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PHYSICAL FACTORS AFFECTING THE PRODUCTION OF AMYLASE FROM *Bacillus* SPECIES ISOLATED FROM NATURAL ENVIRONMENT

WPLYW CZYNNIKÓW FIZYCZNYCH NA AKTYWNOŚĆ AMYLOLITYCZNA *Bacillus* sp. WYZOLOWANYCH ZE ŚRODOWISKA NATURALNEGO

Abstract: The influence of different factors on amylolytic activity *Bacillus cereus* (2 strains) and *Bacillus mycoides* (2 strains), isolated from soil samples and water of Turawa Lake has been studied. Effect of physiological (pH of reaction mixture in the range of 4.0–8.0, temperature of reaction from 30 to 60 °C) and nutritional parameters (the carbon sources and they concentration in medium) were examined spectrophotometrically for their effect on amylase production. The results obtained showed, that in view of all studied factors the least favorable value of pH was 5.0, while in most cases the most favourable for the process of amylase production were following values 7.0 and 8.0. Moreover, it has been stated that the lowest amount of amylase was noted at 30 °C, and the highest at 60 °C. In case of amylolytic activity, in view of all temperatures the most effective strain was *B. mycoides* A134 whereas the least *B. mycoides* G3. The best results of amylase production have been achieved for all remaining strains in two cases – on maltose and glucose medium at all tested temperatures. Additionally, soil occurring strains preferred lower concentration of potato starch (*ie* 1 %) when compared with water strains which favoured higher concentration (5 %).

Keywords: *Bacillus cereus*, *Bacillus mycoides*, amylase, physical factors

In recent years intensive studies have been conducted in order to look for new possibilities of environment protection with a view to reducing the chemicals, which contaminate the environment. One of the methods used is the application of bio-preparations, bioutilizers based on microbial populations or their enzymes isolated from natural environment – soil, water in the processes of bioremediation, biodegradation, biostimulation and plant protection [1, 2]. Amylases (E.C.3.2.1.0) are an important group of biotechnologically valuable enzymes hydrolysing starch molecules to dextrin, maltose and/or glucose. They are produced by plants, animals and microorganisms, of

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which the last group remains in the centre of attention. Many kinds of bacteria possess the ability to produce the useful enzymes or secondary metabolites, among others bacteria of *Bacillus* spp. [3–5]. They are particularly interesting due to their easy cultivation and high production of extracellular enzymes, high adaptability to adverse environmental conditions and its wide area of potential application. Furthermore, with the advent of new frontiers in biotechnology, the spectrum of the applications of amylase has been widened up to many other fields such as analytical, medicinal and clinical chemistries, as well as their widespread use in the industries such as textile, detergents, paper, food, brewing, pharmaceutical and animal feed [6–11]. According to literature data [6, 10, 12–15] they are varied in terms of their enzymatic activity, that depends on the species of microorganisms and the culturing conditions *eg* pH of the medium, temperature, composition of the growth medium (the source of carbon, nitrogen, phosphate, metal ions and their concentration). Interactions of these parameters are reported to have a significant influence on the productive synthesis of these enzymes. Therefore, they are research being carried out continually, with the purpose of find new, more and more productive strains, able to synthesise a considerable quantity of enzymes. Among the various species of *Bacillus*, *B. licheniformis*, *B. amylo-liquefaciens* and *B. subtilis* are used most frequently in the commercial production of amylases [3, 7, 9, 16]. Reference data show that *B. cereus* and *B. mycoides* might be involved in future biotechnological and industrial applications [8, 17]. The purpose of conducted research was to assess the production of amylase by *Bacillus* strains and optimize the parameters for their production.

Materials and methods

The bacterial strains used in this study were *Bacillus* species, previously isolated from soil samples (2 strains marked as *Bacillus cereus* A96 and *Bacillus mycoides* A134) and water of Turawa lake (2 strains marked as *Bacillus cereus* G10 and *Bacillus mycoides* G3).

