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EVALUATION OF CULTURE REQUIREMENTS FOR CELLULOSE PRODUCTION BY EGYPTIAN LOCAL ISOLATE ALONGSIDE REFERENCE STRAIN GLUCONACETO-*BACTER HANSENII* ATCC 23769

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ABSTRACT

Cellulose demonstrates unique properties and suitable for many different applications. In the present study an acetic acid bacterial strain has been isolated from rotten apple and investigated as cellulose producer. The strain was identified using morphological, biochemical characterization and 16s rRNA gene sequencing and named as *Komagataeibacter hansenii* (*K. hansenii* AS.5) under identity percentage 99%. Culture conditions for BC production by AS.5 were screened and compared with reference one *Gluconacetobacter hansenii* ATCC 23769 under static condition. Optimization of key production parameters has been carried out using OVAT (One Variable At Time) approach. Effect of media composition, inoculum size, pH, temperature, incubation time, different carbon and nitrogen sources were evaluated. The highest production of cellulose (3.75 g/l) was obtained after 10 days, 8% inoculum size, incubation 25◦C by *K. hansenii* AS.5 using Yamanaka medium with glucose and yeast extract as a sole carbon and nitrogen source, respectively. On contrast, *G. hansenii* ATCC 23769 exhibits the maximal BC production (2.18 g/l) under the modified GEM medium composed of mannitol and yeast extract as the optimum carbon and nitrogen source after 7 days at 25 ◦C and inoculum size 6%. It is clearly noticed the Cellulose production by the local isolate is higher than the reference one by 1.7- fold.

Key words: Cellulose production, Egyptian isolates, Culture requirements, OVAT.

1. INTRODUCTION

Cellulose is the most abundant biopolymer, renewable and biodegradable produced in the earth with 180 billion tons per year in nature (Engelhardt, 1995). BC is a bacterial-based homopolymer of β(1→4) D-glucopyranose units intertwined by intermolecular hydrogen bonds with the formula $[(C_6H_{10}O_5)n]$ (Huang et al., 2014), Plant cellulose (PC) and BC have a much the same chemical structure (Wan et al., 2006). However, bacterial cellulose is contrasting from PC in some physicochemical and mechanical properties, including fibrils where, bacterial cellulose are 100 times thinner than that of PC, making it more porous, finer structure (nanoscale microfibrils < 10 nm in width), higher purity (free from hemicellulose and lignin), longer fiber length (polymerization degree between 2000 and 6000), higher crystallinity, higher water absorbing and holding capacity, higher tensile strength, strong biological adaptability, nontoxic and non-allergenic (Iguchi et al., 2000; Bäckdahl et al., 2006; Chawla et al., 2009; Ul-Islam et al., 2012). Therefore, BC represents a potential alternative to plant-derived cellulose and a promising material for many applications (Iguchi et al., 2000). These include a thickening agent and food stabilizer (Shi et al., 2014), food packaging (Spence et al., 2010), biomaterial for manufacturing cosmetics (Kawaguchi and Nakamura, 2007), artificial skin (Kingkaew et al., 2014), artificial blood vessels or tissue engineering (Scherner et al., 2014), preparation of optically transparent films (Palaninathan et al., 2014) and electric conductors (Müller et al., 2012). Bacteria of the family *Acetobacteraceae* are most commonly used for BC production, mainly bacteria from the genus *Komagataeibacter* and usually strains of the species *Komagataeibacter xylinus* and *Komagataeibacter hansenii* (previously known as *Gluconacetobacter xylinus* and *Gluconacetobacter hansenii*). However, other species of this genus have also been reported to harbour cellulose-producing strains, such as *Komagataeibacter swingsii, Komagataeibacter rhaeticus and Komagataeibacte rmedellinensis* (Santos et al., 2014). Other genera such as *Agrobacterium, Aerobacter, Achromobacter, Alcaligenes, Azotobacter, Rhizobium, Pseudomonas, Sarcina, Salmonella*, etc. have ability to produce cellulose (Huang et al., 2014). Moreover, cellulose producing strain can also be isolated from different sources such as fermented fruit juice (Lin et al., 2016; Kim et al., 2017), kombucha tea (Rozenberga et al., 2016), vinegar (Tabaii

and Emtiazi, 2015), wine (Bıyık and Çoban, 2017), orange pulp (Tyagi and Suresh, 2013) and rotten apple (Hungund and Gupta, 2010). Gram-negative bacteria in *Komagataeibacter* (former *Gluconacetobacter* and *Acetobacter*) genus are mainly cellulose producer these bacteria are isolated from different sources, strictly aerobic and produce BC as an extracellular product at the air liquid interface of the growth media at pH with in 3 and 7 and temperatures ranged from 28 to 30◦C (Lin et al., 2016; Machado et al., 2016). *G. hansenii* ATCC 23769, a Gram negative bacterium and has ability to produce BC at the air liquid interface in growth medium, and has long been used as a model system for production of BC and a reference strain in the various studies (Lee et al., 2015; McManus et al., 2016). One of the bacterial cellulose application problems in industry is its low productivity. So, OVAT method was applied to obtain the optimal culture conditions for highest production of bacterial cellulose. Several studies describe the optimization of cellulose production using OVAT such as media composition, carbon, nitrogen source, pH, temperature and incubation time, etc (Zahan et al., 2015b; Bıyık and Çoban, 2017; Molina-Ramírez et al., 2017). The purpose of this study, production of cellulose through isolation of bacterial producer strains from Egyptian local sources as a source of microbial contaminant. Only one isolate has ability to produce cellulose which compared with the *G. hansenii* ATCC 23769 as a reference strain by evaluation of culture conditions requirements for both strains.

