ISOLATION AND CHARACTERIZATION OF CELLULOSE NANO FIBER PRODUCING BACTERIAL STRAIN FROM FERMENTED FRUITS

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ABSTRACT

A bacterial cellulose (BC) producing strain isolated from fermented fruit. Twenty BC producing bacteria were isolated from each the isolation sources (fermented fruits). The most potent strain was identified to be *Komagataeibacter xylinus* SB3.1 based on several morphological characteristics, biochemical tests and 16srRNA. The *Komagataeibacter xylinus* SB3.1. was produce BC within pH 4–9 and exhibit maximum BC production (2.4 g/L) at pH 6 in under static conditions for 7 days. The structure of BC produced from the tested strains was assayed by scanning electron microscope it was revealed the diameter of thin ribbons ranged from 34.34 nm to 39.16 nm and exhibits higher porosity (81.5%).In comparison with the specimen from model BC producer, *Gluconacetobacter xylinus* 10245. Based on these analyses, the isolated *Komagataeibacter xylinus* SB3.1 can efficiently produce BC, which can be applied for industrial manufacturing with potential features.

Keywords: Bacterial Cellulose; *Komagataeibacter xylinus* , Nanocellulose , Acetobacter and fermented fruits.

1.Introduction

Bacterial cellulose (BC) is a promising natural polymer belongs to specific products of primary metabolism **(Retegi *et al*.2010).** Cellulose is synthesized by bacteria belongs to the genera of *Acetobacter, Rhizobium, Agrobacterium, Psuedomonas and Sarcina* **(Vu *et al*. 2008).** Many strain of *A. xylinum* are capable of producing cellulose in varying amounts and growing on wide varieties of substrates like glucose, sucrose, fructose, invert sugar, ethanol and glycerol **(White and Brown 1989).** Cellulose production by *Acetobacter xylinum* had been noted both in static as well as agitated cultures **(Chao *et al.* 2000).** The most efficient producer is gram-negative and acetic acid bacteria , *Acetobacter xylinum* (reclassified as *Komagataeibacter xylinum*) **(Yamada *et al.*2011)** .The bacteria was applied as a model microorganism for basic and applied studies on cellulose. *Acetobacter xylinum* is widely distributed in nature and is a common contaminant in the industrial production of vinegar by *Acetobacter aceti*. *Acetobacter xylinum* has been isolated from rotting fruits, vegetables and by fermenting coconut water **(Jagannath *et al.*2008)**

Presently BC is receiving great attention and being widely investigated as a new type of scaffold material due to its fine fiber network, biocompatibility , high water holding capacity , high tensile strength **(Putra *et al.*2008)** , high crystalline , high degree of polymerization, high purity , elasticity, durability, non -toxic and non- allergic **(Hei,1999,Backdahl *et al*.2006, Sherif and Kazuhiko 2006,El-Saied *et al*.2008,Liet *et al*.2009,Marzieh and Ali 2010,Denise *et al*.2011).** In food applications the BC was used as an additive, emulsifier, dietary fiber, edible preservative and as a barrier against bacterial growth **(Pacheco *et al*. 2004, Denise *et al.* 2011).** Recently, BC is used in many special applications such as a scaffold for tissue engineering of cartilages and blood vessels **(Yamanaka *et al.*1990, Klemn *et al.*1999 and 2001),** as well as for artificial skin for temporary covering of wounds **(Krystynowicz and Bieleck 2001).** Purified and dried BC was converted to a membrane to be used in the separation processes such as ultrafilteration, gas permeation and vapor permeation, and used in paper manufacture **(Luz *et al.*2006, Kuan *et al.*2009).**

The aim of the current investigations was to a new BC-producing strain from fermented fruit juice. The isolated strain was characterized based on colony morphology, specific biochemical tests and 16S r-DNA sequence analyses. The cellulose production ability of isolated strain was compared using six different types of culture media. The BC production abilities at different initial pHs were investigated. The materials properties of the produced BC such as morphology, and porosity were also evaluated.

2. Materials and Methods

Chemicals and Reagents

All chemicals used in present investigation were analytical grade and purchased from Hi-media, Sigma- Aldrich, Ranbaxy and Merck.

2.1. Fermented fruit juice preparation

Various fruits (pineapple, apple and guava) were purchased from local market in Cairo Egypt. Approximately 200 g of fruit dices were added to 1 L water containing 100 g of granulated sugar and 100 g of brown sugar. The mixed solution was stored at room temperature with a cover. After 10 days, a gelatinous membrane floated on the surface of the solution.