Determination of amylase production

In the research, *Bacillus* species producing amylase were screened by means of starch hydrolysis procedure followed by a 48 h cultivation at 30°C on modified Waksman medium (consisting of 1.0 g/dm³ NaCl, K₂HPO₄ 1.0 g/dm³, (NH₄)₂SO₄ 2.0 g/dm³ and CaCO₃ 5.0 g/dm³) [18] with an addition of different carbon source. The effect of potato starch (P), corn starch (C), and maltose (M) at concentrations ranging from 1 % to 5 %, and 1 % glucose (G) was determined, that were added into the basic culture medium. The medium (50 cm³) was inoculated with *Bacillus* inoculum with an optical density of 2.0 at 560 nm, next incubated at 30 °C on a rotary shaker at 110 rpm for 48 h. The supernatant of the culture was collected after centrifugation at 4000 rpm for 20 min and subjected to assessment of amylase activity. The amylase activity was estimated on the basis of the reduction in blue color intensity resulting from enzymatic hydrolysis of soluble starch as a substrate following the modified method of Fennala [19] as described previously [20]. Enzymatic activity (U/ cm³) was examined spectro-

photometrically and was defined as the amount of the enzyme which hydrolyzed 1 mg of starch per 1 cm³ of the extract solution over 30 min of incubation.

Effect of pH and temperature

In order to determine the appropriate pH range of enzyme activity, 0.1M buffers were applied. For pH 5.0 and 6.0 was used citrate buffer, while for pH 7.0 and 8.0 phosphate buffer. In order to evaluate the effect of various temperature on amylase production, the reaction mixtures were incubated at temperatures ranging from 30–60 °C.

Results and discussion

In the presented paper, 4 bacterial strains of *Bacillus* genus were screened for their ability to synthesize amylolytic 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, enzymatic activity depended on the medium composition, pH and the temperature, while individual *B. cereus* and *B. mycooides* strains showed varied activity in exocellular amylases production.

In an extracellular amylase biosynthesis the most active strain was *B. cereus* A96 at pH 5.0. The highest amylase amounts were recorded in a maltose growth medium, especially at its highest concentration amounting 5 % (1.58 U/cm³). Whereas the lowest amylase amounts were noted in case of water occurring strains, of which *B. mycooides* G3 did not produce amylase under the above mentioned pH conditions. None of the strains under study showed enzymatic activity in a glucose medium, similarly to media with 1 % and 2.5 % potato starch and 2.5–5.0 % maltose media (Fig. 1).

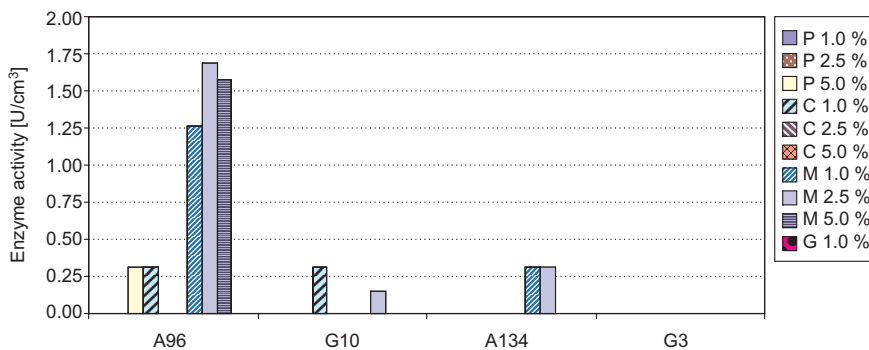


Fig. 1. The influence of pH 5.0 on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycooides* (A134, G3)

At consecutive pH value equal to 6.0, it has been displayed that out of all *Bacillus* sp., the water strains demonstrated the highest enzymatic activity, particularly *B. mycooides* marked as G10. The growth medium containing the highest concentration of potato starch (5 %) turned out to be a favourable medium for this strain but at a lower concentration (1 %) for *B. mycooides* G3. Both strains decomposed starch in the amount

of 1.22 U/cm^3 and 1.03 U/cm^3 respectively. Whereas in the medium containing corn starch these strains behaved inversely. The lowest amylase activity was found in case of *B. mycooides* A134, nearly 2–4 fold decrease in amylase level was observed in the potato (2.5 %) and the maltose (5 %) media, compared to the other strains (Fig. 2).

