Original Article

APPLIED FOOD BIOTECHNOLOGY, 2016, 3(1): 35-41
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Comparison of Bacterial Cellulose Production among Different Strains and Fermented Media

*Maryam Jalili Tabaii and Giti Emtiazi**

Department of Biology, Faculty of Science, University ofIsfahan, Isfahan, Iran.

Abstract

The effect of different carbon sources on bacterial cellulose production by *Gluconacetobacter xylinus* (PTCC 1734) and two newly isolated strains (from vinegar) under static culture conditions was studied. The production of bacterial cellulose was examined in modified Hestrin-Shramm medium by replacing D-glucose with other carbon sources. The results showed that the yield and characteristics of bacterial cellulose were influenced by the type of carbon source. Glycerol gave the highest yield in all of the studied strains (6%, 9.7% and 3.8% for *S*, A_2 strain and *Gluconacetobacter xylinus* (PTCC 1734*)*, respectively*)*. The maximum dry bacterial cellulose weight in the glycerol containing medium is due to A_2 strain (1.9 g l^{-1}) in comparison to *Gluconacetobacter xylinus* as reference strain (0.76 g l^{-1}). Although all of the studied strains were in *Gluconacetobacter* family, each used different sugars for maximum production after glycerol (mannitol and fructose for two newly isolated strains and glucose for *Gluconacetobacter xylinus)*. The maximum moisture content was observed when sucrose and food-grade sucrose were used as carbon source. Contrary to expectations, while the maximum thickness of bacterial cellulose membrane was attained when glycerol was used, bacterial cellulose from glycerol had less moisture content than the others. The oxidized cellulose showed antibacterial activities, which makes it as a good candidate for food-preservatives**.**

Article Information

Article history:
Received 18 Nov 2015
Revised 6 Dec 2015
Accepted 16 Dec 2016

Keywords:
Bacterial cellulose,
Carbon sources,
Fermentation media,
Gluconacetobacter,
Optimization

Correspondence to:
Giti Emtiazi
Department of Biology, Faculty of Science, University of Isfahan, Isfahan, IR Iran
Tel: + 98-311-7932457
Fax: + 98-311-7932456
E-mail: emtiazi@sci.ui.ac.ir

1. Introduction

In food science, polymers can improve food quality and safety. Bacterial cellulose (BC), produced by *Gluconacetobacter (G.) xylinus,* is a promising polymer that has been widely accepted as one of the stiffest multifunctional biomaterials in food industry [1]. BC contains sets of parallel glucan chains linked with highly regular intra- and inter-molecular hydrogen bonds [2]. The unique ultrafine reticulated structure of BC offers interesting chemical and physical properties such as biocompatibility, high water holding capacity, high tensile strength, high crystaline structure, high purity, high degree of polymerization, elasticity, durability, stability, non-toxicity, hydrophilicity, good sorption ability for liquids, non-allergenicity, biodegradability, and rheological properties. Recently, BC is receiving great attention, and being widely investigated as a new type of polymeric material [3, 4]. BC and its derivatives have a multitude of applications in food industry [5-8]. In food applications, BC is used as an additive, emulsifier, dietary fiber, edible preservative and as a barrier against bacterial growth [9]. Furthermore, it has potential application as thickening, gelling, stabilizing and water-binding [6]. BC is traditionally used to make Nata de coco, an indigenous dietary fiber of South-East Asia that is served as gelatinous cube with chewy like textural properties. Mesomya et al. (2006) reported that Nata de coco has the ability to reduce the consumer's blood lipid level [10]. Processing of BC with sugar alcohol could change the texture of the gelatinous gel. It has been shown that the texture of BC, when processed with sugar alcohols, is comparable to grape, making it suitable for salads, low calorie desserts and other food items [11]. Despite its enormous potential in various applications [5-10], the low yield and the high cost of

BC production are the main drawbacks that hinder its industrial implementation [7].
With screening of high-yield strains, optimization of medium composition, and selection of suitable cultivation methods, we can enhance the yield of BC production. Since, the carbon source is one of the most important parameters involved in the BC production; various research have been conducted on carbon sources to increase BC production. Depending on bacterial strain, the best reported carbon sources are different [1].

