Response surface optimization for cellulose production from agro industrial waste by using new bacterial isolate *Gluconacetobacter xylinus* C18

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Abstract In the present investigation, a low-cost medium prepared from molasses and corn steep liquor was used for the bacterial cellulose production by using an isolated bacterial strain. This bacterium, identified as *Gluconacetobacter xylinus* C18, was isolated from Indian fruit waste (rotten grapes). The process of cellulose production from the isolated bacterial strain was optimized using response surface methodology based on the central composite rotatable design. The optimum parameters for maximum bacterial cellulose production (4.34 g/L) obtained were sugarcane molasses concentration 10.77% (w/v) supplemented with 12.47% (v/v) corn steep liquor concentration at 31 °C, pH 6.5, and incubation time of 172 h. The structure of cellulose was characterized and confirmed by using SEM and FT-IR spectroscopy.

Keywords Bacterial cellulose · Scanning electron microscopy · Fourier transform infrared spectroscopy · Response surface methodology

Introduction

Cellulose is one of the most popular biopolymer in view of its biodegradable and environmental friendly nature. Some of the common genera of bacteria that produce the bacterial cellulose are *Acetobacter aceti*, *A. xylinum*, *A. pasteurianus*, *Rizobium*, *Sarcina*, and *Agrobacterium* [1–3]. Although these are well known to produce cellulose, some other genus such as *Komagataeibacter hensini*, *K. xylinum*, and *K. swingsii* also produce the cellulose [4, 5]. *Gluconacetobacter xylinus* is a gram-negative, aerobic, and rod-shaped bacterium, which efficiently synthesizes the cellulose using agro-industrial byproducts. The bacteria can consume multiple sugars at a time and synthesize additional bacterial cellulose using agro-industrial byproducts due to the presence of reducing and non-reducing sugars [6]. *Gluconacetobacter* sp. is capable of synthesizing higher amounts of cellulose in the form of extracellular ribbon pellicles. However, some genera such as *Rhizobium* and *Agrobacterium* produce cellulose in the form of short fibrils. Bacterial cellulose is free from hemicelluloses and lignin and found in pure form. Moreover, it has several unique properties which differ from plant cellulose in terms of high crystallinity, high mechanical strength, high water-holding capacity, and ultra-fine pure nanofibril network. Due to these properties, bacterial cellulose have a wide range of applications in the medical field, for example, as a wound dressing material, an antimicrobial agent, an artificial blood vessel, and a drug delivery material [7, 8].

Many researchers have shown interest in minimizing the cost of cellulose production by replacing the costly synthetic media with cheap agro industrial wastes generated from various food industries. The different wastes used as a substrate for bacterial cellulose production include pineapple waste,

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watermelon waste, mango waste, coffee cherry husk, sugar cane molasses, and corn steep liquor [9, 10]. Amongst all, sugarcane molasses and corn steep liquor are reported to give maximum yield; they are, therefore, used as potential carbon and nitrogen sources. To develop efficient fermentation, there is continual search for a new bacterial isolate that can efficiently ferment the agro-industrial byproducts.

Conventional practice of single-factor optimization by maintaining other factors at an unspecified constant level cannot provide a desirable explanation on the effect of all the variables in the process. The optimization of process parameters using the conventional method of changing one independent variable while keeping others at a fixed level is time-consuming and expensive for a large number of process variables [11]. Therefore, there is a need for a mathematical and statistical method to study the effect and interactions among the variables during the fermentation process. A response surface methodology (RSM) is such a technique that allows the study of interactions among the variables simultaneously during the fermentation process.

Keeping the above concerns in view, in the present investigation, the RSM is used to optimize process parameters involved in the cellulose production using agro-industrial wastes from *G. xylinus* C18 strain.

