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Isolation and Characterization of Cellulose - Producing Bacteria from Local Samples of Iran

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Abstract: Bacterial cellulose is produced by several species of *Acetobacter* and because of its unique properties; it has advantages over plant cellulose. Today, this biopolymer has numerous applications at many industrial fields. In this research, samples from different sources of Iran were collected and cultivated in an enrichment liquid medium for growth of acetic acid bacteria. After purification the pellicle-forming bacteria were approved for cellulose production by a modified enzymatic hydrolysis approach. Based on cellulose hydrolysis standard curve, 8 isolates were able to produce cellulose. 4B-2 isolate was selected as the best cellulose producer by 11.98 g/l. Morphological, physiological and biochemical characteristics of the strain was examined. The 16srRNA gene was amplified using universal eubacteria specific primers. According to sequencing of approximately 1500 bp of 16S rRNA gene and comparing the sequences with existing data in GenBank and BIBI databases, 4B-2 isolate was belonging to the *Acetobacter spp. Acetobacter* 4B-2 is comparable with other industrial strain and its production is higher than some of them, so it can be used at industrial applications.

Key words: Bacterial Cellulose · Enzymatic hydrolysis · 16S rDNA sequencing

INTRODUCTION

Cellulose is an extracellular polysaccharide which is synthesized as long β-1,4 glucan chains that form the microfibrils commonly observed in cellulose-synthesizing organisms [1]. The bacterial cellulose produced by certain species of *Acetobacter* has unique properties [1] and its use is promising in many industrial fields [2]. This biopolymer is produced by some species of acetic acid bacteria which occur mainly in sugary, acidic and alcoholic habitats [3].

There is a lack of knowledge on a screening method of cellulose-producing bacteria and their diversity in Iran. This paper dealt with the screening of these bacteria from different local materials collected from various regions of Iran by an enzymatic hydrolysis method. Since molecular methods are commonly used in bacterial phylogenetic studies and identification of bacterial species [4-5], 1500 bp of 16S rRNA gene of all isolates was amplified, sequenced and compared with the sequences available in gene databases.

MATERIALS AND METHODS

Isolation and Screening of Cellulose-Producing Bacteria: Different local samples including vinegar, beer, fruits, fruit juices, alcoholic beverages and flowers were collected from all over Iran (Khuzestan, Isfahan, Lorestan, Fars and Tehran Provinces, Iran) in the period from April to August, 2008. 100 µl of liquid samples or 1 g of the solid samples were cultured into 200 ml of an enrichment medium (%10 D-glucose, 1% yeast extract, 5% ethanol, amphotericin B 2500 unit/ml, pH 5) and incubated at 28ºC, at 150 rpm for 3-5 days. 100 µl of the cell cultures were streaked on plates containing 5% glucose, 0.5% yeast extract and 1.5% agar. The plates were incubated for 2 days at 28ºC. After the primary isolation, all isolates were incubated in Hestrin-Schramm medium (2% glucose, 0.5% peptone, 0.5% yeast extract, disodium phosphate 0.27%, 0.115% citric acid; pH adjusted to 6.0) at 30°C for 2 days [2]. The medium was modified by simply replacing D-glucose with other carbon sources.

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Enzymatic Hydrolysis of Cellulose: Enzymatic hydrolysis of cellulose was applied for confirmation of cellulose production. After purification of the produced pellicles of each isolates [1, 6], the pellets were washed by resuspention in excess of distilled water followed by resuspension in 200 ml NaAc buffer, pH 5 and stored at 5°C. Enzymatic hydrolysis experiments were carried out in tubes by incubating 0.5 ml of pellicle treated suspension (10mg/ml) in 0.05 M NaAc buffer, pH 5.0 with 200µl of each of the following enzymes; 0.5U (1, 1, 4 -β- D-endoglucanase from *Trichoderma reesei* (EC 3.2.1.4.) (Sigma, Aldrich) and 1, 1, 4- β- D- exoglucanase from *Aspergillus niger* (EC 3. 2. 1. 91) (Serva). The reaction was initiated by addition of enzyme followed by vortex mixing for 10s and stopped at selected times by addition of 1ml of DNS (Dinitrosalicylic acid) reagent. The cellulose residue was collected by centrifugation (13000g, 5 min) and the concentration of the reducing sugars in the suspernatant was determined by the anthrone/sulfuric acid method using d-glucose as standard and absorption measurements at 546 nm (Perkin-elmer junior model 35 spectrophotometer).