2. MATERIALS AND METHODS

2.1 Isolation of cellulose producing microbes: Ten (gm or ml) samples of rotten fruits (Banana, Apple, Palm, Guava, Orange, Tangerine, Peach, Strawberry, Date and Pineapple) and rotten vegetables (Lemon and Tomato) and rotten Vinegar were inoculated in 90 ml of a modified Hestrin and Schramm medium (MHS) in a 250-ml flask contains: (g or ml/l) D-glucose 20, peptone 5, YE 5 g/l, sodium hydrogen phosphate dibasic 2.7, citric acid 1.15, acetic acid 2, ethanol 5 and Nystatin 2 as antifungal. The liquid MHS medium contained rotten samples were incubated statically at 30°C for 7 days. After incubation time, the flasks with white pellicle covering the surface of liquid medium were selected as a positive result for microbial cellulose production. The culture broth of the selected flask was serially diluted with 0.9% NaCl (w/v) and 0.1 ml of each dilution was spread on GEY agar, which composed of: (g/l) D-glucose 20, YE 10, ethanol 5 ml, calcium carbonate 3 and agar 20. The agar plates were incubated at 30°C for one week. After the time consumed, the colonies with a clear zone around were selected and inoculated in test tube containing 5 ml of HS medium composed of: (g/l) D-glucose 20, peptone 5, YE 5, sodium hydrogen phosphate dibasic 2.7, citric acid 1.15, then incubated at 30°C for one week (Hestrin and Schramm, 1954). Subsequently, the purified isolated colonies with a white pellicle on the surface of test tube were stored at 4°C as cellulose producer microbes for further study. On the other hand, *G. hansenii* ATCC 23769 achieved from American Type Culture Collection was used as a reference strain in this study.

2.2 Standard inoculum preparation: A freshly isolated single colony was culture in HS medium (5ml) and then incubated at 30°C for 2 days at 200 rpm as standard inoculum preparation.

2.3 Purification of Cellulose: The resulting pellicle was harvested and washed many times with distilled water to remove the residues of medium components. Afterwards, the pellicle was then treated by 0.5% NaOH at 90°C for 30 min, to remove microbial contaminants and other impurities immobilized on the films, and then washed by distilled water until a neutral pH of washed liquid was reached. In the end, the purified microbial cellulose was dried at 70°C over-night until constant weight (Hsieh et al., 2016). The dry-weight, yield and productivity of cellulose were determined.

2.4 Parameters of microbial cellulose production: Productivity and yield were calculated according to Aytekin et al., (2016) and Mohammad kazemi et al., (2015) using the following equations:

Yield % = (Cellulose dry weight g/l)/ (Original sugar g/l) x 100

Productivity % = (Cellulose dry weight g/l)/ (Production time d) x 100

2.5 Identification of the selected isolate

2.5.1Genetic identification: The cellulose producing isolate was identified using partial 16s rDNA sequences analysis. Based on salting out method, genomic DNA was isolated from pure culture according to (Miller et al., 1988). The amplification of the 16S rRNA gene from the genome of isolate under investigation was performed through the polymerase chain reaction (PCR) (Mullis et al., 1986), using universal degenerate primers designed to amplify the full length (1500 bp) of the 16S rRNA gene according to the *E. coli* genomic DNA sequence. The purified PCR product was sequenced using dideoxy chain termination method (Sanger et al., 1977**).** This was done using

ABI PRISM model 3730 automated DNA sequencer at Sigma for Scientific Research and big dye terminator ready reaction mix. The sequences were assembled using BioEdit Sequence Alignment Editor Program (Hall, 1999) and comparative sequence analyses were performed using ClustalW. BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to assess the similarity and phylogenetic tree was constructed with MEGA software version 4.0.2 (Tamura et al., 2007).

2.5.2 Morphological and biochemical identification: The most potent isolate which producing microbial cellulose was undergone to further identification such as morphological observation by scanning electron microscopy (SEM) (JEOL JSM 6360 LA, Japan) at laboratory center- City of Scientific Research and Technological Application. Gram′s characteristics via Gram′s staining Kit according to standard protocols, and physio-biochemical characterization through analytical profile test using available commercially Kit (Macrobact GNB 24E Kit) Oxoid according to the manufacture instruction with reference to Bergey′s Manual of Systematic Bacteriology.

2.5.2.1 Enzymes profile of the selected strain: Qualitative screening for different enzymes production by the selected strain was done by plate assay method. Agar plates were prepared by incorporating the substrate as 1% gelatin (protease), 0.2% soluble starch (amylase), 0.2% tributyrin and Tween 20 (lipase/esterase), 0.2% carboxymethyl cellulose (cellulase), 0.2% xylan (xylanase), 0.2% uric acid (uricase), 0.5% lactose (beta galactosidase) and 0.01% guaiacol (laccase) in HS medium at 30°C for 48 hrs. After incubation time the results were recorded according to the clear zones or color of colony.

