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Authors	Kuniyoshi, Taís Mayumi;Mendonça, Carlos Miguel Nóbrega;Borges Vieira, Viviane;Robl, Diogo;Gombossy de Melo Franco, Bernadette Dora;Todorov, Svetoslav Dimitrov;Tomé, Elisabetta;O'Connor, Paula Mary;Converti, Attilio;Araújo, Welington Luiz;Palmeiras Vasconcellos, Lívia Paula Silva;de Mello Varani, Alessandro;Cotter, Paul David;Rabelo, Sarita Cândid;de Souza Oliveira, Ricardo Pinheiro
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Pediocin PA-1 production by *Pediococcus pentosaceus* ET34 using non-detoxified hemicellulose hydrolysate obtained from hydrothermal pretreatment of sugarcane bagasse

Taís Mayumi Kuniyoshi[a†], Carlos Miguel Nóbrega Mendonça[a†], Viviane Borges Vieira[a], Diogo Robl[b], Bernadette Dora Gombossy de Melo Franco[c], Svetoslav Dimitrov Todorov[de], Elisabetta Tomé[f], Paula Mary O'Connor[g,h], Attilio Converti[i], Welington Luiz Araujo[j], Lívia Paula Silva Palmeiras Vasconcellos[k], Alessandro de Mello Varani[l], Paul David Cotter[g,h,m], Sarita Cândida Rabelo[n], Ricardo Pinheiro de Souza Oliveira[a*]

[a]*Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, 05508-000 São Paulo, Brazil.*

[b]*Department of Microbiology, Immunology and Parasitology, Federal University of Santa Catarina (UFSC), 88040-900, Florianópolis, Santa Catarina, Brazil.*

[c]FoRC (Food Research Center)*, Food and Experimental Nutrition Department, School of Pharmaceutical Sciences, University of São Paulo, São Paulo 05508-000, Brazil.*

[d]*Food and Experimental Nutrition Department, School of Pharmaceutical Sciences, University of São Paulo, São Paulo 05508-000, Brazil.*

[e]*ProBacLab, Advanced Convergence, Handong Global University, Pohang, Gyeongbuk 37554, South Korea.*

[f]*Instituto de Ciencias y Tecnología de Alimentos. Escuela de Biología. Universidad Central de Venezuela. Apartado 47.097 – Caracas 1041 A, Venezuela.*

[g]*Food Bioscience Department Teagasc Food Research Centre, Moorepark, Fermoy, Cork, T12 YN60 Ireland.*

[h]*APC Microbiome Ireland, University College Cork, T12YT20, Cork, Ireland.*

[i]*Department of Civil, Chemical and Environmental Engineering, Genoa University, I-16145 Genoa, Italy*

[j]*Microbiology Department - Biomedical Sciences Institute, University of São Paulo, São Paulo 05508-900, Brazil.*

[k]*Brazilian Biorenewables National Laboratory (LNBR), National Research Center for Energy and Materials (CNPEM), Campinas, Brazil.*

[l]*Department of Technology, School of Agricultural and Veterinary Sciences, Faculdade de Ciências Agrarias e Veterinária, São Paulo State University, Jaboticabal, SP, 14884000, Brazil*

[m]*School of Microbiology, University College Cork, T12YT20, Cork, Ireland.*

[n]*Department of Bioprocess and Biotechnology, College of Agricultural Sciences, São Paulo State University, 18610-034, São Paulo, Brazil.*

[†] *Taís M. Kuniyoshi and Carlos M. N. Mendonça shared first co-author*

**Corresponding author: rpsolive@usp.br*

Abstract

Listeria monocytogenes is one of the foodborne pathogens of most concern for food safety. To limit its presence in foods, bacteriocins have been proposed as natural bio-preservatives. Herein, a bacteriocin was produced on hemicellulose hydrolysate of sugarcane bagasse by *Pediococcus pentosaceous* ET34, whose genome sequencing revealed an operon with 100% similarity to that of pediocin PA-1. ET34 grown on hydrolysate-containing medium led to an increase in the expression of PA-1 genes and a non-optimized purification step sequence resulted in a yield of 0.8 $mg \cdot L^{-1}$ of pure pediocin (purity > 95%). Culture conditions were optimized according to a central composite design using temperature and hydrolysate % as independent variables and validated in 3-L Erlenmeyers. Finally, a process for scaled-up implementation by sugar-ethanol industry was proposed, considering green chemistry and biorefinery concepts. This work stands up as an approach addressing a future proper sugarcane bagasse valorisation for pediocin production.

Keywords: sugarcane bagasse; *Pediococcus pentosaceus*; bacteriocin; hemicellulose hydrolysate.

1. Introduction

Bacterial infections are again becoming an imminent threat to public health worldwide, jeopardizing the extraordinary clinical advances achieved since antibiotic discovery (Tacconelli and Pezzani, 2019). Due to the rise in antibiotic multi-resistance among an increasing number of pathogenic microorganisms, the search for new antimicrobial compounds will become one of the most important

71 scientific issues of the coming years (Mantravadi et al., 2019). In addition to the
72 related medical challenges, the increasing number of disease outbreaks caused by
73 foodborne pathogenic microorganisms, e.g *Listeria monocytogenes* has raised
74 concerns among world public health organizations (Bucur et al., 2018). Thus,
75 alternative natural food additives with anti-*Listeria* properties have been
76 increasingly requested by the food industry for post-processing treatments as this
77 pathogen has the ability to proliferate under refrigerated conditions, high
78 concentrations of NaCl and wide pH range(Bucur et al., 2018).
79 Bacteriocins, a heterogeneous group of antimicrobial peptides that are
80 ribosomally produced by certain bacterial strains (Cotter et al., 2013), are
81 promising bio-preservatives and antimicrobial agents for the food industry and
82 medical areas, respectivelyBacteriocins produced by lactic acid bacteria (LAB)
83 are the most applied in food processes and those present in class IIa are often
84 described as listericidal (Drider et al., 2006). LAB is known for demanding
85 numerous amino acids, peptides, nucleic acids and vitamins (Brinques et al.,
86 2010) leading to, in some cases, their biotechnological use economically
87 unfeasible, even for high added value biocompound production such as
88 bacteriocins.
89 The development of alternative growth media using industrial by-products
90 has been widely studied in order to decrease production costs but also due to
91 environmental policy trends of circular economy. Cheese whey and vinification
92 lees (Rodríguez-Pazo et al., 2018), fish wastes (Vázquez et al., 2019), brewer's
93 spent grain (Paz et al., 2018) and soy whey (Mitra et al., 2010) are some
94 examples of industrial residues used as substrates for bacteriocin production by
95 LAB. Considering that the carbohydrate source accounts for 15 to 60% of

