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EFFECTS OF CULTIVATION PARAMETERS ON BACTERIAL NANOCELLULOSE PRODUCTION BY *KOMAGATAEIBACTER XYLINUS* DSM 2325

Bacterial nanocellulose (BNC) is a high-value biopolymer with promising applications in biomedical engineering, food technology, packaging, and advanced materials. However, industrial implementation remains constrained by relatively low production yields and the sensitivity of cellulose biosynthesis to cultivation conditions. In the present study, the combined effects of temperature, initial pH, ethanol concentration, and sodium lactate concentration on BNC production by *Komagataeibacter xylinus* (*K. xylinus*) DSM 2325 under agitated cultivation conditions were investigated using a four-factor Box–Behnken design coupled with Response Surface Methodology (RSM). Twenty-nine experimental runs were conducted, and BNC yield was evaluated as dry cellulose production. The highest experimental yield (0.446 g/L) was obtained at 30 °C, pH 6, 2% (v/v) ethanol, and without sodium lactate supplementation. Analysis of variance demonstrated that temperature, pH, and sodium lactate significantly affected BNC production, whereas ethanol showed no statistically significant linear effect. The developed quadratic model was significant ($p = 0.0001$) with an R^2 value of 0.7752. Numerical optimization predicted optimal cultivation conditions of 24.4 °C, pH 4.57, 2% ethanol, and 0.04% sodium lactate, corresponding to a predicted yield of 0.767 g/L. However, experimental validation revealed lower cellulose production under optimized conditions than under the standard medium. Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analyses confirmed the successful synthesis of chemically pure BNC with a characteristic cellulose I crystalline structure. Scanning electron microscopy (SEM) was used to evaluate the surface morphology and fibrillar network structure of the bacterial nanocellulose samples. These findings demonstrate the importance of experimental validation when applying statistical optimization approaches to BNC production systems.

Keywords: bacterial nanocellulose, *Komagataeibacter xylinus*, response surface methodology, Box–Behnken design, ethanol, sodium lactate, optimization.

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Komagataeibacter xylinus DSM 2325 штамының бактериялық наноцеллюлоза өндіруіне өсіру параметрлерінің әсері

Бактериялық наноцеллюлоза (БН) – биомедициналық инженерияда, тағам технологиясында, қаптама өндірісінде және заманауи материалдар жасауда кең қолдану әлеуетіне ие жоғары құнды биополимер. Алайда оның өнеркәсіптік деңгейде өндірілуі салыстырмалы түрде төмен өнімділікпен және целлюлоза биосинтезінің өсіру жағдайларына жоғары тәуелділігімен шектеледі. Осы зерттеуде араластырып өсіру жағдайында *Komagataeibacter xylinus* DSM 2325 штамының бактериялық наноцеллюлоза өндіруіне температураның, бастапқы pH мәнінің, этанол концентрациясының және натрий лактаты концентрациясының бірлескен әсері төрт факторлы Бокс–Бенкен жоспары мен жауап беті әдіснамасын (Response Surface Methodology, RSM) қолдану арқылы зерттелді. Барлығы 29 тәжірибе жүргізіліп, БН шығымы құрғақ целлюлоза мөлшері

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бағаланды. Ең жоғары тәжірибелік өнімділік (0,446 г/л) 30 °C температурада, pH 6 мәнінде, 2% (көлем/көлем) этанол қатысында және натрий лактатын қоспай өсірілген жағдайда алынды. Дисперсиялық талдау нәтижелері температураның, pH мәнінің және натрий лактатының BNC өндірілуіне елеулі әсер ететінін көрсетті, ал этанолдың сызықтық әсері статистикалық тұрғыдан маңызды болмады. Құрылған квадратиктік модель статистикалық маңызды болды ($p = 0,0001$), ал детерминация коэффициенті $R^2 = 0,7752$ құрады. Сандық оңтайландыру нәтижесінде 24,4 °C температура, pH 4,57, 2% этанол және 0,04% натрий лактаты жағдайлары оңтайлы деп болжанып, BNC шығымы 0,767 г/л болады деп есептелді. Алайда тәжірибелік тексеру барысында оңтайландырылған жағдайларда алынған целлюлоза мөлшері стандартты қоректік ортаға қарағанда төмен болды. Фурье түрлендірулі инфрақызыл спектроскопия (FTIR) және рентгендік дифракциялық талдау (XRD) нәтижелері химиялық тұрғыдан таза және целлюлоза I типіне тән кристалдық құрылымы бар BNC синтезделгенін растады. Сонымен қатар сканерлеуші электрондық микроскопия (SEM) әдісі арқылы бактериялық наноцеллюлоза үлгілерінің беткі морфологиясы мен фибриллярлық тор құрылымы зерттелді. Алынған нәтижелер бактериялық наноцеллюлоза өндіру жүйелерін оңтайландыру кезінде статистикалық модельдерді міндетті түрде тәжірибелік тексерумен растаудың маңыздылығын көрсетеді.

Түйін сөздер: бактериялық наноцеллюлоза, *Komagataeibacter xylinus*, жауап беті әдіснамасы, Бокс–Бенкен жоспары, этанол, натрий лактаты, оңтайландыру.

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Влияние параметров культивирования на продукцию бактериальной наноцеллюлозы штаммом *Komagataeibacter xylinus* DSM 2325

Бактериальная наноцеллюлоза (БН) представляет собой высокоценный биополимер с широкими перспективами применения в биомедицинской инженерии, пищевых технологиях, упаковочных материалах и современных функциональных материалах. Однако её промышленное производство по-прежнему ограничено сравнительно низкими выходами продукта и высокой чувствительностью биосинтеза целлюлозы к условиям культивирования. В настоящем исследовании с использованием четырёхфакторного плана Бокса–Бенкена и методологии поверхности отклика (Response Surface Methodology, RSM) изучено совместное влияние температуры, начального значения pH, концентрации этанола и концентрации лактата натрия на продукцию бактериальной наноцеллюлозы штаммом *Komagataeibacter xylinus* DSM 2325 в условиях культивирования с перемешиванием. Было проведено 29 экспериментальных серий, а выход BNC оценивали по количеству сухой целлюлозы. Максимальный экспериментальный выход (0,446 г/л) был получен при температуре 30 °C, pH 6, содержании этанола 2% (об. /об.) и отсутствии добавления лактата натрия. Дисперсионный анализ показал, что температура, pH и лактат натрия оказывают статистически значимое влияние на продукцию БН тогда как линейный эффект этанола оказался статистически незначимым. Разработанная квадратичная модель была статистически значимой ($p = 0,0001$) при коэффициенте детерминации $R^2 = 0,7752$. Численная оптимизация предсказала оптимальные условия культивирования: температура 24,4 °C, pH 4,57, 2% этанола и 0,04% лактата натрия, что соответствовало прогнозируемому выходу 0,767 г/л. Однако экспериментальная проверка показала более низкую продукцию целлюлозы в оптимизированных условиях по сравнению со стандартной средой. Анализ методом инфракрасной спектроскопии с преобразованием Фурье (FTIR) и рентгеновской дифракции (XRD) подтвердил успешный синтез химически чистой бактериальной наноцеллюлозы с характерной кристаллической структурой целлюлозы I. Для оценки морфологии поверхности и структуры фибриллярной сети образцов бактериальной наноцеллюлозы была использована сканирующая электронная микроскопия (SEM). Полученные результаты демонстрируют важность экспериментальной валидации статистических подходов при оптимизации процессов производства бактериальной наноцеллюлозы.

Ключевые слова: бактериальная наноцеллюлоза, *Komagataeibacter xylinus*, методология поверхности отклика, план Бокса–Бенкена, этанол, лактат натрия, оптимизация.

