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Comparative Study for Bacterial Cellulose Production Using Egyptian *Achromobacter* sp.

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ABSTRACT

The present study was designed to investigate the production and characterization of bacterial cellulose (BC) from local isolate. The isolate obtained from decomposed apple showed maximum BC production (5.95 g/L) at 5% molasses treated with H_2SO_4 and $Ca_3(PO_4)_2$; pH 6 at 35°C for 120 h. Therefore this strain was identified by chemical, Morphological and physiological tests and confirmed by Blast analysis of the 16S rDNA sequencing. The phylogenetic relationship showed maximum similarity of 99% with *Achromobacter* Sp. The chemical structure of BC was measured by HPLC and FT-IR spectra. The monosaccharides gave glucose as major sugars 98.75%. The morphology of BC was carried out by SEM and TEM; the results indicated that BC produced from *Achromobacter* sp. had pure structure without any other impurities. Finally, the study used a cost-efficient production method for BC from *Achromobacter* sp as Egyptian isolated bacteria.

Keywords: Bacterial cellulose, *Achromobacter* sp., Molasses, Character, TEM, SEM, HPLC

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INTRODUCTION

Bacterial cellulose (BC) is an extracellular excretion that forms aggregated fibrils which crystallize into ribbons and assemble into a thick cellulosic mat known as a pellicle [1]. The cellulose produced in a bacterial cell forms micro fibrils of joined macromolecules in an ultra-fine reticulated structure. During cultivation, 12–70 molecules of B cellulose are ejected from a cell into the media over pores situated about 10 nm apart in a discrete array on the cell surface [2]. After biosynthesis, the cellulose molecules link to each other via hydrogen bonds close the cell surface to produce pure cellulose [3]. Hornung *et al.* [4] report that BC obtained from aerobic fermentation is pure has higher degree of polymerization and high water-holding capacity. Compared with plants cellulose, BC is chemically pure, free of hemi-cellulose and lignin [5-7]. However, commercialization BC production has been limited because of the low yields, meaning many batches need to be processed to produce sufficient material.

Acetic acid bacteria (AAB) have been used from ancient times for vinegar and wine production, cheese. Kutziing's demonstrate that of the nature of "mother of vinegar" first showed that acidification of wine was caused by living organisms called AAB that oxidize ethanol to acetic acid but they are very resistant to acetic acid [8]. Xylinus was first described in 1986 [9,10]. This gram negative bacterium is found in soil and can be frequently be isolated from decaying fruit. It is a strictly aerobe in the family *Acetobacteraceae* [11]. Occur singly, in pairs or in chains and reproduce by binary fission. They have flagella and are motile but do not from end spores. The optimum growth temperature is 25- 30 °C and the optimum pH range is 5.4 to 6.2 [12,13]. Under same growth conditions, *Gluconacetobacter xylinus* from involution forms, which have swollen or elongated filaments. These can atrophy or fragment into shorter pieces, which allow normal cells to be recovered. *G. xylinus* produce BC on the surface of liquid and solid culture media. The gelatinous, leather-like mats formed on the surface of liquid culture media under static culture conditions contain bacterial cells entrapped in a network of cellulose fibers. Under agitated cultivation, pellicle deposition is disrupted and cellulose forms irregular granules, stellate strands and fibrous strand [12,13]. On agar media, *G. xylinus* forms transparent or white, smooth or rough, flat or convex colonies which can have undulate edges. The major problem for commercial production of BC using *G. xylinus* is that this bacterium tends to spontaneously mutate and produce non-cellulose. This occurrence in agitated cultures which were first described by Hestrin and Schramm [14], who isolated three different types of *G. xylinus* cells, based on colony morphology and cellulose biosynthesis efficiency. There are only few reports on the BC production by newer species of *Acetobacter* and other bacterial strains.

Molasses is a by product of the final stage of crystallization of sugar production procedure that can be a promising applicant for being a low cost carbon source in microbial production. Molasses has been used as a carbon source production of different industrial products such as the lactic acid, ethanol, pullulan, xanthan and cellulose [15-19]. Molasses contains carbon sources, nitrogen sources, vitamins, minerals and complex structures which source heterogeneity in medium and have an effect on the cell growth rate. So, many types of molasses treatment have been proposed to prepare the inimitable molasses medium for equal microorganism strains [20]. The main objective of the present work is therefore to provide a novel isolated bacterial strain from fruit residues, which is capable of producing appreciable amounts of BC.

MATERIALS AND METHODS

Collection of Samples

Samples of different fruits, vegetables, and soil were collected from various places of Egypt. All the samples were stored in sterile bags at 4°C for further use.

Isolation of Egyptian bacterial strain can produced Cellulose

Samples (4 g) collected were different natural sources suspended in 95 mL sterile water. Serial dilutions of water samples were plated on nutrient agar plates. After incubation at 30°C for 72 h, cultural logogriphic bacterial colony was obtained. Purification of single colonies was done by dilution streaking on nutrient agar plates. Single colony cultures were maintained on nutrient agar. Each distinct purified colony and inoculated individually in the Hestrin-Schramm (HS) medium [14] containing (g/L), glucose, 20; yeast extract, 5; peptone, 5; Na_2HPO_4, 2.7; citric acid, 1.17; pH 6.0; and then incubated at 30 °C for 5 days in static condition.

The pellicle formed was collected and rinsed with water for three times. It was then treated with 4 N NaOH at room temperature for 24 h. To neutralize NaOH, the pellicle was treated with 6% acetic acid solution. It was again washed with water for three to four times. The purified pellicle obtained was dried at room temperature until a constant weight and then weighed for determining BC production.

Selection and identification of the most potent cellulose producer

The bacterial isolates that showed BC production were inoculated individually in HS medium and kept at 30°C for 5 days. Among these isolates, the most potential cellulose producer was selected by comparing the amount of BC produced (g/L) by each isolate. The most potent BC producer was identified by studying its cultural, morphological, and physiological characteristics and 16S rDNA sequence analysis. The bacterial isolate was gram stained and then its morphology was observed under light microscope optical light microscope (Olympus CH40, U-LH100HG, Japan). For studying cultural characteristics of the isolate, it was streaked onto nutrient agar plates, HS agar plates, and on HS agar plates supplemented with 10% ethanol and 2% $CaCO_3$, and its physiological characteristics of the potential producer was determined by adopting standard method [21].