96 production costs (Straathof et al., 2011), the use of carbohydrates from
97 lignocellulosic biomass as renewable raw material has been proposed for
98 alternative large-scale biotechnological production processes (Du et al., 2012).
99 An option is sugarcane bagasse, the main lignocellulosic biomass generated by
100 the sugarcane industry, which has been explored for the production of a broad
101 group of biotech products due to its high abundance, low cost, renewability and
102 high fermentable carbohydrate content. Nevertheless, no reports, to the best of
103 our knowlegde, have been described on the use of industrial sugarcane residues to
104 produce bacteriocin.
105 Different incremental biomass deconstructive pathways have been used to
106 break down the lignocellulosic matrix (cellulose, hemicellulose and lignin),
107 resulting in a lignocellulosic fraction of varying chemical composition (Vieira et
108 al., 2020, Satlewal et al., 2018). Among the existing fractionation strategies
109 employed in the lignocellulose liquefaction process, the hydrothermal pre-
110 treatment has stood out as a promising low-cost and ecological technology
111 capable of promoting, with short residence times and low sugar degradation
112 profiles(Haldar et al., 2021). The hydrothermal pretreatment process results in a
113 hemicellulosic hydrolysate rich in xylooligosaccharides (XOS) (Santucci et al.,
114 2015) over monomeric sugars, which are mostly obtained in the diluted acid
115 pretreatment (Roque et al., 2019). *Pediococcus pentosaceus* belongs to the LAB
116 group and presents an expressive potential in biotechnology industries (Porto et
117 al., 2017). Studies with this bacterial species have demonstrated a great genetic
118 diversity related to carbohydrate metabolism (Jiang et al., 2020) as well as the
119 existence of a number of XOS/arabinoxylan consuming strains (Han et al., 2021,
120 Jiang et al., 2020).

Here, an isolate named *Pediococcus pentosaceus* ET34 (Tomé et al., 2007) with a potent extracellular listericidal activity (Todorov et al., 2011), was grown in a culture medium containing non-detoxified hemicellulose hydrolysate. Many *Pediococcus* strains are described as pediocin PA-1 producers, genome sequencing of 65 *P. pentosaceus* isolates from different niches revealed that some strains harbour the operons of other class IIa bacteriocins such as penocin A and plantaricin (Jiang et al., 2020). Thus, firstly, this work sought to evaluate the molecular aspects related to bacteriocin production by *P. pentosaceus* ET34. Secondly,process optimization and preliminary scale up studies were carried out and ultimately, an integrated industrial-scale process based on biorefinery practices and the experimental data obtained in the present work was proposed.

This study is a pioneer in the use of lignocellulosic residue to increase bacteriocin production including upstream and downstream prodution aspects. In addition, the standardization of a protocol to purify pediocin PA-1 from HSB containing medium was also explored, which would allow its application in protocols for pathogen controlling in a precise dose/quantity avoiding the emergence of bacteriocin-resistant strains. Undoubtedly, the possibility of producing a pure product with high added value such as pediocin PA-1, using by-product is a relevant achievement within a vast number of challenges that still need to be overcome for PA-1 production via circular economy principles.

2. Materials and Methods

2.1 Bacterial strains

Pediococcus pentosaceus ET34 was previously isolated from smoked salmon (*Salmo salar*) and characterized as bacteriocin producer (Tomé et al.,

2007). *Listeria innocua* CLIST 2711 and *L. innocua* DPC3572 strain were used as bioindicators in the antimicrobial activity assays. Both, *P. pentosaceus* ET34 and *Listeria* strains were cryopreserved at -80°C in their respective media, supplemented with 20% ($v.v^{-1}$) of glycerol.

2.2 Microbial growth conditions and culture media

P. pentosaceus ET34 was grown at 37 ºC under static conditions for 12 to 24 h using MRS-Lactobacilli Broth (Difco Laboratories, Detroit, MI, USA) or LAPTg medium(Testa de Nadal et al., 1997), without glucose (LAPT: 15g peptone, 10g yeast extract, 10g tryptone and 0.1% of tween 80). The culture medium used to optimize pediocin PA-1 production was prepared by mixing a fixed volume of 2x concentrated LAPT broth (50 % of the final culture volume) with a variable volume of the hemicellulose hydrolysate of sugarcane bagasse (HSB) mixed with sufficient sterile deionized water to ensure the remaining 50%. Cells of *L. innocua* CLIST 2711 or *L. innocua* DPC 3572 for antimicrobial activity assay inoculum were grown at 37 ºC under static conditions for 16 to 24 h in BHI medium (Difco Laboratories).

2.3 Genome sequencing and assembly

Genomic DNA of *P. pentosaceus* ET34 was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Paired-end libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA), and sequenced on the MiSeq (Illumina) platform, using the 600-cycle MiSeq reagent version 3 kit (Illumina). The raw

reads were trimmed with Trimmomatic v0.39 using default parameters. The genome was assembled using the SPAdes pipeline, version 3.14.1 , using the *P. pentosaceus* SRCM102736 (CP028259) genome as a guide for repetitive regions assembly resolution. Genome completeness, contamination and strain heterogeneity were analyzed via CheckM, version 0.9.6.The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number JABJXG000000000, and BioProject PRJNA633488. The version described in this paper is version JABJXG010000000.

2.4 Annotation and analysis of genomic data

NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Haft et al., 2018), Rapid Annotation using Subsystem Technology (RAST) Server (Overbeek et al., 2014), and BlastKOALA (Kanehisa et al., 2016) were used for annotation, extraction of comprehensive functional information and reconstruction of metabolic pathways via the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2000). The bacteriocin operon was predicted through BAGEL4 pipeline (Kanehisa et al., 2016), and bacteriocin related proteins were identified through BLASTP analysis (Van Heel et al., 2018). The taxonomic identity of *P. pentosaceus* ET34 strain was confirmed using Average Nucleotide Identity (ANI) approach by OrthoANI tool (Camacho et al., 2009) using seven *P. pentosaceus* complete genomes (CP000422, CP046938, CP023655, CP039378, CP028254, CP028259, and CP023008), *Pediococcus acidilactici* DSM 20284 (NZ_AEEG01000000) and *Lactococcus lactis* subsp. *lactis* Il1403 (AE005176) as outgroups.

