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Utilization of Post-Maceration Liquid from *Spartium junceum* Enzymatic Retting in Biosynthesis of Bacterial Nanocellulose

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Abstract
The idea of the production of high value-added materials from wastes originating from renewable plant biomass has great potential of becoming an integral part of modern biorefineries. Spartium junceum (Spanish broom) is a perennial shrub widespread in the Mediterranean area. Since ancient times it has been used as a raw material for the manufacture of ropes, nets, bags, sails and even high quality yarns, fabric and garments. Waste post-maceration liquid (PML) from Spanish broom enzymatic retting was utilized as a component of different culture media in the biosynthesis of bacterial nanocellulose (BNC) by Gluconacetobacter xylinus (ATCC 700178). The results of the experiments showed that it is possible to use post-maceration liquid as a medium component for the biosynthesis of BNC. It was also possible to reduce the cost of biosynthesis by elimination or reduction of the amount of individual medium components and to obtain BNC with a comparable or higher yield than in the standard culture medium.

Key words: *maceration liquid, Spartium junceum, bacterial cellulose.*

List of abbreviations

DP – degree of polymerization
BNC – bacterial nanocellulose
PML – post-maceration liquid
HS – Hestrin-Schramm culture medium

Introduction

The application of renewable raw products in various branches of industry will be beneficial due to their intrinsic biocompatibility and biodegradability. The production of high value-added materials from wastes originating from renewable plant biomass is an interesting idea, which has the potential of becoming an integral part of modern biorefineries. In order to effectively extract the fibres from Spanish broom twigs, an Italian Research Institute, ARTES, isolated strains and developed an enzymatic retting process which uses own enzymatic cocktail branded as GINEXTRA®. The core aim of the project funded by ARTES which involved the Institute of Biopolymers and Chemical Fibres (IBWCh), was to investigate the appropriateness of post-maceration liquid (PML) obtained from *Spartium junceum* for the production of BNC. An advantage of using PML as a feedstock for the production of bacterial nanocellulose (BNC) is the possibility to utilise low cost waste raw material.

Spartium junceum (the common name is Ginestra - in Italian, and Spanish broom - in English) is a perennial shrub of the *Leguminosae* family, widespread throughout the Mediterranean area, where it spontaneously grows in hilly areas as well as on rocky marine coasts [1]. Its roots are deep and bind the soil, contributing to lower erosion and risk of nutrient leaching. This plant is somewhat adapted to alkaline and salty soils [2]. In ancient times, the Greeks, Romans and Carthaginians used it as raw material for the manufacture of ropes, nets, bags, sails and even clothing [3].

Recently - by enzymatic maceration of *Spartium junceum* twigs, good quality textile fibre was obtained, which was industrially spun and woven (***Figure 1***, see page 46).

Cellulose is one of the most common polysaccharides and is considered an inexhaustible and indispensable source of material for a wide range of applications. Although a major part of cellulose on Earth is produced by plants, some microorganisms e.g. fungi, algae and bacteria are able to produce this polysaccharide [4]. Cellulose secreted extracellularly by some bacterial genera e.g. *Gluconacetobacter*, *Sarcina* and *Agrobacterium* is characterised by many interesting properties. Bacterial nanocellulose (BNC), in contrast to the plant cellulose is devoid of hemicelluloses, lignins and pectins and possesses unique physical and mechanical properties resulting from its three-dimensional nano-sized fibrous network. Last but not least, BNC is biocompatible with human tissues. Among the factors limiting the range of poten-

Figure 1. *Yarns and textiles made of Spartium junceum fibres.*

tial applications of BNC is the relatively high cost of the nutrient medium required for the cultivation of bacteria [5], which may be justified in the case of high value-added products e.g. in biomedical applications. Based on BNC a range of medical products has been developed including dressings for treating burns and trophic wounds, bone implants, temporary skin, artificial blood vessels and scaffolds for tissue engineering. Studies on BNC, however, are also conducted in other fields such as composite materials. Although the mechanical properties of bacterial cellulose do not exceed those of petroleum-based polymers, nano-fibrous composite materials based on BNC and synthetic polymers can be suitable for various technical applications, especially for structural materials.

