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EXTRACTION AND ANTIOXIDANT ACTIVITY ASSESSMENT OF POSTBIOTIC EXOPOLYSACCHARIDES PRODUCED BY SELECTED LACTIC ACID BACTERIA

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Abstract: Lactic acid bacteria strains are frequently involved in obtaining fermented products characterized by an enhanced nutritional and sensorial value. Exopolysaccharides are carbohydrate-based macromolecules produced by selected strains of lactic acid bacteria (LAB) as extracellular postbiotic compounds. The extraction techniques used to obtain high exopolysaccharides yields are extremely important factors that directly impact their future utilization and functional properties. In this work, the exopolysaccharides produced by two LAB strains, belonging to genus *Lactobacillus* and *Leuconostoc*, were extracted from the fermentation medium following two different protocols that employed trichloroacetic acid (TCA) and/ or absolute ethanol. The highest exopolysaccharide extraction yield (956.60 mg/L) was determined after proteins' precipitation with TCA from the fermented medium by *Leuconostoc* spp. MIUG BL40 strain. The crude exopolysaccharides biosynthesized by the strain mentioned above had the highest ABTS radical scavenging activity (28.53%) at 10 mg/mL. Therefore, DPPH radical scavenging rates ranging between 2.99-3.30% were calculated for the studied exopolysaccharides. Overall, the results reported in this work offer promising research perspectives that will be further investigated.

Keywords: lactic acid bacteria; exopolysaccharides; extraction; antioxidant activity

Introduction

Lactic acid bacteria (LAB) strains have been used since ancient times for their ability to enhance the sensorial and nutritional value of foods and beverages. Moreover, some LAB strains biosynthesize extracellular polymeric macromolecules known as exopolysaccharides (EPS) composed of monosaccharides. The EPS are classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS) considering the EPS chemical structure (Rana and Upadhyay,

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2020; Zannini *et al.*, 2016). HoPS are characterized by repeating units of the same monosaccharide; their production starts from fructose or glucose, the biosynthesis being catalyzed by specific enzymes. The main classes of HoPS are α-D-glucans, β-D-glucans, fructans, inulin-type and polygalactans. Branched or linear molecules made of 3-8 different monosaccharides and derivatives are part of HePS. Interestingly, the yield, functionality and chemical nature of the HePS is strongly determined by the fermentation conditions (Rahbar Saadat *et al.*, 2019). EPS are considered postbiotics because they are important extracellularly released compounds with functional properties that contribute to a healthy lifestyle, thus intensively studied (Nataraj *et al.*, 2020; Żółkiewicz *et al.*, 2020).

Various EPS produced by LAB strains belonging to *Leuconostoc* spp., *Weissella* spp., or by the newly taxonomical classified *Limosilactobacillus reuteri*, *Lentilactobacillus parabuchneri*, *Lactilactobacillus sakei* and *Lacticaseibacillus casei* proved to be efficient in the bakery as technological improvers, sugar replacers, or prebiotics (Bikric *et al.*, 2021). Thus, other beneficial implications were determined for the confectionary products or ice creams containing EPS as emulsifiers or stabilizers (Korcz and Varga, 2021). The EPS produced by a probiotic *Limosilactobacillus reuteri* (former *Lactobacillus reuteri*) strain was able to overcome the inflammatory reactions as well as tumors' proliferation, results reported by Chen *et al.* (2019) after in vitro assays. These health-promoting effects were linked to the monomeric composition of the studied EPS in the above-mentioned work. The EPS produced by lactobacilli have diverse immunomodulatory, anticarcinogenic, antimicrobial and antioxidant effects associated with the hydroxyl, phosphate and carbonyl functional groups within their structure (Riaz Rajoka *et al.*, 2020).

Therefore, considering the preoccupations in the field towards healthier food products with improved functionality, EPS derived from LAB strains are promising alternatives to fulfill the current industrial requirements. In this work, two selected LAB strains able to produce EPS by fermenting the modified de Man, Rogosa and Sharpe (MRS) medium were studied regarding the extraction techniques and the antioxidant properties of these biopolymers.

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Materials and methods

Reagents and chemicals

All the culture media, reagents and chemicals used in this study were purchased from Sigma-Aldrich (Steinheim, Germany).

