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Original Article

Characterization of bacterial cellulose produced by *Novacetimonas pomaceti* KMPG_12, and improving prebiotic property by reducing size using high pressure homogenization

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Abstract

This study investigates the production and characterization of bacterial cellulose (BC) by *Novacetimonas pomaceti* KMPG_12, focusing on enhancing its prebiotic properties through size reduction via high-pressure homogenization (HPH). Optimal conditions for BC production were established using Response Surface Methodology (RSM), yielding a maximum dry weight of 9.78 g/L under conditions of 15% glucose, 0.4% peptone, and 4.5% alcohol over a 14-day cultivation period. The produced BC was subjected to HPH at 20,000 psi for eight cycles, significantly reducing fiber size, as confirmed by SEM analysis. The transformation from a compact, net-like structure to a more separated fibrous network at the nanometer scale was observed. FTIR analysis revealed modifications in the BC's molecular framework post-HPH, indicating a disruption of hydrogen bonds and a transition towards a less crystalline structure. The prebiotic potential of HPH-treated BC was assessed through *in vitro* experiments with *Bacillus amyloliquefaciens*, showing a significant enhancement in bacterial proliferation (P=0.0437) compared to untreated BC. This study highlights the potential of BC, particularly when processed to the nanoscale, to serve as a functional ingredient that supports gut health and microbial growth, marking a significant advancement in the development of sustainable and effective prebiotic products.

Keywords: Bacterial cellulose, High pressure homogenization, Prebiotic, Acetic acid, Bacteria, *Novacetimonas pomaceti*

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Introduction

Bacterial cellulose (BC) is a naturally occurring polymer produced by acetic acid bacteria (AAB), including strains like *Gluconoacetobacter xylinus*, *Ga. Hansenii*, *Komagataeibacter kakiaceti*, *K. medellinensis*, and *K. maltaceti*. BC is composed of pure cellulose, which is a polysaccharide made up of glucose monomers linked by β-(1→4)-glycosidic bonds (Nicolas et al., 2021). The repeating unit in this polymer is cellobiose, consisting of two glucose molecules. Unlike plant cellulose, bacterial cellulose lacks other components such as lignin, hemicellulose, and pectin, which are commonly found in the cell walls of plants (Naomi et al., 2020). This purity gives bacterial cellulose distinctive physical and chemical properties, such as high tensile strength, crystallinity, and a remarkable ability to retain water.

BC has widespread attention across various fields due to its exceptional safety and non-toxic nature. Within the medical field, it finds significant application as a biocompatible membrane for tissue repair, including restoring corneal membranes in instances of tissue damage or corneal ulcers (Anton-Sales et al., 2020). Furthermore, BC is an effective wound dressing for injuries caused by burns or scalds. (Portela et al., 2019). BCis increasingly utilized in the food industry, both as a packaging material and as a prebiotic substrate. It also serves as a stabilizing matrix for the delivery of probiotic (Azeredo et al., 2019; Jayani et al., 2020). Moreover, incorporating dietary cellulose as a prebiotic can reduce inflammation in the gastrointestinal tracts of a murine endotoxemia model. This reduction is achieved by decreasing the activity of the transcription factor NF-κB, which in turn lowers the production of pro-inflammatory cytokines, an effect effectively triggered by natural bacterial cellulose (Di Caro et al., 2019).

Further investigations have shown that converting bacterial cellulose into nano-bacterial cellulose (NBC) using techniques like acid hydrolysis significantly enhances its prebiotic properties. Specifically, 125-nanometer cellulose fibers improve gut bacteria activity, increasing beneficial metabolites like short-chain fatty acids (SCFAs) and significantly raising Bifidobacteria populations (Nsor-Atindana et al., 2020). The limitation of small-scale modification using chemicals is environmental toxicity. Other approaches to improve cellulose structure for better prebiotic properties are using cellulase enzyme processes (Boisset et al., 2000; Liu et al., 2023). However, this process is complex; enzymes that can digest BC are limited, time-consuming, and, therefore, unsuitable for industrial production.

This research isolated *Novacetimonas pomaceti* from the ripe grapes' samples. The genus *Novacetimonas* has shown promise in producing bacterial cellulose, a biopolymer with numerous applications in various industries, including food, pharmaceuticals, and textiles. Understanding its cellulose production capabilities can lead to more efficient and cost-effective production methods (Kolesovs et al., 2022). Therefore, this study aimed to use the Response Surface Methodology (RSM) to optimize BC production and enhance its prebiotic properties by applying high-pressure homogenization (HPH) to refine its structure. This study introduces novel aspects of BC application. Using the HPH to reduce BC to the nanoscale significantly improves its prebiotic utility, as confirmed through *in vitro* testing.

Material and Methods

Isolation of cellulose-producing bacteria from ripe grapes

Novacetimonas pomaceti KMPG_12 was isolated from ripe grapes. The ripe grapes purchased from retail supermarkets were cleaned with sterile water three times, then submerged in sterile distilled water contained within a flask. The flask's opening was sealed with cotton to maintain sterility. This setup was left undisturbed until a gelatinous film, indicative of bacterial cellulose, developed on the water's surface. Following the formation of the bacterial cellulose film, known as the Symbiotic Culture of Bacteria and Yeast (SCOBY) (de Miranda et al., 2022), it was carefully separated from the residual fermentation broth for detailed examination. The SCOBY underwent an extensive cleansing, entailing three consecutive washes with sterile distilled water. The SCOBY was chopped into fine pieces post-cleansing and subjected to intense vortexing for 10 minutes. Simultaneously, the supernatant derived from the SCOBY was combined with a saline solution containing 0.85% sodium chloride. The SCOBY supernatant and the original fermentation broth were then cultivated on HS medium, which contains 20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 2.7 g/L sodium phosphate dibasic, 1.5g/L citric acid, with the pH

adjusted to 6 and supplemented with 5 µL/mL of amphotericin B (Supakod and Wongwicharn, 2012). These cultures were subsequently maintained at a steady temperature of 30 °C for seven days for incubation.

