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Short communication

Production of microbial cellulose: Response surface methodology approach

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ABSTRACT

The present work was carried out to test the potential of *Acetobacter aceti* MTCC 2623 for the production of cellulose and optimization of various process conditions. Response surface methodology (RSM) was applied to optimize the process parameters for microbial cellulose production. The optimized parameters for maximum cellulose production (1.73 g/L) and sugar utilization (99.8%) obtained were 2.25% (w/v) glucose concentration, 1.16% (w/v) sodium nitrate concentration, pH 7, 27.5 °C temperature, and 159 h of incubation time. The structure of produced microbial cellulose was established by using FT-IR spectroscopy.

1. Introduction

Microbial cellulose is produced by several genera of bacteria including *Agrobacterium*, *Rhizobium*, *Pseudomonas*, *Sarcina* and *Acetobacter* (Hestrin & Schramm, 1954; Ross, Mayer, & Benziman, 1991). Although all are known to synthesize cellulose, but only *Acetobacter* species can produce sufficient amount of cellulose, and has attracted the attention of researchers.

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and searching for optimum conditions for desirable responses. RSM can identify and quantify the various interactions among different parameters and it has been applied for optimization of medium and process conditions in bioprocesses (Bogar et al., 2003; Panesar, 2008). Keeping this in view, the present investigation was undertaken to apply the response surface methodology on the fermentation of *Acetobacter aceti* MTCC 2623 for the production and to optimize the various process parameters for maximal cellulose production.

2. Materials and methods

2.1. Micro-organism and its maintenance

A. aceti MTCC 2623 was procured from Institute of Microbial Technology Chandigarh, India. The bacterial culture was maintained on growth media containing each (5 g/L) of yeast extract and peptone and (25 g/L) mannitol. The medium was autoclaved, inoculated with bacterial strain and incubated at 28 °C for 24 h.

2.2. Fermentation media

Hestrin–Schramm's medium, which contains (%, w/v) carbon source 2.0, peptone 0.5, yeast extract 0.5, disodium phosphate 0.27, and citric acid 0.115 was used for the production of microbial cellulose (Hestrin & Schramm, 1954). The starter culture (2%) of *A. aceti* MTCC 2623 was added into the flasks containing 100 ml media, and the inoculated medium was incubated at 28 °C for growth for 144 h. Cellulose pellicle was isolated from the surface layer of fermented broth.

2.3. Isolation and purification of cellulose

The cellulose pellicle of each batch was measured after isolation by centrifugation at 5000 rpm or by filtration. Obtained pellicle was boiled in 2% (w/v) NaOH solution for 20 min to remove cells from the cellulose matrix. The cellulose was washed with deionised water until the remaining base is removed. The wet weight was measured by taking the water soaked pellicle after draining for 5 min. The dry weight was taken after drying the film at 35 °C temperature until constant weight (Keshk & Sameshima, 2005).

2.4. Determination of sugars

Reducing and total sugars were estimated by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956) and Miller (1959), respectively.

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Table 1
Experimental design in coded and uncoded forms of process variables and values of experimental data for optimization of cellulose production.