2.6 Optimization of culture conditions: OVAT approach was used to obtain maximum production of BC by selected isolate; various nutritional and physiological parameters were studied such as different media composition, inoculum size, pH, temperature and incubation time, carbon and nitrogen sources. The optimization experiments were performed in triplicate. All the experiments were carried out in static condition for BC production.

2.6.1 Selection of suitable medium composition: Four media for BC production being HS (Hestrin and Schramm, 1954), modified HS (Lee et al., 2015), modified Yamanaka media (Mohammad kazemi et al., 2015) (g/l: D-Glucose 20, YE 5, $(NH4)_2SO4$ 5, K_2PO4 3, MgSO4.7H2O 0.05 and 5ml ethanol) and modified GEM (Hanmoungjai et al., 2007), were used in this study to select the most suitable medium to achieve high BC production. The production process was performed in 250 ml Erlenmeyer flasks containing 45 ml sterile medium, then inoculated with 5 ml from standard inoculum and incubated at 30°C for 7 days at pH 6 under static condition. At the end of incubation time the microbial cellulose pellicle was dried and weighted to determine the dry weight, yield and productivity of cellulose.

2.6.2 Effect of different inoculum size: Different inoculum size (2, 4, 6, 8, 10, 12 and 14%) was tested to investigate their effect for maximal production of cellulose. The production process was performed in 250 ml Erlenmeyer flasks containing 45 ml sterile selected medium for both isolated and reference strains, then inoculated and incubated at 30°C for 7 days at pH 6 under static condition.

2.6.3 Effect of different pHs: The influence of various pHs on cellulose production by isolated and reference strains were studied by marinating the culture media at pH 2, 4, 6, 8 and 10. The production process was conducted in 250 ml Erlenmeyer flasks containing 45 ml sterile medium, each under preferred medium and inoculum, then incubated at 30°C for 7 days at different pHs under static condition.

2.6.4 Effect of different temperatures: To monitor the temperature impacts on cellulose production by studied isolate and reference strains, different temperatures 20, 25, 30, 35 and 40°C were tested under preferred conditions for each strain.

2.6.5 Effect of different incubation time: The effect of various incubation periods, like 5, 7, 9, 10 and 11 days on the BC production by isolated and reference strain was studied each under recommended conditions of previous experiments.

2.6.6 Effect of different carbon source: To study the impacts of different carbon sources on the cellulose production, carbon source like, glucose, fructose, mannitol, xylose, galactose, sucrose and starch were added at 2% concentration.

2.6.7 Effect of different nitrogen source: Yeast extract, peptone, casein and tryptone represented as organic nitrogen source, on the other hand, ammonium nitrate, ammonium sulphate, ammonium chloride and sodium nitrate represented as inorganic nitrogen source, which all were screened as a source of nitrogen for the highest production of cellulose by isolated and reference strain at 0.5% concentration.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of cellulose producing strain: Most of earlier studies describe the production of cellulose by cultivation of *Acetobacter* sp, reclassified as the genus Gluconace-

tobacter, which is a typical isolate obtained from different sources such as rotten fruits (Rangaswamy et al., 2015) , flowers and fermented foods (Park et al., 2003), beverages (Jia et al., 2004), and vinegar (Gullo et al., 2006).

In this study, we obviously directed to isolate bact eria with the ability to produce higher cellulose from different sources: rotten fruits, vegetables and vinegar. Among tested sources a flask contained a rotten apple covered with a white pellicle on the liquid medium surface was selected as positive results for microbial cellulose production. The bacterial isolates related were purified by serial dilution to obtain sixty-one different purified isolates and only one isolate namely No. 2 has been selected as cellulose producer under static conditions then compared with *G. hansenii* ATCC 23769 as a reference strain. Similarly, Park et al., (2003) succeeded in isolation *Gluconacetobacter* sp from rotten apple as cellulose producer after repeated attempts. Moreover, identification of the selected isolate was executed mainly through molecular identification, morphological and biochemical characterization. Analysis of 16s rDNA (partial sequence) reveals that the selected strain (No. 2) showed 99% similarity to the *Komagataeibacter hansenii* sequence. The 16S rRNA gene sequence of *Komagataeibacter hansenii* was deposited in the GenBank under the accession number (MH109871). Subsequently, the isolate was designated as *Komagataeibacter hansenii* AS. 5. A Phylogenetic tree was designed using Clustal X program (Fig 1) and showed that the isolate AS.5 is more related to *Komagataeibacter hansenii*. Generally, different bacteria from many sources can be used as a source of BC such as: *Komagataeibacter rhaeticus* from Kombucha tea (Semjonovs et al., 2017), *Komagataeibacter saccharivorans* from peach (Abdelhady et al., 2015) and *Komagataeibacter intermedius* from fermented fruit juice (Lin et al., 2016). The investigated bacterium *Komagataeibacter hansenii* AS.5 isolated from rotten apple and reference strain were subjected to morphological characterization through SEM (Figs 2), respectively. It was recognized that the *K hansenii* AS.5 is high similar in morphological and biochemical characteristics to the *G hansenii* ATCC 23769 using available commercially kit (Macrobact GNB 24E kit) Oxoid according to the manufacturer's instruction with reference to Bergey's Manual of Systematic Bacteriology as showed in table 1 and 2, respectively. Also, *K. hansenii* AS.5 and *G. hansenii* ATCC 23769 are considered a positive producer of protease, lipase/esterase, xylanase and catalase; respectively, while protease exhibited the most potent enzyme; it had a large clear zone obtained from gelatin hydrolysis followed by catalase. On the contrary, the plate assays performed for amylase, uricase, beta galactosidase and laccase yielded negative results, these results indicated that the both strains hadn't machinery for production of amylase, uricase, beta galactosidase and laccase which enable it to utilize soluble starch, uric acid, lactose and guaiacol respectively. Also, *K. hansenii* AS.5 and *G. hansenii* ATCC 23769 were showed a typical profile for examined enzymes (protease, amylase, lipase, cellulase, xylanase, uricase, beta galactosidase, catalase and laccase).

