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From Bacterial Cellulose Production by *Komagataeibacter xylinus* to Bacterial Cellulose Nanoparticles: A Standardized Enzymatic Approach

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Abstract

Bacterial cellulose (BC) is a renewable biopolymer valued for its exceptional purity, biocompatibility, and mechanical strength, with broad applications in biomedicine and sustainable materials. However, achieving reproducible BC production and downstream processing remains a major challenge. Inoculum preparation is particularly difficult to standardize because cellulose-producing strains form pellicles that sequester cells, making optical density measurements unreliable. In addition, recovery and drying procedures can alter fiber accessibility, and enzymatic hydrolysis conditions are often inconsistently defined and lack proper enzyme activity assessment. These issues contribute to substantial variability in BC-derived nanoparticle yields. This protocol describes the production of BC from *Komagataeibacter xylinus* DSMZ 6513, including culture medium preparation, inoculum generation, and scaling up under static cultivation conditions. It further details BC pellicle purification using NaOH, followed by pulping, freeze-drying, and milling to ensure material stability during storage and use. BC hydrolysis is performed with commercially available cellulase from *Trichoderma reesei*, with enzyme activity quantified prior to each reaction to ensure reproducibility. This standardized approach enables the reproducible production of bacterial cellulose nanoparticles (BCNPs). The protocol also includes minimal morphological characterization methods. By standardizing culture, recovery, and hydrolysis steps, the workflow reduces experimental variability and improves comparability across laboratories. Overall, it provides an accessible and reproducible method for generating BC and BCNPs of consistent quality without the need for specialized instrumentation.

Key features

- Standardized inoculum preparation that does not rely on optical density measurements, ensuring reproducible starting conditions for cellulose-producing strains.
- Controlled recovery and freeze-drying procedures that preserve fiber accessibility and promote consistent enzymatic hydrolysis outcomes.
- Use of commercially available *Trichoderma reesei* cellulase (≥700 units/g), with enzyme dosage defined by measured activity according to a publicly available Megazyme protocol.
- Minimal but robust morphological characterization methods to assess material quality.

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Keywords: Bacterial cellulose, *Komagataeibacter xylinus*, Bacterial cellulose nanoparticles, Enzymatic hydrolysis, Cellulase activity, Nanomaterial characterization, Sustainable biomaterials, Enzyme-assisted nanoparticle synthesis, Antimicrobial material platforms, Dynamic light scattering (DLS), Scanning electron microscopy (SEM), Transmission electron microscopy (TEM)

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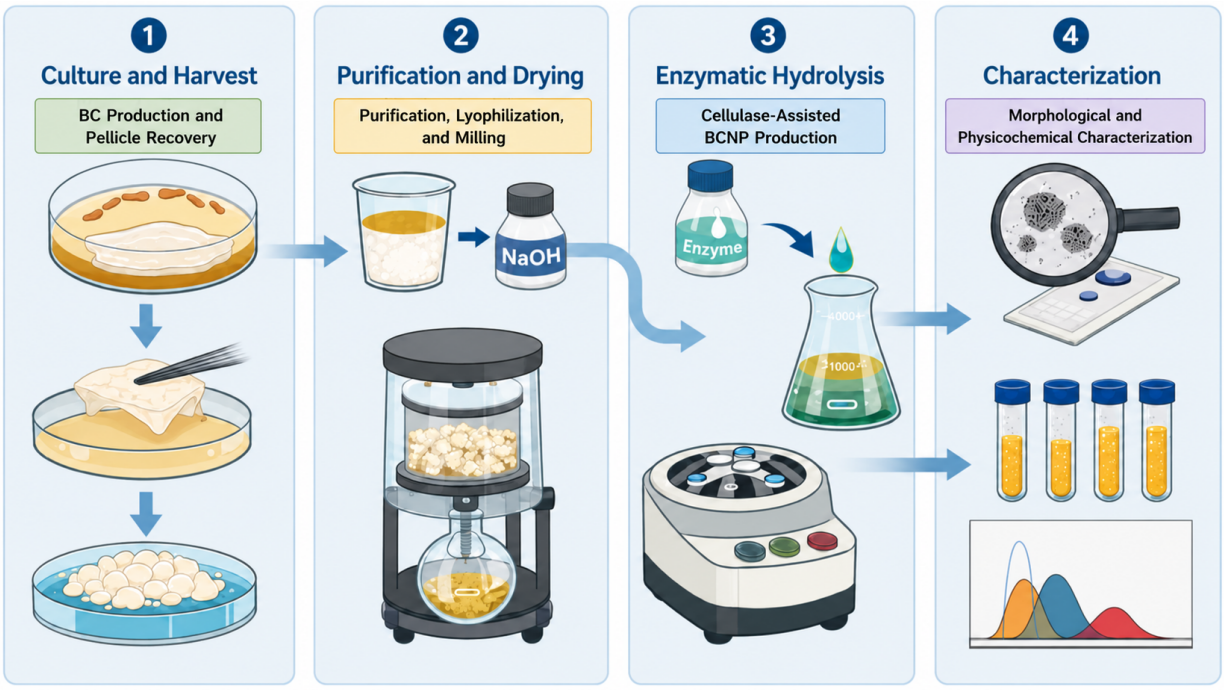
Graphical overview



Background

Nanocellulose has emerged as a versatile class of biomaterials with growing relevance in biotechnology, nanomedicine, and sustainable materials science [1]. Among its various forms, bacterial cellulose (BC) has attracted considerable attention due to its high purity, crystallinity, mechanical strength, and excellent biocompatibility [2,3]. Unlike plant-derived cellulose, BC is synthesized extracellularly by bacteria such as *Komagataeibacter xylinus*, producing a highly ordered three-dimensional nanofiber network free of lignin and hemicellulose. This intrinsic purity simplifies downstream processing and makes BC particularly suitable for biomedical applications [4].

