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Molecular Identification and Characterization of Some Gluconacetobacter Strains Isolated from Some Egyptian Fruits.

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ABSTRACT

Three local isolates of biocellulose (BC)-producing *Gluconacetobacter* strains were isolated from rotten fruits out of 103 isolates. The representative isolates were selected from each of the 20 isolation sources (rotten fruits). Morphological (using Scanning of electronic microscope), physiological and biochemical characteristics examinations were done to identify the local isolates on classical level. 16S rRNA gene sequence analysis test were done were done to identify the local isolates too but on molecular genetic level. Comparison between the reference strain *Gluconacetobacter xylinus* NBRC 3288) A) and the three local strains; *Gluconacetobacter hansenii* strain UAC09 (A2), *Gluconacetobacter sacchari* strain DSM12717 (A3) and *Gluconacetobacter xylinus* E25 (A4) on Morphological, physiological and biochemical characteristics were done. Three accession numbers submission under registration in the EMBL database nucleotide; ID 1742271 for *Gluconacetobacter hansenii strain* UAC09, ID 1742272 for Gluconacetobacter sacchari strain DSM12717 (A3) and ID 1742273 for *Gluconacetobacter xylinus* E25 (A4).

Keywords: Biocellulose-producing bacteria, *Gluconacetobacter*, 16S rRNA. Morphological, physiological and biochemical characteristics.

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INTRODUCTION

Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the Oomycetes. Some species of bacteria secrete it to form biofilms. Cellulose is the most common organic compound on Earth. About 33 percent of all plant matter is cellulose (the cellulose content of cotton is 90 percent and that of wood is 40-50 percent) [1].

The cellulose-producing bacteria include the genera Acetobacter, Rhizobium, Agrobacterium, and Sarcina .Its most efficient producers are Gram-negative, acetic acid bacteria *Acetobacter xylinum* (reclassified as *Gluconacetobacter xylinus* [2]. The gram-negative *Acetobacter xylinum* has been the subject of the most intensive inquiry, which permits a detailed biochemical description of cellulose biogenesis in this organism. This knowledge is rapidly appreciating in value as interest moves to other organisms as well as to novel biotechnological applications [3].

Bacterial cellulose or biocellulose (BC) is extracellular cellulose naturally produced by many species of microorganisms. BC has been considered as an alternative biomaterial since it possesses superior qualities to other cellulose. BC exhibits many unique characteristics which are different from those of other plant celluloses, such as high water-holding capacity (over 100 times of its weight), high degree of crystallinity, great elasticity, high tensile strength, non-drying state, excellent biocompatibility and high purity, because it is free from other contaminating components such as hemicelluloses, lignin or waxy aromatic substances [4-6]. These distinct physical and mechanical qualities have made BC more attractive than other materials well known as alternative materials in food, biomedical and other industries. For food applications, BC has been used as raw materials for Natta de coco, which is a popular dessert in Philippines and other countries, and a dietary drink called Kombucha or Manchurian tea. In biomedical applications, BC is ideal for wound-healing dressing, micro blood vessels and scaffolds for tissue engineering of cartilage and bone [7-8]. In other applications, BC has potential for producing banknote and Bible paper, high performance speaker diaphragms, electronic paper displays, flexible display screens, paint thickeners, make-up pads and anti-aging cosmetics [5-9-11].

Members of the genus *Gluconacetobacter* are divided into two groups, the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group [12]. The former group consists of the non-nitrogen fixers such as *G. liquefaciens* and *G. sacchari* and the nitrogen fixers such as *G. diazotrophicus*, *G. azotocaptans* and *G. johannae*. The latter group consists of the non-BC producers such as *G. europaeus*, and the BC producers such as *G. xylinus*, *G. nataicola* and *G. rhaeticus*. *G. xylinus* (formerly *Acetobacter xylinum*) are the most common species, many strains of which are high cellulose producers. These cellulose-producing bacteria are commonly found in natural sources such as flowers, vegetables, nuts, sugar cane and, in particular, rotten fruits [13-15]. Industrial production of BC using these bacteria is traditionally achieved by using a static cultivation method. BC is produced as white pellicle at the air-liquid interface of a liquid medium. However, this method requires a long cultivation time and large area while in shaking or agitated culture; non-BC producing mutants are produced [16]. Therefore, the improvement of static fermentation process, optimization of culture condition and isolation of highly effective BC-producing

strains are desirable.

