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Nisin production in a chitin-including continuous fermentation system with *Lactococcus lactis* displaying a cell wall chitin-binding domain

Ömer Şimşek

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Abstract The limiting factors in the continuous production of nisin are high amount of biomass loss and low dilution rate application. In this study, a chitin-including continuous nisin fermentation system (CICON-FER) was constructed for high volumetric nisin production using nisin producer *L. lactis* displaying cell wall chitin-binding domain (ChBD) together with chitin in the reactor. In this respect, the highest binding conditions of relevant *L. lactis* cells to chitin were determined. Then the chitin flakes carrying nisin-producing *L. lactis* cells were used within the CICON-FER system at different dilution rates (0.1–0.9 h^{-1}) and initial glucose concentrations (20–60 g l^{-1}). The results revealed that the pH 7 conditions and the use of 100 mM sodium phosphate buffer with 0.1 % Tween 20 and Triton X-100 significantly increased the binding capacity of ChBD displaying *L. lactis* cells to chitin. The constructed CICON-FER system maintained the presence of the ChBD surface displaying *L. lactis* cells in the reactor system until 0.9 h^{-1} dilution rate that resulted in a considerably high level of volumetric nisin production and productivity (10,500 IU ml^{-1} and 9,450 IU ml^{-1} h^{-1}, respectively) with the combination of a 0.9-h^{-1} dilution rate and a 40-g l^{-1} initial glucose concentration. In conclusion, an innovative nisin fermentation system that yielded the highest nisin production thus far and that was feasible for industrial application was created.

Keywords *L. lactis* · Nisin · Chitin · Chitin-binding domain · Continuous fermentation

Ö. Şimşek (✉)
Department of Food Engineering, Faculty of Engineering, University of Pamukkale, Denizli, Turkey
e-mail: omers@pau.edu.tr

Introduction

The use of bacteriocins in food production systems has been considered as an alternative and natural preservation method for ensuring consumer and product safety [1–3]. Nisin is the first identified bacteriocin in *Lactococcus lactis,* classified as a type I (A) lantibiotic. Owing to its broad-spectrum antimicrobial activity, FDA considered it as a "generally recognized as safe" (GRAS) agent (E234), and approved its use in the food industry. Therefore the use of nisin as a natural preservative in the production of many foods such as milk and dairy products, canned foods, and instant soups has become increasingly common [4, 5].

Industrially, nisin is produced through the biosynthesis of *L. lactis* in a batch fermentation system using milk or whey. Consequently, the nisin that is secreted into the fermentation medium is first concentrated, then dried by spraying, and finally reduced to small fragments. In this manner, commercially prepared nisin contains only 2.5 % active nisin, where the remaining product is 74.4 % sodium chloride, 23.8 % milk solids, and 1.7 % moisture [6]. The limited production rate of nisin in fermentation systems (100–200 μg/l) and the high production and purification costs of nisin are the main factors limiting its use in food production. Thus, there is still need for alternative high-yield fermentation systems that would allow nisin production at g/l concentrations [5].

The initial studies for high-yield nisin production at an industrial scale began with batch systems [7]. These studies were later followed by fed-batch [8–11], and continuous systems [12–14] to further improve production yields. Alternative systems have also been proposed to reach a high density of biomass, such as biofilm reactors, which have been shown to result in a significantly shorter lag time on nisin production and easier-to-process fermentation broth

with lower viscosity and biomass content [15, 16]. However, in recent years, innovative nisin production systems have been developed using genetically improved nisin producers. For instance, Wardani et al. [17] was able to shift the metabolic pathway of lactic acid to ethanol to avoid the inhibition of cells by expressing genes that encode the enzymes pyruvate decarboxylase and alcohol dehydrogenase in nisin-producing *L. lactis*. In another study, regulation and resistance genes responsible for nisin biosynthesis were over-expressed in *L. lactis* LL27, which yielded high-yield nisin producers and increased the stability of producers at higher dilution rates within continuous fermentation systems [18, 19]. Furthermore, when the alternative oxidase (*aox*1) gene from *Aspergillus niger* was cloned into the nisin producer *L. lactis* ATCC11454, the constructed recombinant strain could be cultivated to high biomass in a fed-batch fermentation system incorporated with high oxygen concentrations. This high biomass allowed high nisin production (8,000 IU ml^{-1}) during fermentation [20].