Characterization of selected strain by 16S rDNA (Hylogenetic analysis)

The promising isolate was characterized and identified by 16S rDNA gene sequencing using universal primers ITS1 5′-ˊTCCGTAGGTGAACTTTGCGG-3′ and ITS4 5′-ˊTCCTCCGCTTATTGATATGC-3′ [22]. A single discrete PCR amplicon band was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out and then performs 35 amplification cycles at 94°C or 45 sec, 55°C for 60 sec, and 72°C for 60 sec. sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Bio Systems, USA). Sequencing products were resolved on an Applied Bio-systems model 3730XL automated DNA sequencing system. Data were submitted to GenBank database. The DNA sequence was compared to the GenBank database in the national Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the BLAST program [23].

Culture media for cellulose production

HS medium was used for cellulose production and the pH was initially adjusted to 5.5. Then, the medium was sterilized. Slant strain taken from the seed culture and was added into a flask and were incubated at 30°C for 5 days [24].

Detection of produced bacterial cellulose

The dry pellicle was added to 90 mL of 0.1 M acetate buffer pH 5 and 10 mL of 20% cellulase (from novo-enzyme Com.) and incubated at 40°C with shaking for 30 min to hydrolyze BC [25]. Then, the solution was centrifuged at 5000 rpm for 30 min. The precipitate was washed three times by deionized water and dried in an oven at 70°C overnight and then weighed to determine biomass.

Quantification of produced bacterial cellulose

The precipitated BC pellicle was treated with KOH (4%, w/v) at room temperature for one day to remove the bacterial cells and medium components [26]. The pellicle then washed with K_2CO_3 (6%, w/v) at room temperature for one day, the solution was centrifuged at 5000 rpm for 20 min (Sigma-Laborzentrifugen, 2K 215). The obtained cellulose was dried in an oven at 80°C overnight and then weighed, and values were calculated as g/L of the original media.

Factors Affecting BC production

As well as studies on stimulatory factors to optimize and maximize BC production:

Effect of incubation periods

The Modified HS medium (HS medium in addition 1% ethanol) was (50 mL in 250-mL Erlenmeyer flasks) was inoculated using a 48 h on old slant (10^5 CFU/mL) of *Achromobacter sp*. The inoculated flasks were incubated at 30°C either for 24, 48, 72, 96, 120 and 144 h under static conditions.

Effect of pH and incubation temperature

The effect of different pH of media on production bacterial cellulose of was studied by preserve the culture media pH at 4, 5, 6, 7 and 8, and then incubation temperatures 25, 30, 35, 40 and 45°C.

Effect of carbon sources

To study the effect of different carbon sources on the bacterial cellulose production, the following sugars were added to the Modified SH medium (sucrose, glucose, lactose and mannitol) at concentration 2% (w/v).

Effect of nitrogen sources

To investigate the effect of nitrogen source on BC production, further trials were done in 250-mL flasks. Yeast extracts, peptone and (yeast extract and peptone) in the ratio of 1:1 (w/w) and 1: 0.5 (w/w), in addition Ammonium sulfate and Sodium nitrate at equivalent 1%.

Pretreatments of crude molasses

The sugar cane molasses (SCM) was supplied from Sugar and Integrated Industries Company, El-Hawamdia, Giza. Sugar cane molasses was subjected to different pretreatments methods including removal of suspended matter by dilution and centrifuging at 6000 rpm for 20 min; 1N H_2SO_4 this were allowed to stand for 24 h and then centrifuged at 6000 rpm for 20 min [27]; and $Ca_3(PO_4)_2$ 1% [28]. The total sugars concentration following every pretreatment step was determined according to Duboies *et al.* [29] and then diluted with distilled water to an appropriate final carbohydrate concentration.

Characterization of BC

Analysis of monosaccharide composition

For monosaccharide composition analysis BC (0.2 g) was hydrolyzed with 2 mL of 90% formic acid at 105°C in a sealed tube for 6 h. Excess acid was removed by flash evaporation on a water bath at a temperature of 40°C and co-distilled with water (1 mL× 3) [30]. The monosaccharide contents were quantified by HPLC on a Shimadzu Shim-Pack SCR-101N column (7.9 mm x 30 cm), using deionized water as mobile phase (flow rate 0.5 mL/min) as described by Asker *et al.* [31].

Morphological analysis (TEM)

The morphology of BC produced from treated and untreated molasses was investigated by using JEOL, TEM-2100 Electron microscope.

Scanning electro microscope (SEM)

After freeze drying, the morphological investigations (SEM) of produced BC were characterized using scanning electro microscope. The scan viewed in a JEOL JXA- 840 electron probe micro analyzer, Japan.

Fourier transfer infra red (FT-IR) analysis

FT-IR spectra of the BC were obtained using a Nexus 670 FT-IR spectrophotometer (Lelet Co., USA). The spectra of sample in the range of 4000-800 cm^{-1} are investigated.

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RESULTS AND DISCUSSION

Isolation of Egyptian bacterial cellulose producer

Most than 100 bacterial were isolates from different natural origins screened and between those isolated as **MM 7** isolates demonstrated BC production. Occurrence the best number of cellulose producer and maximum production was found in fruits samples. The isolate M15 obtained from decomposed apple exhibited extreme BC production (1.5 g/L) and elected as the maximum effective cellulose producer. In past studies likewise, cellulose producers has been isolated from vinegar, vegetables, and fruits [32-35]. Comparable to the current study Toyosaki *et al.* [36], notified most professional deposition of cellulose producers from fruits and no segregation from soil specimens. In addition, as is the case in this study, Park *et al.* [37] gained *Gluconacetobacter hansenii as* a cellulose producing bacterium from a rotten apple.

Isolation and identification of bacterial cellulose producer

The isolated bacterial types were specified by their morphological, cultural and physiological properties. According to the result the isolate was characterized as *Achromobacter* sp. **Table (1).** The isolate was gram negative, short rods, motile, catalase positive and aerobe which metabolize different sugars such as glucose, fructose and mannose that it uses in cellulose synthesis. The colonies were smooth, circular, reddish brown, convex, and regular shape on the agar media. On agar plate of HS containing $CaCO_3$ and ethanol, a hydrolysis zone was spotted about the bacterial growth, which produced from the $CaCO_3$ solubilition through acetic acid produced via the oxidation of ethanol [34, 38].

