Characteristics of properties of carbohydrase enzyme complexes *Bacillus subtilis* strain

Rinat Mukhammadiev[1,2], *Lenar* Valiullin[1,2*], *Evgenya* Tarasova[1], *Alina* Mukhammadieva[1], *Almaz* Saifullin[1], *Ainur* Yarullin[1], *Ilsiyar* Karimullina[2], *Ilnur* Khusainov[1], and *Rishat* Mukhammadiev[1]

[1] All-Russian Research Institute of Phytopathology, Laboratory of biologically active substances, 143050 Odintsovo, Russia
[2] Federal Center for Toxicological, Radiation and Biological Safety, Laboratory of Cell Cultures and Nutrient Media, 420075 Kazan, Russia

Abstract. In this study, we have obtained two enzymes from the group of carbohydrases, cellulase and hemicellulase, secreted by vegetative cells of the B. subtilis strain, and comparatively characterized their properties in order to determine the possibility of further use of the bacillus and its metabolic products as safe biopesticides Homogeneous cellulase with a specific activity of 69.8 U/mg protein was isolated and purified electrophoretically from the culture liquid of *B. subtilis* by precipitation with ammonium sulfate, ion exchange chromatography, followed by purification by gel filtration. The molecular weight of the native cellulase molecule, determined by SDS-Na-PAGE electrophoresis and gel filtration, was established to be 34.0 kDa, and it was found that the enzyme consists of one subunit. *Bacillus* carbohydrases functioned in the temperature range (from 40 to 60 °C) and pH values (from 5.0 to 6.0). The enzyme was characterized by high stability of pH values from 3.0 to 9.0 (optimum pH 6.0) and temperatures from 20 to 75 ° C (temperature optimum from 53 to 59 ° C), respectively. A comparative study of the properties of cellulase and hemicellulase from the producer B. subtilis revealed differences in their structure and physicochemical properties: cellulase of the strain was a more thermostable and alkali-resistant protein. These results open up prospects for further study of the *B. subtilis* strain and its metabolic products - cellulolytic and hemicellulolytic enzymes, for potential use as an environmentally friendly means for the control of phytopathogens of agricultural crops.

1 Introduction

In the scientific literature there are quite a lot of domestic and foreign works that are devoted to microorganisms that synthesize a huge number of different biologically active substances [1, 2]. Of particular interest to researchers in this area are bacteria with the ability to produce extracellular hydrolytic enzymes [3, 4]. To date, about two hundred such enzymes have been isolated and characterized from various organisms, the mechanism of action of which is based

* Corresponding author: tanirtashir@mail.ru

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

on catalysis of the reaction of cleavage of intramolecular bonds of the substrate with the participation of water [5, 6]. Their unique ability to hydrolyze complex organic substances into simpler constituent parts (monomers) [7] distinguishes hydrolases from other enzymes and makes these proteins invaluable candidates for solving various agricultural problems.

Among the hydrolytic enzymes' carbohydrases, cellulases and hemicellulases, produced mainly by bacteria and microscopic fungi, are the subject of active research [8, 9]. Cellulases and hemicellulases are widely used in various industries [9, 10], including agriculture. The ability of cellulase and hemicellulase to break down components of the cell walls of phytopathogens and participate in their degradation opens up promising prospects for the use of these enzymes as environmentally friendly biopesticides [11, 12]. The use of microorganisms themselves, producers of carbohydrases, together with extracellular cellulolytic and hemicellulolytic enzymes also opens up new opportunities for increasing the efficiency of plant protection from phytopathogens [12, 13]. From the standpoint of optimal biotechnological properties and safety, representatives of the genus *Bacillus* are the most promising in this aspect [3, 6]. Unlike chemical pesticides, carbohydrases act by a fundamentally different mechanism and their use does not lead to the emergence of resistance in pathogenic microorganisms [12]. It should also be noted that for the industrial use of producer enzymes it is necessary to know the features of its physical, chemical and biological properties, which require the production of a homogeneous protein [14]. Obtaining enzymes in a homogeneous state will allow us to draw conclusions about the structure, physicochemical and biological properties, as well as the potential for their use in agriculture.

The purpose of this work is to obtain and characterize cellulolytic enzymes secreted by *B. subtilis* cells and compare them with the properties of hemicellulolytic (xylolytic) enzymes of the producer.

