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Statistical Optimization of Medium Composition for Bacterial Cellulose Production by *Gluconacetobacter hansenii* UAC09 Using Coffee Cherry Husk Extract – an Agro-Industry Waste

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During the production of grape wine, the formation of thick leathery pellicle/bacterial cellulose (BC) at the air–liquid interface was due to the bacterium, which was isolated and identified as *Gluconacetobacter hansenii* UAC09. Cultural conditions for bacterial cellulose production from *G. hansenii* UAC09 were optimized by central composite rotatable experimental design. To economize the BC production, coffee cherry husk (CCH) extract and corn steep liquor (CSL) were used as less expensive sources of carbon and nitrogen, respectively. CCH and CSL are by-products from the coffee processing and starch processing industry, respectively. The interactions between pH (4.5–8.5), CSL (2–10%), alcohol (0.5–2%), acetic acid (0.5–2%), and water dilution rate to CCH ratio (1:1 to 1:5) were studied using response surface methodology. The optimum conditions for maximum BC production were pH (6.64), CSL (10%), alcohol (0.5%), acetic acid (1.13%), and water to CCH ratio (1:1). After 2 weeks of fermentation, the amount of BC produced was 6.24 g/l. This yield was comparable to the predicted value of 6.09 g/l. This is the first report on the optimization of the fermentation medium by RSM using CCH extract as the carbon source for BC production by *G. hansenii* UAC09.

Keywords: *Gluconacetobacter*, bacterial cellulose, RSM, coffee cherry husk, optimization, corn steep liquor

The production of cellulose is a characteristic feature of *Gluconacetobacter* sp. They generally produce cellulose in liquid medium, which forms a floating pellicle. The pellicle comprises cellulose, entrapped cells with other media ingredients. It has a soft texture and high fiber content and is popularly known as "natto" in the Philippines. It is also popular in other Asian countries such as Indonesia, Japan, and Taiwan [12]. Bacterial cellulose (BC) displays many unique properties including high mechanical strength, high crystallinity and an ultrafine pure nanofibril network structure with stability towards chemicals and high temperature. In native state, BC has a higher hydration rate, holding over 100 times its own weight of water. This property is due to the reticulated network of fine fibers, with a diameter of 0.1 μm, which is about one hundredth of plant-derived fibers. It makes the BC far superior than plant celluloses, which are usually associated with hemicellulose and lignin. BC finds its applications in the biomedical field as a temporary substitute for human skin in case of burns, ulcers, scaffold for tissue engineering [18], and other biotechnological fields. It is used as a high-quality speaker diaphragm and as a food bulking agent [6].

The microbial productivity of BC depends on the culture conditions such as carbon and nitrogen sources, pH, and additives such as alcohol and acetic acid. The conventional method of medium optimization, one parameter at a time, is time consuming, expensive, and often leads to misinterpretation of results when there are chances of interactions between different components. Statistical experimental design minimizes the error in determining the effect of parameters and it shows the simultaneous, systematic, and efficient variation of all parameters. Response surface methodology (RSM) is an effective tool for optimizing the process condition that uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equations [1, 19]. It usually involves an experimental design such as central composite rotatable design (CCRD) to fit a second-order polynomial by a least squares technique. An equation is used to describe the test variables, and describe the combined effect of all the test variables in the response [9].

Utilization of less-expensive carbon and nitrogen sources like coffee cherry husk (CCH) and corn steep liquor (CSL)

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can reduce the cost of BC production. CCH is one of the most abundantly available agro-industrial wastes produced after processing of coffee cherry by dry process in many coffee producing areas [27]. The husk contains carbohydrates, proteins, minerals, and high amount of polyphenols [2]. Owing to the presence of unfavorable substances like caffeine, tannins, and other polyphenols in CCH, its use in agriculture has been restricted to a large extent and the disposal of coffee waste presents an enormous pollution problem in the processing units. It was noticed that the area where CCH effluent flows was devoid of any vegetation [23]. In recent years, there has been a renewed trend towards the efficient use of CCH [13]. Application of CCH in the bioprocess on the one hand provides a cheaper alternate substrate and it also helps to solve pollution problems.

The main objectives of the present work were (1) the optimization of BC production from *G. hansenii* UAC09 by RSM and (2) to study the interrelationship among the parameters on the BC yield using mathematical equations and response surface plots.

