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MOLECULAR IDENTIFICATION OF THE MOST COMMON CELLULASE PRODUCING-*BACILLUS* FROM FOREST SOILS

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ABSTRACT

Cellulose, an abundant polysaccharide in nature, is the best carbohydrate storage molecule for production of energy in biological systems. Bacterial and Fungal Cellulases play an important role in hydrolysis and use of cellulose. Endoglucanases hydrolyze glycosidic bonds in cellulose and convert it to glucose. The aim of this study was to isolate and identify new strains of the genus Bacillus from the forest soil, with the ability to produce cellulose. A total of 40 bacterial isolates were collected from soils of the forests located in the West of Mazandaran, Iran, using Grams iodine test and their ability in production of cellulase on carboxymethyl cellulose (CMC agar). The high cellulase-producing strains were selected by measuring the diameter of the zone. The temperature and time optimization were thereafter performed on the strains. The bacterial species were identified by 16S rDNA sequencing in the next stage, and the approximate molecular weight of the enzyme was determined by protein precipitation and SDS-PAGE.In this

study we were identified 5 strains belonging to the 5 Bacillus species *including Bacillus cereus, Bacillus subtilis, Bacillus thuringiensis, Bacillus megaterium* and *Bacillus mycoides*. The highest amounts of the enzyme were produced at 37 °C for 96 hours by all identified species. The highest level of cellulase production belonged to a *Bacillus subtilis* strain with3.8u/ml. The molecular weight of cellulase isolated from this strain was determined to be about 60 kDa. Our result showed that Bacillus species isolated from the forested areas have the high potential in cellulase production, so that these bacteria can directly be used in production of glucose syrup from vegetable fibers and a well as in other industries.

Keywords: Molecular Identification, Bacillus, Cellulases

INTRODUCTION

Bacillus species are aerobic and facultative anaerobic bacteria. They can be produced the hydrolytic enzymes which is used in many industrial processes **[1, 2]**. Cellulose is a linear polymer of glucose units with beta 1-4 glycosidic bonds and is one of the most abundant carbohydrates in nature. Cellulase enzymes are able to hydrolyze glycosidic bonds, and they can be classified based on their function in two categories: endoglucanase and exoglucanase.Beta-1, 4 endoglucanase is one of the most type of cellulase enzymes also called the CMCase **[3, 4]**. Already due to the extent of cellulosic resource and easier and less expensive enzymatic hydrolysis of cellulose than chemical hydrolysis, cellulases are the focus of research. Product of enzymatic hydrolysis of cellulose is usually reducing sugars to form glucose **[5, 6]**. In enzymatic hydrolysis of cellulose there is no environmental pollution and the corrosive actionsocan be seen chemical methods because of the low pH. A variety of bacterial and fungal cellsareable to produce cellulase enzymes for the Lignocellulose biomass hydrolysis. Due to the importance of cellulose, the purpose of this study was to isolate and identify secreting cellulose *Bacillus Spp*. with the industrialization capacity of forest soils in northern Iran.

MATERIALS AND METHODS

Isolation and Screening of Microorganisms

Approximately 50 g of soil were collected from each 35 forest areasof West Mazandaran, Iran. After serial dilution and heat-treated (at 95°C for 5 min), 10^{-5}, 10^{-6} and 10^{-7} dilutions were cultured by spread plate techniques in CMC agar media (pH 7.5) (Hi Media, India). The plates were incubated at 25ºC, 30ºC, 37 ºC, 40ºC for 1, 2, 4, 5 days **[7]**.

Screening for Cellulase-Producing *Bacillus*

For screening cellulose-producing isolated, we used of the Gram's iodine reagent (2.0 gr KI,1.0gr Iodine, 300ml distilled water)and washed the plate and viewed hydrolysis zone around each of colony **[8]**. Also to determine the relative activity of cellulase, the zone diameter of each isolate was measured.

Molecular Identification of cellulase-Producing *Bacillus*

Genomic DNA was extracted from cellulase-producing isolates using conventional methods. Then, the different isolates were identified by High Resolution Melting-Real Time PCR using 16S rDNA genes universal primers according to For ghani and colleagues **[9]**. To identify the species of each isolate, 16S rDNA gene amplification products were sequenced by the Macrogen Company (South Korean)and the sequencing results were aligned using Blast program in NCBI website.

