# INNOVATIVE BIOPOLYMER-BASED HYDROPHILIC MATRICES WITH TAILORED PROPERTIES FOR MEDICAL APPLICATION

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# SCIENTIFIC REPORT

## Stage I - 2015

### DESIGN AND OPTIMIZATION OF THE PROCEDURE USED FOR THE OBTAINING OF EXOPOLYSACCHARIDES (EPS)

***O.I. Biosynthesis and characterization of EPS from lactic acid bacteria***

**A.I.1.** Microbiological biosynthesis of exopolysaccharides from lactic acid bacteria (EPS-LAB)
**A.I.2.** Extraction and purification of EPS-LAB
**A.I.3.** Physicochemical characterizations of EPS-LAB

## A.I.1. Microbiological biosynthesis of exopolysaccharides from lactic acid bacteria (EPZ-LAB)

Nowadays, due to an increased awareness on environmental problems, it has been developed an interest towards biopolymers, in order to develop environmentally friendly materials suited for high performance applications and for their traditional uses.

The trend of orientation towards biopolymers and to design innovative products has led to a global resurgence of interdisciplinary research on *bacterial exopolysaccharides* (EPS). The inherent bioavailability and the apparently non-toxic nature of EPS resulted in their use in many medical applications such as scaffolds in tissue engineering, systems for controlled release of drugs, as well as bandages, making them more attractive compared to polysaccharides derived from plants and microalgae (Nwodo et al., 2012). Generally, these biopolymers include exopolysaccharides in capsular form or in the form of a viscous substance (Wang et al., 2010).

According to current taxonomy, this group of bacteria consists of twelve bacterial genera. However, only few of them are found in food, such as *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Lactobacillus* and *Pediococcus*. These kinds of lactic acid bacteria may biosynthesis dextran, extracellular xanthan, gellan, glucans, pullulan, etc. (Patel et al., 2012). Until recently, dextran was the mainly exopolysaccharide produced by *Leuconostoc* genre. Industrial scale production was focused mainly on dextran produced by *Leuconostoc mesenteroides* and *Leuconostoc citreum* (Miao et al., 2014). It is well known that dextran is used as a substitute for blood plasma in clinical

applications, as a standard in exclusion chromatography, as an ingredient in cosmetic products, bakery products, and in frozen industrial products (Moscovici et al., 2015).

EPS intracellular biosynthesis by fermentation of lactic acid bacteria (EPS-LAB) was considered of interest for this project due to their structural and functional diversity and to their beneficial effects. Factors influencing the biosynthesis of EPS-LAB are the culture medium composition and the incubation conditions, such as the type of carbon source, nitrogen source type, incubation time, agitation rate and temperature of incubation. *The carbon source* is responsible for the type of EPS biosynthesized by the lactic acid bacteria (LAB); the best results were obtained when the glucose from the culture medium was added in a concentration higher than 100 g/l (Grobben et al., 1997). *The nitrogen source* is responsible for the growth of lactic acid bacteria strains and an optimal balance between the carbon and nitrogen source is absolutely necessary to achieve high EPS yields. In order to monitor the EPS-LAB biosynthesis, *the time* of incubation has a significant influence. The maximum of EPS biosynthesis is recorded when LAB are in exponential growing stage, but no further production was observed in the stationary phase (Tayuan et al., 2011). *The agitation rate* is an important factor in the biosynthetic pathway which can affect the rate of multiplication, the medium homogeneity and thus, the availability of the nutrient from the substrate and of the air for the existing microbial species (LAB oxygenation). The incubation *temperature* depends on the type of *Lactobacillus* species, as well as its subsequent use. In particular, for the EPS biosynthesis was noticed that the optimum growing temperature is 33°C (Tayuan et al., 2011).

**a. Isolation and selection of lactic bacteria strains**

In this stage, *34 lactic acid bacteria strains* from different sources were isolated in order to test the ability to produce exopolysaccharides.

The culture media used for the activation, cultivation and conservation of LAB were MRS broth and MRS agar. MRS broth was purchased from Bio Springer (Lesaffre Group Company, France) and as standards there were used sucrose, glucose and fructose (Sigma-Adrich). Ethanol and trichloroacetic acid were purchased from the Chemical Company SA, Romania.

