Influence of Fermentation Condition and Alkali Treatment on the Porosity and Thickness of Bacterial Cellulose Membranes

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Abstract: The object of the present study was to produce bacterial cellulose (BC) membranes and studying the effect of carbon source, time and conditions of inoculation, type of alkali used for purification and the method of drying on it[s] porosity and thickness of the resultant membranes. *Acetobacter xylinum* was isolated from local rotten juice, and used for membrane production. The highest porosity was attained when sucrose was used as a source of carbon compared to glucose, fructose and glycerol. However, fructose, glucose and glycerol resulted in higher pH value for the medium used as medium for bacterial growth. Using glycerol as the sole carbon source gave the highest bacterial cellulose and biomass (g/l) as compared to glucose, fructose and sucrose. Small inoculation led to high porosities and lowest thickness of the resultant membranes. Porosity of membranes was affected by the type of alkali used for the purification of the membranes. Application of K_2CO_3 for purification gave the highest porosity while Na OH gave the lowest porosity. Hot air- drying of the membrane resulted in the lowest porosity as compared with freeze drying method which did not cause any damage to the porosity of the membrane.

Key words: Bacterial cellulose membrane, Thickness and porosity, *Acetobacter xylinum*

Introduction

Bacterial cellulose 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 *A. 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 *Gluconobacter xylinum*) (Yamanaka *et al*.2000) .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 it[s] fine fiber net work, 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*.1999,2001), 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). We believe that culture conditions such as type of strain , temperature of growth , carbon source, pH and the method of gel purification weather it is done by chemical agent, concentration, temperature or exposition time possibly affected the physical properties of the resultant membranes.

The aim of the present work was to evaluate the effect of growth conditions and the methods of purification on some of physicochemical and transparent properties of the resultant membranes.

Materials and Methods:

1- Stock culture

The organism exploited for the production of cellulose in this study was a strain of *Acetobacter xylinum* AJ_3 , which was isolated from local rotten apple juice. The cultures were maintained on tomato agar slants and were reactivated every month.

Tomato medium was composed of 50 g/l glucose, 5g/l peptone, 5g/l yeast extract and 10% by volume tomato juice at pH 6.8. Stock cultures were stored at 5°C according to Marzieh and Ali (2010).

2- Inoculums preparation

A culture medium composed of 5% glucose, 0.5% peptone, 0.5% yeast extract, 0.27%sodium phosphate monobasic and 0.12% citric acid. This medium was autoclaved at 121°C for 15 minutes. After cooling to room temperature, the medium was inoculated with 2.5 ml of the stock culture and incubated in a shaker incubator with the use of 200 rpm set at 30°C for 24h and pH 6.8. The organisms were harvested by centrifugation at 10000 rpm for 30 min and re-suspended in liquid medium to prepare the suspension of bacteria.

3- Membranes formation

Membranes were prepared using different carbon sources including glucose, fructose, sucrose or glycerol (35g/l) with 10g/l yeast extract, 7.5g/l peptone, 10g/l disodium phosphate and 10 ml/l acetic acid. The media was autoclaved at 121°C for 15 minutes, inoculated with 6% of the previously prepared bacteria and incubated at 30 °C for 8 days.

The effect of incubation period was studied using mediums contained glucose as carbon source using 6%of inculcation volume from the fermentation medium after (2, 4, 6, and 8) days. Membranes were also prepared using different percentages of inoculums (2, 4, 6, and 8%). Yield of cellulose, porosity and thickness of the resultant bacterial cellulose membranes were studied. The pH values for each medium were measured during fermentation.

4- Purification

After 8 days of cultivation, BC was harvested and purified by soaking in a solution of 0.5N NaOH at 90°C for 1h to remove the bacterial cell and other medium components. Then after, membranes were purified by Sodium hydroxide, potassium hydroxide, sodium carbonate or potassium carbonate to study it[s] effect on porosity of membranes. The purified bacterial cellulose membranes were then dried either in an air- drying oven at 80°C or by freeze- drying. Porosities of the dried bacterial cellulose membranes were determined, for membranes dried by either hot air or freeze drying methods.

