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Dedicated to Professor Bogdan C. Simionescu on the occasion of his 70th anniversary

BIOPOLYMER BIOSYNTHESIS BY LACTIC ACID BACTERIA STRAIN IN FOUR DIFFERENT CULTURE MEDIA

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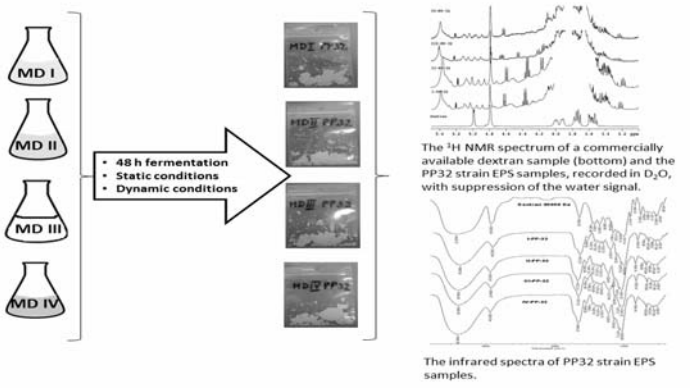
The influence of four different culture media on the exopolysaccharides (EPS) biosynthesis was studied using a new isolated lactic acid bacteria strain. The biopolymer synthesis was strongly influenced by the presence of milk in culture media, yielding 6.1 g/L dry-freezing biopolymer after 48 hours of fermentation. Also, comparatively with normal culture medium supplemented with different sugars, the presence of aqueous fruit extract from *Hippophae rhamnoides* determined an improvement of the EPS biosynthesis, yielding 4.8 g/L freeze-dried biopolymer. The aqueous fruits extract may be used for the EPS biosynthesis, taking into consideration that the obtained EPS amount is comparable with those resulting from the culture media supplemented with milk. The NMR and FTIR analyses revealed that the resulted EPS have dextran structures, with protein residues covalently attached to the structure.



INTRODUCTION

Due to numerous industrial applications such as pharmaceutical, medical and food products and to numerous health benefits, exopolysaccharides (EPS), which are extracellular bio-macromolecules, have received special attention in the latest decade. They are considered to be generally recognized as safe (GRAS) for human health, being an ideal candidate for food industry, having gelling, emulsifying, stabilizing and viscous properties.[1] EPS produced by lactic acid bacteria (LAB) have immune-modulatory, antitumor and anti-inflammatory effects, acting as oxidizing agents, their biological activity and technological applications being determinated by their structural properties.[2]

Dextran is an EPS biosynthesized by several types of lactic acid bacteria such as *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Streptococcus mutants* and *Weissella confusa*. Depending on the strain and the composition of the culture medium, dextran has low or high molecular weight (10-150 kDa).[3] Dextran is a very complex glycan composed of chains of α-D-glucose with α-(1→6) linear links and different percentages of α-(1→4),

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α-(1→3) and α-(1→2) bonds, which depend on the nature of dextransucrase biosynthesized by the microbial strain.[4]

Beside other industrial applications, especially bakeries,[5] dextran has various applications in pharmaceutical and light industries,[6] being used in medicine as antithrombotic agent reducing blood viscosity.[7] Dextran-based nanoparticles have applications in targeted drug therapy, where dextran is used in coating and it can be functionalized.[8] Another application is as protective coating against oxidation of metal nanoparticles.[9]

The biosynthesis of secondary metabolic compounds can be influenced by the presence of vegetal aqueous extracts.[10] *Hippophae rhamnoides L.* (sea buckthorn) berries are very rich in active compounds with various effects on the organism. The major components are already identified, including significant amounts of minerals, vitamins, polyphenols, phytoestrogens, phytosterols, lipids, carotenoids, tocopherols and ascorbic acid.[11] *H. rhamnoides L.* belongs to *Elaeagnaceae* family and is naturally present in Europe and Asia. *H. rhamnoides* berries present antioxidant properties, having a high nutritional value.[12]

The aim of this study was to test the composition of four different culture media on the strain growth. At the same time, after 48 hours of fermentation, EPS was extracted purified and quantified, in order to monitor the biopolymer yield produced by the LAB strain denoted PP-32.

