J Ind Microbiol Biotechnol (2009) 36:409–415
DOI 10.1007/s10295-008-0511-x

ORIGINAL PAPER

Production of nisin Z using *Lactococcus lactis* IO-1 from hydrolyzed sago starch

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Received: 22 June 2008 / Accepted: 3 December 2008 / Published online: 10 January 2009

Abstract A membrane bioreactor for production of nisin Z was constructed using *Lactococcus lactis* IO-1 in continuous culture using hydrolyzed sago starch as carbon source. A strategy used to enhance the productivity of nisin Z was to maintain the cells in a continuous growth at high cell concentration. This resulted in a volumetric productivity of nisin Z, as 50,000 IU l^{-1} h^{-1} using a cell concentration of 15 g l^{-1}, 30°C, pH 5.5 and a dilution rate of 1.24 h^{-1}. Adding 10 g l^{-1} YE and 2 g l^{-1} polypeptone, other inducers were unnecessary to maintain production of nisin. The operating conditions of the reactor removed nisin and lactate, thus minimizing their effects which allowed the maintenance of cells in continuous exponential growth phase mode with high metabolic activity.

Keywords Nisin Z · Bacteriocin · Sago starch · Membrane bioreactor · *Lactococcus lactis IO-1*

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Introduction

It has been demonstrated that *Lactococcus lactis* IO-1 is not only characterized by its high capacity in production of lactic acid (LA), but also for its production of a bacteriocin, which has been identified as nisin Z [12, 14]. The bactericidal effect that bacteriocins have on Gram-positive bacteria, including foodborne pathogens such as *Listeria monocytogenes* and *Bacillus cereus,* and the inhibition of bacterial spore outgrowth enhance the importance of this compound for the food industries. Nisin Z production kinetics in *L.lactic* IO-1 has been widely studied [5, 6, 14, 15, 18]. In order to improve the productivity of nisin Z, a continuous bioreactor with immobilized *L. lactis* IO-1 supported by different materials, both natural and synthetic has been reported [18]. Among the supports tested, ENTG-3800 (polyethylene glycol/polypropylene glycol at 4:1 w/w) gave the best results. Based on this support material and a dilution rate of 0.1 h^{-1}, productivity in the range of 1,500–2,900 AU ml^{-1} was obtained. The highest titers for nisin production employing *L. Lactis* IO-1 have been obtained performing continuous fermentation with a cell recycling system using a ceramic membrane [5]. At a dilution rate of 0.3 h^{-1}, a volumetric productivity of 6×10^5 AU l^{-1} h^{-1} was obtained, corresponding to a bacteriocin titer of 5,860 IU l^{-1}. The use of chemostat cultures to enhance production of bacteriocins has been widely established [3, 4, 7, 8]. Bhugaloo et al. [3] reported on production of the bacteriocin divercin from *Carnobacterium divergens* V41, employing high cell density bioreactors. The productivity was compared in continuous culture with free cells, immobilized cells in alginate beads packed in a plug-flow bioreactor and a membrane reactor. Immobilized cells presented the best results being as high as 100,000 AU l^{-1} h^{-1}. However, the membrane bioreactor failed to be efficient due

to low transmission of divercin through the membranes tested. This failing was due to divercin was adsorptive on the membrane surface, and then the kinetics for its production were difficult to follow. Although the authors could not explain the main reason for this low transmission of the divercin the possible causes were explained as the effect of polarization and fouling of the membrane linked to the presence of high molecular weight material and minerals in the fermentation medium; aggregation of the peptide molecules forming high molecular weight polymers; Interactions between the peptide and/or the aggregates and the membrane material at the surface or within the pores; combination of several of the above points. These points were raised because samples of the fermented medium treated by a simple centrifugation, discarding the microbial cells, enabled a high activity to be determined. Therefore they reasoned that even high adsorption of the divercin on the membrane cell surface was not the reason to have low concentration of divercin in the permeate.