Both BC and its nanoscale derivatives have been widely explored for applications including wound dressings, tissue engineering scaffolds, drug delivery systems, and antimicrobial materials, owing to their large surface area, mechanical stability, and capacity to interact with biological molecules [1,5]. Bacterial cellulose nanoparticles (BCNPs), in particular, represent promising nanocarriers for therapeutic agents. Their nanoscale dimensions and physicochemical properties enable efficient loading of bioactive molecules while maintaining high biocompatibility and environmental sustainability. Multiple strategies have been developed to obtain cellulose nanoparticles from bulk cellulose fibers. Traditional methods often rely on strong acid hydrolysis or other aggressive chemical treatments, which can produce hazardous waste, require extensive purification, and alter the resulting nanoparticles' physicochemical properties [6]. Enzymatic hydrolysis using cellulases has recently emerged as a more sustainable alternative, enabling controlled fragmentation of cellulose macrofibers under milder conditions and reducing environmental impact [5]. Indeed, the successful exploitation of BCNPs in biomedical and biotechnological applications requires reliable control over critical parameters, including particle size, size distribution, morphology, and surface properties, which directly influence their performance as functional nanomaterials. Therefore, standardized and reproducible production strategies are essential to ensure consistent BCNP characteristics and facilitate

their comparison across different studies and their translation toward practical applications.
Recent studies have shown that BCNPs generated through cellulase-mediated hydrolysis can serve as efficient platforms for the delivery of antimicrobial peptides (AMPs), improving peptide stability and enabling the development of sustainable antimicrobial formulations. For example, BCNPs produced from *K. xylinus* were functionalized with a human AMP via non-covalent interactions, yielding nanomaterials that inhibited bacterial growth while maintaining good biocompatibility with human cells [7]. Despite these advances, reproducibility in BC production and processing remains a major challenge. Sources of variability include differences in microbial growth kinetics, nutrient composition, oxygen availability, and cultivation scale, which can affect BC yield, fiber organization, and the final properties of the material. Furthermore, variations in purification strategies and drying procedures may influence cellulose crystallinity, aggregation state, and subsequent enzymatic accessibility. Therefore, the development of standardized protocols that define critical parameters throughout BC production and nanoparticle generation is essential to ensure consistent material quality and facilitate the translation of BC-based nanomaterials into research and industrial applications [3,6]. In particular, inoculum standardization, pellicle recovery, and control of enzymatic hydrolysis conditions can strongly influence nanoparticle yield and morphology. Many published procedures lack standardized approaches for culture preparation, enzyme activity assessment, and downstream handling of BC macrofibers, limiting inter-laboratory reproducibility [3,6].
The protocol presented here provides a standardized workflow for producing BC and generating BCNPs through controlled enzymatic hydrolysis. It includes reproducible procedures for culture preparation, pellicle purification, freeze-drying, and milling, as well as quantification of cellulase activity prior to hydrolysis. Compared with previously described approaches, this protocol emphasizes reproducibility, accessibility, and minimal instrumentation, while remaining compatible with common nanoparticle characterization techniques.
Beyond BCNPs production, this workflow supports a wide range of downstream applications, including antimicrobial formulations, nanocarriers for bioactive molecules, and functional biomaterials for biomedical and biotechnological use.

Materials and reagents

Biological materials

1. Bacterial strain: *Komagataeibacter xylinus* DSMZ 6513, gram-negative, aerobic, non-motile acetic acid bacterium characterized by the ability to produce extracellular bacterial cellulose as a pellicle; German Collection of Microorganisms and Cell Cultures GmbH (Leibniz Institute DSMZ, Germany)
2. Cellulase enzyme: Cellulase from *Trichoderma reesei*, aqueous solution ≥700 units/g (Merck Life Science S.r.l., catalog number: C2730)

Reagents

Note: All chemicals used were standard laboratory-grade reagents suitable for microbiological and biochemical applications.
1. Acetic acid (Merck, catalog number: A6283)
2. Sodium hydroxide (NaOH), pellets, anhydrous (Merck, catalog number: 221465)
3. Peptone (Merck, catalog number: 70028)
4. Yeast extract (Merck, catalog number: Y1625)
5. Disodium phosphate (Na_2HPO_4) (Merck, catalog number: 71699)
6. Citric acid monohydrate (Merck, catalog number: C1909)
7. D-(+)-Glucose (Merck, catalog number: G8270)
8. Agar (for solid media) (Merck, catalog number: A1296)
9. Sodium acetate trihydrate (Merck, catalog number: 29152900)
10. Azo-CM-Cellulose powder (Megazyme, catalog number S-ACMC)
11. Milli-Q water (or equivalent ultrapure water system)

Solutions

1. Hestrin–Schramm (HS) liquid medium (see Recipes)
2. HS agar (see Recipes)

3. 2 M sodium acetate buffer, pH 5.0 (see Recipes)
4. 1 M NaOH (see Recipes)

Note: Solutions for the Azo-CM-Cellulose assay were prepared according to the publicly available Megazyme protocol (Megazyme Ltd., Ireland; catalog number S-ACMC), including all buffers and dilutions required for enzyme activity quantification.

Recipes

1. HS liquid medium, pH 4.5

Reagent	Final concentration	Quantity or volume
Peptone	5 g/L	5 g
Yeast extract	5 g/L	5 g
Na_2HPO_4	2.7 g/L	2.7 g
Citric acid monohydrate	1.15 g/L	1.15 g
D-(+)-Glucose (separately autoclaved)	20 g/L	40 mL concentrated solution*
Milli-Q water	n/a	960 mL
Total	n/a	1,000 mL

*Glucose is prepared as a concentrated solution and autoclaved separately to prevent Maillard reactions.

1. For 1 L of liquid culture medium, in a graduated cylinder, dissolve 5 g of peptone, 5 g of yeast extract, 2.7 g of disodium phosphate, and 1.15 g of citric acid in 960 mL of Milli-Q water. Transfer the solution into a heat-resistant bottle (Sol. A).
2. In a separate clean cylinder, dissolve glucose in Milli-Q water at a concentration of 500 mg/mL and transfer the solution into a heat-resistant bottle (Sol. B). The solution is viscous compared to water but remains fluid and easy to handle.
3. Autoclave Sol. A and Sol. B separately. After sterilization by autoclaving at 121 °C for 20 min, aseptically add 40 mL of sterilized Sol. B to Sol. A and mix thoroughly. This yields 1 L of culture medium with a final glucose concentration of 20 g/L.
4. Use immediately for inoculation or store under sterile conditions at room temperature.