196 **2.5 Pediocin transcript analysis**

197 *P. pentosaceus* ET34 was grown on LAPT medium in the presence or
198 absence of 10% of HSB at 37 ºC, and total RNA was extracted after 4, 8 and 24 h
199 using PureLink RNA Mini kit (Thermo Fisher Scientific Inc., Waltham, MA,
200 USA). The integrity of the total RNA was checked on an agarose gel (1.0%) and
201 quantified by absorbance (260 nm) using a NanoDrop ND-1000
202 spectrophotometer (Nanodrop Technologies, Wilmigton, DE, USA). All the
203 materials used were sterilized and later treated with RNaseZAP™ (Thermo Fisher
204 Scientific) prepared with diethyl pyrocarbonate-treated water to prevent nucleic
205 acid degradation. Residual DNA was removed using DNAse Purelink (Thermo
206 Fisher Scientific). Retrotranscription from mRNA to cDNA was performed with
207 SuperScript™ First-Strand following the manufacturer's recommendations.

208 The amplification reaction was performed separately for each gene in a
209 final volume of 20 µL, containing 5 µL of cDNA (1 ng for *ped*A, *ped*C, *ped*D and
210 0.01 ng for 16S gene, respectively), 0.3 µL of 10 µM of each oligonucleotide and
211 10 µL of the Power SYBR® Green qPCR Master Mix solution (Thermo Fisher
212 Scientific). For each condition tested, technical duplicates of a total of three
213 biological replicates were analyzed. Real time PCR was performed on a StepOne
214 Real-time PCR System (Thermo Fisher Scientific) using an amplification
215 program consisting of an initial denaturation at 95°C for 10 min, followed by 40
216 cycles of amplification at 95°C for 15 s and 60°C for 1 min, and a melting curve
217 analysis (95°C for 1 min, cooling to 60°C for 15 s and a final ramp to 95°C at a
218 rate of 0.3°C·s^{-1}).

219 The relative expression ratio (*R*) was quantified according to the equation
220 1, described by Pfaffl et al., (2001).

$$R = \frac{E_{(target)}^{\Delta C_{Ptarget}(control-sample)}}{E_{(ref)}^{\Delta C_{Pref}(control-sample)}} \quad \textbf{(eq. 1)}$$

The evaluated target gene were those related to pediocin synthesis (*ped*A, *ped*C and *ped*D) and the reference gene the 16S rRNA, as previously described by Fernandez et al., (2014) *P. pentosaceus* ET34 cultured in LAPT medium supplemented with 10% HSB was taken as the test sample, and *P. pentosaceus* ET34 grown in LAPT medium was included as the control. PCR products of target and reference samples were considered to double (PCR efficiency, E=2) in each cycle. Statistical significance analysis of the obtained data was performed using the Minitab 17 Statistical Software (State College, PA, USA: Minitab, Inc., www.minitab.com).

2.6 Antimicrobial activity determination

To evaluate bacteriocin production, aliquots of culture medium were collected and centrifuged at 3000 *g*, 25 ˚C for 10 min. To prevent the antimicrobial effect of organic acids, the pH was adjusted to 6.0-6.5 using 1.0 N NaOH. To inactivate extracellular proteases, the samples were heated at 80°C for 10 min and then filtered to give *P. pentosaceus* ET34 cell free supernatant (CFS). The CFS antimicrobial activity, expressed in arbitrary units (AU) per mL, was quantified indirectly by the drop-agar diffusion method (Sabo et al., 2018) using equation 2:

$$\frac{AU}{mL} = \frac{\pi r^2}{v} \quad \textbf{(eq. 2)}$$

where *r* is the radius of the inhibition halo (mm) and *v* the volume (mL) of CFS applied to Petri plates. Briefly, 20 µL of CFS were pipetted onto the surface of freshly prepared solid BHI culture medium (0.8% agar) containing *L. innocua* CLIST 2711 (~ 10^6 CFU). After absorption, the plates were incubated at 37 ˚C for 24 h, and the inhibition halos measured using a high precision electronic digital Vernier caliper.

2.7 Preparation of non-detoxified hemicellulose hydrolysate

The HSB was obtained by hydrothermal pre-treatment of sugarcane bagasse. Biomass was provided by Ferrari Agroindustrial (Pirassununga, SP, Brazil) and used without comminution and previous washing. The chemical composition of the dry raw material, determined according to Sluiter et al., (2008), was 43.02 ± 0.34% cellulose, 25.46 ± 0.12% hemicellulose, 22.32 ± 0.43% lignin, 4.62 ± 0.25% ash, and 4.23 ± 0.11% extractives.

The hydrothermal pretreatment reaction was performed in the Pilot Plant for Process Development at the Brazilian Biorenewables National Laboratory (LNBR/CNPEM/MCTIC) (Campinas, SP, Brazil). Around 20 kg of raw sugarcane bagasse with 50% ($w \cdot w^{-1}$) moisture content were fed into a 350-L alloy steel reactor (Pope Scientific Inc, Saukville, WI, USA) with 10% ($w \cdot w^{-1}$) solids loading, and the reaction was performed at 190 °C for 10 min, according to Santucci et al. (2015). Thermal fluid was percolated through the jacket heating reactor, which was continuously stirred at 150 rpm. After completion of the reaction, the fermenter was cooled, depressurized, and opened. The solid and liquid fractions were separated with a Nutsche filter (Pope Scientific Inc.) with 140 L capacity. The solid fraction was stored in a cold chamber (18 °C) and used

in other studies, while the liquid fraction, HSB, was concentrated four times in a wiped film evaporator (Pope Scientific Inc.), with up to 50 $kg \cdot h^{-1}$ of water evaporation capacity at 80 °C and 470 mbar.

The resulting concentrate was stored in a cold container for further fermentation studies. Prior to use for the alternative culture medium formulation, a batch of crude HSB was adjusted to pH 6.5, and the resulting precipitate (phenolic compounds from lignin) removed by centrifugation at 6,690 *g* for 20 min.

