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Biocellulose Production by *Gluconacetobacter hansenii* ATCC 23769: Application of Statistical Experimental Designs and Cellulose Membrane Characterization

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IN THIS STUDY, an optimization of the culture parameters used for the production of bacterial cellulose (BC) by *G.hansenii* ATCC 23769 was carried out. This is the first study reported statistical optimization of the fermentation medium by Plackett–Burman Design (PBD) and Box–Behnken Designs (BBD) for BC production by type strain *G.hansenii* ATCC 23769. The effect of seven culture parameters on BC production was evaluated by implementing PBD, where the results revealed that, the most significant variables affecting BC production were yeast extract, temperature and incubation time. Response surface methodology (RSM) using BBD was applied to find out the optimum level of each significant variable. The optimal levels of the three significant components were found to be yeast extract 13 g/l, temperature 26.3°C and incubation time 12 days with a predicted yield 2.91 g/l. According to the results of the PBD and BBD the following medium composition is expected to be optimum (g/l): mannitol 25, yeast extract 13, ethanol 7 ml/l, pH 7, inoclume size 7%, temperature 26.3°C and incubation time 12 days. Characterization of dried BC membrane was carried out to determine the morphological structure and purity by Scanning Electron Microscopy (SEM), crystallinity by X-ray Diffraction (XRD), chemical structure and functional group by Fourier -Transform Infrared spectroscopy (FT-IR), and thermal stability by Thermogravimetric Analysis (TGA). In addition, mechanical properties such as: the Young's modulus, tensile strength, elongation at beak % and thickness of BC membrane obtained from *G.hansenii* ATCC 23769 were determined.

Keywords: *Gluconacetobacter hansenii* ATCC 23769, Bacterial cellulose, Statistical optimization, Characterization.

Introduction

One of the most abundant materials on earth is cellulose, which recognized as the major component of plant biomass than hemicellulose and lignin. It is a polymer of β1,4 linked glucose units with the formula $[(C_6H_{10}O_5)n]$ [1, 2]. Cellulose is generally synthesized by plant and known as plant ccellulose (PC). It is used widely in several applications similar: textile and food [3], as well as that produced by bacterial strains [4]. The BC is an extra polysaccharide produced by various species of bacteria, such as the genera *Acetobacter* (reclassified as *Gluconacetobacter), Agrobacterium, Rhizobium and Sarcina* [5].

The *Gluconacetobacter* sp is a Gram-negative bacterium, and is known to be one of the most effective BC producers [6, 7]. *Gluconacetobacter hansenii* ATCC 23769 (*G.hansenii* ATCC 23769), a Gram negative bacterium, produces and secrets highly crystalline cellulose into growth medium, and has long been used as a model system for production of BC and a reference strain in various studies [8, 9]. PC and BC have a much the same chemical structure [10]. However, BC is contrasting from PC in some physicochemical and mechanical properties, including fibrils where, BC are 100 times thinner than that of PC, making it more porous, finer structure (nanoscale microfibrils < 10 nm in width), higher purity (free from hemicellulose and lignin), longer fiber length (polymerization degree between 2000 and 6000), higher crystallinity, higher water absorbing and holding capacity, higher tensile strength, strong biological adaptability, nontoxic and non-allergenic [11-14]. Therefore, BC represents a potential alternative to plant-derived cellulose and a promising material for many applications [11]. These include a thickening agent and food stabilizer [15], food packaging [16], biomaterial for manufacturing cosmetics [17], artificial skin [18], artificial blood vessels or tissue engineering [19], preparation of optically transparent films [20] and electric conductors [21]. The productivity of BC needs to be spoken to make it economically well-suited. Hence it becomes necessary to optimize the BC production through applying various process improvement strategies. Primarily, various studies have been directed to optimize the medium constituents and cultivation parameters for increasing the BC production [22-26]. One-variable-at-a-time (OVAT) approach is one of the most traditional processes for optimization of BC. In this practice, all parameters have been kept constant while only one parameter of input has been changed. In this approach, the chance is an important factor to find the actual optimum values of studied parameter conditions, which is complicated and time-consuming, especially on multi-variables screening and inherently ignore the interaction between the parameters [27]. Statistical experimental designs, which are also called statistical optimization, can be used to obtain much more reliable data for process optimization and have proved to be more efficient than OVAT method [28]. Statistical experimental designs provide a matrix and efficient strategy for experimentation to achieve certain goals so that many parameters can be simultaneously studied.

Statistical optimization allows quick screening of large experimental domain, and reflects the role of each component and their interactions. The PBD and the RSM can be used to optimize cultivation conditions [29]. The most important advantage of RSM is the ability to design a minimum number of experiments, which lead to making the process less time consuming and reducing the material costs. RSM can be used for achieving the optimum levels of the culture conditions which are found to be most significantly using a minimum number of experiments [30-32]. Recently such statistical designs were used to determine the most significant factors that effect on BC production as well as the optimum levels of introduced significant factors [22, 30, 33-35].

The culture conditions affecting BC production by G.hansenii ATCC 23769, were intensively demonstrated through applying experimental design approaches through this study. Primarily, PBD was applied to screen some factors like: glucose, yeast extract, magnesium sulphate, ethanol, pH, potassium phosphate, inoclume size, incubation temperature and time. Thereafter, RSM was applied to optimize the significant parameters (identified by the PBD) using BBD. Moreover, BC was characterized using SEM, XRD, FT-IR, TGA and mechanical properties were evaluated.

Materials and Methods

Microorganism

The *Gluconacetobacter hansenii* ATCC 23769 (G.hansenii ATCC 23769) strain used in this study was obtained from the American Type Culture Collection and recognized as BC producer. The culture was maintained on Hestrin & Schramm (HS) agar slants, transferred and stored at 4°C in the refrigerator for further study.

Pre inoclume preparation

HS medium, which comprises the following components (g/l): 20 D-glucose, 5 peptone, 5 yeast extract, 2.7 disodium hydrogen phosphate and 1.15 citric acid, was prepared; autoclaved (MC-40, ALP, Japan) at 121 °C for 20 min and inoculated with the *G.hansenii* ATCC 23769, then incubated in shaker incubator (150 rpm) (INNOVA 42 R, Brunswick, USA) at 30 °C for 2 days to activate *G.hansenii* ATCC 23769 [36].