2. HS agar

Reagent	Final concentration	Quantity or volume
Peptone	5 g/L	5 g
Yeast extract	5 g/L	5 g
Na_2HPO_4	2.7 g/L	2.7 g
Citric acid monohydrate	1.15 g/L	1.15 g
Agar	20 g/L	20 g
D-(+)-Glucose (separately autoclaved)	20 g/L	40 mL concentrated solution*
Milli-Q water	n/a	960 mL
Total	n/a	1,000 mL

*Glucose is prepared as a concentrated solution and autoclaved separately to prevent Maillard reactions.

1. For 1 L of solid culture medium, add 20 g of agar to Sol. A (see Recipe 1) before autoclaving.
2. Proceed as for liquid culture medium.
3. After autoclaving, and while still hot, aseptically combine Sol. A and Sol. B. Immediately pour 10 mL into each Petri dish and allow to solidify at room temperature. A volume of 10 mL is intentionally selected because it is sufficient to uniformly cover the bottom of a 90 mm Petri dish while producing a relatively thin agar layer. This facilitates the excision of the agar disc used for inoculum preparation compared with the thicker agar layer obtained using the more conventional pouring volume of 20 mL.
4. It is recommended to prepare multiple Petri dishes at once to avoid re-melting or reheating the solidified medium.
5. After solidification, store Petri dishes at 4 °C under sterile conditions until use.

3. 2 M sodium acetate buffer, pH 5.0

Reagent	Final concentration	Quantity or volume
Sodium acetate trihydrate	2 M	272.2 g
Acetic acid (glacial)	adjust pH	as needed
Milli-Q water	n/a	to 1 L

Sterilize all stock solutions by filtering them through a 0.22 µm membrane under a laminar flow hood. Store all stock solutions at 4 °C. Dilute sodium acetate buffer to a 0.1 M working concentration immediately before use in enzymatic hydrolysis. Check the pH after dilution, as minor variations may affect enzyme activity.

4. 1 M NaOH, pH ~14.0

Reagent	Final concentration	Quantity or volume
Sodium hydroxide pellets (anhydrous)	1 M	40 g/L
Milli-Q water	n/a	to 1 L

Store the 1 M NaOH stock solution at room temperature. Dilute 1:10 to obtain a 0.1 M NaOH solution immediately before use for BC purification.

Laboratory supplies

1. 50 mL Falcon tubes (Merck, catalog number: CLS430829)
2. 100 mL Erlenmeyer flasks (Merck, catalog number: Z744843)
3. Sterile 1 L Erlenmeyer flasks (Merck, catalog number: SLW1135/26D)
4. Autoclavable bottles, 500 mL (Merck, catalog number: DWK61110P-500)
5. Autoclavable bottles, 1 L (Merck, catalog number: DWK61110T1000)
6. Micropipettes (Gilson), P20, P200, P1000 (Colaver, catalog number: 43GI3600)
7. Sterile pipette tips for Gilson P20, P200, P1000 (Merck, catalog numbers: F196087, F196088, F196089)
8. Sterile plastic disposable pipettes, 5 and 10 mL (Merck, catalog numbers: CLS70775N, CLS707710N)
9. Sterile L-shaped plastic loop, disposable (Merck, catalog number: HS8171A)
10. Sterile 90 mm Petri dishes (Merck, catalog number: Z717223)
11. Heat-resistant glass beakers, 250 and 400 mL (Merck, catalog numbers: BR91236, BR91241)
12. 40 mL glass flat-bottom tubes (Merck, catalog number: 27184)
13. Sterile 1.5 mL polypropylene centrifuge tubes (Merck, catalog number: HS4323)
14. 1 L graduated cylinders (Merck, catalog number: CLS3022P1L)
15. Magnetic stir bars (Merck, catalog number: Z745058)
16. Sterile 0.22 µm filters (Merck, catalog number: WHA10463607)
17. Stainless steel forceps (Merck, catalog number: Z168777)
18. Aluminum foil (Merck, catalog number: Z691577)
19. Parafilm M laboratory film (Merck, catalog number: P7793)
20. Disposable microcuvettes for dynamic light scattering (DLS) measurements (Malvern Panalytical, catalog number: ZEN0040)
21. Folded capillary zeta cell (Malvern Panalytical, catalog number: DTS1070)
22. Aluminum SEM pin stub mounts (Merck, catalog number: 933155)
23. Black conductive adhesive tabs for SEM (Ted Pella, catalog number: 16084-3)
24. Round glass microscope slides (Merck, catalog number: 63413)
25. 200-Mesh carbon-coated copper TEM grids (Merck, catalog number: 930369)

Equipment

1. Laminar flow hood (NuAire, Inc. Biological Safety Cabinet, ID 81718, model: NU-440-600E)
2. Chemical fume hood (Dynamika 150, ID 007)
3. Magnetic stirrer with heating plate and temperature probe (Four E'S Scientific Co., Ltd., ID LS52P051052)
4. Freeze-dryer (Lyoques, Hosmotic, ID C61644)
5. Milli-Q water purification system (Merck KGaA, Millipore Sigma, model: Q-Gard 1)
6. Analytical balance (0.1 mg precision) (Mettler-Toledo International Inc., model: XPR204S; ID XPR204S)

7. pH meter (Jenway, Cole-Parmer Instrument Company Ltd., ID 3510)
8. Hot plate with thermocouple (Kartell S.p.A., model: TechnoKartell TK22)
9. Countertop jar blender (Munro Instruments Ltd., catalog number: 800 G)
10. IKA® M 20 universal mill (IKA-Werke GmbH & Co. KG, catalog number: Z645141)
11. Water bath (GFL Gesellschaft für Labortechnik mbH, ID GFL_20001)
12. Orbital incubator (Stuart Equipment, Cole-Parmer Ltd., model: SI600)
13. Vortex mixer (Kartell S.p.A., model: TechnoKartell TK3S; ID 1892)
14. UV lamp (integrated UV sterilization lamp of biological safety cabinet) (NuAire Inc., model: NU-440-600E; ID 81718)
15. Refrigerated microcentrifuge (Thermo Fisher Scientific Inc., ID Fresco 21)
16. Fisherbrand™ Elmasonic Select 30 ultrasonic bath (Thermo Fisher Scientific Inc., FEI Company, Waltham, Massachusetts, USA)
17. Zetasizer Nano ZSP system for DLS and electrophoretic light scattering (ELS) measurements (Malvern Panalytical Ltd., Worcestershire, UK)
18. Sputter coater for SEM sample preparation (Denton Vacuum LLC Desk V, Moorestown, NJ, USA)
19. Scanning electron microscope (FEI Nova NanoSEM 450; Thermo Fisher Scientific Inc., FEI Company, Waltham, Massachusetts, USA)
20. Transmission electron microscope (FEI Tecnai G2 200 kV; Thermo Fisher Scientific Inc., FEI Company, Waltham, Massachusetts, USA)