Materials and Methods

Microorganism

The bacterial culture of *Gluconacetobacter hansenii* UAC09 used in this study was isolated from contaminated grape wine and identified by sequencing the amplified product of 16S rRNA (Accession No. FJ655878).

Culture Media and Cultural Conditions

Hestrin and Schramm (HS) medium (g/l); Glucose (20), peptone (5), yeast extract (5), citric acid (1.15), and disodium hydrogen phosphate (2.7) and the pH of the medium was adjusted to 4.5 [16].

CCH extract; CCH of variety Robusta was collected locally from Kodagu district, India. Cleaned CCH was dried to 12% moisture and ground to 50 mesh size in a plate mill. Powdered CCH was boiled with 1:1 (w/v) distilled water for 30 min. The thick viscous slurry was filtered through muslin cloth to collect the CCH extract. Parameters such as pH, total sugar [15], and total polyphenols [26] of CCH extract were determined.

Sterile HS medium was used for the production of inoculum. One loopful of 24-h-old culture grown on HS slant was inoculated and grown on a incubated rotary shaker (150 rpm) at 27 ± 1^{o}C for 24 h. The resulting suspension was used as inoculum [5% (v/v)] for all the experiments, and inoculated flasks were incubated at 27 ± 1^{o}C in stationary condition for 2 weeks. The experiments were carried out in triplicates (100 ml volume in a 500 ml flask) and the average of results is reported.

Experimental Design

A central composite rotatable design with 5 variables was followed to examine the response pattern and to determine the optimum synergy of variables (Table 1). The variables and their optimized ranges were pH (X_1, 4.5–8.5), CSL (X_2, 2–10%), alcohol (X_3, 0.5–2%), acetic acid (X_4, 0.5–2%), and dilution rate of water to CCH ratio (X_5, 1:1–1:5) for BC production. Each of above variables was investigated at 5 levels (coded); namely, −2, −1, 0, 1, and 2 [3].

The treatment schedule for CCRD shown in Table 1 was arranged to allow for fitting an appropriate regression model using a multiple regression program. CCRD combines vertices of the hypercubes whose co-ordinates are given by a 2n factorial design to provide for the estimation of curvature of the model [5]. Six replicates (treatments 27 to 32, as indicated in Table 2) at the center of the design were used for estimation of a pure error sum of squares. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

Statistical Analysis

A second-order polynomial equation was used to fit the experimental data given in Table 3. The model proposed for the response (Y) was

$$Y=a_o+a_1X_1+a_2X_2+a_3X_3+a_4X_4+a_5X_5+a_{11}X_1^2+a_{22}X_2^2+a_{33}X_3^2+a_{44}X_4^2 +a_{55}X_5^2+a_{12}X_1X_2+a_{13}X_1X_3+a_{14}X_1X_4+a_{15}X_1X_5+a_{23}X_2X_3+a_{24}X_2X_4 +a_{25}X_2X_5+a_{34}X_3X_4+a_{35}X_3X_5+a_{45}X_4X_5+\epsilon \quad (1)$$

where Y is the predicted response for total polysaccharide yield; a_0 is the value of the fitted response at the center point of the design, a_i, a_{ii}, a_{ij} being the linear, quadratic, and cross point terms, respectively; and ϵ is the random error. The coefficients of Eq. (1) were obtained using MATLAB 7.0 software based on the data provided in Table 1 and are presented in Table 2. The *t*-values of the estimates were compared with the tabular value, and the terms having *t*-values lower than the tabular values were omitted [7]. Statistically not significant coefficients were omitted before predicting the response.

The optimization of fitted polynomials for the BC yield was performed by a nonlinear mathematical optimization procedure of the Quattro Pro software package [24]. The optimum conditions were monitored and the results were compared with predicted values.

Purification of Pellicle

Pellicle grown on the liquid surface was harvested once a week under sterile condition, washed thoroughly with water, immersed in

Table 1. Variables and their levels for CCRD.

	Symbols	−2	−1	0	1	2	Mean	SD
pH	X_1	4.50	5.50	6.50	7.50	8.50	6.50	1.000
CSL conc. (%)	X_2	2.00	4.00	6.00	8.00	10.00	6.00	2.000
Alcohol conc. (%)	X_3	0.50	0.88	1.25	1.63	2.00	1.25	0.375
Acetic acid conc. (%)	X_4	0.50	0.88	1.25	1.63	2.00	1.25	0.375
Water husk ratio	X_5	1.00	2.00	3.00	4.00	5.00	3.00	1.000

CSL, Corn steep liquor.
CCRD, Central composite rotatable design.