Recently, Şimşek et al. [21] obtained chitin-binding ability in the nisin producer *L. lactis* N8, due to the lack of immobilization of nisin producers on the support material in continuous fermentation system [12, 14], and in order to reach high biomass levels in the reactor. ChBD of C-terminal chitinase A1 from *Bacillus circulans* was fused to the different length of PrtP (153, 344, and 800 aa) and AcmA (242 aa) anchors, which were then expressed on the cell walls of the nisin producer *L. lactis* cells under the p45 promoter and with the usp45 secretion signal. The acquired chitin-binding ability in nisin producers maintained them in the fermentation environment at repeated medium changes that ensured high levels of nisin production at forward cycles of fermentation [21].

In this study, the highest binding conditions on the chitin-binding ability of nisin-producing *L. lactis* strains that expressed ChBD in the cell wall were initially determined. Then, the CICON-FER system was constructed using *L. lactis* PLAC2 and *L. lactis* PLAC7 strains [21] with the highest binding capacity to chitin. In this system, the nisin-producing behavior of these strains was determined at different dilution rates and sugar concentrations. Eventually, an innovative system for the production of nisin was described, and the most efficient dilution rate and initial glucose concentrations were determined. As such, the prevention of cell loss by the CICON-FER system that normally occurs in continuous fermentation systems, and a very high level of volumetric nisin production could be achieved.

Materials and methods

The microorganisms and growth conditions

The bacterial strains used in this study and their characteristics are shown in Table 1. These strains were obtained from Pamukkale University, Food Engineering Culture Collection (PUFECC, WDCM 1019). *L. lactis* strains were grown at 30 °C in M17 (Merck) supplemented with 0.5 % glucose (M17G). When required, 5 μg ml^{-1} erythromycin was added to the growth medium for *L. lactis.*

Determination of the efficient conditions yielding highest binding of *L. lactis* strains to chitin

Control and recombinant *L. lactis* strains were bound to chitin flakes (Sigma, St. Louis, MO, USA), by using the method described by Wang and Chao [22]. Accordingly, after the relevant strains were grown overnight in M17G, strains were re-inoculated at 10 % in fresh medium and incubated for an additional 4 h. The cells were then collected by centrifuging at 5,000 × *g*, and the upper phase was completely separated. Then, the cell pellets were washed twice with binding buffer (20 mM sodium phosphate, pH 7.0) and dissolved in the same buffer to set the optical density to 0.5 (OD_{600}). Ultimately, 1 g of chitin flakes was added to 10 ml of relevant cell suspension. To determine the effect of temperature on the binding of cell wall ChBD displaying *L. lactis* strains on chitin surfaces, the cell–chitin mixtures were incubated overnight at 4, 22, and 40 °C separately. The pH of the binding buffer was set to 3, 5, 7, and 8 to determine the effect of pH and the cells were

Table 1 Bacterial strains and their properties

Strains	Properties	References
L. lactis N8	Wild-type, nisin Z producer	[31]
L. lactis PLAC1	*L. lactis* N8 harboring pBL01 (including SSusp45-ChBD-PrtP153 fusion sequence under frame of p45 promoter), ery^{+}, nisin Z producer	[21]
L. lactis PLAC2	*L. lactis* N8 harboring pBL02 (including SSusp45-ChBD-PrtP344 fusion sequence under frame of p45 promoter), ery^{+}, nisin Z producer	[21]
L. lactis PLAC3	*L. lactis* N8 harboring pBL03 (including SSusp45-ChBD-AcmA fusion sequence under frame of p45 promoter), ery^{+}, nisin Z producer	[21]
L. lactis PLAC7	*L. lactis* N8 harboring pBL04 (including SSusp45-ChBD-PrtP800 fusion sequence under frame of p45 promoter), ery^{+}, nisin Z producer	[21]

ChBD chitin-binding domain

incubated overnight at 4 °C with these pH values. To determine the effect of sodium phosphate concentration of the binding buffer, 5, 50, and 100 mM were evaluated against water, which was used as the control. To determine the effect of Tween 80 and Triton X-100, relevant agents were added at 0.1 % to the binding buffer.