2.2. Isolation of BC-producing strain

The gelatinous membrane was homogenized using a waring blender 7011HS (Osaka Chemical Co. Ltd., Osaka, Japan), and then added 10% into HS medium at 28 °C, and spread onto Hestrin and Schramm (HS) **(Hestrin & Schramm, 1954)** agar (20 g of d-glucose, 5 g of peptone, 5 g of yeast extract, 2.7 g of Na_2HPO_4, 0.115 g of citric acid and 15 g of agar in 1 L deionic water) for 7 days. The single colony was picked to inoculate into 96-well plate with HS medium (same as HS agar without 15 g of agar) for 7 days. In pH resistant experiment, HS medium was used as base medium and adjusted to the desired final pH value (4–10) with HCl or NaOH. Finally, each cellulose producing strain was selected for further analysis.

2.3. Identification of BC-producing strain

Morphological, physical and biochemical analyses were carried out according to Bergey's Manual of Systematic Bacteriology **(Brenneretal.,2004).** Colony morphology such as Gram staining, production of catalase, production of water soluble pigment, oxidation of acetate or lactate, growth in the presence of 0.35% acetic acid, growth on 3% (v/v) ethanol in the presence of 5% acetic acid, requirement of acetic acid for growth, growth only in the presence of acetic acid, ethanol and glucose, growth on the medium of Carr and Passmore, growth on carbon source ethanol, growth in the presence of 30% (w/v) glucose and production of cellulose were evaluated. *Acetobacter xylinum* was used as the reference strain for biochemical characteristics **(Brenner *et al*., 2004).** The selected strains were also identified by using 16s rDNA sequencing. and genomic DNA was extracted for PCR on 16S rDNA with the forward primer: 5-AGAGTTTGATCCTGGCTCAG-3, and the reverse primer: 5-TACGGCTACCTTGTTACGACTT-3 PCR products were purified and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China) The sequencing results were submitted to BlastN for sequence alignment and homology comparisons against the NCBI GenBank data- base. The 16S rDNA of representative species were used for multiple sequence alignment with ClustalX software and the phylogenetic tree was constructed by MEGA 7.0 based on the Neighbor-Joining method with bootstrapping 1000 times

2.4. Influence of different culture media for cellulose production

Six types of media were tested for comparing the total yield (dry weight basis) of BC by the bacterial isolate. The compositions of various media (pH adjusted to 5-6) are as follows:

HS medium **(Hestrin and Schramm, 1954),**complex medium(COM) **,(Kamide *et al*.,1990)**, Gluconobacteroxydans medium(DSM) **(Timke *et al*.,2005),** sterile distilled water supplemented with ethanol (4%), SEED medium **(Sudsakda *et al*.,2007)**, and glucose-ethanol acetic acid medium(GAM) **(Hanmoungjai *et al,* 2007)**, sterile distilled water supplemented with ethanol (4%), SEED medium **(Sudsakda *et al*.,2007),** and glucose-ethanol acetic acid medium(GAM) **(Hanmoungjai *et al,* 2007),** GEM medium **(Son *et al*., 2002)** were used throughout this investigation for bacterial cellulose production. The optical density of cell growth (O.D/620nm) was measured and the pellicle formed at the air-liquid interface of the production medium was collected and rinsed with water for two to three times. It was then treated with 1 N NaOH at 80°C for 20 min. to neutralize NaOH, the pellicle was treated with 5% acetic acid solution. It was again washed with water for three times. The purified pellicle obtained was dried at 60°C until a constant weight and expressed as g/l dry BC weight, and cellulose yield (%) was calculated, according to **Gamal *et al*. (1991).**

$$\text{Yield (\%)} = \frac{\text{Dry cellulose production (g/l)}}{\text{Original Sugar (g/1)}} \times 100$$

2.5. Effect of initial pH

The selected strain was also incubated into HS medium with different pH conditions (4–10) for 7-day cultivation to confirm the effects of initial pH value for BC production. Porosity was calculated using the equation of **Kouda *et al.* (1997)**

Porosity% = (wet weight – dry weight)/ (wet weight –weight in water) x100. Dried bacterial cellulose membranes were soaked in deionizer water for more than 12h at room temperature, and the weight in water was measured by harnessing the sample in advice which suspended the sample in water **(Al-shamary and Darwash, 2013).**

