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Production, Recovery and Characterization of an Enterocin with Anti-Listerial Activity Produced by *Enterococcus hirae* OS1

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Abstract

Background and objective: Lactic acid bacteria, used in food processing for a long time, can produce various metabolites during their growth, including bacteriocins. These antimicrobials, used as natural bio-preservatives, enhance the food safety. The objective of this study was to assess lactic acid bacteria bacteriocins with anti-listerial activity and optimize their production and recovery process.

Material and methods: Isolate was identified using conventional assays (morphological and biochemical characteristics) and 16S rRNA gene sequencing. Lactic acid bacteria bacteriocins were characterized based on their physicochemical properties (nature, pH stability and thermo-resistance). The production process was based on optimization of media components (growth media and addition of glucose, nitrogen source and tween 20) and culture conditions (temperature, pH, agitation and inoculum size). Furthermore, optimization of the recovery process was studied using ion exchange chromatography on amberlite IRC-50 (effects of resin size and NaCl eluent concentration).

Results and conclusion: *Enterococcus hirae* OS1 was isolated from Moroccan raw cow milk as bacteriocinogenic strain. After optimization of the bacteriocin production process, results showed that the key parameters for increasing of production included temperature of 30°C, pH of 6.5 and inoculum size of 5%. Production with whey-based and economic food-grade substrate allowed high production of enterocin OS1 (1,600 AU ml^{-1}) ($P<0.001$). Simultaneous addition of glucose (1%) and Tween 20 (1%) increased enterocin titer significantly (5,866 AU ml^{-1}) ($P<0.01$). Recovery efficiency increased with use of 70% amberlite IRC-50 resin and elution with 2M NaCl. Indeed, recovery of 75.4% of bacteriocin was achieved in comparison to 15.7% of bacteriocin without optimization. This promises achieving high quantities of enterocin at low costs.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Despite use of modern preservation technologies in food production, the number of foodborne infections is increasing. In fact, 4,362 foodborne outbreaks, including waterborne outbreaks, were reported in EU countries [1]. Nearly 9.6% of cases were reported as strong-evidence food-borne outbreaks [1]. In Morocco, foodborne diseases are increasing in recent years. more than 2,800 cases were reported by the Centre Anti-Poison et de Pharmacovigilance du Maroc [2]. In preventing growth of the pathogenic and spoilage microorganisms in foods, use of chemical preservatives is only recommended for improving food shelf life and safety. However, these preservatives, currently licensed in food industries, are denounced as harmful for human health. Currently, there are public urges to replace chemical preservatives with novel alternatives, especially natural preservatives. Examples of these alternatives with great interests in food safety include bacteriocins and bacteriocinogenic lactic acid bacteria (LAB). Indeed, LAB are categorized as "GRAS" (generally recognized as safe) and their bacteriocins can be classified as natural bio-preservatives [3]. Therefore, LAB bacteriocins can be used

in natural, microbiologically safe, efficiently preserved minimally processed foods.

Nowadays, use of LAB bacteriocins in foods, alone or in combination with physicochemical agents, is considered as an efficient tool to improve their shelf life and sensory properties [4]. However, one of the most common problems includes high costs and availability of the food-grade substrates needed by LAB to efficiently produce bacteriocins. However, neither brain heart infusion (BHI), De Man, Rogosa and Sharpe (MRS) nor complex medium (CM) culture media are economical or food-grade media, in contrast to other culture media such as whey media. To promote studies on efficiency of these bacteriocins in food products, it is recommended to prepare sufficient quantities of media with low costs. Therefore, the aim of this study was to investigate potential LAB bacteriocins with antimicrobial activity against *Listeria* (*L.*) *monocytogenes*, using practical, efficient and simple methods for LAB screening and identification. Due to the controversial effects of some parameters on bacteriocin production, production of bacteriocin was optimized in this study for the time. Moreover, optimization in bacteriocin recovery processes was suggested.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Enterococcus (*E.*) *hirae* OS1, a bacteriocinogenic LAB, was isolated from Moroccan raw cow milk. The *E. faecalis* S5, *Bacillus (B.) cereus* BM1 and *Salmonella* sp. (from the authors' collection), *L. monocytogenes* 4032 and *Staphylococcus (S.) aureus* 81 (from the Spanish Type Culture Collection) were used as indicator strains in antimicrobial activity assessments. All bacterial strains were habitually cultured at 37°C and maintained in BHI agar slants (Biokar Diagnostics, France) at 4ºC.

2.2. Screening for antimicrobial activity

Screening of inhibitory activity in solid media was carried out using agar diffusion and in liquid media using agar well diffusion methods. Plates were incubated under 37°C for 24-48 h. In both methods, *L. monocytogenes* (approximately 8-log CFU ml^{-1}) was used as indicator strain for inhibitory activity. All plates were investigated for zones of inhibition surrounding individual colonies or wells. To eliminate interferences with antagonism due to organic acid production, all culture media were buffered at pH 7.2 using 0.2 M phosphate buffer prepared based on a previously published method [5]. Furthermore, inhibitory effects of hydrogen peroxide were excluded by incubation of the indicator strain under anaerobic conditions using anaerobic jar kit (CO_2 Generating Kit, Don Whitley scientific, UK).

2.3. Morphological characterization and biochemical identification of the isolate

The LAB isolates were characterized morphologically (Gram staining and colonial characteristics in MRS agar) and identified biochemically. The biochemical identification was carried out using catalase test (Solvapur, France), oxidase test (Sigma-Aldrich, Germany) and gas production [6,7]. Moreover, additional assays were used, including growth at pH 9.6 with 6.5% NaCl (Riedel-de Haen, Germany) at various temperatures (10, 15, 37 and 45°C) hydrolysis of esculin in media with 4% bile (Fluka, Biochimika, India) and sodium azide (0.04%) (Polysciences, USA), heat resistance (60°C for 30 min) and sugar fermentation (glucose, lactose, mannitol, sucrose, raffinose, sorbitol and arabinose).

2.4. Molecular identification

2.4.1. Genomic DNA extraction

In general, DNA for the polymerase chain reaction (PCR) amplification was prepared based on a published protocol [8]. The DNA was resuspended in 200 µl of TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8) (Sigma-Aldrich, Germany) and incubated at 55°C for 5 min.

2.4.2. Amplification of 16S rRNA gene using polymerase chain reaction

Fragments of 16S rRNA gene from the isolate were amplified using polymerase chain reaction [9] and 170-8720 Cycler Machine (Bio-Rad, Hercules, CA). The polymerase chain reaction mixture (50 µl) included 5 µl of Taq reaction buffer, 10 µl of Taq enhancer, 3 µl of 1.5 mM magnesium acetate, 2 µl of 0.4 mM of each dNTP (Bioline, Germany), 2 µl of 0.4 M primer WO1 (5'-AGAGTTTGATC [A/C]TGGCTC-3'), 2 µl of 0.4 M primer WO12 (5'-TACGCATTTCACC[G/T]CTACA-3'), 1 U of Master Taq polymerase (Invitrogen, USA) and 1 µl of the template DNA. Amplification was carried out with an initial denaturing step of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 60 s, and a final extension step of 72°C for 2 min. After purification step using Perfectprep Gel Cleanup Kit (Eppendorf, Germany), amplicons were sequenced using ABI Prism Dye Terminator Cycle Sequencing Ready-Reaction Kit (ABI-3100, Applied Biosystems, USA). Sequences of 16S rDNA were analyzed using BLAST [National Center for Biotechnology Information (NCBI) USA].

2.5. Characterization of the antimicrobial substances from Enterococcus hirae OS1

2.5.1. Effects of proteolytic enzymes

To characterize the nature of antimicrobial substances, a proteolytic enzyme preparation (pepsin, papain, proteinase K, chymotrypsin and protease from *Streptomyces griseus* at 50 mg l^{-1}) (Invitrogen, USA) was added to the supernatant (SN) of an overnight cultured *E. hirae* OS1. After incubation at 37°C for 2 h, inhibitory activity of the SN against *L.*

monocytogenes was assessed using well diffusion method. A negative control of the enzyme preparation was assessed as well.