Nisin production in the CICON-FER system

Continuous nisin production with the *L. lactis* N8 and *L. lactis* PLAC2 and PLAC7 strains was carried out in a computer-controlled autoclavable bioreactor system (Minifors B.-Pack-5, InforsHT, Switzerland) having feed and exit peristaltic pumps, in addition to pH and temperature sensors (Mettler Toledo, Columbus, OH, USA) and with base dimensions 395 × 370 × 550 mm (Fig. 1a). The recombinant and control *L. lactis* strains were bound to chitin flakes prior to the fermentation. Two hundred grams of chitin flakes were placed in the reactor after they were washed and drained. Separately, *L. lactis* cells were inoculated as 10 % into the 200 ml M17G and incubated for 4 h where cell pellets were collected by centrifugation. Then, a 2-l cell suspension (OD_{600} 0.5) was prepared using cell pellets, which was placed in the bioreactor system including chitin flakes (Fig. 1b). This bioreactor system was operated overnight with 200 rpm stirring to bind the cells to chitin surfaces. Chitin flakes that remained in the bioreactor system

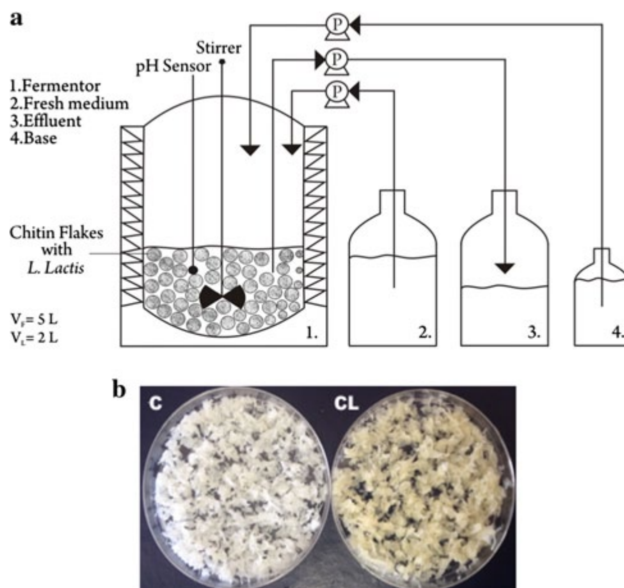

Fig. 1 The schematic representation of CICON-FER system (**a**) and image of chitin flakes used in the CICON-FER system (**b**). V_F fermenter volume, V_L medium volume. *P* peristaltic pump, *C* chitin flakes treated with HCI (6N) and NaOH (5N), respectively, and washed extensively with PBS. *CL* Chitin flakes used in CICON-FER system after washed and subsequently immobilized with nisin producer *L. lactis* cells

were washed thoroughly with 5 l of binding buffer. Next, 2 l of M17G was added to the system, and fermentation was started at 30 °C. After 3 h of fermentation, the feed and exit pumps were turned on and the operational parameters were adjusted to 0.1, 0.3, 0.5, 0.7, and 0.9 h^{-1} dilution rates and to 20, 30, 40, 50, and 60 g l^{-1} initial glucose concentrations separately. For determination of the effects of dilution rates; 10 g l^{-1} glucose was used at feeding medium and as well as to test the initial concentration of glucose; bioreactor was operated at 0.9 h^{-1} dilution rate. After this system was operated at each parameter until three fermentation volumes (approximately 5 l) passed through, 50 ml was withdrawn at 1-h intervals to determine biomass, residual glucose, and nisin production.

Analytical analysis

The level of nisin production was determined using the method proposed by Tramer and Fowler [23]. Producer cells within the samples collected from the fermenter were separated by centrifuging at 8,000×*g* for 5 min. The supernatant was transferred to a new tube and treated at 80 °C for 15 min. This partially sterilized supernatant was then diluted 2^{10} fold using 0.1 % Tween 80 including pH 2.5 sterilized water. Furthermore, the *M. luteus* NCIBM inoculated LB (Fluka, St. Louis, MO, USA) soft agar (0.7 %), which was grown overnight, was then poured onto a previously prepared LB agar surface. Finally, 5 μl of each diluted sample was pipetted onto the relevant LB agar plates and respectively dried and incubated at 30 °C. To measure the final nisin activity of the samples, standard solutions of Nisaplin (Sigma, St. Louis, MO, USA) with concentrations between 1 to 100 IU ml^{-1} were used simultaneously. The measured diameter of inhibition zones obtained from standard solutions and samples were used to determine the ultimate nisin concentrations of the relevant samples.

The released cell density at the samples collected at intervals of fermentation was determined spectrophotometrically (PC Instruments, Klongluang, Pathumthani, Thailand) at 600-nm absorbance. To measure the optical density and biomass equivalence, cell cultures of different optical densities were first centrifuged at 10,000×*g* and washed twice with PBS buffer. The obtained cell precipitates were then dried at 70 °C until their dry cell weight was stabilized. A standard curve was generated for optical density and biomass. By using the equation ($y = 4.3451x - 0.1008$, $R^2 = 0.9994$), the biomass of the samples was calculated based on their optical density values [20].

