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ORIGINAL PAPER

Zineb Khouiti · Jean-Paul Simon

Carnocin KZ213 produced by *Carnobacterium piscicola* 213 is adsorbed onto cells during growth. Its biosynthesis is regulated by temperature, pH and medium composition

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Abstract Carnocin KZ213 is an antilisterial bacteriocin produced by *Carnobacterium piscicola* 213. The effects of pH and temperature were studied during batch fermentation in MRS* medium (modified MRS without ammonium citrate or sodium acetate). The optimal pH for growth is between 6 and 7. The maximum bacteriocin productivity in the supernatant occurs at pH 7. Operating at controlled pH increases the volumetric activity of the free bacteriocin by 8- to 16-fold, compared with uncontrolled pH. No bacteriocin production is observed below pH 6.5. Temperature has a dramatic effect on carnocin KZ213 production. Growth is optimal at 25 °C and 30 °C, although no bacteriocin production is detected at 30 °C. Also, bacteriocin production is observed at 25 °C in MRS*, but not in complex APT broth, where growth is optimal. The presence of glucose as a carbon and/or energy source is important for carnocin KZ213 synthesis. Hence, bacteriocin synthesis is regulated by temperature, carbon source and medium composition. Quantification studies of bacteriocin adsorbed onto producer cells show that the majority of the carnocin KZ213 secreted is adsorbed onto the producer cells during growth. Only 15% of the total bacteriocin produced is detected in the cell-free supernatant at the end of growth.

Keywords Lactic acid bacteria · Carnobacteria · Bacteriocin adsorption · Production kinetic · Temperature regulation · Carbon source

Z. Khouiti (✉) · J.-P. Simon
Unité de BioTechnologie, Institut Meurice,
Haute Ecole Lucia de Brouckère,
CERIA, Bat 2, 1 avenue Emile Gryzon,
1070 Bruxelles, Belgium
E-mail: z.khouiti@mrd.ubt.be
Tel.: +32-2-5267386
Fax: +32-2-5267388

Introduction

Lactic acid bacteria (LAB) have been exploited for many years in the production of fermented foods, because of their ability to produce desirable changes in taste, flavour and texture and to inhibit pathogenic and spoilage micro-organisms. The inhibitory activity of LAB is due to pH decrease, competition for substrates and a variety of antimicrobial compounds, including bacteriocins. Bacteriocins have been defined as a secreted product of bacterial ribosomal synthesis with a narrow spectrum of bactericidal activity, including species close to the producer strain [9]. Threats to public health posed by the presence of pathogenic micro-organisms, such as *Listeria monocytogenes*, in foods and feed and the rejection of chemically preserved foods by consumers have prompted the proposal of using bacteriocins (via bacteriocinogenic starters, or as purified ingredients) as natural food preservatives [19].

Two of the most important aspects in the study of bacteriocins are their production and their purification necessary for their use in the food industry. The complex media commonly used for LAB growth are generally used for this purpose. Bacteriocin production is strongly dependent on pH and temperature [8, 16, 21, 22]. All studies on optimal conditions of bacteriocin production have focused on the free bacteriocin detected in the supernatant fluid. Most of them indicated that the highest bacteriocin contents are obtained at temperature and pH values lower than the optima for growth [8, 13, 14, 21]. Most bacteriocins are produced only during growth of the producing organism and show a decrease in activity at the end of cell growth [4, 14, 20].

Carnocin KZ213, designated previously as a bacteriocin-like inhibitory substance (BLIS 213), is produced by *Carnobacterium piscicola* 213 [10] and is an antilisterial bacteriocin with a narrow inhibitory spectrum acting against closely related species. The stability of its activity over large pH intervals and at high temperatures offers the potential for use in meat and cheese as a

natural preservative. Studies of the optimal conditions for bacteriocin production for direct use as a food preservative or for use of the producer micro-organism as a starter for the in situ production of bacteriocin are of interest to develop an economic process for bacteriocin production. Growth kinetics of *C. piscicola* 213 and its bacteriocin production with respect to medium composition, pH and temperature are presented in this paper.

Materials and methods

Bacterial strains and media

The producer strain *C. piscicola* 213 was isolated from meat [12]. *C. piscicola* 9839 was used as the indicator strain (Laboratory of Microbiology, University of Gent, Belgium). *C. piscicola* 213 was cultured in MRS* (modified MRS [3] broth prepared without ammonium citrate or sodium acetate) [10]. The indicator strain was propagated in APT broth (Difco, Detroit, Mich., USA) at 30 °C.