RESULTS AND DISCUSSION

Aqueous extracts characterization

Identification of total polyphenol content and polyphenol by HPLC-ESI MS analysis

The aqueous extract was characterized by the total polyphenol content using the Folin Ciocalteu method as described previously.[10] Before dissolving the culture medium components and preparing the MD IV (culture medium IV consisting in 55.3 g/L MRS-55.3 and 80 g/L sucrose, dissolved in aqueous extract from *H. rhamnoides* (sea buckthorn) berries), the total polyphenol concentration of the 5 g/L *Hippophae rhamnoides* berries aqueous extract was evaluated at 270.77 mg Gallic Acid Equivalent (GAE)/L. Based on the HPLC-ESI MS analysis, nine polyphenols were identified in the extract, namely: salicylic acid, p-hydroxybenzoic acid, cinnamic acid, protocatechuic acid, p-coumaric acid, caffeic acid, vanillic acid, gallic acid, ferulic acid.

The influence of fermentative conditions on the EPS biosynthesis yield

The obtained EPS amount after the fermentative processes is strongly influenced by the culture medium composition and by the fermentation conditions. These results are in accordance with the literature data (**Table 1**).[13]

The lowest amount of EPS was registered for the fermentation in MD I culture medium, in static conditions, due to the low oxygenation of the LAB strain, while the highest amount was obtained for the fermentation in MD III culture medium which contains the components dissolved in UHT milk, the proper media for LAB development. A remarkable amount was recorded for the fermentation made in MD IV, of 4.8 g/L EPS, an amount comparable with the fermentation made in UHT milk. From these results it may be concluded that aqueous extract from *H. rhamnoides* berries positively influences the biopolymer synthesis and may be used to obtain EPS for further applications. More than that, after the fermentative process, the residual total polyphenol concentration was 261.25 mg GAE/L, which confirms the literature data according to the idea that the polyphenols can be used by the microbial strain for biomass development and secondary metabolic compounds biosynthesis.[10]

Table 1

The EPS amount extracted from the individual culture medium after 48 h fermentation time

Fermentations Conditions	**EPS** from **MD I** ((g/L): MRS-55.3, fructose-40, glucose-40 in water)**,** **g/L**	**EPS** from **MD II** ((g/L): MRS-55.3 and sucrose-80 in water)**,** **g/L**	**EPS** from **MD III** ((g/L): MRS-55.3 and sucrose-80 in UHT milk)**,** **g/L**	**EPS** from **MD IV** ((g/L): MRS-55.3 and sucrose-80 in *H. rhamnoides* aqueous extract)**,** **g/L**
Static fermentations	1.1	2.9	**6.1**	4.31
Dynamic fermentations	2.6	4.42	**5.4**	**4.8**