2.6. Scanning Electron Microscopy (SEM)

BC films were frozen at –80 ºC for 24 hours and freeze-dried for 72 hours. The pellicles were metalized by platinum sputtering and analyzed by scanning electron microscopy (SEM) on a Zeiss DSM-940A microscope at 30 keV. The diameter of 50 nanofibers was determined using the ImageJ program (National Institute of Health-NIH)

2.7. Statistical analyses

Statistical evaluation of all experimental data (variation from basal values) were performed using ANOVA.All pairwise-multiple comparisons were performed using Holm-Sidak test. This test is more powerful to detect differences than Tukey's and Bonferroni's tests and is recommended as the first line procedure for most multiple comparisons testing **(Systat Software, 2011)** SigmaPlot® 12.5 software extended with a statistical package and Graphs were plotted in Microsoft™ Excel® 2013 was used. The graphed values are represented as means and error bars. The error bar represents the standard error means calculated from standard deviations. USA) ($p < 0.05$)

3. Results and Discussion

3.1. Isolation and identification of cellulose-producing strain.

Twenty bacterial strains producing a BC pellicle on the HS medium were obtained from three fermented fruits after streaking on the HS agar medium, colony shapes were shown to be similar to those of reference strains, which was Gram negative. All the isolates were examined for the BC productivity in the HS medium as showen in Fig(1).

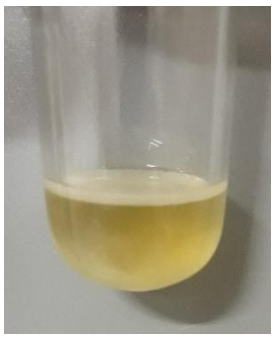

Fig (1): Photograph image of bacterial cellulose pellicle in culture medium HS

Since strain SB3.1 showed the best productivity, it was used in the subsequent studies. As is shown in Fig. (2), under 1000x magnification, the bacterial cells appeared in short rod shape and were Gram- negative. Colony and cell morphology were consistent with the *Gluconobacter* in Manual of Systematic Bacteriology.

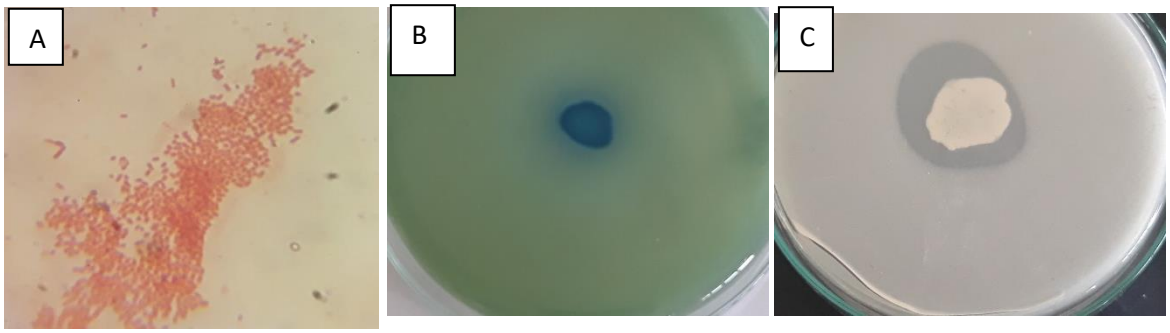

Fig. 2: Cell morphology of strain SB3.1 (A), growth on Passmore medium (B), Growth on Carr medium (C)

In biochemical characteristics analysis, the strain SB3.1 did show the identical physiological and biochemical characteristics as *Acetobacter xylinum* and reference in Bergey's Manual of Systematic Bacteriology **(Table 1).** *Acetobacter xylinum* and reference exhibited minimal BC production when cultured in the medium of Carr and Passmore. However, the strain SB3.1 can produce large amount of BC in Carr and

Passmore medium, suggesting that the strain SB3.1 is a new strain and named as *K. xylinus* SB3.1.