Introduction

BNC is a unique extracellular polysaccharide produced by several acetic acid bacteria, particularly members of the genus *Komagataeibacter (Almeida et al., 2021; Barja, 2021)*. BNC possesses exceptional physicochemical properties, including high purity, mechanical strength, biocompatibility, biodegradability, and water-holding capacity, making it a promising material for biomedical, food, packaging, and engineering applications (Phatchayawat et al., 2025; R et al., 2021). A major advantage of BNC over plant-derived cellulose is its intrinsic purity, as it is synthesized without lignin, hemicellulose, or other plant-associated contaminants (Dáger-López et al., 2024). Among BNC-producing microorganisms, *K. xylinus* is considered one of the most efficient cellulose producers and has therefore become a model organism for studying bacterial cellulose biosynthesis (Ryngajłło et al., 2020; Ryngajłło, Kubiak, et al., 2019). The strain *K. xylinus* DSM 2325 possesses a well-characterized cellulose synthase system and demonstrates the ability to produce highly crystalline cellulose with excellent structural properties (Jang et al., 2019). Nevertheless, large-scale industrial implementation of BNC remains limited by relatively low productivity and the high cost of cultivation media. Consequently, optimization of cultivation conditions represents one of the most important approaches for improving the economic feasibility of bacterial cellulose production (Sayah et al., 2025; Skaradziński et al., 2025). The production of BNC is strongly influenced by environmental and nutritional factors, including temperature, pH, dissolved oxygen availability, carbon source composition, and the presence of metabolic stimulators (Chen et al., 2019). Temperature and pH directly affect bacterial growth, enzyme activity, and the regulation of cellulose biosynthesis pathways (Li et al., 2022). Previous studies have shown that even small deviations from optimal cultivation conditions may significantly reduce cellulose yield and alter the morphology of the resulting material. Therefore, identifying optimal physicochemical conditions is essential for maximizing productivity (El-Naggar et al., 2025; Rackov et al., 2025). In addition to conventional medium components, several metabolic additives have been reported to enhance bacterial cellulose production (Mongkolchat et al., 2026). Ethanol is one of the most extensively studied supplements and has been shown to stimulate cellulose biosynthesis in various *Komagataeibacter* strains (Montenegro-Silva et al., 2024). The beneficial effect of ethanol is commonly attributed to its role as an auxiliary energy source and its ability to redirect carbon metabolism away from gluconic acid formation, thereby increasing the availability of carbon precursors for cellulose synthesis (Pagliardini et al., 2013; Sun et al., 2020). However, the magnitude of this effect is highly strain-dependent and may vary according to cultivation conditions (Barboráková et al., 2025). Organic acids and their salts have also received attention as potential modulators of bacterial cellulose production (Revin et al., 2018; Revin et al., 2021). Sodium lactate can influence cellular metabolism by serving as an alternative carbon source and affecting intracellular redox balance. Furthermore, lactate supplementation may alter medium buffering capacity and cellular energy metabolism, potentially influencing cellulose biosynthesis (Hu et al., 2025; Lasagni et al., 2024). Despite these potential benefits, information regarding the combined effects of sodium lactate and ethanol on BNC production under agitated cultivation conditions remains limited (Kumar & Han, 2021). Agitated cultivation systems offer several advantages over conventional static fermentation, including improved oxygen transfer, enhanced nutrient distribution, and easier scale-up for industrial applications (Zhou et al., 2018). However, agitation also introduces hydrodynamic stress that may affect bacterial physiology, cellulose morphology, and overall productivity (Cáceres et al., 2025). As a result, optimization strategies developed for static cultivation are often not directly applicable to agitated systems. Understanding the interactions among environmental and nutritional factors under agitation therefore remains an important challenge for industrial BNC production (Dayou et al., 2026; Kamal et al., 2022). RSM has become a widely used statistical tool for optimizing complex bioprocesses because it enables simultaneous evaluation of multiple variables and their interactions while reducing the number of required experiments (Kanmani et al., 2013). Among RSM approaches, the Box–Behnken Design (BBD) provides an efficient framework for developing predictive models and identifying optimal cultivation conditions. This methodology has been successfully applied to optimize medium composition, fermentation parameters, and metabolite production in various microbial systems (Behravan & Hashemi, 2021; Sakr et al., 2026). Therefore, the aim of the present study was to investigate the combined effects of temperature, initial pH, ethanol concentration, and sodium lactate concentration

on BNC production by *K. xylinus* DSM 2325 under agitated cultivation conditions. A Box–Behnken experimental design coupled with Response Surface Methodology was employed to evaluate factor interactions, establish a predictive model, and identify optimal cultivation conditions. In addition, the physicochemical properties of the produced BNC were characterized using FTIR, XRD and SEM.

Materials and methods

Microorganism and Cultivation Conditions

The BNC-producing strain *K. xylinus* DSM 2325 was used throughout this study. The strain was maintained on Hestrin–Schramm (HS) agar medium and cultivated aerobically under laboratory conditions.

Batch cultivation experiments were conducted in 500 mL Erlenmeyer flasks containing 100 mL of production medium. The basal HS-medium consisted of glucose (20 g/L), yeast extract (5 g/L), and peptone (5 g/L). The initial pH of the medium was adjusted according to the experimental design using phosphate or phosphate–citrate buffer systems. Ethanol and sodium lactate were supplemented at concentrations specified by the Box–Behnken experimental design, with ethanol varying from 1 to 2% (v/v) and sodium lactate from 0 to 2% (w/v).

For inoculum preparation, *K. xylinus* DSM 2325 was pre-cultivated for 72 h under identical nutritional conditions. Optical density at 600 nm (OD_{600}) may yield inaccurate readings, as bacterial cellulose attenuates the incident light and thereby confounds turbidity-based estimation of cell density. Inoculum pre-cultivation was carried out under static conditions to prevent the shear-induced conversion of cellulose-producing (Cel^+) cells into non-producing (Cel^-) mutants prior to the main agitated experiments (Hur et al., 2020). Subsequently, 5 mL of the seed culture was transferred aseptically into each production flask. Cultivation was performed in an orbital shaking incubator at 120 rpm for 120 h. Depending on the experimental run, temperature and pH values were varied according to the optimization matrix. All experiments were conducted under aerobic conditions, and BNC production was evaluated as dry cellulose yield (g/L) after purification and drying procedures.

Experimental Design and Statistical Analysis

A four-factor Box–Behnken Design (BBD) was employed to evaluate the effects of: Temperature (°C); Initial pH; Ethanol concentration (% v/v); Sodium lactate concentration (% w/v) on BNC production. Response Surface Methodology was applied using Design-Expert 13.0.5.0 software to establish a second-order polynomial model describing the relationship between process variables and BNC yield. Prior to model fitting, the BNC yield response was subjected to a natural-logarithmic transformation, ln (Y + 0.0025), to stabilise variance across the wide range of experimental values. Consequently, the regression coefficients and the mean response value reported below (Table 3) refer to this transformed (ln-scale) response rather than to the untransformed yield in g/L.

The following second-order quadratic regression model was used to describe the relationship between the response and the investigated factors:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$

where Y is the predicted value of the transformed response; X_i и X_j are the coded values of the independent factors. The coefficients for regression, linear, quadratic, and interaction are shown by the symbols β_0, β_i, β_{ii}, β_{ij}, respectively. Analysis of variance (ANOVA) was performed to determine statistical significance. Model adequacy was evaluated using R^2, adjusted R^2, predicted R^2, coefficient of variation (CV), Adeq Precision, and lack-of-fit statistics. All ANOVA computations were performed using Stat-Ease 360 V25.0.1 software.

Bacterial Nanocellulose Recovery and Yield Determination

Following cultivation, BNC was separated from the culture broth by filtration and subjected to an alkaline purification procedure to remove bacterial cells, residual nutrients, and other medium-derived impurities. The collected cellulose samples were treated with 0.1 M NaOH at 80 °C for 1 h under continuous heating. Subsequently, the purified material was thoroughly washed with distilled water until a neutral pH was reached.

Purified BNC samples were frozen and freeze-dried for 48 h at a condenser temperature of -60 °C under full vacuum to obtain a constant dry weight. The dry mass of BNC was determined gravimetrically using an analytical balance. BNC yield was expressed as grams of dry BNC per liter of culture medium (g/L).

The validation experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD). The coefficient of variation (CV) was calculated to evaluate the reproducibility of the cultivation process under different experimental conditions.