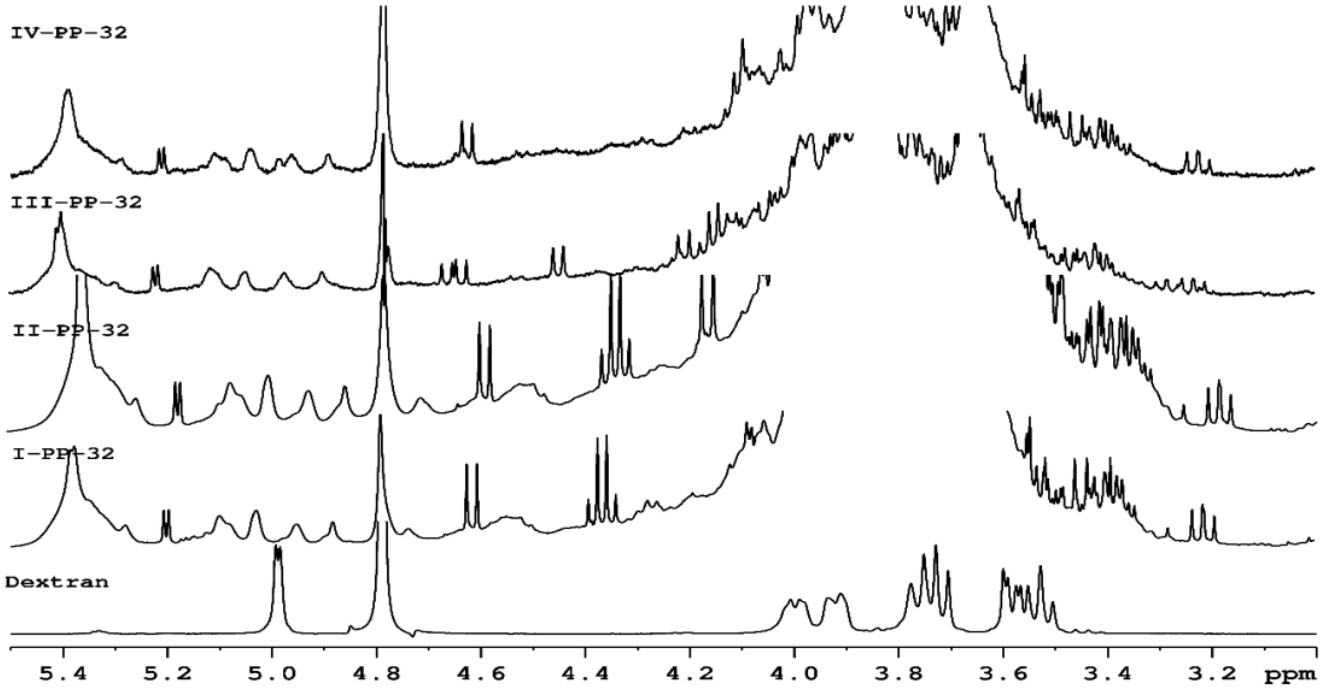

Fig. 1 – The ^{1}H NMR spectrum of a commercially available dextran sample (bottom) and the I to IV PP-32 samples spectra, recorded in D_2O, with suppression of the water signal.

Nuclear magnetic resonance analysis of EPS extracted from fermented culture media

The NMR analysis was performed only for the representative samples. The obtained ^{1}H NMR spectra show the characteristic signals of a complex mixture of exopolysaccharides. Based on previously reported data[14] and the NMR analysis of the commercially available standard, it was deduced that one of the exopolysaccharides present in this mixture is dextran.

The ^{1}H NMR spectrum corresponding to dextran sample is presented in **Figure 1** (bottom spectrum). Several signals belonging to the glucose units can be observed in the region 3.5-4.1 ppm, while the protons from position 1 of the α-(1→6)-linked glucose units appear as a doublet at 4.99 ppm. The same signal, from 4.99 ppm, could be identified in all the ^{1}H NMR spectra of the samples I to IV-PP-32, as exemplified in **Figure 1**, but the intensities are very low. These low intensities indicate a low dextran concentration in the analyzed samples. Moreover, in the ^{1}H NMR spectra of the samples I to IV-PP-32 one can observe more signals in the region 4.86-5.50 ppm, characteristic to different types of anomeric protons. This can suggest the presence of more than one exopolysaccharide.

Fourier-transform infrared spectroscopy (FTIR) analysis of EPS extracted from fermented culture media

The FTIR spectra of I-PP-32, II-PP-32, III-PP-32 and IV-PP-32, are very similar with Dextran 40000 Da spectra (**Figure 2**).

The absorption bands of hydroxyl stretching are observed around 3400 cm^{-1}. The presence of protein residues is indicated by the presence of the amide I and amide II bands (1672 cm^{-1} and 1544 cm^{-1}), which are not registered in the dextran standard spectra. The region between 1490 and 1308 cm^{-1} covers signals from the bending vibrations of CH, CH_2 and OH groups. The sub-band at 1054 cm^{-1} is specific to α-D-glucoses, is mainly given by ν(C2-O2H)[15, 16] and is overlapped by the band observed at 1028 cm^{-1}, which can be assigned to the (C6-O6H) vibrations in H-bonded primary alcoholic groups. A registered intense peak at 1078 cm^{-1} is composed from the overlapped ν(C6-O6H), δ(C4-C5) and δ (C1-H) vibrations.[16, 17] The ν(C-O-C) in glycosidic linkages of α-D-glucopyranoses is observed at 1148-1150 cm^{-1}, being confirmed by the band at 834-835 cm^{-1} and 845 cm^{-1} for standard dextran (ν(C-O)), which indicates the α-(1→3) anomeric configuration.[18] Therefore, all EPS spectra are very similar to that of standard dextran, but with signals of proteins irreversibly bonded to dextran structure.

