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Original scientific paper

ISOLATION, SCREENING AND CHARACTERIZATION OF CELLULOLYTIC BACTERIA FROM DIFFERENT SOIL SAMPLES FROM PELAGONIA REGION

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Abstract

Cellulose is the most abundant renewable natural product in the biosphere, so cellulolytic microorganisms are fundamental for the transformation of cellulose into sugars that are essential nutrients for various organisms and for biofuels. Additionally, since the annual production of cellulose is estimated at 4.0 x 10^7 tons, large quantities of industrial and agricultural cellulosic waste have been accumulated due to inefficient use. Different kind of soils could potentially support several microbes with potent cellulolytic enzyme activities and therefore the exploration of those communities could be useful for biotechnology as well as for ecological conservation.

The goal of this study was to conduct a survey for bacteria with cellulolytic potential, isolated from soils originating from Pelagonia Region. To select microorganisms with cellulolytic potential, qualitative cellulolytic activity was determined by culturing microorganisms in media containing cellulose as the only carbon source. After screening, fifteen colonies were isolated capable of degrading cellulase. Determination revealed the isolates were identified as *Bacillus spp., Bacillus weihenstephanensis, Pseudomonas putida* and *Staphylococcus spp*.

This study gives an overview of the potential microorganism that could be used for cellulose degradation in various biotechnological applications and for sustainable agricultural waste treatment.

Key words: *bacteria, enzymes, agricultural waste, Bacillus*

INTRODUCTION

Cellulose, which is part of the lignocellulosic biomass, is a common and easily accessible polymer in natural environment. It is an organic compound, which is the main component of plant cell walls. It creates the most resistant and stable skeleton built from cellulose fibrils, so called microfibrils and macrofibrils. Hydrogen bonds occurring between the neighbouring hydroxyl groups and Van der Waals forces ensure stabilisation of cellulose fibres and the required conformation of glucose particles. Its content in plants depends on their age, plant type and parts. For example, it is 45-50 % in leafy stems, 40-55 % in woody stems and 15-20 % in leaves (Reddy et al., 2017; Poszytek et al., 2016; Juturu et al., 2014; Eveleigh et al., 2009).

As the main source of carbon generated by photosynthesis, cellulose is a nutrient for cellulolytic microorganisms using lignocellulosic biomass. Cellulose decomposition undergoes through hydrolysis of β-1,4 glycoside bonds. However, due to stabilisation of cellulose microfibres with these bonds, cooperation of several enzymes is required for effective decomposition. Cellulose is mineralised by cellulolytic microorganisms belonging to various groups: fungi, aerobic and anaerobic bacteria (Juturu et al., 2014; Singhania, 2009; Lynd et al., 2002; Bayer, 1998).

Cellulolytic enzymes can be divided into three groups, taking into consideration their structure, enzymatic activity, specificity and

active centres. Cellulolytic enzymes are made of the so-called domains - catalytic and binding carbohydrates (CBM, Cellulose Binding Module) and have a characteristic shape active point, which determines their activity. CMB domain influences binding with cellulose surface and is located on the C-terminus of enzyme, connected with the catalytic domain at N-terminus. These enzymes (endoglucanases and exoglycanases) act in a complex way, resulting in shortening of cellulose chain, single units of cellobiose and glucose. β-glucosidase is part of cellulolytic enzymes complex. The enzyme does not attack cellulose but it decomposes cellobiose and reduces its blocking activity towards cellobiohydrolase and endoglucanase (Lugani et al., 2015; El-Hadi et al., 2014; Berlin, 2013; Horn et al., 2012; Shuangqi et al., 2011; Eveleigh et al., 2009; Singhania, 2009; Lynd et al.; 2002).

Considering this huge significance of cellulase and keeping the above in sight, the present work aimed to isolate and characterize bacterial isolates that were collected from five different soils of different areas of Pelagonia region district with high cellulase-producing ability determining by the zone size diameter around the colony degrading cellulose.

MATERIAL AND METHODS

Collection of soil samples

Soil samples were collected from different locations in Pelagonia region. Samples from five different areas namely leafy forest, winter green forest, stubble, meadow and compost were collected (Figure 1). Samples were collected 15-20 cm below the surface. Soil was collected with a sterile spatula and transported in sterile bags in a refrigerator box with ice to the laboratory of the Department of Microbiology and Microbial Biotechnology at the Faculty of Natural Sciences and Mathematics, where a laboratory was accessed within 24 hours after the material was collected (Figure 2).