This study is aimed to isolation, molecular identification of some local *Gluconacetobacter* strains isolated from some rotten Egyptian fruits. Comparison of Physiological and Biochemical Characteristics of the isolates and one reference strains of *Acetobacter* well be done .All of these strains well be sequenced using 16S rRNA test.

MATERIALS AND METHODS

Isolation of *Gluconacetobacter* Strains.

BC-producing *Gluconacetobacter* isolates in this study were isolated from 20 rotten fruits collected in Egypt using the method modified [15]. Firstly, 10 g of each rotten fruit was transferred into 90 mL of a modified Hestrin-Schramm (HS) medium in a 250-mL flask containing 2.0% D-glucose (w/v), 0.5% peptone (w/v), 0.5% yeast extract (w/v), 0.27% Na_2HPO_4 (w/v), 0.12% citric acid (w/v), 0.2% acetic acid (v/v), 0.5% ethanol (v/v) and 0.01% cycloheximide (w/v) [17]. The flask with rotten fruit and liquid medium was then incubated statically at 30^{o}C for 7 days. After incubation, the flask with white pellicle covering the surface of the liquid medium was selected. The culture broth of the selected flask was serially diluted with 0.85% NaCl (w/v) and 0.1 mL of each dilution was spread on GEY agar, which was comprised of 2.0% D-glucose, 1.0% yeast extract, 5% ethanol and 0.3% $CaCO_3$. The agar plates were incubated at 30^{o}C until colonies were formed. The colonies with a clear zone around were selected and transferred to vials containing 5 mL of HS medium and then incubated at 30^{o}C for 3-7 days. Subsequently, only the vials with white pellicle on the surface were collected for further purification. The pellicles were confirmed by boiling with 0.5N NaOH for 15 min., since they might not be cellulose.

Selection of *Gluconacetobacter* Isolates

The BC-producing isolates with the highest and the lowest yields were selected from each fruit on the basis of BC thickness, yield and appearance. A single colony of each BC-producing isolate was transferred into 5 mL of HS medium in a vial and incubated statically at 30^{o}C for 7 days. The resulting pellicle was harvested and washed three times with distilled water. Subsequently, BC appearance was observed by the naked eye and the thickness was measured. The pellicle was then purified by heating with 2% NaOH at 121^{o}C for 15 min. to remove bacterial contaminants and other residues. Finally, the purified cellulose was dried at 80^{o}C in a hot air oven to constant weight.

Identification of *Gluconacetobacter* Strains

Morphological characteristics (Electron-microscope examination)

The type strain and three local isolates were scanned using a transmission electron microscope (model JEM 1200 EX11 JEO2) (JEOL, Supplier: Blue Star Ltd.) at 25 000_ magnification. Micrographs were recorded to compare the morphological features of the type strain and isolates [18-19].

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Biochemical and Physiological characteristics

The Biochemical and physiological characteristics of the above isolates were determined [20].

16S rRNA gene sequence analysis.

16S rRNA gene sequence analysis according to the method was used [21-22]. A specific fragment for 16S rRNA gene-coding regions was amplified using PCR amplification. Two primers, 800R (5'-TACCAGGGTATCTAATCC-3'; position 800-783), 518F (5'-CCAGCAGCCGCGGTAATACG-3'; position 518-537) (Table 1). The positions in the rRNA gene fragment were based on the *Escherichia coli* numbering system (accession number V00348). A phylogenetic tree for 989 bases was constructed by the neighbour-joining method [23] using MEGA programme (version 4.0) [24] after multiple alignments of the sequences obtained with CLUSTAL W [25]. The distance matrices for the aligned sequences were calculated by the two-parameter method [26]. The bootstrap values at branching points in the phylogenetic tree were calculated with 1,000 replications. A 16S rRNA gene sequence similarity between the type strain of *Gluconacetobacter* species and an isolate was calculated for 989 bases.

Table 1: code, sequence and number of nucleotides of 16S rRNA gene sequence primers test.