Classification of isolated bacteria through 16S rRNA gene sequencing

DNA extraction was carried out using the PureDirex DNA extraction kit (Bio-Helix; Taiwan). The process began with the resuspension of bacterial DNA in 200 µL of TE buffer, followed by heating for 20 minutes to aid in the dissolution. The DNA extraction was then performed in accordance with the instructions provided by the extraction kit. The 16S rRNA gene was amplified using the Polymerase Chain Reaction (PCR) technique, utilizing the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The polymerase chain reaction (PCR) was carried out in a total volume of 50 µL. The reaction mixture comprised 0.2 µM of each primer, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.5 U/µL of Taq DNA polymerase. To this mixture, 2 µL of the DNA sample was added. The PCR conditions were as follows: initial denaturation at 94 °Cfor 5 minutes, followed by 30 cycles of denaturation at 94 °Cfor 50 seconds, annealing at 53 °Cfor 30 seconds, and extension at 72 °Cfor 1 minute. A final extension step was performed at 72 °Cfor 5 minutes to ensure complete synthesis of the PCR products. Following amplification, the PCR products were subjected to agarose gel electrophoresis to evaluate the amplification results. The DNA fragments obtained from PCR were subsequently purified using the same PureDirex kit (Bio-Helix; Taiwan). These purified DNA samples were sent for nucleotide sequencing. The resulting sequences were compared against the GenBank database to identify evolutionary relationships. This analysis was conducted using MEGA Version 11 software.

Production and optimization of bacterial cellulose synthesis

The experimental approach involved using a Box-Behnken Design (BBD) integrated with Response Surface Methodology (RSM) to systematically investigate the principal factors affecting the synthesis of bacterial cellulose (BC). The key factors chosen for optimization were glucose concentration (ranging from 5% to 20%), peptone concentration (ranging from 0.3% to 0.6%), and ethanol concentration (ranging from 3% to 6%). A total of 17 experimental runs were conducted based on the Box-Behnken Design matrix. Each run involved culturing *N. pomaceti* KMPG_12 in a modified HS medium under different combinations of glucose, peptone, and ethanol concentrations. The response variable measured was the dry weight of bacterial cellulose produced (g/L). This systematic approach allowed for the precise identification of optimal conditions for BC production, ensuring a comprehensive understanding of the factors influencing yield. The bacterial strains under investigation were incubated in an HS medium at 30 °C for three days. The turbidity of the bacterial cultures was adjusted to a McFarland standard number 3 with a 0.85% sodium chloride solution, employing a DEN-1B McFarland Densitometer (BioSan, England) for precise measurement. Subsequent to this calibration, 10 mL of the adjusted culture was inoculated into a designated liquid medium (100 mL) with varying concentrations of glucose, peptone, and absolute ethanol as described above.

Pretreatment and refine the structure of BC to the nanometer scale

BC was purified with several washes in distilled water to remove impurities. Following this preliminary cleanse, the BC was treated with an alkaline solution, specifically boiled in 1 M NaOH at a W/V ratio 1:1. This boiling occurred at 70 °C for 2 hours. After the alkaline treatment, the BC was rinsed 2-3 times with distilled water, followed by boiling at 70 °C for 1 hour. After this boiling, the BC underwent 2-3 washes with distilled water. The BC was then stored in distilled water at refrigeration temperatures for 24 hours. Post-refrigeration, it was rinsed 2-3 times, followed by a final boiling in distilled water at 60 °C for 30 minutes. Following this last boiling step, there were 2-3 more rinses. After completing the washing cycles, the BC was homogenized using a laboratory blender (Waring, model 8010BU, USA). The procedure entailed eight cycles of treatment under a pressure of 20,000 psi.

Analysis of physical and chemical properties of BC

For the microstructural study, samples of BC were prepared by freeze-drying. These samples were then affixed onto a stub using carbon tape and subjected to gold sputtering to enhance conductivity. The

morphological characteristics of the BC were analyzed using a field emission scanning electron microscope (FEI-SEM), (model; Quanta 450), at the TSU-E-Lab service system, Thaksina University, Thailand. Images captured were analyzed to determine the fiber diameter of the BC.

Analysis of chemical structure via Fourier Transform Infrared Spectroscopy (FT-IR)

The chemical structure of the BC was investigated using Fourier Transform Infrared Spectroscopy (FT-IR). The freeze-dried BC samples were converted to powder form for this analysis. This technique was employed to identify various bonds and functional groups across a wavelength range of 4000-400 cm^{-1}. The data collected was then graphically represented to illustrate the correlation between wavelength and transmission values. This process was carried out using the Fourier Transform Infrared Spectroscopy (VERTEX 70, Bruker, Germany) at the Office of Scientific Instrument and Testing, Prince of Songkla University, Thailand.