2 Materials and Methods

2.1 Objects of research

The object of the study is the *B. subtilis* 9 strain [14], a producer of enzyme complexes of carbohydrases (cellulolytic and hemicellulolytic enzymes), obtained from the collection of microorganism cultures of the laboratory of feed additives of the Federal Center for Toxicological, Radiation and Biological Safety.

2.2 Cultivation conditions

The culture liquid of the bacillary carbohydrase producer was obtained using a liquid nutrient medium containing enzyme synthesis inducers [14] at a temperature of 37–38 °C under stirring conditions (200 rpm) for 48 hours. After cultivation, the producer's culture liquid was separated from the bacterial biomass by filtering the culture through a sterile nylon cloth and centrifuging at 10 thousand g (15 min, 4 °C). The culture liquid was used to assess hydrolase activity and further purify the target enzymes.

2.3 Isolation and purification of enzymes from culture fluid

Initially, cellulases were isolated by fractionating the proteins of the enzyme-containing supernatant with crystalline $(NH_4)_2SO_4$ in concentrations of 30-80% (v/v) for 24 hours at 4 °C [14, 15]. The sediment was collected by centrifugation at 10 thousand g for 20 min at 4 °C and suspended in a minimum amount of 50 mM Tris-HCl buffer (pH 7.0). Precipitated

proteins were dialyzed using a membrane with a pore size of 12-14 kDa (Orange Scientific, Belgium) against the same buffer with stirring (1 day, 4 °C).
Cellulases were purified from the total proteins using sequential chromatography on DEAE-Sepharose and Sephadex G-100 sorbent columns. Before applying to the column, the dialyzed sample was filtered using sterile Millex tips (0.22 μm pore diameter, Merck Millipore, USA) to release residual substances. Elution of bound enzymes from the ion-exchange sorbent was carried out with a linear concentration gradient $(NH_4)_2SO_4$ 0→1 M in Tris-HCl buffer (pH 7.0), flow rate - 3 ml/min; elution from the gel filtration column - with the specified buffer solution, flow rate - 0.5 ml/min. Fractions in which hydrolase activity was detected were collected and used in further experimental studies. After each stage of isolation and purification of carbohydrases, samples were taken to determine hemicellulase [14] and cellulase activity [16], protein content [13], and to analyze the degree of purity of the target enzymes [15].

2.4 Establishment of molecular mass of enzymes and assessment of their physicochemical properties

Establishment of molecular weight of carbohydrases and assessment of their physicochemical properties was carried out according to the methods we previously described [14, 15].

2.5 Statistical Analyses

The experimental data were statistically processed in the Microsoft Office Excel 2013 software environment. Data on the normality of sample distribution were determined by the Shapiro-Wilk test. Tukey's post hoc test was used for comparison between groups [17]. A level of significance was $p < 0.05$. The average values (M) and standard errors (m) were calculated.

3 Results and Discussion

At the first stage of the study, experiments were carried out to study the effect of different concentrations of ammonium sulfate on the degree of precipitation of cellulases from the enzyme-containing culture liquid of *B. subtilis* strain (figure 1). The highest specific activity and yield of cellulolytic enzymes were observed at 70% saturation of the culture supernatant with ammonium sulfate.

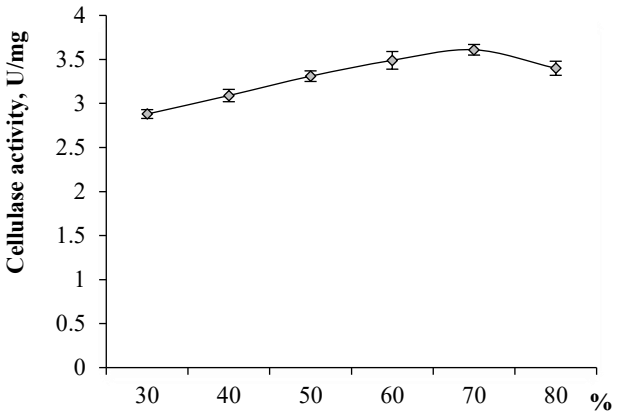

Fig. 1. Effect of ammonium sulfate concentration (%) on the degree of precipitation and specific activity of cellulolytic enzymes of the *B. subtilis* strain

Previous experiments on the isolation of the hemicellulolytic complex from the strain under study made it possible to obtain the highest specific activity and yield of xylanases at 60% saturation of the culture supernatant with ammonium sulfate [14].

