OPTIMIZATION OF BACTERIAL CELLULOSE PRODUCTION FROM SUGAR BEET MOLASSES BY *GLUCONACETOBACTER XYLINUS* NRRL B-759 IN STATIC CULTURE

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We investigated the optimization of bacterial cellulose (BC) production from sugar beet molasses by *Gluconacetobacter xylinus* NRRL B-759 in static culture. The optimization studies were performed using the central composite design (CCD) of response surface methodology (RSM). The independent variables were the molasses concentration, inoculation ratio and culture volume. The dependent variable was BC production yield. From the optimization tests, based on the model developed by RSM-ANOVA, it was found that binary interactions between molasses concentration–culture volume and inoculation ratio–culture volume had the most significant influence on the responses. The optimum conditions were as follows: 78.932 g/L molasses concentration, 12.973% inoculation ratio, and 130.405 mL of culture volume. The obtained BC was characterized by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and elemental analysis. The characterization results obtained in the study revealed that the produced BC exhibited typical FTIR spectrum, elemental composition, and nanofiber structure.

Keywords: bacterial cellulose, sugar beet molasses, *Gluconacetobacter xylinus* NRRL B-759, optimization, static culture

INTRODUCTION

Cellulose is the most abundant biomaterial in the world and is traditionally extracted from plants and their wastes.[1,2] Its annual production amounts to approximately 10^{12} tons.[3,4] According to a market research report, the cellulose fiber market was $20.61 billion in 2015, and it is strongly expected to increase.[5] Though cellulose is mainly extracted from wood pulp, other non-wood resources have also gained ground, such as cotton, algae, ramie, flax, hemp and bacterial cellulose (BC).[4] This biomaterial and its derivatives (for example, cellulose esters and ethers) are used in many different industrial areas, such as in foods, textiles, paper, biomedical materials, pharmaceuticals, membranes, drilling, coatings, and building materials.[2,6-8]

To obtain cellulose from wood or other plants, it must be separated from lignin and hemicelluloses. For this, the raw material is subjected to different treatments, such as chemical, physico-chemical, enzymatic *etc*.[9] However, these synthesis techniques for cellulose production have important disadvantages, for example, acid and alkali treatments used for lignin and hemicellulose removal may cause environmental pollution, the enzymatic route is expensive because of the high cost of enzymes; in addition to the deforestation problem when it comes to wood derived cellulose.

Biosynthesis is a route to produce cellulose using different types of microorganisms, such as algae, fungi and bacteria.[9,10] The most commonly used bacteria in BC production are *Acetobacter*, *Achromobacter*, *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, *Sarcina*, *Zoogloea*, *Salmonella*, *Enterobacter*, *Escherichia*, and some cyanobacteria.[8,11,12] Nano-sized BC is generally synthesized using low-molecular-weight sugars and alcohols and its fiber diameter ranges from 20 to 100 nm.[6] Although BC has the same chemical formula ($C_6H_{10}O_5$) as the cellulose originating from plants, some properties are different. As BC consists of only the glucose monomer, it has extra purity, nanostructure, high water holding capacity or hydrophilicity, high polymerization degree, high mechanical strength

and high crystallinity.[2,13] Due to these features, BC is recommended in many industrial applications, including biomedical products, foods, textiles and electronics.[6,14,15]

The most important parameters in BC production are the carbon and nitrogen sources used, as well as their ratio, the pH, the volume of the culture medium, the inoculum ratio, incubation time and temperature.[7,16] The most often used carbon sources for BC production, as reported in the literature, are monosaccharides (various pentoses and hexoses), disaccharides, such as sucrose and mannose, and polysaccharides, such as dextran and starch; while yeast extract, peptone, casamino acids and corn steep liquor are the major nitrogen sources.[17-21] Sugar beet molasses, a by-product of the sugar industry, were reported by Bae and Shoda[22] to be used as a carbon source to produce BC.