Assessment of potential as a carbon source for probiotics *in vitro*

This study was to assess the capability of BC as a carbon source in promoting the growth of probiotics, with *Bacillus amyloliquefaciens* serving as a model representative probiotic species. The comparative efficacy of BC was evaluated against several established carbon sources, namely xylo-oligosaccharides (XOS), inulin, dextran, and carboxyl methylcellulose (CMC). The proliferation of *B B. amyloliquefaciens* in the presence of these carbon sources was methodically monitored and quantified using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or MTT assay. *B. subtilis* was cultivated in a carbon-free mineral salts medium (MSM), which consisted of 1.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 0.6 g of Na_2HPO_4, 1.0 g of $MgSO_4.7H_2O$, 0.75 g of Citric acid, 0.1 g of Yeast extract, 2.0 g of $C_2H_3NaO_2$, and 2% of the tested carbon source.

Statistical analysis

The comparison of mean results for the carbon source utilized by the bacteria before and after the High-Pressure Homogenization (HPH) process was conducted using a paired t-test analysis. This statistical analysis was performed with the Prism V9.5.1 analysis software.

Results

Isolation and identification of cellulose-producing bacteria from ripe grapes

Cellulose-synthesizing bacteria or acetic acid bacteria were isolated from ripe grapes as mentioned in materials and methods. The acetic acid bacteria (AAB) colonies formed on HS agar were noted for their solid, gelatinous appearance, which is distinct from the creamy texture often observed in other bacterial colonies, and they showed a tendency to adhere to the agar's surface. Through a thorough screening process involving Gram staining, we identified a single isolate, KMPG_12, characterized as a gram-negative short rod (Figure 1A). Further identification through 16S rRNA gene sequence analysis, with comparisons to reference strains from the GenBank database, revealed that isolate KMPG_12 aligns with the genus *Novacetimonas*. It exhibited a 99.37% sequence similarity to the species *N. pomaceti* (Accession No. NR_180086) (Figure 1B). Top left image: Flask containing the bacterial cellulose-producing isolated form ripe grapes; Top middle image: Gram staining of *N. pomaceti* KMPG_12 showing gram-negative, short rod-shaped bacteria; Bottom left image: Colony morphology of *N. pomaceti* KMPG_12 grown on HS agar plates after 72 hours of incubation. B). 16S rRNA gene sequence analysis, with comparisons to reference strains from the GenBank database, revealed that isolate KMPG_12 aligns with the genus *N. pomaceti*. Phylogenetic tree construction by MEGA11 with Maximum likelihood with 1000 bootstrapping.

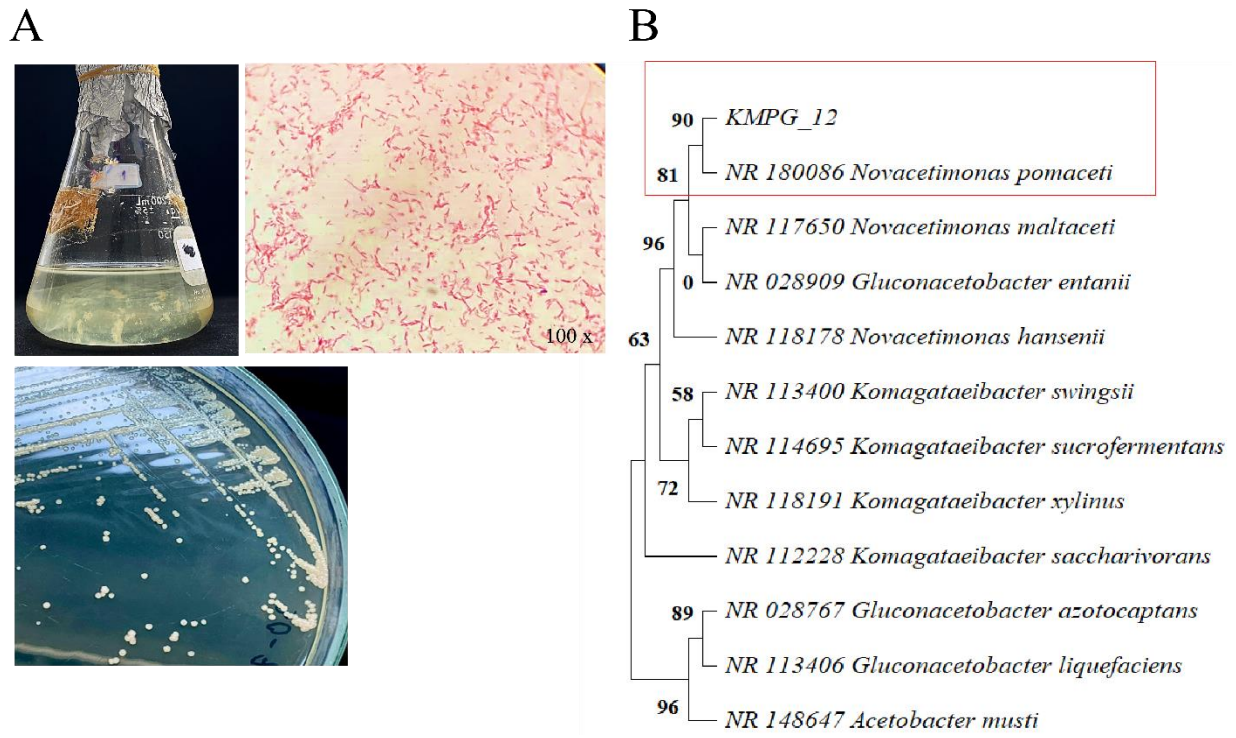


Figure-1. Isolation, morphological characterization, and phylogenetic analysis of *N. pomaceti* KMPG_12. A).