Primer code	Primer sequence	No. of nucleotides
518F	CCAGCAGCCGCGGTAATACG	20
800R	TACCAGGGTATCTAATCC	18

Nucleotide Sequence Analysis

Database searches with determined sequences were conducted by using the BLASTN programs in the GenBank on web site http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences were aligned online by using BLASTN, version 2.2.21+ (on the web site http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=Blast) and the alignments were refined by visual inspection. The nucleotide sequences reported in this study under registered in the EMBL database nucleotide under accession numbers submission; ID 1742271 for *Gluconacetobacter* (A2), ID 1742272 for *Gluconacetobacter* (A3) and ID 1742273 for *Gluconacetobacter* (A4).

BC Production by *Gluconacetobacter* Strains

Bacterial cellulose production of the type strain and three local isolates were determined using the method [27].

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RESULTS AND DISCUSSION

Identification of *Gluconacetobacter* Strains

From the 20 rotten fruits collected, 103 bacterial isolates were obtained as BC-producing candidates. They were then examined for BC production using a modified HS medium. As a result, 25 isolates from 16 fruits were BC-producing bacteria. The most efficient isolates were from the apple with 3 isolates, and the last was from lady finger's banana (*Musa acuminata*) with one isolate, 20 isolates were selected as representative BC-producing strains and divided into four subgroups based on morphological, physiological, biochemical characteristics and 16S rRNA gene sequences. Colonies of the 20 isolates on HS agar plates after 48-hr growth were pale yellow, smooth, viscous, convex, dense, with circular or irregular shape and entire or undulating margin. All the isolates were Gram-negative, rod-shaped or short rod and occurred singly or in pairs. The morphological results obtained are congruent with the results obtained [28] who isolated *Gluconacetobacter* strains from Italian apple fruit.

All the BC-producing isolates showed catalase-positive reactions and growth at pH 3.0-7.0. They grew slowly at pH 3.0, 3.5 and 4.0, but the growth was better at pH 4.5-7.0. Growing in different carbon sources indicated that all the isolates could not grow on sorbitol or methanol medium but grew well on glucose or sucrose medium (data not shown). Testing for acid production in different carbon sources indicated that all the isolates produced acid from D-glucose and D-sorbitol, but 9 out of 20 isolates also produced acid from D-arabinose, L-rhamnose and L-sorbose. From the different phenotypic characteristics obtained (Table 2).

Identification of *Gluconacetobacter* Strains.

Traditional microbial identification methods.

Morphological (Electron-microscope examination) and Biochemical characteristics.

The three local isolate bacterial strains were compared with the type or reference strain in the transmission electron micrographs for size and possession of a peritrichous flagellum; they were found to very similar to the type strain. The cells of all strains studied were Gram-negative and rod-shaped, measured 05–1.0 by 1.0–3,0 mm and were non-motile (Fig 1). They were aerobic, catalase-positive and oxidase-negative. All of these strains did not produce a water-soluble brown pigment on the culture media used, oxidized acetate and lactate, produced acid from ethanol and grew well on mannitol agar and glutamate agar. They grew in the presence of 0.35 % acetic acid in AG medium and grew without acetic acid, but not at 1 or 5 % acetic acid, in AE broth.

Phenotypic characterization of the strains studied was mostly carried out as described previously [20]. Production of acid from ethanol and oxidation of ethanol were examined by clearing around colonies [29]. Growth in the presence of 0.35 % acetic acid was examined by using AG medium. However, *G. xylinus* (A) grew at 1 % acetic acid and (A2) and (A3) grew even at 5 % acetic acid. Production of 5-keto-D-gluconate from D-

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glucose varied with the strain studied. *G.xylinum* (A) strains produced acid from galactitol. *G. xylinus* (A4) produced acid from propan-1-ol. Data of other characteristics are shown in Table (2).

Morphological observations and biochemical tests (Table 2) were performed as recommended in *Bergey's Manual of Systematic Bacteriology* [30].