Nolasco-Hipólito and co-authors developed a bioreactor for lactic acid fermentation (LAF), which was named 'synchronized fresh cell bioreactor' [17]. It consists of a pH-dependent substrate feed system coupled with cross flow filtration and turbidity control. Here, the cells responded synchronously to changes in the dilution rate from the addition of glucose by increasing the rate of biomass formation. This reactor facilitates efficient operation with high cell viability by maintaining the cells in continuous growth mode as if in a permanent exponential growth phase. On the other hand, it has been reported that most bacteriocins show a rather sharp decrease in activity at the end of the exponential growth phase [1, 2, 7, 20] Thus, in order to enhance the productivity of nisin Z the cells must be kept in continuous growth or in a permanent exponential growth phase as in the synchronized fresh cell bioreactor.

This paper deals with the employment of the synchronized fresh cell bioreactor operated at both high cell density and high dilution rate to enhance nisin Z production. Glucose syrup obtained from enzymatic hydrolysis of sago starch was used as the carbon source to reduce operating costs. In addition, in previous report no differences in the comparative LAF kinetics between commercial glucose and glucose from sago starch were found [17].

Materials and methods

Hydrolysis of sago starch

Hydrolysis of sago starch has been described elsewhere [17]. Briefly, two enzymes, Kleistase T10 (Daiwa Kasei, KK. Ltd., Osaka, Japan), an amylase from *Bacillus subtilis* with an activity of 13,100 Lj g^{-1} (Lj = amylase unit), and glucozyme (Amano Pharmaceutical Co., Ltd., Nagoya, Japan) from *Rhizopus delemar* with an activity of 4,200 AU ml^{-1} were used, for the liquefaction and saccharification of the starch. From here on, the word 'glucose' refers to glucose obtained from enzymatic hydrolysis of sago starch, unless otherwise stated.

Bacterial strains

In this study, *Lactococcus lactis* IO-1 (JCM 7638) was used for nisin Z production and *Bacillus circulans* JCM 2504 was used as the indicator strain to determine nisin Z activity. *B. circulans* from the stock cultures at −84°C was thawed and 100 µl was inoculated in 10 ml of Brain Heart Infusion broth (Difco) and incubated at 30°C at static culture. About 1 ml of this culture was used as inoculum for 100 ml of the same broth and incubated at 30°C, at static culture during 18 h in order to have a cell density of ca. 10^7 CFU ml^{-1}. The pH of the broth was 7.3 measured previous sterilization. MRS agar and Lactobacilli Agar (AOAC, Difco) were used for the quantitative determination of bacteriocin titers. MRS agar was prepared by the addition of 1.2% agar (Katayama Chemical) to MRS broth (Oxoid).

Quantitative determination of bacteriocin titers

Nisin Z activity was determined by a modified spot-on-lawn method as described by Ennahar et al. [11]. Briefly, 10 µl twofold dilutions of cell-free filtrate (culture extract) onto a double-layer agar plate comprised of 5 ml of Lactobacilli Agar (inoculated with 50 µl an overnight culture of *B. circulans* at a level of ca. 10^7 CFU ml^{-1}) as a top layer and 10 ml of MRS agar as the bottom layer. After overnight incubation at 30°C, bacterial lawns were checked for inhibition or clearance zones. The titer expressed in activity units (AU) per ml was defined as the highest dilution factor of cell-free filtrate which causes clear zones of inhibition on the indicator lawn.