Materials and methods

Chemicals

All reagents and chemicals used for experimental investigations were of analytical grade and procured from Qualigens, Fine Chemicals, Mumbai (India), Ranbaxy Labs Ltd, Chandigarh (India), Shanti Das (S.D.) Fine Chemicals Ltd, Mumbai (India) Sigma-Aldrich, (USA), Central Drug House (CDH) Chemicals, New Delhi (India) and High Media Chemicals, Mumbai (India). Sugarcane molasses was collected from Bhagwanpura Sugar Mill, Dhuri (India) and corn steep liquor was procured from Sukhjot Starch and Chemicals Ltd, Phagwara (India).

Inoculum preparation

The isolated bacterial culture, identified as *G. xylinus* C18, grown in a medium containing yeast extract (5 g/L), peptone (5 g/L), and mannitol (25 g/L) at 30 °C for 24 h was used as the inoculum for the fermentation process.

Bacterial cellulose production

The basal media (Hestrin–Schramm) used for the production of bacterial cellulose comprised (% w/v) glucose 2.0, peptone 0.5, yeast extract 0.5, disodium phosphate 0.27,

and citric acid 0.115 [12]. The compositions of the media used in this study were modified with sugar cane molasses and corn steep liquor. A pH of 6.5 was maintained by using 1.0 N hydrochloric acid. The sugar cane molasses was treated with 2N H₂SO₄ to hydrolyze the sugar content [13].

The (2%) inoculum of *G. xylinus* C18 was added to a 250 mL Erlenmeyer flask containing 100 mL of fermentation medium; this was then incubated at 30 °C for 168 h under static conditions.

Isolation and purification of bacterial cellulose

The cellulose pellicle mat grown on the air–liquid interface was harvested after 168 h and washed with distilled water to remove media from pellicle and then centrifuged at 5000 rpm to remove excess water. The isolated pellicle mat was boiled with 1 N NaOH for 20 min to remove the bacterial cells and other impurities associated with the cellulose matrix. The harvested cellulose was washed continuously with de-ionized water until neutral pH [14]. The wet weight was measured and then dried at 60 °C until a constant weight was obtained [15, 16].

Estimation of sugars

For the determination of total and reducing sugars, the standard methods were followed [17].

Fourier transform infrared (FT-IR) spectroscopy

The surface properties of the cellulose were examined by FT-IR spectroscopy (Bruker Tensor 27) in a transmittance mode in the range 4000–400 cm^{−1} mode.

Scanning electron microscopy (SEM)

The structural properties of bacterial cellulose produced by the isolate *G. xylinus* C18 were examined by SEM (JEOL NEOSCOPE JCB6000). The freeze dried bacterial cellulose were coated with gold and viewed under 10 kv and 5000× magnification.

Experimental design for process optimization

The experiments were conducted according to central composite rotatable design (CCRD) [18] containing five independent variables—temperature, pH, incubation time, molasses concentration, and corn steep liquor concentration—at five different levels each. The design was generated using Stat-Ease software (Stat-Ease, version 6.01 2001, Stat-Ease Inc., 2021 East Hennepin Avenue, MN 55413) trial version.

On the basis of preliminary trials, the low and high levels of process variables used for the optimization of

bacterial cellulose production from *G. xylinus* C18 isolates were temperature 25 and 45 °C, pH 4.5 and 8.5, incubation time 48 and 240 h, molasses concentration 2 and 18%, and corn steep liquor concentration 4 and 16%. Table 1 shows the CCRD with five variables, pH, temperature, incubation time, and molasses and corn steep liquor concentration, at five levels each along with the observed data for the trial cellulose production and sugar utilization.

Total number of experiments = $2^{\text{No. of variables}} + 2 \times \text{No. of variables} + \text{Central points}$

For five variables, total number of experiments = $2^5 + 2 \times 5 + 8 = 50$

The five different levels for each experiment are coded as $-\alpha, -1, 0, +1, +\alpha$, where $\alpha = (2)^{\text{No. of variables}/4} = (2)^{5/4} = 2.37841$

$$\text{Coded value, } x_i = \frac{2(X_i - \bar{X}_i)}{R_i}$$

Here X_i is the actual setting in the un-coded units of the i th factor, $\bar{X}_i$ is the average of the low and high settings for the i th factor, and R_i is the range between the low and high settings.

