Advanced Manufacturing Engineering for Nano-Bacterial Cellulose (A.M.E.N.)

Within the INEST – Interconnected Nord-Est Innovation Ecosystem
Funded by the European Union – NextGenerationEU (PNRR, CUP B33D24000090004)

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Summary

Progetto AMEN selezionato nell'ambito del Programma di Ricerca e Innovazione dell'ecosistema di innovazione dal titolo "iNEST-Interconnected Nord-Est Innovation Ecosystem" identificato con codice ECS00000043 e finanziato dall'Unione europea – Next Generation EU, Missione 4 Componente 1 CUP B33D24000090004 PNRR_M4C2

1. *Executive Summary*

The *AMEN* project, part of the INEST Interconnected Nord-Est Innovation Ecosystem, aims to revolutionize the production of **nano-structured bacterial cellulose (BC)** through a combination of biotechnology, advanced manufacturing, and digital process control. BC is a versatile bio-based material with exceptional mechanical, optical, and biocompatible properties, making it attractive for **biomedical, cosmetic, packaging, food and energy applications**.

The project supports **EU Green Deal** and **NextGenerationEU** priorities by fostering circular bioeconomy models, low-impact manufacturing, and data-driven industrial transformation. Its goal is to create an **integrated, scalable, and sustainable platform** for bacterial cellulose production with reduced resource consumption and improved process intelligence.

Led by **Bioniks Srl** in collaboration with **Biosphere Srl**, **Microbion srl and Ing. Alberto Benetti**, AMEN combines microbiological innovation, process engineering, and digitalization. The project is organized into three main Work Packages (WPs):

- **WP1:** Optimization and maintenance of microbial strains for cellulose synthesis.
- **WP2:** Development of scalable cultivation and purification systems.
- **WP3:** Digital integration and industrial scale-up through smart bioreactor design.

Together, these work packages form a comprehensive framework for **Advanced Manufacturing Engineering of Nano-bacterial Cellulose**, enabling industrial adoption of this bio-based material.

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2. *Abstract*

Bacterial cellulose, thanks to its mechanical, thermal, purity, and biodegradability properties, represents a promising material for the future. However, to date, its low production yield and high costs limit its industrial use. This project aims to explore the resources and methods necessary to develop an innovative system for bacterial cellulose production that is scalable on an industrial level and environmentally and economically sustainable. The project is divided into three phases. The first phase involves studying bacterial strains that produce cellulose, with the goal of defining usage protocols and optimizing polymer production yield. The second phase focuses on researching production methods to obtain the product in two different formats: film and pellets. In this phase, the feasibility of creating a modular static system and exploring post-production treatment methods will be assessed. The third phase entails designing a prototype of a static bioreactor and its related controlled environment. The prototype will then be validated in a relevant context. Each phase includes activities dedicated to digital transition, ranging from assessing computerized digital systems for studying bacterial metabolism to applying sensors and machine learning techniques in the studied and designed production processes.

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3. *Work Package 1 – Microbial Strain Characterization and Optimization*

3.1. ***Objectives and Scope***

WP1 lays the biological foundation for AMEN, focusing on the selection, maintenance, and optimization of **acetic acid bacteria** capable of producing high-quality bacterial cellulose under consistent, scalable conditions. The goal is to ensure that microbial systems remain robust and efficient when transferred from laboratory to industrial scale. Special emphasis is placed on *Komagataeibacter rhaeticus* and *Novacetimonas hansenii*, two species known for their high productivity and structural control of cellulose nanofibers. Research and optimization in the frame of the AMEN project involved three specific strains proprietary of Bioniks, hereafter referred as *K. rhaeticus* strain 1, *K. rhaeticus* strain 2 and *N. hansenii* strain 1. Such strains were isolated from a microbial consortium of bacteria and yeasts used for kombucha production. Project results report a comparison between BC production with single strains and the complete consortium.

3.2. ***Tasks and Activities***

3.2.1. ***Task 1.1: Protocols for Maintenance and Multiplication***

Description: Establish standardized methods to maintain bacterial strains over time, ensuring reproducibility and purity across experiments and for BC production process.

Core Activities and Results: Development of protocols for long-term storage, regular propagation in liquid media, and contamination control under sterile laminar flow conditions. Rigorous purity checks were implemented through spot plating techniques. These protocols ensure consistent strain behavior and minimize contamination risk during long-term studies. Cultivation media were optimized for cell viability and stable cellulose formation, enabling consistent strain recovery and use in downstream processes.

Different media were tested for propagation and BC production, starting from a chemically defined minimal medium used as baseline for comparison with other media implemented for BC yield optimization in Task 1.2.

The results of this task allowed to define *K. rhaeticus* strain 1 as the most suitable for a scale-up process, due to its adaptability to different conditions and media, lack of mutation and stability. In analysis and tests performed in the successive tasks, priority was given to this specific strain.

3.2.2. ***Task 1.2: Optimization of Growth Media***

Description: Identify the best nutrient formulations and carbon sources to enhance microbial growth and cellulose productivity.