Culture conditions

Bacterial stock cultures were maintained at −80 °C in 40% glycerol. For bacteriocin production, the stock culture was propagated overnight in MRS* broth at 25 °C. The inoculum size was always 1% (v/v). Glucose was prepared, sterilised separately and added to the growth medium as carbon source at the desired concentration.

Influence of the growth medium and incubation temperature on bacteriocin production

The two complex media, APT and MRS* broth (initial pH 6.5) were tested for their ability to support the growth of *C. piscicola* 213 and bacteriocin production. Cultures were incubated at 25 °C, 30 °C and 37 °C for 24 h. Samples were checked at time intervals for growth (calculated from the optical density at 660 nm, OD_{660}), pH and bacteriocin activity. Growth was converted to cell dry weight, using a calibration graph (OD_{660} versus grams biomass per litre). One OD_{660} unit was equivalent to 0.36 g cell dry weight l^{-1}.

Influence of initial pH on bacteriocin production in MRS* medium at 25 °C

MRS* medium was adjusted to pH 5.5, 6.0, 6.5, 7.0 and 8.0 with NaOH (1 M) or H_3PO_4 (5% v/v) before sterilisation. Cultures were incubated at 25 °C and growth and bacteriocin production were monitored.

Bacteriocin production at controlled pH

Production of carnocin KZ213 at controlled pH in MRS* was conducted in a fermenter (Biolab or Biostat ED; B. Braun, Germany) connected to an automatic pH controller. The pH of the medium was adjusted initially to 6.5 and maintained during fermentation by the automatic addition of 4 M NaOH or 10% H_3PO_4. The temperature was held at 25 °C and the culture was agitated continuously at 250 rpm. At time intervals, samples were removed for estimating viable cell counts, pH and bacteriocin activity. Glucose was determined by an enzymatic colorimetric method (GOD-PAP method; Boehringer Mannheim). L-Lactic acid was determined using an enzyme kit (Boehringer Mannheim) and acetate by HPLC on an analytic organic acid column (Aminex HPX-87H; Biorad).

Preparation of the cell-free supernatant

Cells were removed from culture by centrifugation and the supernatant was heated at 70 °C for 30 min in order to kill residual cells and inactivate proteases. Samples were stored at −20 °C for bacteriocin assay and further analysis.

Bacteriocin assay

Bacteriocin activity was assayed by a dilution micromethod in microtitre plates [10]. Two-fold dilutions were prepared in 160 µl of APT broth in 96-well plates (Becton Dickinson Benulux, Aalst, Belgium). Wells were inoculated with 50 µl of an overnight culture of the sensitive *C. piscicola* 9839 strain diluted 10^4-fold (10^4 cells). Microplates were incubated at 30 °C for 16 h. The bacteriocin titre was defined as the reciprocal of the highest dilution that did not give a visible growth of the test organism in 160 µl and was expressed in units of activity (UA) per millilitre.

Bacteriocin adsorption/desorption studies

Bacteriocin adsorbed onto producer cells was desorbed by the method of Yang et al. [23]. Samples of 500 ml from cultures in a fermenter at controlled pH were harvested at time intervals and centrifuged at 9,000 *g* for 20 min. Supernatant and cells were treated at 70 °C for 30 min to inactivate cells and proteases. Cell pellets were washed in a sodium phosphate buffer (5 mM at pH 6.5), and incubated at 4 °C in 50 ml of NaCl solution (100 mM at pH 2.5) for desorption of the bacteriocin bound to the producer cells. The desorbed solutions were dialysed against deionised water overnight in dialysis bags (Spectra/Por CE, molecular weight cut-off: 2 kDa), lyophilised and finally suspended in 5 ml of sodium phosphate buffer (5 mM at pH 6.5). Bacteriocin activity in the cell-free supernatant and desorbed solutions was assayed by the dilution micromethod in the 96-well plates. Pellets corresponding to the desorbed solutions were washed in 10 ml of sodium phosphate buffer and assayed to estimate the bacteriocin still associated with the inactivated producer cells. The adsorbed bacteriocin was the sum of the bacteriocin titre in the desorbed solutions and still associated with cells.