Table 1. Morphological, cultural and physiological characteristics of the bacterial isolate

Characteristics	Bacterial isolate (M15)
Morphology	Gram negative, short rods, non-spore forming
Motile	Motile
Cultural	Circular, smooth, reddish brown
Physiological	Aerobic, catalase positive

The sequence analysis exposed that the isolate M15 was phylogenetically closely related to the genus *Achromobacter.*

Blast analysis of the 16S rDNA sequence of isolate revealed that the selected isolate showed maximum similarity of 99% with *Achromobacter* sp. and *uncultured bacterium.* The Phylogenetic relationship was obtained using national amalgamation by match up wise comparison among the 16S rDNA gene sequence of selected isolate with species. The daydream was constructed for their phylogenetic relationship and it revealed that the isolate *Achromobacter* sp. was clearly placed under separate clusters. The 16S rRNA gene sequences of the bacterial isolates had been subjected to the NCBI GenBank **(Figure 1)**.

Kadere *et al.* [39] scrutinized the amount and concurrence of the dominant spoilage genera of acetic acid bacteria in wine ,coconut by plating the dilution methods already pre-enriched in a basal medium, go after morphological, biochemical, and physiological tests. Five strains of acetic acid bacteria were isolated from wastes. Found that three of which are capable of secretion of cellulose. These strains were identified by numerous morphological, biochemical, and physiological tests while, the results were compared with reference strains *Gluconacetobacter hansenii* DSM 5602 and *Gluconacetobacter xylinus* DSM 46604 [34].

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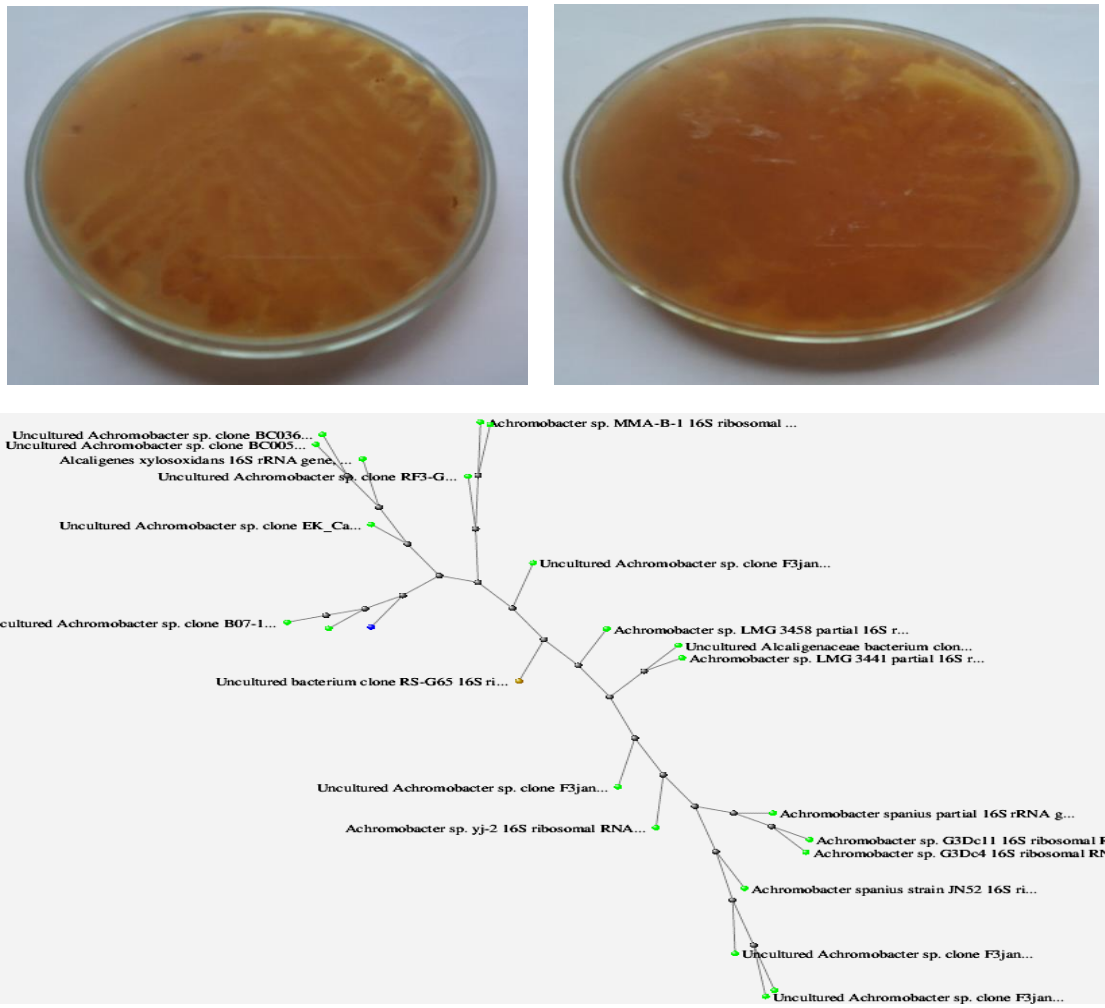

Figure 1: Phylogenetic tree of the partial sequence of 16S rDNA of the local isolate M15 with respect to closely related sequences available in GenBank databases

Effect of different fermentation media on production of bacterial cellulose

To obtain high productivity in fermentation, it is important to identify the best media and process conditions [40]. Bacteria convert media components into biomass and/or use them to synthesis metabolites. Supplying nutrients to the microbes affects products concentration, directly through metabolism and not directly by affecting production rate of the specific metabolite [41. Fermentation medium is important as it can affect production yield volumetric production and process cost [42]. The reason for the slow growth of these aerobic organisms is that the pellicles at the air/liquid interface form an effective barrier between atmospheric oxygen on one surface and the nutrient on the other, reducing the rate at which oxygen penetrates the pellicles to the cells within [443,44]. Production of BC was done in several media for production as reported, Mannitol medium, SH medium and Modified SH medium. *Achromobacter* sp. M15 was vaccinated in these media and brood at 35°C for 7 days. The results exhibited that Modified SH medium including (%) yeast extract, 0.5 ;glucose, 2; peptone, 0.5; Na_2HPO_4, 0.27 and citric acid, 0.117 and ethanol, 1 mL, pH 6 allowed maximum BC yield (2.5 g/L), as paralleled to the HS medium (1.7 g/L). So, Modified HS medium was chosen as basal medium to further optimization studies (**Figure 2)**.