Media and fermentation conditions

Lactococcus lactis IO-1 from the stock cultures at −84°C was thawed and 400 µl was inoculated into 40 ml of the media for its propagation containing in g l^{-1}: commercial glucose (Nacalai Tesque, Inc. Kyoto, Japan) 30; yeast extract (YE, Difco Laboratories, Detroit, MI, USA) 10, and polypeptone (PP, Nihon Seiyaku, Tokyo, Japan) 2.5. The propagation media was incubated at 37°C at static culture. The seed for fermentation was prepared by inoculating the propagation culture into 400 ml of fermentation media containing (in g l^{-1}): glucose (from hydrolyzed sago starch) 40; YE, 10; PP, 2.0. The seed-culture was carry-out in 1 l jar fermenter with a working volume of 400 ml,

incubated at 37°C, agitated at 400 rpm during 12 h. All the media were prepared using tap water, the pH adjusted to 5.5 and sterilized at 12° C for 15 min, Batch culture was initially performed in a 5-l jar fermenter (working volume 4 l) and inoculated with 400 ml (10% v/v) of seed-culture.

When the cells have attained log phase, the broth was concentrated to 400 ml using a cell recycling system to obtain high cell concentration promptly. Once concentrated, this was transferred into a 1-l jar fermenter to initiate the continuous culture, simultaneously feeding the main media. The turbidostat system maintains constant cell concentration by automatic discharge of excess biomass, this volume was counterbalanced by the addition of a glucose-free solution containing only 0.5% YE to further dilute cell concentration to 15 g l^{-1}. The temperature and agitation were controlled at 30°C and 400 rpm, respectively. During the experiment, cell concentration and temperature were changed to 6 g l^{-1} and 37°C, respectively. The pH of the fermentation was maintained at 5.5 by the addition of 10 M NaOH. The experimental set-up is shown in Fig. 1.

Analytical methods

Cell density was measured at 562 nm on a Spectrophotometer (UVIDEC-320 Spectrophotometer, Jasco, Kyoto, Japan) and converted to dry cell weight (DCW) based on the standard calibration curve. Viable counts (CFU ml^{-1}) were based on pour-plate counts on TGC medium (with 1.5% agar), incubated at 37°C for 48 h before counting. Glucose and lactate were determined by HPLC (LC-10AD, RID-6A Refractive Index Detector, Shimadzu, Kyoto, Japan) using an Aminex column (HPX-87H, Biorad, CA. USA) at 50°C with 5 mM H_2SO_4 as the mobile phase (flow-rate at 600 ml min^{-1}).

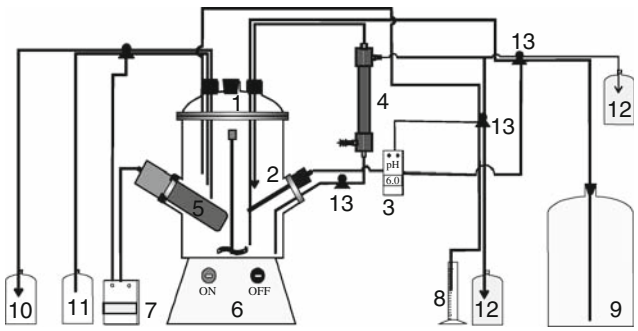

Fig. 1 Schematic diagram of the synchronized fresh cell bioreactor. *1* Fermenter, *2* pH electrode, *3* pH controller, *4* Hollow fiber cartridge, *5* Turbidity probe, *6* Magnetic stirrer, *7* Turbidity controller, *8* NaOH reservoir, *9* Fresh medium reservoir, *10* Glucose-free medium reservoir, *11* Discharged reservoir, *12* Permeate reservoir, *13* Pumps

Results

Nisin production in batch culture

It was reported that nisin Z is produced in the log phase, at the optimum pH and temperature of 5.5 and 30°C, respectively [14]. The time course of LAF using these conditions in our trial (5-l fermentor) is shown in Fig. 2. The presence of bacteriocin was detected right from the beginning of the fermentation and its production was directly related to the increased in biomass production (as CFU ml^{-1}) (data not shown). The titers of nisin Z attained maximum production (8 UA ml^{-1}) after 10 h (exponential phase) and it was realized that high nisin Z production rate could be maintained in continuous culture, aided by high biomass concentration and by maintaining the culture in a