Software and datasets

1. Zetasizer Software 8.02 (Malvern Panalytical Ltd., Malvern, Worcestershire, UK), www.malvernpanalytical.com
2. GraphPad Prism version 8.4.3 for Windows (GraphPad Software, LLC, San Diego, California, USA), www.graphpad.com
3. Microsoft Excel version 365 (Microsoft Corporation, Redmond, Washington, USA), www.microsoft.com

Procedure

A. Culture starter assessment and cellulose production

Note: Quantifying cells for inoculation is challenging for this strain because it produces a solid BC pellicle that traps cells. As a result, optical density measurements are not reliable. The following procedure ensures a uniform and reproducible inoculum.

A1. Solid starter preparation
1. Inoculate 5 mL of HS liquid medium with 10% (v/v) of a 48-h-old mother culture in which a well-formed cellulose pellicle is developed.
2. **(Critical)** Vortex the culture thoroughly before sampling to release cells trapped within the cellulose pellicle (two pulses at approximately 3,000 rpm).
3. Incubate the culture statically at 30 °C for 24 h.
4. After incubation, vortex the liquid starter to resuspend the cells (two pulses at approximately 3,000 rpm).
5. Transfer 1 mL of the liquid culture onto an HS agar plate and spread it using a sterile L-shaped loop; leave the inoculated plates under a laminar flow hood until complete evaporation of the inoculum (approximately 1.5 h at 25 °C under ambient humidity of ~35%) before incubation.
Note: *Using a micropipette is recommended. When aspirating the sample, the pellicle may adhere to the pipette tip. To prevent device damage caused by sudden suction, aspirate slowly and avoid direct contact with the pellicle.*

6. Allow the liquid to evaporate completely under a sterile laminar flow hood (approximately 1.5 h at 25 °C under ambient humidity of ~35%).
Critical: Do not spread the culture during drying. Uniform evaporation under sterile conditions ensures even deposition and promotes consistent growth on the solid medium.

7. Once the agar surface is fully dry, incubate the plate at 30 °C under static conditions for 24 h or until the colonies form a visible, uniform layer.

A2. Cellulose production in liquid medium

Once a uniform starter culture has been obtained, the culture can be scaled up for cellulose production as follows:

1. Using the back end of a sterile yellow P200 pipette tip, excise a small agar disc (approximately 5 mm in diameter) containing bacterial cells from the starter plate.
2. Transfer the agar disc into 5 mL of HS liquid medium in a 50 mL Falcon tube. Incubate statically at 30 °C for 24 h.

Critical: Maintain static incubation at 30 °C through the entire scaling-up process. Using an agar disc from a uniform starter ensures consistent inoculation and homogeneous cellulose production. Do not shake or agitate the culture, as this disrupts proper pellicle formation.

3. Scale up by transferring the culture into HS medium to a final volume of 30 mL in a 100 mL flask. Incubate statically at 30 °C for 24 h.
4. Further scale up by transferring the culture into HS medium to a final volume of 300 mL in a 1 L flask, reaching the conditions for effective cellulose production. Incubate statically at 30 °C for 7 days.

B. Recovery of bacterial cellulose (BC)

After completion of the liquid culture (7 days at 30 °C under static conditions), BC pellicles form at the air–liquid interface. The following procedure describes their recovery, purification, and processing into macrofibers suitable for enzymatic hydrolysis. Representative images of bacterial cellulose production and purification are reported in **Figure 1**.

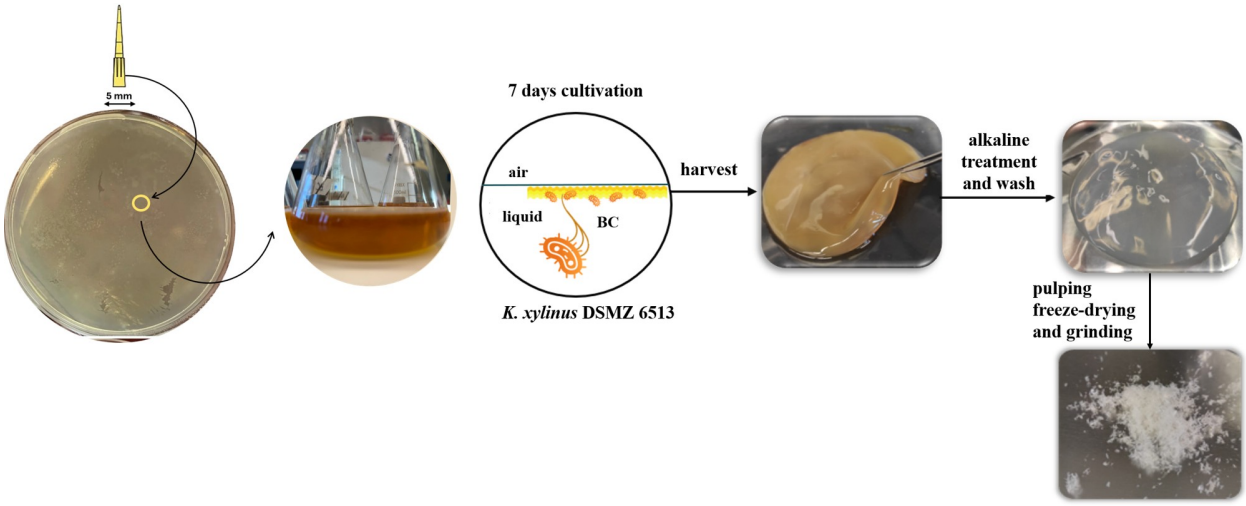


Figure 1. Representative images of bacterial cellulose production and purification. Cells from a *Komagataeibacter xylinus* culture plate are inoculated into Hestrin–Schramm (HS) liquid medium, where a bacterial cellulose (BC) pellicle is produced after 7 days of static incubation. The pellicle is then harvested, purified by NaOH treatment, extensively washed with Milli-Q water, and freeze-dried to obtain purified bacterial cellulose suitable for subsequent enzymatic hydrolysis.