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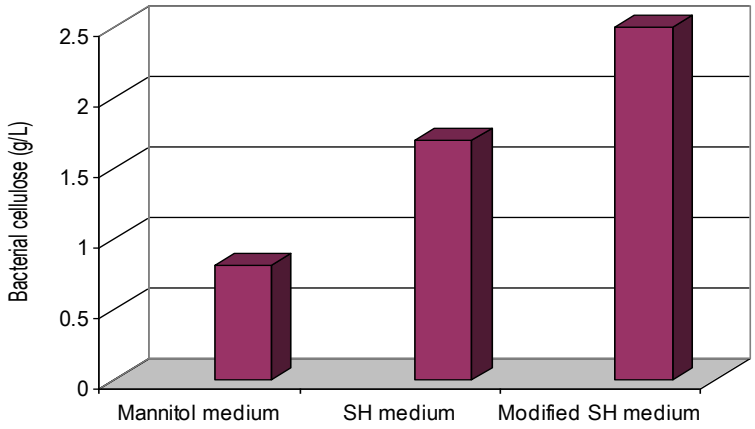

Figure 2: Comparison between *Achromobacter* sp. M15 in production using various media

The present of citric acid led to maximize the production of BC. It is believed that citric acid act by a mechanism in which it is oxidized and thus reduces the consumption of sugars in oxidative reactions [45]. Ethanol or different organic acids are known to induce microbial cellulose production [446, 47]. Ethanol acts as an inducer for BC production like it is capable of creating the reduced form of NADH which lowers the redox potential that should be substantial for optimal BC production [448, 49].

Incubation period in the optimal Medium for BC production

Various physiological and nutritional parameters, an optimal culture medium for BC production was obtained after optimizing. Furthermore, the incubation period time of BC production by *Achromobacter* sp. M15 was done in the optimal medium to time periods extend from 24 to 168 h at 35°C in constant culture condition. 0.2 g/L of BC was produced in the culture medium after 24 h of incubation time, and production gradually increased with time until to reach maximum (3.5 g/L) for 120 h of cultivation, after which the production becomes about constant **(Figure 3).** However, in the case of *G. oxydans* ATCC 15163 have the same trend but less productivity (2.4 g/L) [50]. Different author have acquired extreme BC production in diverse time periods be based on the physiological strain used with culture conditions [51-55].

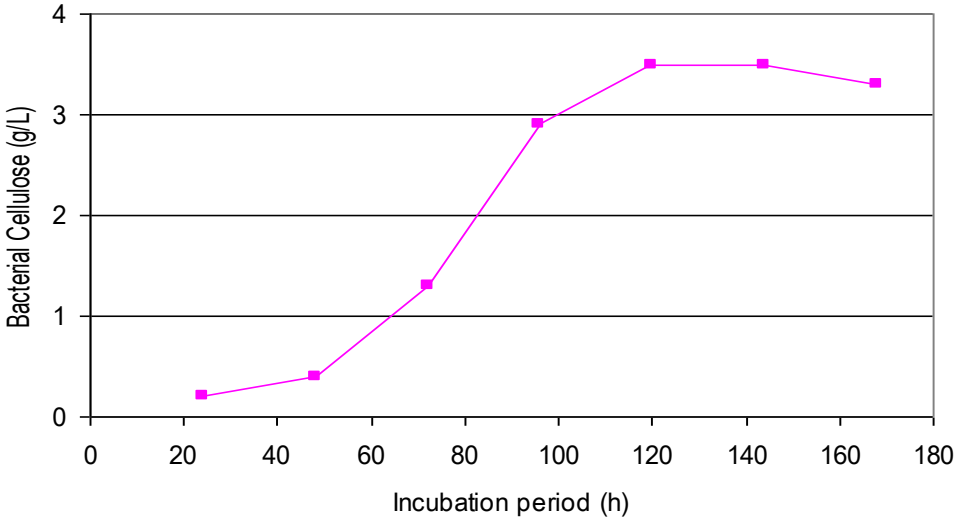

Figure 3: Effect of incubation period on production of BC from *Achromobacter* sp. M15

Effect of different carbon sources on BC biosynthesis

For economic production of BC, growth conditions that produce high BC yields need to be identified along with the how these conditions affect BC morphology and properties. In general, selection of carbon

source is an important agent like it is the major substrate for BC production. Likewise, in studies on parameters simulating the BC production, much awareness has been compensated to carbon origins. Herein, diverse carbon sources at an ultimate concentration of 2% were examined for their effects on the BC production.

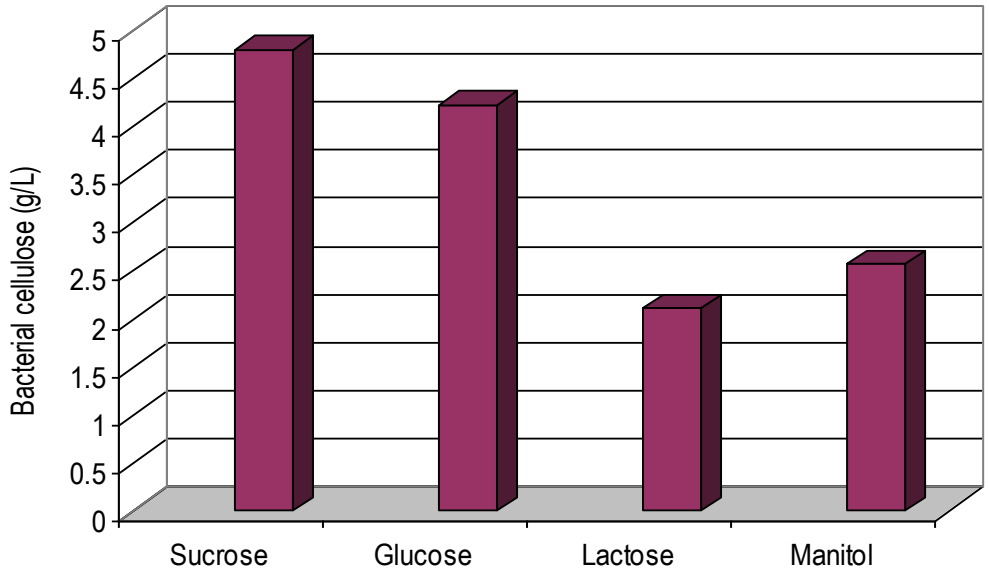

Figure 4: Effect of carbon sources on production of BC from *Achromobacter* sp. M15

Like shown in **Figure (4)**, a top level of BC production was gained from *Achromobacter* sp. M15 when sucrose, glucose were utilized, as carbon source. Between these, sucrose the carbon was found to be the best source for producing maximum BC (4.8 g/L) from *Achromobacter* sp. M15. On the other hand, the remaining sugars, lactose, and mannitol gave lower cellulose yields. New, several studies using different carbon sources, including monosaccharide (glucose, fructose, xylose), disaccharides (sucrose, lactose, maltose), alcohols (ethanol), sugar alcohols (mannitol, serbitol, glycerol, arabitol), and organic acids (citric acid, malic acid) have been done for making extreme BC production [51,56,57]. Glucose is the master carbon source for the production of BC like it not only acts as an energy source but also like a cellulose precursor [37, 55, 58].