Phenotypic Characterization: To identify the isolated bacteria; morphological, physiological and biochemical characteristics of the pure isolates were examined according to the laboratory exercises in microbiology [7] and second edition of Bergey's Manual of Determinative Bacteriology [8].

Phylogenic Characterization

***In vitro* Amplification of the 16s RDNA Gene and Sequencing:** The isolates were cultured in nutrient broth medium and incubated at 30°C for 2 days. The DNA extraction was performed using AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea). The 16S rRNA gene was amplified as described by Weisburg *et al.* [9]. The PCR conditions were started by initial denaturing for 3 min at 95°C and followed by 30 cycles of 1 min at 94°C, 30 S at 62°C and 2 min at 72°C, plus one additional cycle with a final 20-min chain elongation Amplicons were purified and sequenced outside of the laboratory (Tag Copen Hagen A/S). Sequencing was done in two directions.

16S rDNA Sequence Analysis: The PCR products were purified by using High Pure PCR Product Purification kit (Roche Applied Science, Germany). 16S rDNA sequence of the representative isolate from fermented vinegar (4B-2), which was selected as the highest cellulose producing strain was determined by direct sequencing of the PCR-amplified rDNA gene products. The 16S rDNA sequence of isolates was aligned along with selected sequences obtained from the Gen Bank databases. This result was compared with the data obtained from BIBI (Bio Informatics' Bacteria Identification) database [10].

RESULTS

Screening of Bacterial Cellulose Producing Bacteria According to Enzymatic Hydrolysis: Forty two acetic acid bacteria were isolated from the Iranian sources. According to the enzymatic hydrolysis, the purified pellicles of 8 isolates were detected as bacterial cellulose. Based on cellulose hydrolysis standard curve, the final absorption of all the samples were between 0.230-0.260 nm. Approximately 80% of the purified bacterial cellulose of all the isolates was hydrolyzed after 4 h (Figure 1). The results showed the strain isolated from tomato produced the second highest amount of cellulose. The 4B-2 isolate from traditional vinegar produced the highest amount of cellulose by 11.98 g/l from 1.5% sucrose as carbon source [11]. Fruits seem to be poor sources of cellulose-producing bacteria.

Phenotypic Characterization: Morphological and biochemical examinations revealed that 12 out of 44 isolates were members of the genus *Acetobacter*.

16S rDNA Sequencing and Bacterial Identification: Regarding the results of Taxonomic report of BLAST program available online at the National Center for Biotechnology Information, NCBI and the Bio Informatics' Bacteria Identification (BIBI) the 4B-2 isolate was affiliated to the genus *Acetobacter* with 98% similarity to specific species.

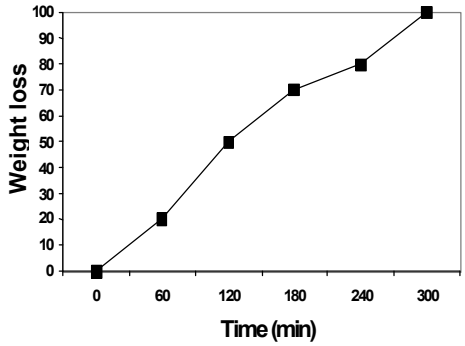

Fig. 1: Hydrolysis of cellulose pellicle produced by strain 4B-2. Alkali treated pellicle (10 mg/ml) was incubated with endo- and exo- glucanase enzyme mixture that contain 1U.

DISCUSSION

Tsuchida *et al*. [12] have described the screening of cellulose producing bacteria by using Buffered Schramm & Hestrin's medium with acid acetic (0.2%), ethanol (0.5%) and cyclohexymide solution (0.01%). According to their research, the most efficient isolates were obtained from fruit samples. In this study, the modified enrichment medium of Seearunruangchai *et al*. [13] and Schwartz *et al*. [14] was used for isolation. Since the enzymatic hydrolysis is a very accurate method for exclusive detection of homopolymer material like cellulose, this method was used for the first time for detection of bacterial cellulose and screening of cellulose producing bacteria. This method was a mixed and modified method of Väljamäe *et al*. [15] and Yongchao *et al*. [16].

We used 16S rDNA sequencing to confirm the biochemical identification of our isolates. According to16S rDNA sequencing, 4B-2 isolate belonged to the *Acetobacter* sp.

In conclusion, *Acetobacter* 4B-2 is comparable with other industrial strains and its production was higher than some of them, so this local strain can be used for improvement of cellulose production in an agitated culture for industrial application.

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