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ORIGINAL PAPER

Continuous nisin production with bioengineered *Lactococcus lactis* strains

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Abstract Nisin production in continuous cultures of bioengineered *Lactococcus lactis* strains that incorporate additional immunity and regulation genes was studied. Highest nisin activities were observed at 0.2 h^{-1} dilution rate and 12.5 g l^{-1} fructose concentration for all strains. Recombinant strains were able to produce greater amounts of nisin at dilution rates below 0.3 h^{-1} compared to the control strain. However, this significant difference disappeared at dilution rates of 0.4 and 0.5 h^{-1}. For the strains LL27, LAC338, LAC339, and LAC340, optimum conditions for nisin production were determined to be at 0.29, 0.26, 0.27, and 0.27 h^{-1} dilution rates and 11.95, 12.01, 11.63, and 12.50 g l^{-1} fructose concentrations, respectively. The highest nisin productivity, 496 IU ml^{-1} h^{-1}, was achieved with LAC339. The results of this study suggest that low dilution rates stabilize the high specific nisin productivity of the bioengineered strains in continuous fermentation. Moreover, response surface methodology analysis showed that regulation genes yielded high nisin productivity at wide ranges of dilution rates and fructose concentrations.

Keywords *Lactococcus lactis* · Nisin · Regulation genes · Immunity genes · Continuous fermentation

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Introduction

Nisin is the only industrially relevant bacteriocin that is produced by some strains of *Lactococcus lactis* and *Streptococcus uberis* [6, 25]. This bacteriocin was approved in 1988 by the U.S. Food and Drug Administration (FDA) for pasteurized cheese spreads. Subsequently, the FDA approved nisin use in other foods such as soups that are heat treated and stored at chilled temperatures [8]. The development of applications for nisin in the food and pharmaceutical industries is limited by low production during fermentation, which eventually results in high product cost. It is therefore essential to develop new production processes that yield high volumetric nisin productivity.

It has been reported that nisin is produced during the exponential growth phase, which in turn corresponds to a high rate of biomass production. Due to this characteristic, nisin production shows primary metabolite kinetics [6, 7, 17]. According to the findings of de Vuyst and Vandamme [6], nisin biosynthesis takes place during the active growth phase and completely stops when cells enter the stationary phase. Therefore, to avoid a stationary phase, the cells can be maintained in a growth phase by using a continuous fermentation system. Continuous culture can also eliminate the substrate inhibition, reduce the production of metabolic out-growth, and favor the stability of bacterial metabolism. In fact, the use of chemostat cultures to enhance production of nisin has been widely established and nisin production

yields improved up to 10-fold as compared with batch cultures [1, 5, 9, 16, 17].

Genetic strategies also enable improved lantibiotic production in the producer strains. For instance, provision of additional copies of the regulatory and immunity genes with high copy number plasmids in the producer strains resulted in greater yields of nisin, lacticin, and subtilin [2, 3, 10]. Similarly, in our recent study, nisin production of three bioengineered strains (LAC338, LAC339, and LAC340) with immunity (*nisFEG*), regulation (*nisRK*), or immunity/regulation (*nisRKFEG*) genes on plasmids produced 24, 45, and 44%, respectively, more nisin than wild-type *L. lactis* LL27 after a 12-h incubation [23]. However, the behavior and the changes in the high production phenotype of these strains during continuous fermentation are unknown. In this study, the bioengineered strains were grown in continuous fermentation at different dilution rates and fructose concentrations for optimization of the nisin production levels.

Materials and methods

Bacterial strains and growth conditions

In this study, wild-type *L. lactis* LL27 and bioengineered strains *L. lactis* LAC338, LAC339, and LAC340 [23] were used for nisin production. *Micrococcus luteus* NCIMB8166 (National Collection of Industrial and Marine Bacteria) was used as the indicator strain to determine nisin activity. *M. luteus* NCIMB8166 was grown in Luria–Bertani broth at 37°C with shaking, whereas *L. lactis* strains were cultivated without aeration at 30°C in M17 broth (Oxoid) containing 0.5% (w/v) glucose (GM17). When needed, GM17 was supplemented with 5 μg ml^{-1} of erythromycin.

In the continuous fermentation, LL27 and bioengineered strains were cultivated in fermentation medium (FM) optimized previously [23] and having the following composition: fructose 10 g l^{-1}, yeast extract 30 g l^{-1}, ascorbic acid 0.5 g l^{-1}, $MgSO_4$ 0.25 g l^{-1}, and disodium hydrogen phosphate 25 g l^{-1}. *L. lactis* cells were cultivated in 10 ml of this medium at 30°C for 16 h before inoculation for continuous fermentation.