BC can be produced by static, dynamic (or agitated/shaking), and bioreactor culture techniques. Although the static culture has the disadvantages of high cost and low rate of production, compared to the dynamic and bioreactor ones, it is a relatively simple technique. Furthermore, the BC produced by static culture has a more suitable particle size range and regularly shaped fibers than that produced by the dynamic and bioreactor culture techniques.[23]

This study aims to optimize some important process conditions of BC production from sugar beet molasses using *G. xylinus* NRRL B-759 in a static culture.

EXPERIMENTAL

Materials and reagents

The molasses used in this study were supplied by Elazığ Sugar Factory in Turkey. Some properties of the molasses samples are given in Table 1. Also, yeast extract (Chemsolute), peptone (Labm), acetic acid (Merck), glycerol (Merck), sucrose (Carlo Erba), and fructose (Carlo Erba) were used. Other chemicals, such as glucose, citric acid, sodium hydroxide, and Na_2HPO_4, were of analytical grade.

Bacterial strain and culture

In this study, *G. xylinus* was used in the production of BC. The *G. xylinus* strain was obtained from ARS Culture Collection (NRRL B-759). This strain was cultured in Hestrin and Schramm (HS)[24] medium, containing 2% glucose, 0.5% peptone, 0.5% yeast extract, 0.27% Na_2HPO_4 and 0.15% citric acid (the percentage values are in w/v ratio). The pH was adjusted to 5, using 1 N acetic acid solution. The HS medium was sterilized by autoclaving at 121 °C for 15 minutes. The lyophilized *G. xylinus* strain was inoculated into 50 mL of sterile HS medium in a 250 mL flask and incubated at 30 °C for seven days. Cellulose production was observed at the end of the 7th day. After cellulose production, a stock culture was prepared from the broth culture. An amount of 100 µl of stock culture was transferred into an Eppendorf tube and 1000 µl sterile glycerol solution (60%) prepared with distilled water was slowly added to the tubes containing the stock culture. These stock cultures were maintained at -80 °C until use in the fermentation experiments.

Fermentation and optimization experiments

The fermentation time of the strain (*G. xylinus* NRRL B-759) chosen in this study was used from our previous work[25] related to the production of BC from molasses using different ethanol ratios. The fermentation time-determining experiments were performed with ten control sets in an incubator at 30 °C for a total of 20 days. The changes in the BC amount produced and glucose concentration in the culture media were measured over time. Both BC (g/L) and glucose concentration (g/L) proved to be independent of time after the tenth day. Thus, the fermentation experiments in the present study were conducted for 10 days.

Table 1
Composition of molasses samples in this study

Parameter	Value
Total solid material content	78.90%
Total sugar content	50.80%
Total dissolved solid material content	60.60%
pH	8.10

In order to optimize the batch fermentation conditions for BC production from sugar beet molasses, RSM based on the CCD was used. The RSM analyses were carried out using three independent variables: the molasses concentration (40-236 g/L), the inoculation ratio (5-15%), and the culture volume

(100-250 mL). The ranges of the selected variables were determined by preliminary experiments based on a literature review. The codes and variation ranges of independent variables in the RSM-CCD for BC production from sugar beet molasses are given in Table 2. Table 3 tabulates the experimental conditions offered by the RSM-CCD according to these levels. The response variable was the BC production percent yield, defined as the ratio of produced BC mass (g) to the initial sugar mass (g) in the sugar beet molasses solution.

A typical fermentation experiment was as follows: any experimental condition in Table 3 was prepared using the related volumes in an autoclavable polypropylene-based culture vessel, with a volume of approximately 700 mL (95 x 100 x 74 mm). This medium was autoclaved at 121 °C for 15 minutes, the *G. xylinus* NRRL B-759 strain was inoculated under aseptic conditions in a laminar air-flow cabinet, and the fermentation broth was kept in an incubator at 30 °C for 10 days.