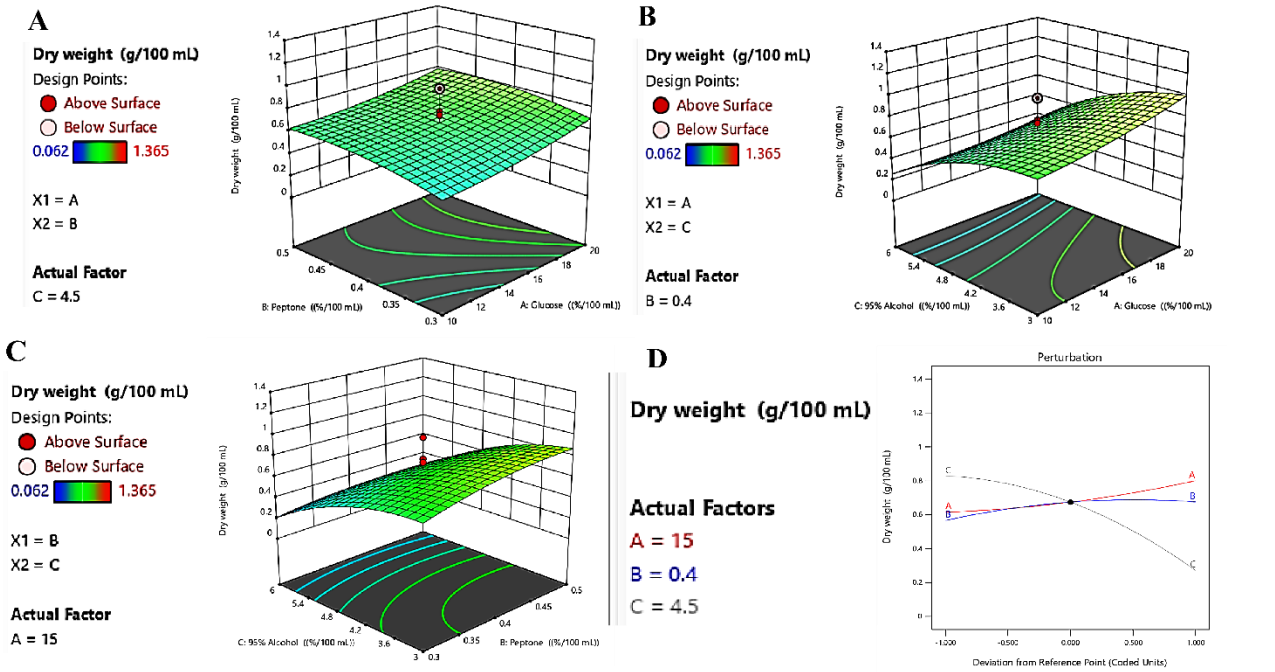


Figure-2. Response Surface Methodology (RSM) plots for the optimization of BC production by KMPG_12 under varying conditions. A) Interaction between glucose concentration and peptone concentration. B) Interaction between glucose concentration and alcohol concentration. C) Interaction between peptone concentration and alcohol concentration. D) the perturbation plot that compares the effect of each factor at a particular point in the design space, showing how deviations from the reference point.

Production and optimization of bacterial cellulose synthesis

In preparation for determining the optimal conditions for BC production using the RSM, isolate KMPG_12 was cultured in an HS liquid medium. This preliminary step was undertaken to assess the isolate's ability to produce BC under standard conditions and to quantify the yield of BC, measured as dry weight, before proceeding with the RSM optimization. The findings indicated that isolate KMPG_12 demonstrated cellulose production capacity, with a peak yield reaching 1.4 g/L.

Consequently, using RSM, KMPG_12 was further investigated to optimize cellulose production conditions at various factors. The optimization process was adjusting three variables: glucose concentration, peptone concentration, and alcohol percentage. The RSM analysis identified that the highest cellulose synthesis was achieved with a medium composition of 15% glucose, 0.4% peptone, and 4.5% alcohol over a 14-day cultivation period (Figure 2). A comparative analysis of the dry weight from each of the seventeen experiments was also carried out. This analysis highlighted those experiments 8 and 12 produced the highest yields of BC, with dry weights of 13.65 g/L, whereas experiment run 12 produced the optimal yield of BC, with 9.78 g/L.

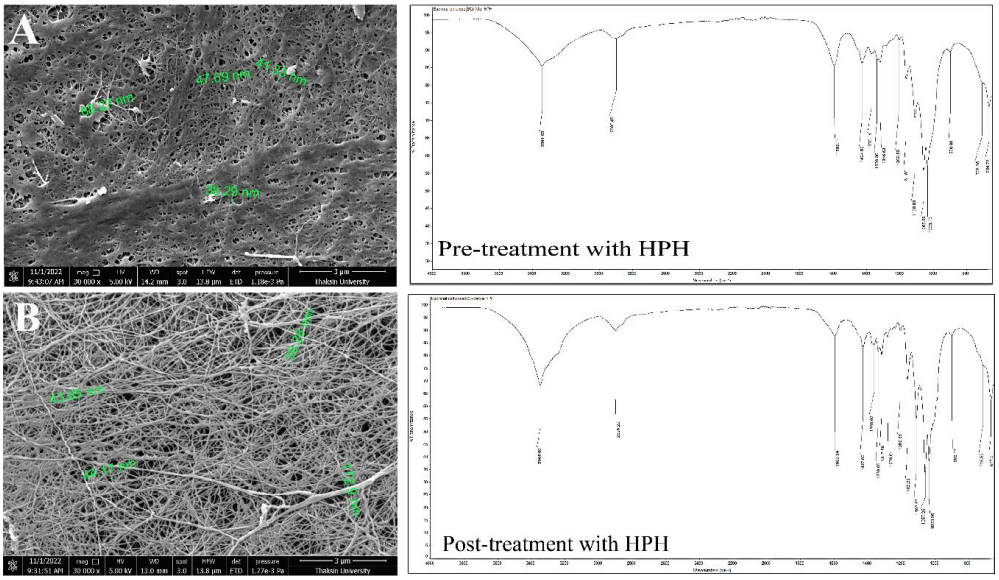
Analysis of physical and chemical properties of BC

