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Utilization of marine algae as a carbon source for bacterial cellulose production by *Gluconacetobacter xylinus*

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ABSTRACT

Bacterial cellulose (BC) has received attention due to its many advantages such as high mechanical strength, purity, crystallinity, liquid-absorbing capabilities, and biodegradability. The most discrepancy associated with BC production is its high cost which higher than the plant cellulose. So, the present study was aimed to improve BC production by using reducing sugar from algae as a cheaper carbon source. Replacing glucose with 20 g/l of reducing sugars extracted from *P. oceanic* increased BC yield to about 5.7g/l, which is higher than on glucose (3.7 g/l). The maximum BC production was obtained at an incubation temperature of 30°C and pH 6.5. According to experimental results of Plackett-Burman design and the steepest ascent methods, the optimal medium for BC production was (g/l); reducing sugar, 30; yeast extract, 7; peptone, 7; Na_2HPO_4, 7.2; citric acid, 0.2, pH 6, for 15 days of incubation at 30°C under static condition. The produced BC was examined and characterized by SEM, FT-IR Spectroscopy analysis, and XRD. This is may be the first report on the optimization of the fermentation medium using marine algal extract as the carbon source for the highest BC production (12.7g/l) by *G. xylinus* ATCC 10245.

INTRODUCTION

Cellulose is considered as the main building material of plants which abundantly available polysaccharide and is a very important substance. Bacterial cellulose (BC) is a natural polymer which is similar in chemical composition but superior in physicochemical properties to plant cellulose (**Ul-Islam *et al.*, 2012**). It has a conceptually different architecture. Its three-dimensional network is formed by fibers 3–5 µm wide which in turn consist of ribbon-like nanofibrils 20–120 nm wide and 3–4 nm thick (**Lee *et al.*, 2014 and Skiba *et al.*, 2019**). BC is preferred over the plant cellulose due to its high purity, degree of polymerization and crystallinity index. It also has water holding capacity and higher tensile strength than that of plant cellulose, making it more suitable raw material for producing high fidelity acoustic speakers, high quality paper and dessert foods (**Enas *et al.*, 2015 and Kwak *et al.*, 2015**). BC can be produced by many

genera of bacteria such as *Alcaligenes*, *Enterobacter*, and *Pseudomonas* (**Shoda and Sugano, 2005**). Among them, *G. xylinum* is the most efficient BC producer that can produce BC in abundance for industrial application (**Brown, 1886**). Its synthesis by the facultative anaerobe *Enterobacter* sp. FY-07 was published by **Ji *et al*. (2016)**. Biosynthesis of BC needs a carbon source, as a monosaccharide (fructose and glucose), a disaccharide (sucrose and maltose) or alcohol (ethanol, mannitol or glycerol) in the production process to produce an extracellular insoluble film. So, these carbon sources are expensive and give low yield (**Phruksaphithak *et al*., 2019**).

Recently, low cost substrates such as food process effluents, hemicelluloses in waste liquor from atmospheric acetic acid pulping (**Uraki *et al*., 2002**), molasses (Bae and Shoda 2004), konjak glucomannan (**Hong and Qui, 2008**), fruit juices (**Kurosumi *et al*., 2009**) and rice bark (**Goelzer *et al*., 2009**) were examined for BC production. Attempts were made to use a new carbon source from marine origin such as algae.

Macroalgae (Seaweeds) are gaining some attention as an alternative renewable source of biomass for the production of many products. They have high contents of carbohydrates (up to 75%) which promoted intensive research towards valorisation of algal components for the production of many important compounds. However, the exploitation of the carbohydrate fraction to produce a range of chemicals and chemical intermediates with established markets is still limited. A number of features of macroalgae make them attractive when compared to terrestrial feedstock crops due to their fast growth rate and large biomass yield, with superior productivity to many terrestrial crops (**Adams *et al*., 2009 and John *et al*., 2011**). They can be grown anywhere and under any weather conditions because they have variety of species with different properties and adaptations ability. Algae are known to produce biomass faster and on reduced land surface as compared with lignocellulosic biomass (**Lee *et al*., 2013**). They contain various types of glucans, which are polysaccharides composed of glucose. These glucans can be hydrolyzed by saccharification enzymes to sugars (**Wang *et al*., 2011 and Borines *et al*., 2013**). So, algael hydrolysate can be used as a carbon source in fermentation processes and considered as a promising alternative for agricultural residues.

In this study, two types of macroalgae were used to produce simple reducing sugars as a first step for BC production. Also, optimize the BC production by *Gluconacetobacter xylinus* ATCC 10245 using *Posidonia oceanica* hydrolysate as substrate was studied. Optimization of fermentation process was applied using experimental designs. Moreover, characterization of BC produced was also carried.

MATERIALS AND METHODS

Bacterial strains

Isolation of marine bacteria from the seawater and sediment samples (Mediterranean Sea, Alexandria coastline, Egypt) was performed by serial dilution and spread plate method. One ml or gram of collected samples was serially diluted in sterilized sea water to get a population range from (10^{-1}) to (10^{-4}). A volume of 1 ml of each dilution was transferred aseptically to glucose yeast calcium carbonate (GYC) agar plates (**Rasooli *et***

***al*., 2010**). The plates were incubated at 30°C ± 2°C for 24-72hr. *Gluconacetobacter xylinus* ATCC 10245 (formely named *Acetobacter xylinum)* was obtained from Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt. The bacterial strain was maintained on GYC agar slants (**Rasooli *et al*., 2010)**, stored at 4°C and subcultured every two weeks to maintain good viability and stability for BC production.

Marine substrates

Sea grass (*Posidonia oceanica*) and green algae (*Ulva lactuca*) were collected from Mediterranean Sea, Alexandria coastline, Egypt, and kindly identified by Dr. Nehal Shams El-Dein, Hydrobiology Lab, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt.The complex carbohydrates in the sea grass were subjected to saccharification and the products were then used as a cheap sole carbon sources for BC production.

Chemical composition of marine algal substrate

It was determined according to the method described by **de Pádua *et al*. (2004)**, Plant Biochemistry Deprtment, Agricultural Research Center, Giza, Egypt.

Pretreatment of different substrates

The dried samples of *P. oceanica* and *U. lactuca* samples were treated with either dilute sulphuric acid (1%) (**Yoon *et al*., 2**012) or sodium hydroxide (1%) (**Solomon *et al*., 1999**). For enzymatic pretreatment, samples were subjected to hydrolases enzymes (produced from *Penicillium chrysogenum* as a control, sea grass was only autoclaved at 121°C for an hour.

Saccharification process using submerged fermentation

Penicillium chrysogenum was grown on slants of Czapek's-Dox agar medium for seven days at 30^{o}C. Spores were scraped with 5.0 ml of sterile distilled water and counted using Haemocytometer. Then, one ml (1×10^{6} spores/ml) was used to inoculate 250-ml Erlenmeyer flasks containing 100 ml of sterilized Czapek's-Dox broth medium containing (g/l): Starch, 20; $NaNO_3$, 2; KH_2PO_4, 1; KCl, 0.5 and 3.0 g of pretreated substrate (used as a sole carbon source). The flasks were incubated for 6 days at 28^{o}C in a rotary shaker (120 rpm) (**Atlas, 1997**). After incubation, the culture was filtred, centrifugated at 5000 rpm for 15 min and the supernatant was used to determine the reducing sugars and measure enzymatic activity (**El-Naggar *et al*., 2014**).The amount of reducing sugars was estimated by dinitrosalicylic acid method (**Miller, 1959**).

Production of BC by *G. xylinus*

The pre-inocula were prepared by inoculating *G. xylinus* cells into Hestrin-Schramm medium (HS) contained (g/l): Glucose, 20.0; yeast extract, 5.0; peptone, 5.0; dibasic sodium phosphate (buffer), 2.7; citric acid, 1.15, pH was adjusted to 6.5with 0.1N HCl or 0.1N NaOH prior to sterilization (**Hungund *et al*., 2013**). The flasks were incubated at 30°C for 24 h in an orbital shaker (120 rpm).One ml of preculture (OD_{600}=1.0) was used to inoculate 30ml of HS medium (contain glucose as a carbon source), HSPO and HSUL (Hydrolysates from the enzymatic hydrolysis of *P.oceanica* and *U. lactuca*,respectively, were used as carbon source instead of glucosein HS medium). Experiments were carried

out in 100 ml flasks containing 30 ml of sterilized medium and incubated for 7 days under shaking condition (120 rpm). At the end of incubation, BC production was estimated (**Andelib and Nuran, 2009**). All experiments were performed in duplicates.