2.5.2. Stability under various pH values

Stability of the bacteriocin produced by *E. hirae* OS1 was assessed at various pH values. The pH of an overnight culture SN was adjusted to 4 [by adding orthophosphoric acid (Scharlau, Spain) in 0.2 M of NaOH], 7 [by adding 0.2 M of phosphate buffer] and 8 [by adding glycine buffer (Amresco, Ohio, USA) in 0.2 M of NaOH]. Samples were stored at room temperature for 2 h. Then, antimicrobial activity of the SN was assessed using well diffusion method against *L. monocytogenes*. A negative control containing buffer alone was assessed as well. Buffers were prepared based on a previously published protocol [5].

2.5.3. Resistance to heat

To assess the chemical thermal resistance, SN containing enterocin OS1 was heated to 60 for 1, 5, 10 and 30 min, to 80°C for 1, 5 and 10 min and to 100°C for 1 and 5 min. The SN was immediately transferred to ice and assessed for inhibitory activity against *L. monocytogenes* using well diffusion method.

2.5.4. Antimicrobial spectra

Inhibitory activity of the enterocin OS1 against indicator strains (*L. monocytogenes, B. cereus, Salmonella* sp., *E. faecalis* and *S. aureus*) was assessed using well diffusion method.

2.6. Cell growth and bacteriocin production of Enterococcus hirae OS1

Cell growth and bacteriocin production of *E. hirae* OS1 in BHI (Biokar Diagnostics) were assessed at 37°C for 24 h. The kinetics of growth was carried out by measuring the optical density at 620 nm using spectrophotometer (MAP-ADA Instrument, Shanghai, China). Moreover, production of enterocin OS1 against *L. monocytogenes* was assessed using well-diffusion method.

2.7. Optimization of the enterocin OS1 production

2.7.1. Quantification of the enterocin OS1

Enterocin titer of the samples was calculated based on a published method [10]. Enterocin OS1 quantification in the preparation of SN was estimated from titration curve achieved by measurement of the diameter of inhibition zones (in mm) against inhibitory activity (in AU ml^{-1}). Inhibition activity of the enterocin OS1 was expressed in arbitrary units per ml (AU ml^{-1}) and defined as reciprocal of the highest dilution producing inhibition against the indicator strain.

2.7.2. Effects of media components and culture conditions

Effects of the incubation temperatures (30 and 37°C), pH (buffered with 0.2 M sodium phosphate buffer at pH values of 6, 6.5 and 7.2), agitation (0, 50, 100 and 200 rpm), inoculum size (2, 5 and 10%, v v^{-1}), and growth media [Luria broth, tryptic soy broth (TSB), BHI broth (Biokar Diagnostics, France) and whey-based media] on enterocin OS1 production were assessed. In addition, whey-based media were supplemented with various sources of nutrients under optimal pre-established culture conditions. These nutrients included glucose (0, 1 and 2%) (Scharlau, Spain), complex nitrogen sources (peptone and yeast extract supplemented at 1%) (Biokar Diagnostics, France) and emulsifier (Tween 20 at 0.1%) (VWR Chemicals, France). During the assessments, production of enterocin OS1 was monitored for 24 h using well-diffusion method. All experiments were carried out in triplicate and results included the average of two independents values.

2.7.3. Optimization of the recovery process

Semi-purified samples of the enterocin were prepared using cation exchange chromatography on amberlite IRC-50 (Janssen Chemica, Belgium). Eluted fractions were dialyzed against distilled water (D.W.) at 4°C for 24 h using 2000-Da molecular weight cut-off membrane (Sigma-Aldrich, Germany) and filtered through 0.45-µm filters (Millipore, USA). Then, enterocin titer (AU ml^{-1}) of the preparation SN was estimated as previously described. To optimize recovery of the enterocin OS1, effects of the resin size (in proportions of 25, 50 and 70%, v v^{-1}) and eluent concentration (1.5, 2 and 2.5 M NaCl solutions) were assessed. Amberlite IRC-50 was added to the culture of *E. hirae* OS1 in proportions of 25, 50 and 70% (v v^{-1}). After agitating at room temperature for 30 min, mixture (culture and resin) was set for 15 min. Then, resin was recovered and well washed with D.W. The elution was then prepared using 2.5 volumes (of resin) of the eluent (NaCl solution). The eluent was assessed at 1.5, 2 and 2.5 M of NaCl solutions (Riedel-de Haen, Germany). Preparations of the enterocin OS1 were manually aliquoted in 15 ml volumes to assess their activity. Moreover, yield of enterocin OS1 was calculated using the following formula:

Yield (%) = 100 × activity recovered (AU ml^{-1}) / total activity (AU ml^{-1})

2.8. Statistical analysis

Average data and standard deviations (SD) were calculated using Excel (Microsoft Corp., USA). Statistical analysis was carried out using SPSS-PC Software v.17.0 (SPSS, Chicago, IL, USA). Results were submitted to ANOVA analysis. Differences were considered significant at $P \leq 0.05$.

3. Results and discussion

3.1. Screening and identification of the isolate with anti-listerial activity

The Moroccan raw cow milk included a LAB population of 6.4×10^8 CFU ml^{-1}. Of the 200 isolates, 20 isolates (10%)

showed anti-listerial activity in solid media; from which, four isolates showed anti-listerial activity in liquid media. The most efficient isolate against *L. monocytogenes* included *E. hirae* OS1, with 19 mm of inhibition zone diameter. Indeed, previous studies reported the presence of potential bacteriocinogenic LAB in Moroccan foods (such as raw milk and cheese) as important sources of bacteriocin producing strains [11,12]. The isolate *E. hirae* OS1 was characterized as catalase-negative, oxidase-negative, cytochrome-negative and Gram-positive cocci. The bacteria antimicrobial activity was inhibited by the proteolytic enzymes (Table 1).

Table 1. Morphological, physiological and biochemical characteristics of *Enterococcus hirae* OS1

Test		Result
Gram reaction		+
Shape		Cocci
Catalase		-
Oxydase		-
Gas production		-
Ability to grow under different conditions	10°C	+
	15°C	+
	37°C	+
	45°C	+
	Presence of 6.5% of NaCl	+
	pH 9.6	+
	Presence of sodium azide (0.04%)	+
Hydrolyze of esculin in presence of bile		+
Heat resistance (60°C for 30 min)		+
Sugar fermentation	Glucose	+
	Lactose	+
	Mannitol	-
	Sucrose	+
	Raffinose	-
	Sorbitol	-
	Arabinose	-
Proteolytic enzymes		+
Identification of isolate OS1		*E. hirae*

The isolate was characterized as glucose positive, mannitol negative, sucrose positive, raffinose negative, sorbitol negative and arabinose negative. Moreover, the isolate was azide resistant, unable to produce gas, able to hydrolyze esculin in bile media and resistant to 60°C for 30 min. The strain also was able to grow under various temperatures (10, 15 and 45°C), at pH 9.6 with 6.5% of NaCl (Table 1). Molecular identification of the isolate *E. hirae* OS1 was carried out using 16S rRNA gene sequence (Table 1). This LAB strain was active against *L. monocytogenes* and *E. faecalis* with inhibition zone diameters of 19 and 17 mm, respectively. However, *B. cereus, S. aureus* and *Salmonella* sp. were not susceptible to the enterocin (Table 2). In general, the *E. hirae* strain produced bacteriocin with anti-listerial activity as primary metabolite in liquid and solid media [12,13]. The inhibitory effect was exclusively against Gram-positive bacteria, including pathogenic species such as *L. monocytogenes* and *E. faecalis*.