Microorganisms and culture conditions

The LAB strains used in this study, respectively a *Lactobacillus* spp. strain (MIUG BL39) isolated from whole wheat flour, and a *Leuconostoc* spp. strain (MIUG BL40) isolated from sesame seeds as previously described (Păcularu-Burada *et al.*, 2020). Both strains were included in the Collection of Microorganisms from the Bioaliment Research Platform, University Dunărea de Jos of Galați, with the acronym MIUG. Previously identified as valuable EPS producers, these above-mentioned strains were routinely reactivated from pure stock cultures in MRS broth medium. After 48 h of incubation at 37℃ in aerobiosis (Binder BF4000, Tuttlingen, Germany), 2% (v/v) inoculum of each LAB strain was used for the fermentation of 500 mL modified MRS broth containing 50 g/L sucrose and 5 g/L glucose, following the protocol described by Zhao *et al.* (2019), taking into consideration for the inoculum an optical density of 1.80 (OD_{600nm}) spectrophotometrically determined (Libra S22 UV-VIS, Biochrom, Cambridge, UK) that corresponds to 10^8 CFU/ mL (Üçok and Sert, 2020).

Exopolysaccharides' extraction protocols

After 48 h of incubation at 37℃ in aerobiosis, prior to the extraction of the EPS from the fermented medium, the enzymes that could determine the EPS structures' degradation were inactivated by heating at 90℃ for 15 min in a water bath (Julabo, Seelbach, Germany) followed by centrifugation (8000 rpm, 4℃) for 10 min (Hettich Universal 320R, Tuttlingen, Germany). The LAB crude biomass was discarded and the resulting supernatant was employed in the EPS extraction protocols (Liu *et al.*, 2019).

Two different extraction protocols were studied. For the first one, proposed by Xiao *et al.* (2020), one volume of cell-free supernatant, obtained as previously described, was mixed with

three volumes of absolute cold ethanol, 1:3 (v/v). This mixture was stored overnight at 4°C and the EPS were separated after centrifugation (8000 rpm, 4°C, 20 min).

For the second extraction protocol, the cell-free supernatant was firstly mixed with 10% (w/v) trichloroacetic acid (TCA), stored overnight at 4°C and then centrifugation was applied (8000 rpm, 4°C, 20 min). The precipitated proteins from the supernatant were discarded. Afterward, the supernatant was mixed with absolute ethanol considering a ratio of 1:3 (v/v), this mixture being as well stored overnight at 4°C. Finally, the EPS were separated after another round of centrifugation using the same conditions as previously stated (Amao *et al.*, 2019).

The crude EPS that resulted from the extractions mentioned above were dissolved in 10 mL ultrapure water and then were subjected to freeze-drying (Martin Christ, Osterode am Harz, Germany) and stored at 4°C in opaque airtight containers. The EPS extraction yields were determined by weighting the crude EPS powders after lyophilization and expressed as mg/L.

Exopolysaccharides' antioxidant activities

Antioxidant activity by DPPH

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity was determined by using a fresh DPPH solution (0.1 mM) in absolute methanol, as described by Abedfar *et al.* (2018) slightly modified by Vasile *et al.* (2020). Precisely, for the antioxidant activity, an aliquot of 100 µL EPS solution (1.50-10.00 mg/mL) in methanol (Sirin and Aslim, 2020) was mixed with 3.90 mL DPPH solution. After 30 min of storage at room temperature in darkness, the absorbances of the samples were determined at a wavelength of 517 nm. Results were expressed as Radical Scavenging Activity, using equation (1):

$$RSA, \% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100 \quad (1)$$

where A_{blank} is the absorbance of the control sample containing methanol and A_{sample} is the absorbance of the analyzed sample containing EPS solution.