In the works of a number of foreign authors, ion exchange columns and weakly alkaline pH buffers are used to purify hydrolytic enzymes from the culture liquid of bacilli [18, 19], since these conditions are the most favorable for their sorption on the surface of the sorbent. In our experiments, a column with DEAE-Sepharose sorbent was used to purify the cellulolytic enzymes of the producer *B. subtilis*. Elution of the total protein sample with a buffer solution with a salt gradient allowed the proteins to be separated into four separate protein peaks: the first peak (I) could be attributed to hemicellulolytic enzymes, and the second peak (II) to cellulolytic enzymes (figure 2a). The protein peak (II), which has cellulase activity, was selected and its protein concentration was determined to calculate the specific enzymatic activity (table 1).

Due to the fact that after using the ion exchange chromatography method we observed the presence of accompanying substances in the protein sample, its further separation was carried out using gel filtration. (figure 2 b).

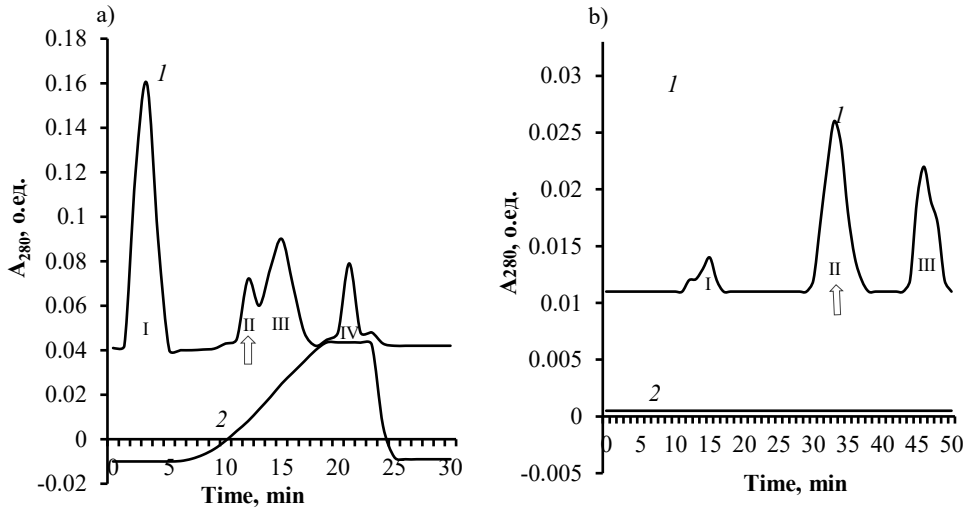

Fig. 2. Chromatogram of proteins of the *B. subtilis* strain, obtained after ion exchange chromatography (a) and gel filtration (b) on columns with DEAE-Sepharose and Sephadex G-100 sorbents, respectively: 1 – protein (peak); 2 – gradient $(NH_4)_2SO_4$. The arrows indicate the protein peak of the enzymes.

Table 1. Purification of enzymes of the cellulase complex from the culture liquid of *B. subtilis*

Purification	Total protein, mg	Total EA*, U	Specific EA**, U/mg	Degree of purification	Yiel d, %
Culture fluid filtrate	27.6	79.8	2.89	1	100
$(NH_4)_2SO_4$ fractionation and dialysis	10.5	36.2	3.5	1.2	45.4
Ion exchange chromatography	0.8	19.0	23.5	8.1	23.8
Gel filtration chromatography	0.1	7.7	69.8	24.5	10.6

* Total enzymatic activity = EA × volume (ml);
** Specific enzyme activity = total EA/protein (mg)

A study of the cellulase activity of the resulting protein peaks after gel filtration chromatography showed that the second peak (II) could be attributed to cellulolytic enzymes. The specific activity of the purified enzymes of the cellulase complex was (69.8±3.1) U/mg (table 1). Purification using gel filtration made it possible to increase the specific activity of cellulolytic enzymes by 24.5 times ($p \leq 0.05$) compared to the activity in the filtrate of the culture liquid of the strain under study. A comparison of this stage of obtaining cellulolytic and hemicellulolytic enzymes showed a difference in the chromatographic spectra of proteins, and the protein sample with hemicellulase was distinguished by a relatively high protein content (0.59 ± 0.02 mg) and more pronounced enzymatic activity (248.9 ± 7.3 U/mg) [14] than the sample with cellulase. Further detection of one bright band in the electropherogram obtained by one-dimensional electrophoresis indicated the homogeneity of the drug (figure 3a).

