Optimization of Bacterial Nano-Cellulose Production in Bench-Scale Rotating Biological Contact Bioreactor by Response Surface Methodology

Baghernіya, Morteza; Babaeipour, Valiollah*[+]; Soleimani, Ali

Faculty of Chemistry and Chemical Engineering, Malek Ashtar University of Technology, P.O. Box 15875-1774 Tehran, I.R. IRAN

ABSTRACT: *The main challenge in bacterial cellulose nanofibers production is low yield and high cost. The aim of this work is to optimize bacterial nano-cellulose production in the bench-scale Rotating Biofilm Contact (RBC) bioreactor using experimental design. At all of the experiments the Acetobacter Xylinum BPR2001 and culture medium molasses – CSL were used. Three effective factors in the three levels including rotation (10, 13, and 16 rpm), aeration (0.2, 0.5, and 0.8 vvm) and disk distance (1, 1.5, and 2 cm) were optimized by response surface experimental design. The optimum conditions of biocellulose production were rotation rate 13 rpm, aeration 0.5 vvm, and disk distance 1.5 cm. The maximum dry weight of bacterial cellulose production reached 11.65 g/L on the 7th day, Which is one of the highest amounts of bacterial cellulose ever reported. Reduced quadratic models were used to final dry weight and moisture content of bacterial cellulose responses. ANOVA results showed the p-values were less than 0.05 that are significant models.*

KEYWORDS: *Bacterial cellulose; Rotating Biological contactor reactor; Optimization; Nano cellulose.*

INTRODUCTION

Microbial cellulose is a polysaccharide biopolymer that is synthesized by some microorganisms such as green algae (Valonia) and some bacteria, principally of the genera *Acetobacter*, such as *Acetobacter xylinum* [1,2]. This material has mechanical properties and unique structure and also has high purity compared with cellulose from plant sources and concerning these characteristics has many applications in different industries [3, 4]. Also, it used in a wide diversity of applied scientific activities such as biomedical and tissue engineering, pharmaceutical, acoustics, textiles, electronics, food products, and paper products [2,5]. Czaja et al. have reported that microbial cellulose can be a vital biomaterial for a wide variety of medical devices and consumer products [2]. Bacterial cellulose is extremely pure and exhibits a higher degree of polymerization and crystallization in respect of the fibrous with lignin, hemicellulose, relatively low glucose concentrations, and waxy aromatic substances [6].

The main constraint of microbial cellulose production is its high cost. The most important strategy to cost reduction is increasing the productivity per unit of time. Several techniques for microbial cellulose production

* *To whom correspondence should be addressed.*
+ E-mail: vbabaeipour@mut.ac.ir
1021-9986/2021/2/407-416 *10/$/6.00*

have been reported, some of which demonstrate a potential tool for economic and commercial bacterial cellulose production: stationary culture (in plastic trays), agitated culture (jar fermenters), cultivation in horizontal fermentation or cultivation in internal loop airlift reactors [7-9].

Researchers have found that rotating biofilm contact (RBC) bioreactor was capable of retaining considerable amounts of attached biomass, which when coupled with a good oxygen transfer capability of the system, could provide successful performance [10]. The RBC generally consists of a series of circular disks mounted on a horizontal shaft. The disks within the RBC are rotated, and alternatively exposed to the fermentation medium and air space. Bacterial cellulose has been conventionally produced by static culture method, which requires a long culture period and intensive man-power, thus resulting in a low productivity [10]. Moreover, cellulose production with continuous agitation and aeration encounters many problems, including spontaneous appearance of Cel^- mutants, which contributes to a decline in polymer synthesis thereby resulting in the lower productivity of bacterial cellulose [10,11].

Cultivation of bacterial cellulose-producing bacteria in an RBC may not have a strong shear stress and an air bubble at the surface of liquid medium, which seems to be excellent in terms of oxygen-transfer ability by which the microorganisms can be readily contacted with air in comparison with stirred-tank bioreactor [11, 12].

Optimization processes may involve the study of many biochemical and physical parameters, including media formulation and parameters. Conventionally, one studied parameter set as variable at time while keeping the other parameters at fixed value. So, obtaining optimum value for each parameter is to some extend difficult. In order to solve this problem in term of process scale-up, statistical analysis offers several advantages over conventional method including being rapid and reliable, helps understanding the interactions among the effective factors and response the total number of experiments. [13,14]. Experiments with two or more factors are encountered frequently. The best way to carry out such experiments is by using factorial design. Factorial design is used primarily for screening significant factors, but can also be used sequentially to model and refine a process. In this study, the cultivation of *A. xylinum* BPR2001 in RBC was attempted to improve the bacterial cellulose production. The effective factor including rotation, aeration and disk distance in the RBC was optimized to maximum production of bacterial cellulose.