Initial production medium and cultural conditions

Modified GEM medium, which comprises the following components (g/l): 20 mannitol, 5 yeast extract, 5ml ethanol at pH 6, was cultured with

6% inoclume of bacterial strain *G.hansenii* ATCC 23769, then incubated (Heraeus B6 incubator, Germany) at 25 °C for 7 days under static mode. These conditions were selected previously through OVAT approach. In this study the former medium was used as standard medium, where further optimization through experimental design was applied to achieve higher quantities of BC.

Screening of the independent variables-Plackett Burman Design (PBD)

PBD was used to identify the most effective variables that significantly influence on the production of BC. PBD have an ability to screen high number of variables reliably, and practically eliminate non-effective variables by investigating the effects of each variable without doing numerous experiments [29]. For *G.hansenii* ATCC 23769 seven independent variables were selected and tested in 12- trails plus 2 trails (repeat of trial12) to determine the high efficiency and accuracy of PBD. The studied variables including media components such as: mannitol, yeast extract and ethanol concentration; while the physical parameters include pH, inoclume size, temperature and incubation time. Each variable has two levels (maximum and minimum value) to determine key factors affecting the BC production. A regression analysis for PBD was used to analyze the results where the dry weight of BC was used as response. All experiments were done in triplicate and the average value was calculated. The signs +1 and −1 represent the high and low levels of the independent variables under investigation (Table 1). In the 14-run PBD each row represents an experiment and each column represents an independent variable (Table 3). All trials were prepared in 250ml Erlenmeyer flasks containing 50ml medium. Plackett-Burman screening design depends on the first order model equation 1:

$$Y = \beta_0 + \sum \beta_i X_i$$

(equation 1)

In this model Y representing the response

(BC dry wt g/l), β_0 is the model intercept, β_i is the variable estimate and X_i represents the variable. The significance of variables was determined by calculating the *p*-value through standard regression analysis.

Optimization of the independent variables- Box–Behnken Design (BBD)

RSM was applied to optimize the significant parameters identified by the PBD using BBD [31]. This optimization process involves three main steps: performing the statistically designed experiments, estimating the coefficients of the structured mathematical model and predicting the response and checking the adequacy of the model. BBD was used to identify the optimum level for each parameter and their mutual interaction on BC production. 13 full factorial BBD plus 2 extra trials at central point (sum 15 trails) were used to investigate individual and synergetic effect of the three selected significant parameters identified through PBD (yeast extract, temperature and incubation time). Each significant variable was studied at three different levels (−1, 0 and +1), where 0 represents the central value of each variable, while +1 and −1 represent the high and low value of each variable, respectively (Table 3). However, the other parameters were kept each at its effective level either positive/ or negative according to regression analysis of PBD experiment (mannitol 25 g/l, ethanol 7 ml/l, pH 7 and inoclume size 7% were set up to culture medium). Thus, in the 15-run BBD each row represents an experiment and each column represents the studied significant variable (Table 4). BC production was taken as response (Y) and culture conditions were taken as independent variables (X). The results were fitted to the following second-order polynomial structured model for three variables:

$$Y = \beta_0 + \beta_1(X1) + \beta_2(X_2) + \beta_3(X_3) + \beta_{12}(X_1X_2) + \beta_{13}(X_1X_3) + \beta_{23}(X_2X_3) + \beta_{11}(X_1)^2 + \beta_2(X_2)^2 + \beta_{33}(X_3)^2 \quad \text{(equation 2)}$$

TABLE 1. Culture conditions and their levels in PBD for BC production.

Symbols of variables	Variables	Low level (-1)	High level (+1)
X1	Mannitol (g/l)	15	25
X2	Yeast extract (g/l)	5	10
X3	Ethanol (ml/l)	3	7
X4	pH	5	7
X5	Inoclume size (%)	5	7
X6	Temperature (°C)	20	30
X7	Incubation time (days)	6	8

Where, Y is the predicted response (BC production), $\circledR_0$ is constant, $\circledR_1$, $\circledR_2$, and $\circledR_3$ are linear coefficients, $\circledR_{12}$, $\circledR_{13}$ and $\circledR_{23}$ are cross product coefficients, and $\circledR_{11}$, $\circledR_{22}$ and $\circledR_{33}$ are quadratic coefficients, Eq. (2) was used to predict the optimum levels of the independent parameters by setting the partial derivative with respect to each independent variable to zero. All experiments were done in triplicate.

Purification of BC

The resulting pellicle was harvested then washed three times with distilled water to remove culture medium residues. Subsequently, BC pellicle was heated in water bath (WB 10, Schutzart DIN, Germany), in presence of 0.5% NaOH solution at 90°C for 30 min, to remove microbial contaminants and other impurities adsorbed on the membranes of BC, then washed 3-4 times with distilled water until a neutral pH of washed liquid was obtained. Finally, the purified BC was dried in oven (T 6, Heraeus, Germany) at 70°C over- night until constant weight [37]. Then the dry weight of BC was determined.

Data analysis

The data of BC g/l was subjected to multiple linear regressions using Microsoft Excel 2007 to estimate *t*-value, *P*-value and confidence levels. The significance level (*P* -value) is determined using the *Students t -test*. Confidence level is an expression of the *P* -value in percent. Optimal value of yield was estimated using the JMP$_{IN}$ program Version: 4.0.4. Three-dimensional plots were drawn for visualization of interaction between significant variables and their optimal values. All experiments were carried out independently in triplicates and the average values are presented.

Characterization of BC membrane

  Scanning Electron Microscope (SEM)

The morphological structure of BC membrane was characterized using scanning electron microscope (JEOL JSM 6360 LA, Japan) observation; the dried BC membrane was mounted and coated with a thin layer of gold nanoparticles in preparation for SEM imaging. SEM experiment

was conducted at an accelerated voltage of 15 kV at a magnification of 5000 X.