1. Carefully remove the BC pellicle from the air–liquid interface using sterile forceps.
2. Transfer the pellicle to a heat-resistant glass beaker.
3. Gently rinse the pellicle with Milli-Q water for 30 min to remove loosely attached cells and residual medium.
4. Prepare a 0.1 M NaOH solution and ensure that the pellicle is completely submerged.

Caution: Always add NaOH to water slowly and under stirring to minimize the risk of exothermic splashing.

5. Treat the pellicle by placing the beaker on a heated shaking plate equipped with thermocouple temperature control. Heat at 60 °C for 4 h, maintaining continuous gentle agitation to promote uniform chemical treatment.
6. Remove and rinse the pellicles extensively with Milli-Q water until the wash solution reaches neutral pH (approximately pH 7.0), ensuring the complete removal of residual NaOH and soluble impurities. The number of washing steps may vary

depending on the amount of residual alkali; therefore, reaching neutral pH is considered the most appropriate endpoint to ensure reproducibility of the purification procedure.
Critical: Multiple washing cycles may be required. Ensure complete removal of NaOH, as residual base can interfere with downstream applications.

7. Pulp the cleaned pellicles using a Munro Instruments 800 G countertop jar blender (maximum speed approximately 22,000 rpm) by applying 10 short pulses. The pellicles should be homogenized in approximately 40 mL of Milli-Q water to obtain uniform cellulose fragments suitable for subsequent freeze-drying.
Critical: Before use, thoroughly clean and sterilize the blender jar and blades to minimize contamination. Avoid prolonged blending, as excessive mechanical treatment may alter the morphology of the cellulose macrofibers.
Note: Resuspend the pulped material in a defined volume of Milli-Q water (e.g., 40 mL) to standardize the preparation across batches. Maintaining a consistent water volume during lyophilization ensures reproducible fiber/flake size and enzyme accessibility. It is noteworthy that pellicles release water during pulping; adjust the final volume accordingly.

8. Freeze the BC suspension (40 mL) overnight at -80 °C and subsequently freeze-dry for at least 24 h.
Note: Do not use oven-drying as it produces a compact, rigid film that reduces enzyme accessibility. Freeze-drying preserves a loose, porous structure ideal for enzymatic hydrolysis.

9. Determine the weight of purified BC by weighing the sample before and after freeze-drying. This provides the cellulose yield for batch comparison and reproducibility assessment.
10. Grind the freeze-dried material to obtain BC macrofibers (flakes) suitable for enzymatic hydrolysis.
Note: Freeze-dried BC was ground using a laboratory coffee grinder by applying approximately 10 short pulses until a homogeneous fibrous powder was obtained. Under the culture conditions described in this protocol, a typical K. xylinus *pellicle weighs approximately 19 g (wet weight), corresponding to approximately 50 mg of freeze-dried BC. The final biomass yield may vary depending on culture conditions and bacterial physiological state. Freeze-dried BC macrofibers can be stored at room temperature in a sealed container to prevent moisture uptake, particularly under humid environmental conditions.*

C. Estimation of cellulase activity

Cellulase activity should be determined and expressed as units/mL prior to hydrolysis.

1. To measure activity, use the Azo-CMC substrate (Megazyme) and follow the procedure described in the corresponding manufacturer's protocol (see General notes).
Note: The enzymatic activity measured for the enzyme batch used in our study was 989.5 U/mL. Since this value is batch-specific, it should be considered as a representative example rather than as a fixed reference value.
Critical: Ensure that cellulase activity is validated under the same experimental conditions used for BC hydrolysis. Specifically, perform the assay using 0.1 M sodium acetate buffer (pH 5.0) and incubate the reaction mixture at 40 °C for the incubation time specified in the protocol. This step is essential to ensure that the measured activity accurately reflects performance under hydrolysis conditions.

D. Enzymatic hydrolysis for BCNP production and recovery

1. Suspend 30 mg of freeze-dried BC macrofibers in 30 mL of 0.1 M sodium acetate buffer (pH 5.0) in a glass flat-bottom tube containing a magnetic stir bar. Sterilize the suspension by exposing it to UV light for 15 min.
Note: The indicated buffer volume ensures that, after enzyme addition, the final BC concentration is 1 mg/mL. UV sterilization is performed prior to the addition of cellulase; therefore, the enzyme is not exposed to UV irradiation during the hydrolysis step, and its activity is not affected by this procedure. UV exposure is used for sterilization and is required if BC is handled under non-sterile conditions, as mold contamination can develop and compromise hydrolysis efficiency.
Critical: BC macrofibers are extremely light and easily dispersed in ventilated environments. Handle the material carefully to avoid loss of sample. Remove gloves during manipulation, if necessary, as static charges may cause macrofibers to adhere to glove surfaces.

2. Incubate the BC suspension at 40 °C until a stable temperature is reached, continuously stirring the suspension at 200 rpm using a magnetic stirrer equipped with a heating plate and a temperature-control probe placed directly in the suspension. Seal the top of the glass tube with Parafilm to prevent solvent evaporation.
3. Based on the concentration of the purchased enzyme solution obtained in section C, prepare a 200 U/mL concentrated stock in 0.1 M sodium acetate buffer, pH 5.0, and pre-incubate the stock at 40 °C in a static water bath.
Notes:
1. It should be specified that the enzymatic activity measured for the enzyme batch used in our study was 989.5 U/mL. Since this value is batch-specific, it should be considered as a representative example rather than as a fixed reference value.
2. Prepare the diluted stock by applying the equation $V_i = \frac{C_f}{Ci} \times V_f$.
For example, if the purchased enzyme solution has an activity of 989.5 U/mL, pipette 606.4 µL of the enzyme into a final volume of 3 mL of 0.1 M sodium acetate buffer.
Critical: It is essential to equilibrate both the substrate and the enzyme before mixing. This ensures consistent reaction timing and avoids temperature fluctuations that may affect enzyme activity.