Analysis of data

The second-order polynomial equation was fitted to the experimental data of each dependent variable as shown below to analyze the experimental data for each independent variable [19].

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

where Y_i = response $\{Y_1$ = cellulose (dry weight, g/L), Y_2 = sugar utilization (%); x_i = independent variables; x_1 = pH; x_2 = temperature (°C); x_3 = incubation time (h); x_4 = molasses concentration (% w/v); x_5 = corn steep liquor concentration (% v/v); β_0 is the value of fitted response at the central point of design, i.e., (0,0,0); β_i , β_{ij} , and β_{ii} are the linear, interactive, and quadratic terms, respectively.

Results and discussion

Diagnostic checking of fitted model and surface plot for cellulose production

The results of second-order response surface model obtained using analysis of variance (ANOVA) for cellulose production are given in Table 2. The high F-value of model 111.052 implies that the model is significant. The linear and quadratic effects of pH, incubation time, molasses

concentration, and corn steep liquor concentration are found to be significant at 5% level of significance ($p < 0.05$) for cellulose production. The interactive effects of x_1x_4 , x_2x_5 , x_3x_4 , and x_4x_5 have a significant effect on cellulose production at 5% level of significance ($p < 0.05$). The value of the adjusted determination coefficient (adjusted $R^2 = 97.82\%$) was high to advocate a high significance of the model.

The relative magnitude of β coefficients revealed that the linear term of corn steep liquor concentration had maximum positive and significant effect ($\beta = 0.396$), followed by incubation time ($\beta = 0.237$), and molasses concentration ($\beta = 0.125$); these indicate that with the increase of these parameters, the cellulose production increased. In contrast, temperature ($\beta = -0.729$) and pH ($\beta = -0.053$) had a negative effect on bacterial cellulose production. In the terms of the interactive effects of the parameters on bacterial cellulose production, the interaction between incubation time and molasses concentration ($\beta = 0.1$) had the maximum positive and significant effect followed by the interaction between pH and corn steep liquor ($\beta = 0.064$) and that between incubation time and corn steep liquor ($\beta = 0.031$). The interaction between molasses and corn steep liquor concentration ($\beta = -0.335$) and temperature and molasses concentration ($\beta = -0.244$) had a negative effect on bacterial cellulose production at 5% level of significance ($p < 0.05$), as indicated in Table 3. The interactive effects of these parameters have also been shown in the Fig. 1(A–D).

Figure 1(A) shows that at low molasses concentration, the cellulose production remained constant with the increase in temperature from 29.5 to 31 °C, but with further increase in temperature, a gradual decrease in cellulose production was observed, because *G. xylinus* bacteria rapidly grow at an optimum temperature of 28–30 °C, synthesizing the desired products [20]. El-Saied et al. [13] reported an increase in cellulose production with the increase in the molasses concentration from 2 to 16% (w/v); thereafter, with further increase, the productivity declined. At higher molasses concentration (13%), the bacterial cellulose production decreased rapidly compared to that at lower molasses concentration with increased temperature above 31 °C. This is possibly due to the fact that the excess molasses leads to accumulation of gluconic acid or lactic acid as intermediate products in the medium during the cellulose synthesis [21, 22].