X-ray Diffraction analysis (XRD)

The crystallinity of dried BC membrane was determined using XRD patterns were collected on an x-ray (Shimadzu XRD- 6000, Japan) diffractometer with a back monochromatic and a Cu anticathode. Radical scan was recorded in the reflection scanning mode at 4 deg per min from 4 to 100^0 2Θ. The tube voltage and current were set to 30 kV and 30 mA, respectively. The divergence slit was set at 1 degree and the antiscatter slit was set at 1 degree. The degree of crystallinity was calculated as follows: CrI=$(I_{200}-I_{am})/I_{200}$ (equation 3)

Where, I200 is the overall intensity of the peak at 2Θ and I_{am} is the intensity of the baseline at $2\Theta=18^0$ [38-40].

Fourier-Transform Infrared Spectroscopy (FT-IR)

The functional groups and chemical bonds of dried BC membrane were investigated using a Fourier transform infrared spectrophotometer (Shimadzu FTIR-84 00 S, Japan) connected to a PC, and data analysis was accomplished using the IR Solution software, version 1.21. For each sample, the scan range was from 4000 to 500 cm^{-1}, using a resolution of 1 cm^{-1}.

Thermogravimetric analysis (TGA)

TGA curves of dried BC were done using a thermogravimetric analyzer (Shimadzu TGA-50, Japan). For thermal decomposition behavior test, cellulose samples were dried at 70 °C before the conduction in a N$_2$ purge (40 mL/min) in a heated temperature from 50 °C to 800 ∘C at a heating rate of 10 °C /min.

 Mechanical properties

The mechanical properties of dried BC membrane was determined by using the universal tensile test machine (Universal Testing Machine, model: AG-I/50 N-10 kn, Japan) operating at a crosshead speed of 2 mm/ min at room temperature. BC membrane was cut into rectangular strips (20 mm x 10 mm) for

TABLE 2. Culture conditions and their levels in BBD for BC production

Symbols of variables	Variables	Low (-1)	Middle (0)	High (+1)
X$_1$	Yeast extract (g/l)	7	10	13
X$_2$	Temperature (°C)	25	30	35
X$_3$	Incubation time (days)	8	10	13

measurement, with a gauge length of 20 mm.. The electronic digital micrometer was used for determination of BC membrane thickness before examination. Three specimens were made to average the results.

Results and Discussion

Screening for nutrients and physical parameters of G.hansenii ATCC 23769 using PBD

The PBD is uncomplicated and quick method for screening a large number of variables in one experiment to evaluate the significant variables affecting the cultural requirements and the production of BC [22, 35]. Table 3 represents the effect of seven independent factors with coded levels on the BC production. A wide variation was shown in the BC produced by *G.hansenii* ATCC 23769 strain throughout 14-different trials of the PBD experiment. The variation ranged from 0.626 - 2.27 g/l BC. This reflects the significant effect of medium composition and other environmental conditions on BC production. The highest BC production (2.27 g/l) was attained in the run number 5, while the lowest yield (0.626 g/l) was observed in the trial number 9. The main effects of the examined factors on the BC production were calculated and illustrated graphically in Figure 1. Based upon the regression coefficients analysis of tested variables; all parameters have a positive effect on BC production. A first order polynomial equation was applied to represent the optimum BC production as a function of the

independent factors. By ignoring the insignificant terms, the following equation of regression in terms of coded factors was obtained:

$Y_{BC\ production} = 1.2949 + 0.0393X_1 + 0.1596X_2 + 0.00797X_3 + 0.01430X_4 + 0.00935X_5 + 0.37835X_6 + 0.15169X_7$ (equation 4)

The statistical analysis of PBD was done by Excel Microsoft Office version 7.0 to show the regression coefficients, main effect, t -values, p-values and confidence level percentages which, are represented in Table 4. Temperature was the most significant variable affecting the BC production at 99.98 % confidence followed by yeast extract at 98.81% confidence, then incubation time at 98.52% confidence. From the confidence level of the variables, it was apparent that temperature, yeast extract and incubation time were the highest significant, therefore they were selected for further study. On the contrary, mannitol, ethanol, pH and inoclume size showed low significant positive effect, thus they were fixed at the high level to increase BC production. The analysis of variance using ANOVA test was generated and summarized in Table 5.

PBD is one of the most commonly used approach for screening factors affecting the production of any bioactive compounds. Bilgi et al [22] and Hegde et al [35] applied this approach to determine the most significant variables that enhance the production of BC as well as decrease

TABLE 3. PBD for 7 variables with coded values, observed and predicted results for BC production.

Trails	Mannitol	Yeast extract	Ethanol	pH	Inoclume size	Temperature	Incubation Time	Observed dry wt of BC g/l	Predicted dry wt of BC g/l
1	-1	-1	1	-1	1	1	1	1.522	1.629047619
2	-1	1	-1	1	1	1	-1	1.532	1.657619048
3	-1	-1	1	-1	-1	1	-1	1.414	1.306952381
4	1	-1	-1	1	-1	1	1	1.552	1.701714286
5	1	1	1	1	1	1	1	2.272	2.055666667
6	-1	1	-1	-1	1	-1	1	1.192	1.175666667
7	1	-1	-1	-1	1	-1	-1	0.682	0.631714286
8	1	1	-1	-1	-1	1	-1	1.748	1.689
9	1	-1	1	1	1	-1	-1	0.626	0.676285714
10	-1	-1	-1	1	-1	-1	1	1.016	0.866285714
11	1	1	1	-1	-1	-1	1	1.126	1.251619048
12	-1	1	1	1	-1	-1	-1	0.881	0.898142857
13	-1	1	1	1	-1	-1	-1	0.883	0.898142857
14	-1	1	1	1	-1	-1	-1	0.89	0.898142857