4. Once both the substrate and enzyme are equilibrated, add 3 mL of the equilibrated enzyme solution to the BC suspension while stirring.
Critical: The reaction starts immediately upon enzyme addition.
Note: After addition, the final concentrations will be 1 mg/mL BC and 20 U/mL enzyme.

5. Incubate at 40 °C under gentle agitation (200 rpm) for 24 h.
6. After the selected reaction time, transfer the reaction mixture into 1.5 mL conical centrifuge tubes and centrifuge at 14,000× *g* for 30 min at 25 °C.
Critical: For the following steps, use centrifuge tubes that are transparent to UV light.
Note: A refrigerated centrifuge is recommended, as sample overheating may cause nanoparticle aggregation. After centrifugation, a white, solid BCNP pellet should be visible.

7. Discard the enzyme-rich supernatant and carefully resuspend the pellet in an equal volume of Milli-Q water to perform a washing step.
Critical: Ensure complete resuspension of the pellet. Use a vortex mixer instead of a micropipette to avoid nanoparticle adsorption onto pipette tips.

8. Centrifuge the suspension again as described in step D6 and discard the supernatant.
9. Resuspend the BCNPs in the same volume of water to obtain a 1× suspension, or adjust the final volume as needed for subsequent experiments.
10. Expose the resuspended BCNPs to direct UV light for 20 min to ensure proper sterilization.
11. Place the resuspended nanoparticles in an ultrasonic bath with room-temperature water and sonicate for 15 min in pulse mode (Fisherbrand™ Elmasonic Select 30 ultrasonic bath operating at a frequency of 37 kHz).
Critical: Ensure that the bath temperature does not increase, as this may promote nanoparticle aggregation.

12. Store the BCNPs at room temperature under standard laboratory conditions and periodically monitor them over time for any signs of aggregation.

E. Dynamic light scattering (DLS) and electrophoretic light scattering (ELS)

1. Dilute the resuspended BCNPs to an appropriate concentration with Milli-Q water; a minimum 1:10 dilution is recommended.
Note: The dilution strongly influences BCNP aggregation. Use a consistent dilution factor when analyzing or comparing different BCNP production batches.
Critical: Ensure the sample is well diluted to avoid particle aggregation during measurement.

2. Transfer 1 mL of the diluted sample into a disposable micro cuvette suitable for DLS or ELS measurements.
3. Measure particle size distribution and zeta potential using a Zetasizer Nano ZSP system. Select *Measure* → *Manual* and define the following parameters:
a. Cuvette: ZEN 0040

b. Dispersant: Water
c. Material: Cellulose
d. Temperature: 25 °C
e. Number of readings: 3
f. Equilibration: 120 s (for DLS measurements)
Note: Results should represent the average of at least three independent experiments, each consisting of three repeated measurements, to ensure reproducibility.

4. Perform data analysis as reported in the Data analysis section.

F. Scanning electron microscopy (SEM)

1. Prepare a suitable dilution of the BCNP sample to ensure that the nanoparticle concentration is suitable for SEM analysis. A 1:20 to 1:50 dilution is recommended for optimal visualization.
Note: It is advisable to test different dilution factors to determine the best conditions for proper imaging.

2. Pipette 15 µL of the diluted sample onto a clean glass slide.
3. Allow the aliquot to air-dry completely on a flat surface at room temperature. This step removes residual solvent and promotes particle adhesion to the surface.
Note: Do not apply heat during drying, as this may alter BCNP coloration.

4. Sputter coating preparation: Using tweezers and an adhesive suitable for SEM sample mounting, affix the glass slide onto aluminum SEM stubs.
5. Coat the surface of the glass slide with a 10-nm thin layer of gold-palladium (Au-Pd) using a sputter coater (Denton Vacuum Desk V).
Note: This step enhances the electrical conductivity of the sample and prevents charging during SEM imaging.

6. Load the coated sample into the SEM chamber and perform imaging using a FEI Nova NanoSEM 450 at an accelerating voltage of 5 kV.
Critical: Use the Everhart–Thornley detector (ETD) and through-lens detector (TLD) to obtain high-resolution images at a high magnification, enabling detailed visualization of BCNP morphology.

G. Transmission electron microscopy (TEM)

1. Prepare a proper dilution of the BCNP sample for TEM analysis. A minimum 1:20 dilution is recommended for proper visualization.
Note: *It is advisable to test different dilution factors to determine the optimal conditions for imaging.*

2. Pipette 4 µL of the diluted sample onto a 200-mesh carbon-coated copper grid.
Critical: Electrostatic forces may attract the grid to the plastic pipette tip. To minimize this risk, dispense the sample slowly and steadily, depositing it directly at the center of the grid. If needed, use clean, sterile tweezers to gently secure the grid on a clean surface for easier handling. Avoid touching the top (sample deposition) surface to prevent contamination or deformation that may impair TEM imaging quality.

3. Allow the sample to air-dry completely on the grid to ensure that the particles adhere properly.
4. Place the fully dried grid onto the TEM sample stage.
5. Perform TEM analysis using a FEI TECNAI G2 200 kV microscope.

Data analysis

DLS and ELS data analysis and visualization

In the *Record View* tab of the Zetasizer software, select all the records that need to be averaged, right-click and choose *Copy*, and paste the data into a new Excel file for automatic analysis. This procedure generates a table as shown in **Figure 2**.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Record	Type	T	Z-Ave	PdI	Pk 1 Mean Int	Pk 2 Mean Int	Pk 3 Mean Int	Pk 1 Area Int	Pk 2 Area Int	Pk 3 Area Int	Scattering Angle
2			°C	d.nm		d.nm	d.nm	d.nm	Percent	Percent	Percent	°
3	667	Size	25	986.6	0.805	290.9	0	0	100	0	0	173
4	668	Size	25	1175	0.77	369.9	0	0	100	0	0	173
5	669	Size	25	1153	0.849	271	0	0	100	0	0	173
6	721	Size	25	743.5	0.602	383.3	68.98	0	94	6	0	173
7	722	Size	25	611.8	0.678	410.6	130.6	0	85.1	14.9	0	173
8	723	Size	25	1539	0.99	353.8	0	0	100	0	0	173
9	Mean		25	1035	0.782	347	28.51	0	97	3	0	173
10	Std Dev		0	333	0.135	55	51.84	0	6	5.7	0	0

Figure 2. Generated Excel table. The image shows an example of an automatically generated table after pasting the selected records into Excel. The subsequent editing step is intended to facilitate data visualization. Values highlighted in red were considered indicative for the analysis of the entire bacterial cellulose nanoparticle (BCNP) suspension (Z-Ave; PdI) or the most abundant BCNP population (Pk 1 Mean Int; Peak 1 Area Int) and were therefore used, when appropriate, for data analysis and result representation.