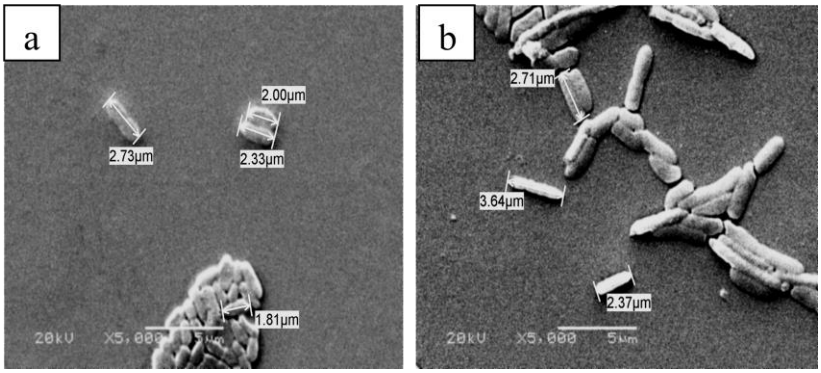

Figure 2: (a) SEM micrographs of *K. hansenii* AS.S with cell size average 2.21 µm and (b) *G. hansenii* ATCC 23769 with cell size average 2.90 µm.

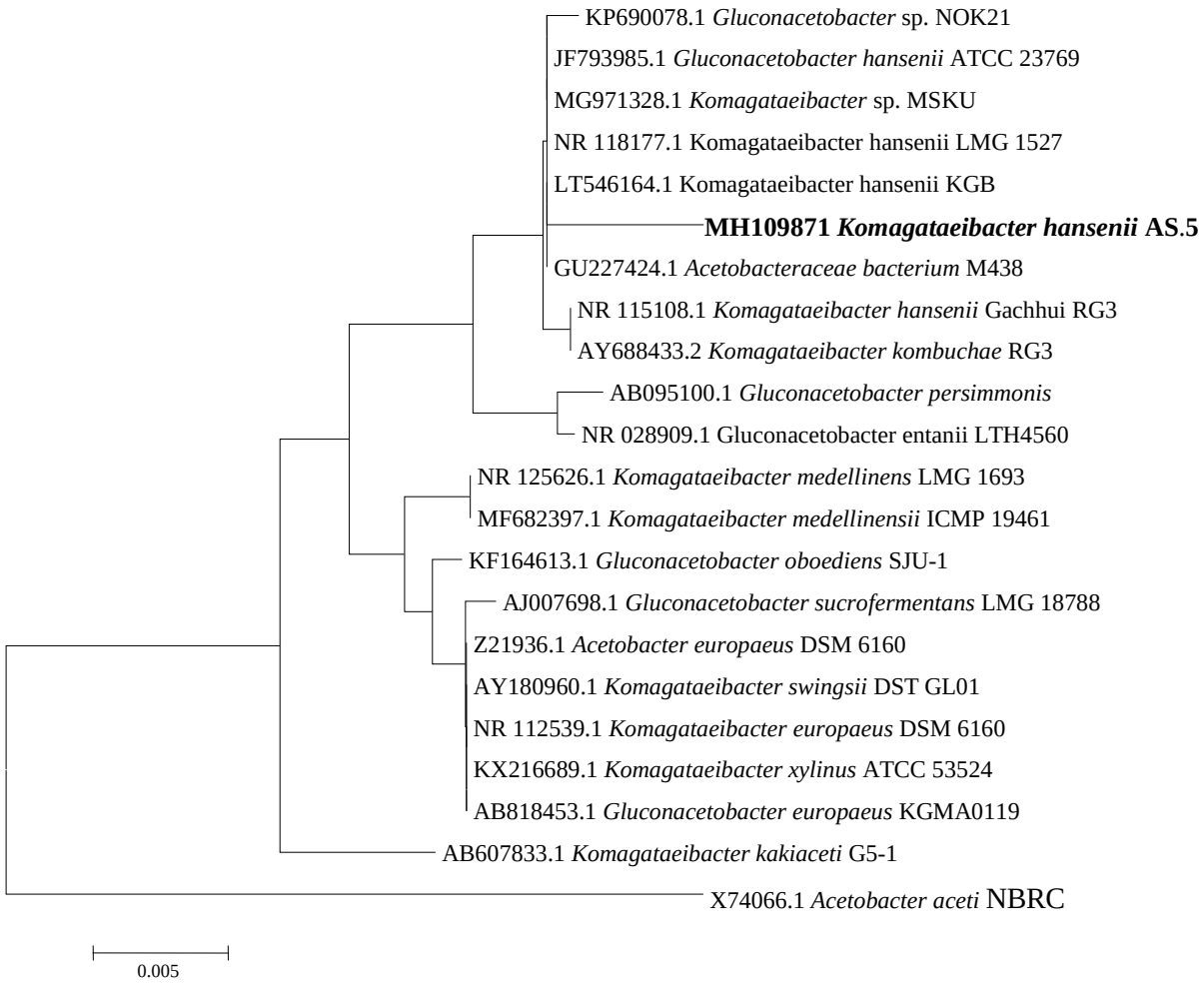

Figure 1: A phylogenetic tree based on 16S rDNA sequences constructed by the neighbor-joining method.

Table 1: Morphological characteristics of *K. hansenii* AS.5 and *G. hansenii* ATCC 23769

Colony morphology	***K. hansenii* AS.5**	***G. hansenii* ATCC 23769**
Configuration	Bacilli	Bacilli
Margin	Entire	Entire
Surface	Smooth	Smooth
Pigment	Yellowish	Cream
Gram reaction	Negative	Negative
Cell shape	Short rods	Short rods
Size average	2.21	2.90
Arrangement	Mono, diplo and few Streptobacilli	Mono, diplo and few Streptobacilli

Table 2: Comparison of the biochemical characteristics of *K. hansenii* AS.5 and *G. hansenii* ATCC 23769

No.	**Test**	**Result**	
		***K. hansenii* AS.5**	***G. hansenii* ATCC 23769**
1	Motility	+	+
2	Nitrate	-	-
3	Lysine	-	-
4	Ornithine	-	-
5	H_2S	-	-
6	Glucose	+	+
7	Mannitol	+	+
8	Xylose	+	+
9	ONPG	-	-

10	Indole	-	-
11	Urease	-	-
12	V-P	+	+
13	Citrate	-	-
14	TDA	+	+
15	Gelatin	-	-
16	Malonate	-	-
17	Inositol	-	+
18	Sorbitol	-	+
19	Rhamnose	+	+
20	Sucrose	+	+
21	Lactose	+	+
22	Arabinose	+	+
23	Adonitol	-	+
24	Raffinose	-	+
25	Salicin	-	+
26	Arginine	-	-
27	Cellulose production	+	+

ONPG=Hydrolysis of o-nitrophenyl-ß-d-galactopyranoside (ONPG) by action of ß- galactosidase, VP test = Voges-Proskauer test

3.2 Culture Conditions Optimization (OVAT): Production of cellulose not depend only on the strain but also on different nutritional ingredients and physical parameters. Accordingly, OVAT technique was carried out to study the following: media composition, inoculum size, pH, temperature, carbon, and nitrogen sources to implement the highest production of cellulose.

