International Journal of Food Microbiology 214 (2015) 54–62

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Production and partial characterization of exopolysaccharides produced by two *Lactobacillus suebicus* strains isolated from cider

Idoia Ibarburu [a], Ana Isabel Puertas [a], Iñaki Berregi [a], Miguel A. Rodríguez-Carvajal [c], Alicia Prieto [b], Mª. Teresa Dueñas [a,*]

[a] Departmento de Química Aplicada, Facultad de Ciencias Químicas, Universidad del País Vasco, Paseo Manuel de Lardizabal 3, 20018 San Sebastián, Spain
[b] Departamento de Biología Medioambiental, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain
[c] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, Profesor García González, 1, 41012 Sevilla, Spain

ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form 19 June 2015
Accepted 9 July 2015
Available online 16 July 2015

Keywords:
Exopolysaccharides
Lactobacillus suebicus
Heteropolysaccharides
Lactic acid bacteria

ABSTRACT

Many lactic acid bacteria synthesize extracellular polysaccharides (exopolysaccharides, EPSs) with a large variation in structure and potential functional properties. Although EPS production can produce detrimental effects in alcoholic beverages, these polymers play an important role in the rheological behavior and texture of fermented products. In this work, EPS production by two *Lactobacillus suebicus* strains, which were isolated from ropy ciders, was examined in a semidefined medium. The existence of priming glycosyltransferase encoding genes was detected by PCR. In addition, the preliminary characterization of the polymers was undertaken. Molecular masses were determined by size exclusion chromatography revealing the presence of two peaks, corresponding to polymers of high- and low-molecular-weight in all fractions. The composition of the EPS fractions was analyzed by gas chromatography–mass spectrometry after acid hydrolysis, revealing that they contained glucose, galactose, *N*-acetylglucosamine and phosphate, although in different ratios, suggesting that a mixture of polysaccharides is being synthesized. We also examined the influence of the sugar source (glucose, ribose, xylose, or arabinose) and pH conditions on growth and EPS production.

1. Introduction

Certain strains of lactic acid bacteria (LAB) produce exopolysaccharide (EPS) with a large variation in composition, molecular mass, and structure. These molecules are secreted into the medium playing an important role in the rheology and texture of fermented foods, enhancing naturally the texture and viscosity, but in addition to being a biothickener, it has been reported that some EPSs can promote antitumoral, immunomodulatory and antimicrobial activity (Werning et al., 2012; Hidalgo-Cantabrana et al., 2012). Additionally, prebiotic effects of several EPS have also been demonstrated (Korakli et al., 2002). These properties generate interest in the food industry due to several LAB species have a "Generally Recognized As Safe" status by the American Food and Drug Association or a "Qualified Presumption of Safety" status by the European Food Safety Authority. However, extracellular polymers also produce deleterious effects to the organoleptic properties of fermented alcoholic beverages, as in cider (Dueñas et al., 1995) and in wine (Llaubères et al., 1990), being the origin of an alteration named oiliness or ropiness characterized by a viscous texture and oily feel. EPSs produced by *Pediococcus parvulus* 2.6 (Dueñas-Chasco et al., 1997), *Lactobacillus* sp. G77 (Dueñas-Chasco et al., 1998) or *Oenococcus oeni* I4 (Ibarburu et al., 2007) isolated from ropy ciders have been characterized. Depending on the chemical composition, the bacterial EPSs are classified as homopolysaccharides (HoPSs), consisting of only one type of monosaccharide and heteropolysaccharides (HePSs) formed by two or more types of monosaccharides. Heteropolysaccharides are usually produced in small amounts by homofermentative and facultatively heterofermentative species (De Vuyst and Degeest, 1999), nevertheless, LAB synthesized a wide variety of HePSs composed of different sugar moieties (glucose, galactose, rhamnose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucuronic acid), molecular mass and structure, whose production are strongly influenced by culture conditions (De Vuyst et al., 2001; Mozzi et al., 2003). To obtain high EPS production, it is necessary to optimize growth conditions, which require an understanding of the different production parameters involved (Velasco et al., 2006). Previous works showed that physical (temperature, pH, oxygen tension) and chemical (carbohydrate source, nitrogen source) factors determined the EPS yield of lactic acid bacteria (Cerning et al., 1994; Degeest et al., 2002). Further, studies for different LAB species conducted on the molecular genetics of HePS biosynthesis suggested that the genetic diversity of the *eps* cluster determinate the structural diversity of HePSs (De Vuyst et al., 2011; Dimopoulou et al., 2014).