The composition of the resulting liquid fraction was then determined by different analytical methods. Monosaccharides (glucose, xylose, and arabinose), cellobiose, acetic, levulinic and formic acids were analyzed using a 1260 Infinity HPLC system (Thermo Fisher Scientific) equipped with a refractive index detector and fitted with an analytical Aminex HPX-87H column (300 mm × 7.8 mm, 9 µm) in combination with a guard column consisting of Micro-Guard Cation PC H Refill Cartridges (Bio-Rad Laboratories Inc., Hercules, CA, USA). The mobile phase consisted of 5 mM sulfuric acid at a flow rate of 0.6 $mL \cdot min^{-1}$. For furfural and hydroxymethylfurfural (HMF) quantification, a reversed-phase HPLC fitted with an Acclaim 120 C18 column (150 mm × 4.6 mm, 3 µm) and a single wavelength UV detector were employed. The mobile phase consisted of a mixture of water-acetonitrile 1:8 ($v \cdot v^{-1}$) with 1% acetic acid ($v \cdot v^{-1}$) at a flow rate of 0.8 $mL.min^{-1}$.

Xylooligosaccharides (XOS) were determined according to Mok and Antal (1992). A quantitative post-hydrolysis with 4% ($w \cdot w^{-1}$) sulfuric acid at 120 °C for 1 h was performed. The XOS content was calculated as the difference between the monosaccharide content measured via HPLC before and after acid

hydrolysis, considering in the mass balance the formed degradation/solubilization products (furfural, HMF, levulinic, acetic and formic acids).

2.8 Experimental design and analysis

To maximize pediocin production by *P. pentosaceus* ET34 using HSB as an alternative low-cost renewable carbon source, a Response Surface Methodology (RSM) based on Central Composite Design (CCD) (Bhattacharya, 2021), was performed, considering temperature and % of HSB as variables.

RSM is a collection of mathematical and statistical techniques used in the development of an adequate functional relationship to optimize a response (output variable) that is influenced by several independent variables (input variables) (Bhattacharya, 2021), employing a smaller number of assays, which can lead to a costs reduction to maximize or minimize some operational response, in our case the antimicrobial activity.

The analysis of the results includes the computation of the linear (L), quadratic (Q), and interaction effects, and the analyses of the variances ascribed to them. The statistical significance of these effects was evaluated by the analysis of variance using the *F* test (Rabelo et al., 2008). A quadratic model was obtained relating each response variable to the significant effects. These models were used to define the conditions that separately and simultaneously maximize the response variable. The Statistica 7.0 software (Statsoft, Inc.,Tulsa, OK, USA) was used to implement all analyses, and the result was illustrated by RSM.

Cells of *P. pentosaceus* ET34 used for the inoculum in the different runs were grown for 12 h after the addition of 100 µL of stock culture into 50 mL of commercial MRS broth in Falcon tube. Biomass was then centrifuged at 2,860 g

for 5 min at 25 °C (Sorvall Lynx 4000, Thermo Fisher Scientific) and washed with saline solution (0.85% NaCl). Based on McFarland standard, the biomass pellet was resuspended at a concentration of 10^7 CFU·mL^{-1}. After 14 h of cultivation, the CFS obtained in each run had its antimicrobial activity evaluated as previously described.

2.9 Scale-up of pediocin PA-1 production

Using the optimized operational conditions optimized, the production of pediocin PA-1 was then evaluated at larger-scale under static conditions in a 3-L Erlenmeyer flask containing 2 L of LAPT + 20.6% HSB culture medium, whose carbon source composition (g·L^{-1}) was glucose, 0.25; xylose, 15.94; arabinose, 17.78; cello-oligosaccharides, 3.42; XOS, 43.85; and arabinose oligomers, 6.35. The concentrations of carbon sources, including those in oligomeric form, lactic and acetic acid, HMF and furfural, were determined over time, as described before. During cultivations, carried out in triplicate, samples were collected every 2 h during the first 14 h and at the end (24 h), and the antimicrobial activity was determined as previously described. All the chemical analyses were performed in triplicate.

2.10 Bacteriocin purification

P. pentosaceus ET34 pediocin PA-1 was purified from CFS by a 4-step process using Amberlite XAD16N hydrophobic resin, SP Sepharose Cation exchange resin, C18 SPE and Reversed phase HPLC. The presence of pediocin PA-1 at each purification step was confirmed indirectly by antimicrobial activity against *L. innocua* DPC3572 and directly by MALDI-TOF MS spectrometry

(Axima TOF^2, Shimadzu Biotech, Wolverton, MK, UK). In the first purification step, the pediocin PA-1-containing CFS was applied to a column containing Amberlite XAD16N resin (Sigma-Aldrich, Saint Louis, MO, USA). After adsorption, the resin was washed with a 20% ethanol solution, and pediocin PA-1 eluted from the column using 70% isopropanol (Sigma-Aldrich) containing 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich), named 70 IPA. The alcohol was evaporated from the 70 IPA eluent by rotary evaporation (Büchi Labortechnik AG, Flawil, Switzerland), and the sample pH was adjusted to 4.4 with 1N NaOH to optimize binding to the SP Sepharose cation exchange column (second purification step). After absorption, the SP sepharose resin was washed with 20 mM sodium acetate at pH 4.4 and 20 mM sodium acetate at pH 4.4 containing 150 mM NaCl to remove impurities, and pediocin PA-1 was then eluted with 20 mM of sodium acetate at pH 4.4 containing 1 M NaCl. In the third purification step, NaCl was removed using a C18 SPE cartridge, and the washing and elution steps were performed as described for the first purification step.

Finally, the bacteriocin was purified to homogeneity by Reversed Phase HPLC using a C12 reverse phase Jupiter Proteo column (250 x 10.0 mm, 4 μm 90Å) running a 25-40% acetonitrile, 0.1% TFA gradient where mobile phase A was 0.1% TFA and mobile phase B 100% acetonitrile with 0.1% TFA. Eluted fractions containing pediocin PA-1 were identified by detection of molecular mass (~4620-4625 Da) using MALDI-TOF MS, and pure fractions were pooled and lyophilized using a Genevac HT 4X lyophilizer (Genevac Ltd., Ipswich, UK).