Analytical methods

1- Measurement of biomass
After the incubation periods of 8 days, the culture broth was centrifuged at 3000 g for 20 min. The bacterial cellulose pellets were added to 90ml (0.1M) potassium acetate buffer (pH 5).Ten ml of 20% cellulolasse solution was added and incubated at 50°C with shaking at 100 rpm for 2h to hydrolyze BC (Kouda *et al*. 1997).Then, the resultant solution was centrifuged at 3000 g for 20 min. The precipitate was dried in an oven at 80°C over night and then weighted to determine the biomass.

2- Porosity
Porosity was calculated using the equation of Kidaoka *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 (Mancini *et al*.2001).

3- Thickness
Thickness of each bacterial cellulose membrane was measured at ten different positions by a thickness gauge, and the values were averaged.

Results and discussion:

Effect of carbon sources on porosity of bacterial cellulose membranes

Figure (1) showed that the highest porosity (80%) of BC membrane was attained when sucrose was used as a source of carbon as compared to glucose, fructose and glycerol which gave lower percentage of porosity 70%, 66% and 65%, respectively. Nakai et al.(1991) warranted that because *A. xylinum* has no sucrose synthetase, therefore, at least four enzymatic steps in the path way from sucrose to get UDP- glucose. On the other hand using sucrose as the sole of carbon source led to limited growth of bacteria as compared to glucose (Table 1).Fewer amounts of microfibriles were produced, which explains the least cell mass, the lowest thickness with highest porosity. Bacterial cellulose membrane had the lowest porosity when glycerol was used as the sole carbon source. Table (1) showed that the lowest pH was attended when sucrose was used as a carbon source. However, fructose, glucose, and glycerol resulted higher pH values proportionally.

When glycerol was used as a source of carbon there might be no gluconic acid production during glycerol metabolism (Jonas and Farah 1998).It was found that when glycerol was used as the sole carbon source, the BC and biomass (g/l) were higher as compared with other carbon sources. The fibrils of BC from glycerol medium were entangled with each other resulting in a denser reticulated structure than those obtained from glucose medium (Jung *et al*.2010). When glucose was used as the sole carbon source, the BC yield and biomass were higher than that for other remaining carbon source. Due to these results and due to the availability of glucose, it was used as the sole carbon source during the following steps of this study.

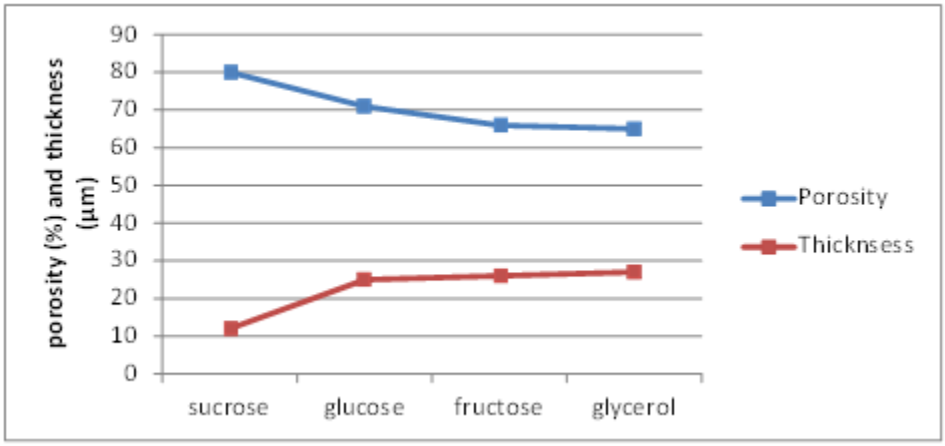

Figure 1. Effect of Carbon source on porosity and thickness of Bacterial Cellulose membrane produced after 8 days of cultivation with 5 % (v/v) inoculation volume

Table 1. The effect of carbon source on bacterial cellulose yield and biomass of *Acetobacter xylinum*.