Table 2. Antimicrobial spectra of *Enterococcus hirae* OS1

Indicator strain	Inhibition diameter (mm)
Listeria monocytogenes	19
Enterococcus faecalis	17
Bacillus cereus	0
Staphylococcus aureus	0
Salmonella	0

3.2. Characterization of the antimicrobial substances from Enterococcus hirae OS1

The antimicrobial substance produced by *E. hirae* OS1 was thermo-stable and sensitive to proteolytic enzymes and preserved its antimicrobial activity against *L. monocytogenes* after heat treatments at 60°C for 30 min (366 AU ml^{-1} of residual enterocin titer) and 80°C for 1 min (100 AU ml^{-1} of residual enterocin titer) (Figure 1A). This antimicrobial substance was stable at pH values of 4, 7 and 8 (166, 100 and 66 AU ml^{-1} of residual enterocin titer, respectively) (Figure 1B). These characteristics (protein nature, thermo stability and pH stability) showed bacteriocin like inhibitory substance (BLIS). Thus, enterocin OS1 was a peptide produced by *E. hirae* OS1, which belonged to the broad family of enterocins.

3.3. Cell growth and bacteriocin production of *Enterococcus hirae* OS1

Cell growth and bacteriocin production of *E. hirae* OS1 were investigated in BHI (Figure 2). The *E. hirae* OS1 was able to grow from 7.9 log CFU ml^{-1} at 0 h to reach maximum of 9.42 log CFU ml^{-1} after 15 h of incubation at 37°C. This was followed by a mild decrease in cell counts (to 9.14, 9.16 and 9.1 log CFU ml^{-1} at 18, 21 and 24 h, respectively); possibly due to the lack of nutrients and cells lysis (Figure 2A). Production of enterocin OS1 began after 3 h during the exponential phase and reached its maximum after 18 h of incubation (366 AU ml^{-1}). This verified the primary metabolite pattern of the antimicrobial substance and its bacteriocin-like nature. However, the inhibitory activity decreased to 333 and 200 AU ml^{-1} at 21 and 24 h of incubation, respectively (Figure 2B). This decrease was attributed to several factors such as adsorption of the bacteriocin molecules by the producing cells, proteolytic degradation caused by liberation of endogenous proteases after destruction of producing cells and/or bacteriocin molecule aggregation [10,14].

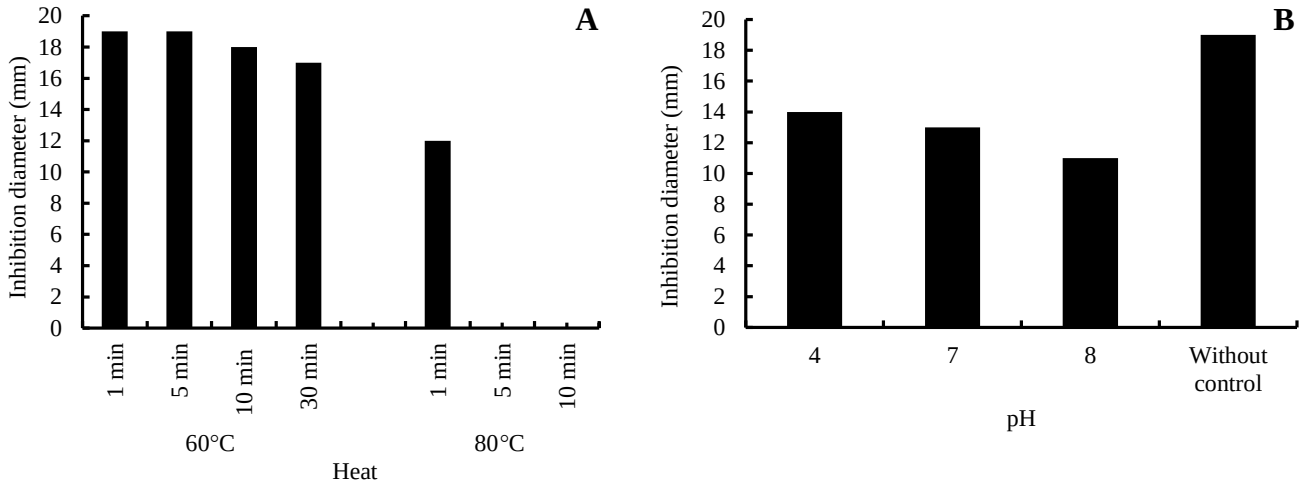

Figure 1. Effects of heat (A) and pH (B) on activity of the enterocin produced by *Enterococcus hirae* OS1 in inhibition diameter (mm)

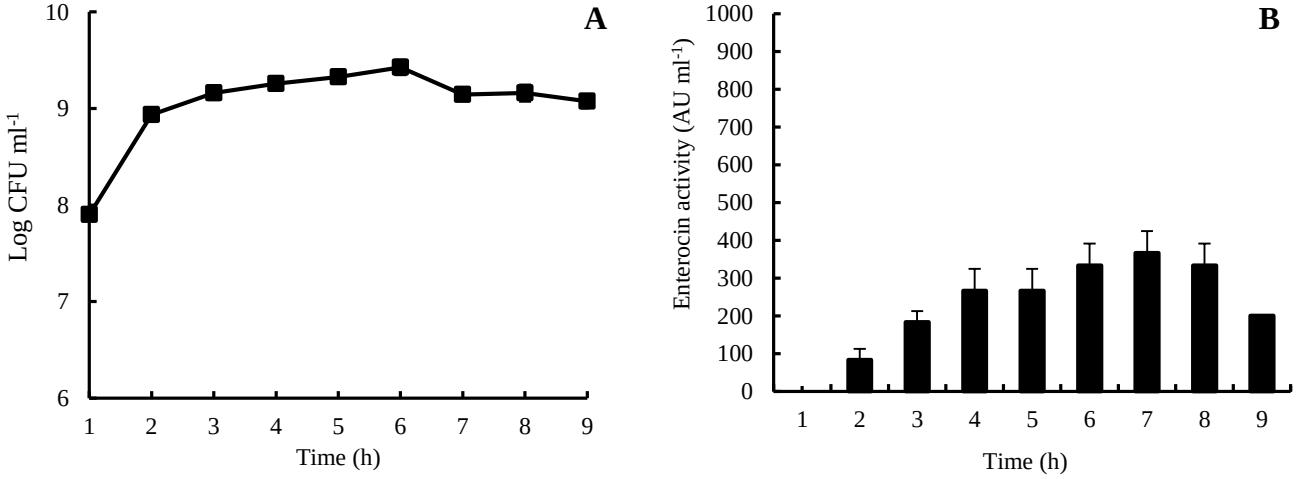

Figure 2. Growth kinetics of (A) and enterocin production (B) of *Enterococcus hirae* OS1 in BHI for 24 h at 37°C. Values are the mean±SD (error bars) of two independent experiments.

3.4. Optimization of the enterocin OS1 production

In general, use of LAB bacterocins as bio-preservatives needs comprehensive studies. Effects of various culture conditions on bacteriocins production are complex that depend on producing strain, culture media, bacteriocin characteristics and others environmental factors such as temperature, pH and salinity. Furthermore, hostile growth environments (e.g. low temperature, low pH, high salinity and stress conditions) were able to improve the production of bacteriocins [15].

3.4.1. Effects of culture conditions and media components

3.4.1.1. Effects of culture conditions

In BHI, *E. hirae* OS1 was able to grow from 7.9 log CFU ml^{-1} to reach maximum of 9.30 and 9.42 log CFU ml^{-1} after 15 h of incubation at 30 and 37°C, respectively, with no significant differences (P>0.05) (Figure 3A). A little decrease in microbial biomass was observed after 18 h of incubation at both temperatures due to lack of nutrients and cells lysis. Results demonstrated that production of enterocin OS1 started after 3 h and reached its maximum of 400 and 366 AU ml^{-1} after 15 and 18 h of incubation at 30 and 37°C, respectively. However, no significant differences were seen for incubation at similar temperatures (P>0.05). Decreases in antimicrobial activity were reported after 18 and 21 h of incubation at 30 and 37°C, respectively (Figure 3B). This could be due to proteolytic degradation caused by cells lysis and bacteriocin absorption by the producing cells [10,14]. These results indicated no correlations between the bacterial biomass and enterocin OS1 production. Indeed, the optimal temperature for the bacterial production (30°C) was not similar to that for the bacterial growth (37°C). This suggested no significant correlations between the growth temperature and bacteriocin production, which was previously described by several studies [10,16,17]. In the current study, effects of buffered media (pH 6, 6.5 and 7.2) on enterocin OS1 production at 30°C were investigated. In all treatments, count of the producing strain increased from 7.8 to approximately 9.3 log CFU after 15 h of incubation at 30°C (Figure 4A). No significant differences were found between the treatments (P>0.05). However, control of pH at 6-6.5 positively affected enterocin OS1 production. Indeed,

higher levels of bacteriocin were achieved after 21 h of incubation for pH 6 (466 AU ml^{-1}) and 15-21 h for pH 6.5 (733 AU ml^{-1}, $P<0.01$), compared to control culture (400 AU ml^{-1} after 15 h of incubation) and pH 7.2 (366 AU ml^{-1} after 21 h of incubation) (Figure 4B); similar to other studies [10,16,18].