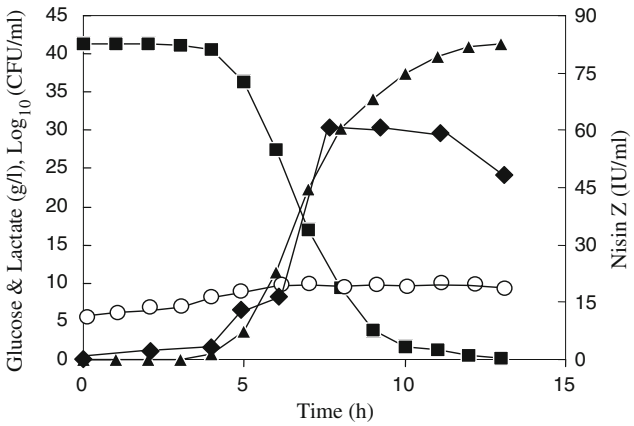

Fig. 2 Time course of batch lactic acid fermentation in 5-liter jar fermenter, using glucose from hydrolyzed sago starch. *open circles* CFU ml^{-1}; *closed squares* residual glucose; *closed triangles* lactate, *closed diamonds* Nisin Z. The culture was conducted by pH-dependent feed using hydrolyzed sago starch as the sole carbon source

Table 1 Kinetic of parameters for nisin production by *L. lactis* IO-1 in the batch culture

Parameter	
Dry cell weight (g l^{-1})	2.76 ± 0.55
Specific grow rate (μ; h^{-1})	0.62 ± 0.18
Sodium lactate concentration (g l^{-1})	20.7 ± 2.2
Glucose consumption rate (g l^{-1} h^{-1})	6.1 ± 2.0
Lactate production rate (g l^{-1} h^{-1})	5.9 ± 1.52
Nisin production rate (AU l^{-1} h^{-1})	8.0 ± 4.0
Colony forming unit (CFU/ml^{-1})	$8.5 \times 10^8 \pm 9.2 \times 10^7$

The culture was conducted by pH-dependent feed using hydrolyzed sago starch as the sole carbon source. The reported parameter values represent the mean ± SD ($\bar{x}$ ± SD) of three repetition of the analysis and correspond to the maximum obtained during exponential phase

permanent exponential phase. Table 1 summarizes the kinetic parameters obtained in batch culture. From these results a specific growth rate of 0.62 l h^{-1} for *L. lactis* IO-1 was calculated. Glucose consumption in log phase was at a rate of 6.1 g l^{-1} h^{-1} with lactate production rate of 5.9 g l^{-1} h^{-1}.

Nisin production in continuous culture

Figure 3 shows the time course of continuous fermentation (1-l jar fermenter) using the synchronized fresh cells bioreactor with nisin Z production after 68 h. It only takes 1.5 h to concentrate 4-l of broth to 400 ml, so target cell concentration was quickly obtained. The turbidity controller was set to control cell concentration at 6 and 15 g l^{-1} with excess biomass discharged as before. Here, dry cell weight (DCW) and viable cell count of 0.7–2 × 10^{10} CFU ml^{-1} remained almost constant throughout the experiment. In order to maintain constant DCW, glucose-free solution at 120 ml h^{-1} (D1) was fed into the fermenter. Feeding of 20 ml h^{-1} (D2) 10 M NaOH was necessary to maintain the pH at 5.5, and to maintain the steady state in the fermenter, substrate (YE and some PP) was fed at 420 ml h^{-1} (D3) to provide glucose and growth factors for cell growth.

