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Cellolytic Activity of Bacteria of the *Bacillus* Genus Isolated from the Soil of Zailiskiy Alatau Slopes

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***Abstract*—**This study was conducted for the investigation of number of cellulolytic bacteria and their ability in decomposition. Seven samples surface soil were collected on cellulose Zailiskii Alatau slopes. Cellulolitic activity of new strains of *Bacillus*, isolated from soil is determined. Isolated cellulose degrading bacteria were screened for determination of the highest cellulose activity by quantitative assay using Congo red, gravimetric assay and colorimetric DNS method trough of the determination of the parameters of sugar reduction. Strains are assigned to: *B.subtilis, B.licheniformis*, *B. cereus and, B. megaterium*. Bacillus strains consisting of several different types of cellulases have broad substrate specificity of cellulase complexes formed by them. Cellulolitic bacteria were recorded to have highest cellulase activity and selected for optimization of cellulase enzyme production.

***Keywords*—**Cellulose-degrading bacteria, cellulase complex, foothills soil, screening.

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I. Introduction

CELLULOLITIC microorganisms play important role in the biosphere by reducing complex polymer cellulose into various economically important products like monomeric sugars, microbial biomass proteins, compost [1]. Microorganisms bring about of the cellulose degradation occurring in nature [2]. Decomposition of cellulose bearing compounds in soils is useful and beneficial biological process that different microorganisms (bacteria and fungi) are involved in [3]. The maximum rate of decomposition occurred by mesopholic microorganisms in aerobic condition [4]. Aerobic bacteria produced numerous individuals and extra-cellular enzymes with binding modules for different cellulose conformations.

Degradation of cellulose in aerobic conditions can be carried out by Eubacteria relating to different taxonomic groups: actinomycetes, myxobacteria, some members of the genus *Pseudomonas and Cellulomonas* [5]-[8]. It is supposed that aerobic bacteria from the genus *Bacillus* have larger perspectives [9]-[11].

These microorganisms, responsible for cellulose decomposition bring about enzymatic hydrolysis of the complex polymer, that is, the enzymes system which involves a group of different enzymes, is collectively known as cellulase. Enzymatic decomposition of cellulose is carried out by using cellulase complex and consists of several stages. Initially the endo-b-1,4-glucanase breaks glycosidic linkages within the cellulose chain that leads to the formation of fragments of relatively large free ends. Then, exo-b-1,4-glucanase catalyses the cleavage of the chain from the end of the cellobiose disaccharide. And, finally, the latter is hydrolyzed to glucose by using b-glucosidase [12].

Of great interest is the specificity of hydrolytic enzymes capable of degrading various cellulosic plant wastes and making them suitable for usage in food applications.

A key feature characterizing the cellulase complex of microorganisms that provides the conversion is ability to deep sugar frication of cellulose and degradation of substrates, in other words, its sugarlytic activity [13]. Therefore, research of scientists and specialists have been directed mainly at seeking for enzyme preparations and their producers, effectively carrying out the hydrolysis of cellulose to glucose, which have already been successfully found among many species of fungi and bacteria [4], [11], [14]. However, only a small number of them are able to synthesize extracellular cellulase and hydrolyze high structural crystalline cellulose.

This was the basis for this study, which aims to examine the possible utilization of cellulose degrading bacteria in foothills soil for highest cellulose activity. This purpose was achieved through different steps: isolation of cellulose degrading Bacillus strains from foothills soil; selection of the potent isolate producing cellulase activity; and determine their spectrum of microbial cellulases.

II. Materials and Methods

Samples collection: Seven surface complex samples from soil were collected on Zailiskiy Alatau slopes. Pre-sterilized screw cap glass vials of 15 ml capacity were used for collection purpose. Randomly selected 10 g soil were scrapped with scapula and collected in separate vials and these were brought to laboratory for isolation of cellulose degrading bacteria. Simples were sieved (2mm) and stored field moist in polyethylene bags at 50 C until analyzed.

Soil subsamples were analyzed for organic carbon, particle size distribution and pH in 1:1 soil to water suspension [3].

Isolation of cellulose degrading bacteria (CDB). Cellulolytic bacteria were isolated from soil samples.

The enrichment cultures method with selective Hutchinson salt solution of (in g / l): Sodium citrate - 1.29; $(NH_4)_2HPO_4$ - 4,75; KH_2PO_4 - 9,6: $MgSO_4$ - 0,18) was used.