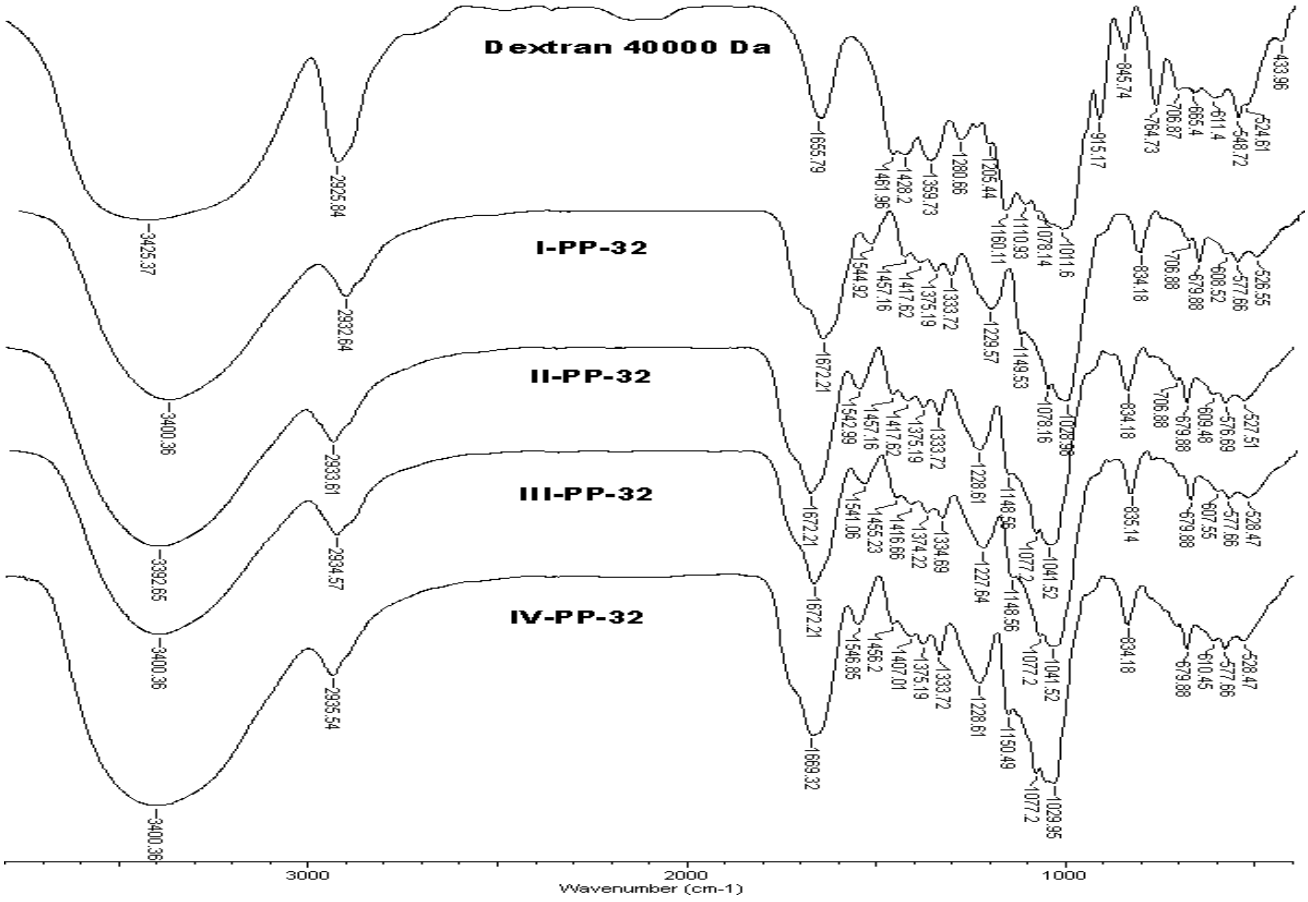

Fig. 2 – The infrared spectra of PP-32 strain EPS samples.

EXPERIMENTAL

Microorganisms

The lactic acid bacteria strain coded PP-32 was isolated from Romanian Rye flour in the laboratories of Centre of Advanced Research in Bionanoconjugates and Biopolymers (IntelCentru) of the "Petru Poni" Institute of Macromolecular Chemistry, Iasi, and kept at -80°C in Man Rogosa Sharpe medium (MRS) supplemented with 20% glycerol.[19]

Fermentation conditions

The lactic acid bacteria strain isolation and purification was made in Petri dishes using MRS agar supplemented with 1% $CaCO_3$ and incubated at 30°C for 48h.[19]

Experimental fermentation was performed in triplicate, in static (S) and dynamic conditions (D) using four different culture media composition denoted MDI, MDII, MDIII and MDIV.

The culture media compositions were as follows:

- MDI (g/L): MRS-55.3, fructose-40, glucose-40, dissolved in double distilled water;
- MDII (g/L): MRS-55.3 and sucrose-80, dissolved in double distilled water;
- MDIII (g/L): MRS-55.3 and sucrose-80, dissolved in UHT milk (with the following composition: energetic value - 44 kcal/100 mL, proteins - 3g/100 mL, lipids - 1.5 g/100 mL (saturates - 0.9 g/100 mL), sugar - 4.5 g/100 mL, calcium ions - 120 mg/100 mL).
- MDIV (g/L): MRS-55.3 and sucrose-80, dissolved in aqueous extract from *H. rhamnoides* (sea buckthorn) berries.