Effect of several nitrogen sources on BC biosynthesis

Cellulose-producing bacteria also need a particular complex nitrogen source. Subsequently, the addition of the carbon source to the nitrogen source was very operative in the BC production [59]. In classify to examine the influence of nitrogen source on BC production; several nitrogen sources at a total nitrogen concentration of 1% (w/v) were tested. As shown in **Figure (5)**, *Achromobacter* sp. M15 grown in the medium including peptone and yeast extract in the ratio of 1:1 (w/w) and 1: 0.5 (w/w) produced highest amount of cellulose 4.85 and 4.25 g/L, respectively. But all the anther nitrogen sources and their combinations appeared minimize BC production.

Peptone (1% w/v)) and yeast extract (1% w/v) gave minimum BC production 2.0 and 2.82 g/L, respectively. While it was the best nitrogen source for *G. oxydans* ATCC 15163 is yeast extract 1% (3.9 g/L) [50]. Results acquired clearly detected that between all the nitrogen sources examined, yeast extract evidence to be the best nitrogen source for cellulose production via *Achromobacterr* sp. M15. Because yeast extract contains considerable nitrogen compounds like many growth parameters; consequently, its addition stimulates the BC production [55,60,61] .

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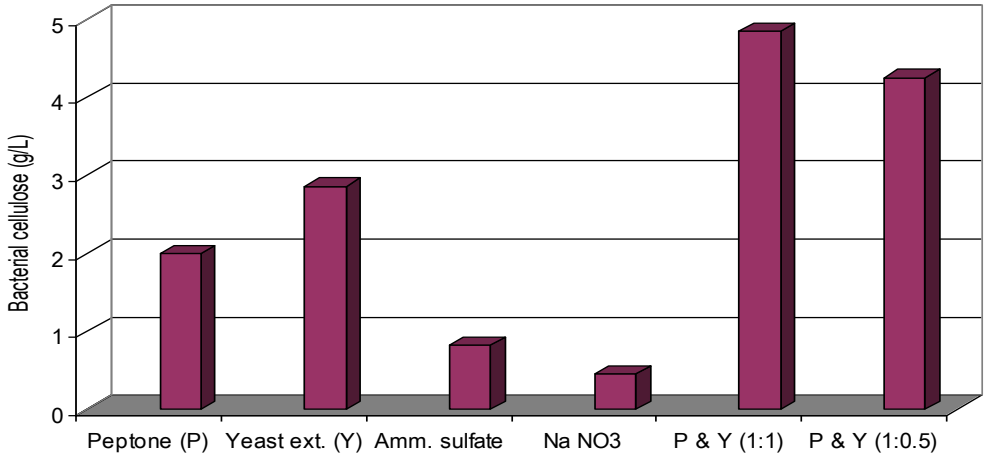

Figure 5: Effect of nitrogen sources on production of BC from *Achromobacter* sp. M15

Effect of pH on BC Production

Fermentation pH is an important factor affects cell growth and BC production so the effect of pH needs to be evaluated. The effect of pH was scrutinized via growing *Achromobacter* sp. M15 at various pH values extend from 4 to 8 [62, 63]. Maximum BC production (4.86 g/L) was obtained at pH 6 **(Figure 6)**, as well as the optimum pH of *G. oxydans* ATCC 15163 was similarly (2.98 g/L) [50]. The optimum C production from bacteria was experimental in the pH extend between 5 and 7; it is moreover explicit from the result gained that at over and below this pH range, BC production reduced. Past studies announce that the optimal pH reach for cellulose production is 4–7 [64,65]. Farthest of the researchers have notified optimum BC production at pH 5 [62,63,66]. Examination of the effect of pH on BC production appeared that the optimum pH depends on the strain physiology which differs from strain to strain. Generally, optimum pH range for the cellulose production via acetic acid bacteria is assumptive to be 4–7 like the acetic acid bacteria grow well in this range [67,68].

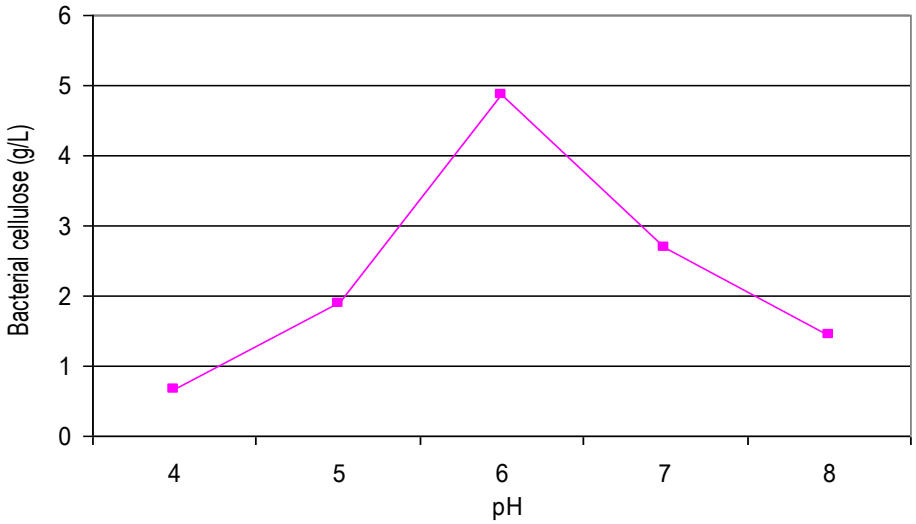

Figure 6: Effect of fermentation pH on production of BC from *Achromobacter* sp. M15

Effect of temperature on BC Production

Bacterial cellulose yield is temperature dependent because temperature affects both cell growth and synthesis of cellulose. It is an essential factor that impacts both the BC production and growth [49]. Thus, in the current study, next after optimizing temperature, pH for extreme BC production was optimized. *Achromobacter* sp. M15 was grown at distinct temperatures 25, 30, 35, 40 and 45 °C, favored maximum BC production (4.91 g/L) at 35°C **(Figure 7)**.