In this work were used two media variants:

1. The Hutchinson agar medium, poured into Petri dishes with filter paper circles (as cellulose source). The lumps of soil were placed on it. The development of microorganisms was considered by hydrolysis zone of the filter paper (after 21 days of incubation). From this zone

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the sample by loop was taken and transferred to fresh nutrient broth containing 0,1% sodium carboxymethyl cellulose (Na-CMC) substrate as carbon source. After overnight incubation at 300C, a 10 ml portion of supernatant fluid were serially diluted up to 107, and then the 103, 104 and 105 dilutions were plated into the mineral salt medium (100 ml) with Na-CMC (0,1%) as carbon source. Plates were incubated at 300C and examined periodically for colonies which showed areas of clearing. Representative colonies were transferred to nutrient broth, allowed to grow for 24 h, diluted and plated again on cellulose agar. This procedure was repeated until a pure culture of the cellulolytic bacteria was obtained. Spread plate technique was followed and colonies appeared on Na-CMC were amended on nutrition media. Again, they were streaked of mineral salts medium with 0,1% Na-CMC media in order to bring pure cultures of CDB.

2. The liquid medium with 0,5% Na-CMC, which soil samples were placed in. Cultivation was carried out in flasks for 15 days, after which plating on agar medium with 0,1% Na-CMC as a carbon source was performed. Streaking plate method was used to obtain pure bacterial strains.

Screening of CDB strains: Isolated cellulose degrading bacteria were screened for determination of highest cellulose activity. Quantitative assay using Congo red Dilution assay was performed where zone of clearance was observed visually by staining plate with 0,1% congo red for 15 minutes and destained with 1 M NaCl. Diameter of clear zone was measured in zone of clearance where cellulose activity occurred. Frech suspension of bacteria which were able to decompose cellulose was used for sporulation, mobility and Gram test.

Semiquantitative gravimetric assay: The essence of this method is to determine the weight loss of cellulose after incubation with bacteria cultures. Two hundred ml of mineral salt broth was prepared and 10 ml of mineral salt broth was dispensed into test tubes. Cellulose sources (filter paper “Filtrak” № 88) was added to the test tubes and sterilized. After sterilization, CDB was inoculated into test tubes, incubated at 300C for 5 to 7 days under the shaking condition.

Cellulolytic activity indicator of strains was the parameter of change of cellulose weight, expressed in percentage from the initial value.

Qualitative assay was performed by determining the amount of reducing sugars liberated by using Dinitrosalicyclic Acid (DNS) method.

To determine the activity of enzymes of cellulase complex 1 ml of culture filtrate was used. For synthesis of enzymes in the medium were added (%): Na-CMC - 0.5, cellobiose - 0.2; cotton wool - 0.5, and filter paper - 0.5. The cotton wool was transferred to hydrocellulose by its treatment with 10 N HCl at room temperature for one day and thorough washing it to neutral pH of water. The filter paper was carefully tore and milled.

These were inoculated with test cultures and incubated at 370C for 48 h. Following incubation, cellulose activity in cell-free culture filtrates were determined by colorimetric assay (with DNS as a substrate) through the determination of the amount of sugars reduction. The cellulose activity was determined by a standard graph with glucose in the concentration range from 0, 5 mg to 10 mg/ml.

Endoglucanase activity was evaluated by the increase of reducing ability of reaction mixture with 0,5% Na-CMC. The activity of C1- and C2- enzymes was measured by the amount of reducing sugars formed from the cotton wool and the filter paper, respectively. Cellobiase activity was determined by high reducing ability of the reaction mixture with 0,2% solution of cellobiose in 1,15 M phosphate buffer.

III. Results and Discussion

Some properties of soils used in these experiments are presented in Table I.

TABLE I
SAME PHYSICAL AND CHEMICAL PROPERTIES OF SOILS USED IN THIS INVESTIGATION

No of soil sample	Soil texture	Organic carbon (%)	pHs (1:1)
1	clay loam	1,36	8,5
2	clay loam	1,30	8,3
3	clay loam	0,98	8,95
4	silty clay loam	6,42	8,88
5	silty clay loam	12,68	8,3
6	sandy loam	0,75	7,1
7	sandy loam	0,88	7,3

In general pH of clay loam and silty clay loam soils is more than that of sandy loam. The pH of saturated extract of clay loam and silty clay loam were about 8,6 and sandy loam was about 7,2. While organic carbon content of silty clay loam soils was eight times more than that of clay loam and twelve times more than that of sandy loam soils.

Some characteristics of cellulololytic aerobic spore-forming bacteria in soil samples are comprised in Table II.