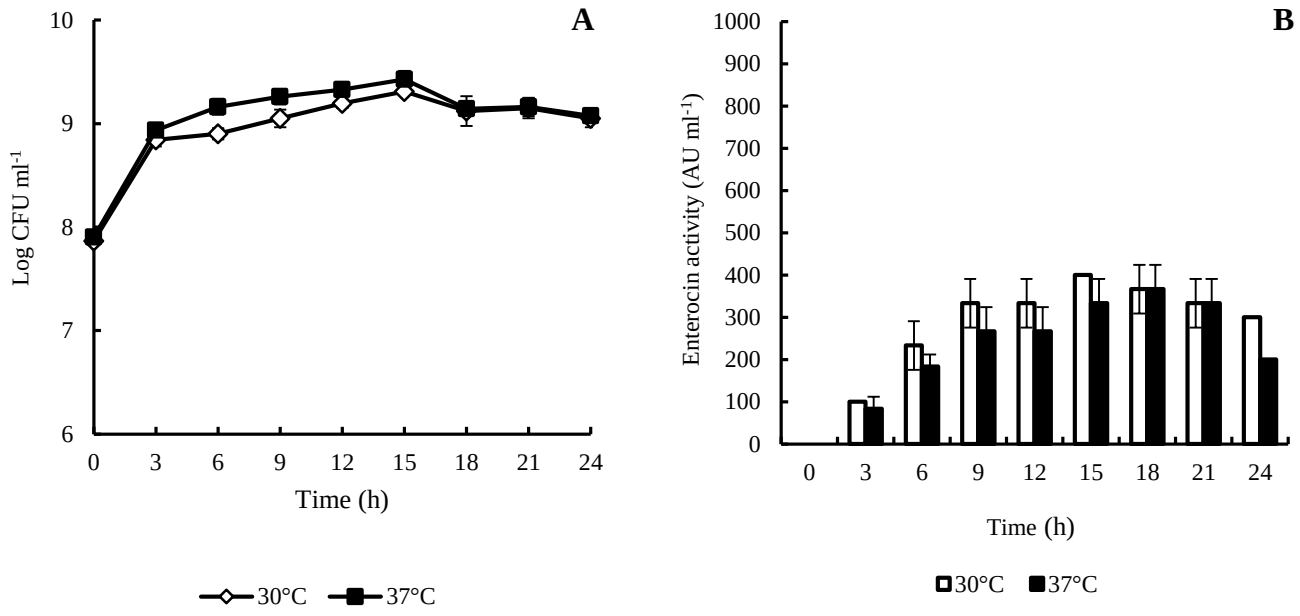

Figure 3. Growth kinetics (A) and enterocin production (B) of *Enterococcus hirae* OS1 in BHI for 24 h at various incubation temperatures. Values are the mean±SD (error bars) of two independent experiments.

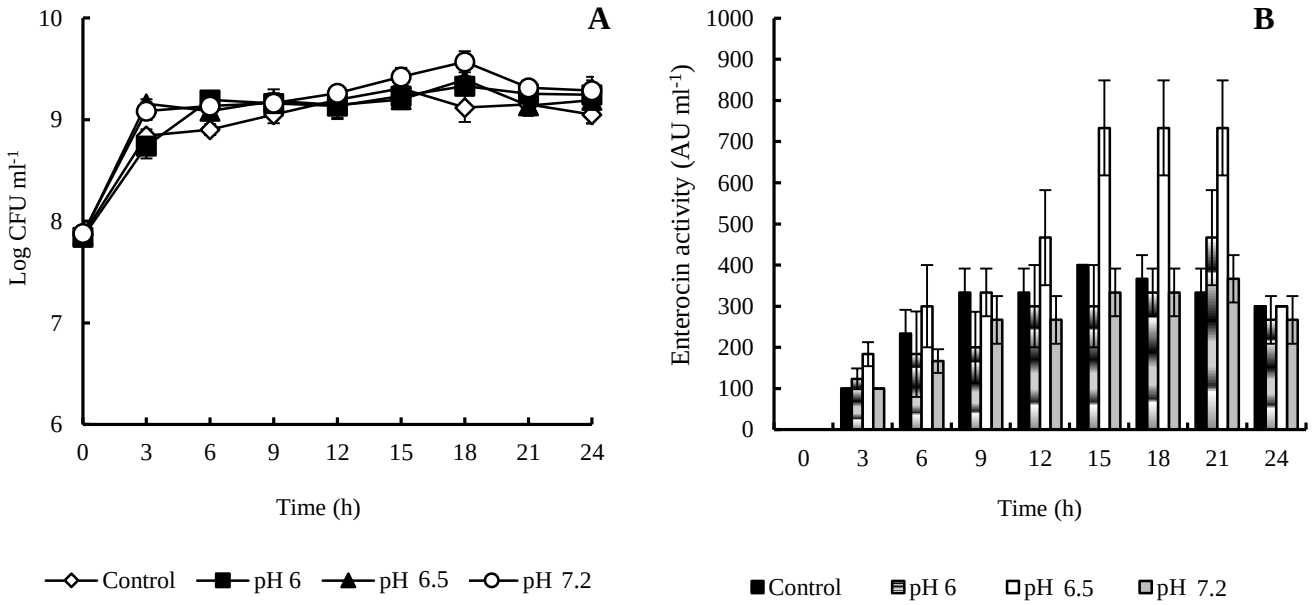

Figure 4. Growth kinetics (A) and enterocin production (B) of *Enterococcus hirae* OS1 in BHI at various pH (6, 6.5 and 7.2) for 24 h at 30°C. Values are the mean±SD (error bars) of two independent experiments.

To adequately prompt production of enterocin OS1, an inoculum size of the producer strain was assessed at 2, 5 and 10% (v v^{-1}) of culture under optimal conditions previously established for *E. hire* OS1 (incubation temperature at 30°C and pH buffered at 6.5) (Figure 5). This approach was appropriate to facilitate implantation of the producer strain in non-sterile culture media such as whey and whey-based media. After 3 h of incubation, no significant differences were found between the inoculums sizes (P>0.05) (Figure 5A). However, the maximum production of enterocin was detected at 18 h of incubation for 2% (400 AU ml^{-1}), 15-8 h of incubation for 5% (733 AU ml^{-1} as the maximum detected), and at 18 h for 10% (666 AU ml^{-1}) (Figure 5B). Effects of orbital agitation (0, 50, 100 and 200 rpm) were investigated under optimal conditions previously established (incubation temperature at 30°C, pH buffered at 6.5 and inoculums size of 5%) (Figure 6). The producing strain grew from 7.9 to 8.7, 9.3, 9.2 and 9.1 log CFU for the control with 0, 50, 100 and 200 rpm, respectively (Figure 6A). Nevertheless, no significant differences were found in cell growth with no agitations (P>0.05). However, the maximum titer of enterocin OS1 was detected after 18 h of incubation for control sample (866 UA ml^{-1}), after 21 h for culture agitated at 50 rpm (933 UA ml^{-1}, the maximum detected), after 18 h for culture agitated at 100 rpm (866 UA ml^{-1}) and after 24 h for culture agitated at 200 rpm (866 UA ml^{-1}) (Figure 6B). Therefore, optimisation of the culture conditions (temperature, pH and agitation) increased enterocin OS1 production from 400 AU ml^{-1} in control culture (with no optimizations) to 933 AU ml^{-1} in culture inoculated at 5% concentration (pH 6.5) and agitated at 50 rpm (P<0.001)