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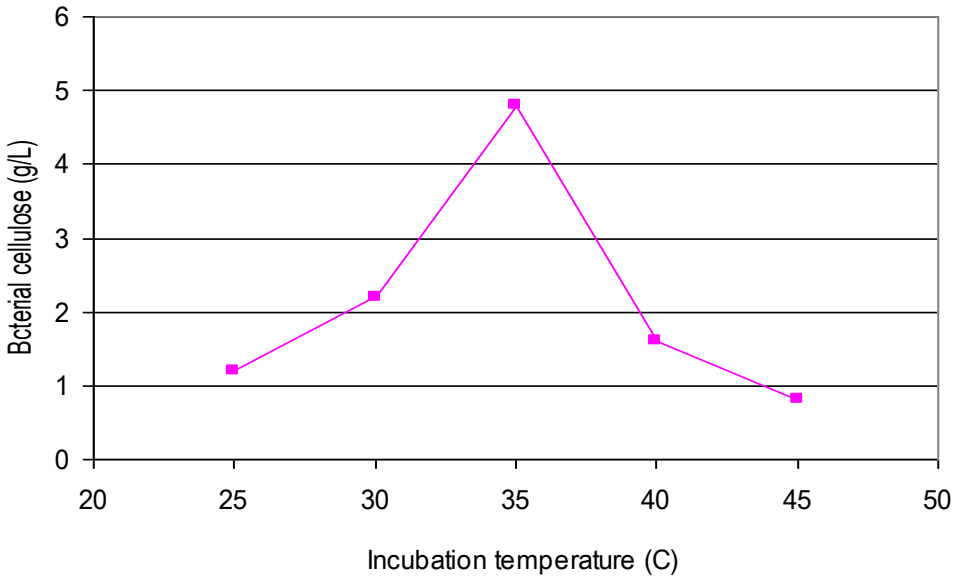

Figure 7: Effect of incubation period on production of BC from *Achromobacter* sp. M15

The local strain was isolated at 37°C, so optimum temperature it was 35°C. While the optimum temperature for *G. oxydans* ATCC 15163 was 30°C [50]. The BC production was found to reduce when the temperature was raised or reduced from 35 °C. Studies detected that the optimal growth temperature for cellulose production is 25–30°C [69,70]. While, in plurality of the studies, the maximum BC production was spotted among 28 and 30°C [14, 61,71,72]. These experimental results explain the best condition implied for BC biosynthesis in case of *Achromobacter* sp. M15, modified SH medium, glucose as carbon source, pH 6 temperature 35°C, yeast extract as nutrient medium and incubation period 5 days. The BC yield was (4.91 g/L) while as using the *G. oxydans* the maximum BC yields in case on using glucose as carbon source was (3.82 g/L) [50]. Our stability studies proved that the isolated strain *Achromobacter* sp. M15 is suitable for BC production in stationary cultures. For production low cost and economic productivity of BC, we used molasses as carbon source.

Treatment of Molasses

Cost of the fermentation medium, 30% of total cost plays a critical role over total cost in microbial fermentations [73]. So, one of the essential portions in the fermentation operations is need a novel cost-effective medium to gain, the highest product of BC. In the majority, pure sugars including glucose, sucrose, mannitol and fructose are used as fermentation media [74,75]. On the other hand, these carbon sources are not economical to use in industrial scale production of BC. New carbon sources for low cost and high cellulose yield needs to be found to produce BC. Molasses was originally pretreated by dilution and centrifugation steps. To increase the clarification and the sucrose degradation, H_2SO_4 and heat treatment were useful and precipitated solid content was then removed by centrifugation. Before using, the treated molasses pH should be adjusted to 6, for bacterial growth. The quantity of reduced sugar in molasses (19.3%) was greater than before pretreatment (23.6%) pointed to production capacity in this study. The presence of this quantity of reducing sugar in the treated molasses drives to less gluconic acid in the medium. Furthermore, molasses contains phenolic compounds; the pluralities of them have guaiacyl and syringyl units that are similar to lignin [76] which are not consumed enough and so the pH varied slightly and enhances the growth and synthesis of BC. Therefore, the presence of carbohydrate (sucrose, glucose and fructose), nitrogen (some amino acids), ployphenolic compounds , and mineral salts, in addition to its minimize cost, gives molasses the edge over using glucose to produce the BC [77]. Molasses have minerals and heavy metals that make toxic effects on microbial outgrowth and product synthesis [78]. To examine the effect of pre-treatment of molasses on BC synthesis, molasses was pre-treated by two distinct methods as aforementioned section pre-treatment molasses was extend at the total sugar concentration of 4.0% (w/v). The H_2SO_4 and $CO_3(PO_4)_2$ treated molasses gave higher BC production emulated with un-treated molasses. The towering BC production **(Table 2)** was acquired with H_2SO_4 + $Ca_3(PO_4)_2$ remedied molasses. While, the over value was minimize by 13% with untreated molasses. This proposes that molasses comprised 151.41% unfavorable substances which may

influence the synthesis of BC. Moreover, suspended impurities and heavy metals of molasses could be taken away by H_2SO_4 treatment, which has been used to raise the fermentative output of polysaccharide [78]. Cellulose output of *Acetobacter oxydans* (ATCC15163) carried out by the pre-treated molasses medium was else comparable with that in the intricate medium having pure glucose like the carbon source [50]. It is well renowned that *Acetobacter* strain; BC producing bacteria oxidize glucose into gluconic acid [14].

Table 2: Effect of pretreatment of molasses on bacterial cellulose production by *Acromobacter* sp.M15

Carbon source	BC (g/L)	Yield against CMS (%)
Crude molasses (CMS)	2.63	0.00
H_2SO_4 treated molasses	3.97	50.95
H_2SO_4 + $Ca_3(PO_4)_2$ treated molasses	6.61	151.41

Cultivation condition: modified media; pH= 6; temperature 35 °C; time 5 days from *Acromobacter* sp. M15.

The transformation of glucose into gluconic acid drives to a significant decline in pH of the culture broth and blocks the synthesis of BC. Therefore, in this study, reduce of BC output in the intricate medium having glucose as the single carbon source may be outcome from the minimum culture pH. In addition, the existence of a minimize amount of glucose in the molasses ingredients would drive to minimal gluconic acid in the molasses medium. It is clear from these results that molasses is a perfect carbon source to backing the BC output. Furthermore experiments on BC output were done by using $Ca_3(PO_4)_2$ treated molasses like a substrate. The maximum BC output by H_2SO_4 treated molasses raised to 43.31% more than that accomplished using un-treated molasses, mentioning that substances in molasses restrained to growth and BC synthesis were taken away to some degree [79, 80].