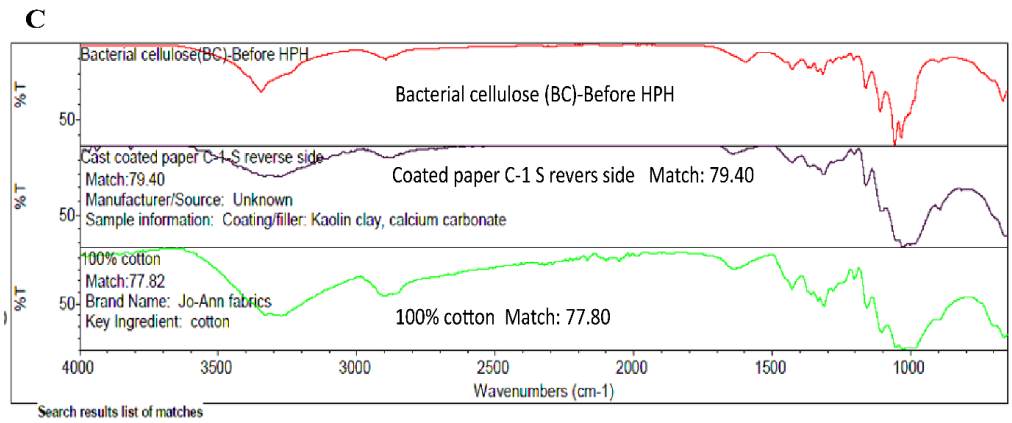
The SEM observations post-freeze-drying revealed that BC fibers formed a highly compact and net-like structure, with fibers extensively overlapping each other. A significant observation was the merging of fibers in most areas, leading to sheet-like formations that lacked individual fiber visibility. The diameter of these fibers was confirmed to be within the nanometer range, highlighting an intricate and finely fibrous network (Figure 3A). To enhance the yield of nanometer-scale fibers, the BC underwent an HPH treatment. Following this treatment, SEM analyses indicated a notable decrease in fiber size and the elimination of flat fiber fusion, showcasing a more separated and distinct fiber structure. Nanometer-sized fibers became more pronounced, demonstrating that the HPH treatment successfully refined the BC's fibrous network into finer structures (Figure 3B). In the study, FTIR was employed to compared the structural changes between pre- and post-HPH treatment of BC. Prior to the application of HPH, the FTIR spectrum of the bacterial cellulose displayed distinct and sharp peaks, indicative of a crystalline structure characterized by well-ordered hydrogen bonds. Notably, the peak in the vicinity of 3340 cm^{-1}, attributed to the OH stretching vibrations, is emblematic of strong intermolecular hydrogen bonding (Figure 3A).

Notable modifications in the FTIR spectrum were observed upon processing the bacterial cellulose to HPH. There was a general broadening and potential slight shifting of the peaks, suggesting alterations within the cellulose's molecular framework. Such changes are probably the disruption of hydrogen bonds, a process instigated by the HPH (Figure 3B). The study's FTIR spectral analysis of BC pre- and post-HPH treatment and its comparison to various standard cellulose sources revealed a notable increase in spectral congruence. The BC subjected to HPH exhibited FTIR profiles that more closely resembled those of the standard reference cellulose samples, indicating a probable enhancement in the structural properties of the cellulose after the HPH process (Figure 3C).

Assessment of potential as a carbon source for probiotics *in vitro*

The study's findings suggest that BC processed to HPH exhibits enhanced prebiotic activity. When used as a carbon source for the growth of B. amyloliquefaciens, HPH-treated BC significantly outperformed untreated BC in promoting bacterial proliferation, as evidenced by the MTT assay results ($P=0.0437$). Notably, the prebiotic efficacy of HPH-processed BC was comparable to that of standard prebiotics (CMC, Inulin, Xylan, XOS and Dextran) included in the analysis and indicated that it was not statistically significant difference between those prebiotics (Figure 4).





Match (%)	Compound Name	Library Name
79.40	Cast coated paper C-1-S reverse side	Paper Materials Library
77.82	100% cotton	Common Materials
77.35	(Methyl sulfoxide)-d6, 100.0 atom % D	Aldrich FT-IR Collection Edition II
77.11	Unbleached Coffee Filter	Common Materials
77.01	US paper money (uninked paper)	Common Materials
76.23	100% Linen	Common Materials
74.93	Paper towel	Common Materials
74.55	Cardboard, 150lb burst	Common Materials
74.05	Bleached coffee filter	Common Materials
73.89	18 point coated carton newsback paper-back side	Paper Materials Library

Figure-3. Scanning Electron Microscopy (SEM) images and FTIR spectra of bacterial cellulose (BC) of the isolate KMPG_12 pre- and post-treatment with HPH. A) Pre-homogenization fibers showing larger fused structures and occasional nanometer-scale fibers. B) Post-homogenization fibers displaying a more uniform and finer fibrous network with clearly visible nanometer-sized fibers. C) FTIR spectra of BC pre-and post- treatment with a high-pressure homogenizer were compared against various standard cellulose.