Recovery and determination of *G. xylinus* BC

After cultivation, the produced BC was washed three times with distilled water and soaked in 0.1 M NaOH at 80^{o}C for 2 h to remove bacterial cells which may be attached to the BC pellicles. The pellicles were washed thoroughly with distilled water for 2–3 times to warrant the complete remove of NaOH and leaving the pellicle at neutral pH. Finally, the purified BC was dried at 60°C until a constant weight is obtained (**Costa *et al*., 2017**).

Characterization of *G. xylinus* BC

Scanning Electron Microscopy (SEM) analysis

The sample was sputtered coated with gold and examined at 15000× magnification for surface view of biocellulose. The same procedure was carried out for dried BC sheet sample, but using 10000× magnification (**Halib *et al*., 2012**). Scanning-electron microscope photographs of the BC were prepared using Scanning Electron microscope, Central Laboratory, Faculty of Science, Alexandria, Egypt.

Fourier Transform Infrared (FTIR) spectroscopy

The BC formed by *G. xylinus* was characterized using FTIR Spectra 2000 (Bruker Vector 22 instrument at room temperature. Powdered forms of the samples were prepared and analyzed over the range of 500-4000 cm^{-1} (**Halib *et al*., 2012**). The FTIR spectroscopy of BC was performed in Central Laboratory, Faculty of Science, Alexandria, Egypt.

X-ray analysis of BC

X-ray diffraction (XRD) patterns of BC membranes were used to examine the crystallinity degree of BC by using a diffractometer (Rigaku Smart lab XRD (Rigaku, The woodlands, TX) using Cu radiation. It was prepared using X-ray diffraction (XRD), Central Laboratory, Faculty of Science, Alexandria, Egypt. The crystallinity percentage was measured by equation of Segal given below.

$$C_{Ir}\ (\%) = \{(I_{002}\text{-}I_{am})/I_{002})\}*100$$

Where, C_{rl} is the relative degree of crystallinity, I_{002} is the maximum intensity (in arbitrary units) of the 002 lattice diffraction, and I_{am} is the intensity in the same units at 28 = 18° (**Costa *et al*., 2017**).

Optimization of BC production

Optimal conditions for BC production by *G. xylinus* were studied. BC production and sugar consumption were measured for different parameters by taking one parameter at one time.

Effect of incubation temperature and initial pH

Cultivation of *G. xylinus* cells was done in a sterile 30 ml of medium at different incubation temperatures (20, 25, 30, 35 and 40ºC) under shaking condition (120 rpm) for determine optimum incubation temperature. For optimum pH, *G. xylinus* cells was cultivated in a sterile 30 ml of medium at initial pH ranging from 4.0- 7.0 at optimum temperature for 7 days under shaking conditions (120 rpm).

Experimental designs for optimization by Plackett-Burman design

The Plackett-Burman experimental design (**Plackett and Burman, 1946**; **Hegde *et al*., 2013 and Suwanposri *et al*., 2014**) was used to identify the major fermentation parameters that affect BC production in liquid cultures. Seven independent variables were screened in eight combinations. For each variable, a high (+) and (-) level was tested. The experimental design is shown in the results and discussion section. All experiments were performed in duplicates and the averages of BC concentration results were treated as the responses.

Analysis for the Plackett-Burman experiment was carried out as follows. First, for all the components, their effect on the response was calculated, which was the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at the low setting (-1) of that factor. The main effect of each variable can be determined using the following equation:

$$\text{Main effect} = (\sum Mi^{+} - \sum M_i^{-})/N$$

Where Mi^{+} and M_i^{-} are the observations of trials where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2. The factor that had no effect would give a value of zero. A main effect figure with a positive sign indicates that the high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. The experimental results were analyzed to extract independently the main effects of these factors; the analysis of variance technique was then applied to determine which factors were statistically significant. The controlling factors were identified, with the magnitude of effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. The Student's *t*-test was employed in order to check the statistical significance of the regression coefficients of the variables (**Farag *et al*., 2015**).

Steepest ascent design

Once the variables having the statistically significant influence on the responses were identified, steepest ascent design was used to optimize the levels of these variables. The signs attributed to the effect total were used to initiate the second phase of the procedure which is known as the steepest ascent method. This experiment was carried out as described by **Bloor and England (1991)**. The steepest ascent method is an effective experimental procedure for moving sequentially along the direction of the maximum increase in the response, and thus, can approach the optimum neighborhood rapidly and efficiently.

The figures in the effect total were divided by the number of trials performed which was equal to 8. Each of the generated figures was called the slope. The slope was then multiplied by the unit of variation used in the factorial experiment (i.e. the extent of the +

and – values used), which was 0.5. The figure generated for each factor was then transformed relative to one of the factors, which was chosen arbitrarily. These final figures for each factor were then progressively added to (if they possessed a + sign) or subtracted from (if they possessed a – sign) the base level concentration of each factor until a reasonable series had been completed, or until one of the factors reached zero. All experiments were performed in duplicate and response values were the averages of the corresponding results.

RESULTS

Chemical composition of algal substrate

The chemical analysis for the algal species (*U. lactuca* and *P. oceanica)* was carried out and the content of organic matter included the carbohydrates, lipids, proteins and ash percentages were detected (Table 1). The carbohydrate was 41.0 ± 3.3% and 35.0 ± 2.8% for and *U. lactuca*, respectively. The total protein content was (16.8 ± 4.1%) for *P. oceanica* followed by *U. lactuca* (18%).

Table 1. The chemical composition of different substrates applied in saccharification process

Component	***P. oceanica***	***U. lactuca***
Moisture content	11.1 ± 0.8	8.8± 0.7
Organic matter	78.0 ± 2.5	73.6 ± 2.5
Carbohydrate	41.0 ± 3.3	35.0 ± 2.8
Lipid	7.0 ± 1.6	9.2 ± 1.3
Protein	18.0 ± 3.5	16.8 ± 4.1
Fibers	12.0 ± 1.6	12.6 ± 1.8
Ash	22.2 ± 0.7	26.4 ± 0.6

Fungal saccharification process for marine substrate

Reducing sugar concentrations (g/l) resulting from enzymatic treatment were estimated (Table 2). The results revealed that *P. oceanic* treated with 1% H_2SO_4, produced the highest concentration of reducing sugar (6.9 g/l) than treated with 1%NaOH (4.4 g/l). Comparatively, *U. lactuca* treated with 1% H_2SO_4 produced lower concentrations of reducing sugar (4.8g/l).

Table 2. Reducing sugar (g/l) resulting from chemical pretreatment of two substrates using *P. chrysogenum*

Substrate	**Treatment**		
	Control	**1% H_2SO_4**	**1% NaOH**
U. lactuca	2.7	4.8	3.5
P. oceanic	3.1	6.9	4.4

Isolation and screening of BC production from different marine bacterial isolates

Twenty two marine bacterial isolates and the reference strain(*Gluconacetobacter xylinus*) were examined for biocellulose producation on three different biocellulose production media (HS medium containing glucose as a reducing sugar, HSF medium contaning fructose and glucose as reducing sugars and HSE medium like HSF but containg also ethanol). The resulsts showed that only the bacterial strain *Gluconacetobacter xylinus* was the most promising for BC production. The data (Fig. 1) realised that HSF medium was the best for highest BC production (3.7 g/l), followed by HSE medium (2.3 g/l), while the lowest production medium was HS medium (1.7g/l).