All culture media were sterilized at 110°C for 30 minutes and inoculated with 30% of fresh inoculum (24 hours) with A_{600nm}-0.5.[20] The samples were incubated at 33°C for 48 hours without pH correction during fermentation, under static and dynamic (at 100 rpm) conditions.[21] Before performing the EPS extraction and purification, the cultures were heated at 100°C for 15 min, in order to inactivate the enzymatic equipment capable of degrading the biopolymer[22] or the polyphenol compound from media.

Aqueous extraction of vegetal materials

The *H. rhamnoides* (sea buckthorn) berries were ground to 1-2 mm and 5 g of the ground material was extracted with 100 mL distilled water in a simple bath system, at 80 ºC for 45 min. The aqueous extraction was repeated until the extract was colourless. All the extraction fractions were collected and cumulated to a final volume of 1000 mL using double distilled water.

Aqueous extracts characterization

Total polyphenol concentration

The aqueous extract was characterized by total polyphenol content using the Folin Ciocalteu method as previously described.[10]

Polyphenol identification by HPLC analysis

The HPLC-ESI MS analyses were carried out using an Agilent Technologies 1200 Series system. The conditions for separation were achieved using a Zorbax SB C18 reverse phase column (4.6 mm x 150 mm, 5 µm particle size) with a column temperature kept at 40ºC, using a 20 µL injection volume, solvent flow 0.6mL/min, elution in gradient: 0% B at 0 min; 7% B at 5-7 min; 38% B at 50-52 min, came-back to 0% B in 10 min and column equilibration at 100% B in 10 min, where (A) is a mixture of 99.9% water and 0.1% formic acid; (B) 99.9% acetonitrile and 0.1% formic acid. The separation process was monitored by UV-VIS DAD detector at 280 nm. The LC system was connected directly to the electrospray ionization source (ESI) in a positive mode, drying gas debit (N_2) 7 L/min, gas temperature 220ºC, nebulizer pressure 25 psig and capillary voltage 4200 V.

EPS isolation and purification

Cells and proteins were removed from the culture media by trichloroacetic acid (TCA) 20% precipitation and centrifugation at 10,000 rpm, for 10 min at 4°C. EPS were separated by precipitation with three volumes of chilled ethanol for 24 hours at 4° C.[21] The EPS were harvested at 12,000 rpm for 15 min at 4°C, washed three times with ethanol and resuspended in double distilled water (DDW). For analysis, EPS samples were coded as follows: I-PP-32 - the PP-32 strain fermented in MD I in dynamic conditions, I-PP-32S - the PP-32 strain fermented in MD I in static conditions, II-PP-32 - the PP-32 strain fermented in MD II in dynamic conditions, II-PP-32S - the PP-32 strain fermented in MD II in static conditions, III-PP-32 - the PP-32 strain fermented in MD III in dynamic conditions, III-PP-32S - the PP-32 strain fermented in MD III in static conditions, IV-PP-32 - the PP-32 strain fermented in MD IV in dynamic conditions, IV-PP-32S - the PP-32 strain fermented in MD IV in static conditions and subjected to lyophilisation. The amount of polymer was expressed in g of dry biopolymer per litre of culture medium. [23]

Nuclear magnetic resonance studies

The NMR spectra have been recorded on a Bruker Avance III 400 MHz spectrometer equipped with a 5 mm inverse detection z-gradient probe, operating at 400.1 MHz for ^{1}H nucleus. The ^{1}H NMR experiments were recorded with suppression of the water signal, using the standard noesy presaturation pulse sequence. The experiments were recorded with TopSpin 2.1 PL6 spectrometer control and processing software. The compounds were dissolved in deuterated water (99.90% D2O, Euriso-top, France) with TSP used as internal standard (TSP – 98% sodium 3-(trimethylsilyl)-[2,2,3,3-d4]-1-propionate, Euriso-top, France). The spectra have been recorded at room temperature. Chemical shifts are reported in ppm and referred to TSP (ref. ^{1}H 0.00 ppm).

Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra were recorded in transmission on a Bruker Vertex 70 spectrometer (Bruker Optics, Germany) at a resolution of 2 cm^{-1}, on KBr pellets and for the data processing OPUS 6.5 software (Bruker Optics, Germany) was used. FTIR spectra were processed with a specialized program from the *SpectraManager* series.

CONCLUSIONS

H. rhamnoides berries aqueous extract influences positively the biopolymer synthesis, and the polyphenols compounds are used by the strain for the metabolic pathway. The FTIR and NMR analyses confirmed the dextran structure with protein impurity permanently bonded to structure of the EPS extracted from PP-32 strain fermentations.

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