Carbon source	Bacterial cellulose Yield(g/l)	Biomass (g/l)	Final pH
Sucrose	4.23	2.10	3.9
glucose	7.52	3.24	4.5
fructose	7.21	3.10	4.1
glycerol	8.52	4.50	5.2

Effect of inoculation volume on porosities and thickness of bacterial cellulose membranes

The effect of inoculation size on porosities and thicknesses of BC membranes is shown in figure 2. Small inoculation led to high porosities and lowest thickness of BC membranes. The results showed that the porosities of bacterial cellulose membranes dropped from 78% to 45% with increasing inoculation volume (v/v) from 2 to 8%.The results showed that the cell growth increased with increasing size of inoculums and that led to increasing BC production . Generally, the production of large number of micro fibrils often led to a compact structure and lower porosity for the BC membrane. This indicated the possibility of production of BC membrane which have wide range of porosity depending on the purpose of BC membrane applications.

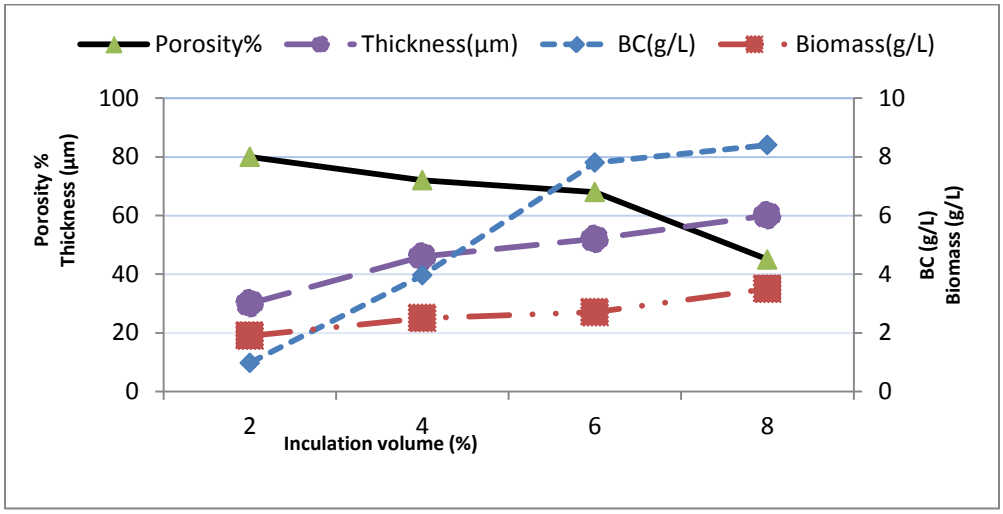

Fig. 2 Effect of inoculation volume on cell growth (Biomass) of *A. xylinum* , porosity, thickness and yields of the bacterial cellulose membranes

The Effect of culture time on porosity and thickness of bacterial cellulose membrane.

Figure 3 showed the effect of culture time on the porosity and thickness of bacterial cellulose membrane. In the first day of cultivation there was no cellulose production. During cultivation, the porosities of bacterial cellulose were decreased successively from the second day to the end of incubation period of 8 days. During cultivation, the yield of BC, cell mass and thickness were increased with increasing time of culture growth, but porosities were decreased due to the accumulation of more fibrils. The secreted cellulose was randomly deposited behind the moving micro-organism to produce certain porosity of cellulose membranes with three – dimensional network. The movement of single cells was caused by the inverse forces of the secretion of cellulose nano fibers. (Hesse and Kondo 2008).

Watanabe and Yamanaka (1995) found that oxygen tension in the gaseous phase under static culture conditions affected network formation of BC, and the density of network in the gelatinous membrane could be controlled. Then by changing oxygen tension we can produce bacterial cellulose membranes with the desired porosities for various applications.