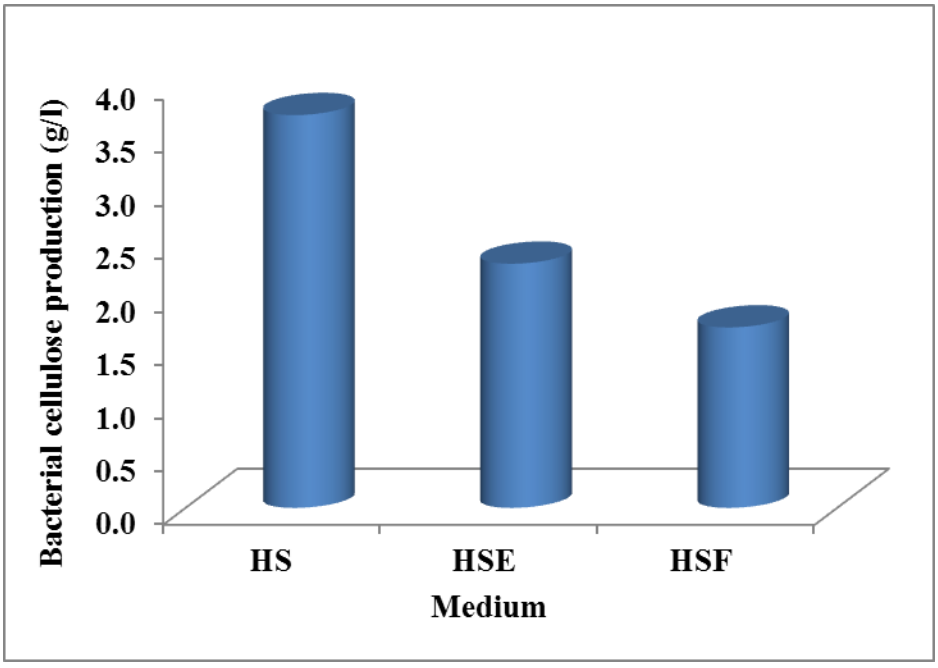


Fig. 1. Effect of different production media on BC production by *G. xylinus*. HS = Hestrin-Schramm medium; HSF = HS fructose medium; HSE = HS ethanol medium**.**

BC production by using the algal biomass as a reducing sugar

The use of a cheaper substrate instead of the commonly used sugar in BC production media (glucose or fructose) might result in a maximum production and lower cost of the final product. In this context, reducing sugars from two marine substrates sea grass; (*P. oceanic*) and green algae (*U. lactuca*) were used as a carbon source in stead of glucose in the HS medium**.** Data (Fig. 2) depict that the HSPO (containing reducing sugars extracted from *P. oceanic* in instead of glucose) was the most promising supporting for the maximal production of BC (5.7 g/l) followed by HS (containing glucose as a carbon source). On the other hand, the HSUL (medium containing reducing sugar from *U. lactuca*) gave the lowest production of BC (0.33 g/l). So, the extract of sea grass (*P. oceanic*) was used as a reducing sugar and a sole carbon source in the fermentation medium in the next experiments.

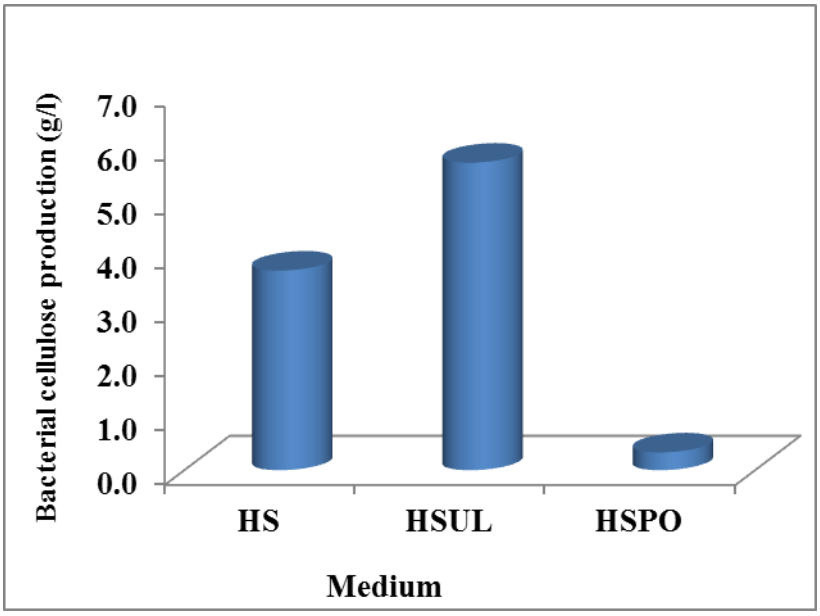


Fig. 2. Production of bacterial cellulose by *G. xylinus* using different media at 30^{o}C and pH 6.5, HS = Hestrin-Schramm medium, HSUL = HS prepared with *U. lactuca* reducing sugars instead of glucose; HSPO = HS prepared with *P. oceanic* reducing sugars instead of glucose.

Identification of the *G. xylinus* BC

FT-IR spectroscopy

One of the most useful methods to identify a chemical compound is the FTIR. The pure cellulose spectrum, distinguishs peaks of 3350 cm^{-1} and shouldering around 3400 cm^{-1} to 3500 cm^{-1} indicates O-H stretching, 2800 cm^{-1} to 2900 cm^{-1} indicates C-H stretching, 1160 cm^{-1} indicates C-O-C stretching and 1035 cm^{-1} to 1060 cm^{-1} indicates C-O stretching. Other fingerprint regions for cellulose are peak around 1431 cm^{-1} indicating CH_2 bending and the peak at 1664 cm^{-1} indicated that carboxylic acid groups exist on the surface. The data (Fig. 3) represents the FT-IR spectra of the *G. xylinus* ATCC 10245 BC showed peaks at 3436 cm^{-1}, 2924 cm^{-1}, 1645 cm^{-1}, 1418 cm^{-1} and 1050 cm^{-1} in comparison to standard , thus FTIR confirming the purity of the BC produced during the current study.

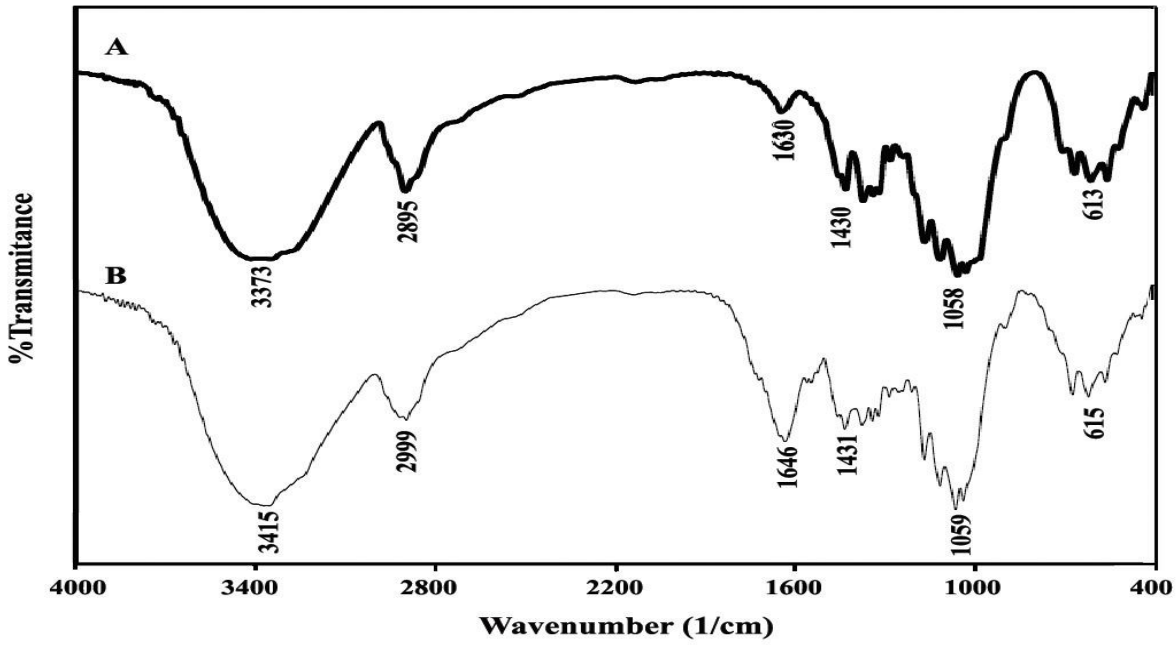
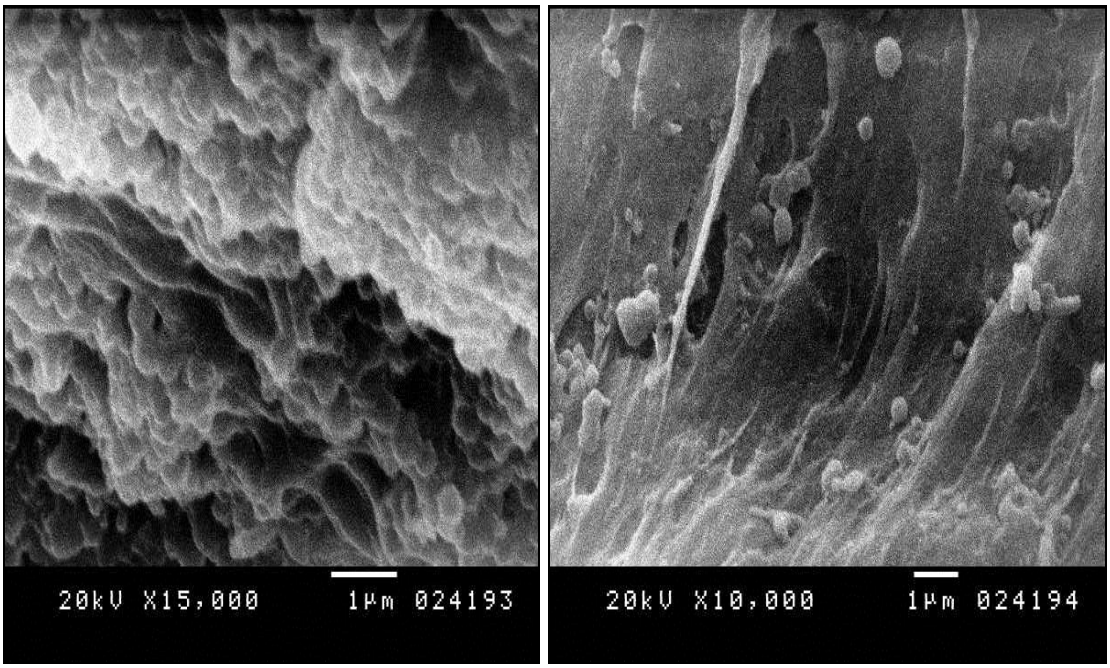