Now attempts to find alternative low-cost culture media for BNC-producing bacterial strains would increase the competitiveness of this unique material, thereby increasing the range of economically justified practical applications, including the field of structural, composite materials.

Despite its enormous potential in various applications, the high cost of BNC production is the main drawback that hinders industrial implementation. The utilisation of industrial wastes and by-product streams as fermentation media could improve the cost-competitiveness of BNC production. In recent years, many studies have focused on developing cost-effective fermentation media for BNC production, such as konjac powder, fruit juices, maple syrup, thin stillage, wheat straw, spruce hydrolysate, crude glycerol from biodiesel production processes, grape bagasse, waste water after plant maceration and acetone-butanol-ethanol fermentation wastewater [6]. Wastewater or liquid obtained after thermal/enzymatic treatment of lignocellulosic materials (for example *Spartium junceum*) consists mainly of hemicelluloses and reducing sugars, and usually has a low content of lignin (≤ 5%). Due to the fact that their composition and structure could potentially yield hydrolysates with high glucose concentrations and low content of inhibitory compounds, this should be advantageous for the bacterial strains used for production of BNC [7]. The great potential of *Spartium junceum* is its extreme resistance to various forms of illness, low request of maintenance, water, ability to grow in poor soils, and above all it is a completely unused and largely diffused plant able to produce high quality fibre and other valuable biomaterials. Through an holistic approach that involves cultivation, bio-refinery, end-product design and manufacturing, as well as selected market–tests, the project may evolve to create a new value chain, expanding the know-how and becoming a model for many other unused European biomasses growing or suitable for cultivation in marginal soils in remote regions, especially those in the east of Europe.

Table 1. *Components of the culture media used in BNC biosynthesis (in 1 liter).*

Component	Culture medium			
	HS (control)	I	II	III
glucose, g	20.0	20.0	20.0	15.0
yeast extract, g	5.0	5.0	5.0	5.0
Bacto™ Peptone, g	5.0	5.0	0.0	0.0
disodium phosphate, anh., g	2.7	2.7	0.0	2.7
citric acid 1-hydrate,g	1.2	1.2	0.0	1.2
96% ethanol,cm^3	20.0	20.0	20.0	20.0
to 1000 cm^3	water	PML	PML	PML

The objectives of this study were to investigate the appropriateness of maceration liquid obtained from *Spartium junceum* for the production of BNC.

Materials and methods

Post-maceration liquid (PML)

PML was obtained from ARTES as a waste of the enzymatic retting processing of *Spartium junceum* stems using a proprietary optimised enzymatic cocktail containing enzymes from the cellulases group, hemicellulase (xylanases, pectinases) and oxidoreductase (laccases) group according to the methodology developed by ARTES. The total solids content in PML was 12.9 g/dm^3 and the pH was 4.52.

- The content of cellulose in PML was determined according to Polish Standard PN-92/P-50092: - Raw materials for the paper industry. Wood. Chemical analysis.
- The contents of lignin in PML was determined according to Kim's method [8].
- The content of hemicellulose in PML was determined according to authors' own gravimetric method: The maceration liquid was centrifuged at 1750 r.p.m. for 30 min. The supernatant was partially concentrated using a vacuum rotary evaporator at 40 °C. Next the supernatant was filtered on a intered glass filter with a pore diameter of 0.2 μm and a mixture of ethyl alcohol and water (4:1 v/v) was added. The ethanol treatment allowed

to precipitate the hemicelluloses, leaving other monomeric carbohydrates in the solution. The precipitate suspension was filtered on a sintered glass filter of 0.2 μm pore diameter and washed successively with ethyl alcohol, methyl alcohol and methyl tert-butyl ether in order to remove monomeric carbohydrate residues. The precipitate was dried in a vacuum oven at 50 °C and weighed [9].

