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ISOLATION AND MOULOCULAR IDENTIFICATION OF *KOMAGATAEIBACTER NATAICOLA* CELLULOSE PRODUCING BACTERIA

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Abstract

This study aims to isolate the bacterial strain *Acetobacter* to produce bacterial cellulose, 23 pure isolates were obtained from rotten apple, pears, vinegar and mother of vinegar by using H-S medium, The isolates were examined morphologically and bio-chemically and proven to belong to *Komagataeibacter sp* and for their cellulose production ability, the highest producing isolate was *Komagataeibacter* Sp. AB3 with a total weight of cellulose 10.2 gm / liter. This isolates was identified by 16 rRNA gene sequencing as *Komagataeibacter nataicola strain RZS01* (accuracy of 100%), which was matched with the sequence of *Acetobacter sp*. ITDI2.1 and recorded in Gene bank under the Accession Number of CP019875.1.

Key words : Bacterial cellulose, *Komagataeibacter nataicola*, 16S rRNA.

Introduction

Acetic acid bacteria (AAB) is a group of bacteria belonging to the family *Acetobacteraceae* The bacteria is widely found on flowers, fruits, and rotten food AAB can act as fermentative organisms A feature that is remarkable of these strains and their ability to survive under extreme environments, like low pH value and high-sugar concentrations, which makes AAB suitable for a lot of industrial applications and this group is obligate aerobes and have the ability to convert ethanol to acetic acid Andrés *et al.,* (2013) Adachi O *et al.,* (2007).

Currently, AAB includes 14 genera, namely *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Komagataeibacter*, *Granulibacter*, *Asaia*, *Acidomonas*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Tanticharoenia*, *Ameyamaea* and *Neokomagataea* Generally Species belonging to the *Gluconacetobacter xylinus group are classified into the novel genus Komagataeibacter in* 2013 Duardo *et al.,* (2016). *Komagataeibacter* species are able to produce and secrete exopolysaccharides (EPS), especially bacterial cellulose BC has been the focus of the research duo to its excellent properties, like high degree of crystallinity, high purity, predominant three-dimensional structure, and superior biocompatibility. Giving to those qualities, BC is used commercially in the development and production of health food, tissue scaffolds, flexible electrode and acoustic speakers Picheth *et al.,* (2017); Wang *et al* (2017); Lee *et al.,* (2014) Gullo *et al.,* (2018) Yamada *et al.,* (2012).

Several attempts have been made to isolate *Gluconacetobacter* sp. from fruits, beverage, flowers, vinegar and fermented foods. Although many more bacteria such as *Agrobacterium*, *Pseudomonas*, *Rhizobium* and *Sarcina* can synthesize bacterial cellulose, *Gluconacetobacter* sp. are proven to have the highest cellulose production capacity Duardo *et al* (2016). The genus Komagataeibacter is characterized morphologically by lack motility and no flagellation, and physiologically/biochemically chemically by growth in the presence of 0.35 % (w/v) acetic acid, production of acetic acid from ethanol, no forming of 2, 5-diketo-Dgluconate from D-glucose and by the oxidation of acetate and lactate to carbon dioxide and water. Strains belonging to the genus Komagataeibacter were mostly isolated from acetous or sugary materials such as vinegar, kombucha,

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fruit and fruit juice Yamada *et al.,* (2012) Adachi O *et al.,* (2007).

Materials and Methods

Bacteria isolated sources

The BC producing microorganism was isolated from locally from different natural sources from local markets such as rotten apples, pears, vinegar and mother of vinegar and soil from Baghdad University College of agriculture.

Culture Media

YGC Medium

The YGC media was prepared as described by the manufacturing company. The media was autoclaved at 121°C and 15 pound/inch^2 for 15 min as described in Son *et al.,* (2001).

Peptone water

It was prepared by dissolving 0.1 gm of peptone in 1000 ml distilled water and was distributed in tubes 9 ml each was autoclaved at 121°C and 15 pound/inch^2 for 15 min**.**

Methodology

Decimal dilutions for the samples were made with peptone water and mixed well with vortex. Isolation was conducted with pour plate method by pouring 1 ml of the proper decimal to sterilized petri dish using YGC Medium, and the dishes were incubated in the incubator at 30c for 48 hours.

The growing colonies were chosen individually and had a clear zone.

Screening isolates for cellulose production

Cellulose producing mediums H-S medium

H-S mediums were prepared as described by the manufacturing company and Ph was adjusted to 6 then using a 250 ml flasks 50ml of the medium was distributed into each flask. The media was autoclaved at 121°C and 15 pound/inch^2 for 15 min.