3. Results and Discussion

370 **3.1 Genomic analysis**

371 Genome sequencing of *Pediococcus pentosaceus* ET34, isolated from food
372 vacuum-packed-cold-smoked salmon (Tomé et al., 2007), yielded a total of
373 2,531,195 high-quality paired-end reads showing a PHRED score above 32. They
374 were assembled in 19 contigs showing N50 of 443-kb and ~80x of coverage,
375 resulting in a draft genome sequence of 1,953,235 bp with an average of 37.7%
376 G+C. CheckM analysis revealed completeness of 99.38%, showing no
377 contamination. According to the PGAP annotation pipeline, the strain harbors
378 1,883 coding genes, 57 tRNAs, 3 ncRNAs, and five copies of the ribosomal
379 operon. Moreover, 50 pseudogenes were identified. An ANI heatmap matrix was
380 built based on the sequences of species closest to the isolate (Figure 1a).

381 It was possible to confirm previous identification (Tomé et al., 2008) of *P.*
382 *pentosaceus* ET34 identity at species level with *P. pentosaceus* (ANI value >
383 98.6%). Based on ANI values, a high similarity was evident between *P.*
384 *pentosaceus* ET34 and strains isolated from food, i.e., *P. pentosaceus*
385 SRCM102736 from soybean paste and *P. pentosaceus* JQI-7 from fermented
386 dairy product. Up to 1,774 CDS were annotated with KEGG Orthology (KO) and
387 Enzyme commission number (EC). BAGEL4 revealed that the *P. pentosaceus*
388 ET34 genome contained the complete pediocin PA-1 operon located in proximity
389 to a number of plasmid-related genes (replication protein and *traA*) (Figure 1b),
390 suggesting that the pediocin operon is present on a plasmid, as previously
391 observed for many other *Pediococcus* spp (Papagianni and Anastasiadou et al.,
392 2009).

393

3.2 Pediocin PA-1 production and relative genes expression by *P. pentosaceus* ET34 in the presence of hydrolysate of sugarcane bagasse

To evaluate the biotechnological potential of using hydrolysate of sugarcane bagasse for pediocin PA-1 production by *P. pentosaceus* ET34, a glucose-free adapted LAPT broth formula supplemented with HSB was prepared. Given the lack of existing knowledge about the tolerance of *P. pentosaceus* ET34 to inhibitory compounds formed and concentrated during HSB production and processing, e.g., furfural and hydroxymethylfurfural (HMF), the initial set of experiments to evaluate pediocin PA-1 production and pediocin PA1-related gene expression was performed using 10% HSB. Aside from the primary carbon source, the adapted culture medium had a similar composition to that normally used for bacteriocin production by LAB (Juarez Tomás et al., 2002). Both, LAPT medium with 10% HSB and without HSB (control) were inoculated with an initial viable cell count of approximately 10^7 CFU·mL^{-1} and incubated at pH 6.0 and 37 ºC for 31 h. Figure 2a shows the *P. pentosaceus* ET34 growth and the variation over cultivation time in antimicrobial activity (Δ antimicrobial activity), i.e., the difference between the inhibitory effects of LAPT + 10% HSB and control cell free supernatants (CFS) against *L. innocua* CLIST 2711.

Since cell viability was not adversely affected by HSB toxicity and anti-*Listeria* activity was improved compared to the control run, it was possible to conclude that the selected HSB percentage provided suitable conditions for *P. pentosaceus* ET34 growth and greatly improved pediocin PA-1 production. The CFS anti-*Listeria* activity progressively increased throughout microbial growth reaching a maximum value of ~6,500 AU·mL^{-1} after 8 h of cultivation. After remaining constant for up to 14 h, the variation in anti-*Listeria* activity decreased

between 15 and 31 h, probably due to the proteolytic action of extracellular proteases produced by *P. pentosaceus* ET34.

To evaluate if the expression of related pediocin PA-1 genes was increased in the presence of HSB, relative expression analysis of pediocin structural (*ped*A), accessory (*ped*C) and transporter (*ped*D) genes was performed on samples collected after 4, 8 and 24 h from both fermented media. Figure 2b shows that the relative expression of *ped*A, *ped*C and *ped*D during *P. pentosaceus* ET34 growth in LAPT medium supplemented with 10% HSB was up-regulated after all tested times, with the highest CFS anti-*Listeria* activity detected after 8 h.

Notably, among the pediocin PA1-related transcripts, *ped*D showed the most pronounced relative expression, consistent with the observations of Fernandez et al., (2014). These results are also in line with the observations made for *P. acidilactici* (Kim et al., 2018) in the presence of inulin nanoparticles, whose *ped*A and *ped*D genes were up-regulated compared to controls, and in which the presence of the prebiotic also triggered the expression of genes associated with stress responses, i.e., *gro*EL, *gro*ES, *dna*K41.

Heterologous expression of pediocin genes in *Escherichia coli* revealed that only *ped*A and *ped*D are essential for pediocin production in its active form (Mesa-Pereira et al., 2018). In this recombinant system, *ped*C, an accessory protein involved in the formation of disulphide bonds, just increased pediocin PA-1 production. Although more in-depth studies are needed to evaluate the exact consequences of up-regulating pediocin PA-1 production by *P. pentosaceus* ET34, one hypothesis could be that the presence of toxic substances in the HSB

443 and/or the prebiotic action of xylooligosaccharides (XOS) may have up-regulated
444 pediocin PA-1 gene expression via stress-related mechanisms.
445
446 **3.3 Pediocin purification**
447 Although the analysis of mRNA transcripts showed a significant increase
448 in the relative expression of the ratio of pediocin PA-1 *ped*A, *ped*C, and *ped*D
449 genes in the presence of HSB, a sequence of purification steps was employed to
450 isolate pediocin PA-1 and analytically confirm the strain ability to produce such
451 bacteriocin. Given the decrease in antimicrobial activity previously observed
452 after 15 h of fermentation, a new CFS was prepared by growing the strain at 37
453 ºC for 14 h in in LAPT medium supplemented with 10% HSB.
454 Pediocin PA-1 partially purified from CFS according to a non-optimized
455 4-step protocol and eluents at the end of each purification step were assayed for
456 antimicrobial activity against *L. innocua* DPC3572. Samples tested positive for
457 anti-*Listeria* activity were then analyzed by MALDI-TOF MS to assess their
458 relative purity (Figure 3).
459 MALDI-TOF MS of the 70 IPA eluent from the first purification step
460 detected a molecular mass of 4625 Da corresponding to that of pediocin PA-1
461 (4624 Da), although several other molecular masses were also detected
462 suggesting that the sample was impure (Figure 3a). This eluent was then further
463 purified by cation ion exchange, and MS analysis of the active 1 M NaCl-
464 containing eluent revealed a mass corresponding to that of pediocin PA-1 (Figure
465 3b). Again, the presence of other masses suggested that this sample still
466 contained impurities. Therefore, salt was removed from the pediocin PA-1-
467 containing eluent by applying it to a C18 SPE column, and MALDI-TOF MS

analysis showed an increased peak corresponding to pediocin PA-1 mass compared to those of contaminants, suggesting an increase in the relative bacteriocin concentration (Figure 3c).