Fermentation conditions

Continuous fermentation runs were carried out in a 3-l (working volume) stirred-tank reactor (Minifors, Switzerland), equipped with instrumentation for the measurement and control of temperature (30°C), pH (6.0, by addition of NaOH), and agitation (100 rpm). The fermenter was inoculated with 1% (v/v) of a 16-h culture, and the fermentation was carried out batchwise until mid-exponential phase (7 h) before continuous feeding of the substrate was started. The volume was kept constant at 1.5 l using a peristaltic pump. Sampling was started after at least three volumes were passed through the vessel. Thereafter, to ensure that the culture had reached a steady state, the optical density of the culture was measured at 600 nm with a spectrophotometer (Shimadzu UV1601, Japan) on samples taken from the medium outlet at 20-min intervals. If the OD values of the samples were found to be stable and did not reveal any statistically significant trend, three other samples were taken at 1-h intervals and used for further analysis.

Analytical methods

For the determination of culture turbidity, culture broths were appropriately diluted with fresh medium and the optical densities were measured at 600 nm using a spectrophotometer (Shimadzu UV1601, Japan). The uncultured media were used as blanks. For cell dry weight (CDW) determination, microcentrifuge tubes were weight-stabilized by heating at 70°C. Portions of culture were dispensed into six weight-stabilized tubes, with 1.5 ml in each, and centrifuged at 12,000 rpm for 15 min, after which the supernatant was removed. The cells were then washed twice with demineralized water and dried in an oven at 70°C for 24 h. Finally, microcentrifuge tubes containing dry cells were weighed, and the CDW calculated.

Nisin titer was measured by the method of Tramer and Fowler [24]. The samples were adjusted to pH 2.0 using a 10 M HCl solution, heated in boiling water bath for 10 min and cooled to room temperature, then centrifuged at 8,000 rpm for 10 min. The supernatant was appropriately diluted with 0.02 M HCl, and the assay was performed using the agar diffusion method with indicator strain *M. luteus* NCIMB8166. A standard curve (50–600 IU ml^{-1}) was plotted using a stock solution of 10,000 IU ml^{-1} nisin (Sigma; nisin content 2.5% w/w). Assays were performed in triplicate, and average results are shown. D-Glucose/D-fructose assay kit (Megazyme, Ireland) was used to assay the residual fructose in the spent medium at different dilution rates and fructose concentrations.

Determination of plasmid stability

The stability of the recombinant plasmids during fermentation was analyzed by measuring the proportion of erythromycin colonies obtained from samples. Twenty colonies from the FM agar plates of each sample were inoculated onto FM agar also containing 5 μg ml^{-1} erythromycin. The stability of recombinant plasmids was calculated by comparing the number of cells growing on

Table 1 Effect of dilution rate on nisin production of LL27 and bioengineered strains during continuous fermentation

	Dilution rates (h^{-1})				
	0.1	0.2	0.3	0.4	0.5
LL27					
Biomass ($g\ l^{-1}$)	2.3	2.8	2.6	2.0	1.7
Fructose consumption ($g\ l^{-1}$)	9.9	9.9	9.8	8.0	7.0
Nisin production ($IU\ ml^{-1}$)	1,020	1,720	1,230	750	260
Nisin productivity ($IU\ ml^{-1}\ h^{-1}$)	102	344	369	300	130
$Y_{N/X}$ ($IU\ mg^{-1}$)	444	614	473	375	153
LAC338					
Biomass ($g\ l^{-1}$)	2.1	2.6	2.5	2.1	1.9
Fructose consumption ($g\ l^{-1}$)	9.9	9.9	9.7	8.2	7.0
Nisin production ($IU\ ml^{-1}$)	1,340	1,730	1,200	640	260
Nisin productivity ($IU\ ml^{-1}\ h^{-1}$)	134	346	360	256	130
$Y_{N/X}$ ($IU\ mg^{-1}$)	638	665	480	305	137
Plasmid instability (%)	1.4	5.4	4.0	13.4	25.1
LAC339					
Biomass ($g\ l^{-1}$)	2.3	2.8	2.5	2.1	1.9
Fructose consumption ($g\ l^{-1}$)	9.9	9.9	9.8	8.6	7.9
Nisin production ($IU\ ml^{-1}$)	1,620	2,150	1,450	730	330
Nisin productivity ($IU\ ml^{-1}\ h^{-1}$)	162	430	435	292	165
$Y_{N/X}$ ($IU\ mg^{-1}$)	704	768	580	348	174
Plasmid instability (%)	1.9	1.4	16.3	21.2	44.7
LAC340					
Biomass ($g\ l^{-1}$)	2.3	2.8	2.5	2.2	1.6
Fructose consumption ($g\ l^{-1}$)	9.9	9.9	9.7	8.6	6.9
Nisin production ($IU\ ml^{-1}$)	1,670	1,940	1,430	750	270
Nisin productivity ($IU\ ml^{-1}\ h^{-1}$)	167	388	429	300	135
$Y_{N/X}$ ($IU\ mg^{-1}$)	726	693	572	340	169
Plasmid instability (%)	6.6	4.7	7.3	11.0	18.0