In this study, effectively production of BC for forthcoming uses, the effect of various carbon sources on the BC production of native strains was investigated.

2. Materials and Methods

2.1. Microorganism and stock culture

Three strains were exploited for the production of cellulose in this work. *G. xylinus (*strain number 1734*)* was obtained from the Persian Type Culture Collection. The two other strains (A_2 and *S*) were recently isolated from the traditionally fermented vinegars in Iran, and according to 16 S rRNA sequencing, these wild type isolates belong to the *Gluconacetobacter sp*. The microorganisms were maintained in the test tubes containing D-glucose (100 g l^{-1}), yeast extract (10 g l^{-1}), peptone (5 g l^{-1}), $CaCO_3$ (20 g l^{-1}), and agar (25 g l^{-1}). The stock cultures were stored at 5˚C to slow down growth and cellulose production.

2.2. Production of BC and culture condition

All chemicals were of analytical grade from Merck Co., Germany. BC was produced statically in Hestrin-Schramm (HS) medium (30 ml) composed of D-glucose (20 g l^{-1}), peptone (5 g l^{-1}), yeast extract (5 g l^{-1}), Na_2HPO_4 (2.7 g l^{-1}) and citric acid (1.15 g l^{-1}) (pH 6.0) at 28˚C for 20 days. D-glucose was replaced by other carbon sources in modified HS media. The prepared 30 mL culture media in 100 mL Erlenmeyer flasks were sterilized by autoclaving and were inoculated at 3% v v^{-1} concentration. In all experiments, triplicate flasks were prepared for each treatment. Primary inocula were prepared by transferring five colonies from the HS medium working culture plate into 30 mL of HS media.

Incubations were performed at 28˚C for 3 days under static conditions. After incubation, the broths were shaken vigorously to (partially) release attached cells from the cellulose pellicles. The resulting cell suspensions were used as inocula in subsequent experiments. Cellulose formation was monitored by the appearance of a white pellicle on the surface of the culture broth. However, the pellicles produced by acetic acid bacteria were not essentially cellulose; thus, an additional purification treatment was required for confirmation of cellulose structure.

The pH of the remaining medium was measured after the cellulose sheets were harvested.

2.3. Purification of BC

The obtained gel-like BC pellicles were purified by washing three times with distilled water. Then they were boiled in a 0.5 M aqueous solution of NaOH for 15 min. Cellulose is resistant to this treatment, and thus the remaining material was accepted as cellulose free from microbial cells and medium components. The obtained BC thin sheets were washed several times with deionized water until the pH of water became neutral. Next, they were afterward stored in deionized water at room temperature prior to use

2.4. Evaluation of BC production

2.4.1. Thickness of BC

Thickness of each dried bacterial cellulose membrane obtained from different carbon sources was measured at 10 different positions by a digital outside micrometer (Accud, China, code: 311-001-01Q), and the values were averaged.

2.4.2. Determination of wet weight /dry weight of cellulose

The wet weight and dry weight of the purified microbial cellulose were recorded. BC production was recorded as dry weight of BC within the volume of medium (g l^{-1}). To determine the dry weight of the cellulose sheets, they were dried at room temperature for three days until their weights became constant.

2.4.3. Yield of BC.

The yield of the biosynthesis process was calculated by Eq. 1:

$$\text{Yield } (\%) = (m/c) \times 100 \qquad \text{Eq. 1}$$

Where, m is the dry weight of BC (g) and C is the weight of carbon source (g) used in the production medium.