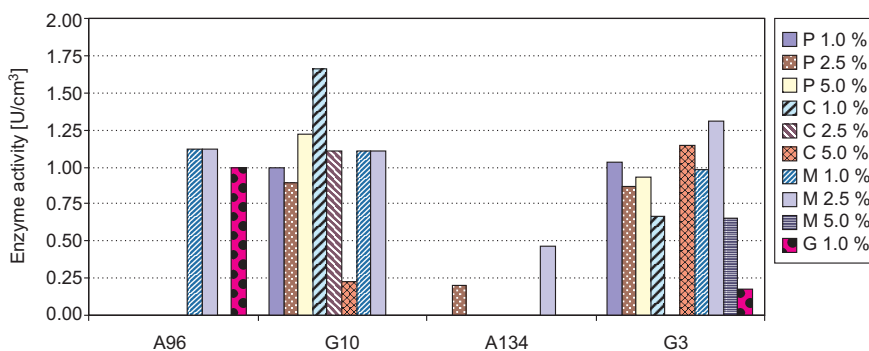


Fig. 2. The influence of pH 6.0 on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycooides* (A134, G3)

In contrast to pH 6.0 the maximum production of amylase was noted at pH 7.0 in case of soil occurring bacteria. The most vigorous strain was *B. cereus* A96 in the medium containing corn starch (from 1.24 U/cm^3 to 1.52 U/cm^3), but *B. mycooides* A134 in the potato (2.5 %) and the maltose (1 %) media, 1.25 U/cm^3 and 1.42 U/cm^3 , respectively. The growth medium containing lower concentration of potato (1 %) turned out to be favourable for water occurring bacteria (strains G3 and G10) and the quantity of hydrolysed starch amounted to 0.95 U/cm^3 (G10) and 1.01 U/cm^3 (G3). The results related to these carbon sources showed that none of the strains produced enzymes in glucose media, similarly to described results obtained at pH 5.0 (Fig. 3).

Amylase activity measured for *B. cereus* strains and *B. mycooides* strains at pH 8.0 showed, that among the first group of the most active was strain marked A96 while among the second group strain marked G3. Among them, *B. cereus* A 96 preferred the

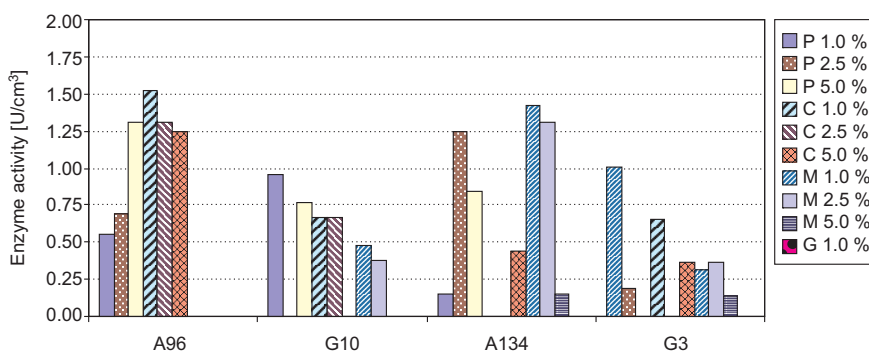


Fig. 3. The influence of pH 7.0 on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycooides* (A134, G3)

highest (5 %) concentrations of potato starch, corn starch and maltose, while *B. mycooides* G3 the lowest (1 %). The enzyme activity ranged from 1.18 (P 5 %) to 1.82 (M 5 %) U/cm³ (A96) and from 0.71 (M 1 %) to 1.50 (P 1 %) U/cm³ (G3). Moreover, glucose medium was sufficient for amylase production only for *B. cereus* A96 (Fig. 4).

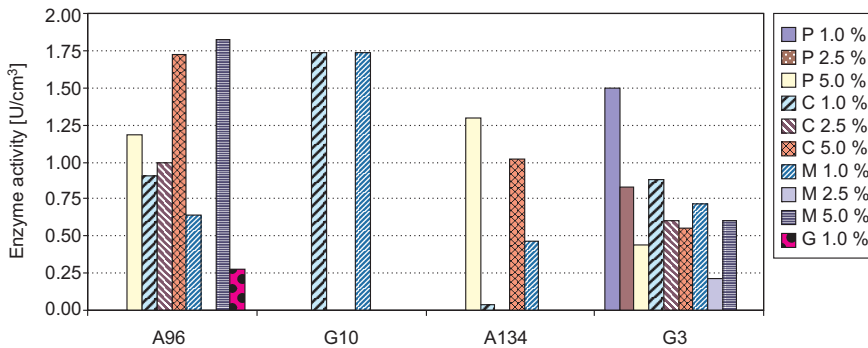


Fig. 4. The influence of pH 8.0 on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycooides* (A134, G3)

For further estimation, in order to determine the influence of temperature on productive abilities of *Bacillus* strains, pH 7.0 was selected.