EXPERIMENTAL SECTION

Microorganism and chemicals

Microorganism used in this study was *A. xylinum* BPR2001, which was obtained from Japan Culture Collection. The culture medium with molasses-CSL treated is introduced, which contains (g/l): molasses treated 150, CSL 160, citric acid 1.15. The culture media were sterilized at 121℃ in an autoclave for 30 min and poured into Erlenmayer flasks. The cultures were incubated at a temperature of 28 ℃, pH of 4.56 and for duration of 7 days. The culture medium used in these experiments is 2.6 L (working volume is 45% of bioreactor volume). All chemicals were analytical grade reagents, and all aqueous solutions were prepared using distilled water.

Bioreactor configuration and startup

A bench-scale RBC bioreactor was used in this study (Fig. 1). The plexiglass bioreactor was fabricated with dimension 15×15×30 cm^3 and a total volume 6.75 L. The RBC bioreactor was operated under mesospheric conditions (24±1℃) and temperature was maintained with an electrical heating tape (heating capacity: 40 W/m) attached to the outside surface of the reactor. All disks were made of poly-etylene and 45% of the disk was immersed in the medium. Disk diameter was 13.5 cm and surface area of each disk was 286.28 cm^2, whereas an effective surface area of each disk was 280 cm^2. Sixteen disks were mounted on a horizontal steel shaft and rotated using a direct driven digital stirrer electric motor.

Bacterial cellulose preparation

The formed thick cellulose membranes on the surface of the disks was harvested with tweezers, and washed with distilled water several times to remove the medium components. Then cellulose membranes treated with 0.1 N NaOH at 80 °C for 30 min in order to dissolve the microorganisms. After these treatments, bacterial cellulose membranes were rinsed again with distilled water until the pH of water became neutral. Purified bacterial cellulose was dried at 80 °C until constant weight was obtained, and then weighed.

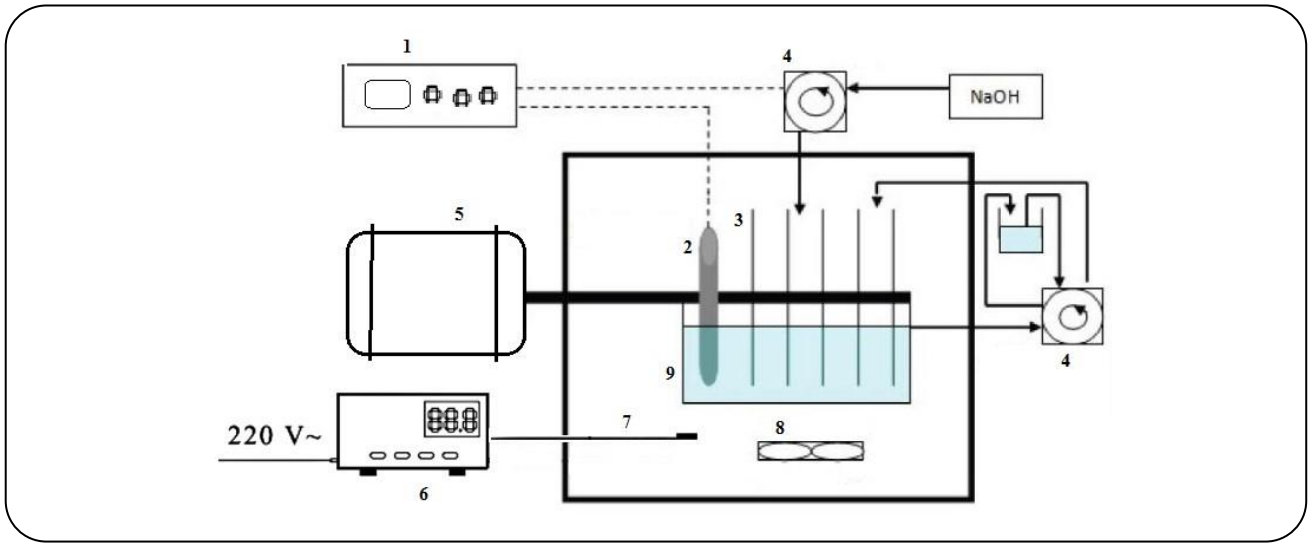

Fig. 1. Schematic diagram of the RBC bioreactor: (1) pH meter (2) probe pH (3) disk (4) peristaltic pomp (5) electromotor (6) temperature controller (7) temperature sensor (8) magnetic stirrer (9) plexiglass bioreactor