Table 1 : Physiological and biochemical characteristics of (*K. xylinus*) SB3.1

	Characteristics	***Acetobacter xylinum (reference strain)***	**SB3.1 Isolated**
1	Gram stain	–	–
2	Production of catalase	+	+
3	Production of water soluble pigment	–	–
4	Growth in the presence of 0.35% acetic acid (pH 3.5)	+	+
5	Growth on 3% (v/v) ethanol in the presence of 5%	+	+
6	Requirement of acetic acid for growth	–	–
7	Growth only in the presence of acetic acid, ethanol and	–	–
8	Growth on the medium of Carr and Passmore	+	(+)
9	Growth on carbon source ethanol	+	+
10	Growth in the presence of 30% (w/v) glucose	+	+
11	Production of cellulose	+	+

comparing with description in Bergey's Manual of Systematic Bacteriology.

The 16s rDNA fragment (1050 bp) of selected strain SB3.1 was amplified by using PCR technique. The analysis of 16s rDNA sequencing indicated that 16s rDNA fragment from the selected strain SB3.1 showed 99% similarity with the sequence of *Komagataeibacter xylinus* NBRC 11664. Hence, the strain SB3.1was identified as member in the *Komagataeibacter* genus **(Fig. 3)**.

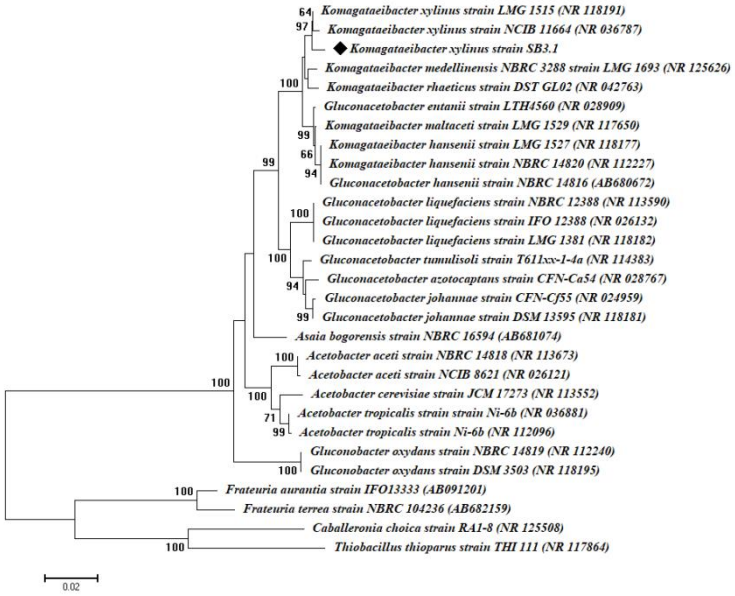

Figure. 3. A phylogenetic tree based on 16S rDNA sequences constructed by the neighbor-joining method.

3.2. Influence of various growth media on the production of bacterial cellulose

The results presented in Fig. (4) reveal that the maximum yield of bacterial cellulose was produced in GEM and COM media. Approximately 12.6 and 11.5 % of cellulose yields were observed, respectively following inoculation with the isolate and incubation for 7 days under static conditions. GEM were selected as the best media for production of cellulose as well as yield.

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 **(Suwanposri *et al.*, 2013).**Similarly, many literatures reported the effect of various growth media on the production of cellulose **(Mohammadkazemi *et al.*, 2015).**

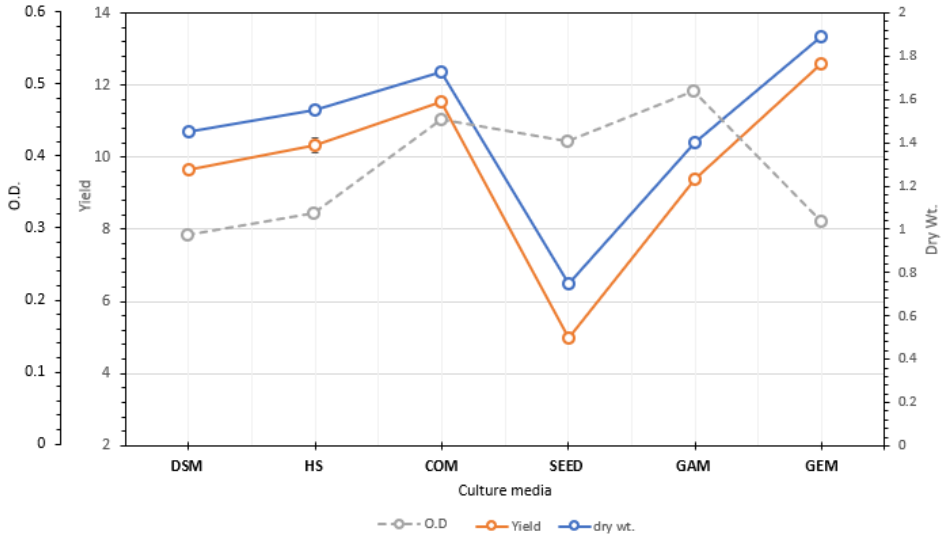

Fig4: Effect of various media on the yield of cellulose produced by *K. xylinus* SB3.1