Coded and uncoded process variables				Responses		
pH (x_1)	Temperature (°C) (x_2)	Incubation time (h) (x_3)	Glucose (%, w/v) (x_4)	Sodium nitrate (%, w/v) (x_5)	Cellulose production (g/L) (Y_1)	Sugar utilization (%, w/v) (Y_2)
(−1) 4.00	(−1) 24.00	(−1) 96.0	(−1) 1.5	(−1) 0.80	0.099	89.10
(+1) 7.00	(−1) 24.00	(−1) 96.0	(−1) 1.5	(−1) 0.80	1.120	96.02
(−1) 4.00	(+1) 32.00	(−1) 96.0	(−1) 1.5	(−1) 0.80	0.085	89.00
(+1) 7.00	(+1) 32.00	(−1) 96.0	(−1) 1.5	(−1) 0.80	0.580	94.50
(−1) 4.00	(−1) 24.00	(+1) 170	(−1) 1.5	(−1) 0.80	0.120	88.10
(+1) 7.00	(−1) 24.00	(+1) 170	(−1) 1.5	(−1) 0.80	1.350	97.00
(−1) 4.00	(+1) 32.00	(+1) 170	(−1) 1.5	(−1) 0.80	0.070	89.40
(+1) 7.00	(+1) 32.00	(+1) 170	(−1) 1.5	(−1) 0.80	0.740	95.05
(−1) 4.00	(−1) 24.00	(−1) 96.0	(+1) 2.5	(−1) 0.80	0.080	89.00
(+1) 7.00	(−1) 24.00	(−1) 96.0	(+1) 2.5	(−1) 0.80	1.250	97.60
(−1) 4.00	(+1) 32.00	(−1) 96.0	(+1) 2.5	(−1) 0.80	0.079	89.80
(+1) 7.00	(+1) 32.00	(−1) 96.0	(+1) 2.5	(−1) 0.80	0.740	96.60
(−1) 4.00	(−1) 24.00	(+1) 170	(+1) 2.5	(−1) 0.80	0.075	89.00
(+1) 7.00	(−1) 24.00	(+1) 170	(+1) 2.5	(−1) 0.80	1.390	97.50
(−1) 4.00	(+1) 32.00	(+1) 170	(+1) 2.5	(−1) 0.80	0.081	89.40
(+1) 7.00	(+1) 32.00	(+1) 170	(+1) 2.5	(−1) 0.80	0.950	96.80
(−1) 4.00	(−1) 24.00	(−1) 96.0	(−1) 1.5	(+1) 1.20	0.090	89.50
(+1) 7.00	(−1) 24.00	(−1) 96.0	(−1) 1.5	(+1) 1.20	1.200	87.00
(−1) 4.00	(+1) 32.00	(−1) 96.0	(−1) 1.5	(+1) 1.20	0.080	88.40
(+1) 7.00	(+1) 32.00	(−1) 96.0	(−1) 1.5	(+1) 1.20	0.700	95.00
(−1) 4.00	(−1) 24.00	(+1) 170	(−1) 1.5	(+1) 1.20	0.055	88.30
(+1) 7.00	(−1) 24.00	(+1) 170	(−1) 1.5	(+1) 1.20	1.540	97.30
(−1) 4.00	(+1) 32.00	(+1) 170	(−1) 1.5	(+1) 1.20	0.060	87.68
(+1) 7.00	(+1) 32.00	(+1) 170	(−1) 1.5	(+1) 1.20	1.090	95.30
(−1) 4.00	(−1) 24.00	(−1) 96.0	(+1) 2.5	(+1) 1.20	0.090	89.70
(+1) 7.00	(−1) 24.00	(−1) 96.0	(+1) 2.5	(+1) 1.20	1.550	99.07
(−1) 4.00	(+1) 32.00	(−1) 96.0	(+1) 2.5	(+1) 1.20	0.100	90.00
(+1) 7.00	(+1) 32.00	(−1) 96.0	(+1) 2.5	(+1) 1.20	1.350	97.90
(−1) 4.00	(−1) 24.00	(+1) 170	(+1) 2.5	(+1) 1.20	0.100	89.00
(+1) 7.00	(−1) 24.00	(+1) 170	(+1) 2.5	(+1) 1.20	1.700	99.00
(−1) 4.00	(+1) 32.00	(+1) 170	(+1) 2.5	(+1) 1.20	0.100	88.30
(+1) 7.00	(+1) 32.00	(+1) 170	(+1) 2.5	(+1) 1.20	1.620	97.01
1.93	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	0.009	76.30
9.07	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.010	95.90
(0) 5.50	18.48	(0) 133	(0) 2.0	(0) 1.00	0.200	88.00
(0) 5.50	37.52	(0) 133	(0) 2.0	(0) 1.00	0.090	86.00
(0) 5.50	(0) 28.00	44.99	(0) 2.0	(0) 1.00	0.380	94.40
(0) 5.50	(0) 28.00	221	(0) 2.0	(0) 1.00	0.700	94.00
(0) 5.50	(0) 28.00	(0) 133	0.8	(0) 1.00	0.300	96.50
(0) 5.50	(0) 28.00	(0) 133	3.2	(0) 1.00	0.710	97.90
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	0.52	0.600	97.00
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	1.47	0.900	97.20
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10
(0) 5.50	(0) 28.00	(0) 133	(0) 2.0	(0) 1.00	1.200	99.10