Table 2
Symbols and levels of independent variables in central composite design (CCD) by response surface methodology (RSM) for bacterial cellulose (BC) production from sugar beet molasses

Symbols	Variables	-α	-1	0	+1	+ α
A	Molasses concentration (g/L)	39.00	78.93	137.5	196.06	236.00
B	Inoculation ratio	5.00	7.03	10.00	12.97	15.00
C	Culture volume (mL)	100.00	130.40	175.00	219.59	250.00

Table 3
Experimental conditions proposed by central composite design (CCD) in response surface methodology (RSM) for bacterial cellulose (BC) production from sugar beet molasses

Sample No	Molasses concentration (g/L)	Inoculation ratio (%)	Culture volume (mL)
1	79	7	130
2	196	7	130
3	79	13	130
4	196	13	130
5	79	7	220
6	196	7	220
7	79	13	220
8	196	13	220
9	39	10	175
10	236	10	175
11	138	5	175
12	138	15	175
13	138	10	100
14	138	10	250
15	138	10	175
16	138	10	175
17	138	10	175
18	138	10	175
19	138	10	175
20	138	10	175

The fermentation medium was adjusted to pH 5 with 0.1 M acetic acid solution. At the end of the fermentation time, the sugar concentration of the fermentation medium and the BC amount produced were measured.

Preparation of BC

After cultivation, the BC produced was separated from the culture medium and washed with distilled water. Then, it was subjected to centrifugation using a NUVE-NF 800 R at 4100 rpm. In order to remove microbial product contaminants, the cellulose floccules produced were washed with 0.1 N NaOH solution in a boiling bath (60 min at 90 °C). After neutralization with 0.1 N acetic acid solution, the purified BC samples were washed with water and dried in an oven (Lab Companion ON-22) at 70 °C until they reached a constant weight.[26]

Sugar analyses

All sugar analyses were carried out using the HPLC technique in this study. For this purpose, the HPLC

(Shimadzu LC-20) device with an RID detector was used. The column, analysis temperature, mobile phase, and flow rates were ICE-COREGEL-87H3, 45 °C, 0.05 M H_2SO_4, and 0.6 mL/min, respectively.

Characterization techniques

Fourier-transform infrared (FTIR) spectroscopy of the BC samples produced under the optimum conditions was performed using an ATI Unicam Mattson 1000 device, with a resolution of 4 cm^{-1} in the wavenumber range from 4.000 to 400 cm^{-1}.

The scanning electron microscopy (SEM) images of the BC samples were obtained using a JEOL JSM-7001F device. The elemental analysis of the BC produced was performed using a LECO-CHNS-932 Elemental Analyzer.

FTIR, SEM and elemental analysis were carried out using samples produced under the optimum experimental conditions proposed by RSM.

RESULTS AND DISCUSSION

Optimization results

The evolution of the BC yield (g/L) as a function of the fermentation time was described in our previous study.[25] Here, the changes in the BC yield (g/L) are considered as a function of the pH during the fermentation time and the revised graph is illustrated in Figure 1. As may be noticed in Figure 1, the initial adjusted value of the fermentation medium of pH 5 decreased to pH 4.3 on the 10th day. Similar pH changes were also found in other studies aiming to produce BC from molasses and HS medium with various *G. xylinus* strains.[27] This decrease in the pH of the fermenting medium might be caused by gluconic acid, which is a by-product.[27-30] It should be remarked that the decrease in pH during this time interval did not inhibit BC production. Also, sugar beet molasses contain a lower amount of glucose than the HS medium. This leads to less gluconic acid formation and, consequently, more BC production in this medium.

To optimize the experimental conditions (sugar beet molasses concentration, inoculation ratio and culture volume), RSM-CCD consisting of a set of 20 experiments, with six replicates at the central point, as given in Table 3, was conducted. The design results showed that the best fit model source was the quadratic model, among the linear, 2FI, and cubic model sources. The data obtained by the P test and Analysis of Variance (ANOVA) for the response surface carried out for the quadratic model suggested are given in Table 4.