Physicochemical Characterization of Bacterial Nanocellulose

The chemical structure and purity of the obtained BNC samples were evaluated using Fourier-transform infrared spectroscopy coupled with attenuated total reflectance (FTIR-ATR). Spectra were recorded on Agilent Cary 630 FTIR spectrometer over the range of 4000–650 cm^{-1} at a spectral resolution of 4 cm^{-1}, with 32 scans averaged for both the background and the sample. Characteristic absorption bands corresponding to hydroxyl (O–H), aliphatic (C–H), and glycosidic (C–O–C) functional groups were identified and compared with previously reported cellulose spectra to confirm the successful production and purification of BNC. The resulting absorbance-versus-wavenumber spectra were processed in OriginPro 2015 V9.2.214 software, including baseline correction and smoothing to reduce high-frequency noise.

The crystalline structure of cellulose I and the crystallinity index of the BNC were evaluated by XRD using a Bruker D8 Advance diffractometer with Cu-Kα radiation (λ = 1.5406 Å). Diffractograms were recorded over a 2θ range of 5–60° at a generator current of 40.0 mA. The diffraction patterns were analyzed to identify characteristic cellulose I crystalline peaks and to evaluate the preservation of the native cellulose structure. The crystallinity index (CrI) was calculated using DIFFRAC.EVA V5.2 software from the integrated areas of crystalline and amorphous regions of the diffractograms and used as an indicator of structural order within the cellulose matrix. The XRD patterns were plotted in Origin Pro 2015 V9.2.214 without additional processing.

The surface morphology and fibrillar network structure of the BNC samples were examined by SEM. Micrographs were acquired on a JEOL JSM-7000 scanning electron microscope. The samples were mounted on aluminum stubs (12/25 mm diameter) using conductive double-sided carbon tape, and excess particles were removed with a gentle stream of dry air; no conductive coating was applied, as imaging was performed in low-vacuum mode. Topographic images were obtained with a low-vacuum secondary electron (LV-SE) detector at 5-10 kV (medium probe size, 4-8 frames averaged), and compositional contrast was recorded with a backscattered electron (BSE) detector at 10-15 kV, over a magnification range of ×100 to ×2,000. The morphology was evaluated in terms of the dimensions of the fibrillar network, pore structure, fiber aggregation, homogeneity, and the presence or absence of residual bacterial cells.

Results and their discussion

Effects of Cultivation Parameters on BNC Production

The effects of temperature, initial pH, ethanol concentration, and sodium lactate concentration on BNC production by *K. xylinus* DSM 2325 were investigated using a Box–Behnken experimental design consisting of 29 experimental runs. Considerable variation in BNC yield was observed among the tested conditions, indicating that cellulose production was influenced by the combined effects of the investigated factors rather than by a single parameter alone. The experimental BNC yield ranged from 0.001 to 0.446 g/L. The highest yield was obtained in run 19, where cultivation was performed at 30 °C, pH 6, 2% (v/v) ethanol, and without sodium lactate supplementation, resulting in a BNC yield of 0.446 g/L. Similarly, run 24 produced 0.407 g/L BNC under cultivation at 30 °C, pH 6, 1% (v/v) ethanol, and without sodium lactate. In contrast, the lowest yields were generally observed at elevated pH values (pH 8) and higher temperatures (38 °C), where cellulose production was almost completely suppressed (Table 1).

The distribution of BNC yields across all experimental runs is presented in Figure 1. The results demonstrate a clear tendency toward enhanced cellulose biosynthesis under moderately acidic conditions and intermediate cultivation temperatures. These observations suggest that *K. xylinus* DSM 2325 is highly sensitive to environmental conditions, particularly pH and temperature, which directly affect cellular metabolism and cellulose synthase activity.

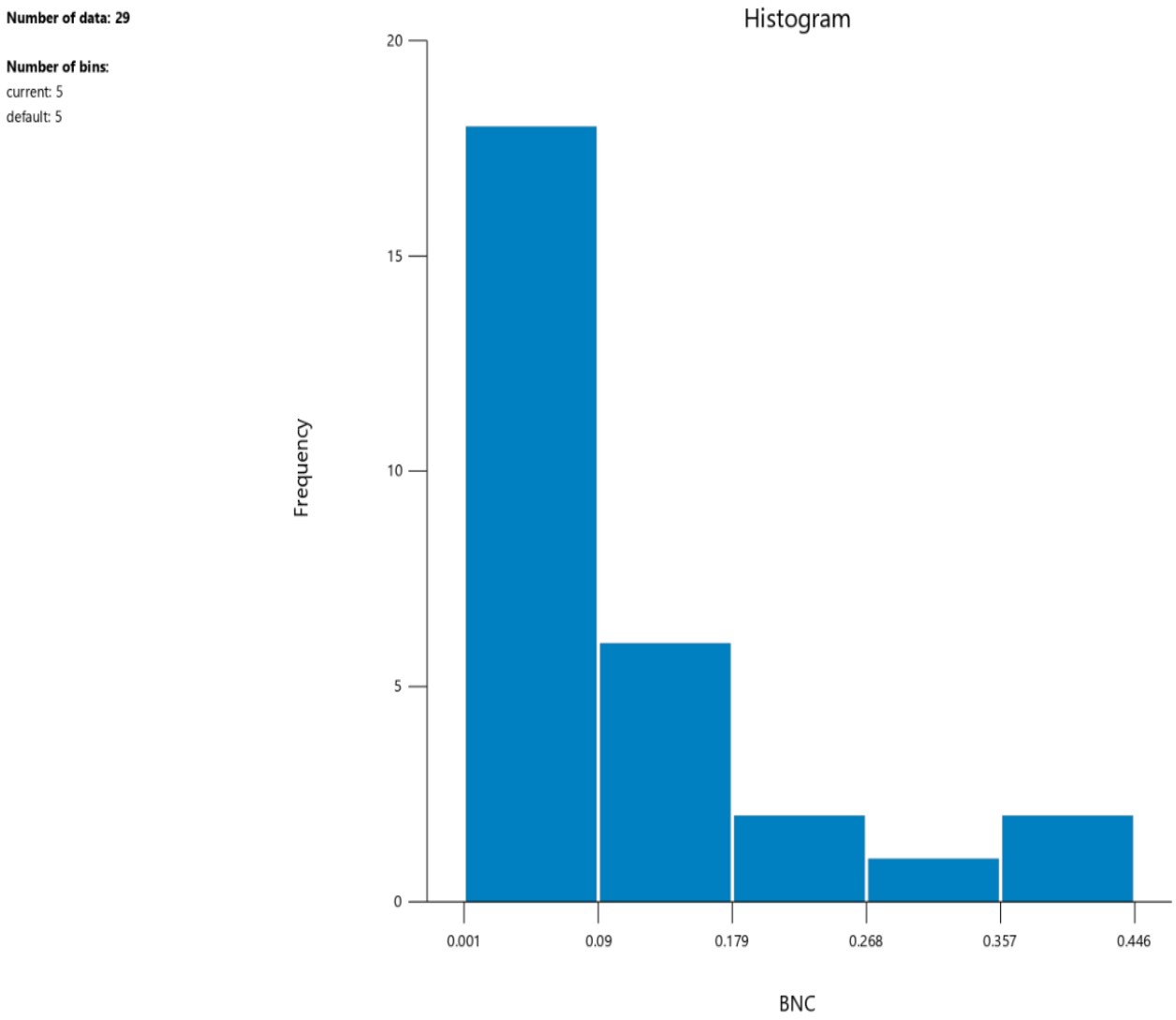
Although ethanol showed no statistically significant linear effect in the ANOVA model ($p = 0.7428$), the highest experimental BNC yields were observed in cultivation runs containing 1–2% (v/v) ethanol and no sodium lactate supplementation. This observation suggests that the influence of ethanol on cellulose biosynthesis may depend on interactions with other cultivation parameters rather than on an independent linear effect. Sodium lactate significantly influenced BNC production according to ANOVA analysis ($p = 0.0470$); however, increased

lactate concentrations did not consistently enhance cellulose yield. The highest BNC production was obtained in the absence of sodium lactate, suggesting that its effect was dependent on interactions with temperature and pH rather than direct stimulation of cellulose biosynthesis.