The genus *Lactobacillus* represents the largest group within the lactic acid bacteria and is also the predominant microbiota in natural fermented products. *Lactobacillus suebicus* is an uncommon obligately

* Corresponding author.
E-mail address: mariateresa.duenas@ehu.es (M.ªT. Dueñas).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.07.012
0168-1605/

heterofermentative organism found in ropy cider (Werning et al., 2006) and in apple and pear mashes (Kleynmans et al., 1989). This species is associated to particular flavors and ripening processes of apple mashes (Nam et al., 2011).

In this work, two EPS-producing strains of *L. suebicus* isolated from ropy ciders were studied to examine the influence of carbohydrate source and pH of the culture medium on growth and EPS production. Taking into account that the technological and biological effects of polysaccharides depend on composition, sugar linkages, and molecular mass, the preliminary characterization of exopolysaccharides was undertaken.

2. Material and methods

2.1. Bacterial strains and growth conditions

The EPS-producing *L. suebicus* strains: CUPV225 (Notararigo et al., 2013) and CUPV226 were isolated from two ropy natural ciders of Basque Country (Spain). Strains were stored at −80 °C in MRS broth containing 20% (v/v) glycerol. Before experimental use, bacteria were propagated in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain) at 28 °C in an atmosphere containing 5% CO_2. For EPS production, strains were grown in a semi-defined (SMD) medium (Marieta et al., 2009) containing (in grams per liter): glucose 20, Casamino Acids (Becton Dickinson, Spain) 5, sodium acetate 5, Difco™ Yeast Nitrogen Base (DYNB, Becton Dickinson, Spain) 6.7, K_2HPO_4 2, $MnSO_4 \cdot 4H_2O$ 0.05, di-ammonium citrate 2, Tween 80 1, adenine, uracyl, xantine and guanine 0.005. Sugars and DYNB were sterilized by filtering them through a 0.45-pore size Minisart (Sartorius Stedium Biotech, Germany) and added to the medium (pH 5.5) after autoclaving.

2.2. Identification of EPS-producer strains

Strains CUPV225 (Notararigo et al., 2013) and CUPV226 were identified by biochemical tests and 16S rRNA gene sequence analysis. The homo- or heterofermentative characteristic was tested as described by Dueñas et al. (1995). Carbohydrate utilization was determined with the API 50CH system (API-BioMérieux, Marcy l'Etoile, France). Additional assays to study the carbohydrate fermentation profiles were carried out in MRS broth without glucose and meat extract, with 0.04% of chlorophenol red as pH indicator, and supplemented with 2% (wt/v) one of the sugars glucose, fructose, sucrose, lactose, galactose, or maltose. Incubation was performed for up 7 days, at 28 °C in an atmosphere containing 5% CO_2. One-milliliter aliquots of MRS cultures were used for DNA extraction. Following centrifugation at 15,800 ×*g* for 5 min, pellets were washed in 1 mL of Ringer's solution (Oxoid) and centrifuged at 15,800 ×*g* for 5 min. The resulting cell pellet was resuspended in 180 μL of an enzymatic lysis buffer containing 20 g/L lysozyme, and incubated for 30 min at 37 °C. DNA was purified by DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. DNA samples were stored at −20 °C until use. For identification of the ropy strains based on 16S rRNA gene sequencing, a fragment of the 16S RNA gene was amplified as previously described (Werning et al., 2006). 16S rRNA sequences were identified with nucleotide identity at the species level higher than 98% by using the BLAST tool of the GenBank DNA database (http://www.ncbi. nlm.nih.gov/).