Isolation and purification of enzymes of the cellulase complex from the culture liquid of *B. subtilis* made it possible to study its physicochemical properties and structure. Estimation of the molecular weight of the purified enzyme by electrophoresis in 12.5% PAGE with SDS-Na made it possible to establish that the resulting cellulase has a molecular weight of (34±1.5) kDa (figure 3a). The molecular weight of the native enzyme, determined by gel filtration

chromatography, was determined within the same limits (Fig. 3b). The monomeric structure of the protein under study is evidenced by data obtained by electrophoresis under denaturing conditions and gel filtration chromatography. The molecular weight of B. subtilis cellulase purified by us was close in weight to exocellulase (23.0 kDa), which was produced by a similar species of bacterium [18], which was also characterized by a single-subunit protein structure. In our previous work [14], we found that hemicellulase from the culture liquid was a monomer (23.0 kDa) and consisted of one subunit.

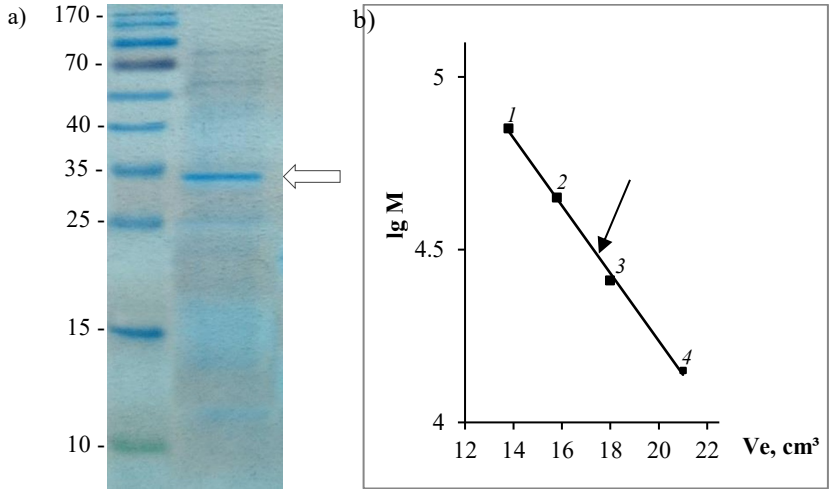

Fig. 3. Molecular mass of *B. subtilis* cellulase: a) electropherogram of the resulting protein preparation, developed with Coomassie R-250 dye: 1 - protein markers of molecular mass, 2 - purified enzyme; b) calibration curve of the linear dependence of the logarithm of the molecular protein mass (lg M) from the volume of its elution from the column (Ve): 1 - bovine serum albumin (70.0 kDa), 2 - ovalbumin (45.0 kDa), 3 - α-chymotrypsin (26.0 kDa), 4 - lysozyme (14.0 kDa); the volume of cellulase elution is indicated by an arrow

The main physicochemical characteristics of hydrolases are their resistance to temperature and pH values, which gives an idea of the optimal conditions for the manifestation of enzymatic activity. In this regard, experiments were carried out to study the influence of these factors on the activity of cellulase of the *B. subtilis* strain (figure 4).