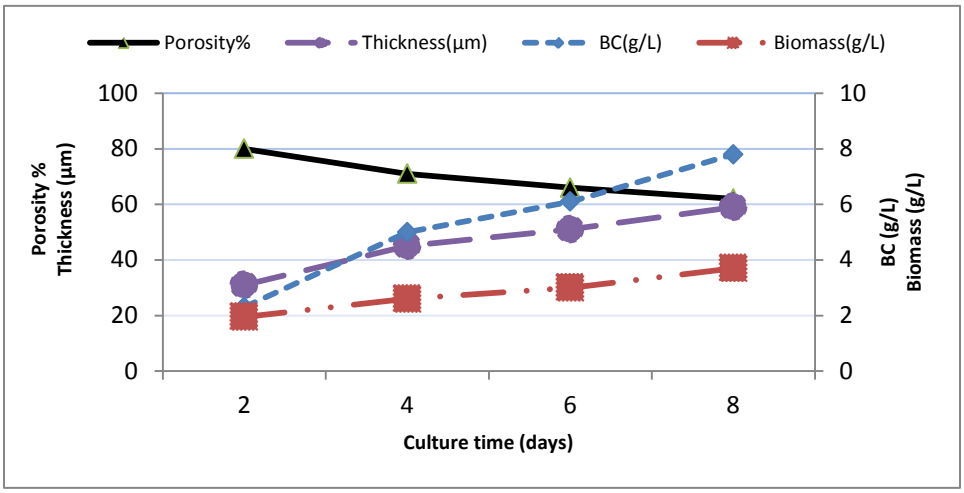

Fig. 3 Effect of culture time on porosity, thickness, yields, of bacterial cellulose Membranes and cell growth (Biomass) of *A. xylinum* AJ3 with inoculation volume of 6% .

Effect of alkali treatment on porosities of bacterial cellulose membranes

Treating bacterial cellulose membranes with various alkaline solutions resulted in increases in the porosity of the membranes (Figure 4).These results indicated that the diameter of membrane's fibrils were affected by alkaline solution in a degree depending on of alkaline solution. Porosities of bacterial cellulose membranes were found to be depending also on the type of alkaline solution and it showed different porosity arranged in descending as K_2CO_3 > Na_2CO_3 > KOH > NaOH. The diameter of NaOH – treated ribbons of BC which had the lowest porosity was in a range of 45 – 130 nm as measured by using microscope stage micrometer, while for K_2CO_3 treated ribbons of BC had the highest porosity and was in a range of 25 – 110 nm. Differences in porosities of BC treated with different alkali probably gave higher swelling fibrils of BC. These results were due to increases in diameter of fibrils especially the membrane that was treated with NaOH, thus effective pore size available in the membranes. (George et al. 2005, Brigid et al. 2009, Weihua et al.2010). By using different alkali treatments resulted in membranes having different porosity which lead to a wide range of membrane applications in the laboratories and in industry.