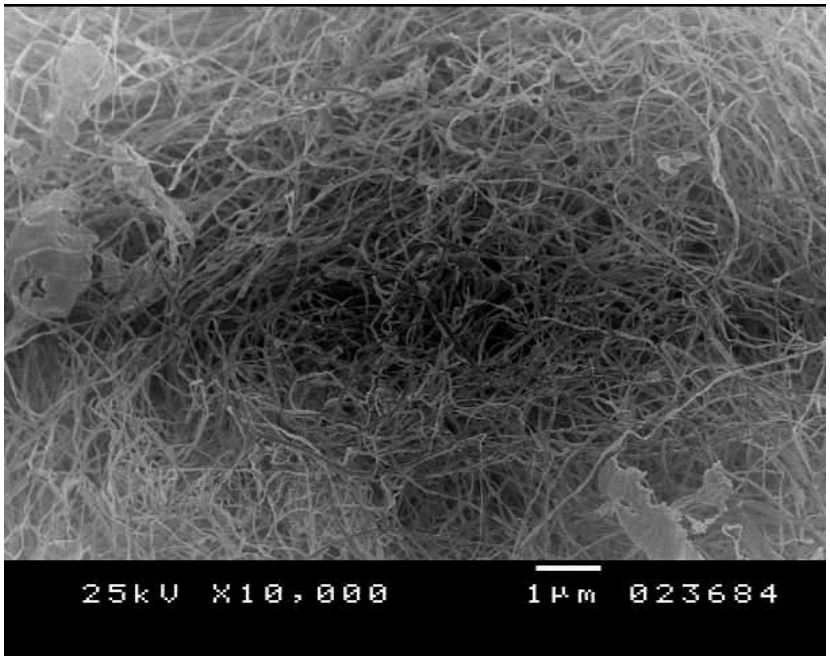
Fig. 3. FTIR spectra of the BC produced by *G. xylinus* during the current study (upper) standard BC (lower)

SEM analysis

SEM analysis was applied to examine the surface features of biocellulose produced by *G. xylinus.* Data in Fig. (4A), exhibit the surface view for biocellulose under 15000 × magnification nanofiberils which can't be observed due to converge by a thick layers so it has well interconnected pore network structure and biocellulose dried sheet under 10000× magnification, the biocellulose was fibrous with irregular size and shape (Fig. 4B). Observation of biocellulose under 10000 × magnification showed the fine cellulose ribbons, sometimes called fibrils. Observation of biocellulose fibril network produced by *G. xylinus* is shown in Fig. 4C.



(A) **(B)**



(C)

Fig. 4. (A) SEM of BC surface produced by *G. xylinus*; (B): SEM of dried BC sheet; and (C): SEM of BC fibril network.

XRD analysis of *G. xylinus* BC

Crystalline index values of BC were calculated from XRD spectra using amorphous region subtraction. The resulting spectra were analyzed and normalized (Fig. 5). The crystallinity for the BC produced from the HSPO medium is 84%. The % crystalline region shift was determined using the following equation C_{Ir} (%) = $\{(I_{002}-I_{am})/I_{002})\}*100$ where 2θ Peaks = (14.5, 16.2, 22.5).

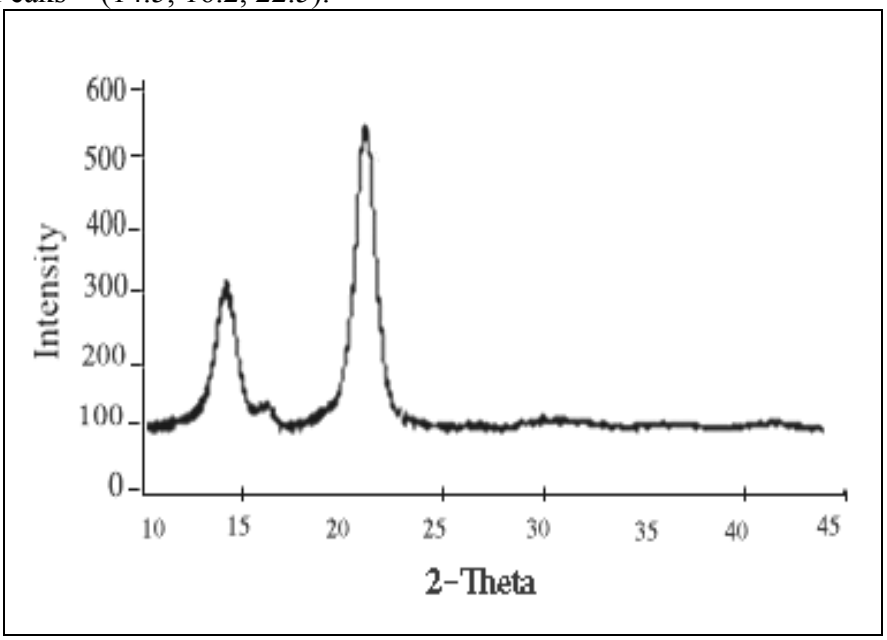


Fig. 5. X-ray patterns of the BC produced by *G. xylinus* during the current study on HSPO medium

Optimization of BC production

Effect of different incubation temperatures and initial pH value

The maximum BC production (5.5 g/l) and sugar consumption (7.2 g/l) were obtained at an incubation temperature of 30°C. Also, the optimum pH for maximum yield of BC (5.7 g/l) and sugar consumption (8.1 g/l) was 6.5 (data not shown).