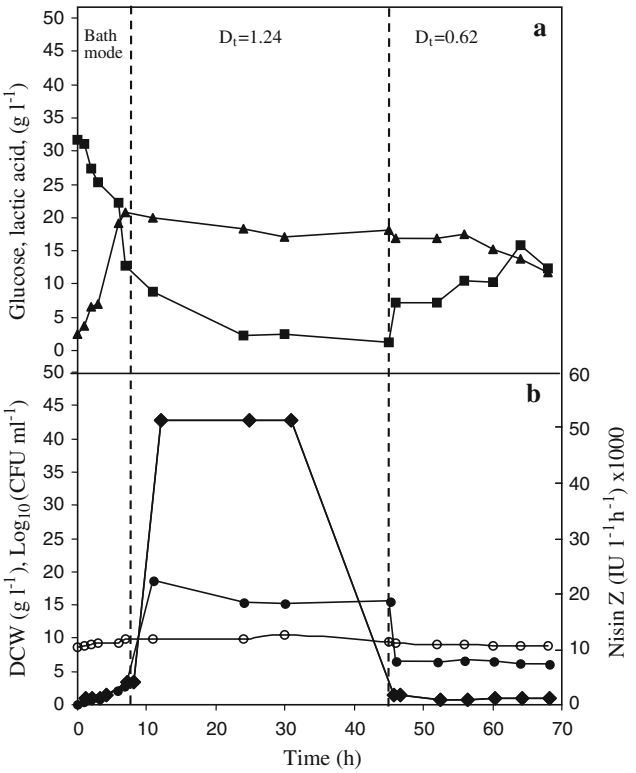

Fig. 3 Time course of lactic acid fermentation at two cell concentrations and two dilution rates in 1-liter jar fermenter. **a** *closed squares* residual glucose; *closed triangles* lactate; **b** *closed circles* DCW; *open circles* CFU ml^{-1}; *opened diamonds* Nisin Z. The *dashed lines* indicate the change of conditions from batch to continuous culture and the change of operating conditions of DCW (from 15.0 to 6.0), and Dilution rate (from 1.24 to 0.62 h^{-1})

Taking into account all these three flows into the fermenter, the total dilution rate (Dt) was: $Dt = (D1 + D2 + D3)/V$ [17]. Where, V is the working volume (the microfiltration module hold up certain volume which must be accounted for the total working volume). Once steady state is obtained, nisin Z production was maintained at a constantly high level of production at a dilution rate of 1.24 l h^{-1} (Fig. 3). Bacteriocin productivity at steady state under these conditions was constant at 50,000 IU l^{-1} (Table 2). It should be noted that this productivity was 6.8 times higher than reported by Chinachoti [5].

Table 3 summarizes the kinetic parameters obtained when the synchronized fresh cell bioreactor was employed at 15 g l^{-1} cell concentration and high dilution rates of 1.24 and 0.62 l h^{1}. From here, it was observed that *L. lactis* IO-1 maintained its viability with volumetric LA productivity maintained at 21.6 g l^{-1} h^{-1} at a dilution rate of 1.24 l h^{-1}. The maximum concentration of LA was 18 g l^{-1} and at this level the effects of product inhibition was not severe for *L. lactis* IO-1. Under these conditions, nisin Z production was maintained almost constant.

In order to assess the stability of the system, this fermentation run was maintained for 34 h, hence a steady state of 40 volume changes was maintained without affecting cell viability and performance of the membrane. Using a total dilution rate (D*t*) of 1.24 h^{-1}, a specific growth rate of approximately 0.27 h^{-1} was obtained, which supports the similar value of 0.21 h^{-1} found by Nolasco-Hipolito et al. [17] at a similar total dilution rate

Table 2 Productivity of bacteriocin in the continuous culture

Time (h)	Nisin activity		
	AU ml^{-1}	IU ml^{-1}	IU l^{-1} h^{-1}
0	6,400	62.5	195.3
1	6,400	62.5	195.3
3	6,400	62.5	195.3
6	1,02,400	1,000	3,125
7	1,02,400	1,000	3,125
11	1,63,8400	16,000	50,000
24	1,63,8400	16,000	50,000
30	1,63,8400	16,000	50,000
45	25,600	250	781.25
52	3,200	31.25	97.6
56	3,200	31.25	97.6
60	6,400	62.5	195.3
64	6400	62.5	195.3
68	6,400	62.5	195.3