- The content of reducing sugars in maceration liquid was determined by the colorimetric method at 540 nm using 3,5-dinitrosalicylic acid (DNS) and a calibration curve prepared for D-glucose, according to the SPR/BBP/15 IBWCh Standard Operating Procedure based on Wood's Emethod [10].
- The content of glucose in the maceration liquid was determined by the colorimetric method at 500 nm using the enzymatic test (BioMaxima S.A.) with glucose oxidase (GOD) and peroxidase (POD) and using a calibration curve prepared for D-glucose.
- The content of protein in the maceration liquid was determined by the colorimetric method at 750 nm using the Folin reagent, according to the SPR/BBP/07 IBWCh Standard Operating Procedure based on the Lowry method [11]
- The total content of residual organic substances was estimated by GC/MS chromatography according to the method developed by Pszonka and Stupinska [12] using Hewlett Packard 5890 series II/5972 GC/MS equipment. Myo-inositol was used as the internal standard.

Culture media

In the biosynthesis process of bacterial nanocellulose (BNC), the standard Hestrin-Schramm (HS) culture medium [13] was used as a control as well as three kinds of culture media in which distilled water was replaced by PML. The composition of the culture media is presented in ***Table 1***.

The biosynthesis of BNC with the use of *Gluconacetobacter xylinum* ATTC 700178 was carried out in sterile flat-bottomed glass vessels with glass covers over 7 days at 30 °C in a water jacketed incubator (***Figure 2.a***). The BNC pellicles obtained (***Figure 2.b***) were washed with distilled water until the complete removal of culture medium components. Next pellicles were soaked in 1% NaOH and autoclaved at 121 °C for 15 min. Then the pellicles were washed with distilled water until neutral pH (***Figure 2.c***).

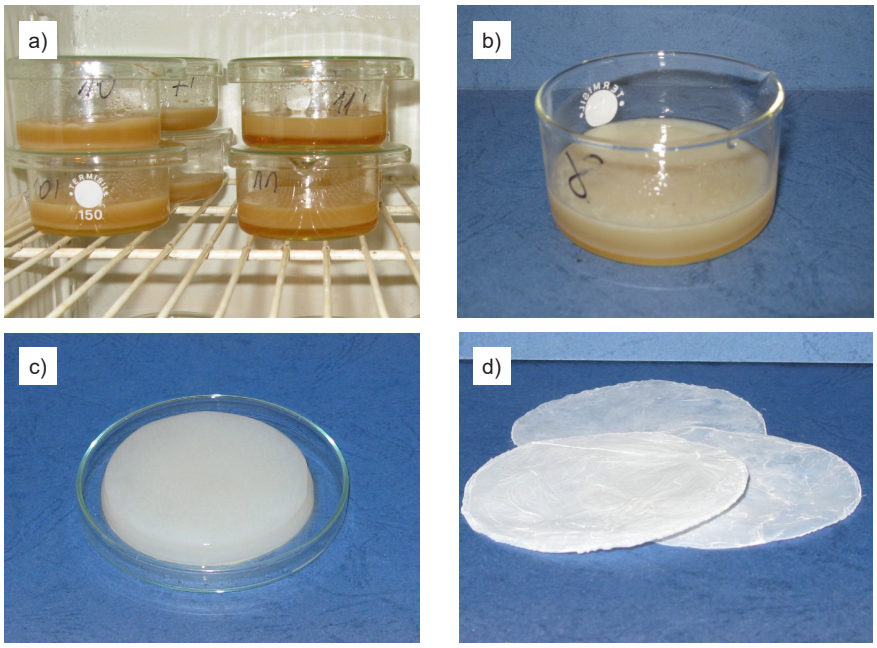

Figure 2. *Bacterial cellulose: a) during biosynthesis, b) pellicles after biosynthesis, c) washed pellicles – wet form, d) pellicles – dry form.*

Bacterial nano cellulose

- The content of alpha-cellulose in BNC was determined according to the TAPPI Standard: T 203 cm-99 - Alpha-, beta- and gamma-cellulose in pulp (1999).

The degree of polymerisation (DP) of BNC was determined according to ISO: 5351 Standard - Pulps - Determination of limiting viscosity number in cupriethylenediamine (CED) solution (2010).