3.2.1 Influence of various media composition on cellulose production: The results (full data not shown) revealed that the maximum yield of cellulose was obtained in modified Yamanaka and HS media (2.76 and 2.48 g/l, respectively), while modified HS and GEM represent a low production of cellulose (0.74 and 0.43 g/l, respectively) using *K. hansenii* AS.5. On the other hand, approximately 1.5 g/l cellulose produced from modified GEM media while 1.3, 1.2 and 1.07 g/l cellulose produced from HS, modified HS and Yamanaka media, respectively using a reference strain (*G. hansenii* ATCC 23769) as BC producer. Ultimately, the modified Yamanaka media and modified GEM were selected as the best media for production of cellulose as well as yield and productivity of cellulose for *K. hansenii* AS.5 and *G. hansenii* ATCC 23769, respectively. Similarly, many literatures reported the effect of various growth media on the production of cellulose (Tyagi and Suresh, 2013; Mohammadkazemi et al., 2015).

3.2.2 Effect of various inoculum sizes on cellulose production: The data represented in table 3 clearly showed that all the inoculum size tested exhibit positive cellulose production but lower and higher values than 8% and 6% inoculum showed a decrease in cellulose production in case of isolated and *G. hansenii* ATCC 23769, respectively. Through this experiment, we can conclude that 8% inoculum size is optimal for the BC production and achieved 2.96 g/l yield compared to other inoculum sizes for *K. hansenii* AS.5. While 6% inoculum size is optimum for BC production and achieved 1.97 g/l yield compared to other inoculum sizes for *G. hansenii* ATCC 23769. Due to appearance of pellicle formation at air liquid inter phase within 48h of incubation in case of cellulose production, the optical density cannot applied as accurate method for determination of growth (Rangaswamy et al., 2015).