Table 3 Kinetic of parameters, high cell density and high dilution rate in continuous fermentation by pH-dependent and cell recycling

Parameter		
Dilution rate (h^{-1})	1.24 ± 0.23	0.62 ± 0.15
Dry cell weight (g l^{-1})	15.0 ± 5.0	6.0 ± 2.0
Lactic acid concentration (g l^{-1})	18.0 ± 3.0	15.0 ± 2.0
Residual glucose concentration (g l^{-1})	4.4 ± 2.9	10.0 ± 2.5
Lactate production rate (g l^{-1} h^{-1})	21.6 ± 4.5	9.3 ± 3.7
Nisin production rate (IU l^{-1} h^{-1})	50,000 ± 0.0	195.3 ± 0.0
Colony forming unit × 10^{10} (CFU ml^{-1})	0.7 ± 0.035	0.5 ± 0.04
NaOH consumption rate (g h^{-1})	9.6 ± 0.7	3.9 ± 1.0
Glucose-free sol. flow-rate (F_3) (g l^{-1} h^{-1})	120 ± 10.0	50.0 ± 7.6
Specific growth rate (μ) = D_3 = (F_3/V)	0.27 ± 0.04	0.11 ± 0.01
Biomass formation rate (μX) (g g^{-1})	4.0 ± 1.0	0.66 ± 0.07
Yield ($Y_{Y/P}$)	0.85 ± 0.11	0.67 ± 0.05

Temperature and agitation were controlled at 30°C and 400 rpm respectively. The values represent the mean ± SD ($\bar{x}$ ± SD) of three repetition of the analysis

(1.1 h^{-1}). The difference was that this specific growth rate of 0.27 h^{-1} was obtained with the goal of LA production.

A production rate of 4 g l^{-1} h^{-1} biomass was achieved and this concentration of biomass was discharged continuously from the fermenter in order to maintain constant the biomass in the fermentor. After 45 h of continuous culture, the fermentation conditions were changed to dilution rate of 0.62 h^{-1}, cell concentration at 6 g l^{-1} and temperature at 37°C to investigate whether a lower cell concentration and higher temperature could maintain high nisin Z productivity (Fig. 3; Table 3). Although the stability of nisin Z production were maintained until fermentation was terminated, the productivity was very low. These results suggest that a higher cell concentration will result in higher nisin production.

The dilution rate of 0.62 h^{-1} was maintained during 12 volume changes, which constituted a steady state for the system. The maximum titer of bacteriocin obtained was 6,400 AU l^{-1}, corresponding to a volumetric productivity of 195.3 IU l h^{-1}. At this dilution rate, biomass production was stabilized (0.66 g l^{-1} h^{-1}, Table 3) as reflected by the steady discharge of the excess biomass by the turbidostat system, supported by feeding of glucose-free solution at 50 ml h^{-1} glucose-free solution. However, under these conditions the volumetric productivity of nisin Z was obviously lower (195.3 IU l^{-1} h^{-1}) compared to at a higher dilution rate of 1.24 h^{-1}.

Effects of temperature on nisin z production

As demonstrated before by Matsuzaki et al. [15], productivity of nisin Z is enhanced at 30°C and pH 5.5 in batch Cultures. Subsequently, the effect of higher temperature at 37°C in continuous culture was studied at biomass concentration of 6 g l^{-1} and a dilution rate of 0.62 h^{-1}. The pH 5.5 and agitation at 400 rpm, remain unchanged. The results obtained from the experiment at dilution rate 1.24 h^{-1} were not reproduced, and nisin Z titers were as low as 195.3 IU l^{-1} h^{-1}. It was assume that temperature plays an important role in activating or promoting the expression of nisin Z production genes to enhance its production. Glucose consumption and LA productivity were also comparatively lower.