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Antioxidant activity by ABTS

For the assessment of the antioxidant activity by ABTS (2,2′- azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), the protocol described by Al-Dhaheri *et al.* (2017) was followed. Briefly, a stock ABTS solution (7.4 mM) was prepared in absolute methanol. Afterward, this solution was mixed with an equal volume of an aqueous solution of potassium persulfate (2.6 mM). After 12-16 h of storage at room temperature in darkness, the absorbance of the mixture was diluted with methanol to reach an absorbance of 0.70 (A_{734nm}= 0.70). For the antioxidant activity by ABTS, a sample of 20 µL EPS solution (1.50-10.00 mg/mL) in methanol was mixed with 2 mL ABTS solution. After 6 min of storage at room temperature in darkness, the absorbances of the analyzed samples were read spectrophotometrically at a wavelength of 734 nm.

For the DPPH and ABTS assays, ascorbic acid (Vc) solution with similar concentrations as the EPS, respectively 1.50-10.00 mg/mL, was used as a positive control sample. Results were expressed as Radical Scavenging Activity following equation (1).

Statistical analysis

Reported results are average measurements (n = 3) ± standard deviations. The statistically significant differences were highlighted by one-way ANOVA followed by the Tuckey test ($p < 0.05$) using Minitab 17 software (Minitab LLC, Pennsylvania, USA).

Results and discussion

Exopolysaccharides' extraction efficiency

Exopolysaccharides' extraction following two different protocols, involving only absolute ethanol on one side, respectively TCA and ethanol on the other side, determined significant differences among the EPS extraction yields ($p < 0.05$), as is shown in Table 1.

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Table 1. EPS extraction yields

LAB strain and extraction protocol	Crude EPS yield, mg/L[1]
MIUG BL39 ET-OH	$403.80^{bA} \pm 7.30$
MIUG BL40 ET-OH	$250.00^{bA} \pm 5.00$
MIUG BL39 TCA-ET-OH	$304.60^{bA} \pm 10.65$
MIUG BL40 TCA-ET-OH	$959.60^{aB} \pm 14.99$

[1] Different lowercase superscript letters denote significant differences between different strains and extraction methods, respectively different uppercase superscript letters denote significant differences between the extraction methods using the same strain.

The amounts of crude EPS extracted ranged between 250 – 959 mg/L. Therefore, the most performant LAB strain regarding the EPS yield was the *Leuconostoc* spp. MIUG BL40. The modified MRS broth medium fermented by this LAB strain, incubated for 48 h at 37℃ that was first subjected to proteins' precipitation by 10% (w/v) TCA was the most efficient extraction protocol. On the contrary, it can be observed (Table 1) that the ethanolic precipitation of the crude EPS was more efficient for MIUG BL39 strain, possibly due to the presence of proteins and other compounds besides EPS. Another reason for these EPS extraction yields is related to the preliminary genus classification of the studied strains. *Lactobacillus* spp. MIUG BL39 strain probably has different nutritional requirements and metabolic features compared with the other strain, respectively *Leuconostoc* spp. MIUG BL40 that influence the process of EPS biosynthesis.

Modified MRS broth, supplemented with 20 g/L glucose, was used by Riaz Rajoka *et al.* (2018) for the EPS production potential for various *Lactobacillus rhamnosus* strains. After 48 h of incubation at 37℃, the EPS were precipitated with ethanol and the results suggested that the most performant strains determined EPS yields between 610 – 737 mg/L. Zhang *et al.* (2019) reported that a *Lactobacillus sanfranciscensis* strain originated from sourdough determined EPS yields between 160-180 mg/L after TCA and ethanol precipitation by a statistical optimization of the fermentation parameters for maximizing the EPS biosynthesis (i.e., the inoculum size, carbon

and nitrogen source, the pH value of the fermentation medium, the incubation time and temperature or specific growth factors).