Analytical methods

The moisture content of the bacterial cellulose and the dry weight production rate were determined using Eq. (1) and (2), respectively. The wet bacterial cellulose was dried at 80 °C.

$$\text{Moisture content}(\%) = \frac{\text{Wet weight}(g) - \text{Dry weight}(g)}{\text{Wet weight}(g)} \times 100 \quad (1)$$

$$\text{Dry weight production rate}\left(\frac{g}{l}\right) = \frac{\text{Dry weight}(g)}{\text{Culture volume}(L)} \quad (2)$$

Experimental design and optimization

Experimental design was performed to investigate the effect of three main parameters including rotation, aeration and disk distance on the process efficiency and also obtaining optimal condition. Each factor in experimental designs based on the general factor was varied at three different levels while the other parameters were kept constant. The range and the levels of the variables investigated in this study are given in Table 1. General factor is essentially a particular set of mathematical and statistical methods for designing experiments, building models, evaluating the effects of variables, and searching optimum conditions of variables to predict targeted responses [15]. The general factorial design allows us to have factors that each has a different number of levels. It will create an experiment that includes all possible combinations of the factor levels. The behavior of the system is explained by the quadratic polynomial empirical model.

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<1}^{3} \sum_{j=1}^{3} \beta i_j X_i X_j + \varepsilon \quad (3)$$

Where, y is the expected value of the response variable, β_0 , β_{ii} , β_{ij} are the model parameters, X_i and X_j are the coded factors evaluated. In this study, y represents the dry weight of the produced bacterial nano-cellulose and moisture content and moisture content percentage in the different empirical models [19, 20].

Confirmation experiments

In order to check the validation of obtained model, an experiment at optimal factor levels was performed and the experimental result was compared with predicted result by the model.

Instrumentation

In this study, functional groups in microbial cellulose were determined by Fourier Transform InfraRed (FT-IR) spectroscopy. Spectra were collected with a spectrometer using KBr pellets. In each case, 1.0 mg of dried microbial cellulose sample and 100 mg of KBr were homogenized using a mortar and pestle, and then pressed into a transparent tablet at 200 kgf/cm^2 for 5 min. The particles

Table 1: Experimental variables at different levels.

Variable	Level (1)	Level (2)	Level (3)
A-Rotation (rpm)	10	13	16
B-Aeration (vvm)	0.2	0.5	0.8
C-Disk distance (cm)	1.5	2	2.5

were analyzed with an FT-IR Spectrometer (Shimadzu 4100) in the transmittance (%) mode in the range 3500–500 cm^{-1}. To study of the sample mineralogy, XRD pattern was done using a Philips X'Pert MPD diffractometer (Netherlands). FE-SEM images of the sample was taken using a Hitachi F4160 (Japan, Tokyo), operating at 25 kV. FE-SEM was used to monitor the morphology of sample before and after bioleaching. Sample was carefully attached to adhesive carbon tubes and a 30 nm thick conductive coating of gold was applied to the surface.

RESULT AND DISCUSSION

Statistical analysis

The general factor design that presents the experimental conditions and their responses are shown in Table 2. In order to investigate the effect of each factor including rotation, aeration and disk distance on the response of the system, analysis of variance (ANOVA) results were calculated as shown in Table 3.

This statistical tool is required to test the significance and adequacy of the model. The Mean Squares (MS) are obtained as: MS = SS/DF, where: SS = Sum of Squares (SS) of each variation source and DF = the respective degrees of freedom (DF). The Fischer variation ratio (F-value) is a statistically valid measure of how well the factors describe the variation in the data about its mean. It can be calculated from ANOVA as: F-value = MS (due to the model variation)/MS (due to error variance). Normally, the data has some variations around its mean value; the greater the F-value from unity, the more acceptable is this variation [17]. In general, the calculated F-value should be several times greater than the tabulated value. In fact, as shown in Table 4, the calculated P-value of the dry weight and moisture model is less than 0.05 which shows that the model is statistically significant.

Fitting models

Eqs. (2) and (3) were obtained from the 27-batch runs using the Design-Expert 7.0 software. By applying multiple regression analysis on the experimental data, the experimental results of the general factor design were fitted with a modified quadratic model polynomial equation. The empirical relationship between dry weight and moisture and the three test variables in coded units obtained by the application of full factorial is given by

$$\text{Dry weight}(g/l) = 5.71 + 0.39\,A + 0.64\,B - 0.67\,C + 0.30\,AB + 0.028\,AC - 0.55\,BC - 4.62\,A2 + 1.79\,B2 \quad (2)$$

$$\text{Moisture content}(\%) = 98.42 - 0.083\,A - 0.073\,B + 0.031\,C - 0.12\,AB - 0.033\,AC + 0.059\,BC + 0.53\,A2 - 0.21\,B2 \quad (3)$$

Where *A* is rotation, *B* is aeration (vvm) and *C* is disk distance (cm). It should be noted that polynomial models are reasonable approximations of the true functional relationship over relatively small regions of the entire space of independent variables [18]. Fig. 2 shows the predicted versus actual data. The clustering of the points around the diagonal line indicates a satisfactory correlation between the experimental data and the predicted values, confirming the robustness of the model [19].