*Lactic acid bacteria isolation and purification* was made in two stages: e*nrichment* (Adnan and Tan, 2007) and c*ulture purification* (Schiraldi et al., 2006).

There were chosen 6 different culture media (rye flour, yogurt, 2 types of flour, milk and homemade cheese) in order to be tested the effect of culture medium composition on lactic acid bacteria growth and production. All 34 LAB strains were tested, and duplicate assays were made to ensure accuracy.

*Selection of fermentation conditions*

In order to test the influence of a culture medium on EPS production, strains were cultivated in two different culture media:

- MRS broth – reference medium,
- MRS agar* - MRS agar supplemented with glucose and fructose.

After preparation and sterilization, the inoculation of the culture medium was performed according to the Malik et al. method (Malik et al., 2009).

*Lactic acid bacteria strain selection*

Following incubation, the EPS-LAB production has been quantified using Paulo et al. procedure (Paulo et al., 2012). After data evaluation there were selected five lactic acid bacteria strains (PP15, PP19, PP29, PP32 and PP34). Selection took into consideration the EPS-LAB amount produced in MRS broth, but also the tensile strength of the samples biosynthesised in MRS agar*.

**b. Biosynthesis of exopolysaccharides by lactic acid bacteria (EPS-LAB)**

*Fermentation conditions – Culture medium*

In order to study the effects of medium conditions, in particular of the carbon source on the growth of lactic acid bacteria and the production of EPS-LAB, MRS broth was supplemented with glucose, fructose and sucrose at a final concentration of 100 g/l (Tayuan et al., 2011).

In this regard, for the fermentation of selected lactic acid bacteria strains there were used two kinds of culture media:

- MD I – containing MRS broth supplemented with fructose and glucose.
- MD II – containing MRS broth supplemented with sucrose.

The culture media was sterilized at 110 °C for 30 minutes and inoculated with 30% fresh inoculum for 48 hours and then the absorbance was read in the region of 0.5 - 600 nm (Liu et al., 2009). Incubation was carried out for 48 hours without pH control, at a temperature of 33 °C, with agitation at low speed (100 rpm), sufficient to keep the culture homogeneous. The obtained culture was treated at 100 °C for 15 minutes to inactivate the enzymes capable of degrading the polymers (Lee et al., 2012). After the inactivation, EPS-LAB were extracted and purified.

## A.I.2. Extraction and purification of EPS-LAB

The extraction and purification protocol of EPS-LAB has been established according to the literature (Liu et al., 2011; Tayuan et al., 2011; Bennama et al., 2012; Palomba et al., 2012). The EPS-LAB amount extracted from different culture media are presented in Table 1.

Table 1. The amounts of EPS-LAB extracted from different culture media

| Culture medium | EPS-LAB, g/l | | | | |
|---|---|---|---|---|---|
| | PP15 | PP19 | PP29 | PP32 | PP34 |
| MD I | 2.48 | 4.19 | 2.80 | 2.60 | 2.60 |
| MD II | 5.05 | 4.63 | 5.18 | 4.42 | 5.20 |

It can be concluded that the optimum culture medium for the growth and the biosynthesis of EPS-LAB was MD II, which contains MRS broth and sucrose.

## A.I.3. EPS-LAB physico-chemical characterization

The isolated lactic acid bacteria strains produced different amounts of exopolysaccharides (EPS-LAB) depending on the type of culture medium used. From all EPS-LAB samples was selected sample PP15 due to its structure with a high elasticity observed in MRS agar *.

**1. Gel permeation chromatography (GPC)**

In order to determine the molecular weight average and the polydispersity index of the EPS-LAB samples it was used gel permeation chromatography (GPC), and the measurements were recorded on a Shimadzu system (PL-GPC 120, Varian). It was injected a dilute solution of the polymer (1 mg/ml) in the solvent system (0.2 M $NaNO_3$, 0.01M $NaH_2PO_4$, pH = 7) with a flow rate of 1 ml/min. The calibration of the system was performed with Pullulan standards (Type P-82, Lot 01101, Denko KK Shodex, Japan).

The detailed compositions of samples I-PP15 and II-PP15 are presented in Table 2, where weight-average molecular weight (Mw, g/mol), number-average molecular weight (Mn, g/mol) and polydispersity index (PD= Mw/Mn) are indicated.