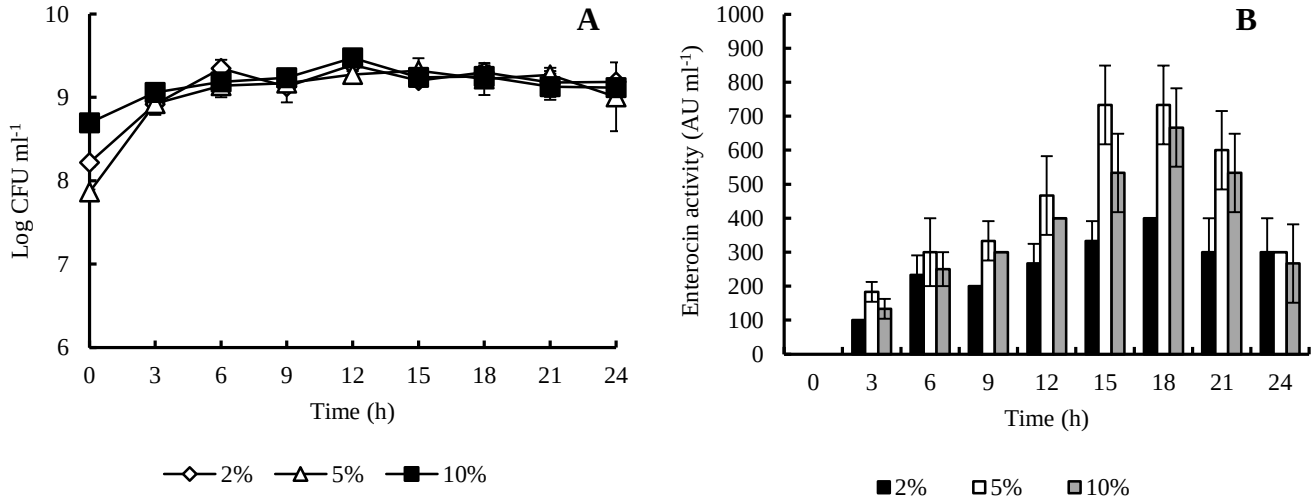

Figure 5. Effects of inoculum size (2, 5 and 10%) on growth kinetics (A) and enterocin production (B) of *Enterococcus hirae* OS1 in BHI within 24 h under optimized culture conditions. Values are the mean±SD (error bars) of two independent experiments.

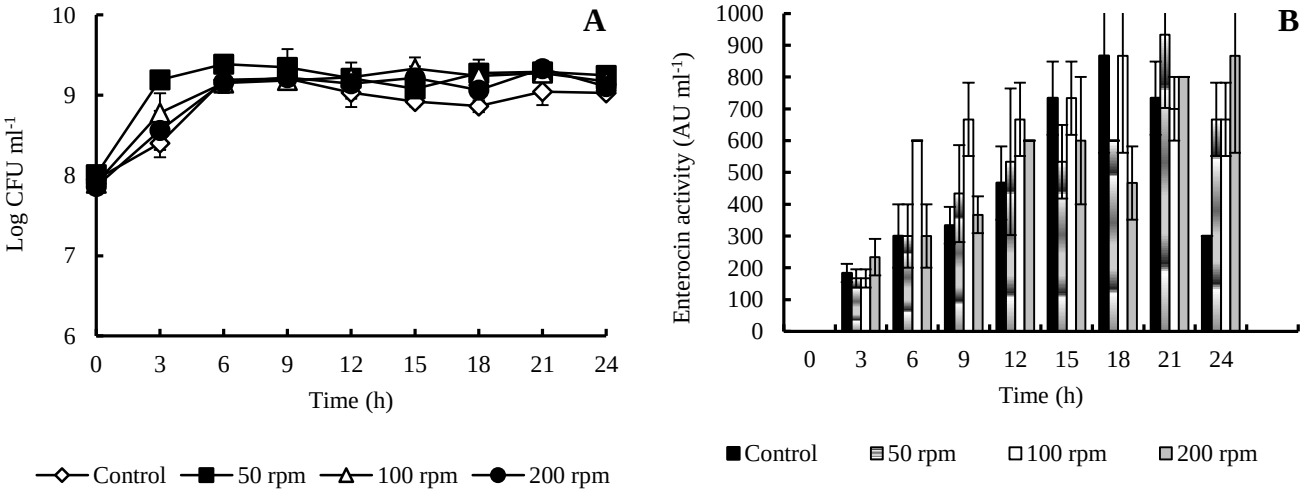

Figure 6. Effects of agitation (0, 50, 100 and 200 rpm) on growth kinetics (A) and enterocin production (B) of *Enterococcus hirae* OS1 in BHI within 24 h at 30°C. Values are the mean±SD (error bars) of two independent experiments.

3.4.1.2. Effects of media components

Development of LAB bacteriocins was disadvantaged by production costs. Several researchers have tried to optimize production process in classical culture media as well as economically food-grade culture media [15-17,19,20]. Indeed, composition of the culture media was a key in LAB biomass and/or bacteriocins production [10,18,21]. Effects of various culture media (BHI, TSB, LB and whey-based media) on cell growth and enterocin OS1 production were carried out with inoculation at 5% concentration (v v^{-1}), pH 6.5 and 30°C (Figure 7). As shown in Figure 7A, higher titers of enterocin were detected in whey-based media with maximum of 1,600 AU ml^{-1} after 9 h. This was significantly higher than the levels of other culture media ($P<0.001$), followed by BHI culture with 933 AU ml^{-1} after 18 h, TSB culture with 600 AU ml^{-1} after 21 h and LB culture with 366 AU ml^{-1} after 15 h of incubation at 30°C (Figure 7B). However, a higher growth of *E. hirae* OS1 was observed in BHI, which showed no correlations between the growth of producing strain and enterocin OS1 production (Figure 5A) [16,22,23]. These results verified that nutrient-rich culture media such as whey (rich in proteins, lactose, minerals, lipids and vitamins), MRS and BHI media are necessary for the better production of bacteriocins. In contrast, TSB and LB are less nutritious media. In particular, results from whey-based media were important because the media offered good opportunity to recycle an important byproduct of dairy industries. Moreover, BHI was not an economical or food-grade compound, contrary to whey. Compared to other media cultures, higher titers of enterocin OS1 were detected ($P<0.001$). These included 800, 1200, 1600, 1333, 1600, 1333, 1333 and 1333 AU ml^{-1} at 3, 6, 9, 12, 15, 18, 21 and 24 h of incubation, respectively (Figure 7B).

In fact, nisin was produced efficiently from *L. lactis* in supplemented whey permeate and reached 1490 and 2560 IU ml^{-1} for non-aerated and aerated continuous cultures, respectively [24]. Under optimal conditions, enterocin AS-48 produced by *E. faecalis* reached 360 AU ml^{-1} using whey-derived substrates (Esprion-300, E-300) [10]. Lacticin 3147 and nisin were produced in large scales at low costs through optimization of culture production or use of heterologous expression systems. Numerous companies such as Novacta Biosystems, Lanthio Pharma and Oragenics, are developing production/recovery processes at large scales for several lantibiotics [25]. The current optimization increased enterocin OS1 production from 933 UA ml^{-1} in BHI to 1600 AU ml^{-1} in whey-based media ($P<0.01$), which could considerably decrease production costs of the enterocin. The annual worldwide production of whey is estimated as 180-190 million tons. This major by-product of dairy industries represents serious economic and environmental problems, because nearly 50% of this quantity are not processed [26]. However, bacterial growth and bacteriocin production were not developed on whey-based substrates. Indeed, *L. lactis* and *P. acidilactici* produced lower levels of biomass and bacteriocin than those in MRS broth did [27].