the cost of production medium. Ordinarily, the most effective parameters increasing BC production varied, this based on the strain and media composition. Hegde et al [35] reported the most effective parameters for BC production by *Gluconacetobacter persimmonis* were observed as glucose, yeast extract and peptone when used standard HS medium. X Zeng, DP Small and W Wan [34] reported the most effective parameters for BC production by *Acetobacter xylinum* BPR 2001were maple syrup concentration, incubation period, inoclume size and shaking speed using fructose based medium. Bilgi et al [22] reported the most effective parameters for BC production by *Gluconacetobacter xylinus* were incubation period, protein concentration and inoclume size using Carob-haricot bean medium. From the confidence level of studied variables, it was apparent that yeast extract, temperature

and incubation time were chosen as the most significant variables needed to be optimized for BC production by the investigated strain. In a confirmatory experiment, to evaluate the accuracy of PBD, a medium, which expected to be optimum of the following composition:(g/l: mannitol 25, yeast extract 10, ethanol 7 ml, pH 7, inoclume size 7%, temperature 30°C and incubation time 8 days to achieve 2.34 g/l BC dry wt, thus it was used as basal medium for BBD.

Optimization of significant culture parameters with RSM based on BBD

BBD (a RSM) was followed to find out the optimum level of each of the most significant independent variables to achieve a maximum production of BC by using *G.hansenii* ATCC 23769. The results obtained from PBD explained the three most significant variables that given the highest efficiency on BC production [yeast extract
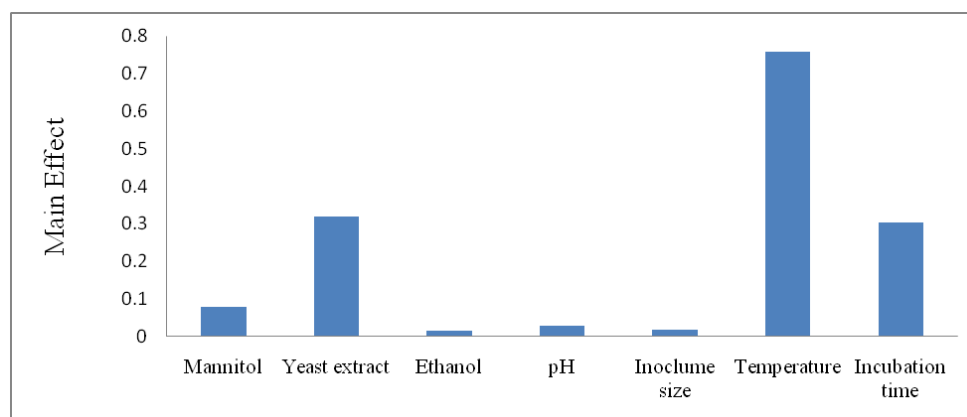

Fig. 1. Effect of culture conditions on BC production by *G.hansenii* ATCC 23769 based on PBD

TABLE 4. Statistical analysis of PBD showing coefficients values, main effect, t- and p –values and confidence level % for each variable on BC production.

Variables	Coefficients	Main Effect	*t* Statistical	*P*-value	Confidence Level
Intercept	1.29497619	-	-	-	-
Mannitol	0.039357143	0.078714286	0.87884853	0.413290925	58.67090751
Yeast extract	0.159642857	0.319285714	3.564839318	0.01185773	98.81422704
Ethanol	0.00797619	0.015952381	0.178109049	0.864498666	13.55013338
pH	0.014309524	0.028619048	0.31953295	0.760155299	23.98447012
Inoclume size	0.009357143	0.018714286	0.208945839	0.841403706	15.85962941
Temperature	0.378357143	0.756714286	8.448748933	0.000150122	99.98498776
Incubation time	0.151690476	0.303380952	3.387261938	0.014725267	98.52747329

TABLE 5. Analysis of variance (ANOVA) for PBD on BC production by *G.hansenii* ATCC 23679.

Variables	Degree of Freedom (DF)	Sum of Squares (SS)	Mean Square (MS)	*F*- Ratio	*P*-value
Regression	7	2.59	0.37	14.29	0.002
Residual	6	0.15	0.02		
Total	13	2.74			
Multiple R	0.97				
R Square	0.94				
Adjusted R Square	0.87				
Standard error	0.16				

(X_1), temperature (X_2) and incubation time (X_3)] were examined at three different levels coded -1, 0 and +1 as shown in Table 2.

As can be seen in Table 6, the design matrix of the variables in coded units with the experimental results of the BC production. All cultures were performed in triplicate and the average of the observations was used. Presenting experimental results in the form of surface plots three dimensional response surface curves against any two independent variables, while keeping the other variables at their middle levels. Figure 2 showed that higher levels of yeast extract support high BC yield level. On the other hand, higher levels of the BC yield were attained with a decreasing the temperature especially when yeast extracts and incubation time levels are higher. Moreover, for predicting the optimal point, within experimental constrains, a second-order polynomial equation was fitted to the experimental results (non-linear optimization algorithm) of BC production.

$Y_{BC\ production}$ = 1.71533 + 0.2715 X_1 - 0.9 X_2+ 0.19 X_3 - 0.2685 X_1X_2 + 0.0165 X_1X_3 - 0.0995 X_2X_3 + 0.21808 X_1^2 - 0.8539X_2^2+ 0.02808

(equation 5)

where Y represents BC production (g/l), and X_1, X_2 and X_3 are yeast extract, temperature and incubation time, respectively. Statistical analysis using JMP$_{IN}$ program Version: 4.0.4 for ANOVA for the whole quadratic model of response surface is presented in Table 7. The regression model fit the data well, as shown by the significance level of 'total regress' ('Prob > F; 0.0023), non-significance of 'lack of fit' ('Prob > F = 0.0027) and the higher R^2 (0.999) in the Box–Behnken