Finally, reversed phase HPLC was used to purify the sample to >95% purity, and HPLC fractions deemed pure by MALDI-TOF MS (Figure 3d) were pooled and lyophilized to give 0.8 mg L^{-1} of culture medium. Although, the purification protocol was adopted simply to validate the production of pediocin PA-1 by *P. pentosaceus* ET34, it proved suitable for purifying this bacteriocin from a complex culture medium to a high purity degree. As the purification protocol used was generic and not optimized for pediocin PA-1, some of the bacteriocin was lost in the column washing steps during purification, meaning there is potential to further improve yield.

3.4 Optimization of small-scale cultivation conditions

The temperature and percentage of HSB in the medium were selected as the independent variables to maximize small-scale pediocin PA-1 production by *P. pentosaceus* ET34. Depending on the operating conditions, antimicrobial activity ranged from 4142 to 14,464 AU·mL^{-1} and peaked at 34 °C and 22.1% HSB.

Figure 4a shows the Pareto chart of standardized effects, considering the significant effects at 90% level of significance. It is possible to see that the largest effect was the linear one of %HSB, followed by the linear effect of temperature, both positively influencing the antimicrobial activity. In general, the interaction factors had less significant effects on the antimicrobial activity, and all effects (linear, quadratic, and interaction factors) were significant.

493 Table 1 depicts the analysis of variance (ANOVA) for the model of
494 antimicrobial activity. It can be seen that the model exhibited a high correlation
495 coefficient and can be considered statistically significant with 90% of confidence
496 according to the F-test, as it presented calculated F-values greater than the listed
497 ones (Rabelo et al., 2008). Also, it do not present evidence of lack of fit, as the
498 calculated values by the F-test for the lack of fit were much smaller than the
499 listed values. Therefore, the experimental data were accurately fitted by the
500 quadratic model represented by Equation 3:
501
502 $$\textbf{Antimicrobial activity}\left(\frac{\textbf{AU}}{\textbf{mL}}\right) = \mathbf{2988.96\ C - 840.15\ C^2 + 2003.83\ T - 634.95\ T^2 -}$$
503 $$\mathbf{619.67\ CT + 11231.67} \qquad \textbf{(Eq. 3)}$$
504 where C and T are the coded values of the HSB percentage and the temperature,
505 respectively.
506
507 The proposed quadratic model (Eq. 3) was then used to plot the response
508 surface (Figure 4b) and for optimization, purposes select the operating conditions
509 capable of maximizing the response. The temperature of 36.8 ºC and 20.6% HSB
510 in the medium were identified as the optimal conditions, with an estimated
511 antimicrobial activity of 14,132 AU·mL^{-1} (Figure 4b). An independent
512 experiment performed under the predicted optimal conditions confirmed that they
513 ensured an anti-*Listeria* activity almost coincident with that achieved in the run
514 number 6 (14,465 AU mL^{-1}), thereby validating the predictability of the selected
515 model.
516 The result of the optimization run was in line with those performed for
517 relative genes expression evaluation, in which pediocin-related transcripts were
518 up-regulated. Moreover, it was corroborated by observations made in previous

studies, where bacteriocin production was described as associated with defense mechanisms subject to the effects of pH, pressure, temperature, O_2 concentration, and cell density (Papagianni and Anastasiadou, 2009). In this sense, it is possible to infer that the simultaneous increase in both variables could have triggered internal cell control mechanisms related to bacterial stress.

3.5 Pediocin PA-1 production scale-up

To highlight possible scale-up effects on bacteriocin production, Figure 5 shows lactic acid concentration, *P. pentosaceus* ET34 CFS anti-*Listeria* activity and concentrations of carbon sources over time during cultivation carried out in 3-L Erlenmeyer flask under static conditions (Figure 5a). Lactic acid production was detected only after 4 h of cultivation (1.23 $g.L^{-1}$), increased up to a maximum value of approximately 3 $g.L^{-1}$ after 8 h, and remained almost constant until the end of the run ($p > 0.05$ between 8 and 24 h). Given the growing interest in lactic acid as a future green key feedstock in the production of a broad group of chemicals like oxygenated compounds (e.g., acrylic acid), green solvents (e.g., 2,3-pentanedione) and bioplastics (e.g., poly-lactic acid), its use could be considered from a biorefinery perspective.

The CFS anti-*Listeria* activity was first detected after 6 h of cultivation (3451 $AU{\cdot}mL^{-1}$) and then increased progressively up to 9963 $AU{\cdot}mL^{-1}$ after 24 h. Although *P. pentosaceus* is a homofermentative lactic acid-producing bacterium, a small production of acetic acid was observed during *P. pentosaceus* ET34 growth (from 1.67 to 2.2 $g.L^{-1}$), as a likely result of arabinose metabolization following glucose depletion after approximately 4 h (Figure 5b). Indeed, arabinose consumption occurs likely due to the activity of citrate metabolism