The quantity of glucose consumed by *L. lactis* strains in the CICON-FER system was determined using the glucose determination kit (Biovision, Milpitas, CA, USA). As such, samples were first diluted with the kit solution, and the kit

protocol was then applied to determine ATP levels according to a measurement standard.

Statistical analysis

All quantitative data, unless otherwise stated, are presented as the means of triplicates with error represented by standard deviation. Significant differences between bacterial numbers of controls and treated samples were determined by the analysis of variance ANOVA procedure and compared with Tukey's comparison test using Minitab program (Minitab 14; Minitab Inc., Minneapolis, MN, USA) at the 5 % level.

Results

Highest binding conditions for *L. lactis* strains to chitin flakes

The binding capacity of ChBD-expressing *L. lactis* strains (PLAC2 and PLAC7) to chitin flakes increased parallel to the gradual increase in pH (Table 2). In fact, the binding capacity increased 48 % for *L. lactis* PLAC2 and 55 % for *L. lactis* PLAC7 with the pH changing from 3 to 8. However, no significant differences ($p > 0.05$) were observed in the binding capacity of the strains at pH values of 7 and 8. These results indicated that the expressed ChBD in *L. lactis* strains was more effective for binding to chitin flakes under neutral and partially alkaline conditions. Similarly, changes in the concentration of the sodium phosphate buffer also affected ($p < 0.05$) the binding capacity of *L. lactis* cells. In the case that pure water was used, there was no difference ($p > 0.05$) in the binding capacity of *L. lactis* N8 and ChBD-expressing *L. lactis* cells. An increase in the sodium phosphate concentration in the buffer significantly improved ($p < 0.05$) the binding capacity of *L. lactis* PLAC2 and PLAC7 strains. For instance, 100 mM sodium phosphate yielded more than twofold higher binding capacity for these strains. The addition of 0.1 % Tween 20 and Triton X-100 to the binding buffer also provided a significant increase in the chitin-binding capacity of *L. lactis* strains. With the use of Tween 20 and Triton X-100, the binding capacity of *L. lactis* PLAC2 was 90 and 96 %, where this value was 93 and 98 % for *L. lactis* PLAC7, respectively. In spite of these results, the binding temperature did not affect the chitin-binding capacity of *L. lactis* strains (Table 2).

Nisin production of *L. lactis* strains in the CICON-FER system

A chitin-including continuous nisin fermentation system (CICON-FER) was constructed for the production of nisin with *L. lactis* PLAC2 and PLAC7 strains, both of which demonstrated high chitin-binding capacity by displaying ChBD on their cell wall structure (Fig. 1a). The significance of this system was including submerged chitin flakes immobilized with *L. lactis* cells. The ratio of chitin flakes required in the reactor system was determined with

Table 2 Effect of different conditions on the binding of nisin producer *L. lactis* strains

Binding conditions	*L. lactis* (binding %)				
	N8	PLAC1	PLAC2	PLAC3	PLAC7
pH					
3	29 ± 7.2^{aa}	38 ± 4.7^{aa}	40 ± 3.1^{ba}	31 ± 3.7^{aa}	42 ± 2.1^{ba}
5	48 ± 6.1^{ab}	56 ± 5.3^{ab}	64 ± 3.7^{bb}	47 ± 4.7^{ab}	73 ± 3.8^{cb}
7	33 ± 6.7^{aa}	54 ± 4.4^{bb}	76 ± 4.1^{cc}	43 ± 5.5^{ab}	96 ± 3.7^{dc}
8	34 ± 5.2^{aa}	44 ± 5.5^{ab}	78 ± 3.5^{bc}	39 ± 4.1^{ab}	95 ± 4.9^{cc}
Temperature					
4 °C	22 ± 3.7^{aa}	31 ± 5.1^{aa}	84 ± 4.4^{ba}	30 ± 3.9^{aa}	95 ± 4.5^{ba}
22 °C	19 ± 2.9^{aa}	28 ± 3.5^{aa}	79 ± 5.8^{ba}	35 ± 4.3^{aa}	91 ± 3.5^{ba}
40 °C	29 ± 4.1^{ab}	41 ± 4.8^{bb}	77 ± 5.1^{ca}	38 ± 4.2^{ba}	93 ± 5.5^{da}
Buffer (mM)					
0	21 ± 4.8^{aa}	34 ± 5.7^{aa}	39 ± 3.8^{aa}	39 ± 5.5^{aa}	37 ± 5.9^{aa}
5	44 ± 5.8^{ab}	50 ± 4.5^{ab}	68 ± 4.6^{bb}	48 ± 5.7^{aa}	73 ± 4.8^{bb}
50	47 ± 5.8^{ab}	62 ± 5.3^{bb}	83 ± 4.9^{cc}	56 ± 6.3^{bb}	91 ± 4.2^{cc}
100	34 ± 3.9^{ab}	56 ± 6.1^{bb}	90 ± 5.7^{cc}	46 ± 5.2^{ba}	96 ± 4.8^{cc}
Surface active reagents					
Triton X-100	39 ± 4.8^{aa}	50 ± 4.2^{ba}	90 ± 3.1^{ca}	51 ± 4.7^{ba}	96 ± 3.2^{ca}
Tween 20	35 ± 3.9^{aa}	46 ± 4.5^{aa}	93 ± 3.4^{ba}	56 ± 5.4^{ca}	98 ± 1.9^{ba}