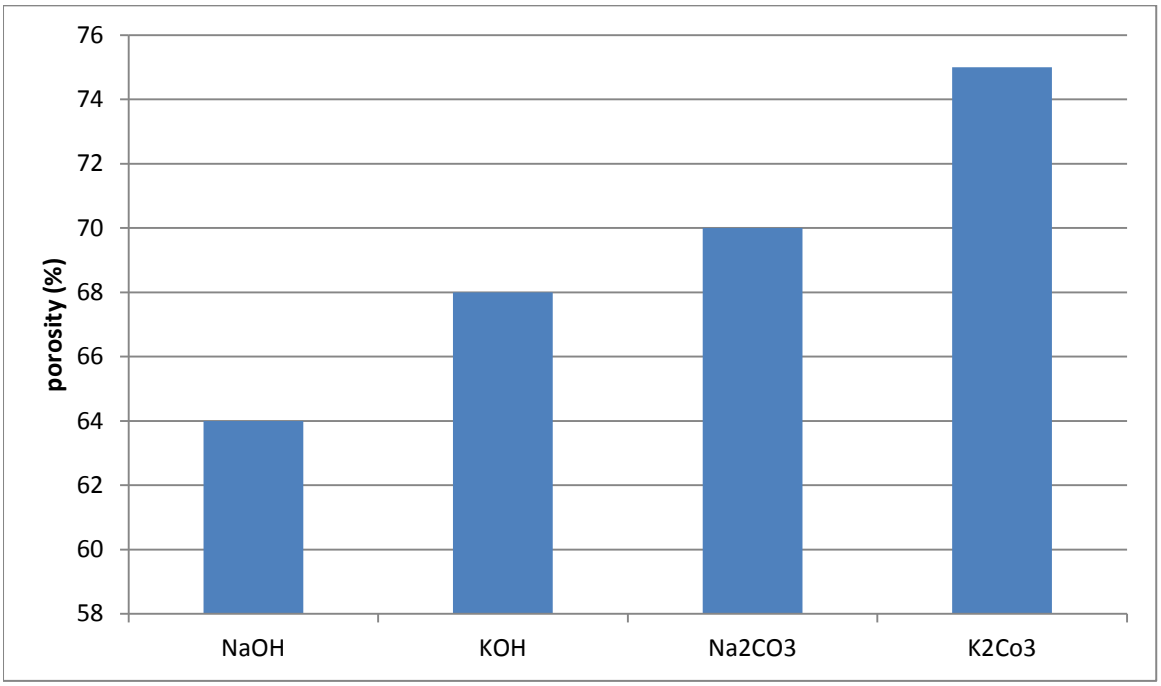

Fig-4- Porosity of bacterial cellulose membranse treated with different alki solution.

Effect of drying method on porosities of bacterial cellulose membranes:

Method of drying of BC membranes affected porosity of the membrane. Membranes which were dried by hot air had the lowest porosity (62%) as compared to freeze – drying method (88%) (Fig. 5). Freeze – drying was effective in preventing the shrinkage of pores during drying (Marabi and Saguy 2004, Svensson *et al*. 2008).The collapse was very severe when hot air drying was used for drying of the high water content materials of the membrane, while the collapse was usually negligible when freeze drying method was used for drying similar membranes (Karathanos *et al*. 1996).

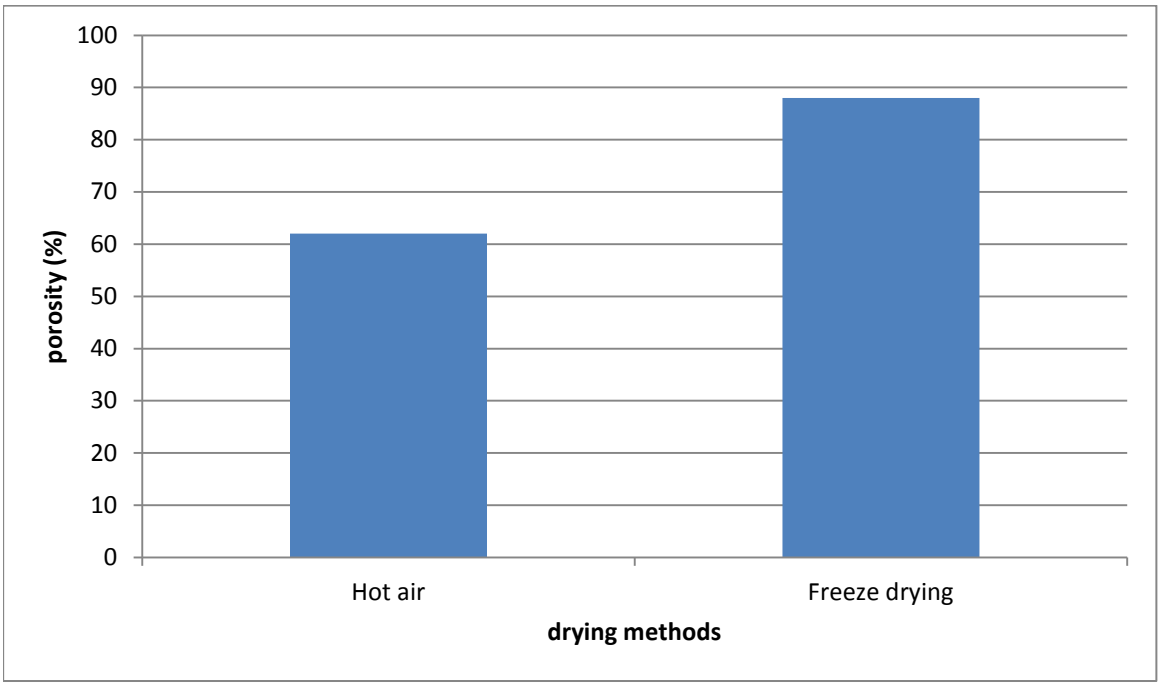

Fig. 5 Effect of drying method on porosity of bacterial cellulose membranes.

