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Occurrence and Activity of the Chitinolytic Bacteria of *Aeromonas* Genus

M. Swiontek Brzezinska, W. Donderski

Department of Water Microbiology and Biotechnology, Institute of Ecology and Environmental Protection, Nicholas Copernicus University 87-100 Toruń, Poland

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Abstract

A study was carried out on the occurrence and activity of chitinolytic planktonic bacteria of *Aeromonas sp., Aeromonas hydrophila* and *Aeromonas salmonicida* species, isolated from lake Jeziorak. Among the identified strains decomposing chitin the most abundant were *Aeromonas sp.*. All the investigated strains showed maximum chitinolytic activity at pH 6.0. An increase in chitinolytic activity was observed that occurred along with temperature growth (10° - 40°C) and colloidal chitin concentration in the medium (0.5 - 2.5%). Their decomposing activity was most intense after a 192 h incubation time. No strain displayed activity after a 48 h incubation time.

Keywords: chitinolytic bacteria, planktonic bacteria, chitinases

Introduction

Chitin is a polysaccharide, a linear polymer composed of N-acetyloglucosamine monomers connected by glycoside β - 1,4 bonds [14]. It forms an outer skeleton of arthropods, protozoa, molluscs, nematoda, crustaceans and is present in the cell walls of many fungi [25].

Biological degradation of chitin is accompanied by endo- and egzo- enzymes known as chitinases (EC 3.2.1.114) and β-N-acetylohexaminidases (EC3.2.1.52) [19]. Enzymes participating in chitin degradation are produced not only by organisms containing chitin in their body but by bacteria, upper plants and mammals where chitin is not present on a regular basis [5, 10, 15, 18]. Bacteria produced chitinases play a significant role in maintaining the matter cycle through making chitin usable biologically [25].

Material and Methods

Study Area

The study was carried out on planktonic bacteria isolated from biofilm, epilimnion, metalimnion and hypolimnion of lake Jeziorak. Lake Jeziorak is situated in Hawskie Lake District and makes a part of the Vis-tula-Drwęca catchment area. The lake surface makes 32.3 km^2, length - 27.5 km, the mean width - about 1.2 km and the maximum depth - 12.0 m; the mean depth is about 5.7 m. It is a post-glacial lake of meridian-like placement and belongs to eutrophic water bodies [2, 13].

Sampling and Isolating of Chitinolytic Bacteria

Water samples for chitinolytic bacteria isolation were obtained between June and November 1997. Water was sampled from surface microlayer (biofilm), epilimnion and hypolimnion by means of a tube sampler of our own construction. The 300 μm thick water layer sampled from biofilm was collected by means of Garret's net with 200 μm mesh size. For bacteria isolation a ferrum-peptone medium was used after Ferrer, Stapert and Sokolski [11] and a friction inoculation was applied. After a 10-day incubation time at 20°C, 30 strains were removed every time (every month and every water layer under investigation) and then their chitin decomposition ability was inspected by means of colloidal chitin spot inoculation of permanent culture consisting of the following: peptone (peptobak) - 0.5 g, iron gluconate - 0.1 g, ammonia sulphate - 0.1 g, iron sulphate - 0.1 g, colloidal chitin - 7g

dry weight tap water - 1.0 dm^3, pH - 7.2-7.4. Colloidal chitin had been prepared according to the formula by Lingappa and Lockwood [17]. After a 12-day incubation time at 20°C diameters of bright halos around colonies were measured to assess the bacteria ability to decompose chitin. After identification and having their chitinolytic activity checked, 51 strains were picked to be reproduced on slants of iron-peptone medium including 0.5% of coloidal chitin for 72 hours at 20°C. Those strains were kept in a refrigerator at +/- 4°C, and were inoculated anew on fresh medium every month.

Identification of Chitinolytic Bacteria

Identification of the investigated chitinolytic strains was performed following the pattern suggested by Allen, Austin and Colwell [1], Shewen, Hobbs, Hodgkins [24] for freshwater bacteria.

Evaluation of Bacterial Chitinolytic Activity

Bacterial strains identified as *Aeromonas sp.*, *Aeromonas hydrophila* and *Aeromonas salmonicida* were preincubated for 72 hours at 20°C on slants containing ferrum-peptone medium, and then washed off with 2.5 cm^3 sterile buffer water [7]. The obtained suspension (0.5 cm^3) was inoculated onto 15 cm^3 doses of liquid ferrum-peptone medium of pH 7.0. The bacteria were incubated at 20°C until the phase of their logarythmic development was captured and optic density (OD) of the culture marked on the "Marcel's 330 Pro" spectrophotometer at 565 nm wavelength. Having rinsed the bacteria with sterile buffer water, a suspension was prepared having E=0.3 absorbency capacity (which is equal to cell contents of about 10^9 / cm^3).