Methodology

Sterilized H-S Medium was inoculated with the isolates by adding 5ml of each isolates to the 50ml H-S medium and inoculates were incubated in 30 c for 7 days.

Biochemical examination of the isolates

Catalase Test, Forming Acids and Gasses from Glucose Consumption, Oxidize Ethanol to CO_2 and H_2O, Motion Test and Pigments Production test were carried as in (6).

Preservation of isolates

Preservation mediums were prepared according to Wajdi (1966) by dissolving the components in table 1 in 1000 ml distilled water and distributed in tubes and autoclaved at 121°C and 15 pound/inch^2 for 15 min and tubes were sloped to make a slant.

Table 1: Isolates preservation medium.

Weight	Component
10 gm	Glucose
30 gm	Yeast Extract
20 gm	Agar

Quantitative screening of bacterial producing isolates

Bacteria count media

YGC medium (excluding calcium carbonate).

Inoculum preparation

Inoculum was prepared by conducting decimals to the cellulose producing isolates using sterilized distilled water distributed in tubes 9 ml each, pour plate method was conducted Petri dishes were incubated at 30 c for 48 hours; colonies were counted by colony counter, and the proper dilution was chosen.

Cellulose production

5ml of the proper dilution (10^7 CFU /ml) of the bacteria isolates used to inoculate the sterilized H-S medium flask and incubated at 30 for 7 days.

Cellulose was obtained according to Son, *et al.,* (2001) by taking out the cellulose of the medium after an incubation period, washing cellulose with distilled water 3 times and shocking the cellulose in NaOH 0.5 molar and water bath at 90c for 1 hour, then bacterial cellulose was washed with distilled water few times to get rid of the NaOH effect and sustain Ph balance. Wet bacterial cellulose was weighed and weighing process was repeated after drying the cellulose at 50c until the weight was sustained.

Molecular identification

The local bacteria isolate was cultured on H-S broth at 30°C for 24 hours. One ml of the media culture was centrifuged at 13000 x g for 1 min. (the supernatant was discarded). A molecular biology kit from Wizard genomic DNA Purification kit USA was used to extract the DNA from the isolate according to the kit extraction method. DNA purification was estimated by Nano drop.

Polymerase chain reaction (PCR)

PCR was used to amplify the 16S rRNA of the rRNA gene. The forward primer 5'-AGAGTTTGATCC

Sequence	Primer
'5'-AGAGTTTGATCCTGGCTCAG-3'	Forward
5'-TACGGTTACCTTGTTACGACTT3'	Reverse

Table 3: Master mix amplification compounds.

Compounds in the master mix	Vol.(µl)
Master mix	12.3
Forward primer	1
Reveres primer	1
DNA extract	2
Nuclease free water	8.2
Total volume	25

TGGCTCAG-3' and reverse primer 5'-TACGGTTACCTTGTTACGACTT3' were used in PCR (Table 1).

The numbers of nitrogen bases were 20 in forward primer and 22 in reverse primer. The amplification was carried in 25 µl which then added to the master mix that was supplied by the Promega Company (Table 2).

The master mixture was mixed for few seconds using vortex. The tube was placed in PCR thermo cycler. The device was programmed according to table 4 and the amplification was taken place to amplify the extracted DNA. By the end of the reaction time, 5 µl of 16S rRNA amplifying product was withdrawn for electrophoresis assy.

The Electrophoresis of DNA amplification products on agarose gel

The PCR products were loaded on 1.1% agarose gel using a horizontal electrophoresis. 5 µl of PCR products and 2 µl of loading buffer for every 5 µl of DNA extracts. The mixture was mixed well and located into the sample gel wells. The sample was subjected to electrophoresis assay for 1 hour, 100v/mAmp for 75min.in order to initiate the movement toward negative and positive poles. The DNA bands were detected by using UV light Tran's illuminator device.

Table 4: PCR program.

Cycles	Time	Temperature	Step	No.
1 cycle	5 min.	95R"C	Initial Denaturation	1
	30 sec.	95R"C	Denaturation	2
35 cycles	45 sec.	55R"C	Annealing	3
	1min .	72R"C	Extension-1	4
1 cycle	7min.	72R"C	Extension Final	5
	10 min	10R"C	Holding	6

Results and Discussion

23 bacterial isolates were obtained locally from different sources included rotten apples and pears, vinegar and, other of vinegar table 5 using YGC medium as isolation medium and it was observed that the bacterial isolates had formed clear zones around them duo to calcium carbonate digestion made by bacteria growth and the bacteria ability to produce acetic acid. For pure colonies a repeated transfer of the colonies forming the cleat zone to the YGC medium until pure colonies were obtained (Table 5).