Table 2. Relevant molecular parameters of I-PP15 and II-PP15

| **Samples** | | **Mw, g/mol** | **Mn, g/mol** | **Polydispersity, PD** |
|---|---|---|---|---|
| I-PP15 | Fr. 1 | $2.8 \times 10^{8}$ | $1.0 \times 10^{8}$ | 2.87 |
| | Fr. 2 | $6.9 \times 10^{6}$ | $6.2 \times 10^{6}$ | 1.11 |
| | Fr. 3 | $4.0 \times 10^{5}$ | $2.9 \times 10^{5}$ | 1.34 |
| | Fr. 4 | $1.6 \times 10^{4}$ | $1.2 \times 10^{4}$ | 1.38 |
| | Fr. 5 | $2.7 \times 10^{2}$ | $2.1 \times 10^{2}$ | 1.24 |
| II-PP15 | Fr. 1 | $4.7 \times 10^{8}$ | $1.4 \times 10^{8}$ | 3.30 |
| | Fr. 2 | $7.8 \times 10^{6}$ | $7.0 \times 10^{6}$ | 1.10 |
| | Fr. 3 | $4.1 \times 10^{5}$ | $2.7 \times 10^{5}$ | 1.51 |
| | Fr. 4 | $3.2 \times 10^{2}$ | $2.6 \times 10^{2}$ | 1.24 |

It can be noticed that the sample I-PP15 presents five characteristic fractions with different molecular weights. For the fraction with the highest molar mass ($2.8 \times 10^{8}$ g/mol) the PD is 2.87, which indicate a polydisperse polymer. For the other fractions the PD values are around 1, which indicate the presence of monodisperse polymers. Taking into account the fact that Fr. 1 of samples I-PP15 and II-PP15 have extremely high molecular weights and are polydisperse polymers, it is assumed that the EPS-LAB are forming complex aggregates (Hwang et al., 2013).

## 2. Thermogravimetric analysis (TGA)

Generally, the thermal degradation of polymeric materials is a complex process that is achieved through simultaneous chemical reactions and physical changes (Ahmed et al., 2013). The thermogravimetric analysis (TG) and differential thermogravimetry (DTG) of EPS-LAB sample were performed on a STA 449F1 Jupiter NETZSCH equipment.

The behavior during the thermooxidative destruction of I-PP15 sample is shown in Figure 1.

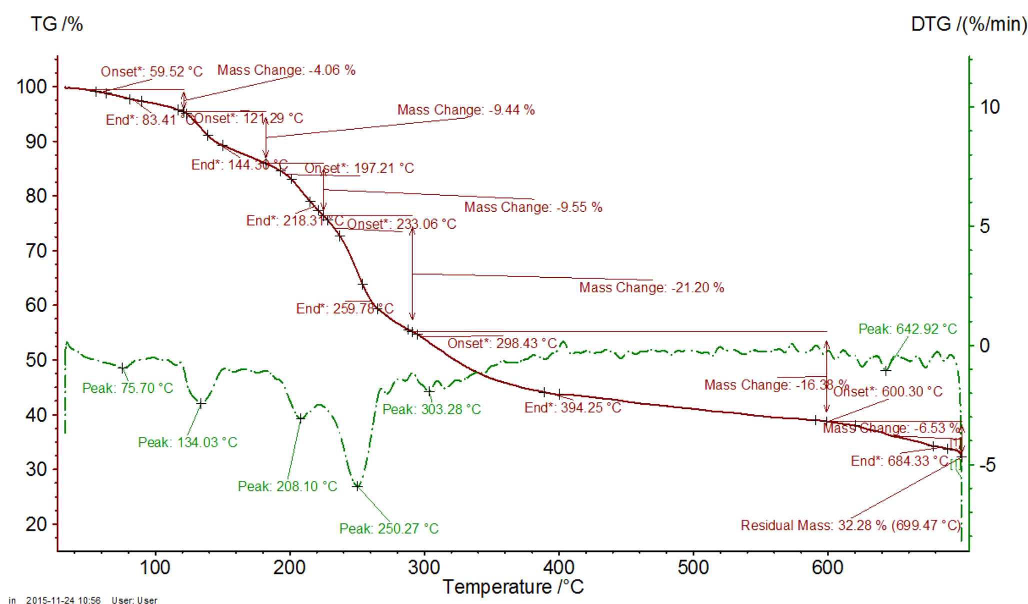


Figure 1. Thermal behaviour of sample I-PP15

In region 60-83 ºC appears an endothermic peak characteristic to the dehydration process, a process that is accompanied by a loss of 4.06% of total mass (Table 3). The second process appears in the range of 120-400 °C, which corresponds to the cleavage of glycosidic linkages with the formation of levoglucosan from the monomer unit. Regarding the sample II-PP15 (figure not shown), its thermal degradation followed a degradation profile similar to the I-PP15 sample.