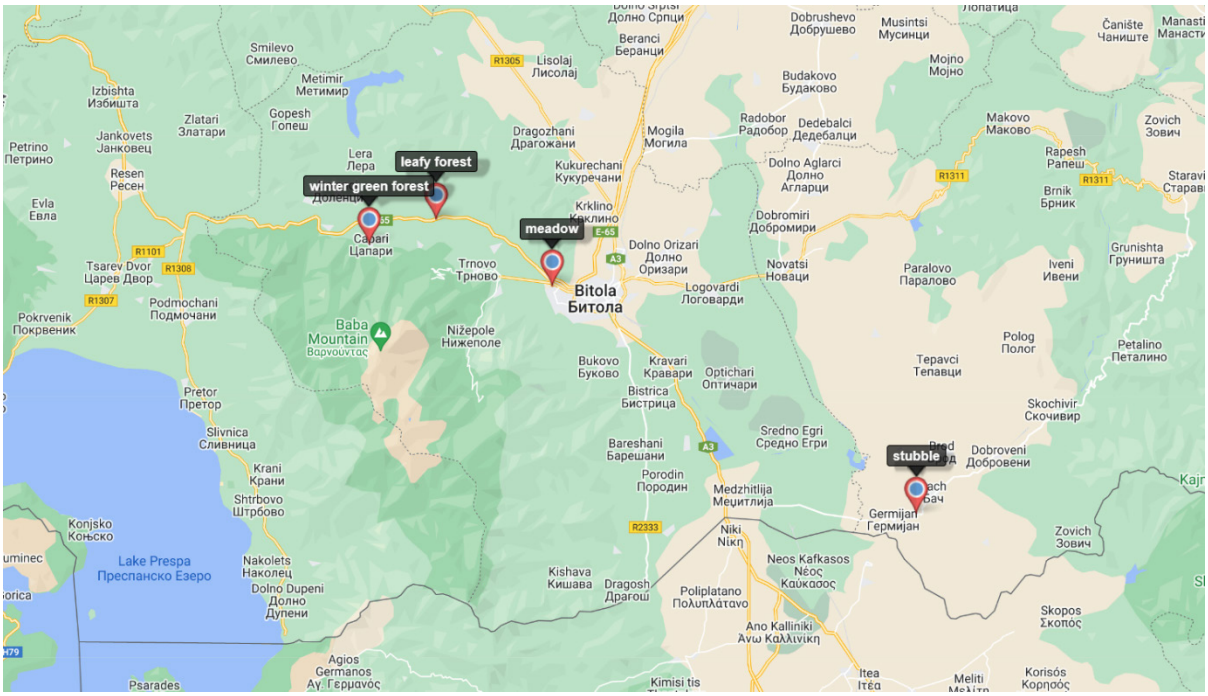

Figure 1. Locations of different areas namely leafy forest, winter green forest, stubble, meadow.

Figure 2. Soil sample collection

Screening of cellulolytic bacteria

In CMC agar plates, pure cultures of bacterial isolates were individually transferred. CMC agar plates were flooded with 1% Congo red and allowed to stand for 15 min at room temperature after 48 hours incubation. For counterstaining the plates, one molar NaCl was thoroughly applied. Around growing bacterial colonies, clear zones were appeared that was indicating cellulose hydrolysis taken place. For identification and cellulase production, the bacterial colonies were selected that having the largest clear zone (Gomashe et al., 2013) (Figure 3).