In another study, the ethanolic precipitation of the EPS produced by a *Lactobacillus fermentum* strain after 48 h of incubation at 30°C lead to more than 450 mg/L EPS. The extension of the fermentation time up to 72 h increased the EPS yield, more than 800 mg/L being reached (Ale *et al.*, 2016). The works mentioned above support our results regarding the EPS extraction yields reported in this work (Table 1). Moreover, the EPS production capacity of the LAB can be different between strains and fermentation conditions. The protocols employed in the EPS extraction may have, as well, an important contribution to the final results. Almalki (2020) and Cirrincione *et al.*, (2018) emphasized that the most common carbohydrates used as carbon sources for EPS biosynthesis are glucose, maltose and sucrose, along with minerals (Cu, Ca and Zn). The ratio between the EPS-containing cell-free supernatant and different extraction solvents was studied by Oleksy-Sobczak *et al.* (2020) and Nachtigall *et al.*(2021) to conclude that, depending on the LAB strain used, two volumes of acetone or one volume of ethanol mixed with the fermented medium determined EPS yields higher than 650 mg/L. The commercial starter culture of *Lactobacillus acidophilus* LA5 produced 349.82 mg/L EPS after 12 h of incubation at 42°C (Amiri *et al.*, 2019), whereas supplementation of the MRS broth medium with serine, isoleucine, methionine and vitamins determined EPS yields between 1.57 - 9.02 g/L (El-Dein *et al.*, 2021). The wild LAB strains possess genes that control the enzymatic production of EPS starting from different sugars available in the fermentation medium. This behavior was observed by Adebayo-Tayo and Fashogbon (2020) when a comparative analysis was carried out between two *Lactobacillus delbrueckii* strains, one of them being a wild strain, while the other was subjected to metabolic engineering techniques. Therefore, the wild *Lactobacillus delbrueckii* was the most productive regarding the EPS yield, respectively 5.91 g/L after ethanolic precipitation.

Even though the MRS broth medium is frequently employed in EPS production, its main disadvantage is its complex composition that could determine the overestimation of EPS yields. Fortunately, it was reported that this culture medium could offer satisfactory results when TCA precipitation is involved in the EPS extraction. This primary precipitation of proteins and other interferants from the MRS broth can contribute to the maximization and an accurate evaluation

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of the EPS yields (Pintado *et al.*, 2020). The impurities from the crude EPS produced by a *Leuconostoc mesenteroides* strain after the fermentation of a culture medium containing glucose and sucrose were reduced when TCA precipitation was carried out (Li *et al.*, 2020). Contrary, in our study, the *Leuconostoc* spp. strain MIUG BL40 had the highest EPS yield, respectively 959.60 mg/L, when the extraction protocol consisted of TCA precipitation followed by the ethanolic extraction.

Exopolysaccharides' antioxidant properties

The evaluation of the antioxidant activities by DPPH and ABTS assays were evaluated, as shown in Table 2 and Figure 1. As such, the extraction protocol, and the LAB strains determined different results for the studied antioxidants. The EPS produced by the *Lactobacillus* spp. and *Leuconostoc* spp. strains could not scavenge the DPPH radical in most cases, except the EPS originated from the *Lactobacillus* spp. MIUG BL39, when a 10 mg/mL concentration determined a radical scavenging activity of 2.99%. On the contrary, a smaller amount, respectively 7.50 mg/mL, was necessary to reach a radical scavenging activity of 3.30% when the *Leuconostoc* spp. MIUG BL40 was the EPS-producing strain (Table 2).

Table 2. Antioxidant activity of the crude EPS determined by DPPH assay

LAB strain and extraction protocol		DPPH, Radical Scavenging Activity, %[2]				
		MIUG BL39 ET-OH	MIUG BL40 ET-OH	MIUG BL39 TCA-ET-OH	MIUG BL40 TCA-ET-OH	Vc
Concentration, mg/mL	**1.50**	n.d.	n.d.	n.d.	n.d.	$90.04^{A} \pm 1.17$
	3.50	n.d.	n.d.	n.d.	n.d.	$90.28^{A} \pm 0.83$
	7.50	n.d.	$3.30^{b} \pm 0.81$	n.d.	n.d.	$90.98^{aA} \pm 0.17$
	10.00	n.d.	n.d.	$2.99^{b} \pm 0.81$	n.d.	$91.21^{aA} \pm 0.05$

[2] Different lowercase superscript letters denote significant differences in the same row, respectively different uppercase superscript letters denote significant differences in one column.