Table 2. Treatment schedule for five-factor CCRD and response in terms of polysaccharide yield.

Exp. No.	pH	CSL conc. (%)	Alcohol conc. (%)	Acetic acid conc. (%)	Water to husk ratio	Polysaccharide yield (mg/100 ml)	Predicted yield (mg/100 ml)
	X_1	X_2	X_3	X_4	X_5	Y	
1	−1	−1	−1	−1	1	420.00	424.96
2	1	−1	−1	−1	−1	493.00	494.17
3	−1	1	−1	−1	−1	548.00	554.58
4	1	1	−1	−1	1	465.50	472.19
5	−1	−1	1	−1	−1	551.00	548.17
6	1	−1	1	−1	1	428.50	425.78
7	−1	1	1	−1	1	417.00	419.69
8	1	1	1	−1	−1	521.00	519.89
9	−1	−1	−1	1	−1	504.00	505.67
10	1	−1	−1	1	1	425.50	427.28
11	−1	1	−1	1	1	409.50	416.69
12	1	1	−1	1	−1	540.50	543.89
13	−1	−1	1	1	1	414.00	411.78
14	1	−1	1	1	−1	509.50	503.49
15	−1	1	1	1	−1	516.50	515.89
16	1	1	1	1	1	414.00	413.50
17	−2	0	0	0	0	486.33	480.13
18	2	0	0	0	0	479.67	480.83
19	0	−2	0	0	0	470.00	474.61
20	0	2	0	0	0	513.00	503.36
21	0	0	−2	0	0	523.67	509.47
22	0	0	2	0	0	480.00	489.16
23	0	0	0	−2	0	487.00	481.80
24	0	0	0	2	0	451.33	451.50
25	0	0	0	0	−2	561.33	562.72
26	0	0	0	0	2	375.67	369.25
27	0	0	0	0	0	503.00	502.01
28	0	0	0	0	0	500.00	502.01
29	0	0	0	0	0	498.00	502.01
30	0	0	0	0	0	501.00	502.01
31	0	0	0	0	0	501.00	502.01
32	0	0	0	0	0	504.00	502.01

CSL, Corn steep liquor.
CCRD, Central composite rotatable design.

1N NaOH for 1 day at ambient temperature to remove the cells and other impurities embedded in the pellicle, and rinsed thoroughly with water until a neutral pH was attained in the drained water. The pellicle was pressed between two filter papers and dried at 60°C until the constant weight was obtained [14, 25].

FTIR Spectral Analysis

The BC film produced using the optimized conditions was purified and characterized by FTIR spectroscopy (RAMAN Nicolet 5700).

Results and Discussion

Optimization of Variables

Production of BC is a characteristic feature of *Gluconacetobacter* sp. The initial total sugar of the CCH extract at 1:1 dilution was 5.3%, with the total polyphenol content at 0.8% and a pH of 4.68. BC production was observed in the presence of polyphenols, indicating the ability of the isolate to mitigate the toxic effect of polyphenols. *G. hansenii* UAC09 has the ability to grow and produce a high amount of BC from CCH extract, which contains high sugar/pectin and high polyphenols [20, 22]. However, earlier study by other workers indicated that the total polyphenols above 0.6% retard the growth of *Gluconacetobacter* sp. (11). Our present study indicates the efficiency of the native isolate to produce BC in the presence of 0.8% polyphenols.

The estimated coefficients for BC yield are presented in Table 3. The response obtained under different combinations of variables and defined experimental design (Table 1 and 2) were analyzed using the analysis of variance (ANOVA)

Table 3. Estimated coefficients of the fitted second-order polynomial representing the relationship between the response and the process variable.