Light scattering techniques can be used as a preliminary tool for particle size characterization. It should be noted that DLS was originally developed for the analysis of spherical particles. Therefore, when anisotropic particles such as rod-like nanocellulose are analyzed, the instrument software reports an equivalent hydrodynamic diameter corresponding to the hydrodynamic behavior of a sphere with similar diffusion properties rather than the actual particle dimensions. Consequently, the random orientation of anisotropic nanoparticles in suspension can broaden the apparent size distribution, making it challenging to obtain low polydispersity index (PdI) values. Despite these limitations, DLS remains a valuable technique for the preliminary characterization of nanoparticle suspensions and for identifying the predominant particle populations.
The results reported in **Figure 2** are graphically represented in **Figure 3**. Although the Z-average diameter was 1,035 ± 333 nm, indicating a broad apparent particle size distribution, this parameter is strongly influenced by the presence of larger particles and aggregates because light scattering intensity increases with particle size. Consequently, the Z-average mainly reflects the overall heterogeneity of the suspension. This interpretation is supported by the high polydispersity index (PdI = 0.78 ± 0.14), which indicates that the sample exhibits a broad size distribution. For anisotropic materials such as rod-like nanocellulose, the intensity-based size distribution provides more informative insight into the predominant particle population. As shown in **Figure 2**, the main intensity peak (Peak 1 Mean Intensity) was centered at 347 ± 55 nm, indicating that the predominant population consists of nanoparticles with an equivalent hydrodynamic diameter in the range of approximately 350–400 nm. Furthermore, Peak 1 accounted for 97% ± 6% of the total scattered intensity, indicating that this population dominated the scattering signal under the measurement conditions. The difference between the Z-average diameter and the main intensity peak size suggests the presence of a small fraction of larger fibers or aggregates within the suspension. Because light scattering intensity is strongly dependent on particle size, these larger structures disproportionately influence both the Z-average diameter and the PdI. Consequently, although the Z-average and PdI provide useful indicators of sample heterogeneity, the Peak 1 diameter and its relative intensity offer a more informative description of the predominant nanoparticle population, indicating that the suspension is mainly composed of nanoparticles with an equivalent hydrodynamic diameter of approximately 350–400 nm. Because of the anisotropic morphology of nanocellulose and the broad size distribution of the suspension, DLS measurements should be regarded primarily as a descriptive characterization tool rather than as an absolute measure of particle dimensions. Replicate measurements are therefore essential to assess the reproducibility of the analysis and to account for sample heterogeneity. In this context, reporting the mean value together with the corresponding standard deviation is generally sufficient to describe the variability of the analyzed suspension. DLS analyses were performed using two independent biological replicates, each analyzed through three repeated instrumental measurements. Inferential statistical analyses are of limited relevance when the objective is the physicochemical characterization of a single sample, but become appropriate when comparing different formulations, treatments, or processing conditions.

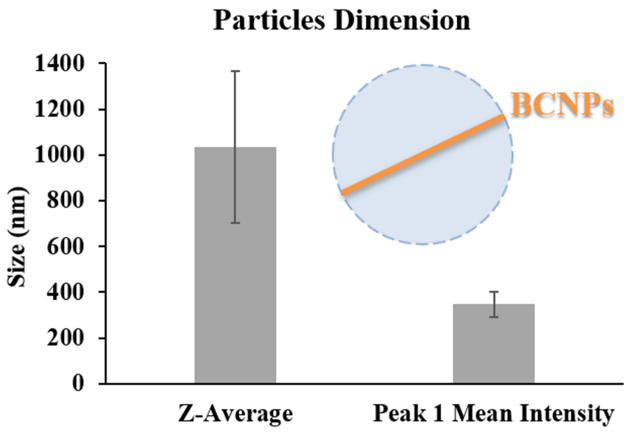


Figure 3. Representative dynamic light scattering (DLS) output for bacterial cellulose nanoparticle (BCNP) characterization. The Z-average diameter (Z-Average) represents the average hydrodynamic diameter of the overall particle population (schematically represented by the blue circle with the orange diagonal line), whereas the Peak 1 Mean Intensity corresponds to the size of the predominant BCNP population detected in the sample. Together with the polydispersity index (PdI), these parameters are used to evaluate particle size distribution and sample homogeneity.

When the objective is to compare different samples, statistical analyses may be performed according to the experimental design using GraphPad Prism. A representative GraphPad Prism data table and a graph layout used for the statistical analysis are shown in **Figure 4**.

Critical: Enter all raw data into the data table before performing any statistical analysis. This step is essential to ensure the appropriate application of statistical tests and accurate interpretation of the results.