TABLE II
CONTENT OF AEROBIC SPORE-FORMING BACTERIA IN FOOTHILLS SOIL

Soil texture	Average number of aerobic spore-forming CDB	Gram reaction	Morphology
clay loam	34	Gram positive	spore-forming bacillus
silty clay loam	48	Gram positive	spore-forming bacillus
sandy loam	25	Gram positive	spore-forming bacillus

The number of cellulololytic aerobic spore-forming bacteria in silty clay loam and clay loam soil samples was more than that in sandy loam soil samples. Soil organic matter is utilized as carbon and energy resources of heterotrophic bacteria. Higher number of cellulolytic bacteria in silty clay loam soil can be attributed to higher organic carbon content of this soil type.

Although a large number of microorganisms are capable of degrading cellulose, only a few of these microbes produce

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significant quantities of cells- free enzymes capable of completely hydrolyzing crystalline cellulose in vitro.

Investigations were carried out with 250 strains of Bacillus genus isolated from soil. Screening of the strains consisted of two stages. At the first stage isolates obtained were plated on solid Hutchinson agar medium with 0,1% Na-CMC to confirm their ability to metabolize cellulose, and determine the level of that activity by measuring diameter of enlightenment zones of coloring around colonies grown after staining plates with Congo red dye (Table III).

TABLE III
ENDOGLUCANASE ACTIVITY OF BACTERIA ISOLATED FROM SOIL

№ of soil sample	Total amount of CDB	Number of active strains according to Na-CMC (diameter of hydrolysis zone, mm)			
		0-10	10-15	15-20	20-25
	250	30	81	99	40
1	32	4	11	12	5
2	37	5	13	12	7
3	34	0	17	15	2
4	49	7	14	22	7
5	47	2	10	18	12
6	25	8	8	0	3
7	26	4	8	20	4

According to available literature data [10], hydrolysis zone within 10 mm indicates a low enzyme activity, in the range of 10-20 mm - about its intermediate level and only zone of more than 20 mm indicates its high level of activity. It appeared that 30 isolates possess low activity, 180 - average, and only 40 - high. Thus, the first stage of screening showed that the majority of studied strains hydrolysing soluble CMC are capable to form a complex of extracellular cellulases. Strains with average activity, as well as those that showed no activity were excluded from the experimental samples .

On the second stage of screening of strains colonies around which there were zones of CMC hydrolisys of more than 20 mm, cellulolytic activity was determined by gravimetric method. Their analysis also showed that in quantitative terms enzyme composition of the cellulase complex produced by bacteria during cultivation on cellulose, varies widely.

As a result, 47 strains were selected, using which more than 18-20% mass loss filter has been noted. We can assume that this is a "resolution limit" of this method. Therefore, along with this in further studies, method of endoglucanase activity testing according to increase of reducing ability of the reaction mixture with the corresponding cellulose-containing substrate will be also used.

It should be noted, that enzymatic hydrolysis of cellulose by bacteria is carried out under the cellulase complex action consisting of different types of cellulases [12], [13]. Particular interest is the specificity of hydrolytic enzymes capable of degrading various cellulose containing plant wastes and making them usable in biotechnological applications.

While degradation of CMC different coloring of zones was observed: first pale yellow ones appeared, and in 5-6 hours around the colonies in the center of these zones blue color appeared, which may be explained by different activity of cellulolytic enzymes, various usually by molecular weight, presence of carbohydrates and amino acids. It is considered that this is extremely typical for cellulase producers [10]. According to some researchers, the manifestation of yellow zones is associated with the formation of glucose, and blue zones - pentose and / or cellooligosaccharides [11]. Thus, the results of experimental studies have shown that extracellular bacterial cellulases of studied strains were active against not only soluble cellulose derivatives such as CMC, but also hydrolyzed insoluble (filter paper) cellulose. In this regard, on the next stage of work the ability to synthesize and produce cellulase complexes by selected strains was determined.

These strains were tested at presence of endoglucanase and cellobiase activity. For this purpose a cup test with Congo red used. The results obtained allowed to divide 47 studied strains into 4 groups according to their ability to degrade different cellulose substrates:

1. Not using cellobiose - 6 strains;
2. With low cellobiase activity - 9 strains;
3. With average cellobiase activity - 7 strains;
4. Actively degrading not only CMC but cellobiose as well - 25 strains.

It should be noted that endoglucanase complex consists of three types of enzymes:

– Cx-enzyme that breaks CMC;
– C1-enzyme that breaks cotton wool;
– C2-enzyme that breaks down the filter paper [12].