	Estimated coefficients	Standard error	*t*-value
a_0	502.006	3.370	148.954[a]
a_1	0.174	1.725	0.101[ns]
a_2	7.188	1.725	4.167[a]
a_3	−5.077	1.725	−2.943[b]
a_4	−7.577	1.725	−4.393[a]
a_5	−48.368	1.725	−28.043[a]
a_{11}	−5.381	1.560	−3.449[b]
a_{22}	−3.256	1.560	−2.087[c]
a_{33}	−0.672	1.560	−0.431[ns]
a_{44}	−8.839	1.560	−5.666[a]
a_{55}	−9.006	1.560	−5.772[a]
a_{12}	5.156	2.112	2.441[c]
a_{13}	−4.281	2.112	−2.027[c]
a_{14}	4.594	2.112	2.175[c]
a_{15}	8.031	2.112	3.802[b]
a_{23}	−9.719	2.112	−4.601[a]
a_{24}	−1.969	2.112	−0.932[ns]
a_{25}	−3.156	2.112	−1.494[ns]
a_{34}	−1.031	2.112	−0.488[ns]
a_{35}	−3.719	2.112	−1.76[ns]
a_{45}	−1.594	2.112	−0.754[ns]

[a]Significant at 0.1% level, [b]Significant at 1% level, [c]Significant at 5.0% level, [ns]Not significant at 5% level.

appropriate to the experimental design (Table 4), which indicated that the sum of squares due to regression (first- and second-order terms) was found to be significant ($p < 0.5$) and lack of fit was not significant ($p > 0.5$). The high value (0.98) of coefficient of determination (R^2) suggested that the model is a good fit. The R^2 is the proportion of variability in response values explained or accounted for by the model. The optimum conditions for the maximum production of BC are listed in Table 5.

The effects of pH, concentrations of CSL, alcohol, and acetic acid, and dilution of water to CCH ratio on BC production are reported by the coefficients of second-order polynomials. Some of the response surfaces based on these coefficients are shown in Fig. 1. The response surfaces indicated complex interactions between the variables. Based on the initial results, optimum levels were set. At optimum levels of pH (6.64), CSL (10%), alcohol (0.5%), acetic acid (1.13%), and water to CCH ratio (1:1), after 2 weeks of fermentation, the amount of BC produced was 6.24 g/l (Table 5).

Response Surface Plotting

The response surfaces for BC yield are shown in Fig. 1. The response surfaces indicated the complex interaction between the two variables. The other three variables were kept at the optimum levels (Table 5).

Effects of pH and CSL Concentration on Polysaccharide Yield

The polysaccharide yield for all levels of pH increased with increase in CSL concentration (Fig. 1A). The probable reason for the increase in yield with increase in CSL may be due to the presence of factors that promote cellulose synthesis [4]. Matsuoka *et al*. [8] have reported 4.04 g/l BC by using CSL in basal medium from *Acetobacter xylinum* subsp. *sucrofermentans* BPR2001. The presence of lactate and methionine in CSL are effective components for enhanced cellulose production [8]. Lactate stimulates cell growth by promoting the TCA cycle, and it also generates energy by the oxidation reaction from lactate to pyruvate,

Table 4. Analysis of variance for the fitted second-order polynomial model and lack of fit for biomass yield as per CCRD.

Source of variation	df	Sum of squares	Mean sum of squares	F-value
Regression				
First-order terms	5	59,384.14[a]	11,876.83	2,194.52
Second-order terms	15	8,998.98[a]	599.93	110.85
Total	20	68,383.12		
Residual				
Lack of fit	6	759.77[ns]	126.63	23.40
Pure error	5	27.06	5.41	
Total error	11	786.83		
Grand Total	31	69,169.95		
Coefficient of determination (R^2) =			0.989	

[a]Significant at 5% level, [ns] Not significant.
CCRD, Central composite rotatable design.

Table 5. Feasible optimum conditions and predicted and experimental value of response at optimum conditions.

	pH X_1	CSL conc. (%) X_2	Alcohol conc. (%) X_3	Acetic acid conc. (%) X_4	Water husk ratio X_5
Coded value	0.14	2.00	−2.00	−0.32	−2.00
Uncoded	6.64	10.00	0.50	1.13	1:1
		(g/l)			
Predicted value		6.09			
Experimental value		6.24±0.05			

CSL, Corn steep liquor.