The *first letters* refer to the difference ($p < 0.05$) for strains at each parameter and the *second letters* refer to the significance ($p < 0.05$) for each applied parameter at each strain

preliminary trials. Therefore, the best proportion of chitin flakes to be used in the bioreactor was 10 % (w/v), which allowed an appropriate medium-chitin flakes balance. If a value higher than this relevant ratio was used, chitin flakes absorbed the majority of the medium. To compare the effectiveness of nisin production of *L. lactis* PLAC2 and PLAC7 strains in the CICON-FER system, *L. lactis* N8, which has no specific ability to bind to chitin, was also used in both the CICON-FER and continuous fermentation system without chitin flakes. This strain was inoculated to a cell density up to 0.5 (OD_{600}) in the fermentation system.

Both in the CICON-FER and continuous fermentation system without chitin, the biomass of *L. lactis* N8 increased until the dilution rate was 0.3 h^{-1}; however, in forward dilution rates, biomass reduced ($p < 0.05$) dramatically (Table 3). On the contrary, *L. lactis* PLAC2 and PLAC7 strains displaying ChBD on their cell wall structure had higher biomass in the CICON-FER system than the *L. lactis* N8. Furthermore, the biomass obtained at higher dilution rates (0.7 and 0.9 h^{-1}) for *L. lactis* PLAC2 and PLAC7 strains was found similar to the biomass measured at lower dilutions (0.1 and 0.3 h^{-1}) ($p > 0.05$), implying that the cells were not diluted at forward dilution rates. In the CICON-FER system with high dilution rates (>0.5 h^{-1}), *L. lactis* N8 consumed glucose at relatively low levels (<5 g/l). However, *L. lactis* PLAC2 consumed the initial glucose completely up to 0.5 h^{-1}, where the consumption slightly reduced ($p < 0.05$) after 0.7 h^{-1}. However, *L. lactis* PLAC7 consumed the initial glucose completely at all dilution rates. In both the CICON-FER and the system without chitin, the highest amount of nisin production (2,220 and 2,210 IU ml^{-1}, respectively) for *L. lactis* N8 was at 0.3 h^{-1}. However, as expected, forward dilution rates also led to lower nisin production in this strain ($p < 0.05$). The highest specific nisin production of *L. lactis* N8 was 666 IU ml^{-1} h^{-1} in CICON-FER and was 636 IU ml^{-1} h^{-1} in continuous nisin fermentation without chitin at 0.3 h^{-1}. In comparison, *L. lactis* PLAC2 and PLAC7 produced the highest levels of nisin (4,920 and 5,520 IU ml^{-1}, respectively) at 0.3 h^{-1}, while the highest amount of nisin production per hour in these strains (2,709 and 4,581 IU ml^{-1}, respectively) was observed at 0.9 h^{-1}. At *L. lactis* PLAC7, nisin production was significantly ($p < 0.05$) maintained until to the 0.9 h^{-1}. The nisin production yield per glucose was determined as similar level after 0.3 h^{-1} for *L. lactis*

Table 3 Effect of dilution rate on nisin production of *L. lactis* strains at CICON-FER system