model for BC production. These results indicate that this model is fitted well with the experimental data. The R^2 implies that the sample variation of 99.9% for BC production is attributed to the factors and also indicates that only 0.1% of the total variation is not explained by the model. Additionally, the optimal levels of the three components as obtained from the maximum point of the polynomial model were estimated using the JMP$_{IN}$ program Version: 4.0.4. and found that the coded values +1, -0.741 and +1 are the optimum for yeast extract, temperature and incubation time, respectively. Yeast extract is the best nitrogen source for BC production this is because yeast extract contains abundant nitrogen compounds as well as many growth factors and its addition into medium might be stimulated BC production [35]. Yeast extract was reported as one of the most significant parameters and the high level was selected as optimum one in many studies [34, 35]. These results are in agreement with the present work. Through, experimental design *G.hansenii* ATCC 23769 achieved higher production of BC in short time than applying traditional optimization method. Accordingly, *G.hansenii* ATCC 23769 under experimental design achieved BC 2.85 g/l where other studies using the same strain obtained BC 1.35 and 0.15 g/l from glucose and galactose respectively [7] and 1.19, 1.35, 1.33 and 1.21 g/l from different volume of carrot juice media [41]. Accordingly, the formula of the optimized medium (g/L) is as follows: mannitol 25, yeast extract 13, ethanol 7 ml/l, pH 7, inoclume size 7%, temperature 26.3°C and incubation time 12 days.

Verification of model

To determine the accuracy of the quadratic polynomial, a verification experiment was carried

out under the predicted optimal conditions using optimized medium of the following composition (g/l): mannitol 25, yeast extract 13, ethanol 7 ml/l, pH 7, inoclume size 7%, temperature 26.3°C and incubation time 12 days. The estimated BC production was 2.85 g/l, where the predicted value from the polynomial model as 2.91 g/l. Thus, these results described a high degree of accuracy (97.9%) which is an evidence of the model validation. Furthermore, the yield of BC increased 1.3 fold compared with initial production (2.19 g/l).

Comparison of BC Production before and after optimization

The *G.hansenii* ATCC 23769 exhibits 1.3 folds increase of BC yield after optimization by using statistical experimental design when compared with the production before optimization using GEM media, as can be seen in Table 8.

Characterization of BC membrane
   SEM analysis
   The morphological structure of BC membrane

TABLE 6. BBD for 3 variables with coded values; observed and predicted results for BC production.

Trail	X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	X_1X_1	X_2X_2	X_3X_3	Observed dry wt of BC g/l	Predicted dry wt of BC g/l
1	-1	-1	0	1	0	0	1	1	0	1.622	1.4395
2	1	-1	0	-1	0	0	1	1	0	2.696	2.5195
3	-1	1	0	-1	0	0	1	1	0	0	0.1765
4	1	1	0	1	0	0	1	1	0	0	0.1825
5	-1	0	-1	0	1	0	1	0	1	1.592	1.5165
6	1	0	-1	0	-1	0	1	0	1	2.108	2.0265
7	-1	0	1	0	-1	0	1	0	1	1.782	1.8635
8	1	0	1	0	1	0	1	0	1	2.364	2.4395
9	0	-1	-1	0	0	1	0	1	1	1.242	1.5000
10	0	1	-1	0	0	-1	0	1	1	0	-0.1010
11	0	-1	1	0	0	-1	0	1	1	1.978	2.0790
12	0	1	1	0	0	1	0	1	1	0.338	0.0800
13	0	0	0	0	0	0	0	0	0	1.71	1.71533
14	0	0	0	0	0	0	0	0	0	1.702	1.71533
15	0	0	0	0	0	0	0	0	0	1.734	1.71533

TABLE 7. Analysis of variance (ANOVA) for response surface quadratic model obtained from experiments to maximize BC production by *G.hansenii* ATCC 23769.

| Source	Sum of Squares	DF	F Ratio	Prob > F
Model	10.699152	9	19.3183	0.0023
X1	0.5896980	1	9.5828	0.0270
X2	6.4800000	1	105.3019	0.0002
X3	0.2888000	1	4.6931	0.0825
X1X2	0.2883690	1	4.6861	0.0827
X1X3	0.0010890	1	0.0177	0.8994
X2X3	0.0396010	1	0.6435	0.4589
X_1^2	0.1756074	1	2.8537	0.1520
X_2^2	2.6923336	1	43.7512	0.0012
X_3^2	0.0029120	1	0.0473	0.8364
Residual				
Lack Of Fit	0.30713200	3	369.1490	0.0027
Pure Error	0.00055467	2		
Total Error	0.30768667	5		
C. Total	11.006838	14		
R^2		0.999		
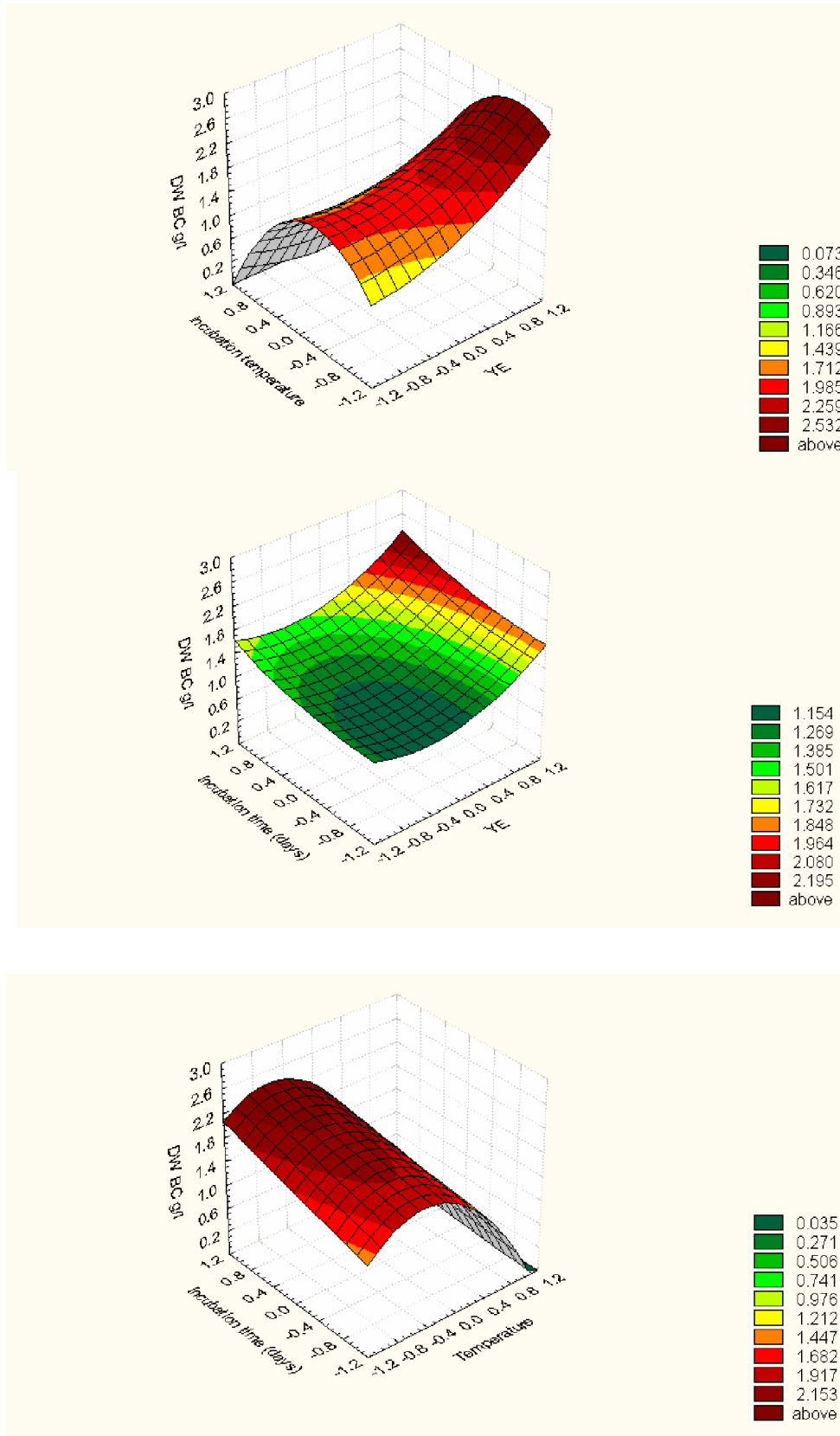
Fig. 2. Three-dimensional surface plots representing the effect of yeast extract, temperature and incubation time on the BC yield (g/L) by *G.hansenii* ATCC 23769.