Figure 1(B) shows that with the increase in the corn steep liquor concentration, the bacterial cellulose production gradually increased. This is possibly due to the composition of corn steep liquor, which is rich in natural protein and small amounts of mineral and vitamins, providing nutrients for bacterial cell metabolism [23]. With the increase in pH from 5.5 to 6.5, the bacterial cellulose

Table 1 Experimental design variables and values of experimental data in coded and uncoded forms for the optimization of bacterial cellulose production from *G. xylinus* C18

Temperature °C X_1	pH X_2	Incubation time (h) X_3	Molasses conc. (%w/v) X_4	Corn steep Liquor conc. (%w/v) X_5	Cellulose production (g/L)(y_1)	Sugar utilization (%w/v)(y_2)
(+1)35.6	(−1)5.6	(−1)103.6	(+1)13.3	(−1)7.4	1.30	83.0
(−1)29.3	(+1)7.3	(−1)103.6	(+1)13.3	(−1)7.4	3.00	95.6
(+1)35.6	(+1)7.3	(−1)103.6	(+1)13.3	(−1)7.4	1.12	83.0
(−1)29.3	(−1)5.6	(+1)184.3	(+1)13.3	(−1)7.4	3.85	99.3
(+1)35.6	(−1)5.6	(+1)184.3	(+1)13.3	(−1)7.4	1.85	80.0
(−1)29.3	(+1)7.3	(+1)184.3	(+1)13.3	(−1)7.4	3.60	99.3
(+1)35.6	(+1)7.3	(+1)184.3	(+1)13.3	(−1)7.4	1.58	80.0
(−1)29.3	(−1)5.6	(−1)103.6	(−1)6.60	(+1)12.5	3.31	95.0
(+1)35.6	(−1)5.6	(−1)103.6	(−1)6.60	(+1)12.5	2.22	85.0
(−1)29.3	(+1)7.3	(−1)103.6	(−1)6.60	(+1)12.5	3.60	96.0
(+1)35.6	(+1)7.3	(−1)103.6	(−1)6.60	(+1)12.5	2.50	87.0
(−1)29.3	(−1)5.6	(+1)184.3	(−1)6.60	(+1)12.5	3.70	99.4
(+1)35.6	(−1)5.6	(+1)184.3	(−1)6.60	(+1)12.5	2.53	87.0
(−1)29.3	(+1)7.3	(+1)184.3	(−1)6.60	(+1)12.5	3.86	99.31
(+1)35.6	(+1)7.3	(+1)184.3	(−1)6.60	(+1)12.5	2.70	95.0
(−1)29.3	(−1)5.6	(−1)103.6	(+1)13.3	(+1)12.5	3.25	95.1
(+1)35.6	(−1)5.6	(−1)103.6	(+1)13.3	(+1)12.5	1.25	83.0
(−1)29.3	(+1)7.3	(−1)103.6	(+1)13.3	(+1)12.5	3.25	92.5
(+1)35.6	(+1)7.3	(−1)103.6	(+1)13.3	(+1)12.5	1.25	83.0
(−1)29.3	(−1)5.6	(+1)184.3	(+1)13.3	(+1)12.5	3.92	99.31
(+1)35.6	(−1)5.6	(+1)184.3	(+1)13.3	(+1)12.5	1.86	87.0
(−1)29.3	(+1)7.3	(+1)184.3	(+1)13.3	(+1)12.5	3.86	99.31
(+1)35.6	(+1)7.3	(+1)184.3	(+1)13.3	(+1)12.5	1.81	87.21
(−2.378)25.0	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.82	99.21
(+2.378)40.0	(0)6.5	(0)144.0	(0)10.0	(0)1.00	0.62	74.0
(0)32.5	(−2.378)4.5	(0)144.0	(0)10.0	(0)10.0	2.02	93.0
(0)32.5	(+2.378)8.5	(0)144.0	(0)10.0	(0)10.0	1.25	99.21
(0)32.5	(0)6.5	(−2.378) 48.0	(0)10.0	(0)10.0	1.45	82.0
(0)32.5	(0)6.5	(+2.378)240.0	(0)10.0	(0)10.0	3.16	91.1
(0)32.5	(0)6.5	(0)144.0	(−2.378) 2.0	(0)10.0	1.50	89.0
(0)32.5	(0)6.5	(0)144.0	(+2.378)18.0	(0)10.0	2.60	87.0
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(−2.378) 4.0	1.95	90.0
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(+2.378)16.0	3.86	97.0
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.21
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.21
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.21
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.21
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.21
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.21
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.2
(0)32.5	(0)6.5	(0)144.0	(0)10.0	(0)10.0	3.85	99.2
(+1)35.7	(−1)5.7	(−1)103.6	(+1)13.4	(−1)7.4	1.30	83.0
(−1)29.3	(+1)7.3	(−1)103.6	(+1)13.4	(−1)7.4	3.00	95.6
(+1)35.7	(+1)7.3	(−1)103.6	(+1)13.4	(−1)7.5	1.12	83.0
(−1)29.3	(−1)5.7	(+1)184.4	(+1)13.4	(−1)7.5	3.85	99.3
(+1)35.7	(−1)5.7	(+1)184.4	(+1)13.4	(−1)7.5	1.85	80.0
(−1)29.3	(−1)7.3	(+1)184.4	(+1)13.4	(−1)7.5	3.60	99.3