2.5. Experimental design

A second-order central composite design (CCD) in the form of a face-centred cube (FCC) with five factors (pH, temperature, incubation time, glucose concentration and salt concentration) at three levels and three replications each was used for cellulose production and sugar utilization (Table 1). The second order polynomial equation was fitted to the experimental data of each dependent variable as follows (Obeng, Morrell, & Napier-Munn, 2005):

$$Y_i = \beta_0 + \sum_{i=1}^{n}\beta_i x_i + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\beta_{ij}x_i x_j + \sum_{i=1}^{n}\beta_{ii}x_i^2 \quad (1)$$

where Y_i = response variable (Y_1 = cellulose production, Y_2 = sugar utilization); x_i's and x_j's represent the coded independent variables (x_1 = pH, x_2 = temperature, x_3 = incubation time, x_4 = glucose concentration, x_5 = sodium nitrate). β_0 was the value of fitted response at the centre point of design, i.e. point (0, 0, 0), and β_i, β_{ii} and β_{ij} were the linear, quadratic and cross-product regression coefficients, respectively.

2.5.1. Analysis of data

The multiple regression analysis was conducted for fitting the model represented by equation to the experimental data. Maximization and minimization of the polynomial thus fitted was performed by numerical techniques and mapping of the fitted responses was achieved using Stat-Ease software (Stat-Ease, version 6.01 2001, Stat-Ease Inc., 2021 East Hennepin Avenue, MN 55413) trial version.

2.6. FT-IR spectroscopy

The surface properties of the cellulose were examined by a Perkin-Elmer S2000 Fourier transform infrared spectrometer (FTIR,

Table 2
Regression summary and ANOVA table for cellulose production for uncoded values of process variables.

Source	β-Coefficient	Sum of squares	DF	Mean square	F-Value	Prob > F
Model	1.207	13.965	20	0.698	17.620	<0.0001
pH	0.459	9.130	1	9.130	230.414	<0.0001
Temperature	−0.084	0.307	1	0.306	7.743	0.0094
Incubation time	0.060	0.157	1	0.157	3.966	0.0559
Glucose	0.075	0.244	1	0.244	6.158	0.0191
Sodium nitrate	0.076	0.256	1	0.255	6.458	0.0166
pH * pH	−0.115	0.735	1	0.735	18.549	0.0002
Temp * temp	−0.179	1.789	1	1.789	45.156	<0.0001
Time * time	−0.109	0.667	1	0.667	16.851	0.0003
Glucose * glucose	−0.115	0.745	1	0.745	18.807	0.0002
Sodium nitrate * sodium nitrate	−0.072	0.292	1	0.292	7.370	0.0110
pH * temp	−0.102	0.335	1	0.335	8.463	0.0069
pH * time	0.060	0.116	1	0.116	2.943	0.0969*
pH * glucose	0.068	0.149	1	0.149	3.761	0.0622*
pH * sodium nitrate	0.083	0.218	1	0.218	5.512	0.0259
Temp * time	4.5×10^{-3}	6.66×10^{-4}	1	6.66×10^{-4}	0.016	0.8977
Temp * glucose	0.029	0.028	1	0.028	0.717	0.4038
Temp * sodium nitrate	0.029	0.027	1	0.027	0.687	0.4136
Time * glucose	-9.1×10^{-3}	2.7×10^{-3}	1	2.7×10^{-3}	0.068	0.7959
Time * sodium nitrate	0.011	4.09×10^{-3}	1	4.09×10^{-3}	0.103	0.7502
Glucose * sodium nitrate	0.041	0.053	1	0.053	1.361	0.2528
Residual		1.15	29	0.040		
Lack of fit		1.15	22	0.052		
R^2	0.9240					

* Non-significant at 5% level of significance.

Nicolet Magna IR 560) in an attenuated total reflectance (ATR) mode (Wan et al., 2007).

3. Results and discussion

3.1. Diagnostic checking of fitted model and Surface plot for cellulose production

The analysis of variance (ANOVA) results (Table 2) indicated that quadratic regression to produce the second order model was significant which was revealed from P-value (R^2 = 92.40%). The model F-value of 17.62 also implies that model is significant. The value of adjusted determination coefficient (adjusted R^2 = 87.15%) was high to advocate a high significance of the model. The magnitude of P-value from Table 2 indicated that linear terms like pH, temperature, glucose and sodium nitrate have significant effect on cellulose production except incubation time. The equation of the model fitted for cellulose production in the actual form of process variables after eliminating the non significant terms is:

Cellulose production (g/L) = −12.66 + 0.744 * pH + 0.630 * Temperature + 0.015 * incubation time + 0.740 * glucose + 0.448 * sodium nitrate − 0.051 * pH^2 − 0.011 * temperature^2 − 8.007 * incubation time^2 − 0.463 * glucose^2 − 1.812 * sodium nitrate^2 − 0.017 × pH * temperature + 0.087 × pH * time + 0.091 × pH * glucose + 0.275 × pH * sodium nitrate

{Degree of freedom = 20; F-value = 17.620; P-value = <0:0001; R^2 = 0.9240}

The data indicated that the increase in pH and incubation time resulted in increase in cellulose production (Fig. 1). It has been observed that at lower pH and incubation time, minimum cellulose production was observed but with increase in incubation time and pH, cellulose production also increased. This indicated that alkaline pH is favourable for cellulose production because of minimum conversion of glucose in to gluconic acid that increases cellulose production (Pourramezan, Roayaei, & Qezelbash, 2009). With increase in incubation time cellulose production also increased, but decreased after 168 h. With increase in glucose and sodium nitrate, there was increase in cellulose production (Fig. 2). However, excess sugar in fermentation media lowered down the cellulose yield because of conversion of excess of glucose into gluconic acid

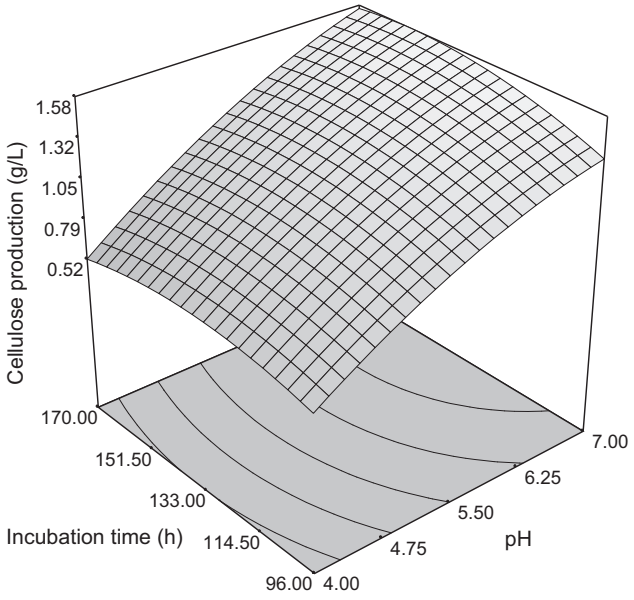

Fig. 1. Effect of incubation time and pH on cellulose production.

which is the intermediate product of cellulose production by *Acetobacter* species (Vandamme, De Baets, Vanbaelen, Joris, & De Wulf, 1998)

In interactive terms, glucose and sodium nitrate had positive effect on cellulose production and it was significant at 10% level. This may be due the reason that an optimum C/N ratio is required for maximum cellulose production (Teresa, 1999). The addition of extra nitrogen source favours the biomass production but can decrease cellulose production (Matsuoka, Tsuchida, Matsushita, Adachi, & Yoshinaga, 1996).

3.2. Diagnostic checking of fitted model and surface plots for sugar utilization

The results of second order responses in the form of Analysis of variance (ANOVA) are given in Table 3. The model F-value of 500.80

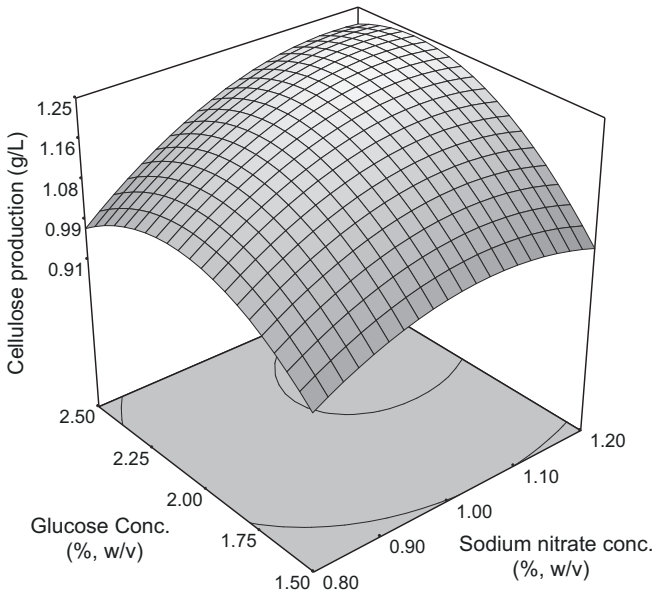

Fig. 2. Effect of glucose concentration and sodium nitrate on cellulose production.