The relatively high R^2 and adjusted R^2 (R^2adj) values that presented in Table 3 indicate that the modified quadratic model for the dry weight and moisture percentage is capable of representing the system under the given experimental conditions.

Three dimensional response plots

The Dry weight of bacterial cellulose production

Fig. 3 represents the three-dimensional response surfaces of the relationship between different parameters. Fig. 3 (a,b) indicates that the shape of predicted response surface is like a saddle. The eigenvalues and eigenvectors indicate the shape of the response surface.

Positive eigenvalues direct an upwards curvature, and negative eigenvalues direct a downward curvature. Therefore, all positive eigenvalues indicate an estimate stationary in a minimum, and all positive eigenvalue

Table 2: Experimental plan based on general factor and the results.

Run	A:Rotation (rpm)	B: Aeration (vvm)	C: Disk distance (cm)	Dry weight (g/l)	Moisture (%)
1	16	0.2	1.5	2.70	98.82
2	10	0.8	2.5	0.90	99.07
3	10	0.5	2	1.55	98.94
4	10	0.5	1.5	1.76	98.90
5	13	0.8	1.5	11.65	97.94
6	16	0.8	1.5	4.88	98.47
7	13	0.5	2.5	4.08	98.55
8	13	0.8	2.5	7.83	98.17
9	13	0.8	2	8.74	98.06
10	13	0.5	2	4.30	98.40
11	16	0.5	1.5	1.98	98.90
12	10	0.5	2.5	1.13	98.98
13	16	0.2	2.5	2.02	98.90
14	16	0.5	2	1.73	98.92
15	13	0.2	2.5	6.88	98.07
16	10	0.2	2.5	1.97	98.87
17	16	0.5	2.5	1.43	98.86
18	16	0.2	2	3.08	98.71
19	10	0.2	1.5	2.61	98.73
20	13	0.5	1.5	5.75	98.53
21	10	0.8	1.5	3.10	98.78
22	16	0.8	2	3.32	98.43
23	10	0.2	2	1.88	98.80
24	13	0.2	2	6.10	98.45
25	10	0.8	2	2.22	98.97
26	13	0.2	1.5	6.85	98.40
27	16	0.8	2.5	2.98	98.54

Table 3: ANOVA for response surface models applied.

Response	Model				ANOVA		
		Source	S.S.	Df	M.S.	F Value	Prob > F
Dry weight (g/l)	Reduced quadratic model	Model	170.252	8	21.281	22.983	< 0.0001
		A-Rotation (rpm)	2.712	1	2.712	2.929	0.1042
		B-Aeration (vvm)	7.376	1	7.376	7.966	0.0113
		C-Disk distance (cm)	8.086	1	8.086	8.733	0.0085
		AB	1.095	1	1.095	1.183	0.2910
		AC	0.009	1	0.009	0.010	0.9203
		BC	3.672	1	3.672	3.966	0.0618
		A^2	127.984	1	127.984	138.220	< 0.0001
		B^2	19.314	1	19.314	20.858	0.0002
		Residual	16.667	18	0.925		
		(R^2=0.91	R^2adj=0.87)				
Moisture (%)	Reduced quadratic model	Model	2.373	8	0.296	23.616	< 0.0001
		A-Rotation (rpm)	0.124	1	0.124	9.889	0.0056
		B-Aeration (vvm)	0.096	1	0.097	7.718	0.0124
		C-Disk distance (cm)	0.016	1	0.017	1.342	0.2617
		AB	0.160	1	0.160	12.795	0.0022
		AC	0.013	1	0.01	1.034	0.3226
		BC	0.041	1	0.041	3.296	0.0861
		A^2	1.658	1	1.658	132.021	< 0.0001
		B^2	0.262	1	0.261	20.834	0.0002
		Residual	16.667	18	0.925		
		(R^2=0.91	R^2adj=0.87)				

Table 4: Verification of the model at optimum condition.