Chitinolytic Activity Measurement

The bacterial suspension in amount 0.5 cm^3 prepared as above, was introduced into 100 mL Erlenmayer flasks containing 20 cm^3 of semi-liquid medium composed of the following: peptone (peptobak) - 1.0 g, iron sulphate - 0.1 g, ammonia sulphate - 0.1 g, yeast extract - 0.1 g, colloidal chitin - 2%, tap water - 1.0 dm^3, pH 7.0 which underwent incubation covering the influence of temperature (10°C, 20°C, 30°C, 40°C), incubation time (48h; 96h; 144h; 192h; 240h; 288h), pH (5.0; 6.0; 7.0; 8.0; 9.0) and colloidal chitin concentration in the medium within the range of 0.5%; 1.0%; 1.5%; 2.0%; 2.5%. Culture environment reaction (pH) was corrected by means of phosphate and citric buffer. The activity of the produced chitinases (crude enzymes) expressed in amount of mmol of N-acetyloglucosamine released during chitin hydrolysis was determined after Reissig, Strominger and Leloir [21]. 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 post-culture liquid/h). Enzymatic protein contents (in post-culture liquid) was determined by means of the Bradford method [6].

Results

Table 1 presents the number of chitinolytic strains able to decompose chitin. It shows that the most abundant group was formed by bacteria determined as *Aeromonas sp.* (8 strains). They occurred most abundantly in the biofilm (5 strains); in epilimnion (3 strains). In metalimnion and in hypolimnion none were recorded. The strain of *Aeromonas salmonicida* was recorded only in epilimnion and hypolimnion, while the strain of *Aeromonas hydrophila* was found only in epilimnion and hypolimnion, however, the strain of *Aeromonas hydrophila* was recorded only in metalimnion and hypolimnion.

Table 1. Number of strains decomposing colloidal chitin.

Name of strain	Surface microlayer	Epilimnion	Metalimnion	Hypolimnion	Total
Aeromonas sp.	5	3	0	0	8
Aeromonas salmonicida	0	2	0	1	3
Aeromonas hydrophila	0	0	2	1	3
other strains	13	3	14	7	37
Number of strains studied	120	120	120	120	

The study results on pH influence on chitinases activity produced by *Aeromonas* bacteria are presented in Table 2. They revealed the strains to have had their greatest chitinolytic activity at pH 6.0, whereas the lowest at pH 9.0, while the strains of *Aeromonas sp. Aeromonas hydrophila* showed no activity at all at that pH.

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

Name of strain	pH				
	5	6	7	8	9
Aeromonas sp.	0.0028* 0.0019**	0.0052 0.0118	0.0030 0.0013	0.0031 0.0017	0.0000 0.0000
Aeromonas salmonicida	0.0008 0.0009	0.0510 0,0214	0.0055 0.0027	0.0049 0.0021	0.0006 0.0010
Aeromonas hydrophila	0.0003 0.0009	0.0061 0.0087	0.0057 0.0036	0.0031 0.0029	0.0000 0.0000

Explanations:
* - total activity in µml N - acetylglucosamine / cm^3 / h,
* * - specific activity in µmol N - acetylglucosamine / mg enzyme proteins / h.

The study on temperature affecting the *Aeromonas* bacteria chitinase activity (Table 3) gave evidence that it increased along with temperature growth and it was at its highest total and specific activity at 20°C, the lowest at 40°C. The *Aeromonas salmonicida* strain was most active in terms of chitinases at 30°C and the lowest at 40°C; whereas at *Aeromonas hydrophila* strain, the greatest chitinolytic activity was noted at 40°C while the lowest - at 10°C.

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

Name of strain	Temperature [°C]			
	10	20	30	40
Aeromonas sp.	0.0028* 0.0046**	0.0038 0.0049	0.0035 0.0049	0.0017 0.0024
Aeromonas salmonicida	0.0019 0.0016	0.0055 0.0022	0.0056 0.0028	0.0015 0.0006
Aeromonas hydrophila	0.0023 0.0027	0.0031 0.0019	0.0033 0.0027	0.0035 0.0029

Explanations:
* - total activity in µmol N - acetylglucosamine / cm^3 / h,
* * - specific activity in µmol N - acetylglucosamine / mg enzyme proteins / h.