L. lactis	Dilution rate (h^{-1})				
	0.1	0.3	0.5	0.7	0.9
N8 (without chitin)					
Biomass (g l^{-1})	2.80^{a}	3.40^{b}	1.60^{c}	1.40^{c}	1.20^{c}
Glucose consumption (g l^{-1})	5.87^{a}	5.44^{a}	3.88^{b}	2.19^{b}	2.10^{b}
Nisin production (IU ml^{-1})	1,150^{a}	2,120^{b}	650^{c}	340^{d}	150^{e}
Nisin productivity (IU ml^{-1} h^{-1})	115	636	325	238	135
$Y_{N/G}$ (IU mg^{-1})	195.91	389.70	167.52	155.25	71.43
N8					
Biomass (g l^{-1})	3.20^{a}	3.10^{a}	2.00^{b}	1.70^{b}	1.40^{c}
Glucose consumption (g l^{-1})	6.19^{a}	6.37^{a}	4.37^{b}	3.66^{c}	3.42^{c}
Nisin production (IU ml^{-1})	1,250^{a}	2,220^{b}	1,250^{a}	650^{c}	340^{d}
Nisin productivity (IU ml^{-1} h^{-1})	125	666	625	455	306
$Y_{N/G}$ (IU mg^{-1})	201.93	348.51	286.04	124.32	89.47
PLAC2					
Biomass (g l^{-1})	4.70^{a}	4.40^{a}	4.20^{a}	3.50^{b}	3.10^{c}
Glucose consumption (g l^{-1})	9.51^{a}	9.45^{a}	9.27^{a}	8.57^{b}	6.43^{c}
Nisin production (IU ml^{-1})	2,530^{a}	4,920^{b}	4,480^{b}	3,530^{c}	3,010^{c}
Nisin productivity (IU ml^{-1} h^{-1})	253	1,476	2,240	2,471	2,709
$Y_{N/G}$ (IU mg^{-1})	266.03	520.63	483.28	411.90	468.14
PLAC7					
Biomass (g l^{-1})	3.10^{a}	3.50^{a}	4.10^{b}	3.90^{b}	3.50^{a}
Glucose consumption (g l^{-1})	9.89^{a}	9.63^{a}	9.57^{a}	9.44^{a}	9.53^{a}
Nisin production (IU ml^{-1})	2,530^{a}	5,520^{b}	4,750^{c}	5,090^{b}	5,090^{b}
Nisin productivity (IU ml^{-1} h^{-1})	253	1,656	2,375	3,563	4,581
$Y_{N/G}$ (IU mg^{-1})	255.81	573.21	496.34	539.19	534.10

Data are the average of the replicates and standard deviations are lower than 2 %. *Different letters at each row* refer to the significantly difference ($p < 0.05$). The initial glucose concentration at feeding is 10 g l^{-1} at each dilution rate

PLAC7 having the ability to produce the highest amount of nisin in the CICON-FER system (Table 3).

The effect of using different initial glucose concentrations (20–60 g l^{-1}) on nisin production of *L. lactis* PLAC2 and PLAC7 was determined at 0.9 h^{-1} within the CICON-FER system (Table 4), due to the high level of glucose consumption in this system. Accordingly, the determined biomass for both strains was nearly the same level in the initial glucose concentration; however, a slight decrease ($p < 0.05$) in the biomass amount was observed at the high initial glucose concentration (>40 g l^{-1}). Additionally, all cells other than *L. lactis* PLAC7 consumed the same level ($p > 0.05$) of glucose up to 40 g l^{-1} concentration when the consumption amount decreased after this concentration ($p < 0.05$). However, glucose consumption of *L. lactis* PLAC7 increased until the initial 50 g l^{-1} glucose. *L. lactis* N8 produced a low level of nisin at all used initial glucose concentrations, parallel to the low level glucose consumption at 0.9 h^{-1}. *L. lactis* PLAC2 and PLAC7 strains produced similar amounts ($p > 0.05$) of nisin between glucose concentrations of 20 and 50 g l^{-1}, while nisin production decreased with high glucose concentrations in these strains. Accordingly, the highest nisin production (10,500 IU ml^{-1}) was produced by *L. lactis* PLAC7 at 40 g l^{-1}. For this strain, specific nisin production was 9,450 IU ml^{-1} h^{-1}, which is a high production rate. However, nisin production per gram glucose of strain was measured interestingly low at maximum specific production levels (Table 4).

Discussion

In this study, the effect of different binding conditions on the binding of cell wall ChBD displaying *L. lactis* strains previously constructed by Şimşek et al. [21] to chitin surfaces was first investigated and then a CICON-FER system was constructed with the established results. This CICON-FER system was operated at different dilution rates and initial glucose concentrations allowing to test the performance of the ChBD expressing nisin producing *L. lactis* strains.