Table 3. Thermal degradation data corresponding to EPS-LAB samples

| **Sample** | **I-PP15** | | | | **II-PP15** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Ti, ºC** | **Tmax, ºC** | **Tf, ºC** | **Δw, %** | **Ti, ºC** | **Tmax, ºC** | **Tf, ºC** | **Δw, %** |
| Peak I | 59 | 75 | 83 | 4.06 | 62 | 77 | 98 | 3.85 |
| Peak II | 121 | 134 | 144 | 9.44 | 125 | 134 | 141 | 8.80 |
| Peak III | 197 | 208 | 218 | 9.55 | 163 | 172 | 175 | 3.40 |
| Peak IV | 233 | 250 | 259 | 21.20 | 197 | 219 | 234 | 11.57 |
| Peak V | 298 | 303 | 394 | 16.38 | 235 | 247 | 261 | 15.04 |
| Peak VI | 600 | 643 | 684 | 6.53 | 609 | 671 | 675 | 6.36 |
| r, % | 32.28 | | | | 30.37 | | | |

From the thermogravimetric study of samples I-PP15 and PP15-II it can be noticed that in the range of 100-300 ºC is produced an important mass loss (Δw,%), due in particular to the depolymerisation reactions of EPS-LAB, by breaking the C-C or CO bonds. The residue obtained after thermal degradation (r, %) is an expression of the sample's crystallinity degree. Thus, the diminishing of the crystallinity determined a decrease of the activation energy and an increase of the residue quantities (Ciolacu et al., 2006). In the present case for sample I-PP15 was obtained a residue of 32.28%, while for II-PP15 was recorded a lower residue (30.37%). These data confirm the fact that sample II-PP15 has a more crystalline structure than I-PP15.

### 3. Differential Scanning Calorimetry (DSC)

DSC measurements of samples I-PP15 and II-PP15 were performed with Maia F3 200 DSC (Netzsch, Germany) equipment. In the region of 20-140 °C, the DSC measurements of EPS-LAB recorded an endothermic peak characteristic for the dehydration process (Figure 2). Considering that the water absorbed by the EPS-LAB occupies almost the entire area of the amorphous structure it can be noticed that the endothermic peak area is directly proportional to the amorphous fraction of EPS-LAB. Thus, from the areas of the endothermic curves can be noticed that sample II-PP15 (81.65%) has a more crystalline structure than sample I-PP15 (93.44%).

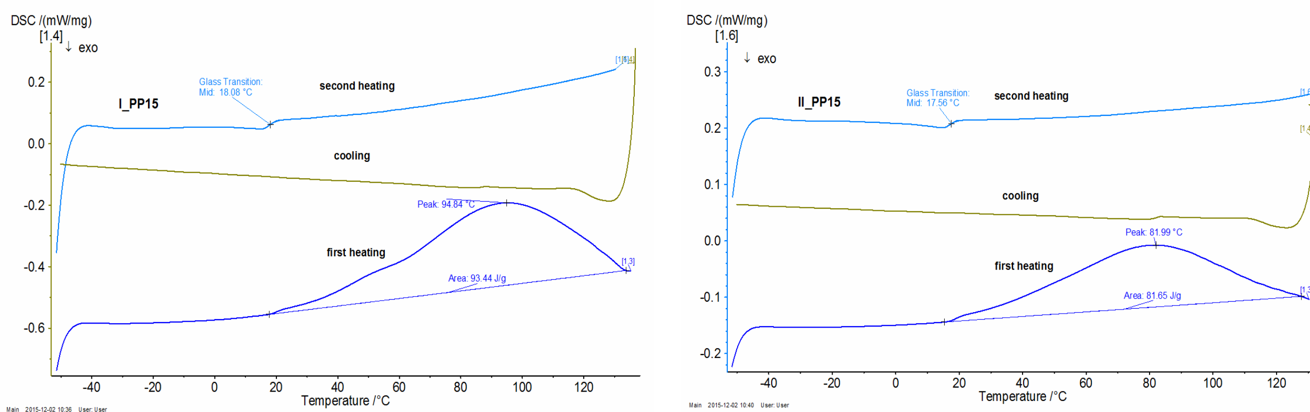


Figure 2. DSC curves of I-PP15 and II-PP15 samples

In addition, both structures have values similar to glass transition areas, which indicate a high flexibility of the chain segments, probably due to the presence of structures with the lowest possible degree of organization.