The results of the study on colloidal chitin concentration affecting chitinases produced by the investigated strains are presented in Table 4. They show that chitinase activity grew along with the chitin concentration increase. *Aeromonas sp.* and *Aeromonas hydrophila* showed the greatest activity when accompanied by a 2% concentration, while *Aeromonas salmonicida* - at 1.5%. Table 4 also shows that *Aeromonas sp.* displayed their greatest specific activity at 1.0% chitin concentration, and *Aeromonas hydrophila* - at as little as 0.5 %.

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

Name of strain	Colloidal chitin [%]				
	0.5	1.0	1.5	2.0	2.5
Aeromonas sp.	0.0032* 0.0040**	0.0032 0.0046	0.0034 0.0024	0.0036 0.0012	0.0030 0.0020
Aeromonas salmonicida	0.0043 0.0010	0.0057 0.0011	0.0062 0.0012	0.0055 0.0062	0.0037 0.0048
Aeromonas hydrophila	0.0022 0.0040	0.0024 0.0036	0.0032 0.0026	0.0036 0.0024	0.0030 0.0022

Explanations:
* - total activity in µmol N - acetylglucosamine / cm^3 / h,
* * - specific activity in µmol N - acetylglucosamine / mg enzyme proteins / h.

The study on incubation time affecting chitinase activity presented in Figs. 1 and 2 gave evidence on the strains showing an increase in chitinolytic activity with incubation time growth. No activity was noted after a 48 h incubation time. The maximum activity of the strains was noted after 192 h of incubation. What the figures also show is the fact that the highest total and specific activity was displayed by *Aeromonas sp.* while the lowest was by *Aeromonas salmonicida.*

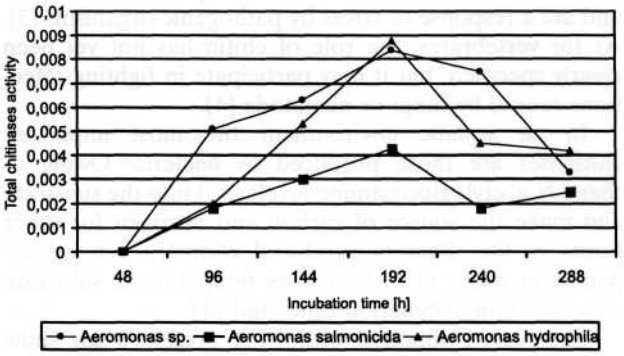

Fig. 1. Effect of incubation time on total chitinase activity (µmol N - acetylglukosamine / mL / h).

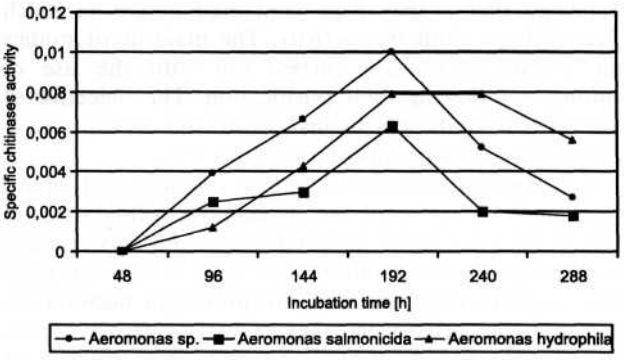

Fig. 2. Effect of incubation time on specific chitinase activity (µmol N - acetylglukosamine / mg enzyme proteins / h)

Discussion

Chitinases are enzymes that directly decompose chitin [16]. The final products of chitinase catalysis are short molecular sections of N-acetyloglucosamine, such as chitibioses, chitotrioses and chitotetroses and also small amounts of N-acetyloglucosamine [15, 22, 23].

Chitinases are produced by bacteria [14, 23], actinomycetes [18], fungi [20], yeast and plants [5]. Chitinases are also synthesised by protozoa, nematoda, snails and arthropodans. There has been recent news about chitinase activity found in human serum and leucocytes [10].

In bacteria, chitinase release N-acetyloglucosamine from chitin, which makes a source of carbon but still more of nitrogen. For plants they play a defensive role

and are a response to stress by pathogenic organisms [3]. As for vertebrates, the role of chitin has not yet been clearly specified, but it may participate in fighting infections caused by fungi or nematoda [4].