Results

Optimal conditions of bacteriocin production in the supernatant

The effects of incubation temperature and medium on bacteriocin production are shown in Table 1. Bacteriocin production was obtained only in MRS* medium with glucose at 25 °C. No bacteriocin production was observed in APT medium nor in MRS* without glucose. However, growth of the producer occurred in APT ($0.40\ h^{-1} < \mu < 0.55\ h^{-1}$) at 25 °C. At 30 °C and 37 °C, bacteriocin was not detected in the cell-free supernatant. Optimal growth was observed at 30 °C. Growth in MRS* medium without glucose was related to the use of amino acids and peptides and the small amount of carbon sources (0.045 g glucose l^{-1}) provided by the complex nutrients in MRS* medium. The use of amino acids is deduced from the increase in final pH (Table 1). The biomass produced in the absence of glucose is not negligible (50%).

Table 1 Influence of medium and incubation temperature on the growth of *Carnobacterium piscicola* 213 and production of bacteriocin in cell-free supernatant after 24 h of fermentation. Cultures were made in flasks of 50 ml total volume at an initial pH 6.5. Glucose (5 g l^{-1}) was added to the MRS* medium (see Materials and methods). APT broth contained 20 g glucose l^{-1}.*UA* Units of activity

Incubation temperature	Biomass (g l^{-1})	μ(h^{-1})	Final pH	Bacteriocin (UA ml^{-1})
MRS* medium (without glucose)				
25 °C	0.511	0.4	6.8	0
MRS* medium				
25 °C	1.04	0.44	4.82	200
30 °C	0.94	0.5	4.65	0
37 °C	0.103	0.37	6.03	0
APT medium				
25 °C	1.152	0.46	4.8	0
30 °C	0.936	0.47	4.8	0

At 25 °C, growth and bacteriocin in MRS* medium production stopped early because of the pH decrease (Fig. 1). Growth and production kinetic parameters are compared in Table 2. The maximum bacteriocin titre was observed at pH 7.0 and pH 8.0, (400 UA ml^{-1} after 24 h of growth), but the maximum growth rate was obtained between pH 6.0 and pH 6.5 ($\mu = 0.5\ h^{-1}$). At pH < 6.0, no bacteriocin production was observed (< 25 UA ml^{-1} after 24 h of growth). The initial pH decrease affected the final titre of bacteriocin in the supernatant more than the biomass. In contrast, glucose utilisation decreased with the decrease in the initial pH (Fig. 1, right part). After 24 h, growth stopped although more than 50% of the glucose was still present in the growth medium at low pH (6.0, 5.5; Table 2).

Bacteriocin produced in the supernatant and adsorbed onto *C. piscicola* 213

Figure 2A shows that *C. piscicola* 213 grew rapidly during the first 8 h at 25 °C. The maximum bacteriocin titre (3,200 UA ml^{-1}) was detected after 12 h (early stationary phase) and bacteriocin could be detected after 3 h (early exponential phase). Only 25% of the maximum yield was produced in the supernatant at the exponential growth phase. The rate of glucose consumption increased in the early stationary phase, coinciding with the increase in lactate and acetate (Fig. 2B) and carnocin KZ213 production (Fig. 2A). A decrease in bacteriocin activity was observed in the supernatant later in the stationary phase, when glucose was completely depleted from the medium.

The concentration of bacteriocin adsorbed onto cells increased during fermentation; and a maximum of 8,960 UA ml^{-1} was observed after 24 h (Fig. 3). Free bacteriocin in the supernatant fluid was markedly less (1,600 UA ml^{-1} after 24 h). Of the total bacteriocin produced, 30% of the free bacteriocin was quantified in the exponential phase, 40–50% at the end of the growth phase and 15% in the stationary phase.

Discussion

Batch studies without pH control show the importance of choosing growth conditions for bacteriocin production. *C. piscicola* 213 grows and produces carnocin KZ213 in MRS* medium at 25 °C or less (data not shown). However, the optimal growth conditions are observed at 30 °C and pH 6.5 ($\mu = 0.5\ h^{-1}$). In the case of bacteriocin produced by *C. piscicola* 213, temperature had an important regulatory effect on its biosynthesis. One example of a temperature-sensitive bacteriocin biosynthesis was published by Diep et al. [5]. They demonstrated that the biosynthesis of sakacin A occurred at 25 °C and 30 °C but not at higher temperatures (33.5–35.0 °C). The temperature regulation of sakacin A biosynthesis occurred at the transcription level; and the reduced bacteriocin production at high temperatures was related to a reduced synthesis of the inducer peptide. Additionally it is known that high temperature enhanced the genetic instability of the plasmid carrying the bacteriocin genes [11]. However, the loss of carnocin KZ213 production by *C. piscicola* 213 at 30 °C could not be due to a genetic determinant instability, because the non-producing bacterial cells (at 30 °C) produced bacteriocin when they were shifted to 25 °C (data not shown). *C. piscicola* 213 was isolated from refrigerated meat [12]; and its ability to produce bacteriocin at low temperature gives this organism a selective advantage over other psychrophilic bacteria which may be associated with food spoilage. This bacteriocin-producing strain may be used to control *L. monocytogenes* that grow at low temperature.