## 4. Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR spectra were recorded using a Bruker Vertex 70 in the frequency range 4000-400 $cm^{-1}$ with 4 $cm^{-1}$ resolution and 24 scans. The obtained spectra were processed using SpectraManager.

In Figure 3 are presented the spectra recorded for samples I-PP15 and PP15 II.

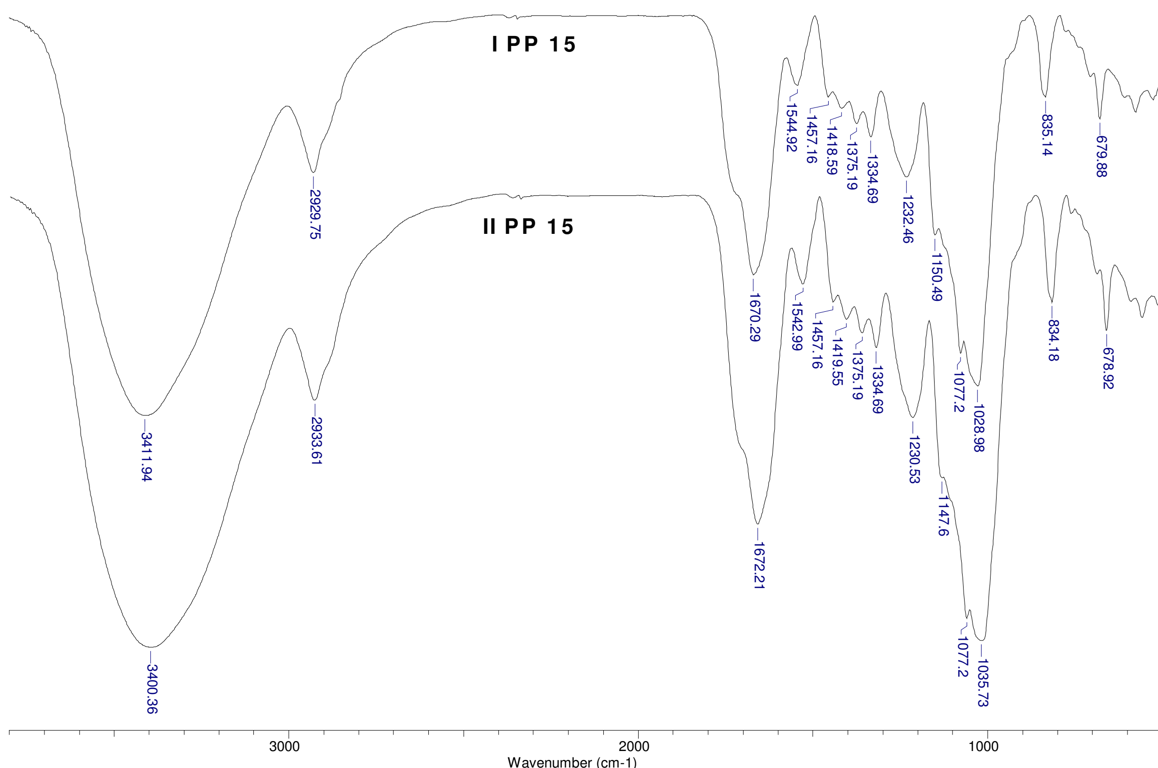


Figure 3. FTIR spectra for samples I-PP15 and II-PP15

The broad band situated in the region 3100-3600 $cm^{-1}$ are due to the fundamental frequencies of OH-stretching vibration; at 3411 $cm^{-1}$ for I-PP15 and 3400 $cm^{-1}$ for II-PP15 (Chen et al., 2013). The absorption bands that appear between 2700-3000 $cm^{-1}$ are assigned to C–H stretching vibration (Xiao et al., 2014), and for EPS-LAB are situated at 2930 $cm^{-1}$ (I-PP15) and at 2934 $cm^{-1}$ (II-PP15). Usually, in region 1500-2000 $cm^{-1}$ appears a peak assigned to absorbed water, at 1670 $cm^{-1}$, which is associated with the OH