Response	Target	Prediction	Confirmation experiment (%)	95% CI low	95% CI high
Dry weight rate production (g/l)	Maximize	9.39	11.65	8.44	10.35
Moisture (%)	Minimize	98.04	97.95	97.93	98.15

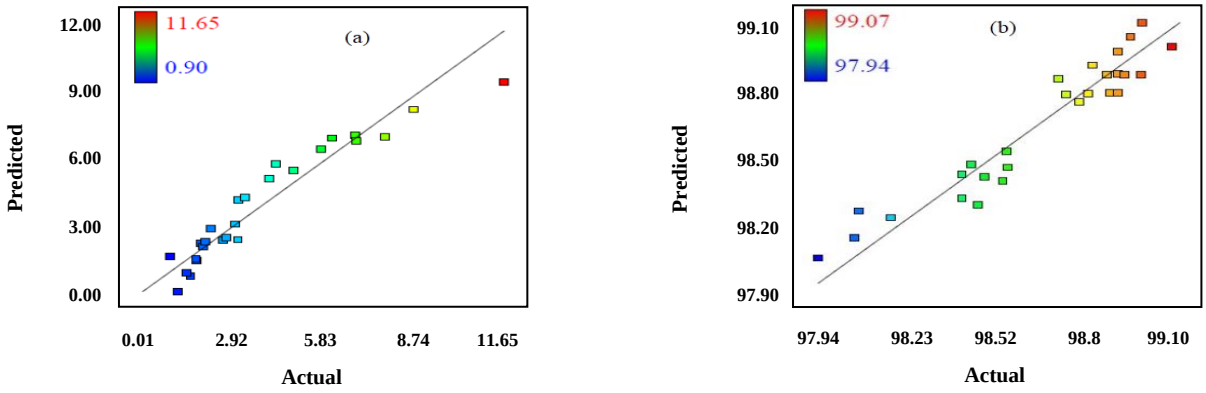

Fig. 2: Predicted vs. actual values for (a) dry weight of the bacterial cellulose rate (b) moisture content.

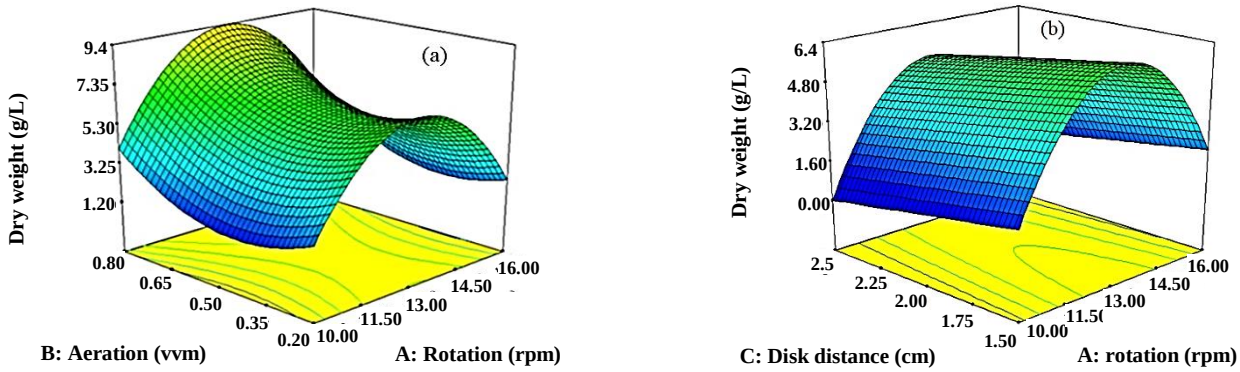

Fig. 3: Three dimensional plots of the interactive effect for the dry weight of the bacterial cellulose rate: (a) effect of rotation and aeration at the constant disk distance 1.5 cm and (b) effect of rotation and disk distance at the constant aeration 0.5 vvm.

indicate a maximum and mixture of positive and negative eigenvalues indicate a saddle point [20]. There is clearly in Fig. 3 a combined effect of rotation and aeration at the constant of disk distance 1.5 (cm). The maximum dry weight production (9.4 g/l) was observed for rotation of 13 rpm and aeration of 0.8 vvm. It is obvious that increasing aeration has positive effect of dry weight of bacterial cellulose production that was also showed by *kim et al.* [10]. The relationship between rotation and disk distance for dry weight production (g/L) showed in Fig. 3b. According to this figure, a maximum dry weight of bacterial cellulose production higher than 6.4 g/L was observed at 13 rpm and disk distance 1.5 cm at the constant aeration of 0.5 vvm. According to this figure amount of dry weight will be increased rotation from 10 rpm to 13 rpm and in the bigger than 13 rpm the amount of dry weight decreased.