A concentration of 0.2 mg/mL EPS from the *Lactobacillus acidophilus* LA5 commercial starter determined a radical scavenging activity of 4% for DPPH (Amiri *et al.*, 2019). As such, these low scavenging activities of the EPS using the DPPH radical could result from the poor

solubilization of the EPS in methanol, respectively the insufficient purification or the chemical nature of these EPS may be the reason for such outcomes. Andrew and Jayaraman (2020) stated that the antioxidant properties of the EPS are strongly linked to their structure (linear or branched) and composition (type and amount of reducing sugars or hydrogen bonds). The scavenging power depends on the presence of functional groups in the structure of the EPS, these functional groups being important hydrogen donors (Xing *et al.*, 2018). Another reason for the above-reported results summarized in Table 2 is related to the volumes of sample and DPPH solution used to assess the antioxidant activity by DPPH. Enhanced antioxidant potential was reported in the literature for various EPS produced by LAB strains; thus, the volume of EPS or DPPH solution used was higher than ours (Adebayo-Tayo and Fashogbon, 2020; El-Dein *et al.*, 2021). Furthermore, Zhang *et al.* (2016) observed an enhanced antioxidant activity by DPPH after the sulfation of the EPS produced by a *Lactobacillus plantarum* strain. It can be concluded that the methods used for the extraction of the EPS or their further processing can have a positive or negative impact on the analyzed properties, depending on various intrinsic factors.

The DPPH radical scavenging activity of a heteropolysaccharide composed of mannose, galactose, fucose and glucuronic acid produced by a *Lactobacillus plantarum* strain was characterized by 60% inhibition until a concentration of 2 mg/mL, another significant increase being observed for a concentration of 10 mg/mL, respectively 80% DPPH radical scavenging activity (Liu *et al.*, 2019). The DPPH scavenging activity determined by the ascorbic acid (Vc) in our work (Table 2) is comparable with the findings from the literature. The DPPH radical inhibition increases along with the concentration of ascorbic acid. Riaz Rajoka *et al.* (2018) and Adebayo-Tayo and Fashogbon (2020) reported the same concentration-dependent behavior. The differences between the analyzed samples and the control sample (Vc) were statistically significant ($p<0.05$).

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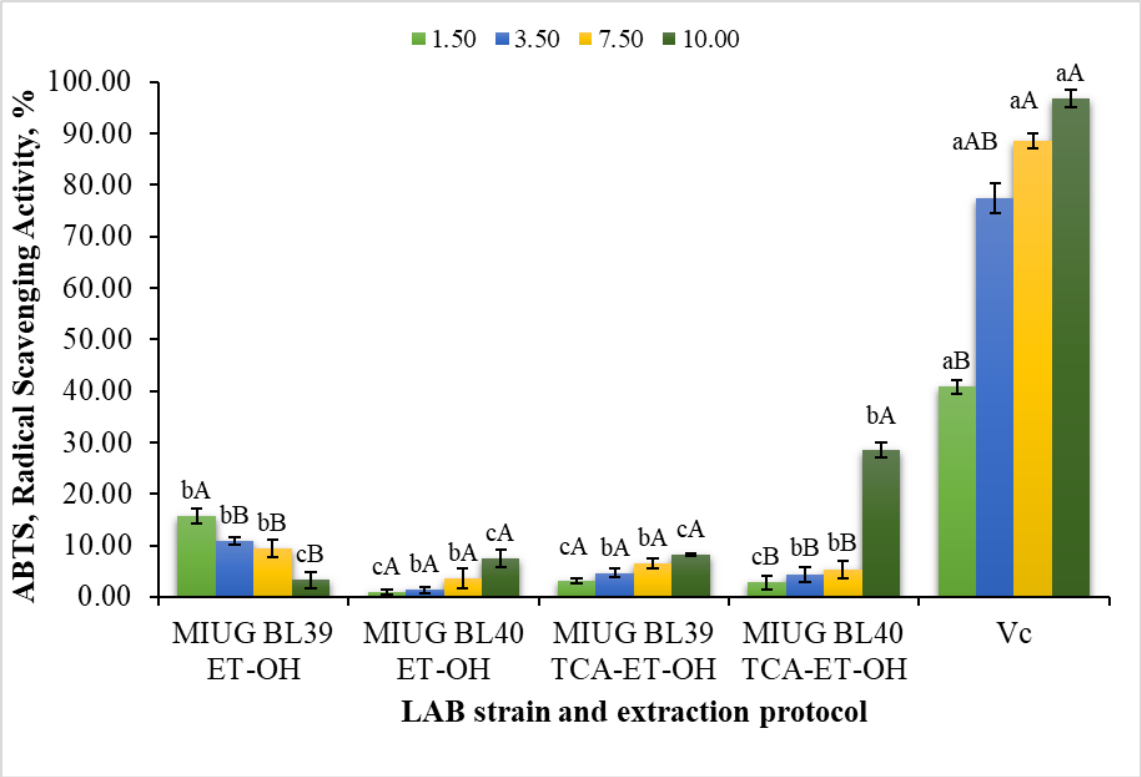