A slight reports have demonstrated that the sugar concentration in molasses could effectiveness metabolisms output [81]. The production of BC based on the initial concentration of overall sugar in molasses. BC production rose with the raise of initial total sugar concentration up to 5% (w/v), but then reduced further than this amount. Top BC yield gained was (5.95 g/L) at an initial total sugar concentration of 5%. Otherwise, informed Lazaridou et al. [17] reported that descend in polysaccharide output performed with rise sugar concentrations in the medium is possibly consequent osmotic influences; a minimize level of water activity as well as plasmolysis incidents could reduce the outgrowth rate and polysaccharide synthesis.

Characterization of BC

HPLC analysis

The monosaccharide of BC hydrolysate was measured by HPLC, wherein glucose as major sugars (98.75%) in case of *Acromobacter* sp. M15 **Figure (8)**.

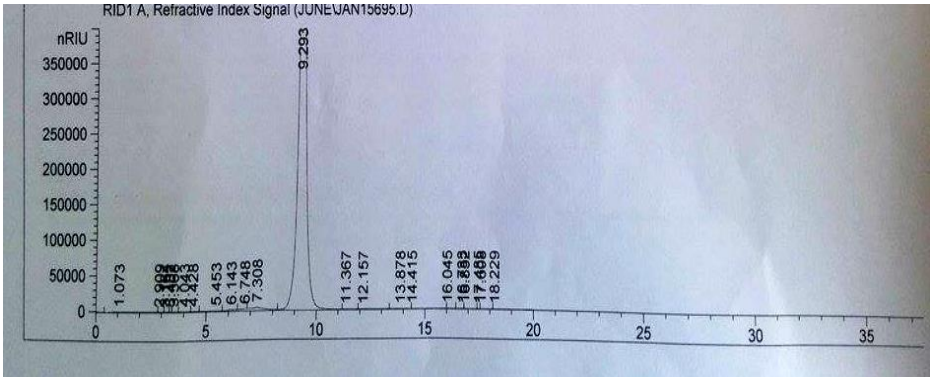

Figure 8: HPLC analysis of BC hydrolyzed from *Acromobacter* sp. M15

BC is chemically perspicuous, free of unwanted components such as hemicelluloses and lignin has high polymer crystallinity and high degree of polymerization that characterizes it from other shapes of cellulose [82, 83].

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FT-IR spectra

The FT-IR spectra of BC production by treated and untreated molasses as carbon source are shown in **Figure (9)**. The FTIR which show in the following three figures present indicates to bacterial cellulose. The absorption peak at 3427 -3438 cm^{-1} spectrum as broad band indicates to the free O-H stretching vibration of the OH groups in cellulose molecules [84], anther absorption peak at 2856 cm^{-1} and 2922 cm^{-1} indicates to the stretching vibration of the C-H group, the absorption peak at 1628-1631 cm^{-1} is attributed to the O-H bending of the absorbed water, the absorption peak at 1436-1447 cm^{-1} indicates to saturated CH_2. The peak at 1300-1313 cm^{-1} in this sample has been relayed to the bending vibration of C-O bond in polysaccharide of aromatic rings. The peak at 1077-1084 cm^{-1} is consequent the C-O-C of pyranose ring stretching vibration. In addition, the treatment process led to an increase in the intensity of 1396 cm^{-1} (asymmetric stretch of C-O-C and CH_2 deformation). we concluded that the bacterial cellulose produced on using glucose or molasses is agree with standard bacterial cellulose, and the highest yield was obtained on using treated molasses with H_2SO_4 then $Ca_3(PO_4)_2$.

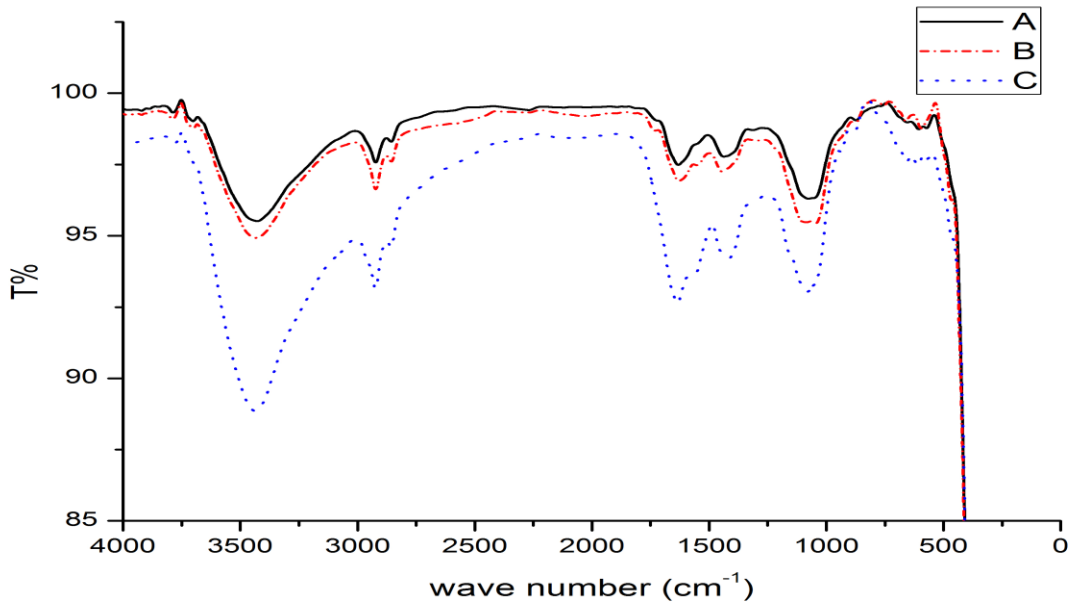

Figure 9: FTIR of BC produced under different treatment of molasses (a) unwashed, (b) KOH washed (c) KOH + K_2CO_3 reated

Scanning electron microscope (SEM) and TEM

Scanning electron microscopy (SEM) *w*as used to monitor morphology and BC pellicles structure. The BC produced by isolate*d s*train *Acromobacter* sp. M15 had the distinguished network structure of pellicles-shaped like the structure of the BC produced by *G. oxydans*, and it was composed of pure cellulose without any other impurities. We succeeded to produce BC using *Acromobacter* sp. M15 **Figure (10 &11)**. The surface morphology of produced BC owns a reticular lager with distribution channels and a uniform size a strict structure and a soft surface in all production [85]. The sub fibrils of cellulose are constantly ejected from linearly ordered pores at the superficies of the bacterial cell, crystallized into micro fibrils and forced deeper into the growth medium.