Table 3: Effect of different inoculum size on the production of cellulose

Inoculum Size %	*K. hansenii* AS.5				*G .hansenii* ATCC 23769			
	BC dry Wt g/l	Yield %	Productivity %	Glucose remaining g/l	BC dry Wt g/l	Yield %	Productivity %	Glucose remaining g/l
2	2.284	11.4	32.6	0.00	1.818	9.09	25.9	0.10
4	2.812	14	40.1	0.00	1.902	9.51	27.1	0.08
6	2.904	14.5	41.4	0.00	1.970	9.85	28.1	0.02
8	2.960	14.8	42.2	0.00	1.898	9.49	27.1	0.20
10	2.832	14.1	40.4	0.00	1.578	7.89	22.5	0.13
12	2.724	13.6	38.9	0.00	1.710	8.55	24.4	0.11
14	2.654	13.2	37.9	0.00	1.751	8.75	25	0.07

3.2.3 Impact of various pH on cellulose production: The pH plays a significant role in cell growth and BC production. The cellulose production was observed at pH ranged from 4 to 8 for both isolated and reference stain as can be seen in table 4. On the other hand, cellulose and cell growth cannot be achieved at pH 2 and 10 for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769. By this investigation, we can surmise that pH 4 is optimum for cellulose production and achieved 2.97 g/l which is maximum yield compared to other pH values for *K. hansenii* AS.5. While pH 6 it is optimum for cellulose production and achieved 1.90 g/l yield relative to other pH values for *G. hansenii* ATCC 23769. The maximum cellulose production of *K. hansenii* AS.5 reached 2.97 g/l when the pH was set at 4, approximately 2-fold than those from *G. hansenii* ATCC 23769 (1.55 g/l) at the same pH. At pH 6 the cellulose production of *K. hansenii* AS.5 attained to 2.88 g/l which was higher 1.2fold than those from *G. hansenii* ATCC 23769 (1.90 g/l) at the same pH. Several studies showed that the pH value range for cellulose production was about 4–9 (Iguchi et al., 2000; Lin et al., 2016) and the optimum pH for cellulose production varies with the bacterial strains, but was usually attributed to a neutral to slightly acidic pH range (Bielecki et al., 2005). Castro et al. (2012) confirmed that a new isolated *Gluconacetobacter* genus exhibited highly tolerant to low pH, and provided the highest cellulose production at pH 3.5, while *K. hansenii* AS.5 produces cellulose at pH 4. Lin et al., (2016) reported that the *G. xylinus* 23769 produces cellulose ranged from 0.64 to 1.4 g/l at pH between 4 to 9 under incubation from 4 to 9 days using HS medium, while the present study achieved the maximum production of cellulose ranged from 1.55 to 1.90 g/l at pH from 4 to 8 after 7 days using GYPE medium by *G. hansenii* ATCC 23769.

Table 4: Effect of different pH on production of cellulose

pH	***K. hansenii* AS.5**				***G. hansenii* ATCC 23769**			
	BC dry Wt g/l	**Yield %**	**Productivity %**	**Glucose remaining g/l**	**BC dry Wt g/l**	**Yield %**	**Productivity %**	**Glucose remaining g/l**
2	0	0	0	19.5	0	0	0	19.2
4	2.976	14.8	42.5	0.07	1.557	7.78	22.2	0.06
6	2.884	14.4	41.2	0.13	1.905	9.52	27.2	0.02
8	2.454	12.2	35	0.15	1.811	9.05	25.8	0.05
10	0	0	0	18.2	0	0	0	19

3.2.4 Effect of different temperatures on Cellulose production: Cellulose production and cell growth were directly affected by temperature. To study the effect of various temperature on cellulose production by isolated and *G. hansenii* ATCC 23769, temperature vary from 20 to 40∘C (with unit increase of 5∘C) was examined as shown in table 5. The results indicated that the *K. hansenii* AS.5 exhibits cellulose production (1.69 to 3.20 g/l) at temperatures ranged from 20 to 35∘C, but no cellulose production and cell growth when temperature reached to 40∘C. As well as, *G. hansenii* ATCC 23769 produces BC (1.35 to 2.06 g/l) at temperature ranged from 20 to 30∘C, and production within the range of 35 and 40∘C was not observed. The optimum temperature for high cellulose production (3.20 and 2.06 g/l) is 25∘C for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769 respectively. The optimum temperature supplies the bacterium with a enough energy which improve the cellulose biosynthetic pathway to transform glucose into cellulose. The *K. hansenii* AS.5 exhibits 4.0 fold higher cellulose production than *G. hansenii* ATCC 23769 under the same temperature. Zahan et al., (2015a) reported that, at incubation temperature of 40°C, there is no significant growth appeared of *A. xylinum* 0416 and cellulose production as well. This is probably due to the harsh and inappropriate environment created by the incubation at this temperature, these data are similar with data obtained from *K. hansenii* AS.5. This could be attributed to the facts that the glucose was transformed into other product than BC such as gluconic acid via direct oxidation during production process which finally lead to significantly reduced in the pH of production medium. The pH decreases during production because of the accumulation of by-products like gluconic, acetic or lactic acids (Zahan et al., 2014). Son et al. (2001) reported that at incubation temperature of 35°C and / or above, the bacteria do not multiply even in an optimal medium due to denaturation of cell components such as nucleic acids and proteins, this lead to the growth of cells and BC production were not observed under incubation temperature of 35°C and above, this may be matching to the results obtained from *G. hansenii* ATCC 23769 in this work.