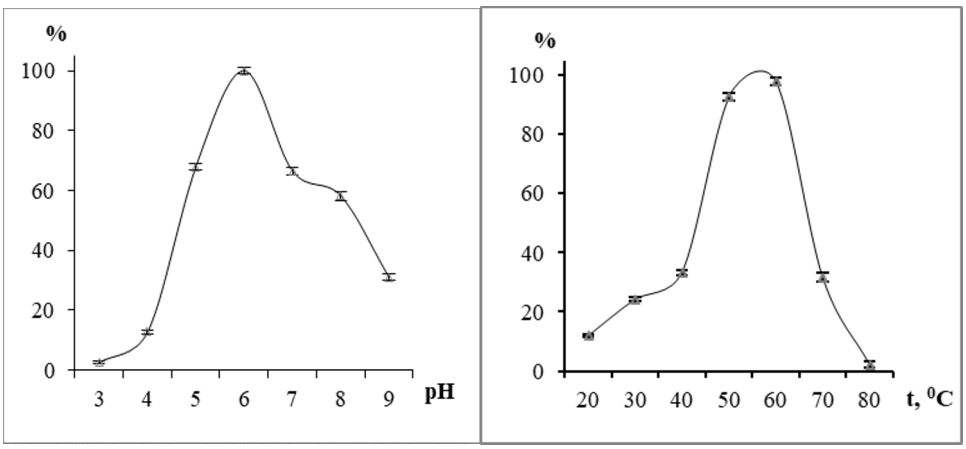

Fig. 4. Effect of pH (a) and temperature (b) on the activity (%) of cellulolytic enzymes of *B. subtilis*

The pH optimum and temperature of action of the cellulolytic enzymes of the *B. subtilis* strain, like many other bacterial hydrolases of this group, were 6.0 units and 58 °C, respectively. At values of these parameters below or above the optimal values, a significant drop in the level of enzyme activity or complete inactivation of enzymes was noted. In the course of previous studies, we discovered the maximum activity of hemicellulolytic enzymes at a pH of 5.0 and a temperature of 50 °C.

The results of experiments on the comparative characterization of the obtained enzyme complexes of carbohydrases based on the *B. subtilis* strain are presented in table 2.

Table 2. Basic physicochemical properties of enzyme complexes of carbohydrases based on *B. subtilis* strain

Enzyme	Molecular mass, kDa	Number of subunits	Optimum	
			pH	T, °C
Cellulase	34.0 ± 1.5	1	6.0 ± 0.3	56.0 ± 2.6
Hemicellulase	23.0 ± 1.0	1	5.0 ± 0.2	49.0 ± 2.1

From this we can conclude that, despite the fact that the compared carbohydrases were low molecular weight and monomeric proteins, the molecular weights of cellulase (34.0 kDa) and hemicellulase (23.0 kDa) were different. The monomeric structure of the protein with one subunit was characteristic of carbohydrases from the bacteria *Bacillus subtilis* YJ1 [18], *Lysinibacillus fusiformis* TB7 [19], and *Bacillus coagulans* ST-6 [20]. The enzymes were characterized by stability of pH values from 3.0 to 9.0 and temperatures from 20 to 75 °C. In general, the temperature and pH optimum values we established for the activity of purified carbohydrases from the bacteria under study are in agreement with the corresponding values for these groups of bacillary enzymes, which are described in the literature [16, 18].

4 Conclusions

Thus, the *B. subtilis* strain was successfully used to obtain enzyme complexes of carbohydrases (cellulolytic and hemicellulolytic enzymes). Multi-stage purification, which included fractionation with ammonium sulfate and various chromatographic methods, allowed us to obtain electrophoretically homogeneous cellulase, which is a monomeric protein with a molecular weight of (34.0 ± 1.5) kDa, from the culture liquid of B. subtilis. The optimal conditions for the action of the resulting producer cellulase were in slightly acidic pH values (pH 6.0 ± 0.3) and in the moderate temperature range (56.0 ± 2.6 °C).

Based on the results obtained, a comparative description of schemes for the production of enzyme complexes of carbohydrases was carried out. In both schemes, anion exchange and gel filtration chromatography made it possible to remove ballast proteins and significantly increase the level of specific enzyme activity. However, differences between the two enzymes were observed in the chromatographic spectra of the proteins obtained after gel filtration. Further comparative study of the properties of cellulase and hemicellulase from the *B. subtilis* strain also revealed differences in their structure and physicochemical properties. The high physicochemical characteristics of the enzyme complexes of carbohydrases of the B. subtilis strain provide grounds for the prospects of cellulolytic and hemicellulolytic enzymes of the producer for use in solving various agricultural problems, including as environmentally friendly preparations for the control of phytopathogens of agricultural crops.