As seen in the ANOVA results in Table 4, the quadratic model terms are significant due to probe > F value (<0.0001) less than 0.05. Thus, the significant model terms were A, B, C, AC, BC, A^2, and C^2. Because the values of the model terms AB and B^2 are greater than 0.1000, these terms are not significant. According to the ANOVA test, some statistical parameters calculated from RSM-CCD for BC production from sugar beet molasses are given in Table 5. Table 5 illustrates that the statistical parameter values of the quadratic model for the optimization of BC production in this study are very good. The standard deviation is low, while the R^2 value is high. From the ANOVA tests carried out in this study, the lack of fit is not significant, which is a good result. In the RSM-ANOVA analyses, the Adeq Precision value is used to measure the signal/noise ratio.

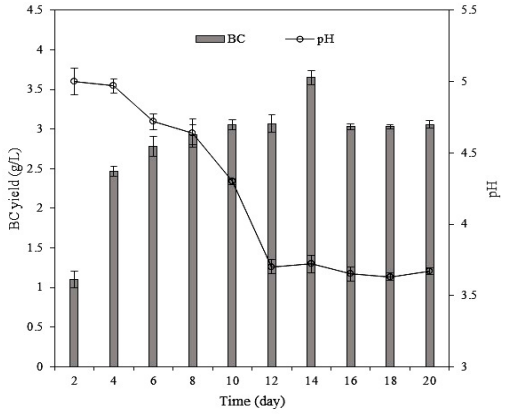

Figure 1: Bacterial cellulose (BC) production (bar chart) and pH change (line plot) of *G. xylinus* in HS medium at 30 °C

Table 4
Analysis of variance and P-test for bacterial cellulose (BC) production from sugar beet molasses

Source	Sum of squares	Mean square	F value	p-value Prob > F	
Model	78.890	10.700	76.160	< 0.0001	Significant
A-Molasses concentration (g/L)	33.160	33.160	236.020	< 0.0001	
B-Inoculation ratio (%)	2.980	2.980	21.230	0.0006	
C-Culture volume (mL)	8.320	8.320	59.230	< 0.0001	
AC	5.760	5.760	41.020	< 0.0001	
BC	0.760	0.760	5.430	0.0381	
A^2	12.550	12.550	89.130	< 0.0001	
C^2	13.520	13.520	96.240	< 0.0001	

Table 5
Statistical parameters calculated by response surface methodology (RSM) ANOVA for optimization of bacterial cellulose (BC) production

Std. Dev.	0.370	R^2	0.978
Mean	4.660	Adj. R^2	0.965
C.V. %	8.050	Pred. R^2	0.910
PRESS	6.880	Adeq. Precision	25.448

It is sufficient if the value is greater than 4. From the ANOVA analysis, the ratio was calculated as 25.448. Thus, an adequate signal was obtained and the model can be used to navigate the design space. The difference between the Adj. R^2 (0.965) and Pred. R^2 (0.910) values is less than 0.2. This result indicates that the recommended model is in agreement with the experimental data. CV% value, another ANOVA parameter, in Table 5 is 8.05. If the CV value of a model is less than 10%, it is considered to be reproducible.[31] Thus, the model is reproducible.

The expression of the final equation (except for the non-significant parameters in the model, *i.e.* AB and B^2) in terms of actual factors generated by the design program is as follows:

$$BC\ Yield\ (\%) = 40.369 - 0.158A - 0.250B - 0.255C + 3.249x10^{-4}AC + 2.328x10^{-3}BC + 2.707x10^{-4}A^2 + 4.846x10^{-4}C^2$$

In this equation, the units of the model parameters used are g/L for molasses concentration, volumetric percentage for inoculation ratio, and mL for culture volume.