Soil occurring bacteria, both *B. cereus* A96 and *B. mycooides* A134, produced the highest amount of amylase in medium containing 1 % potato starch in a full range of temperature. When *B. cereus* G10 is considered, the highest amounts were noted in P 1 % only at temp. 30 °C and 50 °C while in P 5 % at temp. 40 °C and 60 °C. The lowest amount of amylase were obtained in case of all the strains at 2.5 % concentration of potato starch when compared with all tested temperatures. Besides, among the four tested strains, the most productive were *B. cereus* G10 and *B. mycooides* A 134. The optimal temperature for amylase production occurred to be 60 °C for soil strains and 60 °C or 50 °C for water strains, that they showed the maximum activity (Fig. 5).

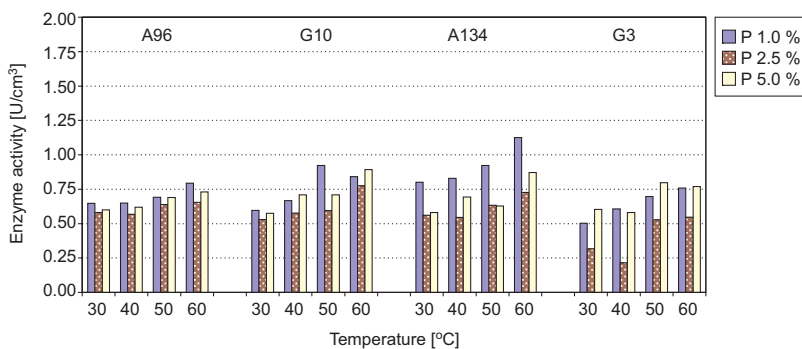


Fig. 5. The influence of temperature on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycooides* (A134, G3) grown in potato starch media

The effect of temperatures and corn starch on amylase production by *Bacillus* strains is presented in Fig. 6.

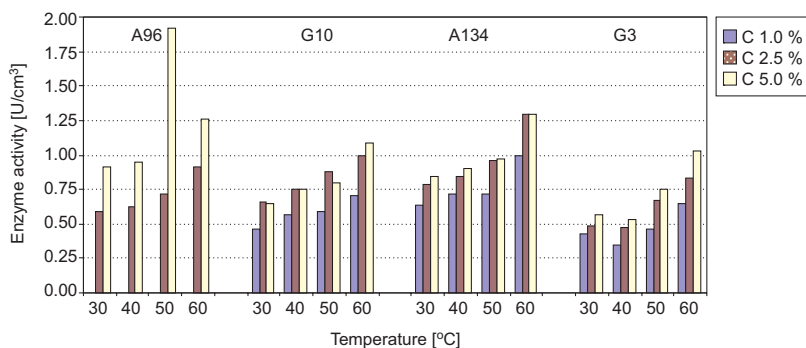


Fig. 6. The influence of temperature on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycoides* (A134, G3) grown in corn starch media

The growth medium containing 5 % corn turned out to be a favourable medium for amylase production by all soil occurring strains and one water strain *B. mycoides* G3, while for water strain *B. cereus* G10 they were C 2.5 % and C 5 % media. However, the lowest amylase activity was found in 1 % corn medium for both *B. mycoides* strains and *B. cereus* G10 as well. Only *B. cereus* A96 did not show amylase production at said concentration. It was found that the highest level of amylase was obtained for all strains at 60 °C and also among *B. mycoides* the highest ability to synthesize amylase was noted for strain marked A 134 (Fig. 6).

The growth medium with maltose appeared to be the most favourable in the biosynthesis process of the amylases compared to all tested media. Based on the obtained results, the highest amount of amylase was observed at 5 % maltose concentration in case of both *B. mycoides* strains and *B. cereus* A96, while at 2.5 % maltose in the event of *B. cereus* G10, in a full temperature range. Maximum amylase production for all strains was obtained at 60 °C, although *B. cereus* G10 also revealed activity in the remaining temperatures with the results similar to the ones at 60 °C. Among all strains, *B. cereus* G10 and *B. mycoides* A134 presented the highest enzymatic ability (Fig. 7).