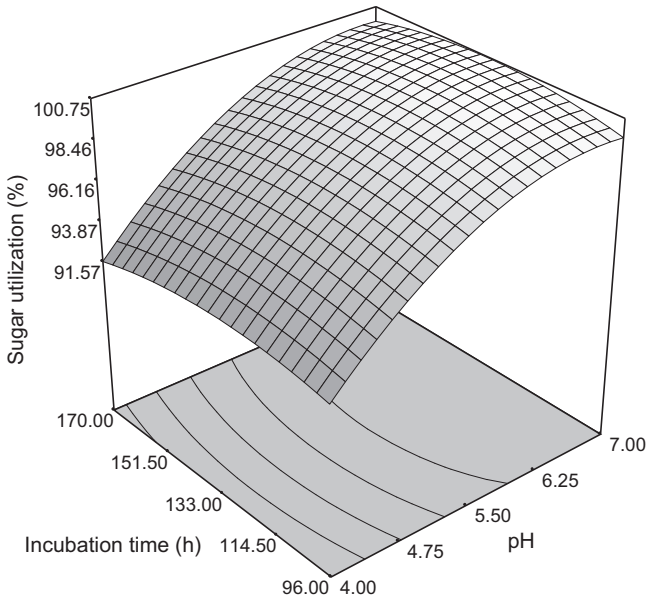

Fig. 3. Effect of incubation time and pH on sugar utilization.

implies that model is significant. The value of adjusted determination coefficient (adjusted R^2 = 99.71%) was high to advocate a high signfinace of the model. This suggested that the model accurately represents the data in the experimental region. The magnitude of *P*-value from Table 3 indicates that all linear terms had significant effect on sugar utilization.

The equation of the model fitted for sugar utilization in the actual form of process variables after eliminating the non significant terms is

Sugar utilization (%) = −82.94 + 13.68 * pH + 7.98 * Temperature + 0.18 * Time + 2.26 * Glucose + 22.02 * Sodium nitrate − 1.03* pH^2 − 0.1353 * $Temperature^2$ - 6.51 * $Time^2$ − 1.44 * $Glucose^2$ − 9.48 * Sodium $nitrate^2$ − 0.066 * pH × Temperature + 3.73 * pH × Time + 0.39 * pH × Glucose + 0.87 * pH × Sodium nitrate − 0.25 * Temperature × Sodium nitrate − 0.021 * Time × Sodium nitrate + 1.26 * Glucose × Sodium nitrate

{Degree of freedom = 20; *F*-value = 500.797; *P*-value = <0.0001; R^2 = 0.9971}

From Table 3 it was revealed that linear terms of pH had maximum positive effect on sugar utilization (β = 3.95), followed by glucose (β = 0.51) and sodium nitrate (β = 0.11). In interactive terms including pH and glucose, pH and sodium nitrate, pH and incubation time, temperature and glucose and glucose and sodium nitrate all had positive β values with significant effect on sugar utilization.

It has been observed (Fig. 3) that the increase in incubation time and pH, resulted in increase of sugar utilization. It may be due to the reason that pH influences the physiology of micro-organism by affecting the nutrient solubility, uptake of nutrient, change in enzyme activity and oxidative reductive reaction so optimum pH range required for sugar utilization. The quadratic and interaction terms of all the process parameters, have negligible effects on sugar utilization, compared to the linear terms of process variables. It can

Table 3
Regression summary and ANOVA table for sugar utilization for uncoded values of process variables.