2.3. PCR detection of the eps genes

Amplification reactions of the *eps* gene were performed using different primers that target homopolysaccharide (*gtf*, *deg*, *lev*) and heteropolysaccharide (*epsD/E*, *epsA*, *epsB*, *epsEFG*, *epsF*) genes. The primers used are shown in Table 1. The PCR conditions were carried out according to the literature. The amplified products were purified by using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations. DNA strands of amplicons were sequenced at the sequencing service SGIker of University of Basque Country (UPV/EHU). Similarity searches were performed with the BLAST algorithm available at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

2.4. Fermentation conditions

Batch fermentation assays were carried out in semi-defined medium (SMD) without pH control in fully filled 250 mL screw-cap flasks during 72 h at 28 °C in an atmosphere containing 5% CO_2. Overnight cultures grown at 28 °C in MRS containing glucose (20 g/L) were inoculated until an absorbance (at 600 nm) value of 0.2. The effect of pH on growth and EPS production was analyzed at initial pH values of 4.8 and 5.5 at 28 °C in an atmosphere containing 5% CO_2, for 111 h in *L. suebicus* CUPV226 and for 72 h in CUPV225. Fermentations at controlled pH of two independent cultures of the *L. suebicus* CUPV226 strain were also performed in a 3 L fermenter (BIOFLO 110 Fermentor/Bioreactor, New Brunswick Scientific). Cultures were carried out in SMD broth at pH 5.5 that was adjusted with 5 N NaOH. The temperature was kept at 28 °C and agitation was set to 50 rpm. Samples from cultures were taken at different times between 0 and 111 h. The plate dilution method was used to monitor cell viability on MRS agar plates, incubated at 28 °C for 5–6 days in an incubator with 5% CO_2 and reported as CFU/mL. To evaluate their influence on growth and EPS production, glucose,

Table 1
Primer pairs used to screen for HoPS and HePS genes.

Primer	Sequence[a] (5′→3′)	Gene target	Expected fragment size (bp)	Reference
gtf fw	CGGTAATGAAGCGTTTCCTG	Glycoslyltransferase	417	Werning et al. (2006)
gtf rev	GCTAGTACGGTAGACTTG			
deg fw	GAYAAYWSNAAYCCNRYNGTNC	Glucansucrase	660	Kralj et al. (2003)
deg rev	ADRTCNCCRTARTANAVNYKNG			
lev fw	GAYGTI TGG GAYWSI TGGC	Levansucrase	800	Tieking et al. (2003)
lev rv	TCITYYTCRTCISWIRMCAT			
eps D/E fw	TCATTTTATTCGTAAAACCTCAATTGAYGARYTNCC	Priming glycosyltransferase	189	Provencher et al. (2003)
eps D/E rv	AATATTATTACGACCTSWNAYYTGCCA	(*L. casei* group and *S. thermophilus*)		
eps A fw	TAGTGACAACGGTTGTACTG	EPS regulation/	784	Low et al. (1998)
eps A rv	GATCATTATGGACTGTCAC	(*S. thermophilus*)		
*eps*B fw	CGTACGATTCGTACGACCAT	EPS chain length determination	1150	Deveau and Moineau (2003)
*eps*B rv	TGACCAGTGACACTTGAAGC	(*L. lactis*)		
*eps*EFG fw	GAYGARYTNCCNCARYTNWKNAAYGT	Priming glycosyltransferase	1600	Mozzi et al. (2006)
*eps*EFG rv	TGCAGCYTCWGCCACATG	(*L. delbrueckii* subsp. *bulgaricus*, *L. helveticus*)		
*eps*F fw	ACCAGATATTGTACATTGTC	Branching galactosyltransferase	571	Low et al. (1998)
*eps*F rv	TGTCATAGGCTGTCACAAC	(*S. thermophillus*)		

[a] Y = C or T; R = A or G; W = A or T; K = G or T; S = C or G; M = A or C; V = A, C, or G; N = A, C, G, or T; I = inosine.