Optimization of BC using Plackett-Burman experimental design

Plackett-Burman design is an efficient technique for medium component optimization. This design includes factorial design and combined analyses, builds models to evaluate the effective factors and study their interaction and select optimum conditions in limited number of experiments. The chosen levels of the culture components are given in Table 3. The design was applied with seven different variables and hence 8 different growth conditions are presented in Table 3. As shown in Table 4 the experimental results (response) demonstrate that trial no. 3 supported the highest BC production by *G. xylinus.*

Table 3. Factors examined as independent variables affecting BC production and their level in the Plackett-Burman design

Variables (g/l)	**Symbol**	**Level**		
		+1(High)	**0(Basal)**	**-1(Low)**
Reducing sugar	RS	30	20	10
Peptone	Pep	7.0	5.0	3.0
Na_2HPO_4	NP	3.7	2.7	1.7
Yeast extract	YE	7.0	5.0	3.0
Citric acid	CA	2.2	1.15	0.2
Incubation period (day)	IP	10.0	7.0	4.0
Shaking (rpm)	SS	150	120	Static

Table 4. The applied Plackett –Burman design matrix for 7 cultural variables

Trial no.	**Variables**								
	NP	**IP**	**RS**	**Pep**	**CA**	**YE**	**SS**	**Bacterial cellulose production (g/l)**	**Sugar consumption (g/l)**
1	+1	-1	-1	+1	-1	+1	+1	4.0	5.2
2	+1	+1	-1	-1	+1	-1	+1	4.5	7.9
3	+1	+1	+1	-1	-1	+1	-1	7.3	8.6
4	-1	+1	+1	+1	-1	-1	+1	5.0	8.1
5	+1	-1	+1	+1	+1	-1	-1	4.5	5.7
6	-1	+1	-1	+1	+1	+1	-1	5.0	7.3
7	-1	-1	+1	-1	+1	+1	+1	2.5	6.6
8	-1	-1	-1	-1	-1	-1	-1	2.5	3.7
9	0	0	0	0	0	0	0	5.7	8.2

The main effect of each variable, based on the BC production was estimated as the difference between both averages of measurements made at the high level (+) and the low level (-). Data represented graphically in Fig. 6, indicate that the main variables which positively affected BC production were dibasic sodium phosphate, incubation period, reducing sugar (sea grasses), peptone and yeast extract. On the other hand, citric acid and shaking showed a negative effect. Corresponding to a statistical confidence of 80%, and hence any component showing a statistical confidence equal and/or higher than 80% was considered significant.

The *t*- test for each effect allows an evaluation of a probability, *P*, which showed that the confidence level of the calculation of incubation period and dibasic sodium phosphate was 97% and 87%, respectively (Table 5). These results pointed out that the incubation period and dibasic sodium phosphate were the most significant variables.

Table 5. Statistical analysis of the Plackett-Burman experimental results

Variables (g/l)	Bacterial cellulose production		
	Main effect	*t*- value	% of confidence
Na_2HPO_4	1.325	1.270	87
Incubation period (day)	2.075	2.550	97
Reducing sugar	0.825	0.730	-
Peptone	0.425	0.366	-
Citric acid	-0.575	-0.500	-
Yeast extract	0.575	0.500	-
Shaking (rpm)	-0.825	-0.730	-

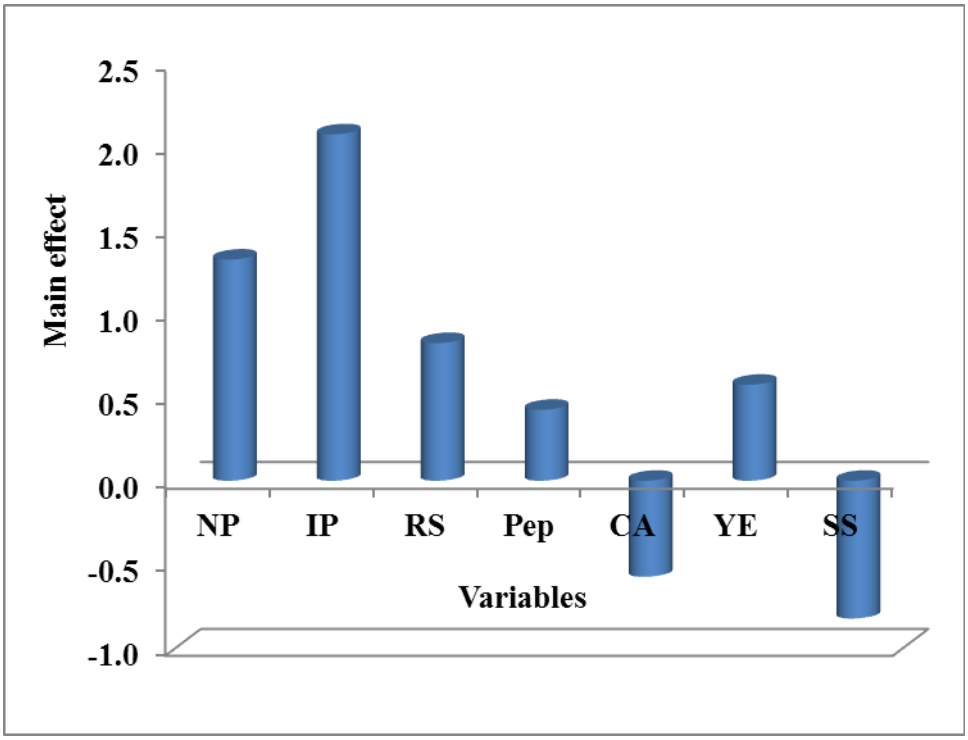


Fig. 6: Main effect of different medium components on BC production by *G. xylinus*

The second phase factorial experiment (steepest ascent method)

Based on the results of the Plackett–Burman design, Na_2HPO_4 and incubation period were chosen as the independent input variables which significantly affected BC production. In order to approach the optimum medium formula for the BC production from *G. xylinus* ATCC 10245, a statistical design known as the steepest ascent method was applied to improve performance. The path of steepest ascent is the direction in which dibasic sodium phosphate, incubation period increases most quickly. The other factors were omitted as variables from the model. Therefore, reducing sugar, peptone and yeast extract all trials were fixed at their + level and citric acid and shaking in all trials was fixed at their - level. The effect of interaction of the two significant variables was also considered in the applied method and accordingly, both were optimized throughout a single experiment. Since the two factors had identical figures of the effect total parameter

in complete factorial design, the relative concentration change unit of Na_2HPO_4 was 0.7 and the change unit of incubation period was 1day (Table 6). Assessed Na_2HPO_4up to 3.7 g/l and incubation period up to 10 days respectively resulted in increased the BC production. It was found that applications higher than 7.2 g/l and 15 days respectively had a negative effect for the BC production (Table 7).

Table 6. Statistical analysis of the second phase factorial experiment (steepest ascent method)

Factor	**Variables**	
	Na_2HPO_4	**Incubation period**
Effect total	1.325	2.07
Slope	0.2	0.258
Change unit	0.1	0.13
Relative change unit	0.7	1

Table 7. Experimental results according to the Steepest Ascent method

Trial no.	**Variables**		**Bacterial cellulose production (g/l)**	**Sugar consumption (g/l)**
	Na_2HPO_4 (g/l)	**Incubation period (day)**		
1	1.6	7	7.3	7.0
2	2.3	8	7.5	7.3
3	3.0	9	8.5	7.7
4	3.7	10	9.7	8.0
5	4.4	11	10	8.3
6	5.1	12	10.6	8.3
7	5.8	13	11.3	8.5
8	6.5	14	11.7	8.7
9	7.2	15	12.7	9.3
10	7.9	16	11.9	8.6

Validation of the model

In order to verify the obtained results and to evaluate the accuracy of two statistical designs, a verification experiment was carried out. The predicted near optimum medium and far from optimum levels of the independent variables were examined and compared to the basal condition. The applied near optimum condition, resulted in 12.7 g/l when using Steepest Ascent method (≈2.5-fold) increase in the BC production when compared to the basal medium formulation within incubation period of 15 days. So, the following optimized medium composition was postulated to maximize BC production (12.7g/l) and contained (g/l): reducing sugar, 30; yeast extract, 7; peptone, 7; dibasic sodium phosphate (buffer), 7.2; citric acid, 0.2 under static condition within incubation period of 15 days.

DISCUSSION

Bacterial cellulose (BC) is anextracellular cellulose naturally produced by many microorganisms and considered as an alternative biomaterial since it possesses superior qualities to other cellulose. The BC exhibits many unique characteristics which are different from those of other plant celluloses. Compared to plant cellulose, the nanofibril network of BC has interesting properties such as excellent water holding capacity, high degree of polymerization, high crystallinity, high purity, good biocompatibility, and excellent mechanical properties. *G. xylinus* is usually the most employed microorganism for producing biocellulose, it is a gram negative rod-shaped bacteria. HS medium is the chemically defined medium which was most commonly used for BC production (**Hestrin and Schramm, 1954**). The cost of BC production is an important aspect for the industrial applications, several studies have focused on reducing the cost of BC production by using low cost fermentation media to replace the common expensive HS medium (**Molina-Ramirez *et al*., 2018 and Revin *et al*., 2018**).