Moisture content

Fig. 4a shows the interaction between rotation and aeration at the constant disk distance of 1.5 cm. According to this figure, the amount of moisture decreased by the increasing rotation from 10 rpm to 13 rpm and the increasing aeration from 0.2 vvm to 0.8 vvm. The shape of predicted response surface is like a saddle. It is clear from Fig. 3b that the rotation 13 rpm and the disk distance 1.5 cm at the aeration 0.5 vvm minimum have moisture content 98.3%.

Process optimization using desirability functions

It should be noted that the goal of optimization is to find a good set of conditions that will meet all of the goals. The goals are combined into an overall desirability function. Desirability is an objective function that ranges from zero outside of the limits to one at the goal.

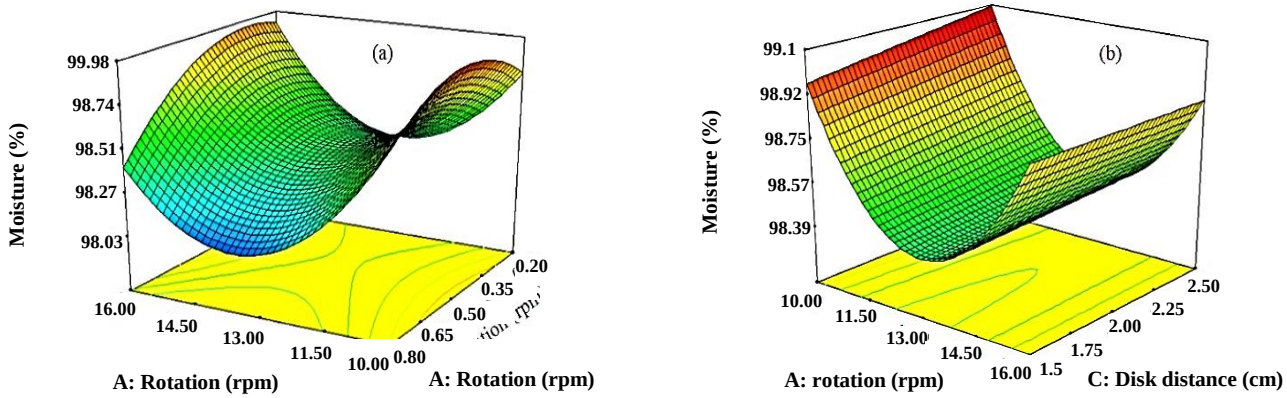

Fig. 4: Three-dimensional plots of the interactive effect for the moisture content of the bacterial cellulose: (a) effect of rotation and aeration at the constant disk distance 1.5 cm and (b) effect of rotation and disk distance at the constant aeration 0.5 vvm.

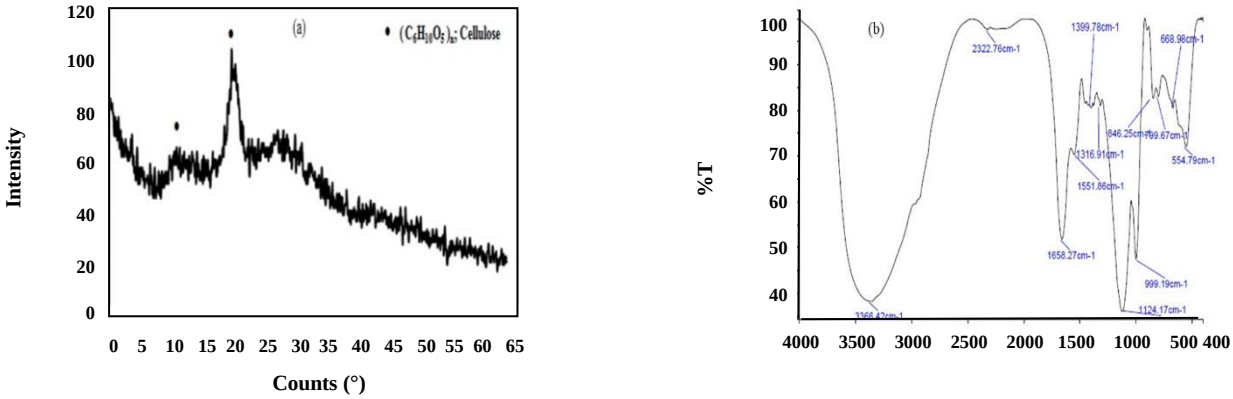

Fig. 5: (a) XRD of bacterial cellulose (b) FT-IR spectra of bacterial cellulose.