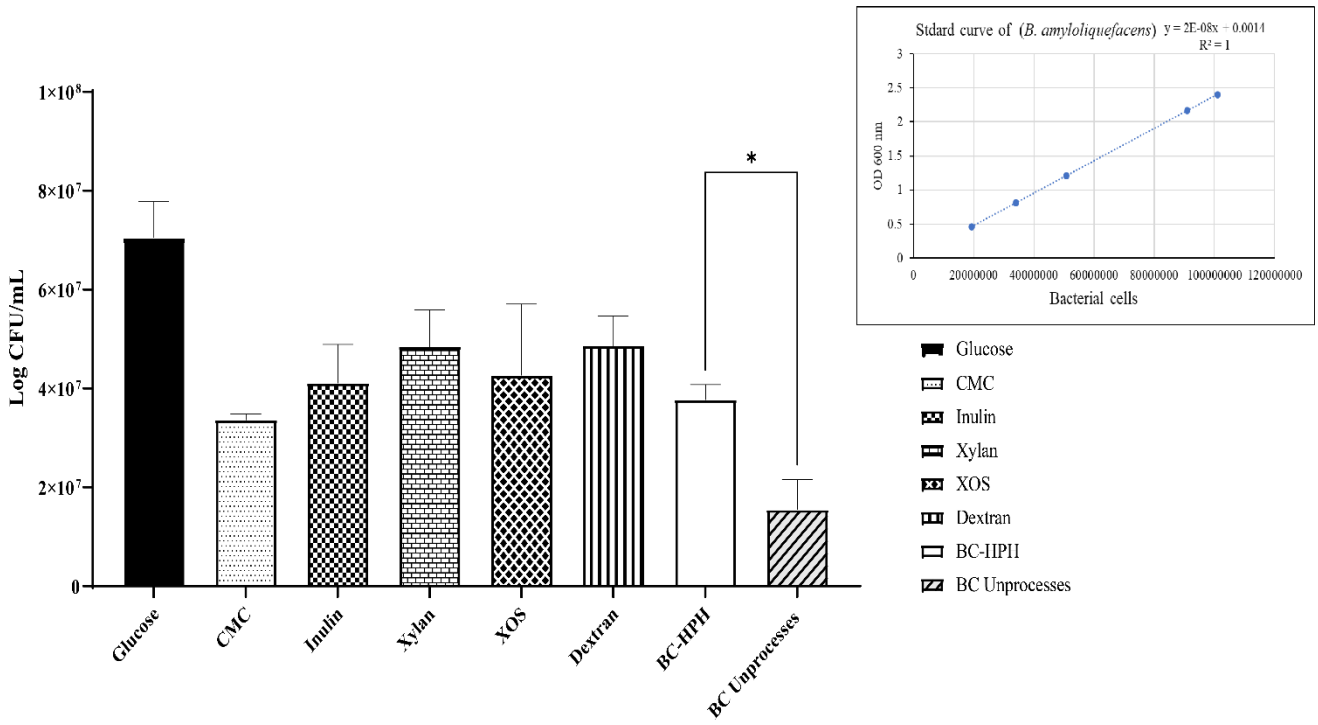


Figure-4. Bar graph and standard curve illustrating the growth of *B. amyloliquefaciens* with different prebiotics. The bar graph compares the bacterial proliferation when cultured with untreated BC, HPH-treated BC, and various standard prebiotics (left to right). The inset standard curve (top right) correlates OD values to bacterial cell counts. * p=0.0437

Discussion

This study explores the use of bacterial cellulose (BC) sourced from *Novacetimonas pomaceti* (KMPG_12), a strain of acetic acid bacteria (AAB), for prebiotic purposes. We hypothesized that reducing BC to the nanoscale would enhance its prebiotic properties.

The phylogenetic analysis of *N. pomaceti* KMPG_12 based on 16S rRNA gene sequencing reveals significant genetic distinctions from other bacterial strains known for producing bacterial cellulose. Comparative analysis with strains from the GenBank database shows that KMPG_12 aligns closely with the genus *Novacetimonas*, exhibiting a 99.37% sequence similarity to the species *N. pomaceti* (Accession No. NR_180086). The phylogenetic analysis of *N. pomaceti* KMPG_12 reveals significant insights into its relationship with other bacterial cellulose-producing strains within the *Komagataeibacter* genus. The recent reclassification of several *Komagataeibacter* species into the new genus *Novacetimonas*, based on phylogenomic and comparative genomic analyses, underscores the importance of genetic distinctions in understanding cellulose biosynthesis capabilities. The reclassification of species such as *K. hansenii*, *K. cocois*, *K. maltaceti*, and *K. pomaceti* into the novel genus *Novacetimonas* highlights significant genetic differences that are also reflected in their cellulose biosynthesis genes (bcs genes) (Brandão et al., 2022).

This study also focused on determining the optimal conditions for BC production. This was achieved by utilizing Response Surface Methodology (RSM) to adjust the influencing variables in the synthesis process. The research specifically varied the concentrations of glucose, peptone, and alcohol, as these factors are known to impact BC production significantly. The study found that the best BC production with N. pomaceti was achieved using a specific mix of 15% glucose, 0.4% peptone, and 4.5% alcohol over a 14-day period, yielding approximately 9.78 g/L (0.978 g/100 mL) in dry weight.

Acetic Acid Bacteria are known for their proficiency in oxidizing a wide array of substrates, including carbohydrates, alcohols, organic acids, and nitrogen sources, and accumulating metabolic byproducts in

their growth medium. (Qiu et al., 2021). The high BC production levels by *N. pomaceti* could be attributed to the study's strategic use of a diverse nutrient mix in the culture medium. Specifically, the combination includes glucose, alcohol and peptone, which may synergistically enhance the bacterium's ability to synthesize bacterial cellulose effectively.