Several studies have focused on reducing the cost of BC production by using low cost fermentation media to replace the common expensive HS medium (**Molina-Ramirez *et al*., 2018 and Revin *et al*., 2018**). The cost of the fermentation medium represents a critical aspect for the commercial production of BC. The cheaper substrate used instead of the commonly used sugars as glucose or fructose might result in a lower cost of the final product. In this context, marine substrate sea grass (*P. oceanic*) has been used as a source of reducing sugar. It was collected from Mediterranean Sea, Alexandria coastline, Egypt, and used as a substrate of reducing sugars and carbon source for BC production by *G. xylinus* ATCC 10245. However, most of these materials must be hydrolyzed with acid (**Hong and Qiu, 2008**) or enzymatically (**Park *et al*., 2006**) before their use in fermentation. Enzymatic hydrolysis of cellulose micro-fibrils (found in the cell wall of algae) releases glucose which involved the synergistic action of cellulase enzyme (**Teeri, 1997**). Utilization of enzymes is common to achieve maximum biomass hydrolysis (**Duarte *et al*., 2009**) and the hemicellulose and cellulose components break down to reducing sugars (**Dien *et al*., 2008**).

The obtained results showed that the amount of total reducing sugar concentrations that were produced during the hydrolysis of green alga and sea grass were varied under different conditions and the highest sugar production (6.9g/l) was produced from sea grass (*P. oceanic*) which subjected to enzymatic pretreatment (acid hydrolysis) using enzymes.

The BC fibril network produced by *G. xylinus* was observed by using SEM analysis. The fibers were loosely arranged with large pores in biocellulose. Moreover, a randomly distributed condensed network of fibrils was observed. A magnified image (1 µ) further clarified that the fibrils were thin and possessed plenty of gaps between them. Similar observation has been previously reported by (**Tsouko *et al*., 2015 and Ullah *et al*., 2016**). SEM images of biocellulose fibers, showed more delicacy. The higher delicacy of the biocellulose fibers could influence its properties such as water-holding capacity, thermal

stability and mechanical strength. For instance bacterial cellulose has a higher tensile strength, mold ability and water holding capacity (**Jung *et al*., 2005**).

The FT-IR spectra of the BC from HSPO medium showed peaks at 3436 cm^{-1}, 2924 cm^{-1}, 1645 cm^{-1}, 1418 cm^{-1} and 1050 cm^{-1}, thus confirming the purity of the biocellulose produced during the current study which are also in agreement with other observations (**Shah *et al*., 2010 and Ullah *et al*., 2016**).

BC is a semi-crystalline material that usually produces three major characteristic crystallinic peaks when examined through XRD (**Ullah *et al*., 2016**). The XRD spectrum for indicated the presence of two distinct peaks at 2θ 14.5^{o} and 22.5^{o} along with a weak middle peak at 16.2^{o}, corresponding to the (101), (002), and crystallinic planes of the cellulose I-β structure, which is in agreement with previous reports (**Ul-Islam *et al*., 2014 and Khan *et al*., 2015**). The degree of crystallinity was calculated from the relative integrated area of the crystalline and amorphous peaks (C_{Ir} (%) = $\{(I_{002}-I_{am})/I_{002})\}*100$. The ratio of the crystalline and amorphous regions varies from sample to sample and depends on various conditions, including the cellulose source, microorganism, medium composition, and processing conditions (**Shezad *et al*., 2010**). The relative crystallinity of BC was 84% which are in good agreement with many publications (**Keshk and Sameshima, 2006 and Tsouko *et al*., 2015**).

The effect of temperature on BC production by *G. xylinus* was examined. The maximum production of BC (5.5 g/l) was detected at the temperature 30ºC. On the other hand, the lowest BC production (2.0 g/l) was obtained at 40°C. The production of BC increased by increauing incubation temperature from 20-30^{o}C because the energy which supply to the cells to converet the glucose to cellulose was increased (**Pourramezan *et al*., 2009**). Also, the enzymes needs for conversion of glucose to cellulose work at optimum temperature between 28°C to 30°C (**Pourramezan *et al*., 2009**). Our results are in agreement with those reported by **Chawla *et al.* (2009)**, **Raghunathan, (2013), Mohammad *et al*. (2014)** and **Voon *et al*. (2016)**.

The pH of the culture medium is a vital and critical factor for BC productivity (**Coban and Biyik, 2011**). Our resusts revealed that the optimum pH with high BC production (5.7 g/l) was found at pH 6.5. Also, the lowest yield (1.7 g/l) was detected at pH 4.5. The pH decreases during fermentative production because of the accumulation of gluconic, acetic or lactic acids in the culture broth (**Raghunathan, 2013; Esa *et al*., 2014 and Liu *et al*., 2015**). Many studies are in good agreement with our results which reported that the optimum pH value for maximum BC production was in range of 4–7 (**Reinitati *et al*., 2017**) and varies with the bacterium strains, but usually attribute to be within a neutral to slightly acidic pH range (**Coban and Biyik, 2011; Lee *et al*., 2014 and Lin *et al*., 2016**). On the other hand, **Castro *et al*. (2012)** reported that a bacterial strain *Gluconacetobacter medellensis*, was highly tolerant to low pH and an optimum yield (4.5 g/l) was achieved at pH 3.5. Recently, *Komagataeibacter intermedius* FST213-1 which isolated from fermented fruit juice can produce BC within pH 4–9 and a maximum production (1.2 g/l) at pH 8 (**Lin *et al.*, 2016**).

Statistical experimental design methods provide a systematic and efficient means of reaching particular goals and simultaneously studying several control factors. Hence, these methods can be used to examine and optimize the operational variables. The advantages of the factorial design are that much quantitative information can be extracted from only a few experimental trials as well as offer simultaneous study of many factors (**Nawani and Kapadnis, 2005 and Senthilkumar *et al*., 2005**). More recently, **Duta *et al*. (2006)** reported that the results analyzed by a statistical planned experiment are better acknowledged than those carried out by the traditional one variable at a time method.

The results of Plackett-Burman design showed that the variables which positively affected the BC production were; Na_2HPO_4, incubation period, reducing sugars, peptone and yeast extract. Yet, citric acid and shaking showed a negative effect, i.e., low amounts of these compounds in the culture medium increased BC yield.
Carbon source plays an important role in BC biosynthesis because it was used as a sole source of energy and help in polymerization process (**Zhou *et al*., 2007**). Reducing sugar from seagrass (used as a sole carbon source) had a positive effect on BC production (**Usha *et al*., 2011 and Hegde *et al*., 2013**). Nitrogen source is a main component of proteins necessary in cell metabolism, and comprises 8–14% of the dry cell mass of bacteria (**Ramana *et al*., 2000 and Chawla *et al*., 2009**). Peptone and yeast extract which used as a nitrogen source which had a positive effect on BC production. Similar observation has been reported by **Raghunathan, (2013)** and **Mohammad *et al*. (2014).**

In addition, the presence of Na_2HPO_4 significantly enhanced the formation of the BC because phosphate considered as an essential component for BC production (**Keshk and Sameshima, 2006 and Raghunathan, 2013**). On the other hand, high concentration of citric acid (2.2 g/l) negatively affected the production of BC because high level of organic acid could have a lethal effect on bacterial cell (**Hegde *et al*., 2013**).

The results showed that dissolved oxygen had a negative effect on BC production by *G. xylinus*. Our results are in agreement with **Tantratian *et al*. (2005)** who found that high dissolved oxygen in the medium increased the gluconic acid content when glucose was used as a carbon source and can reduce cellulose production. Also, a higher BC production by many bacteria are produced in a static culture than in shaked (**Kurosumi *et al*., 2009 and Wu and Liu, 2012**).
In order to approach the optimum medium formula for BC from *G. xylinus*, a statistical design, the steepest ascent method (**Box *et al*., 1978**) was applied to improve performance. The path of steepest ascent is the direction in which incubation period and Na_2HPO_4 increases most quickly. Assessed Na_2HPO_4 up to 3.7 g/l and incubation period up to 10 days, respectively, resulted in the increase of BC production. It was found that applications higher than 7.2 g/l and 15 days, respectively had a negative effect for BC production. In line with our results,

Many authors used Plackett-Burman experiment, Box-Behnken design, and Central Composite Design and found that BC yield was increased than before using statistical designs (**Bilgi *et al*., 2016**). **Hegde *et al*. (2013)** used Plackett-Burman design and a Central Composite Design (CCD) for optimization of significant factors for BC

production and found that glucose, yeast extract and peptone were the most significant factors.

