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Influence of Physical and Chemical Factors on the Activity of Chitinases Produced by Planktonic Bacteria Isolated from Jeziorak Lake

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Abstract

Studies on the influence of physical and chemical factors on the activity of chitinases produced by planktonic bacteria isolated from Jeziorak Lake were carried out. The results evidenced maximum activity of bacterial strains at pH 6.0 and at 10°C and 30°C. Among the strains studied, higher activity was recorded along with an increase in colloidal chitin concentration. The highest activity was recorded at 2% concentration of chitin. The majority of strains hydrolyzed chitin most intensely after 192 h of incubation, whereas no strain revealed chitinases activity after 48 h.

Keywords: chitinolytic bacteria, planktonic bacteria, chitinases

Introduction

Chitin is an unbranched polysaccharide composed primarily of 3-1,4 linked N-acetyloglucosamine and occasionally glucosamine residues [10]. It makes part of outer arthropod skeletons and also is one of components of fungi and some yeast's cell walls [11].

Chitin enzymatic hydrolysis for its component monomers runs with the assistance of specific chitinolytic enzymes. Transmo and Harman [21] isolated three groups of chitinolytic enzymes: chitinases cutting the chitin at random at different points of N-acetyloglucosamine polymer: chitobiosidases operating after chitinases and releasing chitobiasis off split fragments, and N-acetyl-β-glucosamidases hydrolyzing chitobiasis for monomers. N-acetyloglucosamine and its decomposition products: glucosamin, acetic acid and ammonia are released during the process of chitin decomposition [13]. They are used by many different organisms as a source of carbon and nitrogen. Chitinolytic enzymes are produced by a large group of organisms: fungi, bacteria [17], vertebrates [9], invertebrates [19] and plants [15].

Materials and Methods

Object of Study

The present study focused on planktonic bacteria isolated from the pelagic zone of Jeziorak Lake in summer (22nd July, 1997). Jeziorak Lake is located in Hawskie Lake District and belongs to the Wista-Drweca river-basin. The lake surface is 32.3 sq. km., length 27.5 km, mean width about 1.2 km, maximum depth 12.0 m, mean depth about 5.7 m. The lake is a eutrophic water body [7].

Chitinolytic Bacteria Isolation

Planktonic bacteria were isolated with the use of iron-peptone medium according to Ferrer, Stapert, Soko-lski [5]. The bacteria ability to decompose colloidal chitin was tested on a stable medium with colloidal chitin composed of: peptone (peptobak) - 5g, iron gluconate - 0.lg, ammonia sulphate - 0.1 g, iron sulphate - 0.lg, colloidal

chitin - 7 g dm., agar - 15 g, tap water - 1.0 dm^3, pH 7.2 - 7.4. Colloidal chitin was prepared according to Lingappa and Lockwood [12]. Isolated bacteria strains were transplanted onto the medium, then after 2, 4, 6, 8, 10, and 12 days of incubation at 20°C the diameter of light-coloured patches around colonies were measured to assess the bacteria ability to decompose chitin. Among chitin decomposing bacteria strains, 10 strains were used for further investigations on the activity of the chitinases. Those strains were multiplied on the slant with iron-pepton medium with the addition of 0.5% colloidal chitin for 72 h at 20°C and later were stored in a refrigerator and kept at ± 4°C for further experiments; they were transfer red into a fresh medium every month.

Chitinolytic Bacteria Identification

The studied strains were identified according to methods used for freshwater bacteria by Allen, Austin and Colwell [1].

Inoculum Preparation

The chitinolytic bacteria were pre-incubated on the iron-pepton slant for 72 h at 20°C and then washed by 2.5 cm^3 of sterile buffer water [3]. Liquid iron - pep ton medium doses of 15 cm^3 and pH 7.0 were then inoculated by 0.5 cm^3 of the obtained suspension. The bacteria were incubated at 20°C until the phase of their logarithm development was determined and the optic density (OD) of the culture was determined on a "Marcel s 330 Pro" spectrophotometer, at a wave length of 565 m. A suspension was made of bacteria rinsed with sterile buffer water, with absorption ratio E=0.3, which is equivalent to the cell contents of about $10^9/cm^3$.