As quantitative estimation of these enzymes and cellobiase activity, a method that is based on determination of reducing sugars produced by the action of cellulase complex on such substrates as Na-CMC cellobiose, cotton wool, filter paper is used. Cultivation of bacteria was performed in flasks on a shaker at 30 ° C for 72 hours in synthetic Hutchinson medium. For the synthesis of the studied enzymes as a sole carbon source were used (%): Na-CMC (carboxymethylcellulose sodium salt) - 0.5; cellobiose - 0.2, cotton wool - 0.5, and filter paper - 0.5. Content of formed reducing sugars were determined by reaction with DNS.

The results of experimental studies have shown that extracellular bacterial cellulases of studied strains showed activity not only towards soluble cellulose derivatives such as CMC, but also to hydrolyzed insoluble one (cotton wool, filter paper), and cellobiose. The activity of these enzymes is determined by amount of reducing sugars in reaction mixture and shown in Table IV.

Investigated strains hydrolyzed CMC, cotton wool, filter paper with the release of glucose in the medium in the range from 0.24 ± 0.01 to 0.56 ± 0.03 mg / ml. However, cellobiose was most intensively cleaved by the enzymes secreted on the cellulose with the release of the greatest amount of glucose - from 0.55 ± 0.02 to 1.61 ± 0.01 mg / ml.

Thus, 6 strains (13%) showed the ability to decompose only the Na-carboxymethylcellulose, 7 strains (15%) showed average cellobiase activity and 9 strains (19%) - lower. 25 strains (53%) synthesized actively not only endoglucanase but cellobiase. Endoglucanase enzyme activity of these strains

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ranges from 0.24±0.01 to 0.52±0.02 mg / ml, cellobiase - 0.55±0.02 to 1.61±0.01 mg/ml. 12 strains among all strains studied showed the highest activity: C-17, C-10, C-7, Pp-5, Zh-25, Zh-23, Zh-7, NP-9, NP-7, NP-1, P-5, P-2.

TABLE IV
ACTIVITY OF CELLULASE COMPLEX ENZYMES, PRODUCED BY BACTERIA ON DIFFERENT SUBSTRATES

Strain	Glucose, mg /ml			
	Na-CMC	Cellobiose	Filter paper	Cotton wool
P-2	0,46+0,02	1,55+0,01	0,38+0,01	0,44+0,01
P-3	0,50+0,03	1,48+0,02	0,41+0,02	0,46+0,02
P-5	0,48+0,02	1,46+0,01	0,39+0,02	0,47+0,01
P-10	0,49+0,04	0,57+0,02	0,47+0,03	0,43+0,01
NP-1	0,46+0,02	1,53+0,01	0,45+0,01	0,52+0,01
NP-7	0,48+0,02	1,34+0,03	0,43+0,03	0,43+0,03
NP-9	0,35+0,02	1,60+0,01	0,36+0,01	0,42+0,02
NP-10	0,24+0,01	1,54+0,02	0,36+0,01	0,37+0,01
Zh-5	0,37+0,02	1,47+0,01	0,46+0,01	0,25+0,01
Zh-7	0,55+0,03	1,50+0,02	0,43+0,02	0,56+0,03
Zh-21	0,44+0,03	0,59+0,02	0,52+0,02	0,50+0,02
Zh-23	0,50+0,01	1,60+0,04	0,42+0,03	0,46+0,02
Zh-25	0,55+0,02	1,61+0,01	0,46+0,02	0,56+0,01
Zh-27	0,35+0,02	0,55+0,02	0,45+0,01	0,47+0,01
Pp-3	0,28+0,02	1,15+0,04	0,44+0,01	0,44+0,03
Pp-5	0,47+0,01	1,17+0,02	0,42+0,02	0,47+0,02
C-1	0,45+0,01	0,60+0,01	0,49+0,02	0,29+0,02
C-2	0,39+0,02	0,86+0,02	0,45+0,01	0,28+0,03
C-7	0,51+0,01	1,34+0,01	0,43+0,01	0,52+0,01
C-9	0,42+0,03	1,13+0,01	0,29+0,02	0,47+0,02
C-10	0,49+0,02	1,46+0,02	0,43+0,02	0,48+0,01
C-13	0,46+0,01	0,93+0,01	0,42+0,03	0,46+0,03
C-17	0,48+0,04	1,38+0,04	0,38+0,01	0,47+0,02
C-18	0,32+0,02	1,27+0,02	0,33+0,01	0,45+0,01
CL-11	0,45+0,02	1,12±0,01	0,28±0,03	0,46±0,03