Table 1 continued

Temperature °C X_1	pH X_2	Incubation time (h) X_3	Molasses conc. (%w/v) X_4	Corn steep Liquor conc. (%w/v) X_5	Cellulose production (g/L)(y_1)	Sugar utilization (%w/v)(y_2)
(+1)35.7	(−1)7.3	(+1)184.4	(+1)13.4	(−1)7.5	1.58	80.0
(−1)29.3	(−1)5.7	(−1)103.6	(−1)6.6	(−1)12.5	3.31	95.0
(+1)35.7	(−1)5.7	(−1)103.6	(−1)6.6	(−1)12.5	2.22	85.0

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

Source	B coefficient	Sum of squares	df	Mean square	F-value	p value prob > F
Model		56.0746	20	2.80373	111.0516	<0.0001
Intercept	3.849667					
x_1	−0.72981	23.07008	1	23.07008	913.7719	<0.0001
x_2	−0.05336	0.123344	1	0.123344	4.885463	0.0351
x_3	0.237502	2.443203	1	2.443203	96.77169	<0.0001
x_4	0.125047	0.677287	1	0.677287	26.82636	<0.0001
x_5	0.396705	6.816494	1	6.816494	269.9913	<0.0001
x_1x_2	−0.01438	0.006613	1	0.006613	0.261911	0.6127*
x_1x_3	−0.0325	0.0338	1	0.0338	1.338768	0.2567*
x_1x_4	−0.24438	1.911013	1	1.911013	75.6924	<0.0001
x_1x_5	−0.03938	0.049613	1	0.049613	1.965078	0.1716*
x_2x_3	−0.0075	0.0018	1	0.0018	0.071295	0.7913*
x_2x_4	−0.04813	0.074113	1	0.074113	2.935487	0.0973*
x_2x_5	0.064375	0.132613	1	0.132613	5.252587	0.0294
x_3x_4	0.1	0.32	1	0.32	12.67473	0.0013
x_3x_5	0.03125	0.03125	1	0.03125	1.237767	0.2750*
x_4x_5	−0.33563	3.604613	1	3.604613	142.7734	<0.0001
x_1^2	−0.28845	4.623377	1	4.623377	183.1252	<0.0001
x_2^2	−0.39186	8.532836	1	8.532836	337.9731	<0.0001
x_3^2	−0.27342	4.154231	1	4.154231	164.543	<0.0001
x_4^2	−0.3185	5.636947	1	5.636947	223.2712	<0.0001
x_5^2	−0.16735	1.556328	1	1.556328	61.64386	<0.0001
Residual		0.732165	29	0.025247		

* Non-significant at a 5% level of significance

production increased, but further increase in pH, a decrease in the bacterial cellulose production was observed. This might be due to the fact that an optimum pH range is required for oxidative reductive reaction and that pH influences the physiology of bacteria by affecting the nutrient solubility uptake [15]. Hutchens et al. [24] studied cellulose production using *Gluconacetobacter hensanii* ATCC 10821 in a glucose medium and indicated the maximum cellulose production at pH 6.5. The use of corn steep liquor also helps to maintain the pH of the medium because of its buffering capacity, as reported by Noor et al. [25]