Core Activities and Results: Comparative testing of glucose, mannitol, and glucose syrup media. The optimal medium composition balanced carbon availability and nitrogen supplementation, improving cellulose yield while maintaining pH stability. Ethanol addition at 1-2% v/v (depending on the strain) was shown to redirect metabolic flux toward cellulose synthesis, resulting in higher product consistency and uniformity. Results also indicated that *K. rhaeticus* strains (1 and 2), although belonging to the same species, exhibited differential substrate preferences, with strain 1 thriving on glucose and strain 2 on mannitol. However, strain 1 was capable of producing BC with adequate yield with both carbon sources, confirming the election of this strain as the main candidate to be used in a scaled up industrial system.

3.2.3. ***Task 1.3–1.4: Integrated Study of Metabolism and Genome***

Description: Explore the genetic and metabolic basis of cellulose production to guide future strain improvement strategies.

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Core Activities and Results: Literature review and genomic sequencing, de novo assembly and annotation of the three strains identified key genetic elements regulating cellulose synthesis and possible targets to improve cellulose yield through strain engineering and metabolism control. The cellulose synthase operon (*bcsA–D*) and associated regulators were prioritized as targets for overexpression, while competing pathways were highlighted for possible suppression.

3.2.4. ***Key Results***

- Protocols and operating procedures established for bacterial maintenance, multiplication, and inoculation.
- Optimized media with improved yield for BC production.
- Identification of high-value genomic targets for future strain engineering and metabolism control.

3.2.5. ***Interdependencies and Contributions***

Results from WP1 informed **WP2 process engineering**, ensuring reliable biological performance under both static and agitated fermentation conditions.

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4. *Work Package 2 – Process Engineering and Production Methods*

4.1. *Objectives and Scope*

WP2 translates microbial potential from WP1 into **scalable bioprocesses**. It examines static and agitated cultivation approaches, downstream separation and purification strategies, and initial digital integration. The objective is to optimize parameters such as temperature, aeration, and pH control while designing processes compatible with automation.

4.2. *Tasks and Activities*

4.2.1. *Task 2.1: Static Production of Bacterial Cellulose*

Description: Develop a reproducible static cultivation system for film-like cellulose production.

Core Activities and Results: Production trials in glass jars and stainless-steel trays using a microbial consortium composed by bacteria and yeasts were carried out, replicating traditional kombucha-like fermentation. Static cultivation in glass jars used for pre-industrial production enabled to obtain uniform cellulose films up to 17 cm diameter and 7 mm thick. The tray-based system established a scalable foundation for modular bioreactors. Controlled environmental conditions—temperature at 26 °C, stable humidity, and adequate oxygen diffusion—ensured consistent yield. The layout developed starting from a single tray served as the conceptual basis for the modular bioreactor prototype developed later in WP3. Successively, cultivation in improved jar systems were carried out with single strains ensuring a closed and sterile environment.

4.2.2. *Task 2.2: Agitated Bioreactor Cultivation of Bacterial Cellulose*

Description: Assess cellulose production under dynamic, controlled conditions in a closed bioreactor environment, for pellet or dispersed cellulose forms.

Core Activities and Results: Fermentation trials in a 4 L agitated bioreactor compared different impeller designs (Rushton and marine propellers) and media. Trials were conducted both with a complete bacteria and yeasts consortium (kombucha-like fermentation) and with single strains. Parameters such as agitation speed (RPM), aeration rate (L/min), and oxygen control were monitored in real-time thanks to the sensors embedded in the bioreactor structure. Tests revealed that mechanical agitation improves oxygen distribution but can reduce yield due to shear effects. Optimal conditions were achieved at 100 RPM, 0.6 L/min aeration and single strain inoculum. Final cellulose yield after purification was higher in single strain trials than in those performed with the microbial consortium, illustrating how the choice of inoculum influences BC bioprocess efficiency. However, agitated cultivation resulted in lower overall productivity than static methods, probably also affected by material loss during the separation and purification phase. Nevertheless, the process offered valuable insights into oxygen and nutrient dynamics, guiding sensor placement and digital control development for WP3.

4.2.3. *Task 2.3: Post-Production Separation and Purification*

Description: Design separation and purification processes that ensure high cellulose yield and purity. Specifically, purification protocols were developed to remove microbial residues and impurities from wet cellulose films and pellets.

Core Activities and Results: Two approaches were tested— one method based on traditional alkaline washing and neutralization and one advanced physical purification. The optimized method, developed specifically for pellet and dispersed cellulose form, achieved 98–99% purity and removed color residues effectively, producing cellulose ready for post-processing and functional testing. Purity was verified by the absence of live microorganisms post-sterilization. Residual cellular debris remains a target for optimization in future automation phases. The work defined critical

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parameters for integrating purification into an automated workflow. The separation and purification implementation process, especially in the case of pellets and dispersed cellulose forms, is particularly important to avoid material loss and subsequent lower yield during the downstream phase.