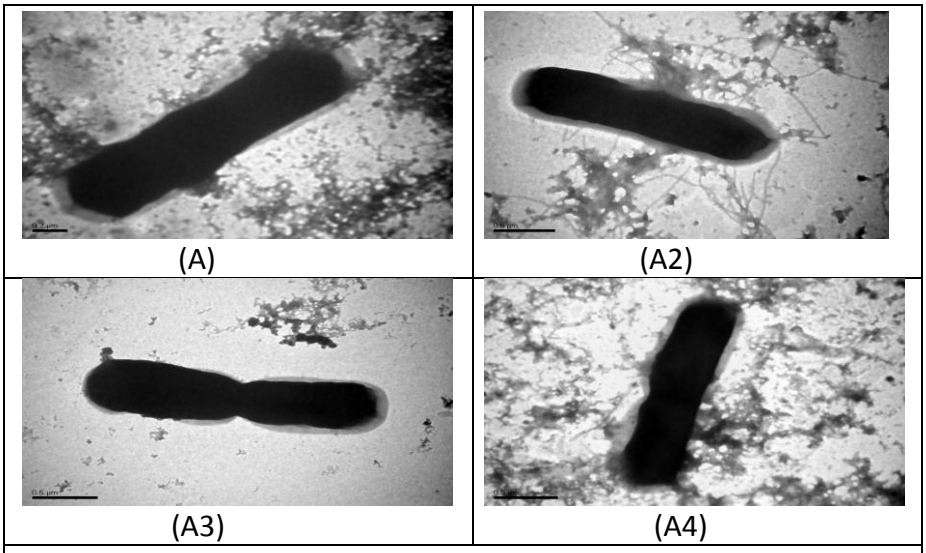

Figure 1: Scanning electron microscope image of the four Gluconacetobacter strains; The type strain (A) and three local; (A2), (A3) and (A4).

Table 2: Comparison of the biochemical characteristics of the reference strain (A) and three Gluconacetobacter strains; (A2), (A3) and (A4).

No.	Test	Strain			
		A	A2	A3	A4
1	Indole test	-	-	-	-
2	Methyl red test	+	+	+	+
3	Voges Proskauer test	-	-	-	-
4	Citrate utilization	-	-	-	-
5	Urease	-	-	-	-
6	Nitrate reduction	-	-	-	-
7	Ornithine decarboxylase	+	+	+	+
8	Production of acid from:				
	-D-glucose	+	+	+	+
	-Sucrose	+	+	+	-
	-Fructose	-	w	-	-
	-Lactose	-	-	-	- -
	-Galactose	+	+	w	+
	-Maltose	-	-	-	W
	-Mannose	+	+	+	+
	-Xylose	+	+	+	+
	- L-sorbose	-	-	-	-
	- starch	-	-	-	-
9	Catalase test	+	+	+	+
10	H_2S production	-	-	-	-
11	water-soluble brown pigment	-	-	-	-
12	BC production	+	+	+	+

+ = positive, - = negative and w = weak response.

Molecular identification (16S rRNA gene sequence test)

Using of PCR technique to detect 16S rRNA and sequencing of the resulted products can be used as specific markers for the identified bacteria and the three local isolates. Phylogenetic analysis based on 16S rRNA sequence showed high levels of sequence similarity and closely related between the type strain *G. xylinus* (model A) and *Gluconacetobacter xylinus* NBRC 3288 up to 99% with accession number 1624. At same trend, each of the three isolates models A2, A3 and A4 showed high levels of sequence similarity and closely related with *Gluconacetobacter hansenii* UAC09, *Gluconacetobacter sacchari* DSM 12717 and *Gluconacetobacter xylinus* E25 up to 96, 95 and 98% with accession numbers; 1548, 1501 and 1610, respectively (Fig 2 and 3) [31].

By comparing between our three isolates and reference bacteria which were identified at molecular level and registered in EMBL database, similarity alignment around 97% in average for that, we used the traditional microbial identification methods (morphological and biochemical tests) to confirm and identify our isolates and registered the 16S rRNA sequences as a novel sequences at molecular level [32].

Phylogenetic analysis based on 16S rRNA gene sequences shows that all the four model A, A2, A3 and A4 BC-producing isolates belong to the *Gluconacetobacter* genus (Figure 2). In the present study, the BC-producing bacterial isolates are identified as *Gluconacetobacter* as follow*;* Model (A) the reference strain was *Gluconacetobacter xylinus* NBRC 3288, Model (A2) was *Gluconacetobacter hansenii* UAC09, Model (A3) was *Gluconacetobacter sacchari* DSM 12717 and Model (A4) was *Gluconacetobacter xylinus* E25. However, it is remarkable in the present study that any strains assigned to *G. xylinus* and *Gluconacetobacter* were isolated from fruits collected in Egypt, This phenomenon is in good accord with previous work on the diversity of acetic acid bacteria in Egypt.