An increase in the cellulose production was observed with the increase in the incubation time up to 184 h, followed by a decrease in the productivity beyond this time 184 h (Fig. 1C); this may be due to non-or less availability

of nutrients [26]. Similarly, when the molasses concentration was increased up to 10%, the maximum cellulose production (3.4 g/L) was observed; with further increase, a decreasing trend was observed. This may be attributed to the fact that molasses have some quantity of heavy metals and minerals that might have harmful effects on bacterial growth and product synthesis [13, 27]. Therefore, the amount of extra molasses in the fermentation medium affects the bacterial growth and product synthesis. The effect of molasses and glucose on bacterial cellulose production by *A. xylinum* had been investigated by Keshak and Shameshima, in which the maximum cellulose was obtained at pH 3.8 by using molasses as the carbon source [28]. On other hand, Frag et al. [29] reported maximum bacterial cellulose by using treated molasses and *Achromobacter* sp after 120 h.

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

Source	Response	2	Sugar utilization				
ANOVA for response surface quadratic model							
Analysis of variance table (partial sum of squares—type III)							
	β coefficient	Sum of squares	df	Mean square	F-value	p value prob > F	
Model		2444.321	20	122.216	53.73212	<0.0001	Significant
Intercept	99.22322						
x_1	−5.95331	1535.119	1	1535.119	674.9129	<0.0001	
x_2	0.50492	11.04257	1	11.04257	4.854853	0.0357	
x_3	1.310522	74.38991	1	74.38991	32.70542	<0.0001	
x_4	−0.82784	29.68372	1	29.68372	13.05041	0.0011	
x_5	0.798105	27.58962	1	27.58962	12.12974	0.0016	
x_1x_2	0.54	9.3312	1	9.3312	4.102449	0.0521	
x_1x_3	−0.71063	16.15961	1	16.15961	7.104552	0.0124	
x_1x_4	−0.89125	25.41845	1	25.41845	11.17519	0.0023	
x_1x_5	1.076875	37.10911	1	37.10911	16.31497	0.0004	
x_2x_3	0.410625	5.395613	1	5.395613	2.372174	0.1344	
x_2x_4	−0.58375	10.90445	1	10.90445	4.794127	0.0368	
x_2x_5	0.310625	3.087613	1	3.087613	1.357465	0.2535	
x_3x_4	−0.02063	0.013612	1	0.013612	0.005985	0.9389	
x_3x_5	1.21125	46.94805	1	46.94805	20.64065	<0.0001	
x_4x_5	−0.10813	0.374113	1	0.374113	0.164478	0.6880	
x_1^2	−2.21369	272.3107	1	272.3107	119.721	<0.0001	
x_2^2	−0.53431	15.86416	1	15.86416	6.974657	0.0132	
x_3^2	−2.22341	274.7079	1	274.7079	120.775	<0.0001	
x_4^2	−1.96708	215.0195	1	215.0195	94.53303	<0.0001	
x_5^2	−0.99481	54.99388	1	54.99388	24.17798	<0.0001	
Residual		65.96176	29	2.274544			
Lack of Fit		65.96161	22	2.998255	139,918.6	<0.0001	Significant
Pure error		0.00015	7	2.14E − 05			
Cor total		2510.283	49				

* Non-significant at a 5% level of significance

The interactive effect of corn steep liquor and molasses concentration observed from Fig. 1(D) indicated an increased cellulose production with increase in the corn steep liquor concentration up to 12.5%, followed by a decreasing trend with further increase in the corn steep liquor concentration. Jung et al. [30] reported that with increasing molasses and corn steep liquor concentration, the cellulose production also increased, and the maximum bacterial cellulose (3.12 g/L) was obtained after 8 days of incubation. Again, when the molasses concentration was increased up to 10%, there was an increase in the cellulose production, after which with further increase, the productivity of cellulose declined (Fig. 1D).