In the present study, addition of glucose (1 and 2%) to whey-based media was studied. Production of enterocin OS1 increased significantly ($P<0.05$) when *E. hirae* OS1 was grown with 1% of glucose. The enterocin increased from 800, 1200, 1600, 1333, 1600, 1333, 1333 and 1333 AU ml^{-1} in control with no glucose addition to 1600, 2133, 2933, 4266, 3733, 3733, 2666 and 2133 AU ml^{-1} in whey media with 1% of added glucose within 3, 6, 9, 12, 15, 18, 21 and 24 h, respectively (Figure 7C). However, increased glucose concentration to 2% decreased enterocin production, compared to control with no glucose. The maximum titer of enterocin OS1 decreased from 1600 AU ml^{-1} in control with no added glucose to 1133 AU ml^{-1} in whey with 2% of added glucose (respecting to 4266 AU ml^{-1} in media supplemented with 1% of glucose, $P<0.001$) (Figure 7D).

Inhibitory effects of 2% glucose on bacteriocin production through catabolic repression, osmotic stress and/or rapid acidification of media were frequency refereed [10,19,21]. In contrast, stimulation effects of high concentrations of glucose were reported [18]. Under pre-established optimal culture conditions (whey-based media supplemented with 1% of glucose under agitation at 30°C, pH 6.5), the whey-based media was supplemented with various nitrogen sources (peptone and yeast extract at 1% of concentrations) or emulsifier (Tween 20 at 0.1% of concentration). The maximum titer of bacteriocin (5866 AU ml^{-1}) was observed after 15 h of incubation in culture supplemented with Tween 20 in contrast to 4266 AU ml^{-1} detected after 12 h of incubation in control with no emulsifiers (Figure 7D). This represented an improvement of 13.6 times respecting to the culture with no optimizations ($P<0.001$). However, none of the complex nitrogen sources increased enterocin OS1 production (data not shown). Positive effects of the emulsifiers on bacteriocin production were reported. Emulsifiers such as Tween can remove and/or prevent adhesion of the bacteriocin molecules to producer cells [20]. Therefore, bacteriocin molecules were released into the extracellular media and increased the bacteriocin titers. In contrast, other researchers reported that Tween 80 in growth media significantly decreased bacteriocin production from *Lactobacillus pentosus* by more than 50% [23]. To optimize bacteriocin production processes, it is necessary to assess all factors for every bacteriocin.

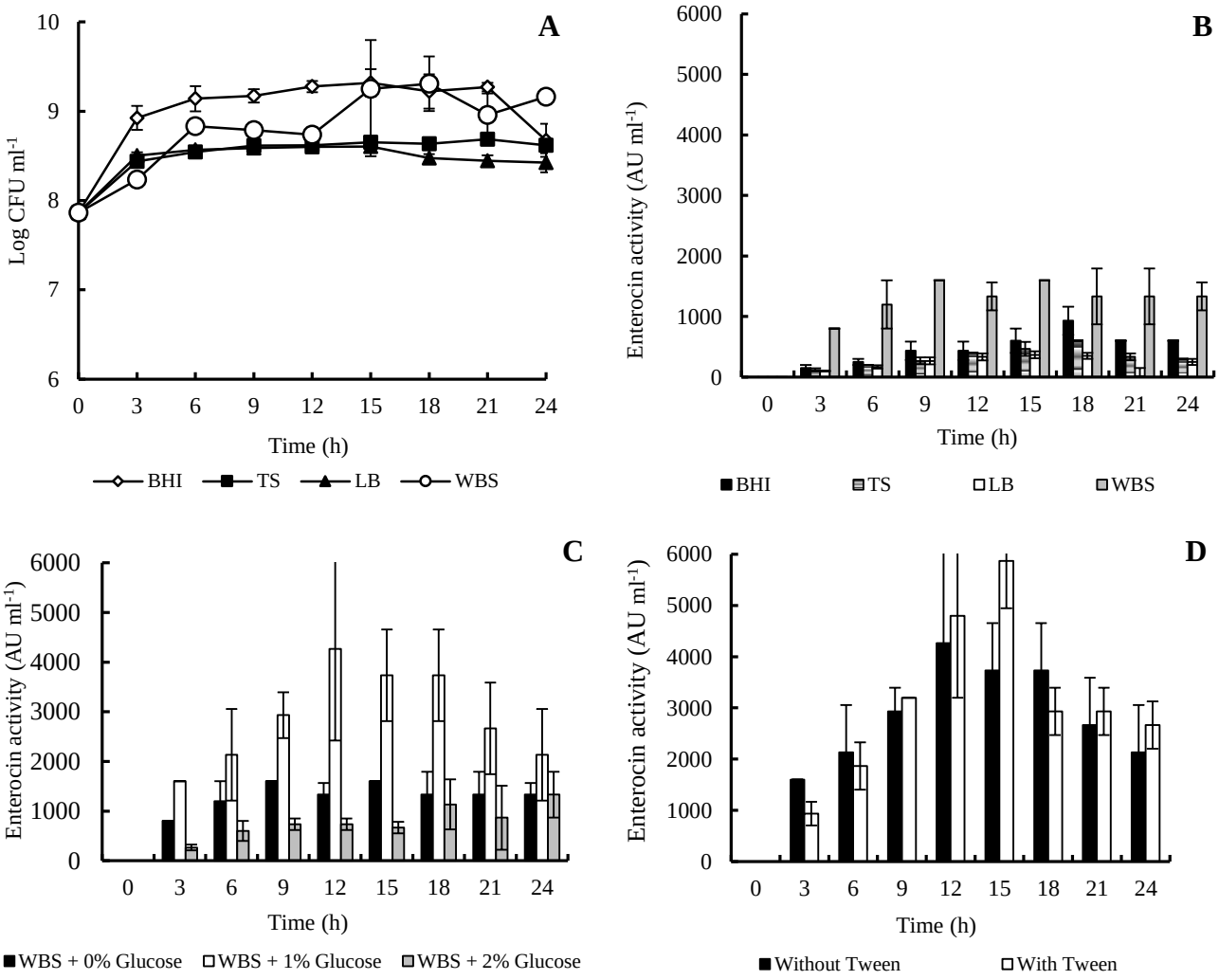

Figure 7. Effects of various parameters on enterocin production within 24 h of incubation at 30°C. Effects of various culture media (BHI, TS, LB and whey-based media) on growth kinetics (A); Effects of various culture media on enterocin production (B); effects of glucose concentrations (0, 1 and 2%) on enterocin production (C); and effects of 0.1% Tween 20 on enterocin production (D). Values are the mean±SD (error bars) of two independent experiments.

3.4.2. Optimization of the recovery process

Based on the cationic nature of bacteriocins, the most common method used for the recovery and purification of these peptides included use of adsorption on cation exchange matrices and their subsequent elution. Generally, several cation exchange resins are used. Enterocins AS-48 and F-58 were recovered using CM-25 carboxymethyl-cellulose gels [10,17,28], and enterocins P, A and B using sepharose gel [29,30]. This process was coupled in some cases with an ultrafiltration step to achieve a higher degree of purification [31]. So, the recovery process of enterocin OS1 was optimized in the current study using cation exchange chromatography and amberlite IRC-50. Effects of resin quantity on bacteriocin recovery was investigated using addition of amberlite at 25, 50 and 70% (v v^{-1}) at optimized culture conditions. Elution from the column was prepared by 1.5 M NaCl solution. Quantity of the recovered bacteriocin increased with increasing resin (Figure 8). In fact, the best yield of semi-purified enterocin OS1 included 52.2% of enterocin recovered with 70% of resin, compared to 39.2 and 15.7% for 50 and 25% of resin, respectively (Figure 8A). In addition, various concentrations of NaCl (1.5, 2 and 2.5 M) were assessed in the elution. Using 70% of resin, 75.4% of the enterocin in culture media was recovered with 2 M of NaCl and 39.2% with 1.5 M of NaCl. A significant decrease in enterocin yield was observed with 2.5 M of NaCl (Figure 8B). Hence, increased quantity of amberlite increased the negative groups that allowed binding to the bacteriocin, and consequently, increased quantity of bacteriocin molecules adsorbed to the resin. However, a significant decrease in yield was observed when 2.5 M of NaCl was eluted. This denaturing effect of NaCl at high concentrations was reported during elution. Indeed, yield of nisin Z resulted in decreases in high concentrations of NaCl [32].