On the basis of 16S rDNA sequences and taxonomic characteristics, they have proposed the strain as *Gluconacetobacter* [22] have reported isolation of *Gluconacetobacter xylinus* from fruits sample and studied the factors affecting BC production.

Database searches with determined sequences were conducted by using the BLASTN programs in the GenBank on web site http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences were aligned online by using BLASTN, version 2.2.21+ (on the web site http://blast.ncbi.nlm.nih.gov/Blast.) and the alignments were refined by visual inspection. The nucleotide sequences reported in this study under registered in the EMBL database nucleotide under accession numbers submission; ID 1742271 for *Gluconacetobacter hansenii strain* UAC09 *(*A2*),* ID 1742272 for *Gluconacetobacter sacchari* strain DSM 12717 (A3) and ID 1742273 for *Gluconacetobacter xylinus* E25 (A4)*.*

BC Production by *Gluconacetobacter* Strains

The production of bacterial cellulose not varied with strains studied. Cellulose production was reported to be useful for the separation of acetic acid bacteria, particularly the reference strain *G. xylinus* in the old descriptions of acetic acid bacteria [33] but the

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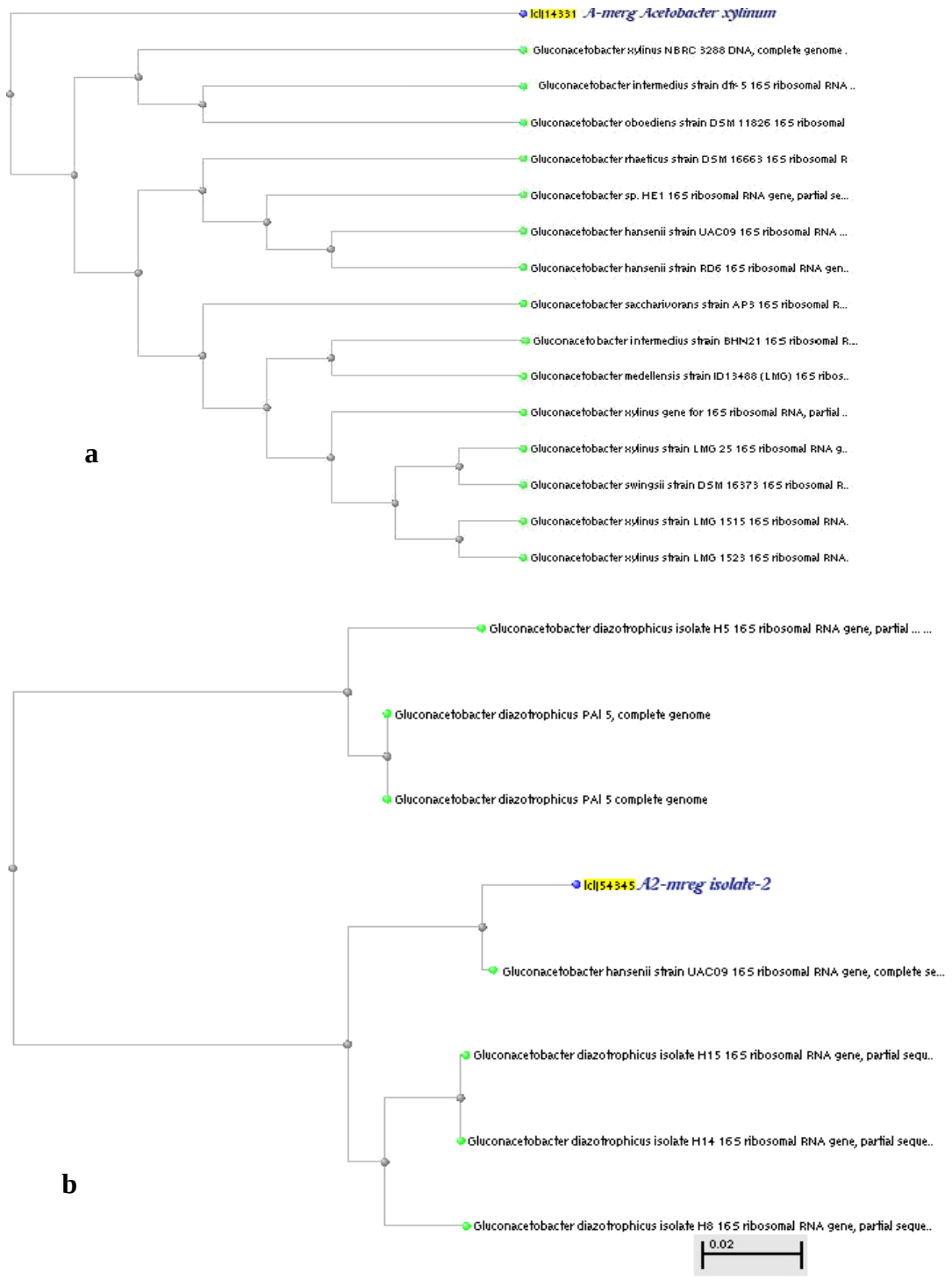