L. lactis strains (PLAC2 and PLAC7) displaying cell wall ChBD with different arrangements had higher binding capacity to chitin surfaces along with the increase of PrtP anchor similarly expressed by Şimşek et al. [21]. The

Table 4 Effect of different initial glucose concentration on nisin production of *L. lactis* strains at CICON-FER system

L. lactis	Initial glucose concentration (g l^{-1})				
	20	30	40	50	60
N8 (without chitin)					
Biomass (g l^{-1})	1.50^{a}	1.50^{a}	1.60^{a}	1.10^{b}	0.90^{b}
Glucose consumption (g l^{-1})	2.77^{a}	2.54^{a}	2.38^{a}	1.39^{b}	1.21^{b}
Nisin production (IU ml^{-1})	150^{a}	120^{a}	150^{a}	80^{b}	70^{b}
Nisin productivity (IU ml^{-1} h^{-1})	135	108	135	72	63
$Y_{N/G}$ (IU mg^{-1})	54.15	42.52	56.72	57.55	57.85
N8					
Biomass (g l^{-1})	3.20^{a}	3.10^{a}	2.00^{b}	1.70^{b}	1.40^{c}
Glucose consumption (g l^{-1})	4.19^{a}	4.27^{a}	4.37^{a}	3.66^{b}	3.42^{b}
Nisin production (IU ml^{-1})	350^{a}	350^{a}	250^{b}	190^{c}	190^{c}
Nisin productivity (IU ml^{-1} h^{-1})	315	315	225	171	171
$Y_{N/G}$ (IU mg^{-1})	83.53	81.97	57.21	51.91	55.55
PLAC2					
Biomass (g l^{-1})	4.70^{a}	4.40^{a}	4.20^{a}	3.50^{b}	3.10^{b}
Glucose consumption (g l^{-1})	7.55^{a}	7.45^{a}	7.20^{a}	7.50^{a}	6.40^{b}
Nisin production (IU ml^{-1})	3,550^{a}	3,900^{b}	3,400^{a}	3,550^{a}	3,010^{c}
Nisin productivity (IU ml^{-1} h^{-1})	3,195	3,510	3,060	3,195	2,709
$Y_{N/G}$ (IU mg^{-1})	470.20	523.49	472.22	473.33	470.31
PLAC7					
Biomass (g l^{-1})	5.90^{a}	6.20^{a}	5.80^{a}	5.50^{a}	4.70^{b}
Glucose consumption (g l^{-1})	19.89^{a}	28.53^{b}	35.77^{c}	39.48^{c}	30.23^{b}
Nisin production (IU ml^{-1})	9,980^{a}	10,100^{a}	10,500^{a}	10,100^{a}	7,610^{b}
Nisin productivity (IU ml^{-1} h^{-1})	8,982	9,090	9,450	9,090	6,849
$Y_{N/G}$ (IU mg^{-1})	501.75	354.01	293.54	255.82	251.74

Data are the average of the replicates and standard deviations are lower than 2 %. *Different letters at each row* refer to significant differences ($p < 0.05$). Fermentations were carried out at 0.9 h^{-1} dilution rate

binding capacity of these strains significantly increased ($p < 0.05$) parallel to the increase in pH values and salt concentration within the buffer. Studies on the structural characteristics of the ChBD have shown that the region responsible for binding contains aromatic amino acids, which bring the isoelectric point to 9.0 [22, 24, 25]. This feature possibly increases the binding capacity of *L. lactis* cells expressing ChBD when the pH and sodium phosphate concentration increase. In addition, the presence of salt under binding conditions may maintain the osmotic balance of the cells, which increase their viability and binding stability to chitin [22]. The binding characteristics associated with pH gradient as well as salt concentrations are considerably significant for the production of nisin under fermenter conditions [7, 18]. Likewise, in order to avoid the effects of lactic acid accumulation on the cells during nisin production, and due to the physiology associated with cellular nisin production, the pH of the reactor was maintained at 6. On the other hand, substrates with high salt concentrations are used in the production of nisin.

The main characteristic of the CICON-FER system is using submerged chitin flakes with immobilized nisin-producer *L. lactis* cells in the reactor. One of the interesting results observed during this fermentation system was although the natural *L. lactis* N8 strain was gradually diluted from the system at higher dilution rates (>0.3 h^{-1}), *L. lactis* PLAC2 and PLAC7 strains displaying cell wall ChBD remained stable in the reactor mediated with chitin. The main finding supporting this result and observation was the higher biomass achieved by the *L. lactis* PLAC2 and PLAC7 strains within the CICON-FER system in comparison to the *L. lactis* N8. Additionally, other indications were high levels of glucose consumption by the PLAC2 and PLAC7 strains at higher dilution rates (Table 3), and the maintenance of nisin production. Although the doubling time of *L. lactis* is dependent on many growth factors, optimal conditions are estimated as approximately 45 min [26]. Therefore, in systems without immobilization, high dilution rates can lead to a washing effect, and hence to a loss of cells prior to the growth of daughter cells. As reflected by the results of this study, the CICON-FER system contains chitin, ensuring the cells remain within the reactor.