Table 5: Effect of different temperature on production of cellulose

Temperature ∘C	*K. hansenii* AS.5				*G. hansenii* ATCC 23769			
	BC dry Wt g/l	Yield %	Productivity %	Glucose remaining g/l	BC dry Wt g/l	Yield %	Productivity %	Glucose remaining g/l
20	1.696	8.48	24.2	0.00	1.354	6.77	19.3	0.07
25	3.206	16	45.8	0.035	2.066	10.3	29.5	0.05
30	2.944	14.7	42	0.05	1.895	9.47	27	0.04
35	1.496	7.48	21.3	5	0	0	0	14.82
40	0	0	0	13.82	0	0	0	17.76

3.2.5 Effect of different incubation period on cellulose production: To obtain maximum cellulose production from isolated and *G. hansenii* ATCC 23769, different incubation time was studied range from 5 to 11 days (with unit increase of 2 days). Data represented in Fig. 3 showed that the cellulose production was observed in all incubation time, but, 10 days cultivation time is considered the optimal time and achieved 3.57 g/l yield by *K. hansenii* AS.5. On the other hand, 7 days incubation time is optimum for cellulose production by *G. hansenii* ATCC 23769 and achieved 2.08 g/l yield. The cellulose production approximately stable after the optimum incubation time for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769. We can report that the *K. hansenii* AS.5 produces cellulose higher than *G. hansenii* ATCC 23769.

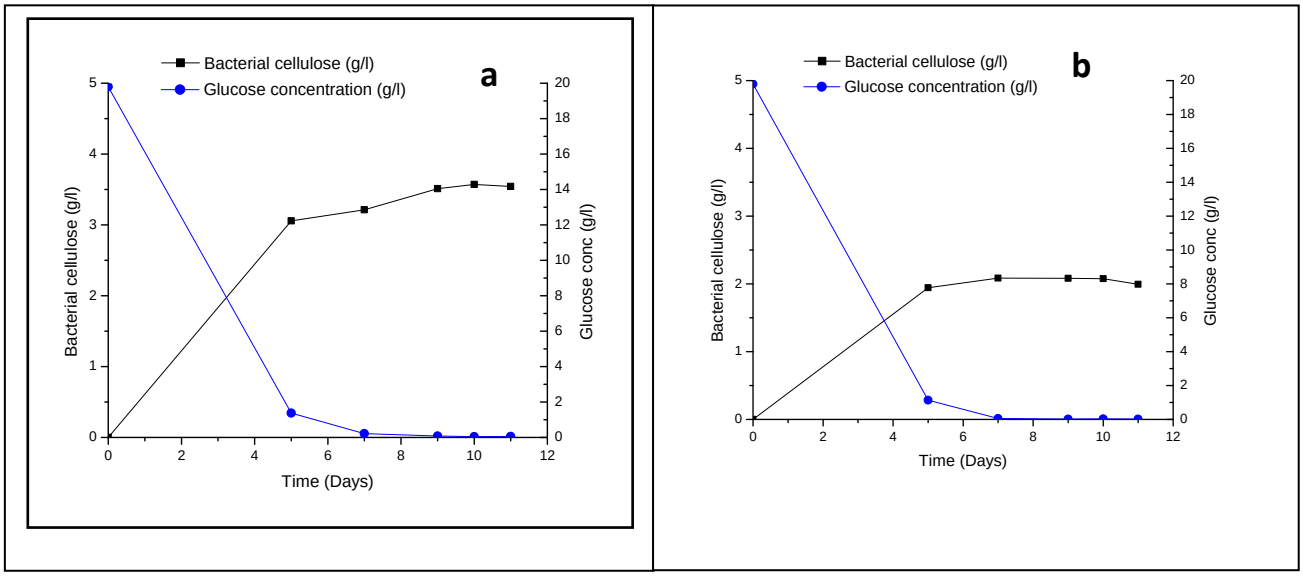

Figure 3: (a) Effect of different incubation time on production of BC by *K. hansenii* AS.5 and (b) *G. hansenii* ATCC 23769.

3.2.6 Effect of various carbon sources on cellulose production: To investigate the effect of carbon source on BC production, carbon sources like mono, di and polysaccharide such as glucose, fructose, mannitol, xylose, galactose, sucrose and starch were supplemented at 2% (w/v) in a suitable medium.

The results are described and presented in table 6 for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769. The maximum BC production (3.50–2.93 g/l) was observed in glucose followed by fructose, respectively for *K. hansenii* AS.5. However, *G. hansenii* ATCC 23769 exhibits maximum cellulose production in mannitol (2.17 g/l) followed by glucose (2.07 g/l). In the present study, *G. hansenii* ATCC 23769 shows the ability to use a wide variety of carbon sources for cellulose production and D-mannitol seems to be the most suitable carbon source. These results are in good agreement with previous reports that cellulose production by *Gluconacetobacter* strains isolated from various sources produces the highest yield in a medium comprising D-mannitol (Panesar et al., 2009, Suwanposri et al., 2013). Yodsuwan et al., (2012) reported that the *Acetobacter xylinum* strain TISTR 975 exhibits high production of cellulose from mannitol as the optimum carbon source when used Yamanaka medium as stander medium, while the glucose is the greatest carbon source for high cellulose production by *K. hansenii* AS.5 when used modified Yamanaka medium. Glucose as a simple carbon source, play an