Enzyme Activity Assay

Cellulase activity was determined by Bernfield's method using Measurement of reducing sugar **[10]**. After culturing isolates in CMC broth for 5 days of incubation at 37°C with shaking at 150 rpm, the cultured broth was centrifuged at 10000 × g for 10 min (Boeco centrifuge, Germany) for preparation of supernatant.Endo-β-1, 4-glucanase activity was determined by incubation of 1ml of 1% CMC in 100 M phosphate buffer (pH 4.8)with 1ml of supernatant at 50°C.After 30 min reaction, 2 mL of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 5 min to stop the reaction.The resulted samples were then cooled to room temperature and measured the absorbance at 540 nm (A540) **[11, 12]**. One unit ofendo-β-1, 4-glucanase activity is the amount of enzyme that could hydrolyze CMC and release 1 μg of glucose within 1 min reaction at 50°C.

Determination of Cellulase Molecular Weight

After enzyme concentration by acetone on raw supernatant, partial cellulase Molecular weight was determined using SDS-PAGE **[13]**. For electrophoresis, protein sample mixed to protein sample buffer (Bio-Rad, USA) and boiled at 100°C for 2 min. Then sample along with protein size marker (Prestained Protein Marker, Thermo Scientific, USA) were loaded on SDS-PAGE (resolving gel: 15%; stacking gel: 5%) by using a Vertical electrophoresis system (Akhtarian, Iran) for 3 h with a 120 Volt and then gel stained by R250 Coomassie Brilliant Blue (20278, Thermo Scientific, USA).

RESULT AND DISSCUSION

After culturing the soil samples of 35 different forest areas in West Mazandaran on CMC agar medium and Gram staining, we

screened 40 spore-bearing gram-positive bacilli isolates **(Figure 1)**. Among 40 isolates, 24 isolates indicated cellulose hydrolysis zone **(Figure 2)**. Also, all of isolates showed best of enzyme activity in 37ºC for 96h.

Based on the HRM-RT PCR results, 24 isolates were divided into 5 different groups. After sequencing and alignment 2 isolates of each group randomly, were identified the five group members **(Table 1)**.

Following the preparation of the standard curve using different concentrations of glucose via DNS method, was determined reduction sugar levels each of raw supernatant species by spectrophotometry **(Table 2)**. As can be seen in **Table 2**, *Bacillus subtilis* Isolate was highest cellulase activity. The molecular mass (M) of purified cellulose from *Bacillus subtilis* Isolate was determined 66 kDaby SDS-PAGE **(Figure 3)**. This MW was heavier than of other endo-glucanases (25-45 kDa) obtained from *Bacillus spp* **[13]**.

Generally cellulase enzymes to capable digest the cellulose polymer both Endo and Exo. All cellulose digests the beta 1-4 glycosidic bond.Endo-β-1,4-glucanase activity to the polymer amorphous regions of cellulose and thus they can be measured by soluble cellulosic substrates such as carboxymethyl cellulose **[14]**.

In this study, we were able to identify five different strains of *Bacillus* species from forest soils in the north of Iran with the ability to produce secretoryEndo-β-1, 4-glucanase.The primary screening method used in this study was able to identify strains producing Endo-β-1,4-glucanase through culture diluted samples in medium containing carboxymethyl cellulose as carbon source, and then stained colony with iodine. Also measurement of enzyme activity to DNS method was determined the effectiveness Cellulase activity of all five strains. *Bacillus subtilis* isolate in this study revealed the highest enzymatic activity at 37°C for 96 hours. Our study showed that among *Bacillus* species, species *Bacillus subtilis*is better candidate for cellulase enzyme production.

CONCLUSION

The present work was carried out to isolation and molecular identification of the most common cellulase-producing bacillus residing in the forest soil. Our result showed that among of the five different spore-bearing bacillus isolates, Bacillus subtilis isolate had highest enzymatic activity.