Table 3 summarizes the kinetic parameters of this experiment. It is observed that the specific growth rate was 0.11 h^{-1}, and it is possible to assume that although the volumetric productivity of LA and glucose consumption rate were low, these were sufficient to maintain a biomass production rate of 0.66 g l^{-1} h^{-1}. Nevertheless, in this case, high cell concentration is not associated with high nisin Z production.

Discussion

Continuous fermentation of lactic acid bacteria is currently employed to produce a number of microbial metabolites, e.g. bacteriocin [3–5, 8], lactic acid [17]. Continuous productions of bacteriocins using free and immobilized cell technology are a relatively recent approaches in bacteriocin research and is not extensively reported in the literature [3, 8, 18]. In this study the bioreactor for continuous production of bacteriocin was employed to enhance the productivity of nisin Z produced by *L. lactis* IO-1.

It has been reported that most bacteriocins are produced during the exponential growth phase, which in turns correspond to high rate of biomass production. Due to this characteristic, bacteriocin production shows primary metabolite kinetics [1, 10]. According with the finding of De-Vust and Vandamme the nisin biosynthesis took place during the active growth phase and completely stopped when cells entered the stationary phase [9]. Therefore to avoid a stationary phase in this study one of the strategies was to maintain the cell in continuous growing by continuous culture at high dilution rate. Under these conditions higher nisin titters were obtained comparing our results

with those obtained in previous works [5, 6, 14, 15, 18] by in almost 6.8 times higher values [5]. Therefore, we found that is possible to achieve high nisin Z production once the goal of maintaining cell growth in a permanent exponential phase is accomplished.

The results of this study presented the same pattern found by Bhugaloo and co-authors in the sense that there was a disparity between batch and continuous mode of bacteriocin production which can explained by the fact that the desired peptide is limited to the metabolism of the growing cells and the amount of peptides is directly proportional to the biomass produced; therefore, supplying fresh medium continuously eliminates the problem linked to substance limiting growth in non-renewed cultures [3]. With the use of the synchronized fresh cell bioreactor this condition was covered satisfactorily.

There is evidence that the production of several bacteriocins in lactic acid bacteria is regulated by inducer peptide pheromones that specifically interact with their cognate bacterial receptor [10, 16, 19] and is affected both by nutrient availability and nisin inhibition [13]. However, in our study the operating conditions of the synchronized fresh cell bioreactor allowed the removal of nisin and lactate from the fermenter by means of high dilution rate hence the inhibitory effects of the end products were minimum. On the other hand, it has been reported that under stress conditions the production of bacteriocins is stimulated and enhanced by the addition of some inducers such as ethanol, Tween-80, Ca^{+2}, or NaCl [15, 16]. In this study 10 g l^{-1} YE and 2 g l^{-1} PP was employed and no other amendments were necessary to maintain the nisin Z production.

In the natural environment, microorganisms display defense mechanisms against each other through the production of metabolites that can display bactericidal or bacteriostatic effects. In heterogeneous microflora, the bacterium with the strongest bactericidal or bacteriostatic compounds (antibiotics, bacteriocins, LA, acetic acid) will survive and grow faster until the exhaustion of the available substrate. Competition in the environment could be very strong if substrates are available and if detection of any chemical signal that induces over-expression of the genes for bacteriocin or any other bacteriostatic compound were unavailable. Therefore the addition of some compounds for example ethanol, perhaps mimics the presence of an ethanol-producing microorganism, and this will act to stimulate bacteriocin production due to competition to increase the population and consumption of nutrients.

Conclusion

High cell viability and removal of LA is imperative to improve either LA or nisin productivity. High dilution rates along with high cell density were exhibited to be a suitable combination in order to enhance nisin production. This can be improved further if the cells are perpetually in the log phase.

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