After eliminating the non-significant terms from the model, the actual form of process variable was fitted in the equation for bacterial cellulose production.

$$\begin{aligned} \text{Cellulose production (g/L)} = & -67.216 + 2.006 \times X_1 + 7.215 \times X_2 + 0.0535 \times X_3 + 1.749 \times X_4 + 0.998 \times X_5 \\ & - 0.029 \times X_1^2 - 0.554 \times X_2^2 - 1.678E - 04 \times X_3^2 - 0.028 \times X_4^2 - 0.026 \times X_5^2 - 0.0230 \times X_1X_4 + 0.030 \times X_2X_5 \\ & + 7.365E - 04 \times X_3X_4 - 0.0395 \times X_4X_5 \\ \{ \text{Degree of freedom} = 20; \text{F-value} = 111.05; p \text{ value} \leq 0.0001; R^2 = 0.9782 \} \end{aligned}$$

Diagnostic checking of fitted model and surface plot for sugar utilization

The results of the second-order response model obtained by ANOVA for sugar utilization are given in Table 3. The high F-value of model 53.73 depicts that the model is significant. The effect of linear and quadratic terms of

Fig. 1 (A) Effect of temperature and molasses concentration on cellulose production. (B) Effect of pH and corn steep liquor concentration on cellulose production. (C) Effect of incubation time and molasses concentration on cellulose production. (D) Effect of molasses concentration and corn steep liquor concentration on cellulose production. (E) Effect of incubation time and corn steep liquor concentration on sugar utilization

temperature, pH, incubation time, molasses concentration, and corn steep liquor concentration are significant at a 5% level of significance ($p < 0.05$). The p value indicated that the linear and quadratic terms of all variables have a significant effect on sugar utilization. The interactive terms of x_1x_3 , x_1x_4 , x_1x_5 , x_2x_4 , and x_3x_5 have a significant effect on cellulose production ($p < 0.0001$). The value of adjusted $R^2 = 0.9556\%$ was high enough to promote a high significance of the model.

Incubation time with the positive β value ($\beta = 1.31$) has the maximum positive effect on sugar utilization, followed by corn steep liquor concentration ($\beta = 0.798$) and pH ($\beta = 0.504$). With the increase in incubation time, an

increased sugar utilization will be observed. The relative magnitude of negative β values ($\beta = -5.953$) for temperature indicates that the temperature has more pronounced effect on sugar utilization as compared to molasses concentration ($\beta = -0.827$). The negative sign indicates that with increase in temperature and molasses concentration, reduction in sugar utilization will be observed. In terms of the effect on sugar utilization, the interaction between incubation time and corn steep liquor ($\beta = 1.211$) had the maximum positive effect, followed by between temperature and corn steep liquor ($\beta = 1.076$), temperature and pH ($\beta = 0.54$), pH and molasses concentration ($\beta = 0.410$), and pH and corn steep liquor

Fig. 2 FT-IR spectrum of bacterial cellulose

($\beta = 0.310$). The interaction between temperature and molasses concentration ($\beta = -0.891$), temperature and incubation time ($\beta = -0.710$), pH and molasses concentration' ($\beta = -0.583$) had a negative effect on sugar utilization at a 5% level of significance ($p < 0.05$). This indicates that with the increase in these interactive parameters, the overall sugar utilization decreases.

The effects of incubation time and corn steep liquor concentration are presented in Fig. 1(E), which indicate that with the increase in incubation time and corn steep liquor concentration, the sugar utilization for cellulose production increased, and then some small decline was observed. This may be due to the fact that the corn steep liquor is rich in complex nitrogen protein and some vitamins [31]. Incubation time also affected the sugar utilization. As the incubation time increased up to 164 h, the sugar utilization also increased, because of the exhaustion of the sugar present in the medium, which might have been consumed by the bacteria for growth as well as cellulose production. A similar result has been reported by El-Saied et al. [13].