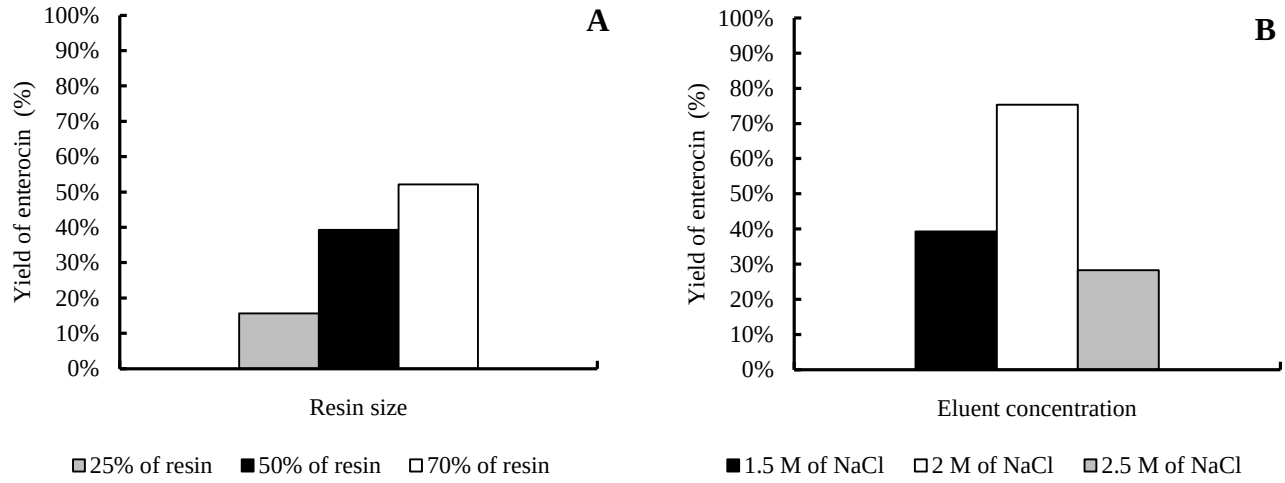

Figure 8. Effects of resin size (25, 50 and 70%) (A) and eluent concentration (1.5, 2 and 2.5 M of NaCl) (B) on bacteriocin recovery

4. Conclusion

In conclusion, this study has shown that anti-listerial enterocin OS1 by *E. hirae* from Moroccan raw cow milk can be produced efficiently in economic substrates (whey). In addition to its food-grade nature, whey is much more economical than other media such as MRS and BHI for bacteriocin production (approximately 0.016 € kg^{-1} instead of 140 € kg^{-1}). The key parameters in optimization of enterocin OS1 production included stabilization of pH at 6.5, simultaneous addition of 1% glucose and 0.1% Tween 20 and, to a lesser extent, 5% inoculum size and agitation. Furthermore, recovery of enterocin OS1 was improved easily to 75.4% (3.6-fold increase) with NaCl elution at adequate concentrations. Thus, this protocol can promise high-quantity production of enterocin at low costs for several applications. However, broad studies are necessary to further characterize this enterocin.

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6. Conflict of interest

The authors declare no conflict of interest.

References

1. EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. EFSA J. 2016: 14(12): 4634. doi:10.2903/j.efsa.2016.4634.
2. CAPM. Centre Anti Poison et de Pharmacovigilance du Maroc. 2015. Maladies d'origine alimentaire. Toxicologie Maroc 27 - 4ème trimestre. Accesible at: http://www.capm-sante.ma/uploads/documents/34.pdf
3. Yost CK. Biopreservation. Encyclopedia of Meat Sciences, Second Edition. Academic Press. 2014: pp 76-82. doi: 10.1016/B978-0-12-384731-7.00111-2.
4. Ananou S, Maqueda M, Martinez-Bueno M, Valdivia E. Biopreservation, an Ecological Approach to Improve the Safety and Shelf-Life of Foods. In: Communicating Current Research and Educational Topics and Trends in Applied Microbiology. Formatex, Badajoz. 2007: pp. 475-486.
5. Gomori G. Preparation of Buffers for Use in Enzyme Active Studies. In: Methods in Enzymology, Colwick SP, NO. Kaplan, Academic Press, New York. 1955. doi: 10.1016/0076-6879(55)01020-3
6. Yost CK. Biopreservation. Encyclopedia of Meat Sciences, Second Edition, Academic Press. 2014: pp 76-82. doi: 10.1016/B978-0-12-384731-7.00111-2.
7. Carr FJ, Chill D, Maida N. The lactic acid bacteria: A literature survey. Crit. Rev Microbiol. 2002; 28: 281-370. doi: 10.1080/1040-840291046759
8. Martin-Platero AM, Valdivia E, Maqueda M, Martinez-Bueno M. Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. Anal Biochem. 2007; 366: 102-104. doi: 10.1016/j.ab.2007.03.010
9. Tahri Joutey N, Bahafid W, Sayel H, Ananou S, El Ghachtouli N. Hexavalent chromium removal by a novel *Serratia proteamaculans* isolated from the bank of Sebou River (Morocco). Environ Sci Pollut Res Int. 2014; 21: 3060-3072. doi: 10.1007/s11356-013-2249-x
10. Ananou S, Munoz A, Galvez A, Martinez-Bueno M, Maqueda M, Valdivia E. Optimization of enterocin AS-48 production on a whey-based substrate. Int Dairy J. 2008; 18: 923-927. doi: 10.1016/j.idairyj.2008.02.001
11. Khedid K, Faid M, Mokhtari A, Soulaymani A, Zinedine A. Characterization of lactic acid bacteria isolated from the one humped camel milk produced in Morocco. Microbiol Res. 2009; 164: 81-91. doi: 10.1016/j.micres.2006.10.008

12. Achemchem F, Cebrian R, Abrini J, Martinez-bueno M, Valdivia E. Maqueda M. Antimicrobial characterization and safety aspects of the bacteriocinogenic *Enterococcus hirae* F420 isolated from Moroccan raw goat milk. Can J Microbiol. 2012; 58: 596-604. doi: 10.1139/w2012-027

13. Cavicchioli VQ, Camargo AC, Todorov SD, Nero LA. Novel bacteriocinogenic *Enterococcus hirae* and *Pediococcus pentosaceus* strains with antilisterial activity isolated from Brazilian artisanal cheese. J Dairy Sci. 2017; 100: 2526-2535. doi: 10.3168/jds.2016-12049

14. Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: Safe, natural antimicrobials for food preservation. Int J Food Microbiol. 2001; 7: 1-20. doi: 10.1016/S0168-1605(01)00560-8

15. Leroy F, Vankrunkelsven S, De Greef J, De Vuyst L. The stimulating effect of a harsh environment on the bacteriocin activity by *Enterococcus faecium* RZS C5 and dependency on the environmental stress factor used. Int J Food Microbiol. 2003 ; 83: 27-38. doi: 10.1016/S0168-1605(02)00316-1

16. Luana RF, Bruna RBP, Eliezer MPE, Janaina DSN. Influence of growth conditions on production of Klebicin K and Raoultellin L, two antimicrobial substances against gram-negative pathogens. Int J Pharm. Biol Arch. 2011; 2: 1095-1099.