The last medium, with 1 % glucose, was promoting extracellular amylase production. The highest amount of amylase in this medium was obtained at 30 °C in case of both *B. mycoides* strains, but the lowest amounts were recorded at higher temperatures (50 °C for A134 and 60 °C for G3). A different situation appeared in case of *B. cereus* strains. The highest activity was observed at 40 °C for strain marked A96 and at 60 °C for G10, whereas the lowest at 50 °C for the both strains. The quantity of hydrolysed starch fluctuated around 1.95 U/cm³ in case of all strains (Fig. 8).

A number of reports available in the literature indicate that the biosynthesis of various extracellular enzymes or secondary metabolites depends on many external factors such as the type and concentration of the substrate, the presence of easily

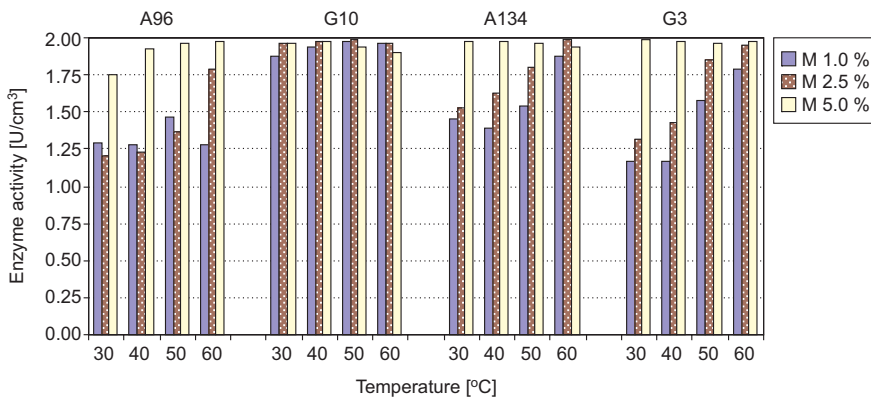


Fig. 7. The influence of temperature on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycooides* (A134, G3) grown in maltose media

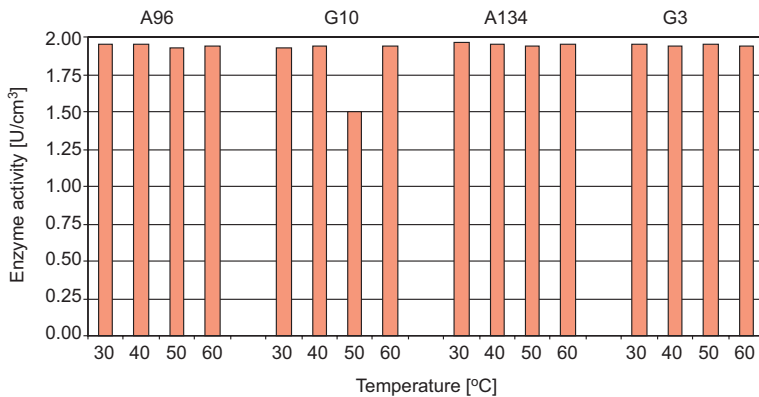


Fig. 8. The influence of temperature on the production of extracellular amylases by *Bacillus cereus* (A96, G10) and *Bacillus mycooides* (A134, G3) grown in glucose (1%) medium

available sources of carbon and enzyme activator, pH, the temperature but also on the genus and even the strain of microorganisms. Earlier studies of many researchers reported that amylases are active in a wide range of pH 5–11 and temperatures between 30–80, although their study have shown different optimal conditions for amylase activity depending on *Bacillus* strains [3, 21–24]. Annamalai et al [17] have shown that the optimal pH of amylase activity for *B. cereus* ranged between 8–11. In contrast to the present study, *B. cereus* MTCC 1305 revealed amylase at pH 5.0 [8] but in another *B. cereus* strain maximum amylase production was observed at pH 7.0–8.0 [12]. Slightly different results have been obtained in our study. In most cases, the most suitable pH was 7.0, though *B. mycooides* G3 and *B. cereus* G10 showed also high activity at pH 6.0 (Fig. 2, 3). Additionally, the highest activity of almost all strains under study have been noted at 60 °C, although amylases proved to be active in slightly lower temperatures ranging from 30 to 60 °C. Similarly, the following results for amylase activity have

been reported by others in case of: *B. cereus* – 55–65 °C [8, 17], *B. megaterium* – 60 °C [25] and higher in case of: *B. amyloliquefaciens* 40 °C [26] and *Bacillus sp.* 35 °C [5], *B. cereus* 30 °C [24] or 40 °C [12]. Studies Deb et al [27] revealed that optimum pH, temperature of enzyme activity of *B. amyloliquefaciens* was 6,5 and 60 °C respectively. Other investigator reported that maximum amylase production occurred at pH 6.0 and temperature 37 °C [28]. Viswanathan et al [29] has evaluated the amylolytic activity of three strains of *Bacillus*, such as *B. subtilis*, *B. cereus* and *B. megaterium* and showed that this latter was found to be the best amylase producer. Maximum enzyme production was observed in pH 7.0 and temperature 37 °C.