To check the adequacy and reliability of the models obtained from the ANOVA test results, the residuals and the normal probability are commonly used.[32] For this purpose, the predicted values versus the actual values, the externally studentized residuals versus the predicted BC production yield (%), the externally studentized residuals versus the run number, and the normal distribution probability charts of the studentized residuals were obtained, as illustrated in Figure 2 (a-d) for BC production from sugar beet molasses. As seen in Figure 2a, the experimental and predicted data points are close to the diagonal line. Figures 2b and 2c show that there is a random distribution of points along the x-axis between +3.894 and -3.894. These graphs indicate that constant variance was observed through the response range. Due to the fact that the points on the normal distribution probability chart shown in Figure 2d form a straight line and the errors in this graph were distributed normally, the model developed is sufficient.

The 3D and contour graphs of the molasses concentration–culture volume are shown in Figure 3 to account for binary interactions of the factors. The 3D response surface graph (Fig. 3a) shows that a minimum point surface chart was obtained.[33] Therefore, the maximum BC production yield from sugar beet molasses can be obtained at minimum or maximum points. From Figure 3a, it is understood that the effect of the molasses concentration on the BC production yield is higher than that of the culture volume. The BC yield had almost no change with varying culture volume at high molasses concentrations, but decreased with increasing culture volume at low molasses concentrations.