Conclusions

This work indicated the possibility of getting the required porosity and thickness by varying the production media and purification conditions. It is also concluded that bacterial cellulose production as a polymer is very wide field for further studies and investment. It is also economical to produce it from local date syrup and molasses.

References

Vu, T. N. ; Bernadine, F.; Michael, J. G. and Gary, A. D. (2008). Characterization of cellulose production by a *Gluconacetobacter xylinum* strain from kombucha. *Curr Microbiol*, *57*:449-453.

Jagannath, A. ;Kalaiselvan, A. ; Manjunatha, S. ;Raju, P. and Bawa, A. (2008). The effect of pH, sucrose and ammonium sulphate concentration on the production of bacterial cellulose (Nata-de-coco) by *Acetobacter xylinum*. *World Microbiol Biotechnol.24*:2593-2599.

White, D.G. and Brown, R.M.(1989). Prospects for the commercialization of the biosynthesis of microbial cellulose. In: Schuerech, C (*ed*) Cellulose and Wood-Chemistry and Technology. Wiley, New York.

Chao, Y.; Ishida, T.; Sugano, Y. and Shoda, M. (2000).Bacterial cellulose production by *Acetobacter xylinum* in 50 L internal- loop air lift reactor. *Biotechnol.Bioeng.68*;345-352.

Putra, A.; Kakugo, A.; Furukawa, H.; Gong, J.P. and Osada, Y. (2008). Tubular bacterial cellulose gel with oriented ibrils on the curved surface. *Polymer 49*;1885-1991.

Denise, M.; Rosilene, A.; Adenise, L. and Gilvan, W. (2011). Application of bacterial cellulose conservation of minimally processed fruits. *Revista Brasileira de Tecnologia Agroindustrial, 5*(1):356-366.

Marzieh, M. and Ali, Y. (2010). Investigation of physiochemical properties of the bacterial cellulose produced by *Gluconacetobacter xylinum* from date syrup. World Academy of Science, Engineering and Technology 68.

Li, M.; Tian, X. and Chen, X. (2009). Modeling of flow rate, pore size, and porosity for the dispensing-based tissue scaffolds fabrication. *Manuf. Sci. Eng. 131*(3):34501-34505.

El-Saied, H; Ahmed, I.; Altaf, H.; Nagwa, A. and Dina, E. (2008). Production and characterization of economical bacterial cellulose."*Economical Bacterial Cellulose" Bio Resources, 3*(4):1196-1217.

Sherif, K. and Kazuhiko, S. (2006).The utilization of sugar cane molasses with/without the presence of oligosulfonate for the production of bacterial cellulose. *Appl. Microbiol. Biotechnol., 72*:291-296.

Bäckdahl, H.; Helenius, G.; Bodin, A., Nannmark, U.; Johansson, B.R. and Risberg, B. (2006). Mechanical properties of bacterial cellulose and interactions with smoth muscle cells. *Biomaterial, 27*(9):2141-2149.

Hei, CL. (1999). Reduced production of microbial cellolose caused by aggregation of *Acetobacter xylinum* under shaking culture conditions observation by scanning electron microscope. *Applied Chemistry, 3*:92-95.

Retegi, A.; Gabilondo, N.; Pena, C.; Zuluaga, R.; Castro, C.; Ganan, P. and Delacaba, K. (2010). Bacterial cellulose films with controlled microstructure- mechanical property relationships.*Cellulose, 17*:661-669.

Yamanaka, S.; Ishihara, M. and Sugiyama, J. (2000). Structural modification of bacterial cellulose. *Cellulose, 7*:312 – 225.

Pacheco, J. ; Yee, S. ; Zentella, M. and Marvan, E. (2004). Cellulosa bacteriana en Gluconacetobacter xylinum:Biosintesisy Aplicaciones. Revista Especializada en *Ciencias Quimico – Biolōgicas, 7*(1):18 - 25.

Yamanaka, S.;Watanabe, K. and Suzuki, Y. (1990). *European patent* 0,396,344A_2.