Table 1
Box–Behnken experimental design matrix and observed and predicted bacterial nanocellulose yields

№	Temperature, °C	pH	Ethanol, % (v/v)	Sodium lactate, % (w/v)	BNC, g/l	Predicted response value, g/L
1	30	6	1,5	1	0.029	0.092
2	30	4	2	1	0.156	0.093
3	30	8	1	1	0.001	0.004
4	22	4	1,5	1	0.137	0.203
5	22	8	1,5	1	0.003	0.000
6	22	6	2	1	0.130	0.080
7	30	6	1,5	1	0.065	0.092
8	38	6	2	1	0.003	0.006
9	30	4	1	1	0.225	0.091
10	30	6	2	2	0.294	0.054
11	30	6	1,5	1	0.081	0.092
12	30	6	1,5	1	0.076	0.092
13	30	6	1,5	1	0.120	0.092
14	38	6	1,5	0	0.001	0.012
15	38	4	1,5	1	0.002	0.000
16	30	4	1,5	2	0.006	0.021
17	30	8	1,5	2	0.001	0.006
18	22	6	1,5	2	0.011	0.037
19	30	6	2	0	0.446	0.191
20	22	6	1,5	0	0.112	0.136
21	30	8	2	1	0.001	0.007
22	38	6	1,5	2	0.001	0.002
23	30	4	1,5	0	0.235	0.376
24	30	6	1	0	0.407	0.156
25	38	6	1	1	0.003	0.005
26	30	6	1	2	0.108	0.043
27	30	8	1,5	0	0.002	0.004
28	38	8	1,5	1	0.008	0.000
29	22	6	1	1	0.082	0.065

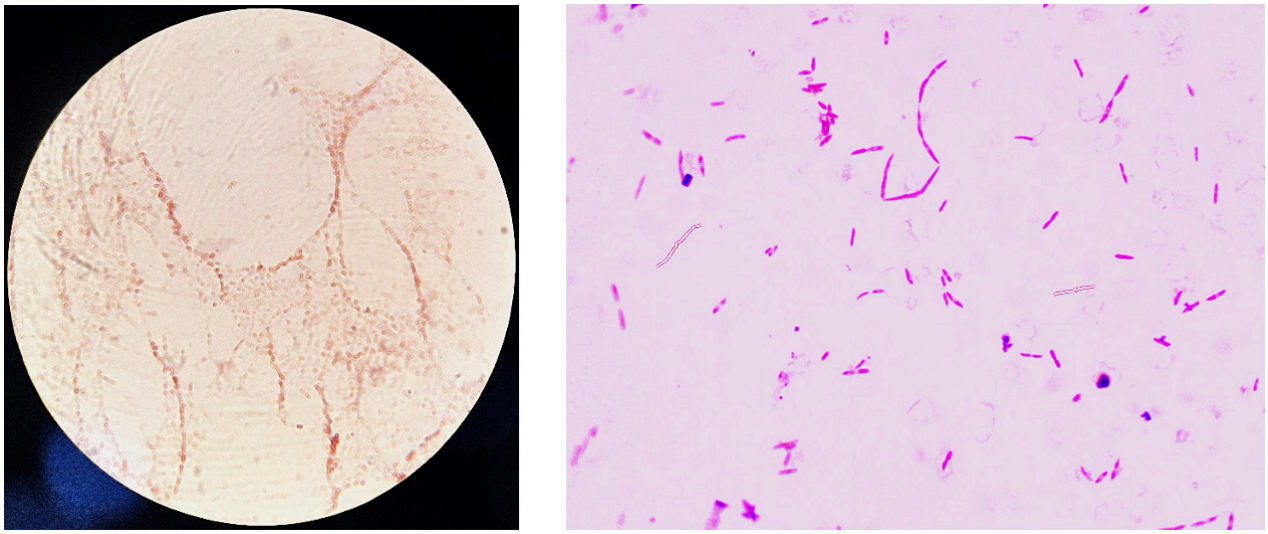
Figure 1
Yield distribution of bacterial nanocellulose obtained under different experimental conditions



Comparison of bacterial morphology under different cultivation conditions revealed notable differences in cell shape and organization. Cells cultivated under agitated conditions exhibited a more elongated morphology compared with those grown under static conditions. This observation may reflect physiological adaptation to hydrodynamic stress and altered oxygen transfer conditions. Furthermore, agitation influenced the physical appearance of the produced cellulose, resulting in dispersed cellulose aggregates rather than the continuous pellicles typically observed during static cultivation (Figure 2).

The results indicate that BNC production by *K. xylinus* DSM 2325 is strongly dependent on the interaction between physicochemical and nutritional factors. The substantial variation in yield observed among experimental runs justified the subsequent application of response surface methodology to identify statistically significant factors and determine the optimal cultivation conditions for maximizing BNC production.

Figure 2
Morphological comparison of K. xylinus DSM 2325 cultivated under static and agitated conditions



Development of the RSM Model

Analysis of variance (ANOVA) demonstrated that the developed quadratic model was statistically significant ($p = 0.0001$), confirming its suitability for describing the relationship between cultivation parameters and BNC production (Table 2). Among the investigated variables, temperature and pH exerted the strongest influence on cellulose biosynthesis, with both factors showing highly significant effects ($p < 0.01$). Sodium lactate also significantly affected BNC production ($p = 0.0470$), whereas ethanol concentration alone did not exhibit a statistically significant linear effect within the investigated range. Nevertheless, the highest experimental yields were achieved in the absence of sodium lactate, indicating that the statistically significant effect of this factor was not necessarily associated with increased cellulose production.

The quadratic model describing the nonlinear relationships between the response and the studied factors, with statistically significant values of $p = 0.0001$ and $F = 7.28$, is represented by the following equation:

$$\hat{Y} = Ц\,2.36 - 1.13A - 1.27B + 0.0972C - 0.6203D + 1.02AB + 0.0903BC + 0.7697BD - 1.37A^2 - 1.27B^2,$$

where $\hat{Y}$ is the predicted value of the transformed response, ln (BNC+0,0025); *A* is the temperature (°C); *B* is the initial pH; *C* is the ethanol concentration (v/v); *D* is the sodium lactate concentration (w/v).

Table 2
ANOVA for the quadratic response surface model

Source of variation	Sum of squares	Degrees of freedom, df	Mean square	F-value	p-value	Note
Model	67.02	9	7.45	7.28	0.0001	Statistically significant
A-Temperature	15.28	1	15.28	14.93	0.0010	Statistically significant
B-pH	19.32	1	19.32	18.89	0.0003	Statistically significant
C-Ethanol	0.1134	1	0.1134	0.1108	0.7428	Not statistically significant
D-Sodium lactate	4.62	1	4.62	4.51	0.0470	Statistically significant
AB	4.16	1	4.16	4.07	0.0580	Not statistically significant
BC	0.0327	1	0.0327	0.0319	0.8601	Not statistically significant
BD	2.37	1	2.37	2.32	0.1445	Not statistically significant