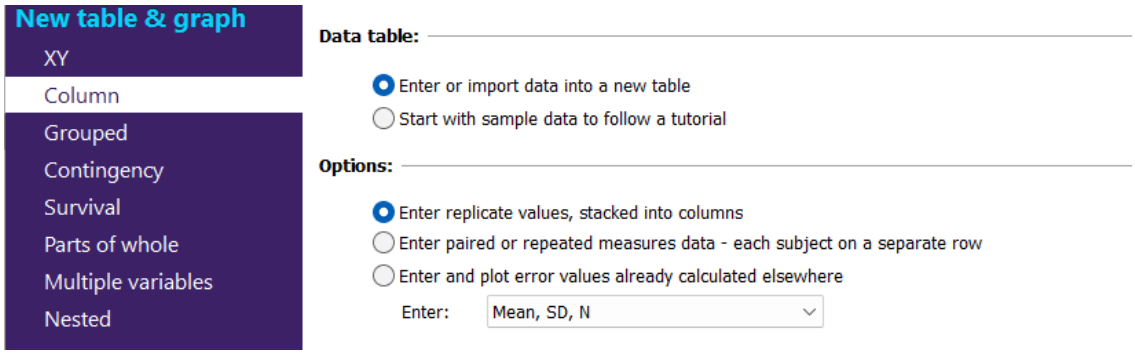


Figure 4. Representative GraphPad Prism data table and graph layout for statistical analysis. When comparing two independent groups (e.g., control and treated samples), differences can be evaluated using an unpaired two-tailed Student's t-test. However, alternative statistical approaches, including paired tests or one-way ANOVA, may be more appropriate depending on the experimental design, number of groups, and type of comparison. An example workflow based on an unpaired two-tailed Student's t-test is provided below.

Select *Analyze* → *t test* to evaluate the significance of the deviation with respect to the control sample. Then, set the parameters as reported in **Figure 5**.

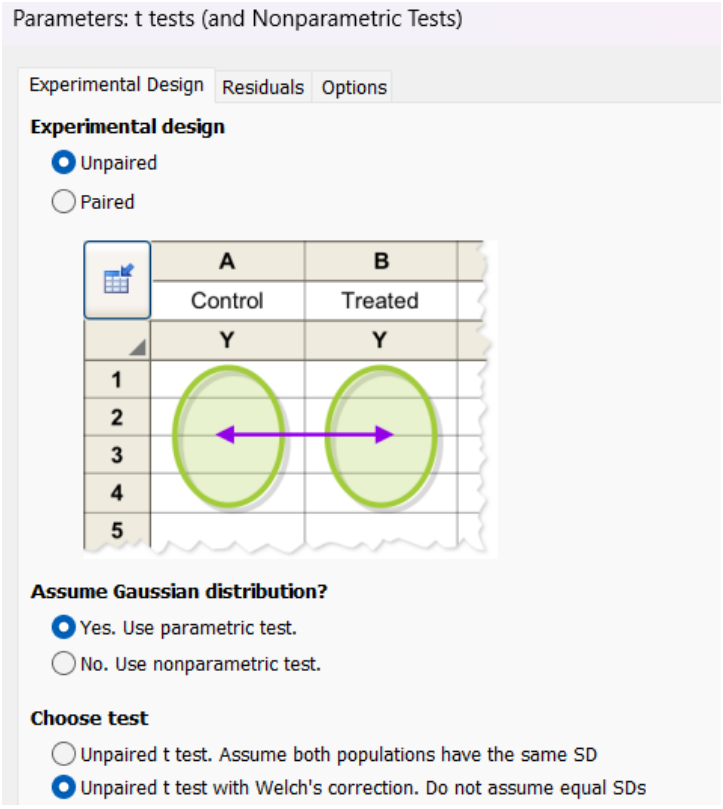


Figure 5. Unpaired two-tailed t-test settings in GraphPad Prism software

Validation of protocol

The protocol described here was experimentally validated in [7], in which BCNPs were produced and characterized using the same experimental workflow. Enzymatic hydrolysis was monitored by DLS through time-course analyses under dynamic conditions (3–24 h), demonstrating the progressive fragmentation of bacterial cellulose and the formation of BCNPs, and allowing optimization of the hydrolysis process (Figure 2A and Tables 2 and S2 from [7]). Nanoparticle morphology was further assessed by SEM and TEM, confirming the formation of nanoscale cellulose particles and revealing their expected tendency to aggregate during sample preparation (Figure 2B, C from [7]). Finally, different centrifugation conditions were evaluated to optimize nanoparticle recovery, identifying 10,000× *g* as the most suitable condition, providing a reproducible balance between recovery efficiency, particle size distribution, and sample homogeneity (Figure 2D and Table 3 from [7]). Overall, these results support the robustness and reproducibility of the protocol for the preparation of BCNPs suitable for downstream physicochemical characterization and functionalization.

General notes and troubleshooting

1. **Culture media preparation:** When preparing culture media, autoclave the glucose separately to preserve its chemical integrity and ensure consistent bacterial cellulose production. Avoid prolonged heating of protein–glucose mixtures, which may lead to unwanted color changes or chemical modifications.
2. **Culture starter preparation and reproducibility:** The culture starter procedure described here avoids relying on optical density measurements, providing a more reproducible distribution of cells. It is important to track the amount of cellulose produced: whenever a new solid starter is prepared, confirm that the cellulose yield is comparable to that obtained with previous starters. Although this strategy minimizes variability between cultures, overall reproducibility still depends on the consistency and precision of the operator. Monitoring cellulose production also serves as a functional indicator of culture health, helping to detect non-producing mutants or suboptimal growth conditions.

3. **Cellulase activity estimation:** Reagents and procedures for cellulase activity estimation are intentionally not included to avoid redundancy with publicly available methods. Detailed instructions can be found in the manufacturer's documentation at Megazyme Cellulase Assay Protocol.
4. **Processing BCNPs and water quality:** When processing BCNPs, always use Milli-Q water. Note that the water absorbs atmospheric CO_2 and therefore becomes slightly acidic (approximately pH 5.5). Check the pH before use to ensure consistent nanoparticle suspension behavior.
5. **Enzyme inactivation and nanoparticle stability:** The effect of UV exposure on residual cellulase activity has not been evaluated in this protocol. High-temperature treatments to inactivate cellulase are avoided because they may cause irreversible aggregation of bacterial cellulose. Once recovered, nanoparticle size remains stable or may gradually increase over time due to spontaneous aggregation.

Acknowledgments

The original research paper in which the protocol was described and validated is Schibeci et al. [7].

Author contributions

Conceptualization, M.S., A.A.; Investigation, M.S., R.G., E.P., B.D.V.; Writing—Original Draft, M.S., R.G., A.A.; Writing—Review & Editing, M.S., R.G., A.A.; Funding acquisition, A.A.; Supervision, A.A.

Competing interests

The authors declare that there are no financial or non-financial competing interests related to this work.

Ethical considerations

This study did not involve human subjects or animal models; therefore, no ethical approval or informed consent was required.

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