CONCLUSION

This work presented the production of BC pellicles with an alternative, low-cost medium containing reducing sugars drived from marine algae. Algae are composed of lignocellulosic materials and produces sugar and can be used as a sole carbon source in the fermentation medium for the production of BC. So, in future BC can be produced using the simultaneous saccharification fermentation (SSF) method effect the production of BC nanofibers. The SEM, FTIR and XRD indicated that the pure BC produced by *G. xylinus* was cellulose and comosed of nanofibrils. Also, optimization of BC production using Plackett-Burman experiment and Steepest Ascent method (≈2.5-fold) increase in the BC production when compared to the basal medium formulation. So, this work may open doors for various researches in coming generation.

REFERENCES

Adams, J.; Gallagher, J. and Donnison, I. (2009). Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. J. Appl. Phycol., 21:569–74.

Andelib, Y.A. and Nuran, D. A. (2009). Isolation of cellulose producing bacteria from wastes of vinegar fermentation. Proceedings of the world congress on engineering and computer science, San Francisco, USA.

Atlas, R.M. (1997). Handbook of Microbiological Media. 2nd edition. Lawrence C.Parks. (Ed).

Bae, S. and Shoda, M. (2004). Bacterial cellulose production by fed-batch fermentation in molasses medium. Biotechnol. progress, 20(5):1366-1371.4

Bilgi, E.; Bayir, E.; Sendemir-Urkmez, A. and Hames, E.E. (2016). Optimization of bacterial cellulose production by *Gluconacetobacter xylinus* using carob and haricot bean. Int. J. Biol. Macromol., 90:2-10.

Bloor, S. and England, R.R. (1991). Elucidation and optimization of the medium constituents controlling antibiotic production by the cyanobacterium *Nostoc muscorum*. Enzy. Microbiol. Technol., 13:76–81.

Borines, M. G.; de Leon, R. L. and Cuello, J. L. (2013). Bioethanol production fromthe macroalgae *Sargassum* spp. Bioresource Technol., 138: 22–29.

Box, G.E.P.; Hunter, W.G. and Hunter, J.S. (1978). Statistics for experimenters: An introduction to design, data analysis and model building. David, J. Balding, Noel A.C. Cressie, Nicholas.I. Fisher.Iain. M. Johnstone, J.B. (Eds). Wiley and Sons, N.Y. pp. 653.

Brown, A.J. (1886). On acetic ferment which forms cellulose. J. Chem. Soc., 49:432-439.

Chawla, P.R., Bajaj, I.B.; Survase, S.A. and Singhal, R.S. (2009). Fermentative production of microbial cellulose. Food Technol. Biotechnol. (2):107–124.

Çoban, E.P. and Biyik, H. (2011). Evaluation of different pH and temperatures for bacterial cellulose production in HS (Hestrin-Scharmm) medium and beet

molasses medium. Afri. J. Microbiol. Res., 5:1037-1045.

Costa, A.F. S.; Almeida, F. C. G.; Vinhas, G. M. and Sarubbo, L. A. (2017). Production of bacterial bellulose by *Gluconacetobacter hansenii* using corn steep liquor as nutrient sources. Frontiers in Microbiol., 8:1-12.

de Pádua, M.; Fontoura; P. S. G and Mathias, A. L. (2004). Chemical composition of *Ulvaria oxysperma* (Kützing) bliding, *Ulva lactuca* (Linnaeus) and *Ulva fascita* (Delile). Braz. Arch. Biol. Technol., 47 (1): 49-55.

Dien, B.S.; Ximenes, E. A.; O'Bryan, P. J.; Moniruzzaman, M.; Li, X. L.; Balan, V. and Cotta, M. A. (2008). Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol. Bioresource Technol., 99(12):5216-5225.

Duarte, L.C.; Silva-Fernandes, T.; Carvalheiro, F.; Gírio, F.M. (2009). Dilute acid cellobiohydrolases. Trends in Biotechnol., 15(5):160-167.

Duta, F.P.; De Franca, F.P. and Lopes, L.M.D. (2006). Optimization of culture conditions for exopolysaccharides production in *Rhizobium* sp. using the response surface method. Electron. J. Biotechnol., 9:391-399.

El-Naggar, M.M.; Abdul-Raouf, U.M.; Ibrahim, H.A.H and El-Sayed, W.M.M. (2014). Saccharification of *Ulva lactuca* via *Pseudoalteromonas piscicida* for biofuel production. J. Energy Nat. Res., 3(6): 77-84.

Enas, A.H.; Hemmat M.A.; Sohir, S.A. and Sara, M.A. (2015). The characterization of bacterial cellulose produced by *Acetobacter xylinum* and *Komgataeibacter saccharovorans* under optimized fermentation conditions. British. Microbiol. Res. J., 9:1-13.

Esa, F.; Tasirin, S.M. and Abd Rahman, N. (2014). Overview of bacterial cellulose production and application. Agric. Sci. Procedia. 2: 113 – 119.

Farag, A. M.; Hassan, S. W.; Beltagy, E. A. and El-Shenawy, M. A. (2015). Optimization of production of anti-tumor L-asparaginase by free and immobilized marine *Aspergillus terreus*. Egyp. J. Aquatic Res., 41(4):259-302.

Goelzer, F. D. E.; Faria-Tischer, P. C.S.; Vitorino, J. C.; Sierakowski, M.R. and Tischer, C.A. (2009). Production and characterization of nanospheres of bacterial cellulose from *Acetobacter xylinum* from processed rice bark. Mate. Sci. Enginee.: C, 29(2): 546-551.

Halib, N.; Mohd, C.I.; Mohd, A. and Ishak, A. (2012). Physicochemical properties and characterization of *Nata de* Coco from local food industries as a source of cellulose. Sains Malaysiana, 41:205–211.

Hegde, S.; Bhadri, G.; Narsapur, K.; Koppal, S. and Oswa, l. P. (2013). Statistical optimization of medium components by response surface methodology for enhanced production of bacterial cellulose by *Gluconacetobacter persimmonis.* J. Biopro. Biotech., 4:142.

Hestrin, S. and Schramm, M. (1954). Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. Biochem. J., 58(2): 345–352.

Hong, F. and Qui, K. (2008). An alternative carbon source from konjac powder for enhancing production of bacterial cellulose in static cultures by a model strain *Acetobacter* aceti subsp. *xylinus* ATCC 23770. Carbohydr. Polym., 72(3): 545-549.

Hungund, B.; Prabhu, S.; Shetty, C.; Acharya, S.; Prabhu, V. and Gupta, S.G. (2013). Production of bacterial cellulose from *Gluconacetobacter persimmonis* GH-2 using dual and cheaper carbon sources. J. Microb. Biochem. Technol., 5:31-33.

Ji, K.; Wang, W.; Zeng, B.; Chen, S.; Zhao, Q.; Chen, Y.; Li, G. and Ma, T. (2016). Bacterial cellulose synthesis mechanism of facultative anaerobe *Enterobacter* sp. FY-07. Sci. Rep., 6: 21863.

John, R.P.; Anisha, G.S.;Nampoothiri, K.M. and Pandey, A. (2011). Micro and macroalgal biomass: a renewable source for bioethanol". Bioresour Technol., 102:186–93.

Junaidi, Z.; Shahril, M. and Siew, K.W. (2012). Enhancement of biocellulose production in mixed medium culture. In: Malaysian International Conference on Trends in Bioprocess Eng., 1-11.

Jung, J.Y.; Park, J. K. and Chang, H.N. (2005). Bacterial cellulose production by *Gluconoacetobacter hansenii* in an agitated culture without living non-cellulose producing cells. Enzy. Microb. Technol., 37:347–354.

Keshk, S. and Sameshima, K. (2006). Influence of lignosulfonate on crystal structure and productivity of bacterial cellulose in a static culture. Enz. Microb. Technol., 40: 4-8.

Kurosumi, A.; Sasaki, C.; Yamashita, Y. and Nakamura, Y. (2009). Utilization of various fruit juices as carbon source for production of bacterial cellulose by *Acetobacter xylinum* NBRC 13693. Carbohydr. Polym., 76:333–335.

Kwak, M.H.; Kim, J.E.; Go, J.; Koh, E.K.; Song, S.H.; Son, H.J.; Kim, H.S.; Yun, Y.H.; Jung, Y.J. and Hwang, D.Y. (2015). Bacterial cellulose membrane produced by *Acetobacter* sp. A10 for burn wound dressing applications. Carbohydr. Polym., 122:387–398.