Ability of bacterial isolates to produce cellulose

Table 6 shows 7 isolates were distinguished for their abilities to produce bacterial cellulose using H-S Medium mas is seen in table and other isolates showed negative ability to produce cellulose as it has seen in table the number of the cellulose producing isolates that were obtained from rotten pears apples, vinegar and mother of vinegar were 1,2,1,3. Gu and Catchmark, (2012) indicated that cellulose production by genus Komagataeibacter and Gluconoacetobacter by forming a membrane on the surface of the liquid medium after growth in static culture or balls or irregular lumps in agitated culture.

Initial diagnosis of bacterial isolates producing cellulose

Initial diagnosis of bacterial isolates were based on culture and morphological tests and some biochemical tests. In table 7 colonies are shown in a creamy color and with a convex round shape and a smooth surface, bacterial cells a positive gram stained reaction. The bacterial cells had a rod short shape with individual or pairs or a cluster of cells.

Table 5: *Komagataeibacter* isolation sources and number of clear zone forming isolates and isolation percentage.

Isolation	Number of Clear Zone Formed Isolation	Isolation	Percentage
Rotten Apples	5	AE1,AE2,AE3,AE4,AE5	21.73
Rotten Apricot	4	AB1,AB2,AB3,AB4	17.39
Vinegar	8	AR1,AR2,AR3,AR4,AR5,AR6,AR7,AR8	34.78
Mother of Vinegar	6	AJ1,AJ2,AJ3,AJ4,AJ5,AJ6	26.06

Table 6: Isolates ability to produce cellulose.

Isolate ability for cellulose production	Isolation cod
+	AR5
-	AE1,AE2,AE3,AE4
+	AB1,AB3
-	AB2, AB4
+	AR5
-	AR1,AR2,AR3,AR4, AR6,AR7,AR8
+	AJ1, AJ3, AJ6
-	AJ2, AJ4,AJ5

As it has shown in table that the chemical test shows the bacteria belong to the genus *Gluconacetobacter* it was catalase, acid forming and gas forming positive duo to glucose consumption and ethanol oxidation to CO_2 and H_2O but gave a negative result for the movement and pigment production.

Quantitative screening of cellulose producing isolates

Table 8 shows the difference between isolates ability to produce cellulose showing isolate AB3 surpasses all the others by cellulose production reaching 10.2 gm / liter using H-S medium for production**.**

Molecular Identification

DNA extraction

The DNA was extracted from isolate AB3 the most efficient cellulose producing isolate and the purity of DNA was examined by Nano Drop with a purity of 1.9 which is adequate for Polymerase Chain Reaction (PCR) process. Green (6) reported that the PCR did not need a large quantity of DNA which may instead produce unlimited amplifying products. On other hand, an adequate quantity of DNA may reduce the accuracy.

Table 8: Quantitve screening of bacterial cellulose isolates.

Isolation Number	Produced Cellulose g/L
AR5	154
AB1	158
AB3	204
AR5	190
AJ1	160
AJ3	168
AJ6	182

Polymerase Chain Reaction (PCR)

A PCR for the local AB3 for 16S rRNA gene was carried out. The electrophoresis on 1% agarose show (by using U.V detector), that there was a clear band represents the genes amplifications (Fig. 3). The molecular size of gene amplification band was over 1300 bp comparing with ladder size at the same conditions, which refers to the prime binding to the complete sequence in DNA pattern.

Sequence analysis of amplification products

The sequence of nitrogen bases, of the 16S-r RNA gene, for the local bacterial isolate AB3 was studied by sending the amplification products to the Korean company Macrogen (Fig. 4). The nitrogen bases sequence (1400 base-pair) which was taken from the local isolate sequence (of the present study) is shown in (Fig. 5). The BLAST program has been used to find out the similarity of gene with the bank information (NCBI). The results showed that there is a match between isolation and 100%

Tabke 7: Culture and morphological characteristics and biochemical test.

Culturing Properties	Notes
Colonies' Pigment	Creamy White
Colonies Shape	Round
Colonies Height	Convexed
Colony's Outer Perimeter shape	Smooth and Uniform
Morphological Properties	Notes
Reactivity of Cells to Gram Stain	Negative
Shape of Cells	Short Bacillus
Cells' Group	Shows as individuals, pairs or chains of cells
Chemo Biological Tests	Notes
Catalase Test	Positive
Forming Acids and Gasses from Glucose Consumption	Positive
Oxidize Ethanol to CO_2 And H_2O	Positive
Motion Test	Negative
Pigments Production	Negative

with global isolation sequences global Registered on the NCBI website and registered in the United States of America Which belong to *Komagataeibacter nataicola* strain RZS01.

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