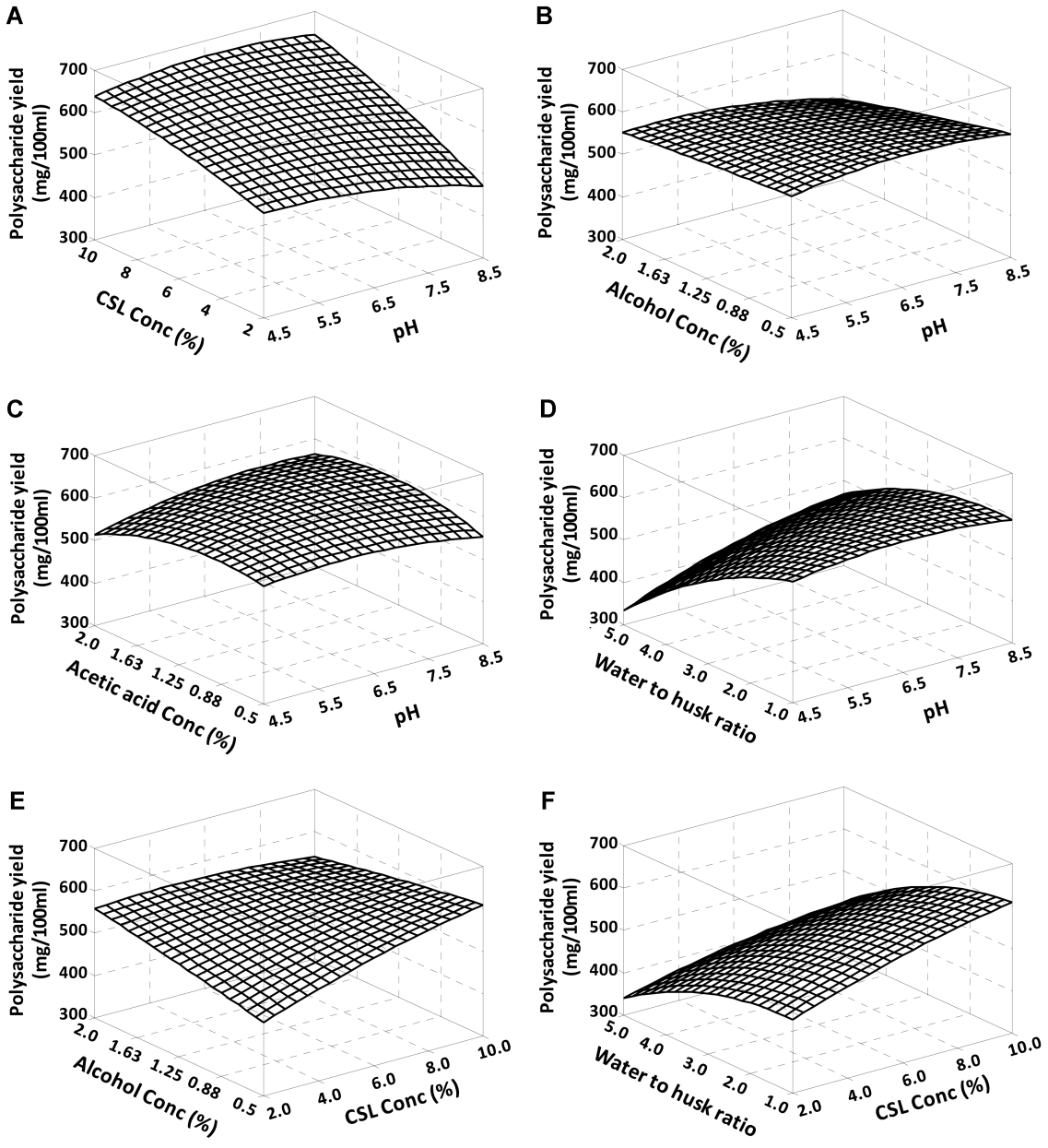

Fig. 1. Response surfaces showing the effects of corn steep liquor (CSL) and pH (A); alcohol and pH (B); acetic acid and pH (C); water to husk ratio and pH (D); alcohol and CSL (E); water to husk ratio and CSL (F) on bacterial cellulose production.

resulting in an increase of cellulose production. At the lowest level of CSL concentration, the yield also increased with an increase in pH. On the contrary, at the highest level of CSL concentration, pH had no effect on yield (Fig. 1A).

Effects of pH and Alcohol Concentration on Polysaccharide Yield

At the lowest level of alcohol concentration, no marked change was observed in the yield of polysaccharide with an increase in pH. However, at the highest level of alcohol concentration, it remained constant up to a certain pH and further increase in pH resulted in a decrease in the yield (Fig. 1B). Similarly, at the lower level of pH, the yield did not change with an increase in alcohol concentration. On the contrary, at a higher level of pH, the yield decreased with increase in alcohol concentration (Fig. 1B). This may be due to increase in the residual acetate, which inhibits the cell growth [10].