Source	β-Coefficient	Sum of squares	DF	Mean square	*F*-Value	Prob > *F*
Model	99.071	1219.874	20	060.993	500.797	<0.0001
pH	3.958	678.789	1	678.789	5573.299	<0.0001
Temperature	−0.387	006.490	1	006.490	053.290	<0.0001
Incubation time	−0.119	000.615	1	000.615	005.049	0.0324
Glucose	0.511	011.337	1	011.337	093.085	<0.0001
Sodium nitrate	0.112	000.546	1	000.546	004.487	0.0428
pH * pH	−2.323	300.101	1	300.101	2464.022	<0.0001
Temp * temp	−2.164	260.416	1	260.416	2138.187	<0.0001
Time * time	−0.892	044.215	1	044.215	363.036	<0.0001
Glucose * glucose	−0.361	007.269	1	007.269	059.684	<0.0001
Sodium nitrate * sodium nitrate	−0.379	007.997	1	007.997	065.661	<0.0001
pH * temp	−0.399	005.096	1	005.096	041.841	<0.0001
pH * time	0.207	001.373	1	001.373	011.278	0.0022
pH * glucose	0.299	002.874	1	002.874	023.597	<0.0001
pH * sodium nitrate	0.263	002.220	1	002.220	018.234	0.0002
Temp * time	−0.018	000.010	1	000.010	000.089	0.7672*
Temp * glucose	0.125	000.502	1	000.502	004.125	0.0515
Temp * sodium nitrate	−0.200	1.292	1	001.292	010.608	0.0029
Time * glucose	−0.093	0.279	1	000.279	002.293	0.1407*
Time * sodium nitrate	−0.157	0.790	1	000.790	006.491	0.0164
Glucose * sodium nitrate	0.126	0.512	1	000.512	004.208	0.0493
Residual	3.53		29	0.12		
Lack of fit	3.53		22	0.16		
R^2	0.9971					

* Non-significant at 5% level of significance.

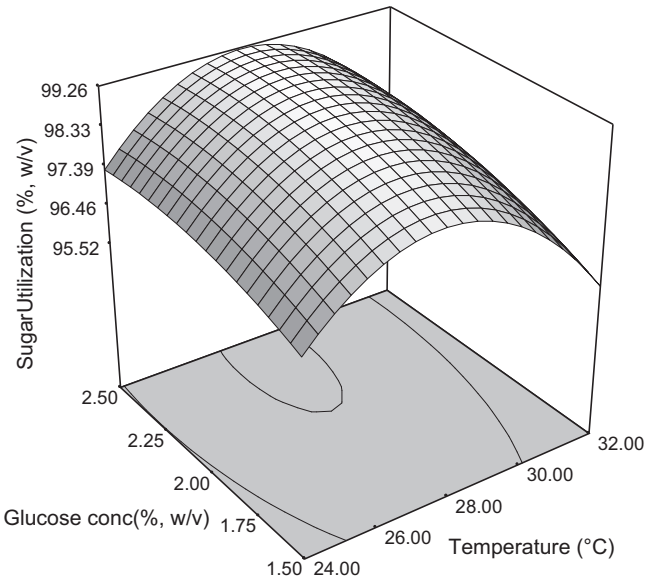

Fig. 4. Effect of glucose concentration and temperature on sugar utilization.

be observed from Fig. 4 that the increase in temperature resulted in increase in sugar utilization within the range 26–30 °C and after this range it slightly decreased.

3.3. Optimization process for cellulose production

Graphical multi-response optimization technique was adopted to determine the workable optimum conditions for the production of cellulose. Numerical optimization was done for the determination of optimum conditions of glucose 2.25% (w/v), sodium nitrate 1.15% (w/v), pH 7, temperature 27.47 °C and incubation time 158.8 h. The values of process variables, the predicted value of cellulose production and sugar utilization was found to be 1.74 g/L and 99.8%, respectively. The results of optimization were confirmed by conducting the experiment in triplicate at the above optimzed values with ±0.2% deviation in activity values. The similar cellulose production were found with *G. hansenii*, when glucose was provided as carbon source and obtained pH range was suitable for cellulose production (Kongruang, 2008; Park, Jung, & Park, 2003).

3.4. Elucidation of structure of microbial cellulose

The structure was assigned and established on the basis of following band in IR spectrum. The IR spectrum showed several strong bands due to OH stretching in the region of 3853–3256 cm^{-1} (presence of hydroxyl functional group). Several bands typical for cellulose were shown in the region of 1500–1235 cm^{-1} due to in plane bending vibrations of CH_2, CH, OH groups. The presence of two strong bands at 1235 and 1081 cm^{-1}, due to C–O and C–O–C asymmetric stretching and symmetric stretching, respectively, typical of cellulose, further proved the structure.

4. Conclusions

In this study, Response surface methodology was successfully employed to investigate the main and the interaction effects of the process variables that are important for the production of microbial cellulose. The pH, incubation time and temperature were the most important factors affecting the performance of the process. The optimum operating conditions obtained from RSM to achieve maximum cellulose production and sugar utilization were glucose concentration 2.25% (w/v), sodium nitrate concentration 1.15% (w/v), pH 6.9 ≈ 7, temperature 27.47 °C and incubation time 159 h.

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