544 enzymes, e.g., phosphotransacetylase (EC 2.3.1.8) and acetate kinase encoded
545 within the *P. pentosaceus* ET34 genome. Of the different carbon sources
546 available, *P. pentosaceus* ET34 was only able to consume the free glucose and
547 arabinose present in the culture medium, while concentrations of XOS,
548 arabinoxylan and monomeric xylose remained constant up to the end of
549 cultivation. The presence of genes encoding enzymes involved in arabinose and
550 glucose consumption, e.g., L-arabinose isomerase (EC 5.3.1.4) and L-ribulose
551 phosphatase 4-epimerase (EC 5.1.3.4), as well as in glycolysis and pentose
552 phosphate pathway found in the *P. pentosaceus* ET34 genome is consistent with
553 the observations made on carbon source consumption.
554 Although the ability to consume prebiotics such as XOS is a characteristic
555 of only a small group of LAB strains, the presence of genes encoding XOS
556 transportation proteins and degrading enzymes (e.g., arabinofuranosidase and
557 xylanases) has been identified in different *P. pentosaceus* strains (Lei et al.,
558 2018). The absence of genes encoding enzymes related to xylose metabolisms as
559 well as XOS and arabinoxylan hydrolysis [e.g., α-1,2- arabinofuranosidase (EC
560 3.2.1.55), 1,4-β-xylosidase (EC 3.2.1.37), endo-1,4-β-xylanase (EC 3.2.1.8),
561 xylose isomerase (EC 5.3.1.5), D-xylose-reductase (EC 1.1.1.307), D-xylulose
562 reductase (EC 1.1.1.9)] is consistent with the inability of *P. pentosaceus* ET34 to
563 consume xylose and arabinoxylans. The final anti-*Listeria* activity was found to
564 be 1.45 times lower than that detected in the early stages of the bioprocess.
565 Since the predicted optimum conditions were experimentally validated at
566 small scale, the lower performance of *P. pentosaceus* ET34 in the scaled-up run
567 may be ascribed to a combination of detrimental factors affecting the interactions
568 between the cellular machinery and the extracellular environment (e.g.,

heterogeneity in a larger volume, absence of mixing under static conditions, and mass transfer limitations). While satisfactory for early bioprocess development, multi-scale analysis should be considered when using scale-up cultivation systems in order to perform a reliable future industrial pediocin PA-1 production process.

3.6 Pediocin PA-1 integrated production process

Composed of cellulose, hemicellulose, and lignin, as well as other minor constituents, sugarcane bagasse is considered the most abundant byproduct of the sugar and alcohol agroindustry. Given its origin and renewable character, such a biomass stands out as a cheaper alternative to conventional sugar sources (Østby et al., 2020), without entering into direct competition with food chain and/or affecting arable land management. Herein, an integrated process is proposed that covers the application of expanded conversion platforms based on the concomitant transformation of sugarcane bagasse into a vast range of products and co-products, focused on the biotechnological production of pediocin PA-1 (Figure 6).

Given the importance of recycling hazardous volatile organic solvents and reducing waste produced during the development of greener and more sustainable processes (Welton, 2015), such aspects were also taken into account in the assessment of environmental friendliness and economic viability of future processes. The unit steps explored experimentally in the present work are represented in green and include: (i) sugarcane bagasse pre-treatment, (ii) hydrolysis of the hemicellulose fraction, (iii) pediocin PA-1 production, (iv) downstream processing, and (v) polishing.

The proposed additional steps are depicted in dashed lines and include: lignin recovery, cellulose hydrolysis and bioethanol production (in yellow), as well as arabinoxylan hydrolysis, production of acetic acid, furfural and HMF, recovery of xylose-rich streams for further chemical and/or bioconversion (in pink). A loading of 20 kg of raw sugarcane bagasse composed, on a dry basis, of ($w \cdot w^{-1}$) 43.03 ± 0.33% cellulose, 25.46 ± 0.21% hemicellulose, 16.81 ± 0.12% lignin, 15.23 ± 0.22% extractives, and 1.18 ± 0.09% ash [50% ($w \cdot w^{-1}$) moisture content] was subjected to a hydrothermal pre-treatment reaction at 190˚C for 10 min.

After the initial biomass processing, the obtained solid and liquid fractions were separated by a Nutsche filter (69.9 ± 2.5% of pretreatment yield). The cellulignin, solid fraction obtained after pretreatment was composed, on a dry basis ($w.w^{-1}$) of 57.50 ± 1.11% cellulose, 15.50 ± 0.89% hemicellulose, 24.05 ± 0.44% lignin, and 1.00 ± 0.22% ashes. Insights from the literature can be used for its exploitation. For instance, a first processing step based on the enzymatic hydrolysis was substantiated using cellulase cocktails (Zhuang et al., 2015). Lignin was recovered by filtration for further conversion into chemicals and/or different biomaterials. Furthermore, the obtained cellulose hydrolysate was used for the productions of second generation bioethanol (Abdeshahian et al., 2020) or different high added value biocompounds (Dietrich et al., 2019). The filtered hemicellulose hydrolysate, composed of 0.23 $g.L^{-1}$ glucose, 4.22 $g.L^{-1}$ cello-oligosaccharides, 6.57 $g.L^{-1}$ xylose, 1.03 $g.L^{-1}$ arabinose, 22.93 $g.L^{-1}$ arabinoxylan, 0.55 $g.L^{-1}$ formic acid, 0.09 $g.L^{-1}$ HMF and 1.10 $g.L^{-1}$ free acetic acid may then be mixed with LAPT culture medium and further used.

618 The production of pediocin PA-1 can be conceived through microbial
619 cultivation employing two possible alternative approaches: in the former
620 approach *P. pentosaceus* ET34 would be used in pure culture, even considering
621 its inability to consume xylose and arabinoxylans, while in the latter in co-culture
622 with other pediocin-producing strains capable of consuming different carbon
623 sources. The latter approach aimed at increasing pediocin PA-1 production would
624 be based on synergistic microbial interactions attributable to competition among
625 strains for nutritional resources. Such synergism was previously demonstrated for
626 a co-culture of *P. pentosaceus* 147 and *Lactobacillus plantarum* LE27 in cheese
627 whey broth, which showed a significant increase in bacteriocin production
628 compared with cultivations performed employing pure cultures (Gutiérrez-Cortés
629 et al., 2018).
630 Such effects were corroborated by the presence of *ped*B gene located in
631 the pediocin PA-1 operon of pediocin-producing strains, which confers self-
632 immunity to this membrane-disrupting bacteriocin and allows their growth in
633 mixed microbial cultures. A large genome study on 65 *P. pentosaceus* strains
634 revealed the presence of putative operons involved in different carbohydrate
635 transport and utilization among strains as well as the presence of a pediocin PA-1
636 operon in 12 of them (Jiang et al., 2020).
637 A pediocin PA-1 pre-purification/concentration step using a cross-flow
638 ultrafiltration system with membranes of different cut-offs can be proposed as a
639 first downstream step. Outlet streams containing pediocin PA-1 would be filtered
640 after biomass separation using a 10 kDa cut-off membrane to eliminate high
641 molecular weight contaminants present in the fermented medium.