stretching (Ciolacu et al., 2011). In the range of 1300-1500 $cm^{-1}$ it can be noticed the band at 1457 $cm^{-1}$, assigned to a symmetric $CH_2$ bending vibration and the bands at 1334 $cm^{-1}$, assigned as C-OH bending in plane (Ryden et al., 2013). The band at 835 $cm^{-1}$ for sample I-PP15 and 834 $cm^{-1}$ for sample II-PP15 is characteristic to the valence vibration of β-D-glucan (Goo et al., 2013).

## 5. Nuclear magnetic resonance ($^1$H-NMR)

$^1$H-NMR spectroscopy was done by using a Bruker Avance III NMR spectrometer with a multinuclear sample head with reverse detection. The samples were dissolved in deuterated water ($D_2O$). $^1$H-NMR spectra of I-PP15 and II-PP15 samples are presented in Figures 4 and 5.

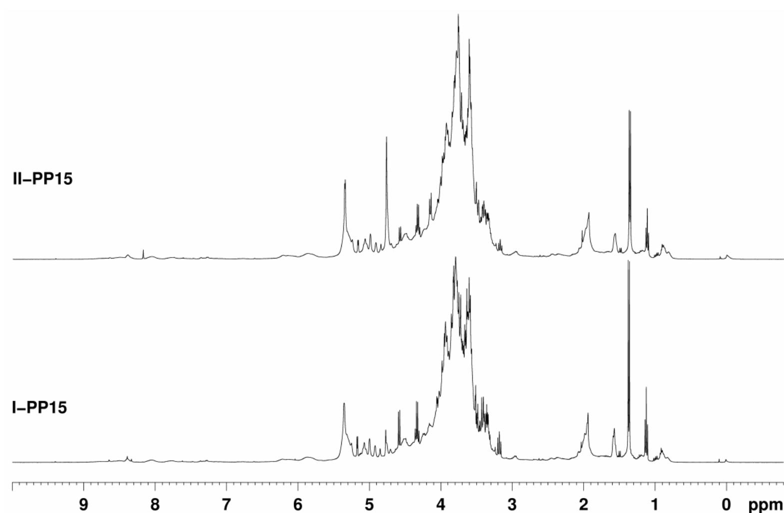


Figure 4. $^{1}$H-NMR spectra of I-PP15 and II-PP15, recorded in $D_2O$ at 400 MHz

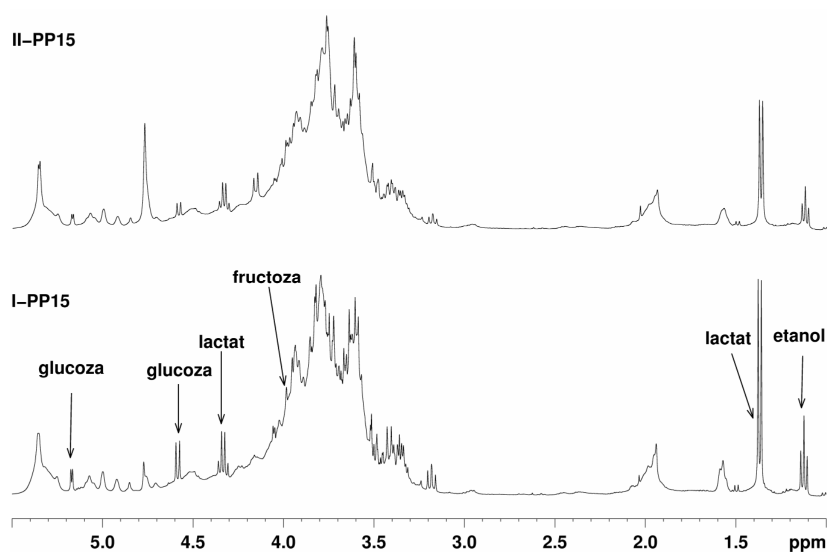


Figure 5. $^{1}$H-RMN spectra of I-PP15 and II-PP15 (range: 1-5.5 ppm).