In order to assess differences in the molecular structure of BNC samples FTIR spectra were recorded on an ATI Mattson Genesis series spectrophotometer. The powdered samples were mixed with an analytical grade KBr and then pressed into discs. The spectra were recorded in the wavelength range of 4000 – 400 cm^{-1}, with 16 scans.

Morphology of the samples was assessed using scanning electron microscope – FESEM type Quanta 200 (FEI).

Research results and discussion

Chemical composition of PML

Chemical composition of PML is presented in ***Table 2***.

The main components of PML were (in decreasing order): reducing sugars, protein, hemicellulose lignin and glucose.

Biosynthesis process of BNC

Media I - III were modifications of standard Hestrin-Schramm medium in which water was replaced by maceration liquid, Modifications of the culture medium in individual variants are given below:

- Medium I - water was replaced by the maceration liquid, thus the amount of reducing sugars in the medium increased from 20 to 24.5 g/dm^3 (in-

Table 2. *Chemical composition of PML.*

Component	Amount, g/l
Hemicellulose	3.53
Lignin	2.14
Protein	3.72
Reducing sugars (expressed as glucose)	3.79
Glucose	0.26
Aliphatic hydrocarbons (C10-C18)	Total: 0.025
Butylene acetate	
Dimethylbenzene	
Other benzene derivatives including vanillin	
Lupanine (CAS-550-90-3)	
N-Methylcytisine (CAS-486-86-2)	
Cyclo(leucylprolyl) (CAS-5654-86-4)	

Table 3. *Initial and consumed amount of some compounds in G. xylinus culture medium.*

Culture medium	Initial		Consumed	
	reducing sugars/ glucose, g/dm^3	protein, g/dm^3	reducing sugars, %	protein, %
HS (control)	20.0/20.0	5.0	74.6	53.4
I	24.5/20.4	9.1	61.8	60.2
II	24.5/20.4	4.1	64.8	52.5
III	20.4/15.4	4.1	61.4	59.1

Table 4. *Analysis of bacterial cellulose (BNC) after biosynthesis.*

Culture medium	BNC yield, mg/ml culture medium	Cellulose content in wet BNC pellicles, %	Surface density of dry BNC pellicles, g/m^2	α -cellulose content, %	DP, -
HS (control)	4.68	0.477	52.97	87.85	2243
I	6.07	0.483	68.73	89.67	2414
II	4.72	0.491	61.94	90.79	1564
III	5.47	0.446	53.46	89.58	2277

cluding 0.4 g/dm^3 of glucose) and the amount of protein increased from 5.0 to 9.1 g/dm^3;

- Medium II - water was replaced by the maceration liquid: no peptone was added hence the maceration liquid was an additional source of protein for bacterial growth. This medium also was devoid of disodium phosphate and citric acid;
- Medium III - water was replaced by the maceration liquid: no peptone was added and the glucose amount was reduced from 20.0 to 15.4 g/dm^3 so that the concentration of protein and reducing sugars in the medium was the most similar to that in the control medium.

The initial an consumed amounts of main compounds in the culture media presents ***Table 3***.

Analysis of the culture medium after bacterial cellulose biosynthesis and some properties of pellicles are presented ***Table 4***.

The results presented in ***Tables 3*** and ***4*** show that the PML can be a good substitute for water as a component of culture media as well as a source of nutrients (proteins, sugars). In the case of a substrate enriched with an additional amount of sugars and proteins (Medium I) by using a PML instead of water, the biosynthesis yield was approximately 30% higher relative to the standard HS medium (control). The use of Medium III (peptone-free) allowed the obtaining of bacterial nanocellulose with improved yields of ca. 17%. Medium II was the closest to the control medium - in this case the biosynthesis yield was almost the same. The content of α-cellulose in pellicles obtained from cultures in the growth medium containing PML is similar to or a little higher than those obtained from cultures in the standard HS medium, which may indicate that bacterial cellulose obtained from cultures in growth medium containing PML is of such high chemical purity as the BNC obtained in standard culture. The cellulose content in wet pellicles was similar in the modified media and in the control HS medium. Only in medium III was the cellulose content in