2.4.4. Moisture content of BC

The moisture content (%w/w) of bacterial cellulose was determined based on the weight loss of BC when dried.

$$\text{Moisture content } \% = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100$$

2.5. Assay of antimicrobial activity

BC sheet was oxidized by hydrogen peroxide for 6 h to obtain hydrogen-peroxide-oxidized BC. Then it was rinsed with deionized water several times. The antimicrobial activity of the oxidized BC was investigated against *Escherichia coli* as the model Gram-negative bacterium, which was pre-cultured at 37˚C to reach a concentration of about 0.5 McFarland standard. BC sheet was cut into discs (1 cm*1 cm) for use. The zone of inhibition test was adopted to evaluate the antimicrobial activity. The test sample was placed on the *E. coli* growth agar plate and incubated at 37˚C for 24 h. The inhibition zone was calculated by measuring the diameter of the

nearest whole millimeter of the inhibited growth around the sample disk.

3. Results and discussion

3.1. Effect of carbon sources on BC production

In this study, the effect of various carbon sources, i.e. monosaccharaides (glucose and fructose), disaccharides (sucrose and food-grade sucrose), sugar alcohols (glycerol and mannitol), whey, and food-grade starch on the dry weight, yield and pH of BC production in three native strains was investigated. As shown in Figure 1, BC productivity of all strains was increased in the presence of glycerol as the sole carbon source. These findings are in agreement with those obtained by others [12-18].

The glycerol led to increased BC yield, approximately 2.2, 8.9 and 1.5 times more than those produced using glucose medium for *S* strain, A_2 strain and *G. xylinus* PTTC 1734, respectively (Figure 2). The BC yields from whey and food-grade starch were low. Although BCs consisted of repeating units of glucose, but the best carbon source patterns for BC production were different among these strains. In *S* and A_2, mannitol and fructose were in the next position after glycerol while in *G. xylinus*, glucose was the second one. Many researchers have found that mannitol gives the highest productivity for cellulose production [19-21].

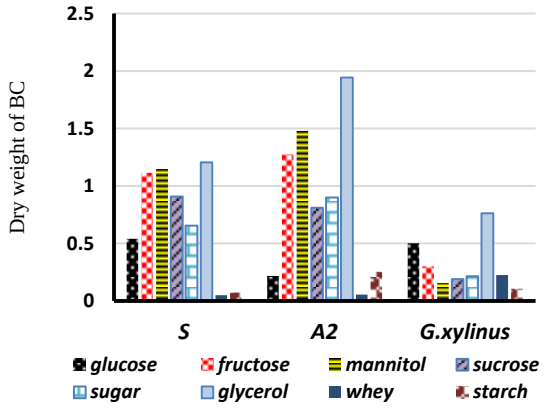

Figure 1. Bacterial cellulose production from various carbon sources

All these results show that the synthesis process of cellulose in bacteria is complex and is affected by many factors. The enhanced BC production could depend on effective utilization of carbon source. Since different bacteria have diverse enzymes and metabolic differences, they can utilize various types of carbon for growth and BC production at different efficiency. Carbon source is key precursor required for glucose synthesis by entering into two main pathways: the pentose phosphate cycle and the Krebs cycle [16].

Glucose was easily transported through the cell membrane and incorporated into the cellulose biosynthetic pathway; however, it was indicated that

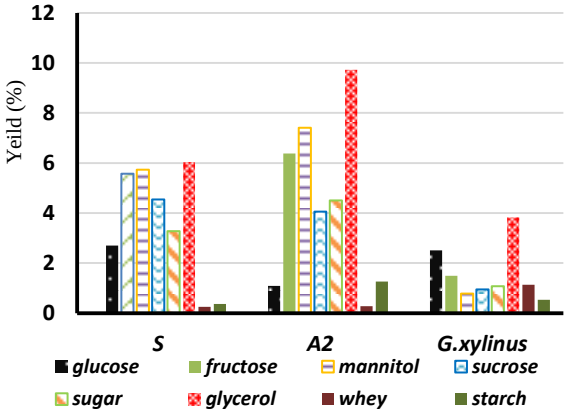

Figure 2. Bacterial cellulose yield (%).

the majority of glucose was converted into the byproduct gluconic acid, which will decrease the pH of the culture, and will ultimately cause lower BC production. In contrast, glycerol switched the pathway from the pentose cycle to the Krebs cycle, which further produced BC without the formation of gluconic acid [16]. This could explain the increased efficiency in BC production and smaller pH fluctuation in our strains when glycerol was used as the sole carbon source compared to glucose. Jung et al. (2010) reported that when glycerol and fructose were as carbon sources, the acidic compounds in the growth medium were utilized through TCA cycle to generate energy and promote cell growth and BC production [18]. Like glycerol, Fructose was able to enter the pentose phosphate pathway or gluconeogenesis pathway, and could easily generate the intermediate (UDP-glucose) for cellulose synthesis [16].