The program seeks to maximize this function. By starting from several points in the design space chances improve for finding the best local maximum are high [17]. The optimum conditions proposed by the model were rotation 13 (rpm), aeration 0.5 (vvm) and disk distance 1.5 (cm) with which the maximum dry weight of 9.39 (g/L) and minimum moisture 98.04% was achieved.

Confirmatory experiments

In order to verification of the optimized conditions given by the model, an experiment was carried out in the optimal condition suggested by the model. Table 4 showed that verification experiment and the predicted values from fitted correlations were in close agreement at a 95% confidence interval. The 95% Confidence Interval (C.I.) is the range in which the process average was expected to fall 95% of the time. These results confirmed the experimental values were determined to be quite close to the predicted values. Under these conditions, the experimental value for the dry weight and moisture percentage was found to be 11.65 (g/l) and 97.95%.

Physicochemical properties

Determination of the mineral content of microbial cellulose was carried out by XRD that was showed in the Fig. 5a. Two domain peaks as labeled in Fig. 5a can be well indexed to cellulose which is in good agreement with the literature values with JCPDS No: 50-2241. To indicate the functional group on the surface of the microbial cellulous, the FT--IR spectrum is shown in Fig. 5b. According to it, there is a strong peak at 3,366 cm^{-1} representing the O–H group, while the peak at 2,918 cm^{-1} indicates the presence of C–H stretching. The appearance of peaks at 1124 cm^{-1} and 999 cm^{-1} indicate the presence

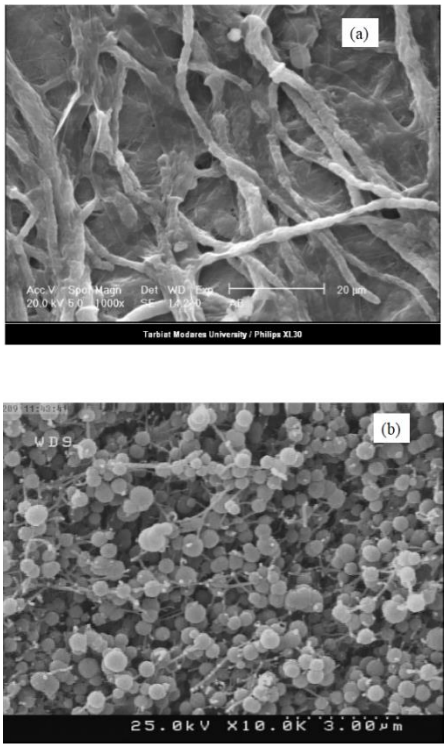

Fig. 6: FESEM of bacterial cellulose powder at different magnification.

of C-C stretching and C-O stretching. Fig. 6 shows the FE-SEM photomicrograph of the microbial cellulose sample in different magnification. Under this observation, the particles of the cellulose powder were fibrous. The average size of the fibrous was 85 nm in accordance with the value in the literature [21, 22].

CONCLUSIONS

The study examined the bacterial cellulose production in the RBC bioreactor. To optimization of effective factors including rotation, aeration and disk distance full factor design was used. ANOVA showed that the reduced quadratic model equations have relatively high R^2 at 95% confidence level. The optimum conditions of removal were rotation 13 rpm, aeration 0.5 vvm and disk distance 1.5 cm. Maximum bacterial cellulose production received to 11.65 g/l with the moisture 97.95 in the 7 day. The amount of bacterial cellulose obtained under optimal conditions from this study is one of the highest bacterial cellulose production ever reported. Physicochemical characteristics such as XRD, FT-IR and FESEM showed the bacterial cellulose has high purification with nanoscale fibrous.

Received : Oct. 30, 2019 ; Accepted : Jan. 14, 2020

REFERENCES

[1] Jonas R., Farah L.F., Production and application of Microbial Cellulose, *Polym. Degrad. Stabil.*, **59**: 101-106 (1998).

[2] Czaja W.K., Young D.J., Kawecki M., Brown R.M., The Future Prospects of Microbial Cellulose in Biomedical Applications, *Biomacromolecules*, **8(1)**: 1-12 (2007).

[3] Krystynowicz A., Czaja W., Wiktorowska-Jezierska A., Gonc¸alves-Mis´kiewicz M., Turkiewicz M., Bielecki S., Factors Affecting the Yield and Properties of Bacterial Cellulose, *J. Ind. Microb. Biotech.*, **29**: 189-195 (2002).

[4] Lin S.P., Calvar I.L., Catchmark J.M., Liu J.R., Demirci A., Cheng K.C., Biosynthesis, Production and Applications of Bacterial Cellulose, *Cellulose*. **20(5)**: 2191-2219 (2013).