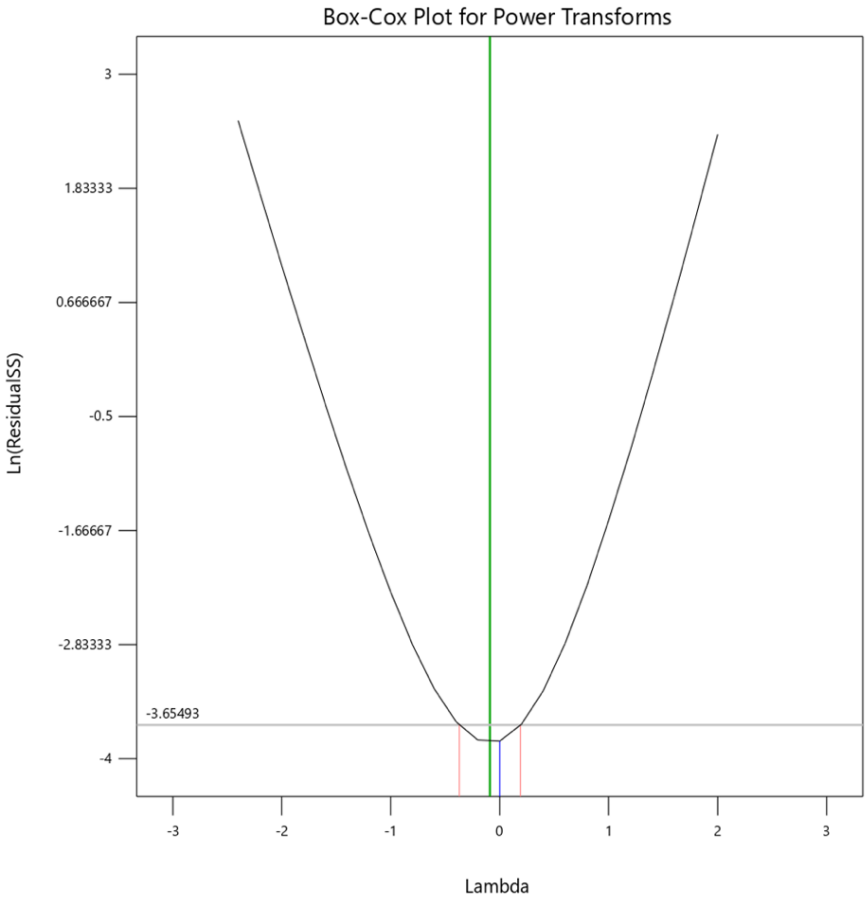
Continuation of the table

Source of variation	Sum of squares	Degrees of freedom, df	Mean square	F-value	p-value	Note
A^2	12.90	1	12.90	12.61	0.0021	Statistically significant
B^2	11.12	1	11.12	10.87	0.0038	Statistically significant
Residual error	19.44	19	1.02	—	—	—
Lack of fit	18.44	15	1.23	4.94	0.0670	Not statistically significant
Pure error	0.9963	4	0.2491	—	—	—
Adjusted total	86.45	28	—	—	—	—

The quadratic model exhibited acceptable goodness-of-fit, with an R^2 value of 0.7752, demonstrating that 77.5% of the observed variation in BNC production could be explained by the selected process variables. The adjusted R^2 (0.6687) indicated satisfactory agreement between the model and experimental data, whereas the predicted R^2 (0.2900) suggested limited predictive accuracy. Nevertheless, an adequate precision value of 9.097, which exceeded the recommended threshold of 4, confirmed that the model provided an adequate signal for navigating the design space.

Prior to model interpretation, a Box–Cox transformation analysis was performed to evaluate the necessity of response transformation. The resulting Box–Cox plot indicated that the selected transformation adequately stabilized variance and improved model performance (Figure 3).

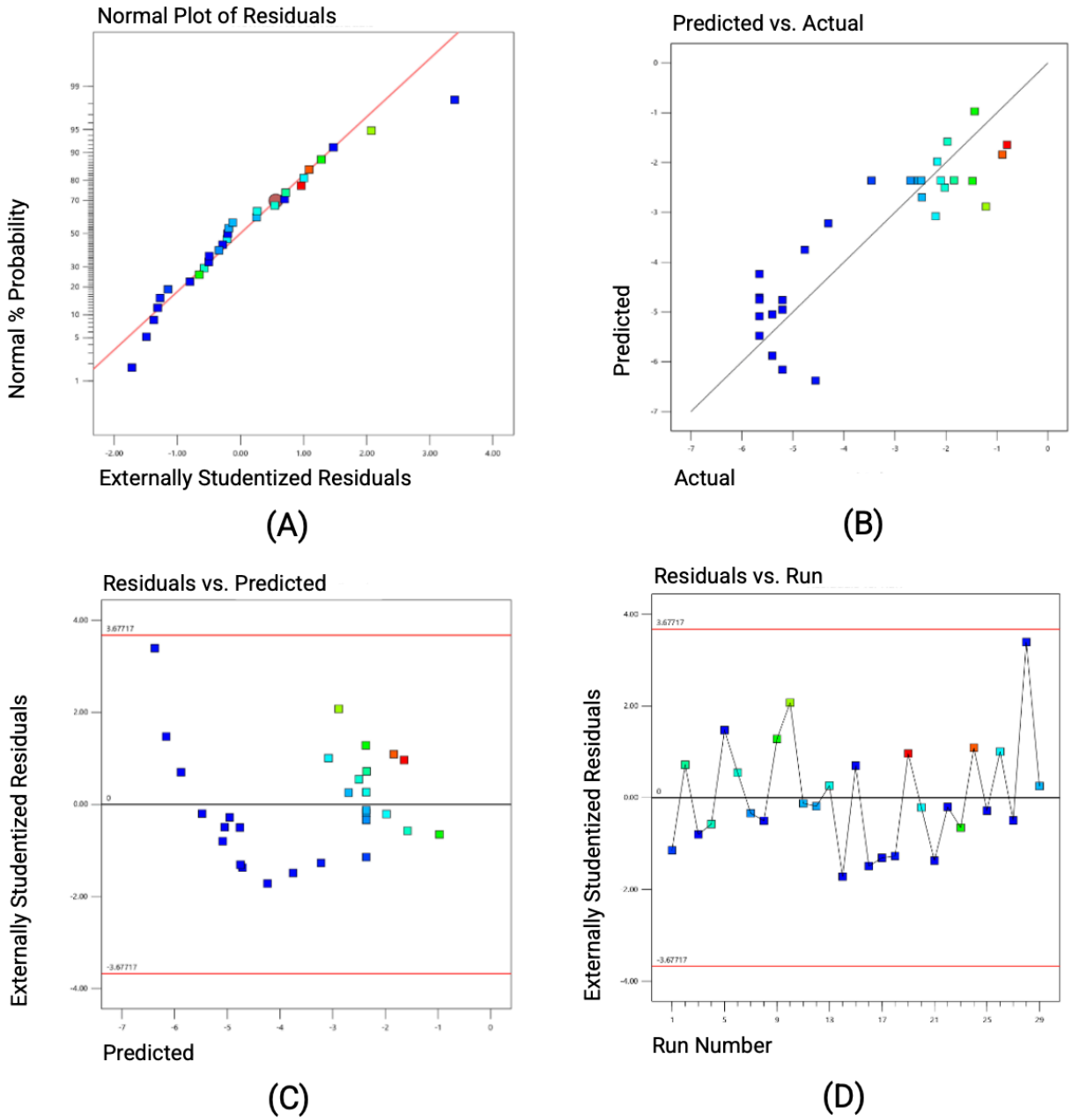
Figure 3
Box–Cox plot for power transformation selection of bacterial nanocellulose yield response



Model adequacy was further assessed using diagnostic plots (**Figure 4**). The normal probability plot of residuals demonstrated an approximately linear distribution, indicating normality of residuals. The predicted versus actual values plot showed reasonable agreement between experimental observations and model predictions. Additionally, residuals were randomly distributed around zero without obvious patterns, suggesting homoscedasticity and independence of experimental errors.