The cellulose synthase enzymes' cascade has different catalytic active sites with different domains, thus different sub species utilize various sugars to produce maximum BC. Figure 3 shows the graphical image of cellulose synthase domains in *Komagataeibacter (K.) xylinus E25*. Four protein domain families are seen: CESA_CelA_like domain (Locat-ion: 146 → 378) (putative catalytic subunit of cellul-ose synthase) belongs to the family of proteins that are involved in the elongation of the glucan chain of cellulose. DXD motif in the catalytic site could be in binding with the metal ion that is used to coordinate the phosphates of the NDP-sugar in the active site. BcsB or bacterial cellulose synthase subunit (Location: 814 → 1421) is of the family that includes bacterial proteins involved in cellulose synthesis. This family encodes a subunit or a regulatory domain that is thought to bind the allosteric activator cyclic di-GMP. This subunit is found in several different bacterial cellulose synthase enzymes. PilZ domain (Location: 569 → 668) is a c-di-GMP binding domain. CelA is cellulose synthase catalytic subunit (UDP-forming) (Location: 41709) [25].

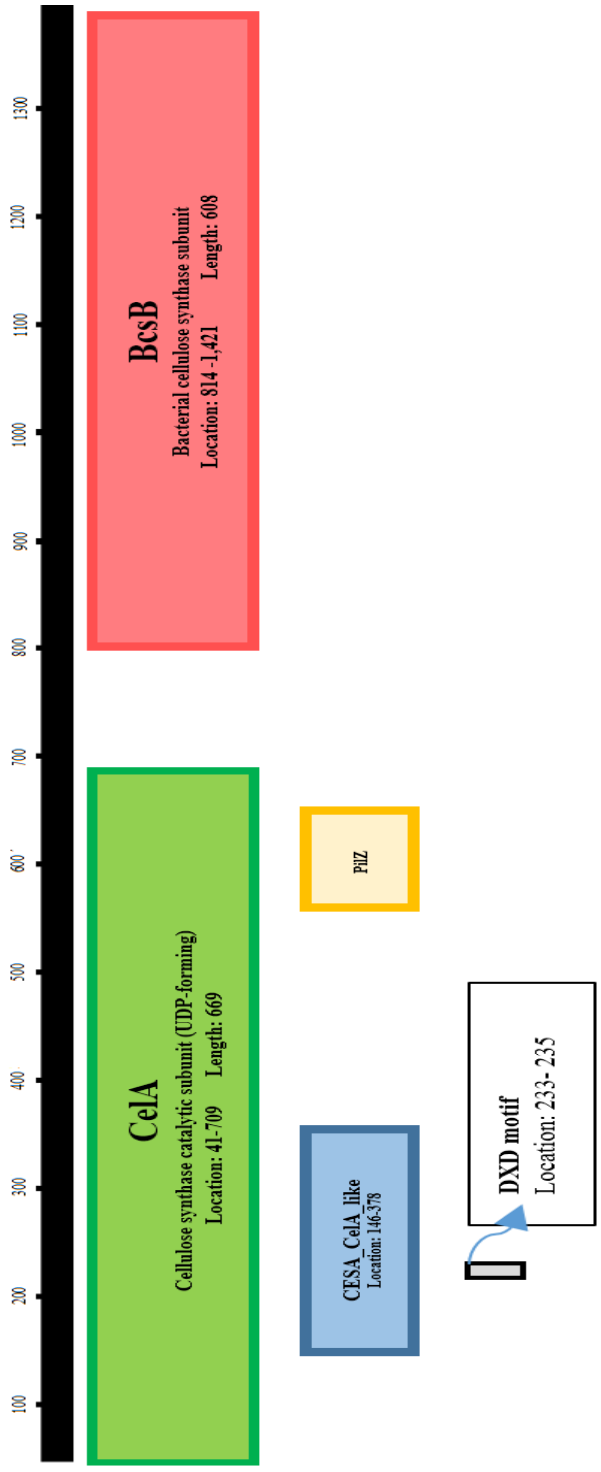

Figure 3. Schematic graphical image of cellulose synthase domains in *Komagataeibacter xylinus* E25 with four protein domain families (CESA_CelA_like, BcsB, PilZ, CelA domains) [25].

Table 1 shows the percentage of similarity in cellulose synthase enzymes with different bacterial sources. The cellulose synthase of *K. xylinus* E25 has 100%, 67%, 68%, and 62% similarity to cellulose synthase from *K. hansenii*, *K. europaeus*, *K. xylinus* NBRC 13693, respectively [25].

Distinct behavior in response to different carbon sources in different cellulose producing bacteria can be due to slight dissimilarity in their cellulose synthase enzymes. However, sucrose and food-grade sucrose also have a positive effect in bacterial cellulose synthesis but they have lower yield. Sucrose needs to be hydrolyzed to glucose and fructose in the periplasm. Perhaps, the relatively low BC production by *G. xylinus* PTCC 1734 was due to the inability of the organism to transport sucrose through the cell membrane.

Mikkelsen et al. (2009) reported that relatively low concentrations of BC were produced by *G. xylinus* ATC 53524 when sucrose was used as the sole carbon source [22].