was investigated by SEM. As can be seen in Figure 3 the purified pellicle and the surface morphology of BC membrane is a uniformly distributed, homogeneous densely packed, network of randomly oriented ribbons of cellulose obtained from *G.hansenii* ATCC 23769, similar to those commonly reported for BC obtained from the seam strain [7, 42]. To remove all impurities as well as bacterial cells for BC membrane vie treatment with NaOH was applied to achieve highly purified BC membrane (Iguchi et al., 2000). The SEM images of the NaOH-treated BC sample (Fig. 3) show that the impurities were almost completely removed as reported by other studies [9, 22].

XRD analysis

XRD pattern demonstrates three relatively intense reflections at 14.72°, 17.01° and 23.12° namely (100), (010) and (110), respectively, which recognized according to the triclinic indexation (Fig.4). These results are in agreement to other studies [9, 41, 43]. Also, BC obtained from the *G.hansenii* ATCC 23769 shows a typical crystalline and amorphous regions form of cellulose I, which is similar to that reported by other studies [44, 45]. This reflects a higher crystallinity of the BC membrane obtained from *G.hansenii* ATCC 23769. According to the method reported by some authors [38-40], the crystallinity index (CI) calculated from the ratio of the intensity of the main peak and the count numbers of the adjacent minimum yielded. CI of BC membrane of *G.hansenii* ATCC 23769 reached to 82.7%

TABLE 8. Comparison of BC production by *G.hansenii* ATCC 23769 before and after optimization.

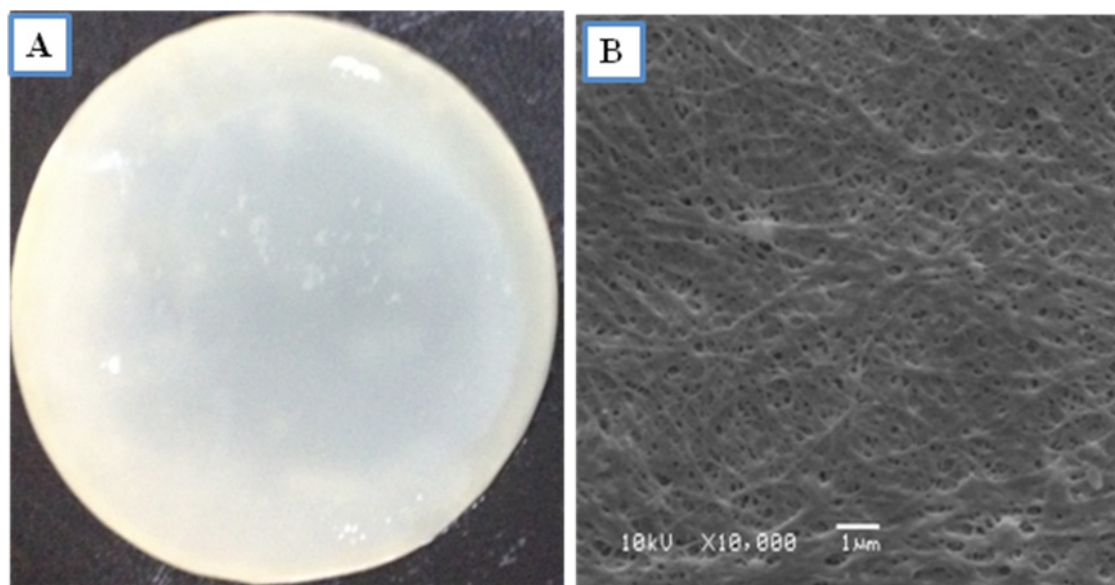| Parameters		*G.hansenii* ATCC 23769	
		Before optimization	After optimization
Media compositions	Mannitol	20 g/l	25 g/l
	Yeast extract	5 g/l	13 g/l
	Ethanol	5 ml/l	7 ml/l
Cultivation conditions	Temperature	25 °C	26.3 °C
	Incubation time	7 days	12 days
	Inoclume size	6 %	7 %
	pH	6	7
Yield of BC	dry wt g/l	2.19	2.85
Fold			1.3

Fig. 3. Images of BC pellicles produced by *G.hansenii* ATCC 23769 after optimization (a) Photograph for visualized BC pellicles and (b) SEM micrograph for BC pellicles.

according to the previous method. This result is nearly similar to some previous studies (under different medium and culture conditions), where CI measured 83 and 84 % using Carrot and HS medium, respectively [41]. Also, CI measured 70 to 80% under different chemical and physical conditions [43] and 77.4 and 78.3% when using galactose and glucose as carbon source [7].