The effects of the initial pH of the medium on BC production were examined. When *K. xylinus* SB3.1 was cultured at 28^{o} C for7 days at various initial pHs of 4, 5, 6, 7, 8, and 9, the BC yield at initial pH 6.0 was the highest with 2.4 g/l (fig 5). On the other hand, BC production decreased noticeably below initial pH 4 and above pH 8. Several studies showed that the pH value range for cellulose production was about 4–9 **(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).**

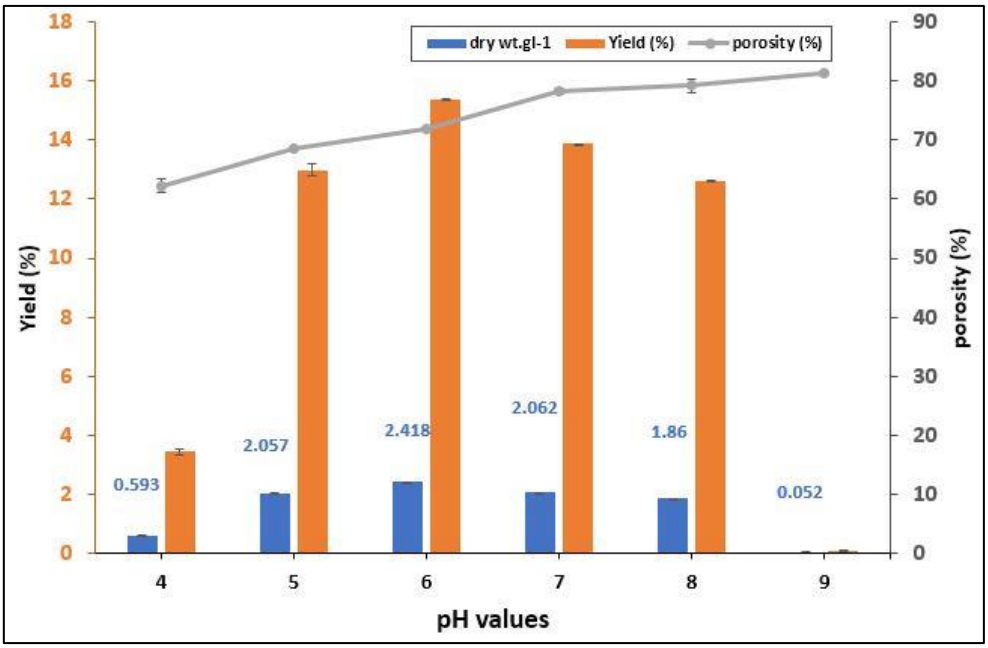

Fig. 5. The effect of variations of pH values on BC yield and biomass by *K. xylinus* SB3.1

SEM results (Fig. 6) provided the surface images of BC produced from *K. xylinus* SB3.1 The morphology of BC samples from *K. xylinus* SB3.1 displayed nano scale network structure (Fig. 4), and its fiber size distribution was around 25–45 nm, slightly smaller than BC from G. xylinus 23769 These results are close to the previous studies (**Luz *etal*., 2006**). Scanning electron micrograph results demonstrated that BC produced from *K. xylinus* SB3.1 is similar to cellulose in morphology.