Finally, since carbon source price plays a key role on the costs of industrial production, low-price ones such as glycerol (obtained as byproduct of biodiesel production) can be promising and abundant carbon source for industrial BC production by these strains. Biodiesel production from animal fats and vegetable oils generates about 10% (w/w) glycerol as the main by-product which can generate many environmental problems [23], whereas glycerol bioconversion to valuable chemicals such as bacterial cellulose could be valuable.

Figure 4 illustrates the thickness of BC with respect to carbon sources. The thickness of BC sheets was 6 to 34 micrometers. In all strains, the maximum thickness of BC was observed in glycerol medium when compared with other substrates (Figure 4). BCs with different thicknesses have distinct application potential. Thus, the control of this parameter can be important for choosing their application, and it is achieved simply by replacing carbon sources. It seems that there is not a direct relationship between the weight and thickness of BCs. For example, in *S* strain, the thickness of BC obtained from fructose and mannitol medium was 27 and 18 micrometers respectively, but BCs from mannitol medium had more dry weight in compare with fructose (Figure 1 and Figure 3). It can probably be related to the distinct structure of BCs produced in different carbon media. The moisture content (%w/w) of BCs produced in different carbon sources was in the range of 90 - 97.6 % (Figure 5).

The maximum moisture content was observed when sucrose and food-grade sucrose were used as carbon sources. Contrary to expectations, while the maximum thickness of BC membrane was attained when glycerol was used, BCs from glycerol had less moisture content than others. It seems that BC membrane had the lower porosity when glycerol was used as the sole carbon source. When sucrose was used as the sole carbon source, the growth of bacteria and the yield of production were limited as compared to other carbon sources (Figure 2). Because sucrose is a disaccharide and needs more enzymes and more complex process to utilize, thus less microfibriles were produced; this can be the explanation of the lowest dried BC thickness with highest porosity, and thus the highest water content of BCs was obtained from the sucrose medium.