FT-IR analysis

FT-IR was used to demonstrate the chemical structure of BC membrane such as functional groups [46]. The FT-IR spectra of BC membrane obtained from *G.hansenii* ATCC 23769 was detected at wave numbers ranging from 500 to 4000 cm^{-1}as can be seen in Fig.5. The band of intense absorption in the BC spectrum at 3345.2 cm^{-1} was attributed to the presence of hydroxyl group (OH) of cellulose type I and is important for elucidating hydrogen-bonding patterns which are in agreement with some literatures [41, 43, 47], strong absorption band at 2896.8 cm^{-1} was also attributed to the presence of stretching of CH this are in agreement with other studies [41, 44, 47]. The cellulose absorption spectrum is the band at 1638 cm^{-1} which has been assigned to carboxyl functional group (C=O) [47]. While 1389.6 cm^{-1} (asymmetric angular deformation of C-H bonds), the band at 1055.6 cm^{-1} to 1066.9 cm^{-1} stretching of C-O-C and C-O-H bonds in secondary and primary alcohols, respectively [41, 43]. The FT-IR features of BC obtained from *G.hansenii* ATCC 23769 in this study are in agreement with other studies using the same strain under different conditions [41, 43].

TGA analysis

Thermal stability of the BC sample may be considered for some applications, and might provide some clues on BC fiber interactions [48]. To determine the thermal stability behavior of BC, TGA was applied. Figure 6 shows the TGA degradation curve of the dried pellicles of BC. Three mass-loss stages can be observed during thermal analysis of the sample. The first stage, occurring from 25 to 100 °C, is contributed to the evaporation of residual water present in the BC membrane and showed a small weight loss. The second stage, occurring in the range of 150–250 °C, is associated with a series of reactions degradation of cellulose, including dehydration, depolymerization of the glycoside units and decomposition of glucose units, followed by the formation of a carbon residue [49]. This second mass-loss stage is contributed with a high loss of weight of BC, which is characterized by the onset temperature (T$_{Onset}$) [50]. The third stage, occurring from 350 to 600 °C, is associated with oxidation and breakdown of carbonaceous residues, yielding gaseous products of low molecular weight and is known as the carbonaceous stage these due to thermal degradation [41, 51]. TG analysis confirmed that BC was very stable and had no degradation up to 250 °C this result are in agreement with a study used *G.hansenii* ATCC 23769 for production of BC membrane [41].

Mechanical properties

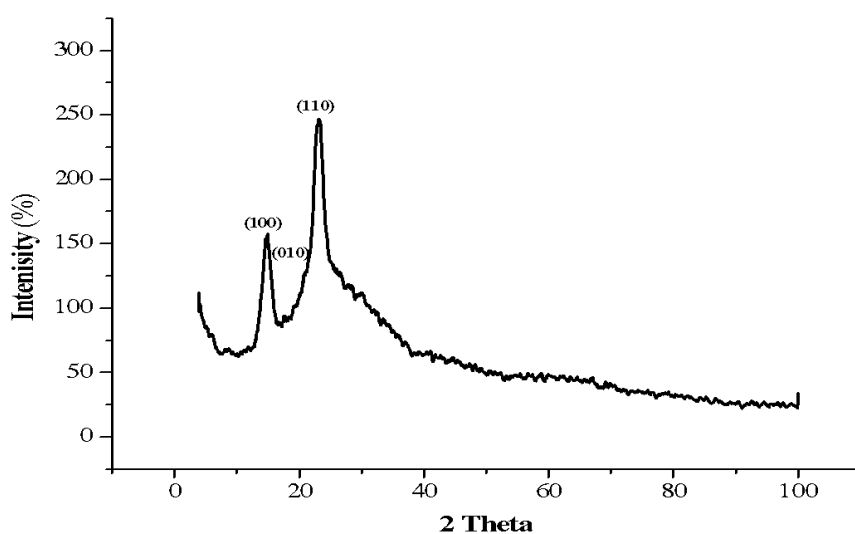Mechanical testing was used to evaluate the inherent mechanical properties as demonstrated in

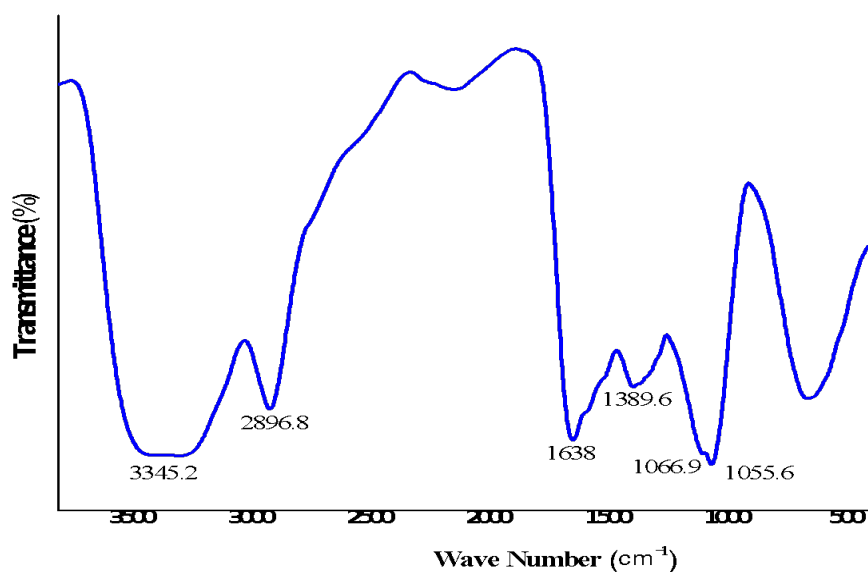**Fig. 4. XRD analysis of BC membrane obtained from *G.hansenii* ATCC 23769.**