In an aquatic environment the most important chitinases are those produced by bacteria. Owing to them, N-acetyloglucosamine is released into the substrate and make the source of carbon and nitrogen for other forms of life. Bacteria-produced chitinase activity depends on many factors such as temperature, substrate concentration, incubation time, and pH.

The results obtained within the present study show their low values, which often cannot be compared to other papers. There are few articles on chitinolytic activity and their occurrence among aquatic bacteria. Bacteria living in a particular environment face various factors which may hamper their activity and even alter it. The experiments under the present study which were carried out on crude enzymes occurring in post-culture liquid indicated that it may have contained inhibitors which slowed down chitinases activity. The majority of studies on activity have been carried out with the use of chitinases following their purification. The objective of the present study was to investigate the activity under conditions almost identical to natural ones.

Laboratory analyses aiming at finding out about plankton bacteria ability to decompose colloidal chitin showed that among the isolated strains only 51 were able to decompose chitin, and among them 14 were *oiAeromonas* genus. Experimenting on chitinolytic bacteria occurrence and activity in lakes of various trophy, Donderski [8] found more abundant occurrence of chitinolytic bacteria in oligotrophic lake Jasne (31.3.%) than in eutrophic lake Jeziorak (15%). The present study also shows that *Aeromonas* bacteria were more abundant in neuston and epilimnion and they were mainly strains of *Aeromonas sp.* One may suppose that *Aeromonas* genus bacteria find better conditions for development in the microlayer and in subsurface water than at greater depths. It has been confirmed by Donderski et al. [9], who in their study worked on bacteria count in neuston and found out that in surface microlayer there occurred majority bacteria of *Aeromonas* genus.

The experiments aimed at finding out about the pH affecting the activity of chitinases produced by *Aeromonas* bacteria have produced data showing that all strains were most active decomposing chitin at pH 6.0. Chitinase activity when produced by *Serratia marcescens* came within pH 4.0 - 7.0, and in the case of *Aeromonas hydrophila H 2330* within pH 5.0 - 8.0 [5]. Studying the chitinase with *Bacillus pabuli Kl,* Frandberg and Schniirer noted their greatest activity at pH 8.0, while Bhushan and Hoondal [5] recorded optimal enzyme activity at pH 8.5 while studying activity of thermostable chitinases isolated from *Bacillus sp. BG-11.* Investigating the activity of chitinases produced by planktonic bacteria of different trophy lakes, Donderski [8] found their maximum values at pH 5.0 or 6.0.

The investigation carried out within the present study aimed at finding the dependence of *Aeromonas* bacteria produced chitinases activity on temperature, showed that the optimum temperature fell between 20° and 40°C. The experiments done by Huang, Chen and Su [14] while looking for chitinase activity at *Aeromonas sp* proved the optimal temperature to be 20-50°C. Optimal temperature at *Bacilus pabuli K1* was noted at 20-40°C [11], while Bhushan and Hoondal [5] determined it to be 50°C in the case of *Bacillus sp BG-11* chitinases.

Studying the influence of temperature on *Aeromonas* bacteria-produced chitinase activity one may come to the conclusion that chitinolytic activity of the investigated strains increased along with chitin concentration growth up to 2% in the medium, only to drop soon after. The present study has brought results showing that the strains of *Aeromonas hydrophila* and *Aeromonas sp.* displayed their greatest activity at 2% chitin concentration., whereas *Aeromonas salmonicida* - at 1.5% concentration.

Studying chitinase activity produced by *Aeromonas sp.,* Huang, Chen and Su [14] recorded optimal activity at a concentration of 1.0 - 2.0%, yet the greatest activity was displayed by the investigated strains at a concentration of 1.5% and 2.0%.

Data on the influence of incubation time on chitinase activity produced by the investigated bacteria showed that they did not display any activity after 48 h. This probably results from the fact that chitin is a high - molecular compound and organisms need a longer time to decompose it completely. In the present study, an increase in activity was recorded along with an increase in incubation time. All strains showed greatest activity following a 192 h incubation time, but this later dropped. It may be assumed that the accumulation of N-acetyloglucosamine resulting from chitin decomposition caused a slow down of further chitinasis production.

The greatest chitinolytic activity *at Aeromonas sp.* was recorded by Huang, Chen and Su [14] after 50 h incubation time. On the contrary, chitinases produced by *Bacillus pabuli K1* achieved their greatest activity after 120 h incubation time [12].

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