Table 1. Similarity comparison of cellulose synthase enzymes

	Max score	Total score	Query cover	E value	Ident	Accession (NCBI Reference Sequence)
Cellulose synthase 2 [*K*.hansenii*]	3242	3242	100%	0.0	100%	WP_003621570.1
Cellulose synthase [*K. europaeus*]	2103	2103	96%	0.0	67%	WP_053322718.1
Cellulose synthase [*K. europaeus*]	2102	2102	96%	0.0	68%	WP_019090488.1
Cellulose synthase catalytic subunit AB[*K. xylinus* NBRC 13693]	1996	1996	97%	0.0	62%	GAO00603.1
Cellulose synthase [*K. oboediens*]	1994	1994	97%	0.0	63%	WP_029329219.1
Cellulose synthase [*G**. sp.* SXCC-1]	1928	1928	98%	0.0	60%	WP_039999744.1
Putative cellulose synthase 2 [*G.* sp. SXCC-1]	1927	1927	98%	0.0	60%	EGG75332.1

* *Komagataeibacter*, ***Gluconacetobacter*

In contrast, glycerol that can be used via two metabolic pathways develops the bacterial growth and produces a denser reticulated structure with decreased porosity. These results are in good agreement with the literature-cited publications [18, 21, 24]. Al-Shamary et al. (2013) showed that when sucrose was used as a source of carbon, the porosity (80%) of BC membrane was higher than that of glucose, fructose and glycerol, which gave lower percentage of porosity 70%, 66% and 65%, respectively [3].

3.3. Antimicrobial activity of oxidized BC

Regarding the assay by zone of inhibition, the oxidized BC exhibited an obvious inhibition zone against the model bacteria, while no inhibition zone was observed for the pure bacterial cellulose. This demonstrates that the antimicrobial activity existed only due to oxidization of BC, and not due to BC itself. Figure 6 shows the inhibition zone of oxidized BC against *E. coli*. The antibacterial activity of the oxidized cellulose makes it a good candidate for food preservation.

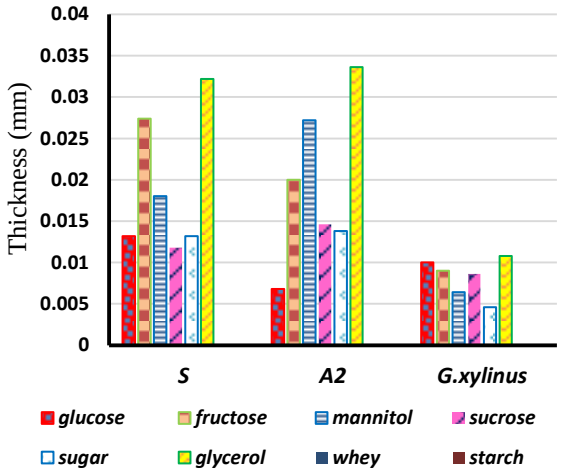

Figure 4. Bacterial cellulose thickness with respect to different carbon sources

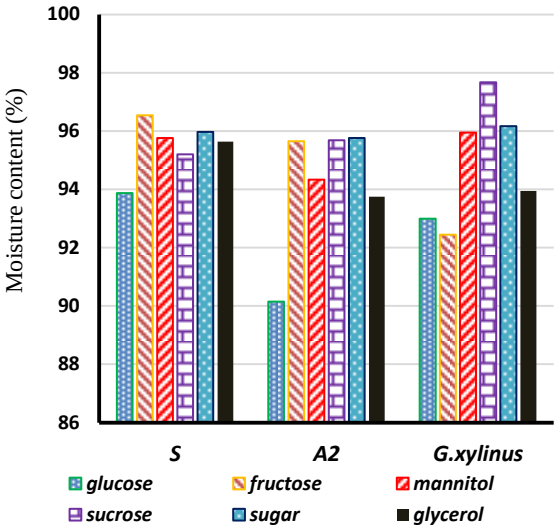

Figure 5. Moisture content of BC

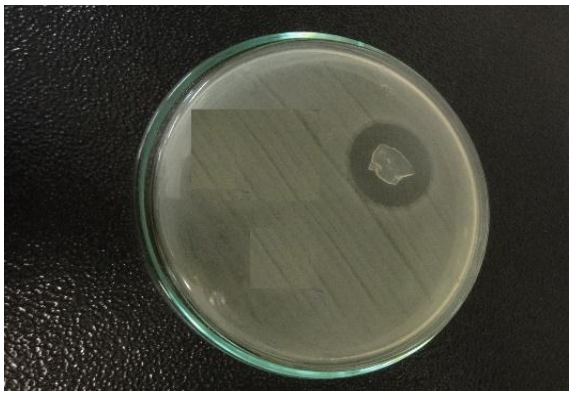

Figure 6. Photograph images of the inhibition zone of oxidized BC against *E. coli*.