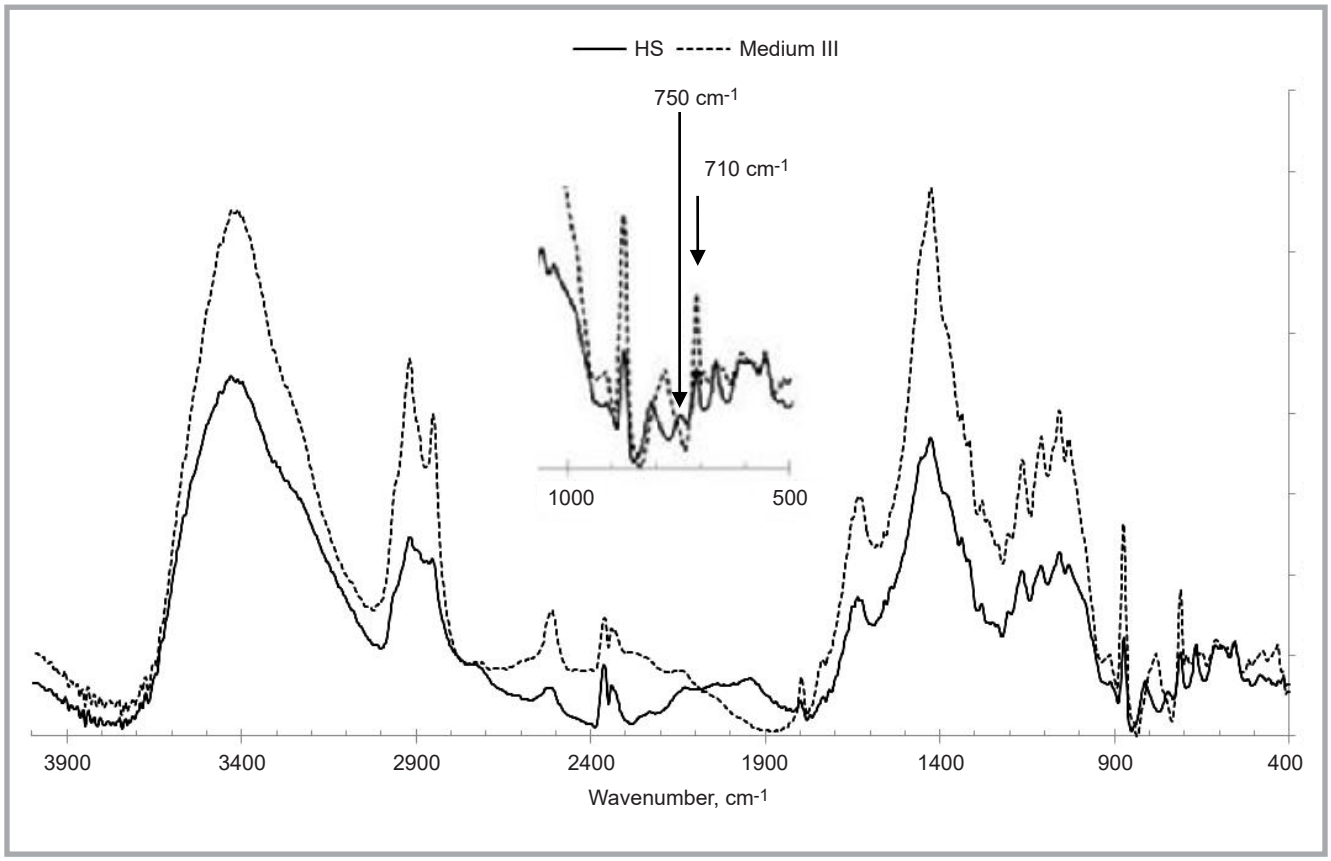

Figure 3. *FTIR spectra of BNC synthesized in standard HS medium and Medium III.*

wet pellicles lower - the structure of bacterial cellulose was looser, hence a higher amount of water amount was contained in the BNC structure.