Figure 2: Phylogenetic tree based on 16S rRNA sequence analysis of the *Gluconacetobacter* strains; reference strain, *Gluconacetobacter xylinus* NBRC 3288 (A) and the local isolate strains *Gluconacetobacter hansenii* strain UAC09 (A2).

local isolate strains; of *Gluconacetobacter hansenii strain* UAC09, *Gluconacetobacter sacchari* strain DSM 12717 and *G. xylinus E25,* also produced cellulose in the present study (Table 2). Therefore, the production of cellulose is not useful for the differentiation of the species in the genus Gluconacetobacter. Pellicles produced by acetic acid bacteria do not

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always mean real cellulose and the production of real cellulose should be confirmed by boiling the pellicles with a dilute NaOH solution [34].

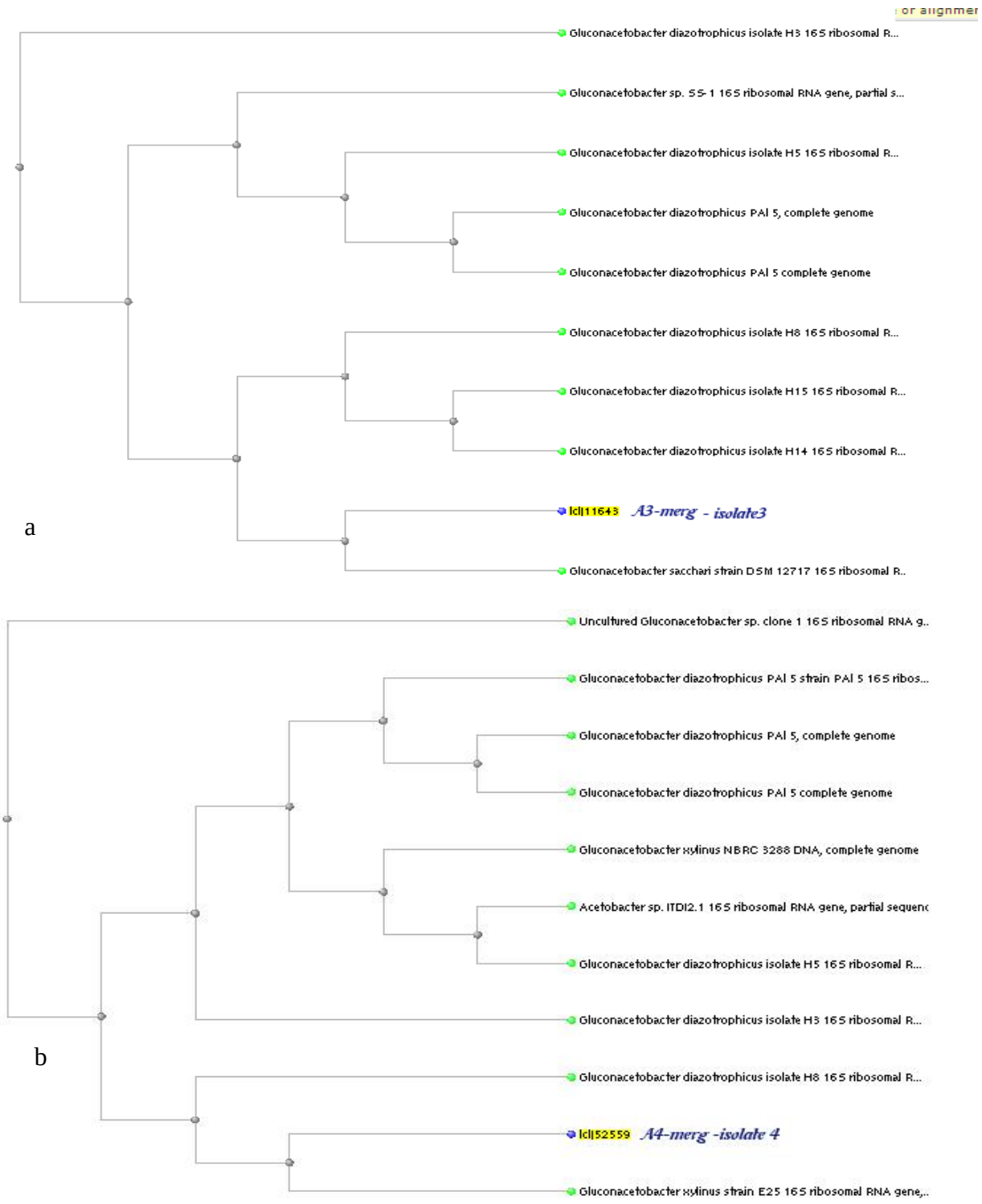

Figure 3: Phylogenetic tree based on 16S rRNA sequence analysis of the two local *Gluconacetobacter* strains; *Gluconacetobacter sacchari* strain DSM12717 (A3) and *Gluconacetobacter xylinus* E25 (A4).

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REFERENCES

[1] Klemn D, Heublein B, Fink H P, Bohn A, Cellulose: Fascinating biopolymer and sustainable raw material, Angew. Chem. Int. Ed., 2005; 44: 3358-3393.

[2] Keshk S, *Gluconacetobacter xylinus*: A New Resource for Cellulose, Egypt.J. of Biotech. 2002; 11: 305-312.

[3] Haigler C H, The functions and biogenesis of native cellulose, 1985, p. 30-83. In R. P. Nevell and S. H. Zeronian (ed.), Cellulose chemistry and its applications. Ellis Horwood Ltd., Chichester, England.