The equation of sugar utilization in terms of the actual form of independent variables after deleting the non-significant terms is as follows:

$$\text{Sugar utilization (\%)} = -120.447 + 11.549 \times X_1 + 2.662 \times X_2 + 0.411 \times X_3 + 7.453 \times X_4 - 3.494 \times X_5 - 0.223 \times X_1^2 - 0.756 \times X_2^2 - 1.365E-03 \times X_3^2 - 0.174 \times X_4^2 - 0.156 \times X_5^2 - 5.583E-03 \times X_1X_3 - 0.084 \times X_1X_4 - 0.135 \times X_1X_5 - 0.206 \times X_2X_4 + 0.012 \times X_3X_5$$

{Degree of freedom = 20; F-value = 53.73; p value ≤ 0.0001 ; $R^2 = 0.9737$ }.

Optimization of process parameters for bacterial cellulose production

The numerical optimization technique was used for the optimization of process parameters for bacterial cellulose production. The optimization was aimed to obtain the maximum bacterial cellulose production from agro industrial waste as carbon and nitrogen sources. The optimum values of variables were calculated by solving the regression equation. The optimized workable conditions for bacterial cellulose production were temperature 31 °C, pH 6.5, incubation time 172 h, sugarcane molasses concentration 10.77%, and corn steep liquor concentration 12.47%. The predicted values of bacterial cellulose production and sugar utilization were 4.43 g/L and 99.4%, whereas the actual values obtained were 4.24 g/L and 99%, respectively. Approximately less than 5% deviation was observed. The experiments were conducted to ensure the results of predicted values in triplicate at the above optimized values.

Morphology and characterization of bacterial cellulose

The FT-IR spectra of bacterial cellulose showing the characteristic absorption bands are given in Fig. 2. The IR spectra showed strong bands at 3347 cm^{-1} due to OH stretching (hydroxyl functional group) and in the range

Fig. 3 Scanning electron micrograph of bacterial cellulose

1431–1395 cm^{-1} due to C=O (carbonyl group) [3, 32]. A strong band in the range 2924 cm^{-1} due to C–H stretching was also observed. Several typical bands for bacterial cellulose were observed in the region 1730–1220 cm^{-1} due to the plane bending vibration of CH_2 , CH, and OH groups. Bands were observed in the region 1220 and 1059–1096 cm^{-1} due to C–O and C–O–C asymmetric stretching and symmetric stretching [33].

The morphological characterization was performed using SEM. The microfibrillar nano-sized structure was observed. The SEM image also confirmed that bacterial cellulose microfibrils were round shaped, as shown in Fig. 3 [3, 34].

Results conclude that bacterial isolate *G. xylinus* C18 has been successfully used for bacterial cellulose production. RSM studies indicated that all the process variables (temperature, pH, incubation time, molasses concentration, and corn steep liquor concentration) strongly influence bacterial cellulose production. Optimization process variables obtained using the RSM are helpful to determine the optimum values of process parameters in order to maximize the bacterial cellulose production by the new isolated *G. xylinus* C18 bacterium. In addition, this method requires a minimum number of experiments as compared to optimizing one factor at one time, thereby saving time and materials. The maximum bacterial cellulose of 4.43 g/L and sugar utilization of 99% was obtained with 10.7% (w/v) molasses and 12.4% (v/v) corn steep liquor at 31 °C, pH 6.5, an incubation time of 172 h, whereas a cellulose production of 4.6 g/L has been reported with higher molasses (17%, w/v) and corn steep liquor (15%, v/v) concentration in previous studies [13].

Agro industrial byproducts such as sugarcane molasses and corn steep liquor are easily available in large amount and can be used as cheap carbon and nitrogen sources for bacterial cellulose production by *G. xylinus* C18 bacterium

and can minimize the cost of production. In addition, the utilization of these byproducts promotes reduction in environmental pollution to some extent. Thus, the outcome of this investigation provides an alternate cost effecting medium for valuable biomaterial.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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