Initial pH had a considerable effect on carnocin KZ213 production. The optimal pH for bacteriocin production was pH 7–8. Bacteriocin activity was not detected in the external medium below pH 6.5. This could not be linked to any instability of the bacteriocin, since the bacteriocin activity was stable at pH 2–8 and at high temperature (121 °C for 20 min) [10]. As we observed, an initial pH decrease affected glucose utilisation. But the presence of glucose as a carbon and energy source was essential to carnocin KZ213 biosynthesis. So the absence of carnocin KZ213 production at low pH (< 6.5) could be related to energy (ATP) saving by the micro-organism for the maintenance of internal pH rather than for bacteriocin biosynthesis. Energy is very important for the translocation of active bacteriocin peptides to the external medium by putative ABC transporters [6]. A possible metabolic regulation of sakacin P biosynthesis by nutrients and energy was discussed by Aasen et al. [1]; and carbon source (sucrose) regulation of nisin biosynthesis has been observed [4]. It was suggested that the carbon source could regulate prenisin-modifying enzymes. Similarly, a high pH was crucial for the maximum yield of bacteriocins from *C. piscicola* LV61 and *C. piscicola* LV17 [2, 18], but the mechanism was not elaborated. Studies on the effect of pH on nisin and pediocin production showed that a

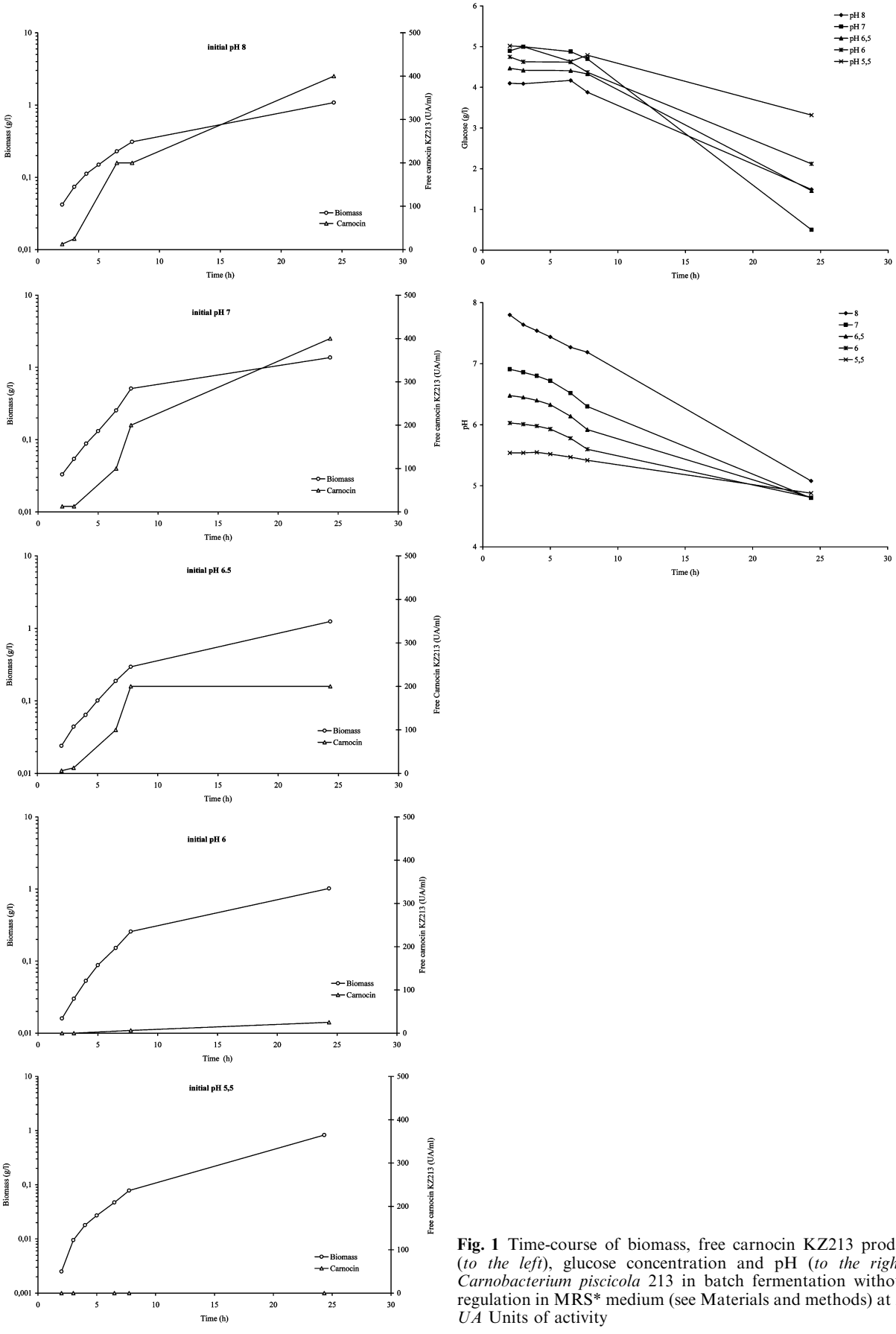

Fig. 1 Time-course of biomass, free carnocin KZ213 production (*to the left*), glucose concentration and pH (*to the right*) for *Carnobacterium piscicola* 213 in batch fermentation without pH regulation in MRS* medium (see Materials and methods) at 25 °C. *UA* Units of activity