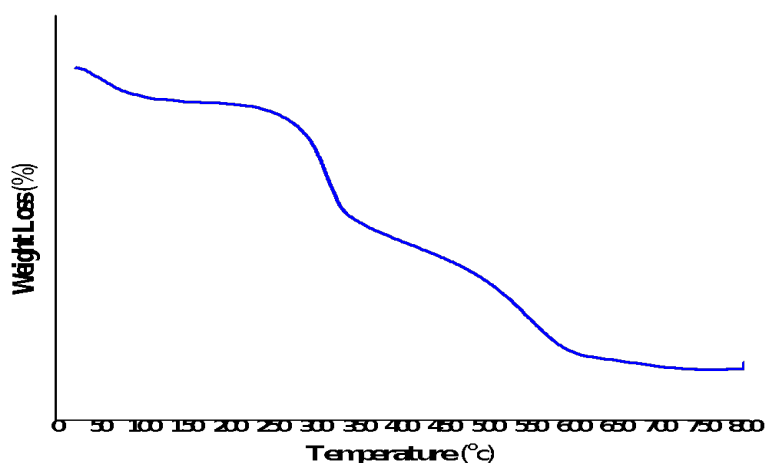**Fig. 5. FT-IR spectrum of BC membrane obtained from *G.hansenii* ATCC 23769.**

Fig. 6. TGA curve of BC membrane obtained from *G.hansenii* ATCC 23769.

Young's modulus, tensile strength, elongation at beak % and thickness of BC membrane obtained from *G.hansenii* ATCC 23769, as can be seen in Table 9. Aytekin *et al* [52] reported that the tensile strength (31 MPa) and Young's modulus (370 MPa) using statistical optimization of culture conditions using RSM by *G. xylinus* FC01. From the data obtained in previous literatures the mechanical properties (tensile strength and Young's modulus) are higher by statistical optimization of culture conditions than non optimized media [52, 53]

Conclusion

This is considered the first reported study focused on optimization of BC production by *G.hansenii* ATCC 23769 strain using experimental design. Firstly, we implemented PBD for the optimization of BC production and screened seven cultivation parameters. From the data obtained from PBD analysis, it was noticed that the yeast extract, temperature and incubation time have significant effect on BC production when compared with other cultivation parameters.

TABLE 9. Mechanical properties of BC membrane obtained from *G.hansenii* ATCC 23769.

Sample	Tensile strength (MPa)	Elongation at beak (%)	Young's modulus (MPa)	Thickness (mm)
BC membrane	48.2	0.42	242.5	0.025

Secondly, we implemented BBD to determine the optimum level of the most significant factors, and found that 13 g/l yeast extract, 26.3°C temperature and 12 days incubation time are the optimal level. Finally, the resulted BC reached to 2.85 g/l by applying the following medium and culture conditions: (g/l): mannitol 25, yeast extract 13, ethanol 7 ml/l, pH 7, inoclume size 7%, temperature 26.3°C and incubation time 12 days. Furthermore, morphological, chemical structure, functional groups, thermal stability and mechanical properties of purified BC were determined.

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إنتاج السليلوز الحيوى بواسطة جلوكناسيتوباكتر هانزينيا CCTA ٩٦٧٣٢: تطبيق التصميمات الأحصائية وتوصيف أغشية السليلوز

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فى هذه الدراسة، تم تحسين المعاملات المزرعية لإنتاج BC بواسطة جلوكناسيتوباكتر هانزينيا ATCC 23769. هذا هو التقرير الأول عن التحسين الإحصائى بواسطة تصميمات Plackett – Burman و Box Behnken – لانتاج BC بواسطة جلوكناسيتوباكتر هانزينيا ATCC 23769. تم تقييم تأثيرسبع معاملات مزرعية على انتاج BC من خلال تنفيذ تصميم Plackett – Burman حيث كشفت النتائج الأكثر تأثيرا على انتاج BC كانت مستخلص الخميرة, درجة الحرارة و فترة التحضين. بإستخدام Response surface methodology تم تطبيق Box – Behnken لمعرفة المستويات المثلى للمعاملات الاكثر تأثيرا على إنتاج BC. تم الحصول على المستويات المثلى من المكونات الثلاثة لتكون مستخلص الخميرة ١٣ غم / لتر ودرجة الحرارة ٢٦,٣ درجة مئوية وفترة الحضانة ١٢ يوما مع العائد المتوقع ٢,٩١ غم / لتر. طبقا للنتائج التى حصل عليها من خلال تطبيق Plackett – Burman و Box – Behnken من المتوقع ان تكون التركيبة المثلى للوسط الغذائى هى (غم/لتر): مانيتول ٢٥ ، مستخلص خميرة ١٣ ، إيثانول ٧ مل / لتر ، الرقم الهيدروجينى ٧ ، حجم الحقنه ٧٪ ، درجة الحرارة ٢٦,٣ درجة مئوية وفترة التحضين ١٢ يوما. بالإضافة إلى ذلك ، تم إجراء توصيف لغشاء BC النقى والجاف لتحديد الشكل الظاهرى والنقاء عن طريق مسح المجهر الإلكترونى ، والبلورة عن طريق حيود الأشعة السينية ، والتراكيب الكيميائية والمجموعات الوظيفية بواسطة التحليل الطيفي بالأشعة تحت الحمراء, والثبات الحرارى والقدرة على الاحتفاظ بالماء بواسطة التحليل الحرارى والخواص الميكانيكية لتحديد معامل يونغ وقوة الشد والإطالة عند القطع ٪ وسماكة غشاء BC الذي تم الحصول عليه من جلوكناسيتوباكتر هانزينيا ATCC 23769.