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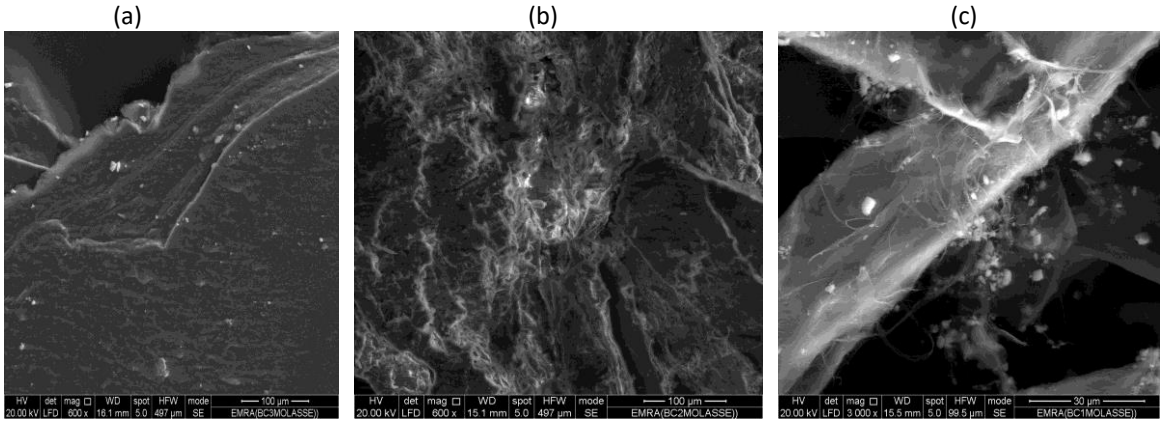

Figure (10). SEM images of BC produced under different treatment of molasses (a) unwashed, (b) KOH washed (c) KOH + K_2CO_3 treated

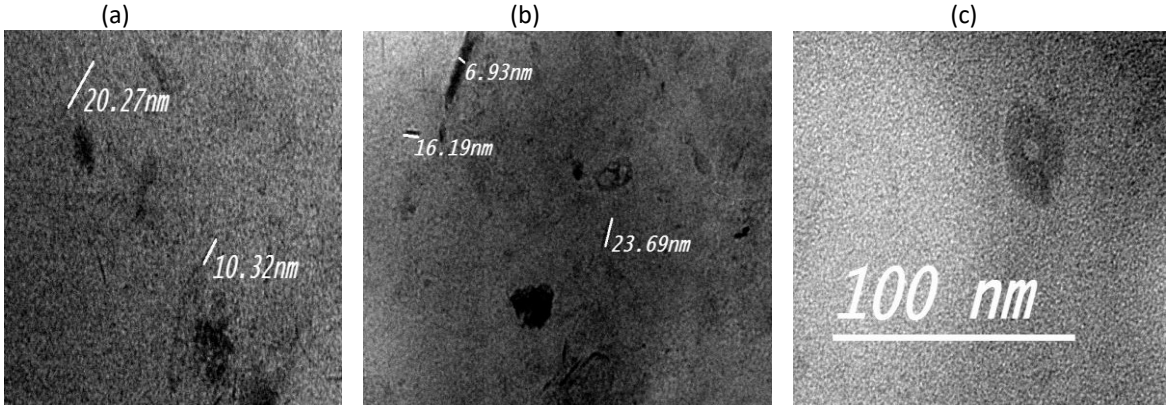

Figure 11: TEM Image of BC produced under different treatment of molasses (a) unwashed, (b) KOH washed (c) KOH + K_2CO_3 treated

The micrographs of BC produced in treatment molasses media extended evidence of the sturdy interfacial adhesion between the BC fibers, as offered by the perfect dispersion through the matrix, without remarkable aggregates. The open structure and great amount of smaller cellulose nano crystal aggregates are seen. Form analysis was made manually in order to characterize between widths, apparent thickness of edged on ribbons saw. The analysis performed did not exhibit the presence of significant divergences between the dimensions of nano fibers obtained. **Figure (12)** show the x-ray diffract gram of BC which demonstrated three characteristic peaks **(Figure 12)** existing at 14.60◦, 16.82◦ and 22.78◦, conforming to (1.10), (1.10) and (2.00) crystal planes. According to Bragg's law, the identity distances of each crystal plane are 0.606, 0.527 and 0.390 nm, indicating the presence of typical cellulose I crystal [86,87].

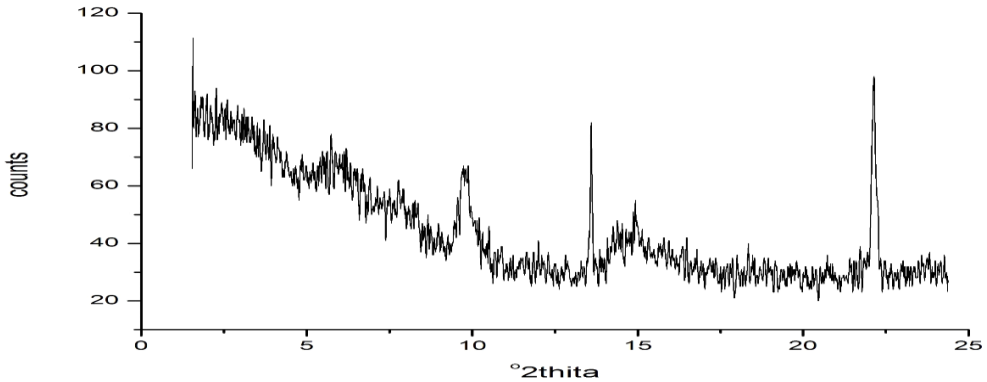

Figure 12: XDR of BC produced from KOH + K_2CO_3 treated

CONCLUSIONS

We used a cost efficient production method; we succeeded to isolate local bacteria from rotten fruit can producer higher quantity of bacterial cellulose using low cost environmentally friendly by product (molasses). Chemically treated molasses demonstrated a higher BC yield than the physically treated molasses. Egyptian isolated strain (*Acromobacter sp.*) yielded maximum quantity of cellulose when compared the American strain (*G. oxydans*) used in the study.

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