Among the carbon sources tested, 5 % maltose and 1 % glucose have been found to enhance amylase production. Lower levels of amylase have been noted in potato medium (Fig. 7, 8). On the other hand Sivakumar et al [12] observed the maximum amylase production in maltose supplemented medium while minimum amylase production in glucose medium. Other studies [30] indicate sucrose as the best carbon source to enhance amylase production by *B. cereus* strain A 26MB. In view of all temperatures the most effective was *B. mycooides* A 134 whereas the least *B. mycooides* G3.

Many researches [8, 13, 21] concluded that the synthesis of amylase was greatly suppressed when the bacteria were grown on sucrose, glucose or fructose and amylase production whereas enhanced when the bacteria were grown on starch. Izvieikova, 2005 and Saxena et al, 2007 showed that in the media rich in available monomers, the bacteria absorb these components, whereas in poor media they produce hydrolytic enzymes to degrade complex molecular substrates. In contrast, there are reports indicating stimulating effect of monosaccharides or disaccharides on amylase production. Anto et al, 2006 and Jamuna et al, 1992 observed that amylase production by *B. cereus* MTCC1305 and *B. subtilis* respectively were higher in the medium with glucose than with potato. Viswanathan et al [29] tested diverse carbon source such as sucrose, starch, glucose and maltose, high production was recorded with starch followed by glucose while the least production of enzyme was noted when sucrose was used as a carbon source.

The selection of an appropriate the culture conditions to determine the metabolic process, as well as the substrate is very important because it enables to find the most active strains useful for the production of biological preparations, which can be used in protecting the environment as well as a useful alternative to chemical methods.

Conclusions

The research proved significant diversity of amylolytic activity of *B. cereus* and *B. mycooides* strains, towards the source of carbon substrates, pH and the temperature analysed in the experiment. Biosynthesis of amylase catalyzed by *Bacillus spp.* at different pH was most varied and depended on the strain and the growth medium. The least favourable pH was 5.0 while in most cases the best noted pH was 7.0. The research enables to state that all tested bacteria hydrolysed starch and the process was intensive for *B. mycooides* G3 at pH 8.0 and *B. cereus* G10 at pH 6.0. Taking into account temperatures range tested, the highest amount of amylase was obtained at 60 °C while

the lowest at 30 °C for all tested strains. Simultaneously, the growth medium with an addition of 5 % maltose and 1 % glucose was most suitable for all tested strains. Considering the temperature and sources of carbon, the most effective strain was *B. mycoides* A 134 whereas the least *B. mycoides* G3.

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**WPLYW CZYNNIKÓW FIZYCZNYCH
NA AKTYWNOŚĆ AMYLOLITYCZNĄ *Bacillus* sp.
WYIZOLOWANYCH ZE ŚRODOWISKA NATURALNEGO**

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Abstrakt: Celem badań była ocena wpływu różnych czynników na aktywność amyloリティczną *Bacillus cereus* (2 szczepy) i *Bacillus mycoides* (2 szczepy) wyizolowanych z gleby i Jeziora Turawskiego. Metodą spektrofotometryczną badano wpływ pH mieszaniny reakcyjnej w zakresie od 5.0 do 8.0 i temperatury w zakresie od 30 °C do 60 °C oraz źródła węgla i jego koncentracji w podłożu na poziom produkowanych amylaz. Stwierdzono, iż najgorszą wartością pH do syntezy amylaz było 5.0, natomiast najlepszą, w większości przypadków pH 7.0. Uwzględniając zakres badanych temperatur, najwyższą aktywność uzyskano w temp. 60 °C, natomiast najniższą w temp. 30 °C dla wszystkich testowanych szczepów. Najbardziej aktywnym szczepem był *B. mycoides* A 134, natomiast najmniej *B. mycoides* G3.

Słowa kluczowe: *Bacillus cereus*, *Bacillus mycoides*, amylazy, czynniki fizyczne