4. Conclusion

In this study, the production of BC using different native BC producing strains from five categories of carbon sources, i.e. monosaccharaides (glucose and fructose), disaccharides (sucrose and food-grade

sucrose), sugar alcohols (glycerol, and mannitol), whey, and food-grade starch was examined. Glycerol gave the highest relative yield (around 9 fold due to A_2 strain) compared to the glucose medium, in all of the strains, followed by mannitol and fructose for two newly isolated strains and glucose for *G. xylinus* (PTCC 1734). No significant differences were between sucrose (Merck) and sucrose in BC production.

Whey and food-grade starch were not suitable carbon sources. Enhanced productivity is associated with a decrease in gluconic acid concentration that was produced during the BC production from glucose as carbon source. This work indicated the possibility of getting the required porosity and thickness by varying the type of carbon source.

It can be concluded that there is no similar pattern of bacterial behavior due to carbon source utilization in BC producing strains. This can help us to select the most appropriate carbon source for BC production; and it is necessary to detect the best carbon source for individual strain. This might be caused by the differences in the metabolic abilities of distinct strains. However, it is necessary to understand the metabolic network and relate it to the production of BC in these strains in order to find a precise answer to the question: *"Why various carbon sources lead to differences in BC productivity and physical properties of cellulose?"*

5. Acknowledgement

The authors thank University of Isfahan for financial support of this research.

6. Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Lin SP, Calvar IL, Catchmark JM, Liu JR, Demirci A , Cheng KC. Biosynthesis, production and applications of bacterial cellulose. Cellulose. 2013; 20: 2191-2219. DOI: 10.1007/s10570-013-9994-3
2. Siro I, Plackett D. Microfibrillated cellulose and new nanocomposite materials: A review. Cellulose. 2010; 17:459–494. DOI: 10.1007/s10570-010-9405-y
3. Al-Shamary EE, Al- Darwash AK. Influence of fermentation condition and alkali treatment on the porosity and thickness of bacterial cellulose membranes. Online J. Sci. Technol. 2013; 3:194-203.
4. Shi Z, Zhang Y, Phillips GO, Yang G. Utilization of bacterial cellulose in food. Food Hydrocolloids. 2013; 35: 539-545. DOI: 10.1016/j.foodhyd.2013.07.012
5. Chawla PR, Bajaj IB, Survase SA, Singhal RS. Microbial Cellulose: Fermentative Production and Applications. Food Technol. Biotechnol. 2009; 47: 107–124. ISSN 1330-9862
6. Sukara E, Meliawati R. Potential Values of Bacterial cellulose for industrial applications. Jurnal Selulosa. 2014; 4: 7 – 16.
7. Esa F, Tasirin SM, Abd Rahman N. Overview of Bacterial Cellulose Production and Application. Agric. Agric. Sci. Procedia. 2014; 2: 113–119. DOI: 10.1016/j.aaspro.2014.11.017
8. Keshk SM. Bacterial Cellulose Production and its Industrial Applications. J Bioprocess Biotech. 2014; 4:1-10. DOI: 10.4172/2155-9821.1000150
9. Denise M, Rosilene A, Adenise L, Gilvan W. Application of bacterial cellulose conservation of minimally processed fruits. Rev Bras Tecnol Agro industrial. 2011; 5(1):356-366. DOI: 10.3895/S1981-36862011000100011
10. Mesomya W, Pakpeankitvatana V, Komindr, S, Leelahakul P, Cuptapun Y, Hengsawadi D, Tammarate P, Tangkanakul P. Effects of Health Food from Cereal and Nata De Coco on Serum Lipids in Human. J. Sci. Technol. 2006; 28 (1): 23-28.
11. Okiyama A, Motoki M, Yamanaka S. Bacterial cellulose II. Processing of the gelatinous cellulose for food materials. Food Hydrocolloids. 1992; 6(5): 479-489. DOI: 10.1016/S0268-005X (09)80033-7
12. Keshk S, Sameshima K. Evaluation of different carbon sources for bacterial cellulose production. Afr J Biotechnol. 2005; 4: 478-482. ISSN: 1684-5315
13. Carreira P, Mendes JA, Trovatti E, Serafim LS, Freire CS, Silvestre AJ, Neto CP. Utilization of residues from agro-forest industries in the production of high value bacterial cellulose. Bioresource Technol. 2011; 102 (15):7354-60.DOI: 10.1016/j.biortech.2011.04.081
14. Segal L, Creely JJ, Martin AE, Conrad CM. An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. Text Res J. 1959; 663 (29): 786-794. DOI: 10.1177/004051755902901003
15. Vazquez A, Foresti ML, Cerrutti P, Galvagno M. Bacterial cellulose from simple and low cost production media by *Gluconacetobacter xylinus*. J. Polym. Environ. 2013; 21:545–554. DOI: 10.1007/s10924-012-0541-3
16. Zhong C, Zhang GC, Liu M, Zheng XT, Han PP, Jia SR. Metabolic flux analysis of *Gluconacetobacter xylinus* for bacterial cellulose production. Appl Microbiol Biotechnol. 2013; 97(14): 6189-6199. DOI: 10.1007/s00253-013-4908-8
17. TsoukoE, Kourmentza C, Ladakis D, Kopsahelis N, MandalaI, Papanikolaou S, Paloukis F, AlvesV, Koutinas A. Bacterial Cellulose Production from Industrial Waste and by-Product Streams. Int. J. Mol. Sci. 2015; 16: 14832-14849. DOI: 10.3390/ijms160714832
18. Jung HI, Jeong JH, Lee OM, Park GT, Kim KK, Park HC, Lee SM, KimYG, Son HJ. Influence of glycerol on production and structural–physical properties of cellulose from *Acetobacter sp. V6* cultured in shake flasks. Bioresour. Technol. 2010; 101: 3602-3608. DOI: 10.1016/j.biortech.2009.12.111
19. Panesar PS, Chavan Y, Bera MB, Chand O, Kumar H. Evaluation of *Acetobacter* Strain for the Production of Microbial Cellulose. Asian J. Chem. 2009; 21(10):99-102.
20. Ramana KV, Tomar A, Singh L. Effectof various carbon and nitrogen sources on cellulose synthesis by

Acetobacter xylinum. World J Microbiol Biotechnol. 2000; 16:245-248.DOI: 10.1023/A: 1008958014270

21. Jonas R, Farah LF. Production and application of microbial cellulose. Polym Degrad Stabil. 1998; 59:101-106. DOI: 10.1016/S0141-3910(97)00197-3
22. Mikkelsen D, Flanagan BM, Dykes GA, Gidley MJ. Influence of different carbon sources on bacterial cellulose production by *Gluconacetobacter xylinus strain ATCC 53524*. J Appl Microbiol. 2009; 107:576–583. DOI: 10.1111/j.1365-2672.2009.04226.x
23. Paulo da Silva G, Mack M, Contiero. Glycerol: A promising and abundant carbon source for industrial microbiology. Biotech Adv. 2009; 27(1): 30-39. DOI: 10.1016/j.biotechadv.2008.07.006
24. Tang WH, Jia SR, Jia YY, Yang HJ. The influence of fermentation conditions and post-treatment methods on porosity of bacterial cellulose membrane. World J Microbiol Biotechnol.2010; 26:125–131. DOI: 10.1007/s11274-009-0151-y
25. Marchler-Bauer A. (2015), "CDD: NCBI's conserved domain database. Nucleic Acids Res. 43 (Database issue):D222-6.Available at: http://www.ncbi.nlm.nih.gov/gene/25559535