Chitinolytic Activity Measurement

0.5 cm^3 of suspension prepared prior to the experiment was transferred into Erlenmayer flask of 100 cm^3 with 20 cm^3 of liquid medium composed of: peptone (peptobak) - 1.0 g, ferrous gluconian - 0.1 g, ammonia sulphate - 0.1 g, ferrous sulphate - 0.1 g, yeast's extract - 0.1 g, colloidal chitin - 2%, tap water - 1.0 dm^3, pH 7.2 - 7.4. The bacteria were incubated at 20°C for 192 h.

The study included the temperature factor (10°C, 20°C, 30°C, 40°C), incubation period (48h, 96 h, 144 h, 192 h, 240 h, 288 h), pH (5.0, 6.0, 7.0, 8.0, 9.0) and colloidal chitin concentration within the range of 0.5%, 1.0%, 1.5%, 2.0%, 2.5%. The incubation environment (medium) reaction (pH) was obtained with the help of 0.1 M NaOH and 0.1 M HCl. Chitinases activity (crude enzymes) expressed by the number of mmole of N-acetyloglucosamine released during the process of chitin hydrolysis was determined by means of the Reissig, Strominger and Leloir method [16]. The activity (U) was expressed as follows: total activity (converted into 1 cm^3 of post-culture liquid/h) and specific activity (converted into 1 mg of protein in the post-culture liquid/h).

Enzymatic protein contents (in the post-culture liquid) was determined by means of the Bradford method [2].

Results

Table 1 presents the ability of planktonic bacteria to biodegrade colloidal chitin. As one may easily see, the most active in chitin decomposition were the strains of *Serratia sp.* and *Erwinia stewartii*, for they made the greatest diameters of light-coloured patches (9 mm). Most bacteria displayed the maximum diameter of light-coloured patches after 192 and 240 h of incubation. No strain, on the other hand, revealed chitinolytic activity after 48 h of incubation.

Table 1. Decomposition of colloidal chitin by planktonic bacteria of Jeziorak Lake.

Name of bacteria	Incubation time					
	48 h	96 h	144 h	192 h	240 h	288 h
Aeromonas sp.	* 0	2	5	7	7	7
Arthrobacter sp.	0	1	3	5	5	5
Flavobacterium sp.	0	1	3	5	5	5
Flexibacter – Cytophaga	0	1	2	5	5	5
Vibrio fluvialis	0	2	3	5	5	6
Aeromonas hydrophila	0	1	3	6	7	7
Erwinia stewartii	0	2	6	8	9	9
Bacillus pumilus	0	1	3	5	5	5
Serratia sp.	0	2	5	8	9	9
Bacillus firmus	0	2	4	5	5	5

Explanation: * - zones of clearing around microbial colonies (in mm)

Table 2 presents the results of investigations on pH influence on the activity of chitinases produced by planktonic bacteria. It is easy to notice that most strains displayed their chitinolytic activity at pH 6.0. The following strains: *Bacillus pumilus, Bacillus firmus* showed the optimum of their activity at pH 5.0. The *Aeromonas* strain displayed greatest total and specific activity at pH 8.0, while the strain of *Flexibacter - Cytophaga* showed its greatest total activity at pH 8.0, while specific activity at pH 5.0. No maximum activity of strains was noticed at pH 9.0 or pH 7.0.

Table 3 presents the results of investigations on temperature influence on the activity of chitinases produced by planktonic bacteria. It is easy to notice that 4 strains displayed their greatest total and specific activity at 10°C *(Flavobacterium sp., Bacillus firmus, Erwinia stewartii, Flexibacter - Cytophaga),* and 4 strains at 30°C *(Aeromonas sp., Vibrio fluvialis, Aeromonas hydrophila, Bacillus pumilus).* Among the investigated strains it was only *Serratia sp.* that displayed the greatest chitinolytic activity at 20°C, while in the case of *Arthrobacter sp.* it was at 40°C.

Table 2. Effect of pH on chitinase activity after 192 h incubation.