Fig. 1. Antioxidant activity of the crude EPS by ABTS assay. Different lowercase superscript letters denote significant differences considering the same concentration and different strains/ extraction protocols, whereas different uppercase superscript letters denote significant differences among various concentrations of the same strain.

For the ABTS assay, the scavenging rates were characterized by a concentration-dependent manner for the EPS produced, on the one hand by the *Leuconostoc* spp. strain (MIUG BL40) or by the *Lactobacillus* spp. strain (MIUG BL39) only when the extraction involved primary precipitation of the proteins with 10% (w/v) TCA, on the other hand, as depicted in Figure 1.

The highest ABTS radical scavenging activities, respectively 15.61% and 28.53%, were determined for the EPS extracted with ethanol from MIUG BL39 strain at a concentration of 1.5 mg/mL, respectively at 10 mg/mL EPS extracted with TCA and ethanol from MIUG BL40. Ayyash *et al.* (2020) studied the biosynthesis of EPS by a *Pediococcus pentosaceus* strain and determined scavenging ratios of 22.30% at a 5.00 mg/mL concentration, respectively 48.90% at 10 mg/mL. Li *et al.* (2020) reported that an ABTS radical scavenging activity of 20% was calculated when a concentration of 2 mg/mL EPS produced by a *Leuconostoc mesenteroides*

strain was used. Higher ABTS radical scavenging activities could be obtained when the crude EPS is purified. As such, an EPS from a *Lactobacillus sanfranciscensis* strain, purified by Thin-Layer-Chromatography (TLC) methods, determined scavenging rates between 30 - 90%. The ABTS scavenging ratios determined for the control samples (Vc) ranging between 40.78 - 96.74% are in agreement with the results reported by other authors; thus, a concentration-dependent manner being observed (Min *et al.*, 2019; Zhang *et al.*, 2019).

Conclusions

LAB strains are able to produce various postbiotic compounds with health-promoting effects. Consequently, some LAB strains biosynthesize the necessary enzymes to convert specific carbohydrates from the culture medium into postbiotic EPS with enhanced functional properties. In this work, two previously selected LAB strains able to produce high yields of EPS, namely *Lactobacillus* spp. MIUG BL39 and *Leuconostoc* spp. MIUG BL40 were tested to identify the most suitable extraction protocol between the ethanolic precipitation alone, and TCA followed by ethanol precipitation. The results highlighted that the extraction methodology had a significant impact on the EPS extraction yield. Considering the LAB strains studied in this work, the highest EPS yield, respectively 959.60 mg/L, was determined after the primary proteins' precipitation with 10% (w/v) TCA and then the EPS precipitation with three volumes of absolute cold ethanol. Moreover, the extraction protocols, as well as the LAB strain determined different scavenging activities for the ABTS radical on the one hand, the highest scavenging ratio (28.53%) being achieved for a concentration of 10 mg/mL EPS solution originated from the fermented medium by MIUG BL40 after 48 h at 37°C. On the other hand, lower DPPH scavenging activities, ranging between 2.99 - 3.30%, were calculated, depending on the EPS producer and extraction method.

In the future, the *Leuconostoc* spp. strain (MIUG BL40) will be used as a valuable EPS producer, considering that the precipitation of the proteins from the cell-free supernatant with TCA represents a practical solution to maximize the EPS yields. Additionally, our future perspectives in this field will focus on designing a sustainable low-price fermentation medium using wastes

from other food industry sectors. The optimization of the biotechnological parameters in order to assure the sustainability and efficiency of the process are targeted.

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