17. Abriouel H, Valdivia E, Martinez-Bueno M, Maqueda M, Galvez A. A simple method for semi-preparative-scale production and recovery of enterocin AS-48 derived from *Enterococcus faecalis* subsp. liquefaciens A-48-32. J Microbiol Methods. 2003; 55: 599-605. doi: 10.1016/S0167-7012(03)00202-1

18. Kim M-H, Kong Y-J, Baek H, Hyun H-H. Optimization of culture conditions and medium composition for the production of micrococcin GO5 by *Micrococcus* sp. GO5. J Biotechnol. 2006; 121: 54-61. doi: 10.1016/j.jbiotec.2005.06.022

19. Von Mollendorff JW, Todorov SD, Dicks LMT. Optimization of growth medium for production of bacteriocins produced by *Lactobacillus plantarum* JW3BZ and JW6BZ, and *Lactobacillus fermentum* JW11BZ and JW15BZ isolated from Boza. Trakia J Sci. 2009; 7: 22-33.

20. Castro MP, Palavecino NZ, Herman C, Garro OA, Campos CA. Lactic acid bacteria isolated from artisanal dry sausages: Characterization of antibacterial compounds and study of the factors affecting bacteriocin production. Meat Sci. 2011; 87: 321-329. doi: 10.1016/j.meatsci.2010.11.006

21. Sahar A, Ramakrishnan NR, Tengku ATI, Shuhaimi M, Rosfarizan M, Hassan MD. Arbakariya BA. Effect of medium composition and culture condition on the production of bacteriocin-like inhibitory substances (blis) by *Lactobacillus paracasei* la07, a strain isolated from budu. Biotechnol Biotechnol Equip. 2011; 4: 2652-2657. doi: 10.5504/BBEQ.2011.0101

22. Giraffa G. *Enterococcus*. In: Encyclopedia of Food Microbiology, Second Edition. Batt CA. and Tortorello ML. Academic Press. 2014: 674-679.

23. Todorov S, Dicks L. Effect of medium components on bacteriocin production by *Lactobacillus Pentosus* ST151BR, a Strain Isolated from beer produced by the fermentation of maize, barley and soy flour. World J Microbiol Biotechnol. 2004; 20: 643-650. doi: 10.1023/B:WIBI.0000043196.09610.de

24. Desjardins P, Meghrous J, Lacroix C. Effect of aeration and dilution rate on nisin Z production during continuous fermentation with free and immobilized *Lactococcus lactis* UL719 in supplemented whey permeate. Int Dairy J. 2001; 11: 943-951. doi: 10.1016/S0958-6946(01)00128-5

25. Dicks LMT, Dreyer L, Smith C, van Staden AD. A review: The fate of bacteriocins in the human gastro-intestinal tract: do they cross the gut-blood barrier? Front. Microbiol. 2018; 9: 2297. doi: 10.3389/fmicb.2018.02297

26. Baldasso C, Barros TC, Tessaro IC. Concentration and purification of whey proteins by ultrafiltration. Desalination 2011; 278: 381-386. doi: 10.1016/j.desal.2011.05.055

27. Guerra NP, Luisa Rua M, Pastrana L. Nutritional factors affecting the production of two bacteriocins from lactic acid bacteria on whey. Int J Food Microbiol. 2001; 70: 267-281. doi: 10.1016/S0168-1605(01)00551-7

28. Achemchem F, Martinez-Bueno M, Abrini J, Valdivia E, Maqueda M. *Enterococcus faecium* F58, a bacteriocinogenic strain naturally occurring in Jben, a soft, farmhouse goat's cheese made in Morocco. J Appl Microbiol. 2005; 99: 141-150. doi: 10.1111/j.1365-2672.2005.02586.x

29. Ennahar S, Asou Y, Zendo T, Sonomoto K, Ishizaki A. Biochemical and genetic evidence for production of enterocins A and B by *Enterococcus faecium* WHE 81. Int J Food Microbiol. 2001; 70: 291-301. doi: 10.1016/s0168-1605(01)00565-7

30. Gutierrez J, Criado R, Martin M, Herranz C, Cintas LM, Hernandez PE. Production of enterocin P, an antilisterial pediocin-like bacteriocin from *Enterococcus faecium* P13, in *Pichia pastoris*. Antimicrob Agents Chemother. 2005; 49: 3004-3008. doi: 10.1128/AAC.49.7.3004-3008.2005

31. Li Y, Xiang H, Liu J, Zhou M, Tan H. Purification and biological characterization of halocin C8, a novel peptide antibiotic from Halobacterium strain AS7092. Extremophiles 2003; 7: 401-407. doi: 10.1007/s00792-003-0335-6

32. Chan-Ick C, Moo-Chang K, Seong-B K, Young-Ho H, Yu-Ryang P. Simple one-step purification of nisin Z from unclarified culture broth of *Lactococcus lactis* subsp. *Lactis* A164 using expanded bed ion exchange chromatography. Biotechnol Lett. 2004; 26: 1341-1345. doi: 10.1023/B:BILE.0000045630.29494.45

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تولید، بازیابی و تعیین ویژگی های انتروسین با فعالیت ضد لیستریایی تولید شده توسط *انتروکوکوس هیرائه* OS1

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چکیده

سابقه و هدف: باکتری‌های لاکتیک ا سید، که مدت‌ها در فرایند مواد غذایی مورد ا ستفاده قرار گرفته‌اند، می‌توانند هنگام رشد متابولیت‌های گوناگونی مانند باکتریوسین‌ها تولید کنند. این ترکیبات ضدمیکروبی، که به‌عنوان نگهدارنده‌های زیستی طبیعی استفاده شده‌اند، ایمنی مواد غذایی را افزایش می‌دهند. موضوع این مطالعه ارزیابی باکتریوسین‌های باکتری‌های لاکتیک اسید دارای فعالیت ضدلیستریایی و بهینه‌کردن فرایند تولید و بازیابی آنهاست.

مواد و روش ها: باکتری‌های جدا شده با روش‌های مر سوم (ویژگی‌های ریخت شنا سی[1] و بیو شیمیایی) و توالی‌یابی ژن 16S rRNA شناسایی شدند. ویژگی‌های باکتریوسین‌های باکتری‌های لاکتیک اسید بر اساس خواص فیزیکوشیمیایی (ماهیت، پایداری pH، مقاومت حرارتی) تعیین شد. فرایند تولید براساس ترکیبات محیط کشت (محیط رشد و افزودن گلوکز، منبع نیتروژن و توئین ۲۰) و شرایط کشت (درجه حرارت، pH، همزدن و میزان تلقیح) بهینه شد. علاوه بر این، بهینه سازی فرایند بازیابی با کروماتوگرافی تعویض یونی بر امبرلیت IRC-50 (اثرات اندازه رزین و شستشو دهنده غلظت سدیم کلرید) مورد مطالعه قرار گرفت.

یافته‌ها و نتیجه‌گیری: *انتروکوکوس هیرائه* OS1 ، به‌عنوان گونه باکتریوسینوژنیک از شیر خام گاو مراکشی جدا شد. پس از بهینه‌سازی فرایند تولید باکتریوسین، نتایج نشان داد که عامل مهم کلیدی برای افزایش تولید درجه حرارت ۳۰°C، pH =۶/۵ و میزان تلقیح ٪ ۵ می‌باشد. تولید اقتصادی و براساس آب پنیر رشدمایه[2]امکان تولید بالای انتروسین OS1 (۱۶۰۰ AU ml^{-1}) ($P<0/001$). افزودن همزمان گلوکز (٪ ۱) و توئین ۲۰ (٪ ۱) تیتر انتروسین را به طور معنی‌داری افزایش داد (۵۸۶۶ AU ml^{-1}) ($P<0/001$). کارایی بازیابی هنگام استفاده از رزین امبرلیت IRC-50 ٪۷۰ و شستشو با سدیم کلرید M ۲ افزایش یافت. به علاوه، بازیابی ٪۷۵/۴ باکتریوسین در مقایسه با بازیابی ٪ ۱۵/۷ باکتریوسین بدون بهینه‌سازی به دست آمد. این امر حصول مقادیر بالای باکتریوسین با هزینه پایین را نوید می‌دهد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- *انتروکوکوس هیرائه*
- انتروسین
- باکتری های لاکتیک اسید
- *لیستریا مونوسایتوژنز*
- بهینه‌سازی
- آب پنیر

***نویسنده مسئول**

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[2] Substrate