Profile spectral data indicated the presence of two types of compounds: low molecular compounds characterized by narrow signals and well-defined macromolecular forms, characterized by broad signals (Figure 4).

Among low molecular compounds there have been identified: **lactate / lactic acid -** characterized by two signals: 1.36 ppm (doublet, coupling constant J = 7.0 Hz) and 4.33 ppm (quartet, coupling constant J = 7.0 Hz), **glucose -** characterized by the two signals corresponding to the anomeric proton from 4.58 ppm (β form, doublet, J = 7.92 Hz), and 5.17 ppm (α form, doublet, J = 3.72 Hz) and **fructose -** characterized by a number of narrow signals in the range from 3 to 4.2 ppm.

The presence of the macromolecular compounds is indicated by broader signals from 3 to 5.6 ppm spectral region (Figure 5), region from 4.4 to 5.6 ppm being characteristic for anomeric protons of such structures**.**

More data and two-dimensional NMR experiments are required for identification of the compounds present in these complex mixtures.

## CONCLUSIONS

The objective of phase I of the MATINOV project was fulfilled. There were selected the optimal conditions for lactic acid bacteria (LAB) to biosynthesize new series of exopolysaccharides (EPS-LAB) and the resulting compounds were isolated, purified and characterized by dedicated methods:

1. 34 strains of lactic acid bacteria from various sources, such as rye flour, yogurt, wheat flour, milk or cottage cheese, were isolated.
2. Lactic acid bacteria selection was done by testing these 34 strains on two types of media: liquid, represented by MRS broth, and solid, represented by MRS agar supplemented with glucose and fructose. Based on this first selection, there were identified five strains best producers of EPS-LAB.
3. For the biosynthesis of EPS-LAB at a large scale, in order to be extracted, purified and quantified, the five strains were further tested in liquid medium, using two types of media improved with different carbon sources, denoted MD I (MRS broth, fructose and glucose) and MD II (MRS broth and sucrose).
4. It has been demonstrated that for all 5 strains of lactic acid bacteria the most suitable medium for the EPS-LAB biosynthesis is MD II, when the maximum yield was 5.18 g EPS-LAB / L of culture medium.
5. GPS analysis showed that EPS-LAB contain polydisperse and monodisperse fractions; also, it is assumed that fractions I with high molecular weight values and polydispersity indices are in the form of complex aggregates of exopolysaccharides.
6. FTIR and $^{1}$H-NMR spectroscopies indicate that it have been biosynthesized EPS-LAB and confirm the estimated structures of the obtained polymers.
7. The thermogravimetric and DSC analysis of the selected samples have revealed the presence of a mixture of exopolysaccharides, along with relatively small amounts of low molecular compounds, possibly fructose and/or glucose.

### References

Adnan A.F.M., Tan I.K.P., Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential, *Bioresour. Technol.*, 98, 1380-1385 (2007).

Ahmed Z., Wang Y., Anjum N., Ahmad H., Ahmad A., Raza M., Characterization of new exopolysaccharides produced by coculturing of *L. kefiranofaciens* with yoghurt strains, *Int. J. Biological Macromol.*, 59, 377– 383 (2013).

Bennama R., Fernandez M., Ladero V., Alvarez M. A., Rechidi-Sidhoum N., Bensoltane A., Isolation of an exopolysaccharide-producing *Streptococcus thermophilus* from algerian raw cow milk, *Eur. Food Res. Technol*., 234, 119–125 (2012).

Chen Y., Mao W., Gao Y., Teng X., Zhu W., Chen Y., Zhao C., Li N., Wang C., Yan M., Shan J., Lin C., Guo T., Structural elucidation of an extracellular polysaccharide produced by the marine fungus *Aspergillus versicolor*, *Carbohydr. Polym*., 93, 478– 483 (2013).

Ciolacu D., Popa V.I., Study on the thermal degradation of cellulose allomorphs, *Cellulose Chem. Technol*., 40, 6, 445-449 (2006).

Ciolacu D., Oprea A., Anghel N., Cazacu G., Cazacu M., 2012, New cellulose-lignin hydrogels and their application in controlled release of polyphenols, *Mater. Sci. Eng. C*, 32, 452–463.