Figure 4

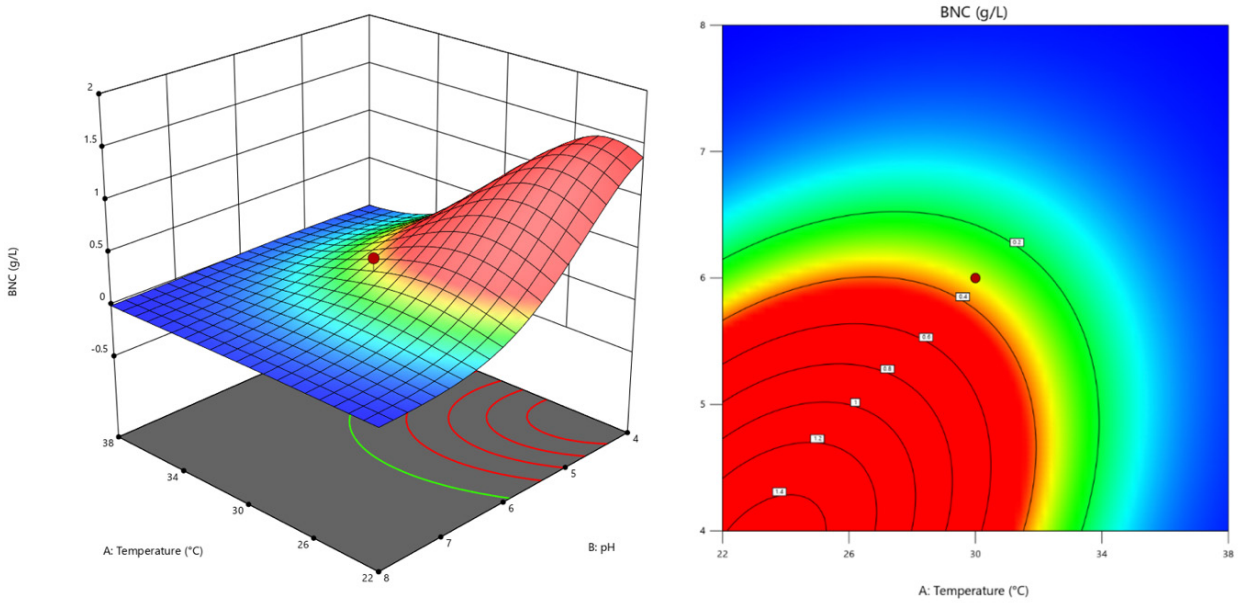
Diagnostic plots of the developed quadratic model: (A) normal probability plot of externally studentized residuals; (B) predicted versus actual values; (C) residuals versus predicted values; and (D) residuals versus run number



Response surface analysis revealed a strong interaction between pH and temperature. Maximum predicted BNC production was observed at moderately acidic pH values and intermediate cultivation temperatures. The response surface plot clearly demonstrated the existence of an optimum region rather than a simple linear relationship between factors and cellulose yield (Figure 5).

Figure 5

Response surface and contour plots illustrating the interactive effects of temperature and pH on bacterial nanocellulose production by K. xylinus DSM 2325 at fixed ethanol (2%, v/v) and sodium lactate (0%, w/v) concentrations



Optimization and Experimental Validation

Based on numerical optimization, the RSM model predicted optimal cultivation conditions of 24.4 °C, pH 4.57, 2% (v/v) ethanol, and 0.04% (w/v) sodium lactate, with a predicted BNC yield of 0.767 g/L.

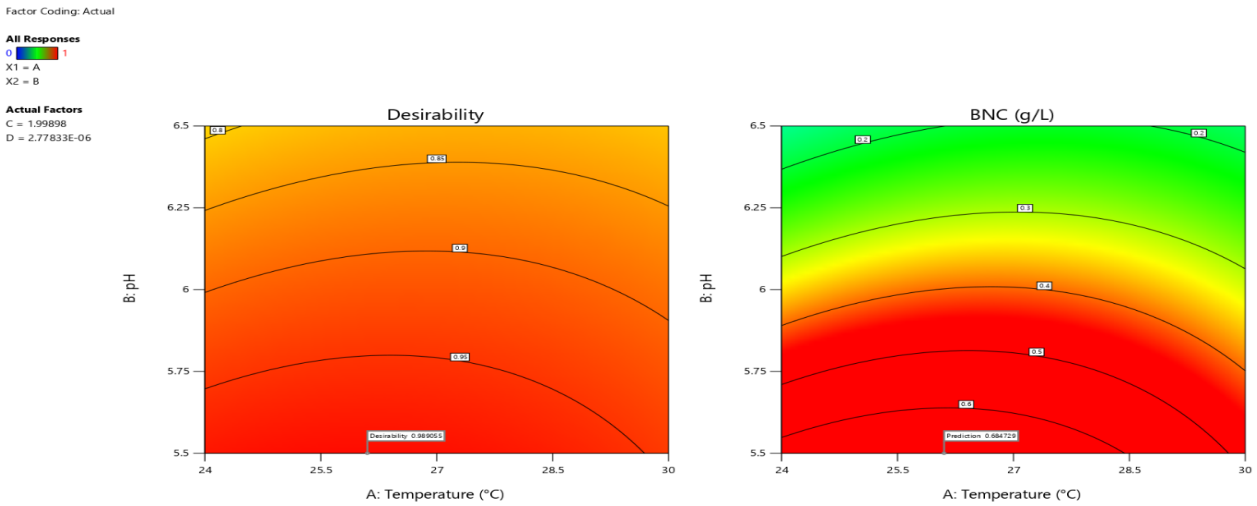
Experimental validation, however, revealed that the predicted optimum did not produce the highest observed cellulose yield. Instead, the standard cultivation medium generated a higher average BNC yield (0.2203 g/L) than the optimized conditions (0.1737 g/L), while also exhibiting considerably lower variability (Table 3).

The coefficient of variation increased from 12.8% under standard conditions to 38.6% under optimized conditions, indicating reduced process reproducibility. These findings emphasize the importance of experimental validation and suggest that the actual optimum may lie within a narrower region around pH 5.5–6.5 and temperatures between 24 and 30 °C. Using this region and the RSM model, the Design-Expert 13 software identified, as the most optimal solution, a combination of factors corresponding to a temperature of 26.0 °C, an initial pH of 5.5, an ethanol concentration of 2% (v/v), and the absence of sodium lactate. Under these conditions, the model predicted a bacterial cellulose yield of 0.685 g/L with a desirability function of 0.989, which fully satisfied the specified optimization criteria within the constrained factor space (Figure 6).

Table 3
Experimental validation of predicted optimum conditions

Experiment series	Batch conditions (with stirring at 120 rpm)	Individual values, g/L	Mean yield, g/L	SD, g/L	CV, %
Standard medium (ST)	Temperature 30 °C. pH 5.7, ethanol 0% (v/v), sodium lactate 0% (w/v)	1) 0.2530 2) 0.2050 3) 0.2030	0.2203	0.0283	12.8
Predicted optimized conditions (OPT)	Temperature 24.4 °C, pH 4.57, ethanol 2% (v/v), sodium lactate 0.04% (w/v)	1) 0.1120 2) 0.2450 3) 0.1640	0.1737	0.0670	38.6

Figure 6
Plot of desirability and bacterial cellulose yield following narrowing of the optimization region

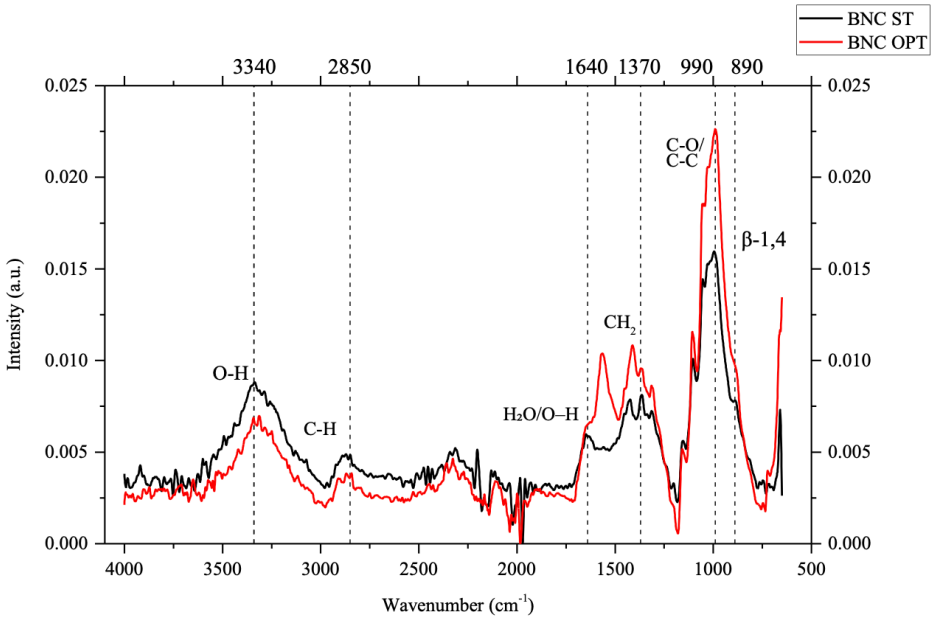


FTIR Characterization

The FTIR spectra of BNC samples produced under standard (BNC ST) and optimized cultivation conditions (BNC OPT) are presented in Figure 7. The spectra included the following main absorption bands: a broad region at 3330–3350 cm^{-1} corresponds to O–H stretching vibrations of hydroxyl groups and hydrogen bonds; the band near 2890–2920 cm^{-1} corresponds to C–H stretching vibrations; the band near 1620–1640 cm^{-1} is associated with the bending vibrations of absorbed water; the bands near 1430–1450 cm^{-1}, 1370–1335 cm^{-1}, and 1160 cm^{-1} are associated, respectively, with CH_2 bending vibrations, C–H/O–H bending vibrations, and C–O–C stretching vibrations of the cellulose backbone.