[5] Islam M.U., Khan T., Park J.K., Nanoreinforced Bacterial Cellulose–Montmorillonite Composites for Biomedical Applications, *Carbohydr. Polym.*, **89**: 1189–1197 (2012).

[6] Ebrahimi E., Babaeipour V., Meftahi A., Alibakhshi S., Effects of Bio-Production Process Parameters on Bacterial Cellulose Mechanical Properties, *J. Chem. Eng. Jpn.*, **50(11)**:857-861, (2017).

[7] Chao Y., Mitarai M., Sugano Y., Shoda M., Effect of Addition p Water-Soluble Polysaccharides pn Bacterial Cellulose Production in a 50-L Airlift Reactor, *Biotechnol. Progr.*, **17**: 781–785 (2001).

[8] Jung J.Y., Khan T., Park J.K., Chang H.N., Production of Bacterial Cellulose by *Gluconacetobacter hansenii* Using a Novel Bioreactor Equipped with a Spin Filter, *Korean J. Chem. Eng.*, **24**: 265–271 (2007).

[9] Sumate T., Pramote T., Waravut K., Pattarasinee B., Effect of Dissolved Oxygen on Cellulose Production by *Acetobacter* sp., *J. Sci. Res. Chula. Univ.*, **30**: 179-186 (2005).

[10] Kim Y.J., Kim J.N., Wee Y.J., Park D.H., Bacterial Cellulose Production by *Gluconacetobacter* sp. RKY in a Rotary Biofilm Contactor, *Appl. Biochem. Biotechnol.*, **137-140(1-12)**: 529-37 (2007).

[11] Hamid M., Babaeipour V., Imani M., Bahrami A., Dissolution and Regeneration of the produced Nano Bacterial Cellulose of Food Industries Wastewaters by a Cost-Benefit Method, *Iran. J. Chem. Chem. Eng. (IJCCE)*, **38(3)**: 9-21 (2019).

[12] Kouda T., Yano H., Yoshinaga F., Effect of Agitator Configuration on Bacterial Cellulose Productivity in Aerated and Agitated Culture, *J. Ferment Bioeng.*, **83**: 371- 376 (1997).

[13] Babaeipour V., Haji Abbas M.P., Sahebnazar Z., Alizadeh R., Enhancement of Human Granulocyte-Colony Stimulating Factor Production in Recombinant *E. Coli* Using Batch Cultivation, *Bioprocess Biosyst Eng.*, **33**: 591–598 (2010).

[14] Jimnez-Contreras E., Torres-Salinas D., Moreno R.B., Baños R.R., López-Cózar E.D., Response Surface Methodology and its Application in Evaluating Scientific Activity, *Scientometrics*, **79(1)**: 201-218 (2009).

[15] Rastegar S.O., Mousavi S.M., Shojaosadati S.A., Sheibani S., Optimization of Petroleum Refinery Effluent Treatment in a UASB Reactor Using Response Surface Methodology, *J. Hazard. Mater.*, **197**: 26– 32 (2011).

[16] Montgomery D.C., "Design and Analysis of Experiments", 6th ed., John Wiley & Sons, Inc., New York (2012).

[17] Seyfi R., Babaeipour V., Mofid M.A., Abarghooi Kahaki F., Expression and Soluble Over-Production of recombinant Scorpine as a Potassium Channel Blocker Protein in *E. coli*, *Biotechnol. Appl. Biochem.* **66(1)**: 119-129 (2019).

[18] Morowvat M. H., Babaeipour V., Rajabi Memari H., Vahidi H., Optimization of Fermentation Conditions for Recombinant Human Interferon Beta Production by *Escherichia coli* Using the Response Surface Methodology, *Jundishapur J. Microbiol.*, **8(4)**: e16236 (2015).

[19] Rastegar S.O., Mousavi S.M., Shojaosadati S.A., Rezaei M., Statistical Evaluation and Optimization of Effective Parameters in Bioleaching of Metals from Molybdenite Concentrate Using *Acidianus brierleyi*, *J. Ind. Eng. Chem.*, **20**: 3096-3101 (2013).

[20] Li Z., Liu Y., Performing Response Surface Analysis Using the SAS RSREG Procedure, *Paper DV02.2012* (2012).

[21] Czaja W., Krystynowicz A., Bielecki S., Brown R.M., Microbial Cellulose-the Natural Power to Heal Wounds, *Biomaterials*, **27**: 145-151 (2006).

[22] Hult E., Yamanaka S., Ishihara M., Sugiyama J., Aggregation of Ribbons in Bacterial Cellulose Induced by High-Pressure Incubation, *Carbohyd Polym.*, **53**: 9-14 (2003).