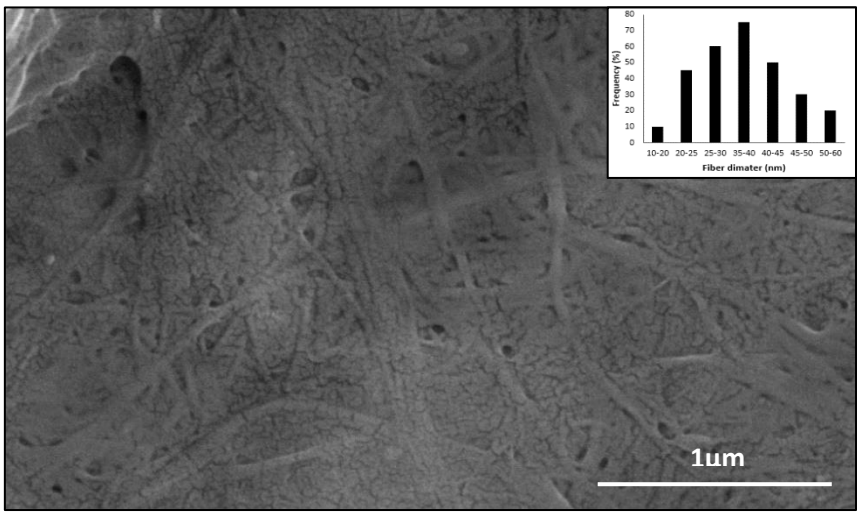

Fig. 6. Scanning electron micrograph (SEM) micrograph of bacterial cellulose produced by *K. xylinus* SB3.1

Conclusion

The isolated strain was identified to be *K. xylinus SB3.1* based on biochemical tests and 16S r-DNA analyses. The bacterial isolate produced cellulose in traditional and modified media. Significant yield of bacterial cellulose was obtained using GEM culture medium and at pH6. Experiments are in progress to characterize bacterial cellulose and optimization of conditions for its enhanced production.

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عزل وتوصيف لسلالة بكتيرية منتجة لألياف النانوسيليلوزالمنتجة من فواكة مخمرة

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تهدف الدراسة الحالية على عزل سلالة بكترية قادرة على انتاج الياف النانو سليلوز البكتيرى , الذى يمتلك العديد من الخصائص ، حيث له قدرة عالية على الاحتفاظ بالماء ومقاومته، كما له مساحة مسامية بين أليافه مما يجعله مصفاة استثنائية. كما أنه متوافق حيويا مع الجسم، فحينما يستخدم كضمادة يتحلل بيولوجيا بسهولة، وهو مستقر حراريا ويتحمل الضغط الميكانيكي بدرجة واسعة من القدرة بسبب ثبات بلّوراته.

وتكمن اهمية البحث فى عزل وتوصيف لسلالة بكتيرية لها القدرة على انتاج السليلوز البكتيرى ذات خصائص عالية ومعدل انتاج عالى مقارنة بالعزلات الاخرى والمعرفة لدى بنك العزلات البكتيرى الامريكي.

وللوصول إلى هذه الاهداف تم عزل عشرين سلاله بكتيريه من الفواكه المخمرة والتى تم جمعها من محلات بيع الفواكه فى محافظة القاهرة. واعتمادا على مقدار انتاج الياف النانو السيلوز البكتيرى تم تحديد السلالة الاكثر فعالية وهى SB3.1. واستنادا الى الصفات المورفولوجية (المستعمرات، الفحص المجهري لشكل الخلية وترتيب الخلايا) وكذلك الصفات الفسيولوجية والبيوكيميائية ثم دعمت النتائج بالتعريف الجزيئي باستخدام 16srRNA، وقد أدت الدراسة إلى تعريفها Komagataeibacter xylinus SB3.1 .كما انه تم دراسة إنتاج الياف النانوسليلوز البكتيرى من خلال التغير فى الرقم الهيدروجينى للوسط الغذائى من 4الى9 واظهرت النتائج أقصى إنتاج الياف السيلوز كان (2.4 جم / لتر) عند الرقم الهيدروجيني 6 في ظل ظروف تحضين ثابتة لمدة 7 أيام. ثم بعد ذلك تم فحص بنية الياف السيلوز المنتجة من السلالات المختبرة عن طريق مسح المجهر الإلكتروني ، وتم الكشف عن أن قطر الالياف يتراوح من 34.34 نانومتر إلى 39.16 نانومتر ويظهر مسامية أعلى (81.5 ٪). بالمقارنة بنتائج مع دراسات لعازلات مشابهةGluconacetobacter xylinus 10245 .

وقد توصلت النتائج إلي أن يمكن للسلاله المعزوله محليا انتاج الياف النانو سليلوز البكتيرى بكفاءة عاليه ، وذات خصائص فريدة والتى ترشح استخدامه و تطبيقه على التصنيع الصناعي مع الميزات المحتملة.

الكلمات المفتاحية : السيليلوز البكتيري، أسيتوباكتر إكسيلينيوم، النانو سيلو، اسيتوباكتر الفاكهة المخمرة