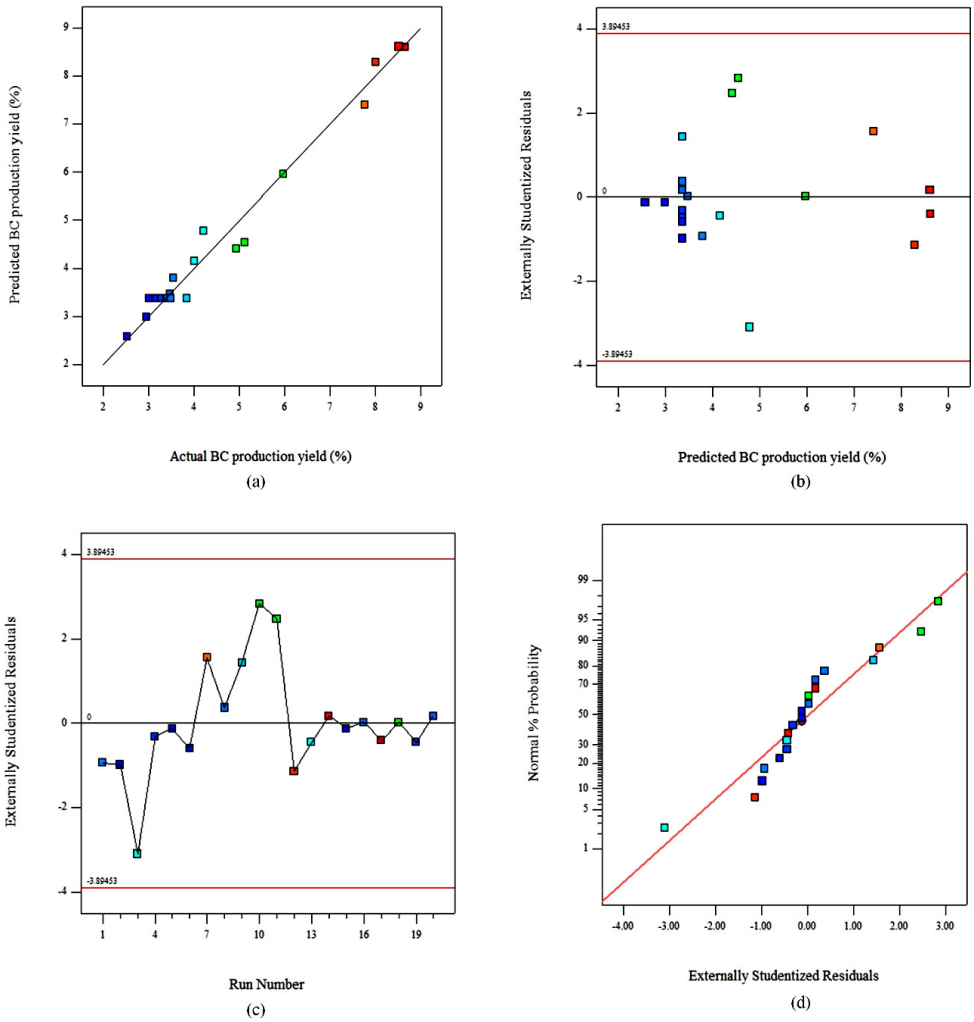

Figure 2: Graphs of experimental *vs*. predicted values (a), externally studentized residuals *vs*. predicted bacterial cellulose (BC) production yield (b), externally studentized residuals *vs.* run number (c), and normal plot of residuals (d) obtained by response surface methodology (RSM) analyses

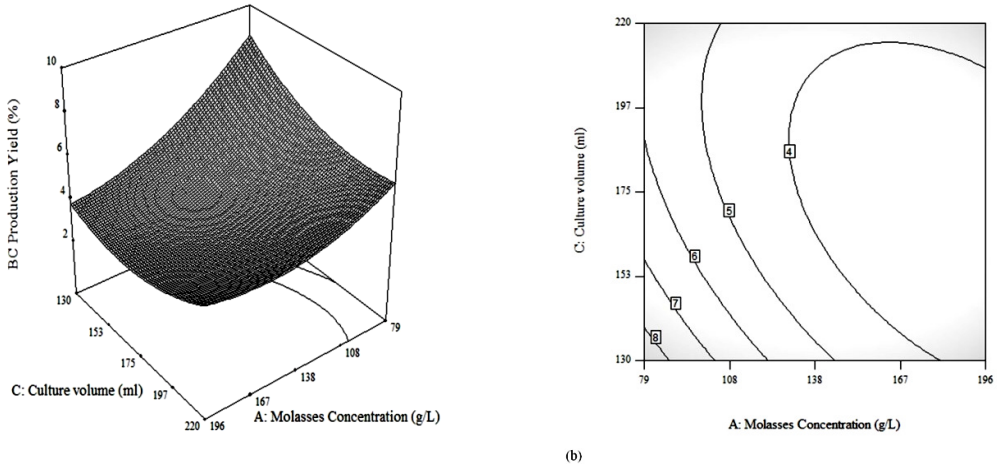

Figure 3: (a) 3D and (b) contour plots for A–C interaction (molasses concentration–cumulative volume)

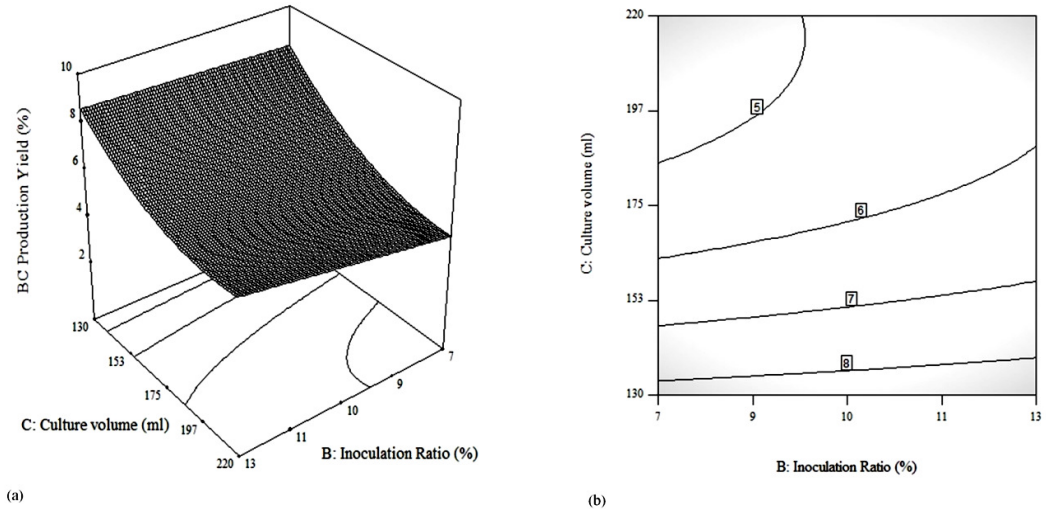

Figure 4: (a) 3D and (b) contour plots for B–C interaction (inoculation ratio–cumulative volume)