Klemm, D.; Udhardt, U.; Marsch, S. and Schumann, D. (1999). " BASYC – Bacterial synthesized cellulose: Miniaturized tubes for microsurgery", *Cellulose Polymer News, 24*(11): 373 – 380.

Klemm, D.; Schumann D.; Udhardt U. and Marsch S.(2001)."Bacterial synthesized cellulose – artificial blood vessels for microsurgery" Progress in *Polymer Science, 26*(9):1561 – 1603.

Krystynowicz, A. and Bieleck, S. (2001). Biosynthesis of bacterial cellulose and it's potential application in the different industries, *Polish Biotetechnol*. News, Copyright by Bio – Tech Consulting.

Luz, D.; Carreno, P.; Luis, A. and Alberto, C. (2006). Effect of culture and purification conditions on physicochemical and transport properties in bacterial cellulose membranes, *Biomaterials, 27*:145 – 151.

Kuan, C.; Jeffrey, M. and Ali, D. (2009) .Effect of different additives on bacterial cellulose production by *Acetobacter xylinum* and analysis of material property. *Cellulose, 16*:1033 – 1045.

Kouda, T.; Yano, H. and Yoshinaga, F.(1997). Effect of agitator configuration bacterial cellulose productivity in aerated and agitated culture. *J. Ferment. Bioeng., 83*:371 – 376.

Marzieh, M. and Ali, R. (2010). Investigation of physicochemical properties of the bacterial cellulose by *Gluconacetobacter xylinum* from date syrup. *World Academy of Science Engineering and Technology, 68*:1248 – 1253.

Neelobon, S.; Jiraporn, B. and Suwannee, T. (2007). Effect of culture conditions on bacterial cellulose (BC) production from *Acetobacter xylinum* TISTR 976 and physical properties of BC parchment paper. *Suranaree J. Sci.- Technol. 14*(4):357

Jonas, R. and Farah, L.F. (1998) .Production and application of microbial cellulose. *Polym Degrad Stab.59*:101 – 106.

Jung, H.I; Jeong J.H ; Lee, O.M.; Park, G.T.; Kim, K.K.; Park, H.C; Lee, S.M.; Kim, Y.G. and Son, H.J. (2010) . Influence of glycerol on production and structural – physical properties of cellulose from Acetobacter sp.V6 cultured in shake flasks. *Bioresour- Technol. 101*: 3602-3608.

Nakai, T.; Tonouchi, N.; Konishi, T.; Kojima, Y.; Tsuchida, T. and Yoshinaga, F. (1999) . Enhancement of cellulose production by expression of sucrose synthetase in *Acetobacter xylinum* . *Proc. Natl. Acad. Sci. USA, 96* (1):14 – 18.

Weihua, T.; Shiru, J.; Yuanyuan, J. and Hongjiang, Y. (2010) .The influence of fermentation condition and post – treatment methods on porosity of bacterial cellulose membrane. *World J.Microbiol.Biotechnol.,26*:125 – 131.

George, J.; Ramana, K.; Sabapathy, S. and Bawa, A. (2005). Physico- mechanical properties of chemically treated bacterial (*Acetobacter xylinum*) Cellulose membrane. *World J. Microbiol. Biotechnol. 21*: 1323 – 1327.

Brigid, A.; Deirdre, M.; Bernhard, W.; Michael, J. and Neal, W. (2009) .Mechanical and structural properties of native and alkali treated bacterial cellulose produced by *Gluconacetobacter xylinum* strain ATCC 53524.*Cellulose. 16*:1047 – 1055.

Marabi, A. and Saguy, I. (2004) . Effect of porosity on rehydration of dry food particulates. *J. Sci. Food Agric., 84*(10)1105 – 1110.

Svensson, A.; Nicklasson, E.; Harrah, T.; Paniaitis, B.and Kaplan, D.L. (2005) . Bacterial cellulose a potentiol scaffold for tissue engineering of cartilage. *Biomaterials, 26*:419 – 431.

Karathanos, VT.; Kanellopoulos, N.K.; Belessiotis, V.G. (1996). Development of porous structure during air drying of agricultural plant products. *J. Food Eng., 29*:167 – 183.