Table 2 Influence of initial pH in MRS* medium on bacteriocin in cell-free supernatant at 25 °C after 24 h of fermentation. Cultures were made in flasks of 50 ml total volume of MRS* broth with 4–5 g glucose l^{-1}

Initial pH	Biomass (g l^{-1})	μ(h^{-1})	Residual glucose (g l^{-1})	Final pH	Bacteriocin (UA ml^{-1})
5.5	0.85	0.43	3.32	4.88	0
6.0	1.04	0.50	2.12	4.81	25
6.5	1.27	0.55	1.46	4.81	200
7.0	1.37	0.47	0.50	4.80	400
8.0	1.08	0.40	1.49	5.08	400

drop in pH is needed for maximum yields [7, 22]. Pediocin production increased after the pH reached 5.0 and below; and this was related to a requirement for low pH for the post-translational processing of prepediocin to active pediocin AcH [17].

Our experiments revealed that bacteriocin is not produced in APT broth under optimal conditions established for MRS* broth. Parente and Ricciardi [15] showed that bacteriocin production is affected by media composition and the type and level of carbon, nitrogen and phosphate sources, cations and surfactants. Our studies showed evidence of carnocin KZ213 regulation by medium composition but the responsible factor is not known. In contrast, *C. piscicola* LV17 produced bacteriocins on APT broth adjusted to pH 6.5 at 25 °C [2].

Studies at controlled pH showed that bacteriocin is continuously synthesised and adsorbed onto producer cells. Its synthesis occurs early during exponential growth and the stationary phase until glucose is depleted. Thus, carnocin KZ213 production is not growth-related. The ratio between free and adsorbed carnocin,