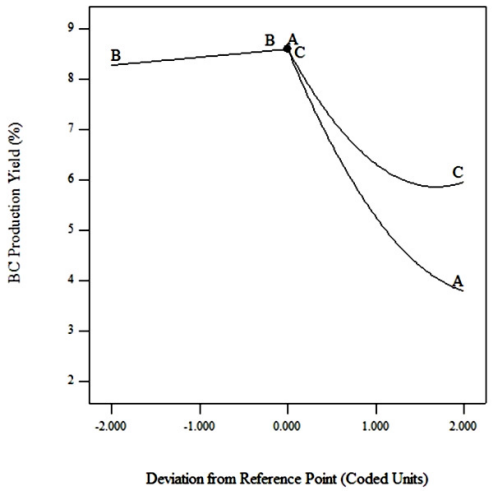

Figure 5: Perturbation graph obtained from response surface methodology (RSM) analyses for bacterial cellulose (BC) production yield from sugar beet molasses

Aytekin *et al.*[34] also showed that BC production decreased with increasing culture volume ratio. Besides, the BC yield increased sharply with increasing molasses concentration in low culture volumes. However, the BC yield slightly increased with increasing molasses concentration in high culture volumes. The contour graph of molasses concentration–culture volume binary interaction given in Figure 3b shows that the maximum BC production is observed at low concentrations and volumes for the examined experimental conditions. Figure 4 illustrates the 3D and contour plots of the inoculation ratio–culture volume binary interaction for BC production from sugar beet molasses. As seen in Figure 4a, the inoculation ratio did not significantly influence BC production within the tested range. A similar result was also obtained in a previous paper[18] focused on BC production from maple syrup. It should be noted that, according to ANOVA results (Table 4), the inoculation ratio did not affect the BC production yield neither as the binary interaction of molasses concentration–inoculation ratio (AB) nor as a quadratic (B^2). From Figure 4a, the BC production yield decreased with increasing culture volume at both low and high inoculation ratio values. The contour chart shown in Figure 4b illustrates that the culture volume must be at low values to achieve a high BC production yield.

The optimal points to obtain maximum BC production yield from sugar beet molasses used in this work proposed by RSM were 78.932 g/L molasses concentration, 12.973% inoculation ratio, and 130.405 mL culture volume. The BC

production yield %, which is the response variable, was 8.604, with 0.993 desirability under the optimum conditions.

In the RSM analyses, the perturbation plots can also be used to compare the effects of all the operating parameters investigated. A perturbation plot of the independent variables used in RSM analyses carried out in this work is presented in Figure 5. As seen in Figure 5, the response variable (BC yield) decreases with the increase in molasses concentration (A) and culture volume (C). However, the effect of inoculation ratio (B) on BC production is not significant. Thus, the molasses concentration and culture volume, individually, are more important parameters than the inoculation ratio in BC production from sugar beet molasses using *G. xylinus* NRRL B-759. This result is supported by the p-values in the ANOVA table (Table 4).

Characterization results

The BC pellicle produced in this study from sugar beet molasses using *G. xylinus* was approximately 95 x 100 mm in size, with 66 µm thickness.