Name of bacteria	pH				
	5	6	7	8	9
Aeromonas sp.	0.0015* 0.0002**	0.0066 0.0009	0.0018 0.0009	0.0010 0.0006	0.000 0.000
Flavobacterium sp.	0.0007 0.0026	0.0033 0.0052	0.0031 0.0036	0.0017 0.0033	0.0002 0.0001
Vibrio fluvialis	0.0010 0.0024	0.0035 0.0069	0.0017 0.0052	0.0005 0.0038	0.000 0.000
Bacillus firmus	0.0122 0.0123	0.0029 0.0020	0.0026 0.0020	0.0025 0.0016	0.0003 0.0001
Arthrobacter sp.	0.0025 0.0010	0.0028 0.0020	0.0028 0.0011	0.0023 0.0011	0.0003 0.0001
Serratia sp.	0.0003 0.0004	0.0556 0.1720	0.0516 0.0382	0.0380 0.0558	0.0066 0.0030
Erwinia stewartii	0.0016 0.0011	0.0028 0.0013	0.0024 0.0012	0.0015 0.0010	0.000 0.000
Aeromonas hydrophila	0.0014 0.0024	0.0018 0.0024	0.0030 0.0034	0.0041 0.0038	0.0006 0.0028
Bacillus pumilus	0.0050 0.0031	0.0041 0.0028	0.0020 0.0028	0.0021 0.0019	0.0030 0.0004
Flexibacter-Cytophaga	0.0016 0.0025	0.0016 0.0019	0.0019 0.0012	0.0021 0.0009	0.0003 0.0004

Explanations: * - total activity chitinases (U/cm^3), ** - specific activity chitinases (U/mg protein)

Table 3. Effect of temperature on chitinase activity after 192 h incubation.

Name of bacteria	Temperature			
	10°C	20°C	30°C	40°C
Aeromonas sp.	0.0006* 0.0012**	0.0017 0.0010	0.0022 0.0011	0.0000 0.0000
Flavobacterium sp.	0.0229 0.0174	0.0031 0.0033	0.0022 0.0015	0.0000 0.0000
Vibrio fluvialis	0.0015 0.0009	0.0027 0.0030	0.0372 0.0243	0.0010 0.0017
Bacillus firmus	0.0069 0.0067	0.0025 0.0018	0.0016 0.0012	0.0016 0.0013
Arthrobacter sp.	0.0010 0.0020	0.0023 0.0021	0.0025 0.0020	0.0053 0.0058
Serratia sp.	0.0016 0.0009	0.0556 0.0382	0.0325 0.0105	0.0011 0.0008
Erwinia stewartii	0.0029 0.0031	0.0028 0.0032	0.0025 0.0019	0.0008 0.0011
Aeromonas hydrophila	0.0030 0.0021	0.0043 0.0068	0.0076 0.0028	0.0000 0.0000
Bacillus pumilus	0.0028 0.0035	0.0036 0.0040	0.0039 0.0045	0.0013 0.0021
Flexibacter-Cytophaga	0.0036 0.0042	0.0016 0.0009	0.0019 0.0008	0.0005 0.0004

Explanations: * - total activity chitinases (U/cm^3), ** - specific activity chitinases (U/mg protein)

Table 4. Effect of colloidal chitin concentration on chitinases activity after 192 h incubation.

Name of bacteria	Colloidal chitin				
	0.5%	1.0%	1.5%	2.0%	2.5%
Aeromonas sp.	0.0006* 0.0004**	0.0019 0.0013	0.0023 0.0015	0.0027 0.0090	0.0013 0.0010
Flavobacterium sp.	0.0010 0.0004	0.0021 0.0009	0.0028 0.0010	0.0031 0.0033	0.0018 0.0011
Vibrio fluvialis	0.0014 0.0011	0.0025 0.0011	0.0026 0.0015	0.0030 0.0016	0.0007 0.0002
Bacillus firmus	0.0024 0.0011	0.0025 0.0014	0.0027 0.0015	0.0029 0.0018	0.0007 0.0006
Arthrobacter sp.	0.0008 0.0009	0.0009 0.0015	0.0017 0.0015	0.0023 0.0021	0.0010 0.0006
Serratia sp.	0.0016 0.0006	0.0017 0.0009	0.0019 0.0009	0.0368 0.0558	0.0028 0.0012
Erwinia stewartii	0.0017 0.0009	0.0024 0.0009	0.0025 0.0011	0.0028 0.0011	0.0007 0.0003
Aeromonas hydrophila	0.0031 0.0016	0.0035 0.0021	0.0043 0.0033	0.0053 0.0033	0.0030 0.0030
Bacillus pumilus	0.0013 0.0005	0.0016 0.0008	0.0022 0.0010	0.0036 0.0035	0.0017 0.0006
Flexibacter-Cytophaga	0.0007 0.0003	0.0008 0.0004	0.0016 0.0010	0.0023 0.0011	0.0003 0.0010

Explanations: * - total activity chitinases (U/cm^3), ** - specific activity chitinases (U/mg protein)

Table 5. Effect of incubation time on chitinase activity.