Effects of pH and Acetic Acid Concentration on Polysaccharide Yield

At the different levels of pH, the yield increased slightly up to a certain extent and then decreased for all the concentrations of acetic acid (Fig. 1C). It is generally accepted that the optimum pH range for cellulose production by this group of bacteria is between 4 and 7 [17]. At the lowest level of acetic acid concentration, the yield was almost constant with an increase in pH. There was a marginal increase in yield at the highest level of acetic acid concentration, with an increase in pH (Fig. 1C).

Effects of pH and Water to Husk Ratio on Polysaccharide Yield

At the highest and lowest levels of pH, the yield was found to decrease with an increase in water to husk ratio (Fig 1D). This may be due to the decrease in the total sugar concentration and other growth factors that influence BC production. Earlier, working on the conventional method of one parameter at a time, authors had observed similar behavior [20]. When the water to husk ratio was lowest, there was no significant change in yield with an increase in pH. However, for the highest water to husk ratio, it was found to increase with increase in pH (Fig. 1D).

Effects of Alcohol and CSL Concentration on Polysaccharide Yield

When the alcohol concentration was lowest, the yield was found to increase with an increase in CSL concentration. No significant change in yield was observed at the highest alcohol concentration (Fig. 1E). For the lowest or highest CSL concentration, the yield was found to increase or decrease, respectively, with an increase in alcohol concentration (Fig. 1E).

Effects of Water to Husk Ratio and CSL Concentration on Polysaccharide Yield

When the water to husk ratio was either lowest or highest, the yield was found to increase with an increase in CSL concentration. However, the increase was more significant at the lowest level of water to husk ratio than at the higher ratio (Fig. 1F). This may be due to the large supply of G6P, a precursor to BC production at higher levels of CSL and sugar [10]. For all the concentrations of CSL, the yield was found to decrease with an increase in water to husk ratio (Fig. 1F).

FTIR Spectral Studies

The FTIR spectrum of the BC produced from CCH medium showed (Fig. 2) the characteristic bands of cellulose, which is similar to BC produced from HS medium. The peak around 1,640 cm^{-1} is due to the H-O-H bending vibration of absorbed water molecules in cellulose. The spectral region 1,162 cm^{-1} is assigned to cellulose C-O-C bridges. The frequency bands at 1,375, 1,335 (O-H plane bending), 1,315 (CH2 wagging), and 1,278 cm^{-1} (CH bending) indicated the presence of crystalline cellulose II. The characteristic predominant bands at 1,428, 1,163, and 1,111 cm^{-1} indicated cellulose I as the major component, and 1,336, 1,317, and 1,281 cm^{-1} indicates, the presence of cellulose II. Hence, these results confirm that the BC produced in the standardized condition was similar to the BC produced in our earlier experiments [21]. This indicates that the composition of the media does not alter the composition of BC. Hence, utilization of cheap, environmentally hazardous agrowaste like CCH to produce BC can lead to the decrease in the cost of production.

The RSM system was applied for optimization of fermentation parameters in order to use CCH, a phytotoxic agrowaste as a fermentation medium for BC production. Various parameters like pH, nitrogen source in the form of CSL, additives such as alcohol, acetic acid, and dilution of CCH were tried at different combination for arriving at the optimum level for maximum BC production. The optimum conditions for maximum BC production were pH 6.64,

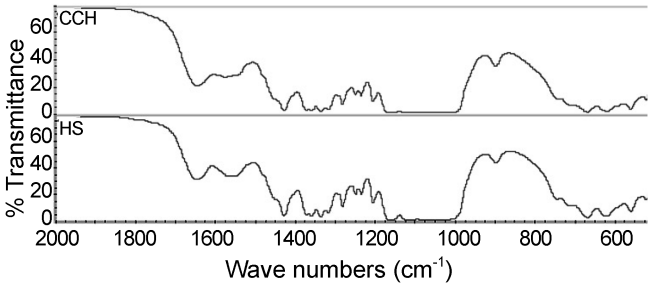

Fig. 2. FT–IR spectrum of bacterial cellulose produced in coffee cherry husk extract and HS medium by *Gluconacetobacter hansenii* UAC09.

CSL 10%, alcohol 0.5%, acetic acid 1.13%, and water to CCH ratio 1:1. Two weeks of fermentation produced 6.24 g of BC/l. This yield was more than the predicted value of 6.09 g/l. These optimized conditions produced more BC than the predicted value, indicating the good fit of the model.

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