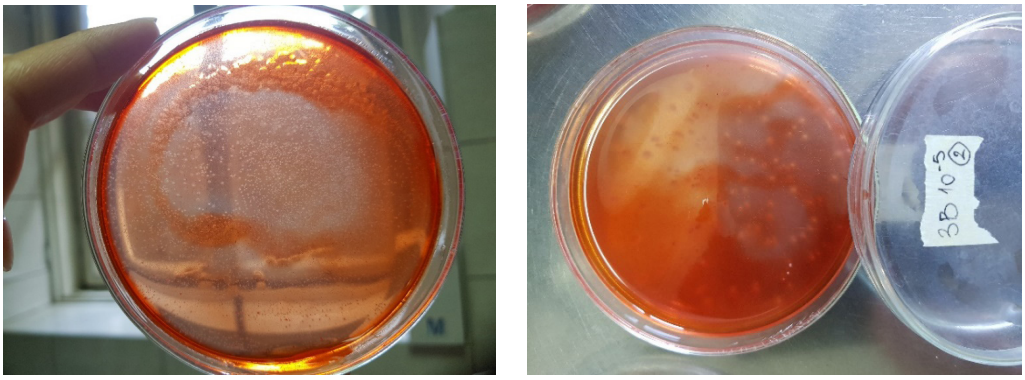

Figure 3. Clear zones around growing bacterial colonies

Secondary screening of cellulolytic bacteria

The selected cellulolytic bacteria isolates were cultured at 37°C at 150 rpm in a selective media composed of KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, and gelatine 2 g, distilled water 1 L and containing Whatman filter paper No.1 (1 × 6 cm strip, 0.05 g per 20 mL) and at pH 6.8–7.2. Broth culture after three days of incubation period was subjected to centrifugation at 5000 rpm for 15 min at 4°C. Supernatant was collected and stored as crude enzyme preparation at 4°C for further enzyme assays. Pellet recovered after centrifugation of broth culture was subjected to gravimetric analysis in order to determine the residual cellulose of filter paper (Tailliez et al., 1989).

Microscopic examination of bacterial morphology

Gram staining

Gram positive (+ve) and Gram negative (-ve) bacteria were distinguished through a differential staining method called Gram staining. Gram (+ve) cells appeared purple and Gram (-ve) cells were pink or red when the cells were examined under the light microscope. The examination of Cell morphology was also done and noted (Gomashe et al., 2013).

Biochemical Characterization

The colonies of the isolates were identified by performing various biochemical tests. Citrate utilization test, methyl red test, Voges-Proskauer (VP) test, Motility test, Catalase test, Triple sugar iron agar (TSI) test were performed, for Gram (+ve) bacteria and Gram (-ve) bacteria. All the tests were carried out according to the standard protocol as described in Bergey's Manual of systematic Bacteriology (Bergey *et al*., 1984).

Microbial identification by MALDI-TOF/ Saramis

Single cell colony from agar plate (incubated from 16-24 h) is transfer to the MALDI steel plate, Axima 384x2.8mm target plate (DE1580TA, Kratos Analytical Limited and Shimadzu Corporation). The cells are immobilized with addition of 1 µl matrix (40 mg/ml α-Cyano-4-hydroxycinnamic acid (CHCA) in water/acetonitrile/ethanol (1:1:1) with 0.03% trifluoroacetic acid) and air-dehydrated within 10–15 min at room temperature. The reference strain Escherichia *coli* DH5α is used as a standard for calibration and as reference for quality control.

The protein mass profiles (spectrum) is obtained using Linear acquisition mode of the MALDI-TOF-TOF mass spectrometer (Axima Performance, Shimadzu Corporation), with laser power of 56 V, frequency of 50 Hz and data acquisition range from 2 000 to 20 000 Da. Peak list obtained in form of ASCII file is transferred directly into the SARAMIS software, where the pattern is compared against the SARAMIS database and subsequently the microorganism is identified up to species level.

RESULTS AND DISCUSSION

Isolation and Screening of Cellulase Producing Bacteria

A total of 15 positive isolates of cellulase producing bacteria were obtained from five different soil samples. Out of these, 15 isolates showed maximum zone of clearance after staining with Congo red dye. A list of all the isolates along with area of clearance zone is given in Table 1. As shown in Table 1, isolates from the soil of winter green forest were coded as 1A 1, 1A 7, 1A 8 and isolates from the soil of leafy forest were coded as 2A 1A, 2A 4A. The bacteria isolated from the soil of meadow were coded as 4A 2, 4A 4, 4A 5, 4A 7, 4B 2. The isolates from the compost were named as 5A 4, 5A 8, 5B 1, 5B 2. The only isolate from the soil of stubble was named as 3B 1. This research indicates that the soil from meadow and compost will be effective in producing cellulase enzyme by different cellulolytic bacteria.

Table 1. Various 15 isolates from five different soil samples

S. no.	Isolates code	Type of soil
1	1A 1	Winter green forest
2	1A 7	Winter green forest
3	1A 8	Winter green forest
4	2A 1A	Leafy forest
5	2A 4A	Leafy forest
6	3B 1	Stubble
7	4A 2	Meadow
8	4A 4	Meadow
9	4A 5	Meadow
10	4A 7	Meadow
11	4B 2	Meadow
12	5A 4	Compost
13	5A 8	Compost
14	5B 1	Compost
15	5B 2	Compost

Secondary screening of cellulolytic bacteria

Secondary screening for cellulase activity on filter paper was found to be highest for 2A 1A, 4A 2, 4B 2 and 5B 1 (Figure 5). A list of all the isolates along with the weight of filter paper after incubation is given in Table 2. Gravimetric analysis shows that maximum and minimum rates of filter paper degradation were 37% and 20%, respectively, estimated at third day of incubation. Figure 4 shows that 5B 1 has highest filter paper degradation rate of 37%. In a result documented by Lu et al., 2004, the data for synergetic cellulose degradation detected in four groups of mixed cultures were only 23.5%, 26.3%, 19.4%, and 24.5%, respectively. Bichet-Hebe et al., 1999, reported the rates of paper degradation ranged from 31 to 60% after 10 days for mixed bacterial populations by gravimetric procedure.