642 Considering a pediocin PA-1 molecular weight of approximately 4.6 kDa,
643 the obtained permeate could be subject to a second filtration step using a 2.0 kDa
644 cut-off membrane, in order to perform bacteriocin concentration and elimination
645 of the main low molecular weight contaminants. The concentrated pediocin PA-1
646 (retentate) could finally be purified by a 4-step sequential chromatographic
647 protocol using a) a hydrophobic interaction column containing XAD resin, b) a
648 Sepharose cation exchange column, c) a C18 solid phase extraction cartridge, and
649 d) a C12 reverse phase HPLC column, as previously shown in the present study.
650 To enhance its stability over time, the pediocin PA-1 could then be lyophilized.
651 Given the high hemicellulose content of final permeate streams obtained
652 by fractionation of pediocin PA-1 according to the first protocol proposed for
653 bacteriocin production (pure *P. pentosaceus* ET34 cultivation process), two
654 different approaches can be proposed: a) a conversion platform based on
655 processes reported in the literature to produce different valuable co-
656 products/building blocks from non-metabolized xylose and arabinoxylans (i.e.,
657 biphasic hydrolysis and dehydration, aldol condensation, furfural–acetone–furfural
658 (FAF) hydrogenation, and H-FAF hydrodeoxygenation) (Olcay et al., 2018), and
659 b) an alternative approach aimed at using recovered raw arabinoxylans as
660 prebiotics in food and feed formulations.
661 The retentate obtained after filtration (using a 10 kDa cut-off membrane)
662 could be submitted to a polishing step, and the recovered arabinoxylans finally
663 lyophilized. As regards the volatile organic solvents to be used in the previously
664 proposed operations/reactions, well-stablished recycling processes can be
665 envisaged, i.e., isopropanol pervaporation (Urtiaga et al., 2006) and THF
666 distillation.

Urtiaga et al., (2006) described the recovery of isopropanol from industrial waste (mainly composed of a water/isopropanol mixture) through the pervaporation process proposed herein, as well as the recycling of THF with a purity degree greater than 96% using a distillation column (40 stages, SS6Mo). It is believed that the proposed integrated process could be applied in a sugarcane biorefinery context as part of a future biomass valorization pathway, especially in countries with a large sugarcane production such as Brazil and India.

4. Conclusions

Pediocin application in industry requires overcoming two bottlenecks: evidence of safety and efficacy required by regulatory agencies, and reduction of pure pediocin PA-1 production costs, which is currently economically unfeasible. In this work, molecular data, process optimization and scale-up have shown that HSB induces the expression of pediocin PA-1-related genes and can be used as a carbon source for ET34 growth. An integrated process that includes from the pretreatment of sugarcane bagasse to the obtaining of pure PA-1 paves the way for the production of this high added-value compound with green biorefinery concepts and practices to reduce process costs.

E-supplementary data of this work can be found in online version of the paper

Conflicts of interest

There are no conflicts of interest to declare.

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Figure captions

Figure 1. OrthoANI heatmap (a) showing the average nucleotide identity of *Pediococcus pentosaceus* ET34 with seven *P. pentosaceus* complete genomes and *Lactococcus lactis* subsp. *lactis* Il1403 (SAMN02603339) genomes were used as outgroups. In b, schematic representation of the bacteriocin operon. The pediocin PA-1 gene cassette consists of four contiguous genes, namely *ped*A, *ped*B, *ped*C, *ped*D, which encode structural, immunity protein, accessory protein, and transporter, respectively.

Figure 2. *Pediococcus pentosaceus* ET34 growth and variation of antimicrobial activity determined over the cultivation time at 37 °C without stirring for 31 h (a). The variation of antimicrobial activity was defined as the difference between the inhibitory effect of cell free supernatant of fermented LAPT + 10% HSB medium and the control, assessed against *L. innocua* CLIST 2711. In b, relative gene expression of *ped*A (yellow), *ped*C (red), *ped*D (green) of *P. pentosaceus* ET34 cells grown in LAPT medium supplemented with 10% HBC (test sample) and *P. pentosaceus* ET34 grown only in LAPT medium (control). Samples were collected after 4, 8 and 24 h.

Figure 3. Mass spectra of the fractions obtained from *Pediococcus pentosaceus* ET34 pediocin purification according to the following sequential steps: a) hydrophobic interaction, b) ion exchange, c) C18 SPE and d) reversed phase HPLC. Pediocin was indirectly quantified by the antimicrobial activity against *L. innocua* DPC3572 of each of the obtained fractions (S: cell free supernatant; FT: "flow through" sample that did not interact with the column; 30E: washing with 30% ethanol; 70IPA: elution with 70% isopropanol and 0.1% TFA; 150 mM: washing with 150 mM NaCl; 1M: elution with 1 M NaCl. d) The mass spectrum corresponds to the lyophilized mixture of the different fractions eluted in HPLC after salts removal using an increasing gradient of acetonitrile.

Figure 4. Pareto chart and response surface plot illustrating the influence of temperature and HSB percentage in the medium on the antimicrobial activity of cell-free supernatant after 14 h of cultivation. The magnitude of each effect is represented in the Pareto chart by a column and a line crossing the columns that indicate how large an effect must be to be considered statistically significant. The effect estimates divided by their standard errors are sorted from the largest absolute value to the smallest one. The vertical line corresponds to a *p*-value of 0.1, which implies a 90% level of significance.

Figure 5. Results of *Pediococcus pentosaceus* ET34 fermentation on LAPT medium supplemented with 20.6% HSB in 3-L Erlenmeyer flask at 36.8 °C. a) Cell-free supernatant antimicrobial activity ($AU \cdot mL^{-1}$) against *Listeria innocua* CLIST 2711 (red columns) and lactic acid (yellow line) and acetic acid (green line) concentrations ($g.\ L^{-1}$) along the cultivation time. b) Arabinoxylan, xylose, cello-oligosaccharide, arabinose, glucose, formic acid, HMF and furfural concentrations ($g.\ L^{-1}$) at the start and after 2, 4, 6, 8, 10, 12 14 and 24 h.

Figure 6. Schematic representation of the planned integrated process for pediocin PA-1 production and purification, and recycling of the main solvents. The isolation of the phenolic compounds from each aqueous phase and reuse of the respective phase-forming components are also illustrated.