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Table 1: Alignment Results of a 380 bp Fragment From 16S rDNA Gene of the 24 Cellulases-Producing Strains

Group	Species
1	***Bacillus subtilis*** **Isolate**
2	***Bacillus cereus*****Isolate**
3	***Bacillus thurngiensis*****Isolate**
4	***Bacillus megaterium*****Isolate**
5	***Bacillus mycoides*****Isolate**

Table 2: Amount of Cellulase Activity the Five Isolated Strains

Species name	Activity (U/ml)
Bacillus cereus**Isolate**	**2.85**
Bacillus subtilis **Isolate**	**3.77**
Bacillus megaterium **Isolate**	**2.36**
Bacillus thurngiensis **Isolate**	**1.88**
Bacillus mycoides **Isolate**	**2.98**

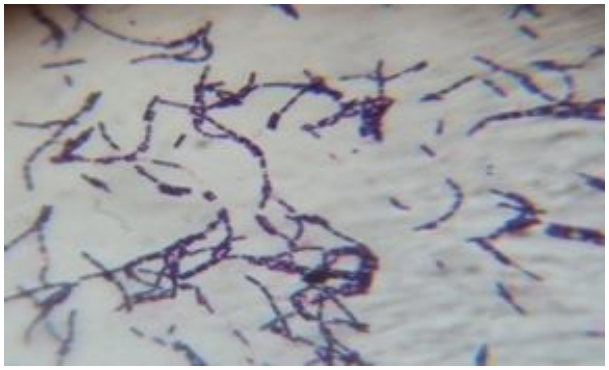

Figure 1: Isolate Gram Staining; Spore-Bearing Gram-Positive *Bacillus*

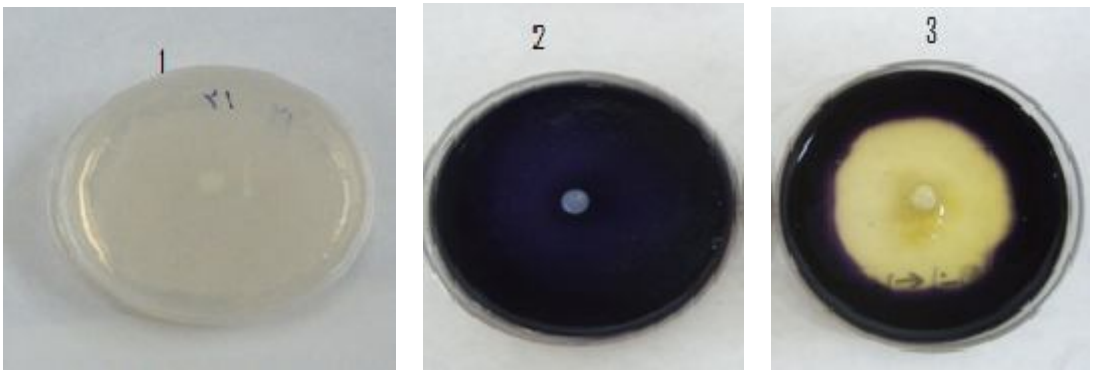

Figure 2: Primery Cellulase Assay With Grams Iodine Reagent. 1) Before Adding the Reagent to Isolate grown. 2) After Adding the Reagent to Isolate Grown, No Cellulase Production Isolate. 3) After Adding the Reagent to Isolate Grown, Cellulase Production Isolate (Hydrolysis Zone)

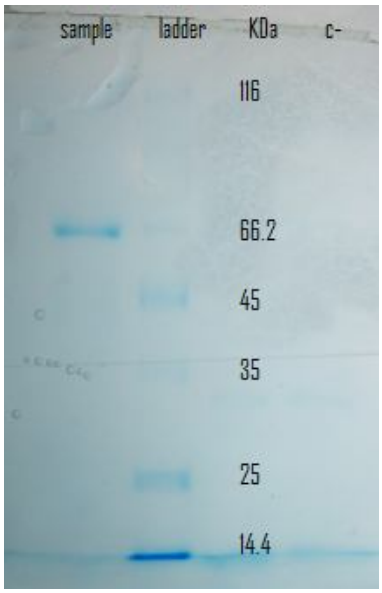

Figure 3: SDS-PAGE of Purified Cellulase From *Bacillus subtilis* Isolate. Left to right: Concentrated Supernatant CMC Medium, Protein Size Markers (Thermo Scientific, USA), Concentrated Supernatant M9 Medium