[4] Ross P, Mayer R, Benziman M, Cellulose biosynthesis and function in bacteria, *Microbiol. Mol. Biol. Rev.* 1991; *55*, 35-58.

[5] Shah J, Brown R M, Towards electronic paper displays made from microbial cellulose , *Appl. Microbiol. Biotechnol*., 2005, *66*, 352-355.

[6] Shoda M, and Y. Sugano Y, Recent advances in bacterial cellulose production, *Biotechnol. Bioproc. Eng.* 2005; *10*, 1-8.

[7] Svensson A, Nicklasson E, Harrah T, Panilaitis B, Kaplan D L, Brittberg M, Gatenholm P, Bacterial cellulose as a potential scaffold for tissue engineering of cartilage", *Biomaterials*. 2005; *26*, 419-431.

[8] Klemm D, Schumann D, Udhardt U, Marsch S, Bacterial synthesized cellulose-artificial blood vessels for microsurgery, *Prog. Polym. Sci.* 2001; *26*, 1561-1603.

[9] Iguchi M, Yamanaka S, Budhiono A, Bacterial cellulose-a masterpiece of nature's arts, *J. Mater. Sci.*. 2000; *35*, 261-270.

[10] Vandamme E J, De Baets S, Vanbaelen A, Joris K, De Wulf P, Improved production of bacterial cellulose and its application potential, *Polym. Degrad. Stabil.* 1998; *59*, 93-99.

[11] Nakagaito A N, Nogi M, Yano H, Displays from transparent films of natural nanofibers, *Mater. Res. Soc. Bull.* 2010; *35*, 214-218.

[12] Yamada Y, and Yukphan P, Genera and species in acetic acid bacteria, *Int. J. Food. Microbiol*.. 2008; *125*, 15-24.

[13] Tsuchida T, Yoshinaga F, Production of bacterial cellulose by agitation culture systems, *Pure Appl. Chem.* 1997; *69*, 2453-2458.

[14] Seto A, Kojima Y, Tonouchi N, Tsuchida T, Yoshinaga F, Screening of bacterial cellulose-producing *Acetobacter* strains suitable for sucrose as a carbon source, *Biosci. Biotechnol. Biochem.* 1997; *61*, 735-736.