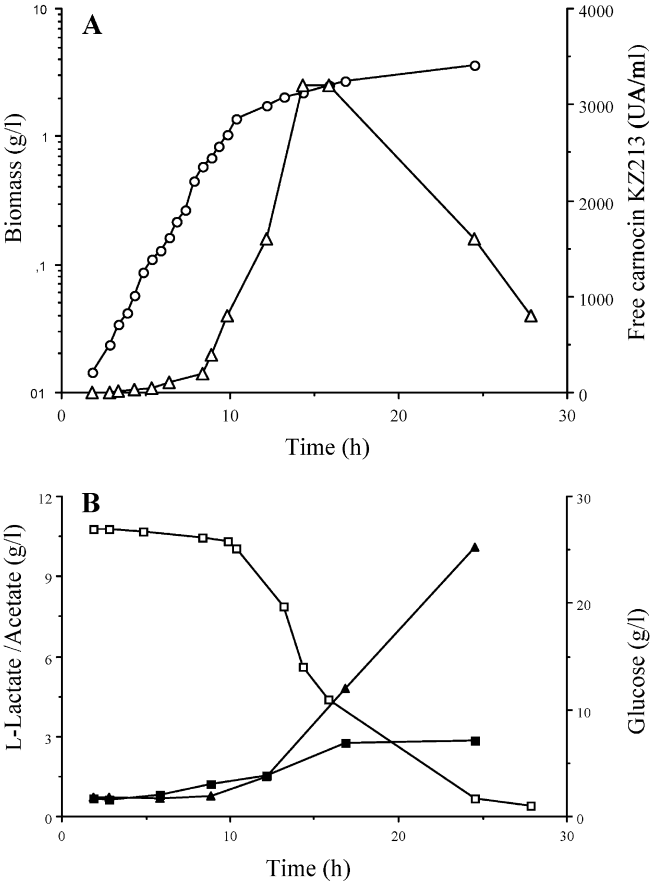

Fig. 2 Growth and bacteriocin (*carnocin*) production (**A**) and acidification (**B**) kinetics by *C. piscicola* 213 at pH 6.5 in MRS* medium at 25 °C. *White circles* Biomass, *white triangles* bacteriocin, *white squares* glucose, *black triangles* L-lactate, *black squares* acetate

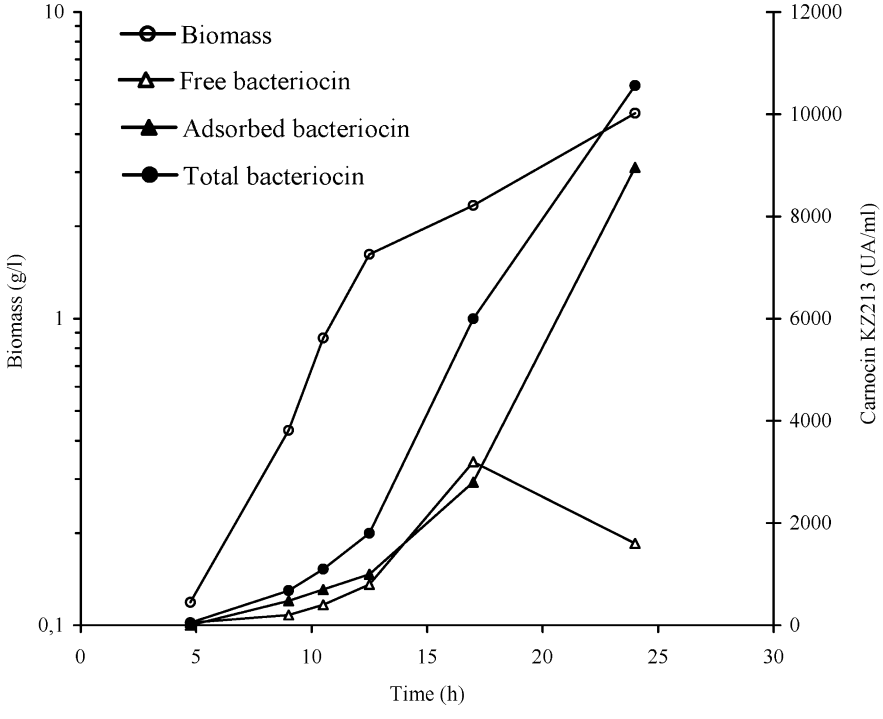

Fig. 3 Production kinetics of the adsorbed carnocin KZ213 (*bacteriocin*) to *C. piscicola* 213 at pH 6.5 and 25 °C. Concentrations of the adsorbed carnocin KZ213 are compared with the corresponding values of free bacteriocin presented in Fig. 2A

however, changes during growth. In fact, a high level of free bacteriocin is observed early during the exponential growth phase and then 80% of the bacteriocin produced is found adsorbed onto cells by the end of growth.

This is the first report on estimating the total bacteriocin synthesised. All previous studies reported the production of bacteriocin only in the cell-free supernatant. For several bacteriocins, production has been optimised by pH-controlled fermentation and the same profile obtained for carnocin KZ213 production (Fig. 2) is observed [14, 16]. A decrease in the bacteriocin titre is usually observed in the supernatant after reaching a maximum under controlled pH conditions. The bacteriocin activity decrease is related either to adsorption onto the producer cells or to proteolytic activity. Hence, the mechanism for the decrease in bacteriocin activity at the end of growth is adsorption onto the producing cells. A primary mechanism of adsorption onto producing cells which may be followed by proteolytic degradation was suggested for enterocin 1146 [14].

In our studies, Yang et al.'s method was very useful for quantifying the bacteriocin still associated with cells, but the desorption method had to be modified, because only 2% of the adsorbed carnocin KZ213 could be recovered from the producing cells [10]. Experiments are now in progress for recovering the total bacteriocin adsorbed onto cells.

As demonstrated in our work, most of the bacteriocin produced is cell-associated and studies are under way for the total recovery of bacteriocin from cells.

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