The results indicate a nuanced interaction between the variables tested for BC production. Specifically, glucose concentration plays a critical role, with increasing levels BC production up to a certain threshold, beyond which the yield stabilizes. This suggests that there is an optimal concentration of glucose that maximizes BC production, and further increases do not contribute additional benefits. Peptone concentration is also a significant factor, positively influencing BC yield, particularly when combined with higher levels of glucose. This synergistic effect underscores the importance of optimizing multiple nutrients simultaneously to achieve maximum BC production. Moreover, the interaction between glucose and alcohol concentrations revealed that the highest BC yield is achieved with an optimal glucose concentration of approximately 15-20 g/100 mL and a moderate alcohol concentration of around 4-5%. The interaction between peptone and alcohol concentrations further emphasizes the complexity of these relationships. Moderate alcohol levels, when combined with increasing peptone concentrations, contribute positively to BC yield. However, excessive alcohol concentration can adversely affect the yield, even in the presence of high peptone levels. This suggests that while alcohol is necessary for optimal BC production, its concentration must be carefully controlled to avoid inhibitory effects.

The production of BC varies significantly across different bacterial strains and nutrient substrates. Although no data have been reported directly comparing RSM with *N. pomaceti*, comparisons with other AAB genera have been performed. However, it is difficult to determine which conditions are best for BC production because different carbon and nitrogen sources were used in different studies and the bacteria were different. A comparison of the study's results with other reported studies is shown in Table 1.

Table-1. Optimization and yield of BC production from various bacterial strains and carbon sources using RSM method

Bacterial Strain	Carbon Source	Method	Cellulose Yield (g/L)	Key Findings	References
Novacetimonas pomaceti KMPG_12	Glucose, Peptone, Alcohol	RSM	9.78 g/L Dry weight	High-pressure homogenization improved prebiotic properties	This study
Komagateibacter saccharivorans APPK1	Ammonium sulphate, ethanol	RSM and artificial neural network (ANN)	475 g/L Wet weight	model development using a hybrid approach of RSM and ANN for enhanced BC production using *Komagateibacter saccharivorans APPK1,*	(Patel et al., 2024)
Komagataeibacter xylinus	Oil palm empty fruit bunch (EFB)	RSM	260 g/L Wet weight	provide insight into the potential use of EFB in producing bacterial cellulose as an eco-friendly alternative to non-biodegradable plastics, using readily available and affordable materials.	(Francis et al., 2024)
	Molasses and corn steep liquor (CSL)	RSM	21.61 g/L Dry weight	highest ever reported for BC production, which was achieved with a more cost-effective culture medium containing molasses and CSL.	(Khanchezar et al., 2024)
Gluconacetobacter entanii	Pecan nutshell	RSM	2.81 g/L Dry weight	Chemical functionalization of BC for enhanced properties	(Dórame-Miranda et al., 2019)
Gluconacetobacter xylinus	Enzymatically hydrolyzed prickly pear peels (PPP)	RSM	6.01 g/L Dry weight	BC production for antimicrobial and packaging applications	(El-Gendi et al., 2023)

Komagataeibacter sucrofermentans	Synthetic media and agrifood side streams supplemented with organic acids and vitamins	RSM	15.7 g/L Dry weight	Raisin and citrus side-streams can be efficiently combined for bacterial cellulose production, enhanced by other vitamin- and phenolic-rich substrates such as green tea.	(Adamopoulou et al., 2024)

Table-2. Comparative Analysis of FTIR Spectra and Structural Characteristics of Bacterial Cellulose from Different Strains

Bacterial Strain	FTIR Peaks (cm^{-1})	Structural Characteristics	Key Findings	References
Novacetimonas pomaceti KMPG_12	3340, 2900, 1429, 1058	Crystalline pre-HPH, amorphous post-HPH	Enhanced solubility and prebiotic properties post-HPH	This study
Komagataeibacter xylinus	3336, 2922, 1431, 1060	High crystallinity, strong hydrogen bonds	Used for eco-friendly materials production	(Francis et al., 2024)
Gluconacetobacter hansenii	3330, 2900, 1420, 1050	Crystalline structure, moderate hydrogen bonds	Effective for wound dressing applications	(Portela et al., 2019)
Komagataeibacter rhaeticus	3345, 2915, 1430, 1065	High crystallinity, robust hydrogen bonds	Applied in biocompatible membranes	(Anton-Sales et al., 2020)
Gluconacetobacter entanii	3335, 2920, 1425, 1055	Crystalline structure, moderate hydrogen bonds	Functionalized for enhanced properties	(Dórame-Miranda et al., 2019)

Bacterial cellulose is renowned for its remarkable properties such as high purity, durability, flexibility, and the ability to retain water, which opens up a spectrum of applications. It's utilized in the medical field for artificial skin, in the cosmetic industry for hydrating face masks and moisturizers, and in the food sector as an alternative to animal-based gelatin (Choi et al., 2022; Lahiri et al., 2021; Wahid and Zhong, 2021). The exploration of BC as a prebiotic is a developing field. Although indigestible by humans, BC's potential as a prebiotic may stem from its role in altering the gut environment or as a vehicle for delivering prebiotics or probiotics (Jayani et al., 2020). Its characteristics like high water retention, biocompatibility, and stability make it a viable option for encapsulating health-promoting compounds to enhance their effectiveness in the digestive system (Charoenrak et al., 2023).

This study also aimed to enhance the prebiotic potential of BC by reducing its size to the nanoscale, a study by previous research (Nsor-Atindana et al., 2020) indicating the limited prebiotic efficacy of larger BC structures. The study's findings aligned with this hypothesis, demonstrating that BC's size reduction through the HPH process effectively reduced its diameter to the nanometer level. Subsequent experiments revealed that this nano-sized BC notably improved the growth of *B. amyloliquefaciens*, used as a test organism, compared to its unprocessed counterpart, indicating a statistically significant enhancement in its prebiotic properties.