Figure 3 presents FTIR spectra of BNC synthesised in standard HS medium and Medium III. Both spectra show several absorption bands characteristic for cellulose: a broad band at 3300 - 3400 cm^{-1} (O-H stretching vibrations of cellulose I), a broad band at around 2900 cm^{-1} (asymmetric stretching of $-CH_2CH$), a band at 1163 cm^{-1} (asymmetric stretching of C-O at a ring), and a 1066 cm^{-1} peak in a broad band (C-O stretching) [14]. According to Czaja et al. [15], peaks at 750 cm^{-1} and 710 cm^{-1} can be assigned to Iα and Iβ cellulose allomorphs, respectively. As can be seen from ***Figure 3***, both peaks are present in the FTIR spectrum of BNC synthesized in the HS medium while in the spectrum of BNC synthesised in Medium III the peak at 750 cm^{-1} characteristic for cellulose Iα disappears. This change in structure may explain the aforementioned differences in the water holding capacity of BNC synthesised in the HS medium and Medium III.

Figure 4 presents SEM micrographs of BNC synthesized in the HS control medium and in Media I - III. All the BNC samples obtained are characterized by a fine, entangled fibrillar network with an average microfibril width of 100 nm.

The results of the study show that it is possible to use PML as a medium component for the biosynthesis of bacterial nanocellulose. It is also possible to reduce the cost of biosynthesis by eliminating or reducing the addition of some components and obtain BNC with a comparable or higher yield than in the standard HS medium. On a laboratory scale, the manufacturing cost of 1 m^2 of BC with the same surface density using the HS medium would decrease by 42% (from 38 € to 22 €) when using a maceration liquid-containing medium. When the manufacturing scale is increased, the price of reagents should decrease, but the proportion would be maintained.