TABLE V
CONTENTS OF INDIVIDUAL CELLULASES IN THE ENZYMATIC COMPLEX OF BACTERIA

Strain	Reducing sugars (mg/ml)			
	C_x-enzyme	Cellobiase	C_2-enzyme	C_1-enzyme
P-2	0,46+0,02	1,55±0,01	0,38±0,01	0,44±0,01
P-5	0,48+0,02	1,46±0,01	0,39+0,02	0,47±0,01
NP-1	0,46+0,02	1,53±0,01	0,45±0,01	0,52+0,01
NP-7	0,48+0,02	1,34±0,03	0,43±0,03	0,43±0,03
NP-9	0,35+0,02	1,60±0,01	0,36±0,01	0,42+0,02
Zh-7	0,55±0,03	1,50+0,02	0,43±0,02	0,56±0,03
Zh-23	0,50±0,01	1,60±0,04	0,42±0,03	0,46±0,02
Zh-25	0,55+0,02	1,61±0,01	0,46+0,02	0,56±0,01
Pp-5	0,47±0,01	1,17+0,02	0,42±0,02	0,47±0,02
C-7	0,51±0,01	1,34±0,01	0,43±0,01	0,52±0,01
C-10	0,49+0,02	1,46+0,02	0,43+0,02	0,48±0,01
C-17	0,48±0,04	1,38±0,04	0,38±0,01	0,47+0,02

Spectrum of cellulase enzyme complex of these strains was identified (Table V).

The ratio of individual cellulases in produced complex of studied strains was different (Table VI).

For a unit there is accepted the least of absolute content value of glucose formed at decomposition of cellulose by each strain separately.

TABLE VI
THE RATIO OF INDIVIDUAL CELLULASES IN THE ENZYMATIC COMPLEX OF BACTERIA

Strain	C_x: Cb: C_2: C_1
P-2	
P-5	1,2: 4 : 1 : 1,2
NP-1	1,2: 3,7: 1 : 1,2
NP-7	1 : 3,4: 1 : 1,2
NP-9	1,1: 3,1: 1 : 1
Zh-7	1 : 4,6: 1 : 1,2
Zh-23	1,3 : 3,5: 1 : 1,3
Zh-25	1,2: 3,8: 1: 1
Pp-5	1 ,2 : 3,5: 1: 1,2
C-7	1,1 : 2,8 :1: 1,1
C-10	1 ,2 : 3,1: 1: 1,2
C-17	1,1 : 3,4: 1: 1,1
	1,3: 3,6: 1: 1,2

The activity of cellobiase and C2-enzyme of all selected bacterial strains were practically identical. As for the ratio of activity of two other enzymes of cellulase complex, the strains NP-1 and NP-9 mostly produced C1-enzyme but the strains NP-7, Zh-25, C-17 - Cx enzyme. For R-2, R-5, C-10, C-7, P-5, Zh-23, Zh-7 strains the ratio of the greatest parameter of C1 and Cx enzymes activity on C1 and Cx enzymes is typical.

Thus, the studied strains present broad substrate specificity they form. Probably, great importance in joint application has not only high activity of individual enzymes, but also their relationship.

The identification of 12 new strains selected by screening was carried out in accordance with the classical requirements. All strains were assigned to the *Bacillus* genus according to their identification based on the determination of cultural-morphological characteristics set (shape and method of connecting cells, tinctorial properties, sporulation, and relation to aeration). Species identification was accomplished by determining biochemical strains characterization (the assimilation of various carbon sources) [12]. Based on the study 4 strains (Pp-5, NP-9, P-5, P-2) were related to *B.subtilis*, 3- *B. licheniformis* (Zh-25, NP-7, C-17), 2 - *B.cereus* (C-10, Zh-7), 3 – *B. megaterium* (C-7, Zh-23, NP-1).

IV. CONCLUSION

According to the research of synthesis capability and release of cellulolytic enzymes in the environment, it can be concluded that the cellulase complex of each of the studied

strains of bacteria of *Bacillus* genus represents a complex system with different ratios of activities of individual components, capable of degrading glucopyranose chains of various substrates. The enzyme composition of cellulase complex produced by bacilli at cultivation on a very difficult to cleave substrste-cellulose, varies widely in accordance with the type or even strain.

Thus, *Bacillus* strains possess an important property - wide substrate specificity of cellulase complexes formed by them, consisting of several different types of celluloses, it means the ability to active degradation of various cellulose derivatives as well as the native crystalline cellulose. The results of this study of component composition and activity of cellulase complex of bacilli will contribute to significant increase in the efficiency and depth of hydrolysis of cellulose-containing materials, in particular, highly structured, the most inaccessible for the usage part of plant wastes of agriculture.

Cellulolyctic bacteria were recorded to have highest cellulase activity and selected for optimization of production of cellulase enzyme.

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