[15] Park J K, Park Y H, Jung J Y, Production of bacterial cellulose by *Gluconacetobacter hansenii* PJK isolated from rotten appl , *Biotechnol. Bioproc. Eng.* 2003; *8*, 83-88.

[16] Valla S, Kjosbakken J, Isolation and characterization of a new extracellular polysaccharide from a cellulose-negative strain of *Acetobacter xylinum*, *Can. J. Microbiol.* 1981; *27*, 599-603.

[17] Hestrin B, Schramm M, Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose, *Biochem. J.*. 1954;*58*, 345-352.

[18] Loganathan P, Sunita R, Parida A K, Nair S, Isolation and characterization of two genetically distant groups of *Acetobacter diazotrophicus* from a new host plant *Eleusine coracana* L, Journal of Applied Microbiology.1999; 87: 167–172.

[19] Trcˇek J, Jernejc K, Matsushita K, The highly tolerant acetic acid bacterium Gluconacetobacter europaeus adapts to the presence of acetic acid by changes in

lipid composition, morphological properties and PQQ-dependent ADH expression Extremophiles.2007; 11:627–635.

[20] Aydin YA, Aksoy N D, Isolation of Cellulose Producing Bacteria from Wastes of Vinegar Fermentation. Proceedings of the World Congress on Engineering and Computer Science. 2009; (I) WCECS, October 20-22, San Francisco, USA.

[21] Yukphan P, Malimas T, Takahashi M, Potacharoen W, Busabun T, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y, Re-identification of *Gluconobacter* strains based on the restriction analysis of 16S-23S rDNA internal transcribed spacer regions. J. Gen. Appl. Microbiol. 2004; 50: 189-195.

[22] Suwanposri A, Yukphan P, Yamada Y, Ochaikul D, Identification and biocellulose production of *Gluconacetobacter* strains isolated from tropical fruits in Thailand. Maejo Int. J. Sci. Technol. 2013; 7 (01): 70-82.

[23] Saitou N, Nei M, The neighbor-joining method: A new method for reconstructing phylogenetic trees, *Mol. Bio. Evol.* 1987; *4*, 406-425.

[24] Tamura K, Dudley J, M. Nei M, Kumar S, MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0, *Mol. Biol. Evol.* 2007; *24*, 1596-1599.

[25] Thomson J D, D. G. Higgins D J, and Gibson T J, CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.* 1994; *22*, 4673-4680.

[26] Kimura M, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, *J. Mol. Evol.* 1980; *16*, 111-120.

[27] Tsuchida, T. and Yoshinaga, F, Production of bacterial cellulose by agitation culture systems. *Pure* & *Appl. Chem.,*1997; (69) 11, 2453-2458.

[28] Dellaglio F, Cleenwerck I, Felis G E, Engelbeen K, Janssens D, Marzotto M, Description of *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., isolated from Italian apple fruit , *Int. J. Syst. Evol. Microbiol*. 2005; *55*, 2365-2370.

[29] Sokollek S J, Hertel C, Hammes W P, Description of *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations. *Int. J. Syst. Bacteriol*., 1998; 48: 935-940.

[30] Bergey D H, Holt J G, Bergey's manual of determinative bacteriology, 9th edn., Baltimore: Lippincott Williams&Wilkins 1994; pp. 71-84.

[31] Lisdiyanti P, Navarro RR, Uchimura T, Komagata K, Reclassification of *Gluconacetobacter hansenii* strains and proposals of *Gluconacetobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov. International Journal of Systematic and Evolutionary Microbiology. 2006;, 56, 2101–2111

[32] Mansour A A, El-Tayeb T S, El-Bordeny N E, Identification molecular characterization and evaluation of some ruminal bacteria that catalyse and degradation plant cellulosic materials. American Journal of Applied Sciences, 2014; 11 (1): 127-136.

[33] Navarro R R, Uchimura T, Komagata K, Taxonomic heterogeneity of strains comprising *Gluconacetobacter hansenii* , *J. Gen. Appl. Microbiol.*, 1999;*45*, 295-300.

[34] Tortora G J, Funke B R, Case C L, Microbiology: An Introduction", 7th Edn., Benjamin Cummings Publishing, San Francisco,2001; pp.69-70.