Name of bacteria	Incubation time					
	48 h	96 h	144 h	192 h	240 h	288 h
Aeromonas sp.	0.0* 0.0**	0.0017 0.0016	0.0019 0.0016	0.0028 0.0025	0.0017 0.0015	0.00100 0.00130
Flavobacterium sp.	0.0 0.0	0.0011 0.0009	0.0028 0.0010	0.0040 0.0033	0.0012 0.0009	0.00092 0.00071
Vibrio fluvialis	0.0 0.0	0.0017 0.00069	0.0022 0.0010	0.0039 0.0021	0.0019 0.0010	0.00190 0.00009
Bacillus firmus	0.0 0.0	0.0046 0.0049	0.0032 0.0040	0.0031 0.0022	0.0031 0.0014	0.00096 0.00066
Arthrobacter sp.	0.0 0.0	0.0017 0.0027	0.0032 0.0027	0.0032 0.0029	0.0020 0.0010	0.00100 0.00087
Serratia sp.	0.0 0.0	0.0010 0.0007	0.0315 0.0290	0.0024 0.0015	0.0013 0.00065	0.00110 0.00048
Erwinia stewartii	0.0 0.0	0.0015 0.0007	0.0035 0.0033	0.0024 0.0033	0.0017 0.0019	0.00140 0.00125
Aeromonas hydrophila	0.0 0.0	0.0020 0.00135	0.0053 0.0043	0.0088 0.0079	0.0042 0.0030	0.00420 0.00100
Bacillus pumilus	0.0 0.0	0.0015 0.0005	0.0025 0.0010	0.0070 0.0050	0.0030 0.0038	0.00150 0.00100
Flexibacter-Cytophaga	0.0 0.0	0.0017 0.0028	0.0020 0.0030	0.0048 0.0052	0.0023 0.0015	0.00100 0.00087

Explanations: * - total activity chitinases (U/cm^3), ** - specific activity chitinases (U/mg protein)

Table 4 presents the results of colloidal chitin concentration on the activity of the chitinases produced by the strains studied. It is clear to see that both the total and specific activities of chitinases grew along with the colloidal chitin concentration increase. The greatest total and specific activities of the strains studied were revealed at 2% concentration of the colloidal chitin; their activity dropping over that percentage.

Table 5 presents the results of the study on incubation time affecting chitinases activity. It evidenced no activity of the strains after 48 h incubation time. The majority of the strains achieved the chitinases maximum activity after 192 h incubation time, with a falling tendency. The greatest total and specific activities of *Bacillus firmus* strain occurred as early as after 96 h incubation time (0.0046 U/cm^3, 0.0049 U/mg protein), only to drop to a minimum after 288 h incubation time (0.00096 U/cm^3, 0.00066 U/mg protein). In the case of *Serratia sp.* the chitinases activity increased until it reached a maximum after 144 h incubation time only to decrease soon after.

Discussion

Being a high-molecular compound, chitin is decomposed mainly by microbes. *Streptomyces griseus* excreted enzymes were divided into chitinasis and chitobiasis [17]. Chitin degradation occurs along with chitinasis attacking polymer at many different points which results in forming small quantities of N-acetyloglucosamine, chitobiosis and chitotriosis in the first place [17]. The presence and importance of chitin destroying organisms is crucial due to its great production in water bodies. Chitinases are produced by a multiplity of organisms: bacteria, fungi, vertaebrates, invertebrates and also plants. However, it is bacteria that seem to be made most responsible for chitin biodegradation.

Chitin degradation depends on many environmental factors. The most important ones seem to be the following: pH, temperature, chitin concentration and incubation time. Studying the chitin degradation rate, Seki [18] found out that bacteria cultures composed of 10^{10} cells in 1 cm^3 of the soil are able to decompose about 30 mg in a day at 25°C. He also calculated that in an ocean, at 15°C chitin gets decomposed within 140 days and at 5°C within 370 days, whereas at below 5°C it took 500 days. At as little as 5°C, Goodrich and Morita [8] found numerous chitin decomposing strains among bacteria isolated from the sea.