HPH is a physical modification technique rather than a biochemical digestion process, which significantly refines the structure of bacterial cellulose (BC) through mechanical shearing. This process reduces the size of cellulose fibers but does not chemically degrade the cellulose polymers into simpler sugars, unlike enzymatic treatments. By altering the physical properties of BC, HPH increases the material's surface area, making it more amenable to enzymatic or chemical digestion (Jin et al., 2015). The decrease in fiber size modifies the aspect ratio and increases the exposure of hydroxyl groups, significantly affecting the polymer's biological activities. Consequently, these changes facilitate more effective interaction between nano-sized BC and microbial cells in the gut, potentially making BC a more potent prebiotic (Nsor-Atindana et al., 2020).

The Fourier Transform Infrared Spectroscopy (FTIR) analysis provided detailed insights into the structural changes in bacterial cellulose (BC) produced by *N. pomaceti* KMPG_12 before and after high-pressure homogenization (HPH). The pre-HPH FTIR spectrum exhibited distinct peaks characteristic of a highly crystalline structure, marked by strong intermolecular hydrogen bonding. Notably, the peak around 3340 cm^{-1}, corresponding to OH stretching vibrations, indicated robust hydrogen bonds within the BC framework. After high-pressure homogenization, the spectrum changes. The peaks may become less sharp or shift slightly, indicating a change in the molecular environment of the cellulose. To provide a comprehensive understanding of the FTIR results, a comparative analysis with other bacterial cellulose-producing strains is presented in the following table 2. This comparison highlights the similarities and differences in the structural characteristics of BC produced by various strains, as revealed by FTIR spectra. To provide a comprehensive understanding of the FTIR results, a comparative analysis with other bacterial cellulose-producing strains is presented in the following table2. This comparison highlights the similarities and differences in the structural characteristics of BC produced by various strains, as revealed by FTIR spectra.

The comparative FTIR analysis reveals that bacterial cellulose from various strains exhibits similar peak positions, indicative of common structural features such as the OH stretching vibrations around 3300-3340 $cm^{-1}$and the C-H stretching vibrations near 2900 cm^{-1}. These peaks are typical for cellulose and reflect the presence of hydrogen bonding and the crystalline structure of the polymer (Anton-Sales et al., 2020; Dórame-Miranda et al., 2019; Francis et al., 2024; Portela et al., 2019) .

However, the extent of crystallinity and the robustness of hydrogen bonds can vary among strains, influencing the functional properties of the produced BC. For instance, the BC produced by *K. xylinus* (Francis et al., 2024), and *K. rhaeticus* (Anton-Sales et al., 2020), shows high crystallinity and strong hydrogen bonds, making them suitable for applications requiring high mechanical strength and durability, such as eco-friendly materials and biocompatible membranes. In contrast, the BC from *N. pomaceti* KMPG_12, post-HPH treatment, exhibits a less crystalline, more amorphous structure. This structural modification enhances the material's solubility and prebiotic properties, making it more effective for promoting probiotic growth (Guo et al., 2022; Ioelovich, 2021; Pirozzi et al., 2023). This transformation is crucial for applications in the food and pharmaceutical industries, where enhanced solubility and bioavailability are desired.

Conclusion

This study successfully demonstrated the potential of *N. pomaceti* KMPG_12 in producing bacterial cellulose (BC) with enhanced prebiotic properties through high-pressure homogenization (HPH). The optimization of BC production conditions using RSM resulted in a peak yield of 9.78 g/L under optimal conditions (15% glucose, 0.4% peptone, and 4.5% alcohol over a 14-day cultivation period). The subsequent HPH treatment significantly reduced the fiber size, transforming the BC into a more amorphous structure, which enhanced its solubility and prebiotic efficacy. Quantitative analysis showed a significant increase in bacterial proliferation ($P=0.0437$) when BC treated with HPH was used as a prebiotic compared to untreated BC. This enhanced prebiotic property of HPH-treated BC positions it as a viable alternative to established prebiotics such as inulin and xylo-oligosaccharides, providing a functional ingredient that supports gut health and microbial growth.

The findings of this study have important implications for sustainable BC production. The use of *N. pomaceti* KMPG_12, a strain capable of producing high yields of BC, combined with the optimization and enhancement techniques employed, provides a model for efficient and cost-effective BC production. The ability to utilize various substrates, including agro-industrial by-products, further supports the sustainability of this production method. The transformation of BC into a nano-sized form through HPH not only improves its functional properties but also opens up new avenues for its application in the food and pharmaceutical industries. This approach aligns with the principles of green technology by reducing waste and enhancing the value of bioproducts through physical, rather than chemical, modifications.

In conclusion, this study contributes to the field of sustainable bacterial cellulose production by demonstrating a method that maximizes yield, enhances functional properties, and utilizes eco-friendly processes. Future research should focus on scaling up this production method and exploring the in

vivo effects of nano-sized BC to fully harness its potential as a functional food ingredient and prebiotic.

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Contribution of Authors

Chaiyod R & Prakit B: Methodology, investigation, formal analysis and manuscript editing
Khongkool K: Investigation, formal analysis and writing of original draft
Chanasit W: Supervision and manuscript editing
Lertworapreecha M: Funding acquisition, conceptualization, supervision, visualization and manuscript editing

All authors discussed results, reviewed the manuscript, provided critical feedback, and approved the final version for publication.

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