly specific towards tributyrin and low towards Tween 60, which proves the process of their biosynthesis to be induced by the lipids.

3. Bacteria of *Bacillus* kind display higher preference of extracellular lipases to hydrolyze ester bonds formed by the long-chain butyric acids (contained by tributyrin) than long-chain fatty acids in Tween 40, 60 and 80.

4. All of tested *Bacillus* strains had the ability to produce the extracellular lipases. In terms of the sources of fatty substrates applied in the experiment the most vigorous strains were *Bacillus cereus G10* and *A96*, and the least vigorous was *Bacillus pumilus Tw3*.

5. There was no clear correlation found between the time of incubation and the lipolytic activity, which indicates that it is specific for particular bacterial strains and depends on both the type of a fatty substrate in the growth medium and the environment of the strain isolation.

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## AKTYWNOŚĆ LIPOLITYCZNA *Bacillus* sp. WYIZOLOWANYCH Z ŚRODOWISKA NATURALNEGO

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**Abstrakt:** W pracy przebadano 10 szczepów bakterii z rodzaju *Bacillus* (*B. cereus, B. firmus, B. mycoides, B. pumilus* i *B. subtilis*) pod względem możliwości syntezy lipaz w podłożach zawierających jako źródło węgla substraty tłuszczowe: tributyrynę oraz Tween 40, 60 i 80. Inkubację podłoży prowadzono przez 15 dni, a odczyty wykonano po 2, 4, 8 i 15 dniach. Wyniki podano jako ilość uwolnionych µmoli kwasów tłuszczowych [µmol] oraz w jednostkach aktywności lipolitycznej U/cm$^3$. Wśród badanych szczepów wykazano zróżnicowanie w poziomie aktywności zewnątrzkomórkowych lipaz w zależności od źródła substratu tłuszczowego i czasu hodowli. Dla większości badanych szczepów maksymalna produkcja lipaz występowała w 15 dniu hodowli. Najefektywniejsze w biosyntezie enzymów okazało się podłoże zawierające tributyrynę, a szczepy *B. cereus A96* i *G10* wyróżniały się najwyższą aktywnością, uwalniając średnio

97,5 oraz 69,375 μmoli kwasów tłuszczowych. Wartość aktywności lipolitycznej wzrastała w trakcie doświadczenia z 0,958 do 1,375 U/cm$^3$ dla szczepu *A96* oraz z 0,625 do 3,125 U/cm$^3$ dla szczepu *G10*. Natomiast, uwzględniając wszystkie testowane podłoża, najmniejszą aktywnością lipolityczną charakteryzował się szczep *B. pumilus Tw3.*

**Słowa kluczowe:** *Bacillus* sp., lipazy, tributyryna, Tween