Peaks in the range of 1050–1030 cm^{-1} indicate C–O stretching vibrations, while a weak band in the range of 896–900 cm^{-1} confirms the presence of β-1,4-glycosidic bonds of cellulose. In addition, low-wavenumber regions near 750 and 710 cm^{-1} were noted, associated with cellulose I allomorphs, where 750 cm^{-1} is attributed to triclinic cellulose Iα and 710 cm^{-1} to monoclinic cellulose Iβ. The absence of pronounced bands in the 1700–1740 cm^{-1} region indicates the absence of residual carbonyl compounds, organic acids, and nutrient-medium impurities. Thus, FTIR analysis confirmed that the obtained samples correspond to the chemical structure of bacterial nanocellulose. Furthermore, compared with the BNC ST sample, the BNC OPT sample showed more intense signals in the fingerprint region (1500–650 cm^{-1}), and especially near 990 cm^{-1}, indicating more pronounced polysaccharide vibrations.

Figure 7
ATR–FTIR spectra of bacterial nanocellulose samples



XRD Analysis

The crystalline structure of BNC was examined using X-ray diffraction analysis. The diffraction patterns obtained for both samples are shown in **Figure 8.** Characteristic diffraction peaks corresponding to cellulose I were observed at approximately 14.5°, 16.5°, 18°, and 22.7° (2θ), confirming preservation of the native crystalline cellulose structure. No additional diffraction peaks associated with contaminating phases were detected, indicating effective purification of the cellulose material.

Quantitative analysis of the diffractograms yielded crystallinity index (CrI) values of 40.6% for the BNC synthesized under standard conditions (BNC ST) and 35.7% for the BNC synthesized under the RSM-predicted optimal conditions (BNC OPT). Thus, although both samples retained the cellulose I structure, cultivation under the optimized condition which contained 2% (v/v) ethanol produced cellulose with a lower degree of crystalline order. This reduction is consistent with reports that ethanol accelerates β-1,4-glucan chain assembly and, together with medium acidification by gluconic acid, can decrease the ordered packing of cellulose microfibrils, thereby increasing the amorphous fraction (Abraham et al., 2025; Fatima et al., 2023). These CrI values are also markedly lower than the 70–90% typically reported for bacterial nanocellulose, which is consistent with the generally low and poorly organized cellulose production observed under agitation in the present study.

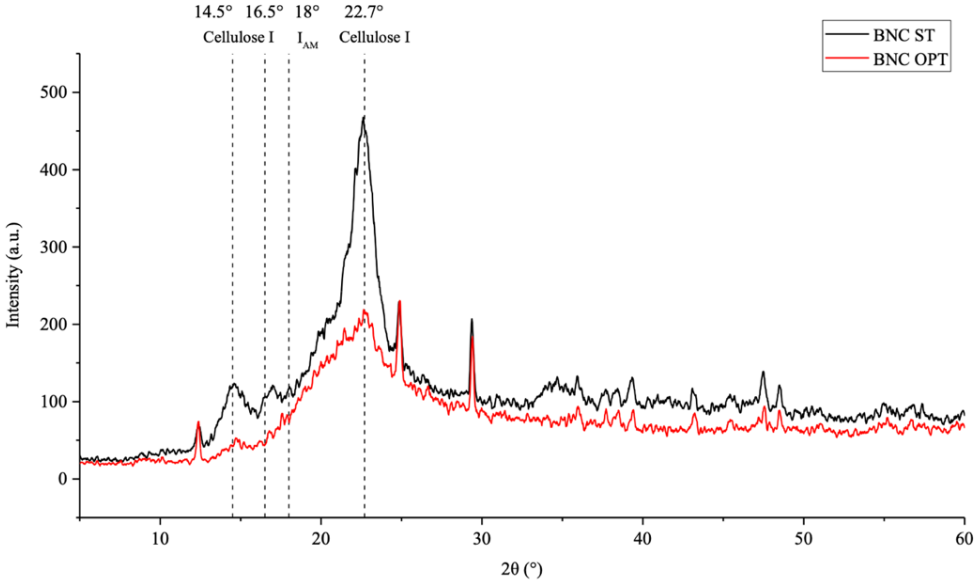
The observed crystalline structure is consistent with previously reported BNC and contributes to its desirable mechanical strength, dimensional stability, and potential applicability in advanced biomaterials.

SEM Characterization

The morphology of freeze-dried BNC synthesized in the standard (ST) and optimized media (OPT) was examined by SEM at identical magnification (×3000). Representative micrographs are presented in Figure 9.

Figure 9a (BNC ST) reveals a compact and highly aggregated morphology. The cellulose matrix appears as dense, coalesced layers with pronounced folds, ridges, and sheet-like structures. Individual nanofibrils cannot be clearly distinguished because they are fused into a continuous film-like network. The interfibrillar porosity is relatively low, indicating extensive fibril aggregation during biosynthesis and subsequent freeze-drying. Several elongated cavities and cracks are also visible, which may have originated from dehydration-induced shrinkage and partial delamination of the cellulose layers. Such compact morphology is commonly associated with reduced oxygen transfer within the cellulose matrix and with the formation of thicker cellulose bundles under cultivation conditions that favor rapid fibril aggregation.

Figure 8
XRD diffractograms of bacterial nanocellulose produced under standard and optimized cultivation conditions



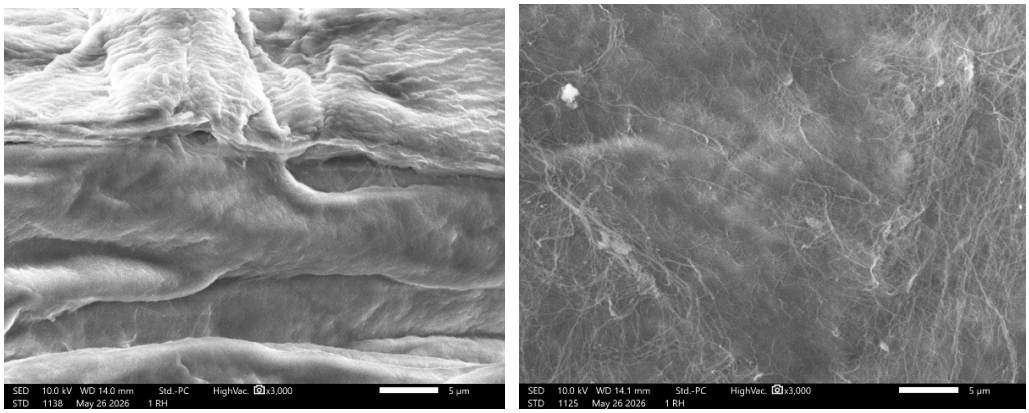
In contrast, Figure 9b (BNC OPT) exhibits a markedly different microstructure characterized by a well-developed three-dimensional nanofibrillar network. Individual ribbon-like cellulose fibrils are clearly visible and form an interconnected porous scaffold. The fibrils appear thinner, more uniformly distributed, and less aggregated than those observed in the standard sample. Numerous interfibrillar voids and channels are present throughout the structure, indicating increased porosity and a greater specific surface area. This morphology is characteristic of high-quality bacterial nanocellulose and is generally considered advantageous for applications requiring efficient mass transfer, water absorption, cell attachment, or loading of functional compounds.