Table 2. Four isolates that showed more cellulase production on secondary screening

Isolate code	Weight of filter paper
Control filter paper	0,0414 g
2A 1A	0,0402 g
4A 2	0,0400 g
4B 2	0,0402 g
5B 1	0,0392 g

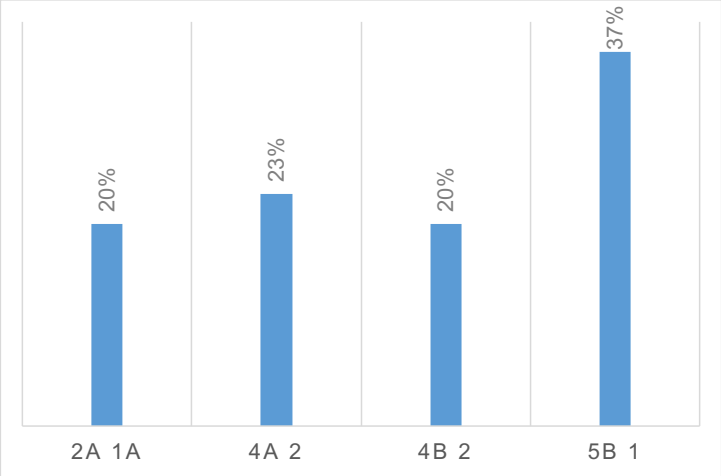

Figure 4. Percent filter paper degradation by various bacterial isolates by gravimetric method. Maximum percentage of filter paper degradation was found to be 37% by 5B 1.

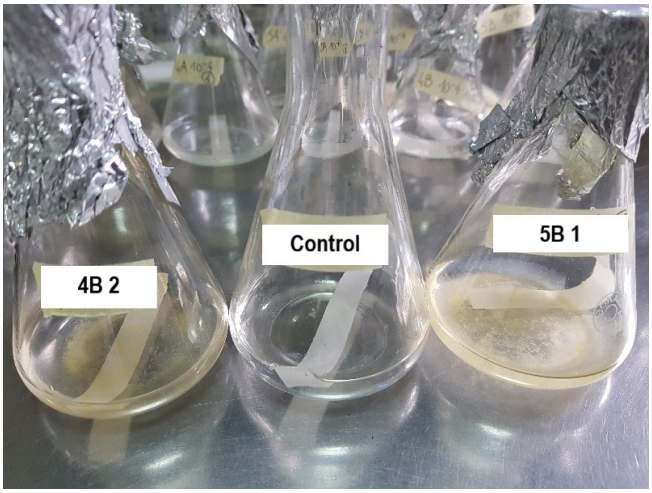

Figure 5. Filter paper degradation by isolates 4B 2 and 5B 1 cultured in selective medium supplemented with Whatman filter paper no.1 (1 × 6 cm strip × 2, 0.05 g per 20 mL) at the end of 96 hours of incubation. Flask control is the control for this experimental set up and does not show any filter paper degradation.

Microscopic Observation of the Isolates

All the isolates were examined microscopically after Gram staining to determine whether the isolates were Gram (+ve) or Gram (-ve) and to observe their arrangements. The results showed that sample 4B 2 was Gram (+ve) and samples 2A 1A, 4A 2 and 5B 1 were Gram (-ve) bacteria (Table 3). The colony characteristics of the isolates were found variable. Microscopic observation of the isolates revealed that most of them are rod shaped and motile. By Gram staining, morphological characteristics of different types of colonies of each sample were recorded. Isolates were identified by their microscopic examination and biochemical reaction.

Biochemical Identification

In this work, 4 isolates were found from 15 samples. All the isolates were subjected to different biochemical tests for their identification (Table 3).