important roles for higher production of secondary metabolites from the specie of mushroom *Pleurotus ostreatust* than other carbon sources (Rana and Dahot, 2017). Most of the researches show that the productivity of cellulose production by *Gluconacetobacter* is influenced by carbon source availability and the aggregation of metabolic by-products that cause adverse growth conditions (Chawla et al., 2009). Molina-Ramírez et al., (2017) expressed that the most efficient carbon source is glucose which achieves 2.80 g/l cellulose at concentration 2% after 8 days by *Komagataeibacter medellinensis*, however the cellulose reached to 3.3 g/l after 15 days when mixed glucose/sucrose used as a carbon source. The present work achieves 3.50 g/l cellulose after 10 days by *K. hansenii* AS.5 at 2% glucose. Other study describe the addition of polysaccharide such as starch on the production media that affect the physical properties of cellulose membrane obtained from *Gluconacetobacter xylinus* BTCC B796 (Sya'Di et al., 2017).

Table 6: Effect of different carbon source on production of BC

Carbon Source	*K. hansenii* AS.5			*G. hansenii* ATCC 23769		
	BC dry Wt g/l	Yield %	Productivity %	BC dry Wt g/l	Yield %	Productivity %
Glucose	3.504	17.5	35	2.079	10.3	29.7
Fructose	2.938	14.6	24.4	2.032	10.1	29
Mannitol	2.396	11.9	19.9	2.174	10.8	31
Xylose	0.918	4.59	7.65	1.122	5.61	16
Galactose	1.174	5.87	9.78	1.146	5.73	16.3
Sucrose	1.644	8.22	13.7	1.102	5.51	15.7
Starch	1.278	6.39	10.6	1.060	5.3	15.1

3.2.7 Impact of different nitrogen sources on cellulose production: The highest production efficiency of cellulose depends not only the carbon source but also on nitrogen source. In this experiment different organic and inorganic nitrogen source was applied. The results represented in table 7 clearly showed that the maximum cellulose production was observed when used organic nitrogen source for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769 than inorganic nitrogen source. As well as, yeast extract exhibits high cellulose production for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769 than other nitrogen source and these data are finding agreement with the previous report (Bıyık and Çoban, 2017). Maximum cellulose production was achieved with yeast extract to obtained cellulose yield of 3.75 and 2.181 g/l for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769, respectively. By this investigation, we can conclude that the *K. hansenii* AS.5 exhibits higher cellulose production 0.5fold than *G hansenii* ATCC 23769 when used yeast extract as a nitrogen source. In different circumstances, the combination of yeast extract and ammonium sulphate as nitrogen source was not significantly affected on production of BC for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769. Çoban and Biyik (2011) reported that a higher cellulose production was achieved in a medium composed of glucose and supplemented with yeast extract as the finest nitrogen source, this data agreement with the present study for both *K. hansenii* AS.5 and *G. hansenii* ATCC 23769.

Table 7: Effect of different nitrogen source on production of BC

Organism	source	Nitrogen source	BC dry Wt g/l	Yield %	Productivity %
K. hansenii AS.5	Organic nitrogen source	Yeast + Ammonium sulphate	2.102	10.5	21
		Yeast extract	3.752	18.7	37.5
		Peptone	0.702	3.51	7.02
		Casein	3.172	15.8	31.7
		Tryptone	0.692	3.46	6.92
	Inorganic nitrogen	Ammonium Nitrate	0.402	2.01	4.02
		Ammonium Sulphate	0.616	3.08	6.16
		Ammonium Chloride	0.194	0.97	1.94
		Sodium Nitrate	0.130	0.65	1.3
G. hansenii ATCC 23769	Organic nitrogen source	Yeast+ Peptone	1.370	6.85	19.5
		Yeast	2.181	10.9	31.1
		Peptone	0.764	3.82	10.9
		Casein	0.836	4.18	11.9
		Tryptone	0.830	4.15	11.8

	Inorganic nitrogen source	Ammonium Nitrate	0.326	1.63	4.65
		Ammonium Sulphate	0.684	3.42	9.77
		Ammonium Chloride	0.251	1.25	3.58
		Sodium Nitrate	0.280	1.4	4

CONCLUSION

An Egyptian local strain (*Komagataeibacter hansenii* AS.5) from rotten apple was selected as BC producer. The production of BC by *K. han senii* AS.5 and reference strain (*Gluconacetobacter hansenii* ATCC 23769) was optimized by OVAT technique. Different media composition, inoculum size, pH, temperature, carbon, and nitrogen parameters were studied for optimal BC production. At the end of optimization, the modified Yamanaka media composed of glucose as a carbon and yeast extract as a nitrogen source at 25∘C, pH 4, with the inoclume size 8% for 10 days achieved 3.75 g/l BC by *K. hansenii* AS.5. While 2.181 g/l BC was achieved when cultivation of *G. hansenii* ATCC 23769 on modified GEM medium with mannitol as a sole carbon and yeast extract as a nitrogen source at 25∘C, pH 6, with the inoculum size 6% for 7 days. We can conclude that, the isolated strain was more effective for BC production than reference strain.

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