4.2.4. *Task 2.4: Digital Integration and Sensorization*

Description: Develop a digital monitoring infrastructure for real-time process control.

Core Activities and Results: Design of an IoT-enabled system for monitoring pH, temperature, and oxygen levels during fermentation. Data were stored in a centralized architecture supporting analytics and predictive control. This digital backbone serves as the data acquisition foundation for the WP3 static bioreactor, linking biological and engineering domains. The environmental data of the controlled fermentation environment generated by the IoT system are accessible through an open platform, designed to ensure transparency and immediate access to the results. These data include the main monitored environmental parameters and represent valuable information for the scientific and technical community. Link to access the platform: Smart Citizen.

4.3. *Key Results*

- Static cultivation confirmed as the most efficient configuration for BC production. A pre-industrial production system in glass jars was setup.
- Agitated systems provided key insights into oxygen management, agitation speed, media and propellers type.
- Separation and purification processes were developed for films and pellets/dispersed cellulose forms. In particular, processes for the latter form included technologies suitable for the scale up.
- Established a robust digital framework for real-time monitoring and process optimization.

4.4. *Interdependencies and Contributions*

WP2's outcomes provided both the **engineering specifications** and **data models** that guided WP3 prototype design and control.

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5. *Work Package 3 – Digital and Engineering Scale-Up*

5.1. ***Objectives and Scope***

WP3 focuses on scaling and digitalizing bacterial cellulose production through the design and validation of a **smart, modular bioreactor** for industrial BC production. It merges mechanical design, sensor integration, and machine learning to ensure process scalability and environmental control.

5.2. ***Tasks and Activities***

5.2.1. ***Task 3.1: Bioreactor Layout Design***

Description: Conceptualize and design a modular cultivation unit suitable for industrial scale-up.

Core Activities and Results: Using parametric 3D modeling tools, multiple geometries having the tray as basic unit were analyzed for operational efficiency. The selected "linear stacking" design maximized surface area utilization and optimized operations and maintenance. Specifically, this layout assured:

- Higher operational ergonomics and ease of maintenance, optimizing both the operator space (corridor) and the tray column space (walls).

- Efficient use of space (up to 18 trays/m²).

- Compatibility with standard fermentation room architecture.

This design includes alternating "master" and "slave" trays. The master units are equipped with embedded sensors for continuous monitoring of environmental parameters, while slave trays maintained passive uniformity. This design allows easy replication across pilot and industrial setups, controlled conditions and sterility.

5.2.2. ***Task 3.2: Controlled Cultivation Environment***

Description: Ensure conditions and parameters stability and reproducibility in the bioreactor and in its relevant environment.

Core Activities and Results: Airflow, humidity, and temperature control systems were engineered to maintain consistent conditions (26–28°C, 50–80% RH) in the closed and controlled relevant environment in which the bioreactor operates. These controlled conditions resulted in consistent cellulose film growth and uniform thickness distribution.

5.2.3. ***Task 3.3: Prototype Construction and Validation***

Description: Build and test a pilot-scale prototype to verify performance against laboratory benchmarks.

Core Activities and Results: The bioreactor prototype was fabricated and tested under operational conditions. Comparative trials demonstrated that film thickness, density, and optical transparency matched those from static lab-scale setups while enabling continuous monitoring. Validation confirmed reproducibility and identified opportunities for automated control feedback loops. Iterative refinements included:

- Optimization of tray geometry for improved oxygen diffusion.

- Validation of sensor calibration and data acquisition accuracy.

- Integration of user interfaces for monitoring and control.

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5.2.4. *Task 3.4: Machine Learning and Data Analytics*

Description: Apply AI and predictive modeling to enhance process efficiency.

Core Activities and Results: Machine learning models were studied to analyze sensor data and predict critical outcomes such as film thickness and yield. Early-stage algorithms demonstrated the feasibility of adaptive control loops, setting the foundation for future autonomous bioprocess optimization.

5.3. *Key Results*

- Functional static bioreactor prototype integrating IoT sensors and smart analytics for BC films production.
- Validated modular architecture enabling stepwise scale-up.
- Machine learning tools demonstrated possible predictive and adaptive capabilities for process optimization.

5.4. *Interdependencies and Contributions*

WP3 integrates biological, process, and digital components developed in WP1 and WP2, transforming laboratory findings into a pilot-scale technological demonstrator.

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6. *Summary and Outlook*

The AMEN project delivers an integrated technological ecosystem for **nano-bacterial cellulose manufacturing**, bridging biology, process engineering, and digital innovation. Through its work packages, AMEN demonstrates how sustainable materials can be produced efficiently using **bio-based, digitally enhanced industrial models**.

Future directions include: - Full automation of purification and material handling. - Integration of AI-based control for autonomous bioreactors. - Industrial upscaling through modular replication. - Development of new BC applications in **biomedical, energy, and green packaging sectors**.

By linking microbiology with digital manufacturing, AMEN sets a benchmark for **European innovation in circular bioeconomy**, advancing sustainability and industrial competitiveness in line with EU strategic priorities.

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