The observed differences between Figures 9a and 9b suggest that cultivation conditions strongly influenced the supramolecular organization of cellulose fibrils. Under the optimized conditions, the lower cultivation temperature (24.4 °C), moderately acidic pH (4.57), supplementation with ethanol (2% v/v), and low sodium lactate concentration (0.04% w/v) likely altered bacterial metabolism and cellulose assembly dynamics. Slower metabolic activity may have promoted a more controlled extrusion and organization of glucan chains, resulting in the formation of thinner and better separated nanofibrils. At the same time, ethanol could serve as an auxiliary energy source, reducing carbon loss through competing metabolic pathways and supporting more uniform cellulose biosynthesis.

The SEM observations are also consistent with the physicochemical characterization results. Although the optimized sample exhibited a lower crystallinity index than the standard sample according to XRD analysis (35.7% vs. 40.6%), it demonstrated a more open and homogeneous fibrillar architecture. This finding indicates that increased fibril individualization and network development do not necessarily correspond to higher crystallinity. Instead, the optimized cultivation conditions promoted the formation of a finer nanofibrillar structure with improved morphological uniformity while partially reducing crystalline ordering.

Overall, SEM analysis confirmed that cultivation parameters significantly affect the microstructure of bacterial nanocellulose produced by *K. xylinus* DSM 2325. The standard medium generated a dense, compact cellulose matrix, whereas the optimized medium produced a more porous and interconnected nanofibrillar network, demonstrating that process optimization influences not only cellulose yield but also the structural characteristics of the resulting biomaterial.

Figure 9
SEM micrographs (× 3000 magnification) of freeze-dried bacterial nanocellulose produced by K. xylinus DSM 2325: (a) BNC synthesized under standard cultivation conditions (BNC ST); (b) BNC synthesized under optimized cultivation conditions (BNC OPT). Scale bar = 5 µm



The present study demonstrated that BNC production by *K. xylinus* DSM 2325 under agitated cultivation conditions was strongly influenced by the interaction of physicochemical and nutritional factors. Among the investigated variables, temperature and pH exerted the greatest influence on cellulose biosynthesis, which is consistent with previous reports indicating that these parameters directly affect bacterial growth, cellulose synthase activity, and carbon metabolism (Chen et al., 2019; Li et al., 2022). The highest experimental BNC yields were observed at 30 °C and pH 6, confirming that moderately acidic conditions favor cellulose formation.

Although ethanol did not exhibit a statistically significant linear effect in the ANOVA model, the highest cellulose yields were obtained in cultivation runs containing 1–2% ethanol. Similar observations were reported by Ryngajłło, Jacek, et al. (2019), who demonstrated that ethanol supplementation modifies the transcriptional profile of *Komagataeibacter* and can enhance cellulose biosynthesis depending on cultivation conditions. Ethanol may reduce carbon loss through gluconic acid formation and improve the availability of precursors for cellulose synthesis. However, the magnitude of this effect appears to be strain-dependent and strongly influenced by environmental conditions.

Sodium lactate significantly affected BNC production according to the statistical model, yet the highest experimental yields were obtained in its absence. This discrepancy suggests that lactate influenced cellulose production indirectly through interactions with temperature and pH rather than through direct stimulation of cellulose biosynthesis. Similar complexity has been reported for other metabolic additives, where statistically significant model effects do not necessarily translate into improved product formation under all cultivation conditions.

The developed RSM model explained approximately 77.5% of the variability in BNC production. Although the model was statistically significant, its predictive performance was moderate, as reflected by the relatively low predicted R^2 value (0.2900). Furthermore, experimental validation did not confirm the predicted optimum. The optimized conditions generated a lower average BNC yield than the standard medium. Similar discrepancies between predicted and experimental optima have been reported in microbial bioprocess optimization studies and are often attributed to biological variability, nonlinear metabolic responses, and limitations of second-order polynomial models (Behravan & Hashemi, 2021; Kanmani et al., 2013)

A further consideration is the generally very low absolute BNC yields obtained in this work (maximum 0.446 g/L), which are well below the values commonly reported for static cultivation of Komagataeibacter (typically several g/L). This is characteristic of agitated cultivation of *K. xylinus*, in which hydrodynamic shear stress promotes the irreversible conversion of cellulose-producing (Cel^+) cells into non-producing (Cel^-) mutants through insertion of an IS element into the bcsA gene (Hur et al., 2020). Such stochastic loss of cellulose-producing capacity is consistent with the elongated cell morphology observed under agitation and with a shift of carbon flux towards soluble polysaccharides such as acetan rather than crystalline cellulose (Zhang et al., 2022). This mechanism offers a biological explanation both

for the predominance of near-zero yields across the design and for the poor reproducibility (high coefficient of variation) that limited the predictive performance of the RSM model.

FTIR analysis confirmed the presence of characteristic cellulose functional groups, while XRD analysis demonstrated the preservation of the cellulose I crystalline structure. The diffraction peaks observed near 14.5°, 16.5°, and 22.7° are consistent with previous reports for bacterial nanocellulose produced by *Komagataeibacter* species. These results indicate that neither ethanol nor sodium lactate supplementation altered the fundamental chemical structure of the produced cellulose. However, the crystallinity index decreased from 40.6% to 35.7%, indicating that the ethanol-containing optimized condition reduced the degree of crystalline order while preserving the cellulose I polymorph. Ethanol at a concentration of 2% (v/v) affected not only the rate of bacterial cellulose biosynthesis by *K. xylinus* DSM 2325 but also the spatial organization of cellulose fibrils, as evidenced by SEM images and a reduction in crystallinity.

The findings indicate that temperature and pH are the primary determinants of BNC production by *K. xylinus* DSM 2325 under agitation, whereas the effects of ethanol and sodium lactate depend on interactions with other cultivation parameters. The study also highlights the necessity of experimental verification when applying statistical optimization methods to biological production systems.

Conclusion

This study systematically evaluated the combined effects of temperature, initial pH, ethanol concentration, and sodium lactate supplementation on BNC production by *K. xylinus* DSM 2325 under agitated cultivation conditions using Response Surface Methodology (RSM) and a Box–Behnken experimental design. The results demonstrated that BNC biosynthesis is governed by complex interactions among environmental and nutritional factors, with temperature and pH emerging as the most influential variables affecting cellulose production. Statistical analysis confirmed the significance of the developed quadratic model, while response surface analysis revealed the presence of a distinct optimum region for cellulose biosynthesis under agitation. Among all experimental conditions, the highest BNC yield (0.446 g/L) was obtained at 30 °C, pH 6, 2% (v/v) ethanol, and in the absence of sodium lactate supplementation. Although sodium lactate was identified as a statistically significant factor in the ANOVA model, its influence on cellulose production was inconsistent, as the highest experimental yields were achieved without lactate addition. Similarly, ethanol did not exhibit a significant linear effect but appeared to contribute to enhanced cellulose production under specific cultivation conditions, highlighting the importance of factor interactions rather than isolated parameter effects. The discrepancy between the RSM-predicted optimum and the experimentally validated results emphasizes the limitations of purely model-based optimization approaches in biological systems. These findings demonstrate that statistical optimization should always be complemented by experimental verification, particularly when working with complex microbial metabolic processes. Furthermore, FTIR and XRD analyses confirmed the successful synthesis of chemically pure bacterial nanocellulose possessing the characteristic functional groups and crystalline cellulose I structure, whereas SEM analysis revealed changes in the morphological structure of the cellulose. The preservation of these structural features indicates that the investigated cultivation parameters affected cellulose yield without substantially altering the fundamental chemical composition or crystallinity of the produced material. Overall, this work contributes to a deeper understanding of the factors regulating bacterial nanocellulose production by *K. xylinus* DSM 2325 under agitated cultivation conditions. The obtained results provide valuable information for future medium optimization studies, bioprocess development, and industrial scale-up of bacterial nanocellulose production. Future research should focus on improving model predictability, investigating additional metabolic stimulators and carbon sources, and evaluating process performance in pilot-scale bioreactor systems to further enhance BNC productivity and economic feasibility.

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Acknowledgements and conflict of interest

The authors thank all collaborators and institutions involved in this study for their support. The authors declare no conflicts of interest.

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