Laboratory tests of chitinases activity of Jeziorak planktonic bacteria proved their low activity. This may result from chitobiose and chitotriose presence in the post-culture substance, and small amounts of hard detectable N-acetyloglucosamine. Besides, the investigations were carried out with the use of crude enzymes which may have caused the existence of the enzyme inhibitors in the culture substance.

The results obtained in the present studies revealed that it was at pH 6.0 that most bacteria intensified their activity to decompose chitin, while *Bacillus pumilus* and *Bacillus firmus* strains revealed optimum activity at pH 5.0. Studying chitinolytic activity at *Bacillus pabuli K 1*, Frandberg and Schniirer [4] found the greatest chitinases activity at pH 8, but found none of it below pH 6. The elevated activity at pH 5 as found in the present study may indicate genetic conditions in particular bacteria species. Testing chitinase activity in *Arthrobacter sp.*, Morrissey [15] found optimum activity at pH 4.9, whereas Monreal and Reese [14] recorded highest activity in *Serratia marcensces* occurring at pH 6.4. On the basis of his investigations, Donderski [4] announced that maximum activity among bacteria isolated from lakes of different trophies occurred at pH 5.0 or 6.0. He recorded no activity at pH 9.0.

Studies on temperature impact on chitinases activity showed that the optimal temperature for most strains was 10°C and 30°C. According to studies by Frandberg and Schniirer [6], maximum activity of *Bacillus pabuli K 1* occurred at 30°C, whereas it did not produce any chitinases at 10°C. The present study proves that chitin hydrolysis is possible at as low temperature as 10°C. One may suppose that low temperature does not hamper the physiological functioning of bacteria, including the enzymatic one in terms of chitin. According to the data obtained from these studies, *Arthrobacter sp.* revealed the highest chitinolitic activity at 40°C. Studying chitinases activity in *Arthrobacter sp.*, Morrissey [15] found their highest activity at 50°C. Monreal and Reese [14] announced optimal temperature of chitin decomposition in *Serratia marcensces* also to be 50°C.

The studies on the influence of colloidal chitin concentration on chitinases activity showed that chitin was correlated with substrate concentration and therefore maximum activity was recorded at 2% chitin concentration in the medium. While analyzing chitinolytic enzyme activity in *Aeromonas sp.*, Huang, Chen and Su [10] found increased chitinase activity along with colloidal chitin concentration growth to 1.5%. Enzyme activity plumetted slightly at 2% chitin. A drop in chitinolytic bacteria activity at 2.5% colloidal chitin concentration, as found in the present paper, may indicate a hampering reaction of colloidal chitin serving as substrate, or accummulation of intermediaries coming from chitin decomposition, which make up a synthesis inhibitor of chitinasis itself. Following the hypothesis by Michaelis and Menten, one may anticipate that the enzymatic reaction rate is directly proportional to substrate concentration when at a low percentage. The greater the concentration the lesser influence of its growth on enzymatic reaction rate [20].

Data concerning the time factor influencing chitinases activity point to the fact that most strains under investigation were able to decompose chitin most intensively after 192 h incubation time, after which figure activity plummeted considerably. One may anticipate that, on one hand, N-acetyloglucosamine is possibly used by bacteria as a carbon and nitrogen source, but on the other greater amounts of accummulated N-acetyloglucosamine may hamper further production of the enzyme. *Serratia sp.* strains were an exception as they reached the highest activity after 144 h incubation time, and *Bacillus firmus* was most active in terms of chitin after 96 h incubation time. Studying the issue of incubation time affecting chitinases activity in *Bacillus pabuli K 1*, Franderg and Schniirer [6] recorded maximum activity after 120 h incubation time at 30°C. On the other hand, Huang Chen and Su [10] found greatest chitinases activity in *Aeromonas*

sp. after 50 h incubation time. During the present study no chitinolytic activity was recorded after 48 h incubation time. Most probably it results from the fact that chitin is a hard-decomposable compound and organisms need a longer time to adapt to this substrate than to other high-molecular compounds in ferms to be able to start the production of necessary enzymes.

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