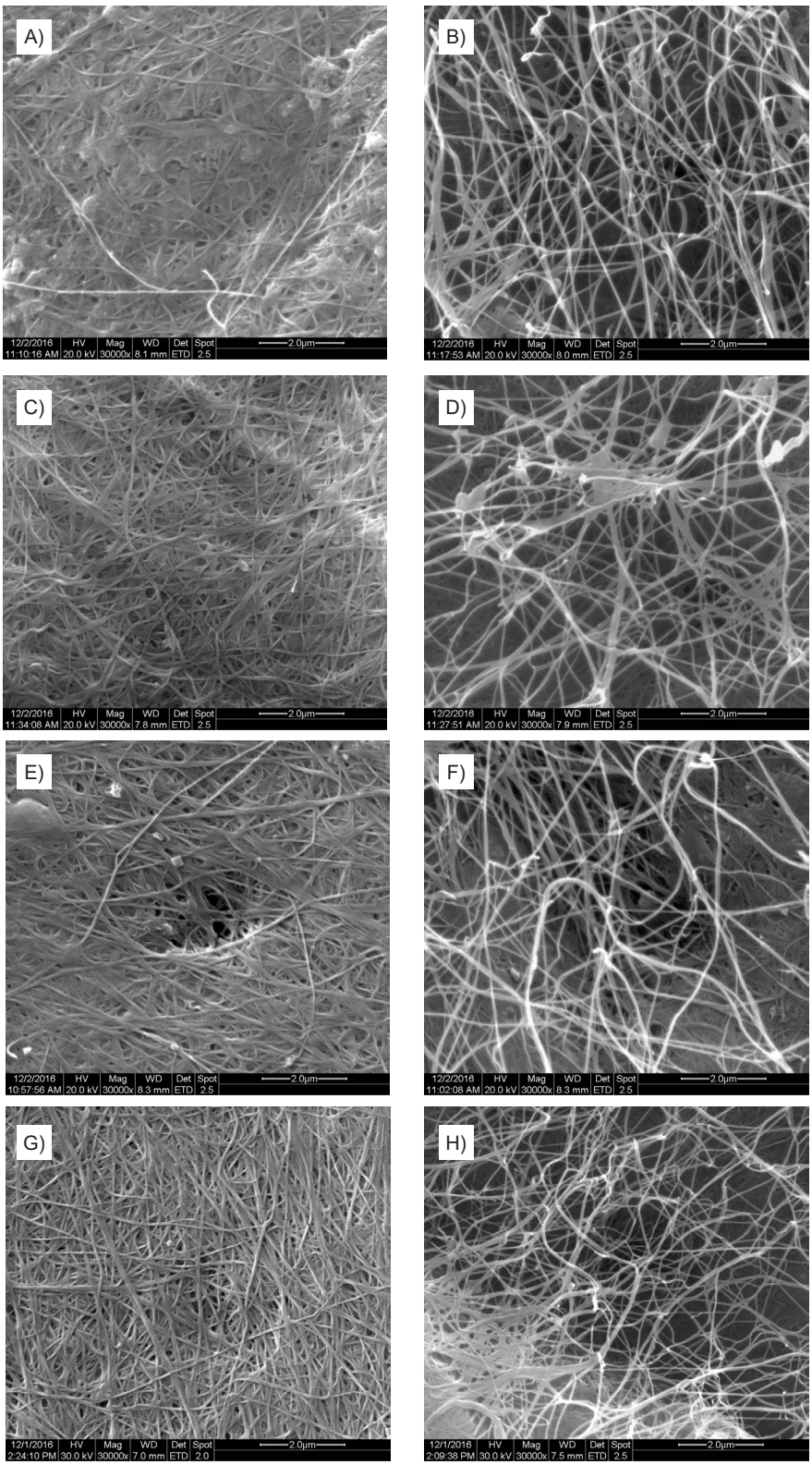

Figure 4. *SEM micrographs of BNC synthesized in different culture media (left - surface view, right - cross-section). A, B - HS, C, D - Medium I, E, F - Medium II, G, H - Medium III.*

Conclusions

Post-maceration liquid obtained from enzymatic retting of *Spartium junceum* can effectively substitute water in the nutrient medium used in the biosynthesis of bacterial nanocellulose with the use of *Gluconacetobacter xylinus* (ATCC 700178). The application of a waste stream from plant biomass processing fulfils the idea of a biorefinery. PML may supplement the culture medium with nitrogen compounds, sugars and microelements. It seems to be possible to lower the cost of BNC biosynthesis by eliminating or reducing the amounts of some components of the experimental culture media and yet obtain BNC with a comparable or higher yield than in the standard HS medium.

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INSTITUTE OF BIOPOLYMERS AND CHEMICAL FIBRES

Team of Synthetic Fibres

The section conducts R&D in melt spinning of synthetic fibres

Main research fields:

- processing of thermoplatic polymers to fibres
  - classical LOY spinning
    - fibres with round and profiled cross-section and hollow fibres
    - special fibres including bioactive and biodegradable fibres
    - technical fibres eg. hollow fibres for gas separation, filling fibres for concrete
  - bicomponent fibres
    - side-to-side (s/s type) self-crimping and self- splitting
    - core/sheath (c/s type)
- processing of thermoplastic polymers to nonwovens, monofilaments, bands and other fibrous materials directly spun from the polymer melt
- assessment of fibre-forming properties of thermoplastic polymers inclusive testing of filterability.

Equipment:

Pilot-scale equipment for conducting investigations in melt spinning of fibres

- spinning frames for
  - continuous fibres 15 – 250 dtex
  - bicomponent continuous fibres 20 – 200 dtex
- drawing frames for continuous filaments 15 – 2000 dtex
- laboratory stand for spun bonded nonwovens, width 30 cm
- laboratory stand for investigation in the field of staple fibres (crimping, cutting line)
- laboratory injection molding machine with a maximum injection volume of 128 cm^3
- testing devices (Dynisco LMI 4003 plastometer, Brabender Plasticorder PLE 330 with laboratory film extrusion device)
- monofilament line for 0.3 – 1 mm diameter of the monofilaments.

Implemented technologies (since 2000):

- texturized polyamide fibres modified with amber for the preparation of special antirheumatic products
- polyolefin hollow fibres for gas separation
- bioactive polypropylene POY fibres
- modified polypropylene yarns
- polyolefin fibres from PP/PE waste.

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