Various continuous fermentation systems reported to date have shown that the highest nisin production could be achieved at 0.2–0.3 h^{-1}. At higher dilution rates, nisin production decreased considerably, parallel to the increase in cell loss [14, 15, 27]. Therefore, the CICON-FER system constructed within this study is an alternative to avoid the washing effect that occurs during fermentation. When the study results were evaluated accordingly, the dilution rates of 0.7 h^{-1} for the *L. lactis* PLAC2, and 0.9 h^{-1} for the PLAC7 could be reached without any loss in nisin production (Table 3). These relevant rates are the highest dilution rates ever used in nisin production for submerged continuous culture fermentations. The better success of the *L. lactis* PLAC7 than PLAC2 at forward dilution rates is due to the displayed ChBD with longer PrtP anchor extending from the cell wall [21].

Glucose concentration affects the production of nisin [7, 18, 27, 28]. It was similarly observed in the CICON-FER system that glucose concentration had an impact on nisin production (Table 4). The high-density cell accumulation within the system also leads to a high level of glucose consumption. Particularly, *L. lactis* PLAC7 completely consumed the available glucose at all dilution rates (Table 3). Thus, the highest level of nisin production among the used glucose concentrations at 0.9 h^{-1} dilution rate was 40 g l^{-1}. Additionally, the calculated yields of nisin production per gram of glucose of the *L. lactis* PLAC2 and PLAC7 strains were, respectively, eightfold and fourfold higher than *L. lactis* N8. One of the interesting details that should be highlighted is despite the fact that *L. lactis* PLAC7 showed high glucose consumption and nisin production, the $Y_{G/N}$ of this strain was relatively low. This difference might have resulted from the low solubility of nisin at relevant high production state.

Different nisin production levels have been reported for various fermentation systems proposed for the production of nisin [9, 12, 14–16, 19, 29]. Among the continuous nisin production systems, the highest volumetric production reported to date was 8,200 IU ml^{-1}, while the highest nisin productivity was 5,730 IU ml^{-1} h^{-1} [29]. Comparing the nisin production systems is not suitable due to the differences in nisin determination methods and medium conditions. However, the CICON-FER system proposed within this study enabled the highest nisin production that has been reported to date in both volumetric and specific production (10,500 IU ml^{-1} and 9,450 IU ml^{-1} h^{-1}, respectively). These results proved that *L. lactis* N8 cells were washed away from the fermentation at the higher dilution rates, while the *L. lactis* PLAC2 and PLAC7 strains remained in the reactor owing to their chitin-binding ability where these cells were thus able to sustain nisin production. Thus, fourfold- and sevenfold-higher nisin production per hour in *L. lactis* PLAC2 and PLAC7 compared to *L. lactis* N8 was reached. Even though the main reason for high nisin production reached in the relevant strains is mainly related to the accumulation of cells in the reactor, the removal of produced metabolites from the reactor may have also contributed to avoiding feedback inhibition on the producer cells. In fact, nisin production is adversely affected by high concentration of lactate and nisin existed in the reactor. In a representative study, nisin production was significantly improved by cycle changing the medium at 1-h intervals during fermentation [30]. On the other hand, high levels of nisin production achieved together with high biomass cell

in the CICON-FER system also contributed to the fact that nisin production by *L. lactis* cells is closely related to cell amount as most of the relevant studies similarly reported [15, 16].

Conclusions

An innovative CICON-FER system was successfully constructed, which could be applicable to industrial nisin production as well as yielding high volumetric nisin production. In this system, the existing chitin flakes prevented cell loss at very high dilution rates, which improved nisin production yield significantly. According to the study results, the highest volumetric nisin production (10,500 IU ml^{-1}) thus far was obtained with the CICON-FER system using *L. lactis* PLAC7 at a 0.9-h^{-1} dilution rate and 40-g l^{-1} initial glucose concentration. Nevertheless, the stability of chitin-*L. lactis* consortia is one of the questions to be answered for long-term applications to test the success of possible industrial usage.

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