The FTIR spectrum of the obtained BC sample is shown in Figure 6. Wang *et al*.[35] stated that a pure cellulose sample exhibits wide absorption bands at 3350 cm^{-1}, which are assigned to O-H stretching vibrations. As seen in Figure 6, the BC produced here has an important peak in this wavelength range. The peak at 2900 cm^{-1} is attributed to the C-H stretching vibrations of aliphatic hydrocarbons.[35,36] Finally, the peaks at 1430 cm^{-1}, 1367 cm^{-1}, and 1055 cm^{-1} correspond to CH_2 symmetrical bending or surface carboxylate groups, CH_2 wagging, ether COC functionalities and C-OH stretching vibrations, respectively.[37]

A SEM image of the BC sample obtained under optimum conditions is illustrated in Figure 7. It reveals the nanofiber structure of the BC, with randomly twisting ribbons. The heterogeneous appearance of the BC surface is due to the fibers alternating in bunches and forming clusters. The reason for forming fiber bunches and clusters is the tight entangling of the fibers due to intra- and inter-molecular hydrogen bonds.[35] The size range of the BC fibers produced under the optimum conditions was from 33 nm to 100 nm. This finding is in agreement with the results reported in previous studies.[38,39]

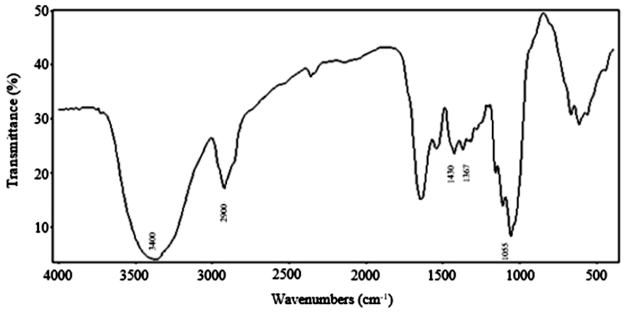

Figure 6: Fourier-transform infrared (FTIR) spectrum of bacterial cellulose (BC) produced from sugar beet molasses under optimum conditions

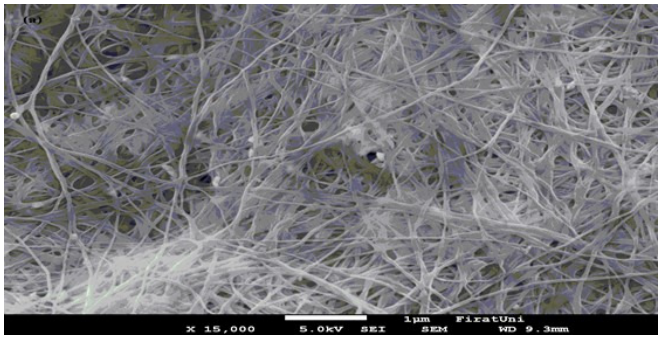

Figure 7: Scanning electron microscopy (SEM) image of bacterial cellulose (BC) produced from sugar beet molasses under optimum conditions

The elemental analysis results showed that the BC sample contained 40.74% C, 6.24% H, 0.76% N, and 0.11% S. These elemental analysis results are similar to those determined for BC by Klem *et al*.[6]

CONCLUSION

This study has presented the optimization of BC production from sugar beet molasses using *G. xylinus* NRRL B-759 in static culture. The data obtained by RSM-CCD methodology were then successfully employed in the experimental studies. The independent variables were molasses concentration, inoculation ratio, and culture volume, while the dependent variable was the BC production yield. As a result, the conclusions reached can be summarized as follows:

- The quadratic model was the best model among the models in the RSM-CCD;
- According to ANOVA, all model parameters, except AB and B^2, were important;
- The statistical parameters, such as R^2, Adj. R^2, Pred. R^2, lack of fit, Adeq. Precision, SD, and CV%, were sufficient for the compatibility of the model;
- The effects of molasses concentration–culture volume and inoculation ratio–culture volume binary interactions on BC production were important;
- The inoculation ratio had a lower effect than the other model variables;
- The optimum conditions were found to be the following: 78.932 g/L molasses concentration, 12.973% inoculation ratio, and 130.405 mL culture volume;
- FTIR results showed that the BC produced has a typical spectrum, compatible with literature data;
- SEM analysis indicated that the BC produced has nanofibers and a heterogeneous structure.

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