Table 3. Morphological and biochemical test results of isolates

S. no.	Isolates code	Gram staining	Citrate utilization test	Methyl red test	Voges-Proskauer test	Motility test	Catalase test	Triple sugar iron test
1	2A 1A	- ve	- ve	- ve	- ve	+ ve	+ ve	- ve
2	4A 2	- ve	- ve	- ve	- ve	+ ve	+ ve	- ve
3	4B 2	+ ve	+ ve	- ve	- ve	+ ve	- ve	- ve
4	5B 1	- ve	- ve	+ ve	- ve	+ ve	+ ve	- ve

Microbial identification by MALDI-TOF/Saramis

Microbiological identification was performed only for 4 isolates that proved to be the most active isolates in the process of cellulose degradation in the in-vitro tests. On the basis of their morphological, biochemical characterization and microbial identification these isolates were confirmed as *Pseudomonas putida* (Isolate 2A 1A, 4A 2 and 5B 1) and *Bacillus weihenstephanensis* (Isolate 4B 2) (Table 4). According to Li et al. (2022), housefly larvae can considerably accelerate the breakdown of cellulose in silkworm faeces. In comparison to the groups (11.5%) without housefly larvae, the cellulose breakdown rate in the groups with housefly larvae reached 58.90% in 6 days. Three new cellulose-degrading bacteria, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus subtilis*, were isolated from silkworm feces substrates. Wang et al. (2022) However, Wang et al. (2022) demonstrated that exogenous cellulose-degrading bacteria (ECDB) boosted the potential activities and interactions of bacterial communities. Bacillus subtilis WF-8, B. licheniformis WF-11, B. cereus WS-1, and Streptomyces nogalater WF-10 were also identified as the ECDB. This degradation technique may be used to test the biodegradation of natural cellulosic fibres if suitable degrading bacteria are available (Lednicka et al., 2000). It has been discovered that many aerobic bacteria degrade cellulose, making them suitable candidates for use in investigations on the biodegradation of natural fibres.

Table 4. Microbial identification of isolates

Isolates code	Familiy	Genus	Species
2A 1A	Pseudomonadaceae	Pseudomonas	putida
4A 2	Pseudomonadaceae	Pseudomonas	putida
4B 2	Bacillaceae	Bacillus	weihenstephanensis
5B 1	Pseudomonadaceae	Pseudomonas	putida

CONCLUDING REMARKS

Treatment of cellulose by cellulase enzyme from different bacterial isolates has attracted the continuing interest of biotechnologists, taxonomists, enzymologists and even some industrialists in their own researches. This research indicates that the soil from meadow and compost will be effective in producing cellulase enzyme by different cellulolytic bacteria.

A total 15 isolates showed cellulase production on primary screening. Ultimately four isolates showing more cellulase production were identified as *Pseudomonas putida* and *Bacillus weihenstephanensis*.

The use of these cellulolytic bacteria as bio-inoculants can be incorporated to enhance organic matter decomposition in soil to increase soil fertility and to minimize the fertilizer application in the area of Pelagonia. These bacteria can also be applied to reduce the environmental pollution and promote sustainable agriculture.

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ИЗОЛАЦИЈА, СКРИНИНГ И КАРАКТЕРИЗАЦИЈА НА ЦЕЛУЛОЛИТИЧКИ БАКТЕРИИ ОД РАЗЛИЧНИ ПОЧВЕНИ ПРИМЕРОЦИ ОД ПЕЛАГОНИСКИОТ РЕГИОН

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Резиме

Целулозата е најзастапена, обновлива, природна материја во биосферата, а целулолитичките микроорганизми имаат фундаментално значење за трансформацијата на целулозата во шеќери кои се основни хранливи материи за различни организми, а исто така и за создавање на биогорива. Дополнително, бидејќи годишното производство на целулоза се проценува на 4,0 x 10^7 тони, се акумулира големо количество на индустриски и земјоделски целулозен отпад поради неговата неефикасна употреба. Различни видови почви можат да поддржат неколку видови микроорганизми со потенцијални целулолитички активности и затоа истражувањето на овие заедници би можело да биде корисно за биотехнологијата, како и за еколошката конзервација.

Целта на ова истражување е да се изолираат и детерминираат бактерии со целулолитички потенцијал, изолирани од почви кои потекнуваат од Пелагониискиот Регион. За да се селектираат микроорганизми со целулолитички потенцијал, квалитативната целулолитичка активност се одредува со култивирање на микроорганизмите во медиуми кои содржат целулоза како единствен извор на јаглерод. По извршениот скрининг се изолирани 15 колонии кои се способни да ја деградираат целулозата. Изолатите се детерминирани како: *Bacillus spp, Bacillus weihenstephanensis, Pseudomonas putida* и *Staphylococcus spp*.

Ова истражување дава преглед на потенцијалните микроорганизми кои би можеле да се користат за деградација на целулозата во различни биотехнолошки апликации и за одржлив третман на земјоделски отпад.

Клучни зборови: *бактерии, ензими, земјоделски отпад, Bacillus*.