Goo B.G., Baek G., Choi D.J., Park Y.I., Synytsya A., Bleha R., Seong D.H., Lee C.G, Park J.K, Characterization of a renewable extracellular polysaccharide from defatted microalgae *Dunaliella tertiolecta*, *Biores.Technol*., 129, 343–350 (2013).

Grobben G.J., Chin-Joe I., Kitzen V.A., Boels I.C., Boer F., Sikkema J., Smith M.R., de Bont J.A., Enhancement of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. Bulgaricus NCFB 2772 with a simplified defined medium, *Appl. Environ. Microbiol*., 64(4), 1333–1337 (1998).

Hwang H.J., Kim S.W., Xu C.P., Choi J.W., Yun J.W., Production and molecular characteristics of four groups of exopolysaccharides from submerged culture of *Phellinus gilvus*, *J. Appl. Microbiol.*, 94, 708–719 (2003).

Lee K.W., Park J.Y., Jeong H.R., Heo H.J., Han N.S., Kim J.H., Probiotic properties of *Weissella strains* isolated from human faeces, *Anaerobe*, 18, 96-102 (2012).

Liu C.T., F.J. Chu, C.C. Chou, R.C. Yu, Antiproliferative and anticytotoxic effects of cell fractions and exopolysaccharides from *Lactobacillus casei* 01, *Mutation Research*, 721, 157–162 (2011).

Malik A., M. Radji, S. Kralj, L. Dijkhuizen, Screening of lactic acid bacteria from Indonesia reveals glucan sucrose and fructansucrase genes in two diferent *Weissella confusa* strains from soya, *FEMS Microbiol Lett*, 300(1), 131–138 (2009).

Miao M., Bai A, Jiang B., Song Y., Cui S. W., Zhang T., Characterization of a novel water-soluble polysaccharide from *Leuconostoc citreum* SK24.002, *Food Hydrocolloids*, 36, 265-272 (2014).

Moscovici M., Present and future medical applications of microbial exopolysaccharides, *Front Microbiol.*, 6(1012), 1-11 (2015).

Nwodo, U.U., Green, E., Okoh, A.I., Bacterial Exopolysaccharides: Functionality and Prospects, *Int. J. Molec. Sci.,* 13, 14002-14015 (2012).

Palomba S., Cavella S., Torrieri E., Piccolo A., Mazzei P., Blaiotta G., Ventorino V., Pepe O., Wheat sourdough from *Leuconostoc lactis* and *Lactobacillus curvatus* exopolysaccharide-producing starter culture: polyphasic screening, homopolysaccharide composition and viscoelastic behaviour, *Appl. Environ. Microbiol.*, 78 (8), 2737-2747 (2012).

Patel S., Majumder A., Goyal A., Potentials of exopolysaccharides from lactic acid bacteria, *Indian J Microbiol.*, 52(1), 3–12 (2012).

Paulo E.M., Vasconcelos M.P., Oliveira I.S., de Jesus Affe H.M., Nascimento R., de Melo I.S., de Abreu Roque M.R., de Assis S.A., An alternative method for screening lactic acid bacteria for the production of exopolysaccharides with rapid confirmation, *Ciência e Tecnologia de Alimento*, 32(4), 710-714 (2012).

Ryden P., Gautier A., Wellner N., Tapp H.S., Horn S.J., Eijsink V.G.H., Waldron K.W., Changes in the composition of the main polysaccharide groups of oil seed rape straw following steam explosion and saccharification, *Biomass Bioenergy*, 61, 121-130 (2013).

Schiraldi C., Valli V., Molinaro A., Carteni M., De Rosa M., Exopolysaccharides production in *Lactobacillus bulgaricus* and *Lactobacillus casei* exploiting microfiltration. *J. Ind. Microbiol. Biotechnol.*, 33 (5), 384–390 (2006).

Tayuan A., Tannock, G. W., Rodtong, S., 2011, Growth and exopolysaccharide production by *Weissella* sp. from low-cost substitutes for sucrose. *African J. Microbiol. Res.*, 5(22), 3693-3701.

Wang Y., Li C., Liu P., Ahmed Z., Xiao P., Bai X., Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from tibet kefir, *Carbohydr. Polym.*, 82, 895–903 (2010).

Xiao Q., Tong Q., Lim L. T., Drying process of pullulan edible films forming solutions studied by ATR-FTIR with two-dimensional correlation spectroscopy, *Food. Chem.*, 150, 267–273 (2014).