Lee, K.Y.; Buldum, G.; Mantalaris, A. and Bismarck, A. (2014). More than meets the eye in bacterial cellulose: biosynthesis, bioprocessing, and applications in advanced fiber composites. Macromol. Biosci., 14(1):10–32.

Lee, J.W.; Deng, F.; Yeomans, W.G.; Allen, A.L.; Gross, R.A. and Kaplan, D.L. (2001). Direct incorporation of glucosamine and N-acetyl glucosamine into exo-polymers by *Gluconacetobacter xylinus* (*Acetobacter xylinum*) ATCC 10245: Production of chitosan-cellulose and chitin-cellulose exo-polymers. Appl. Environ. Microbiol., 67(9):3970-3975.

Lin, S.P.; Huang, Y.H.; Hsu, K.D.; Lai, Y.J.; Chen, Y.K. and Cheng, K.C. (2016). Isolation and identification of cellulose-producing strain *Komagataeibacter intermedius* from fermented fruit juice. Carbohydr. Polym., 151:827–833.

Liu, M.; Zhong, C.; Wu, X.Y.; Wei, Y.Q. and Han, P.P. (2015). Metabolomic profiling coupled with metabolic network reveals differences in *Gluconacetobacter xylinus* from static and agitated cultures. Biochem. Eng. J., 101:85–98.

Liu, M.; Zhong, C.; Zhang, Y.M.; Xu, Z.M.; Qiao, C.S. and Jia, S.R. (2016). Metabolic Investigation in *Gluconacetobacter xylinus* and Its Bacterial Cellulose Production under a Direct Current Electric Field. Front Microbiol., 7: 331.

Miller, G.L. (1959). Use of dinitrosalicylic acid for determination of reducing sugar. Anal. Chem., 31:426–428.

Mohammad, S.M.; Rahman, N.A.; Khalil, M.S. and Abdullah, S.R.S. (2014). An Overview of biocellulose production using *Acetobacter xylinum* culture. Adv. Biol. Res., 8:307-313.

Molina-Ramirez, C.; Castro, C.; Zuluaga, R. and Ganan, P. (2018). Physical characterization of bacterial cellulose produced by *Komagataeibacter medellinensis* using food supply chain wast and agricultural by-products as alternative low-cost feedstocks. J. Polym. Environ., 26: 830-837.

Nawani, N.N. and Kapadnis, B.P. (2005). Optimization of chitinase production using statistics based experimental designs. Process Biochem., 40:651-660.

Nguyen, B.: Flanagan, M.J.: Gidley, G.A. and Dykes. (2008). Characterization of cellulose production by *Gluconacetobacter xylinus* strain from kombucha. Curr. Microbiol., 57:449–453.

Park, S. T.; Kim, E. and Kim, Y.M. (2006). Overproduction of cellulose in *Acetobacter xylinum* KCCM 10100 defective in GDP-mannosyltransferase. J. Microbiol. Biotechnol., 16(6): 961–964.

Phruksaphithak, N.; Kaewnum, C. and O-Thong, S. (2019). Bacterial cellulose production and its applications. Sci. Enginee. Health Studies (SEHS), 13(1):

Plackett, R.L. and Burman, J.P. (1946). The design of optimum multifactorial experiments. Biometric., 33:305-325.

Pourramezan, G.Z.; Roayaei, A.M. and Qezelbash, Q.R. (2009). Optimization of culture conditions for bacterial cellulose production by *Acetobacter xylinum* sp. 4B-2. Biotechnol., 8: 150-154.

Raghunathan, D. (2013). Production of microbial cellulose from the new bacterial strain isolated from temple wash waters. Int. J. Curr. Microbiol. App. Sci., 2:275-290.

Ramana, K.V.; Tomar, A. and Singh, L. (2000). Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter xylinum*. World J. Microbiol. Biotechnol., 16:245–248.

Rasooli, I.; Sharafi, S.M. and Beheshti-Maal, K. (2010). Isolation, characterization and optimization of indigenous acetic acid bacteria and evaluation of their preservation methods Iran. J. Microbiol., 2:41-48.

Reiniati, I.; Hrymak, A.N. and Margaritis, A. (2017). Recent developments in the production and applications of bacterial cellulose fibers and nanocrystals. Crit. Rev. Biotechnol., 37, 510-524.

Revin, V.; Liyaskina, E.; Nazarkina, M.; Bogatyreva, A. and Shchankin, M. (2018). Cost-effective production of bacterial cellulose using acidic food industry by-products. Brazil. J. Microbiol., 49: 151-159.

Senthilkumar, S.R.; Ashokkumar, B.; Raj, K. and Chandra Gunasekaran, P. (2005). Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* in solid-state fermentation using central composite rotary design. Biores. Technol., 96:1380-1386.

Shoda, M. and Sugano, Y. (2005). Recent advances in bacterial cellulose production, Biotechnol. Biopro. Eng., 10:1–8.

Skiba, E.A.; Budaeva, V.V.; Ovchinnikova, E.V.; Gladysheva, E.K.; Kashcheyeva, E.I.; Pavlov, I.N. and Sakovich, G.V. (2019). A technology for pilot production of bacterial cellulose from oat hulls, Chem. Engine. J.

Solomon, B.O.; Amigun, B.; Betikue, T.V.; Ojumu, T. and Layokun, S.K. (1999).

Optimization of cellulase production by *Aspergillus flavus linn.* isolates NSPR 101 grown on bagasse. JNSCHE., 18:61-68.

Surma-Ślusarska, B.; Presler, S. and Danielewicz, D. (2008). Characteristics of bacterial cellulose obtained from *Acetobacter xylinum* culture for application in papermaking. FIBRES & TEXTILES in Eastern Europe, 16:108-111.

Suwanposri, A.; Yukphan, P.; Yamada, Y. and Ochaikul, D. (2014). Statistical optimization of culture conditions for biocellulose production by *Komagataeibacter* sp. PAP1 using soya bean whey. Maejo Int. J. Sci. Technol., 8:1-14.

Szulczyk, K.R. (2010). Renewable and sustainable reviews. Market penetration of biodiesel, 14(8):2426-2433.

Tantratian, S.; Tammarate, P.; Krusong, W.; Bhattarakosol, P. and Phunsri, A. (2005). Effect of dissolved oxygen on cellulose production by *Acetobacter* sp. J. Sci. Res. Chula Univ., 30*:* 179–186.

Teeri, T. T. (1997). Crystalline cellulose degradation: new insight into the function of hydrolysis of wheat straw oligosaccharides. Appl. Biochem. Biotechnol., 153(1-3): 116-126.

Tsouko, E.; Kourmentza, C.; Ladakis, D.; Kopsahelis, N.; Mandala, I.; Papanikolaou, S.; Paloukis, F.; Alves, V. and Koutinas, A. (2015). Bacterial cellulose production from industrial waste and by-product streams. Int. J. Mol. Sci., 16:14832-14849.

Ul-Islam, M.; Khan, T. and Park, J. (2012). Water holding and release properties of bacterial cellulose obtained by in situ and ex situ modification, Carbohydr. Polym., 88:596–603.

Uraki, Y.; Morito, M.; Kishimoto, T. and Sano, Y. (2002). Bacterial cellulose production using monosaccharides derived from hemicelluloses in water-soluble fraction of waste liquor from atmospheric acetic acid pulping.Holzforschung, 56(4): 341-347.

Voon, W.W.Y.; Rukayadi, Y. and Meor Hussin, A.S. (2016). Isolation and identification of biocellulose-producing bacterial strains from Malaysian acidic fruits. Lett. Appl. Microbiol., 62: 428–433.

Wang, X.; Liu, X. and Wang, G. (2011). Two-stage hydrolysis of invasive algal feedstock for ethanol fermentation. J. Integr. Plant Biol., 53:246–52.

Wu, J. M. and Liu, R. H. (2012). Thin stillage supplementation greatly enhances bacterial cellulose production by *Gluconacetobacter xylinus*. Carbohyd. Polymer., 90:116–121.

Yoon, M.; Choi, J.; Lee, J. and Park, D. (2012). Improvement of saccharification process for bioethanol production from *Undaria* sp. by gamma irradiation. Radiation Phys. Chem., 81:999–1002.

Zhou, L.L.; Sun, D.P.; Hu, L.Y.; Li, Y.W. and Yang, J.Z. (2007). Effect of addition of sodium alginate on bacterial cellulose production by *Acetobacter xylinum*. J. Ind. Microbiol. Biotechnol., 34:483–489.