Acknowledgements

The study was supported by the by the Russian Science Foundation under grant № 23-26-00161.

References

1. A. Rani. K.C Saini. F. Bast. S. Mehariya. S.K Bhatia. R. Lavecchia. A. Zuorro. Molecul. **26**. 1142 (2021)
2. D.C. Amaning. P.A. Minkah D.I. Osei K.B. Amankwah S.O. Somuah. Antibiotic. **11**. 285 (2022)
3. I. Danilova. M. Sharipova. Front. Microbiol. **11**. 1782 (2020)
4. L.R. Valiullin. R.S. Mukhammadiev. R S. Mukhammadiev. A.S. Saifullin. A.I. Samsonov. V.G. Gumerov. A.I. Yarullin. M.G. Baryshev. Bio Web Conf. **105**. 06007 (2024)
5. E. Shukla. A.D. Bendre. S.M. Gaikwad. In Hydrolases: IntechOpen. London M. (2022)
6. S. Vojnovic. I. Aleksic. T. Ilic-Tomic. M. Stevanovic. J. Nikodinovic-Runic. Appl. Microbiol. Biotechnol. **108**. 185 (2024)
7. B.S. Velázquez-De. E.M. Hernández-Domínguez. M. Villa-García. G. DíazGodínez. V. Mandujano-Gonzalez. B. Mendoza-Mendoza. J. Álvarez-Cervantes. Catal. **11**. 851 (2021)
8. E.V. Kostylevaa. A.S. Seredaa. I.A. Velikoretskayaa. A.M. Aisinaa. N.V. Tsurikovaa. E.A. Rubtsovab. A.D. Satrutdinovb. A.P. Sinitsynb. Appl. Microbiol. Biotechnol. **57**. 68 (2021)
9. A. Kiribayeva. D. Silayev. A. Abdullayeva. Y. Ramankulov. B. Khassenov. Eurasian J. Appl. Biotech. **1**. 24 (2022)
10. A.P. Sinitsyna. O.A. Sinitsyna. I.N. Zorova. A.M.P Rozhkovaa. Appl. Microbiol. Biotechnol. **56**. 551 (2020)
11. L.R. Valiullin. V.Y. Titova. E.V. Skvortsov. R.S. Muhammadiev. S.Z. Validov. V.Y. Rud. V.V. Davydov. A.P. Glinushkin. IOP Conf. Ser.: Earth Environ. Sci. **663**. 012005 (2021)
12. S.R. Roohallah. V. Masoumeh. H. Mohadeseh. A.B. Essaid. Front. Biosci. *(Landmark Ed.)* **29**. 105 (2024)
13. R. Mukhammadiev. E. Skvortsov. L. Valiullin. A. Cheremisin. S. Zavriev. A. Gerner. R. Mukhammadiev IOP Conf. Ser.: Earth Environ. Sci. **578**. 012009X (2020)
14. L.R. Valiullin. R.S. Muhammadiev. R.S. Muhammadiev. V.I Egorov. A.P. Glinushkin Ach. Sci. Tech. Agro-Indust. Compl. **35**. 66 (2021)
15. R.S. Mukhammadiev. R.S. Mukhammadiev. E.V. Skvortsov. L.R. Valiullin. A.P. Glinushkin. T.V. Bagaeva. Appl. Microbiol. Biotechnol. **57**. 206 (2021)
16. M. Irfan. M. Nadeem. Q. Syed. Protein Pept. Lett. **20**. 1225 (2013)
17. A. Akhmedov. R. Gamirov. Yu. Panina. E. Sokolova. Yu. Leonteva. E. Tarasova. R. Potekhina. I. Fitsev. D. Shurpik. I. Stoikov. Org. Biomol. Chem. **21**. 4863 (2023)
18. L.J. Yin. H.H. Lin. Z.R. Xiao J. Mar. Sci. Technol. **18**. 466 (2010)
19. S.O. Omisore. T.B. Fabunmi. A.O. Ayodeji. O.O. Olaniyi. D.J. Arotupin Heliyon **14**. 11106 (2022)

20. A. Suhaimi. R. Abidin. F. Yusof. A. Roslan. A. Sipat. K. Yusoff. J. Mol. Biol. Biotech. **17**. 27 (2023)