Reported data are means of duplicate cultures

$Y_{N/X}$ Nisin production levels per biomass

the erythromycin + FM agar plates with the number of colonies initially inoculated.

Data analysis

Statistical analysis of the data was performed using Minitab Statistical Software (release 14.0, Minitab, State College, PA). Nisin productions of LL27 and bioengineered strains were optimized with response surface methodology (RSM). In the model analysis, nisin productivity values obtained for dilution rates from 0.1 to 0.4 h^{-1} and fructose concentration from 7.5 to 15.0 $g\ l^{-1}$ were used as a face-centered composite statistical design. The response surface model was fitted to the response variable, namely nisin productivity ($IU\ ml^{-1}\ h^{-1}$).

Results

For continuous nisin production, wild-type strain LL27 and recombinant strains were first cultivated in a batch-wise fermentation system until mid-exponential phase; subsequently feed and evacuation pumps were operated for continuous culture. Nisin production and growth patterns of the relevant cells were evaluated at different dilution rates (0.1–0.5 h^{-1}) and fructose concentrations (7.5–20.0 $g\ l^{-1}$) in this system.

The effect of dilution rates on nisin production in continuous fermentation

Biomass concentrations decreased with increased dilution rates. A dilution rate of 0.2 h^{-1} provided the highest biomass level for all of the strains. The initial fructose concentration (10 $g\ l^{-1}$) was completely consumed at 0.1 and 0.2 h^{-1} dilution rates. However, the residual fructose concentration increased proportionately with higher dilution rates. Therefore, these results indicated that a 0.2 h^{-1} dilution rate was optimum for reaching the highest biomass concentration at an initial fructose concentration of 10 $g\ l^{-1}$ (Table 1).

The nisin production of all strains increased with dilution rates 0.1 to 0.2 h^{-1} but decreased slightly above

0.2 h^{-1}. At this rate, the highest nisin production was achieved for strain LAC339 followed by LAC340, LAC338, and LL27. Furthermore, the 0.2 h^{-1} dilution rate also yielded the highest nisin production levels per biomass ($Y_{N/X}$). However, nisin productivity of all strains at the 0.3 h^{-1} dilution rate was found to be higher than at the 0.2 h^{-1} dilution rate. This result indicated that cells could be maintained in a very active phase as a result of removing the accumulated metabolites at this dilution rate (0.3 h^{-1}). Although 0.3 h^{-1} dilution resulted in the highest nisin productivity, 0.2 h^{-1} was found to be more efficient in terms of nisin production amounts and the other yield parameters for all strains. On the other hand, the instabilities of the recombinant plasmids at 0.1, 0.2, and 0.3 h^{-1} dilution rates were lower than those obtained at 0.4 and 0.5 h^{-1} ($P < 0.05$) (Table 1).

When the nisin production levels of the recombinant strains were compared with the control strain LL27 in the continuous fermentation system at different dilution rates, the recombinant strains were able to produce more nisin than the control strain at the low dilution rates. In particular, LAC339 and LAC340 strains with the extra regulation genes yielded 59, 25, and 18% and 64, 13, and 15%, respectively, more nisin than the control strain LL27 at 0.1, 0.2, and 0.3 h^{-1} dilution rates. This difference disappeared at dilution rates of 0.4 and 0.5 h^{-1} (Table 1). These results indicated that the benefit of the extra regulation and immunity genes [23] was lost at dilution rates above 0.3 h^{-1}. The specific nisin production rates of the strains (Fig. 1a) further supported this conclusion.

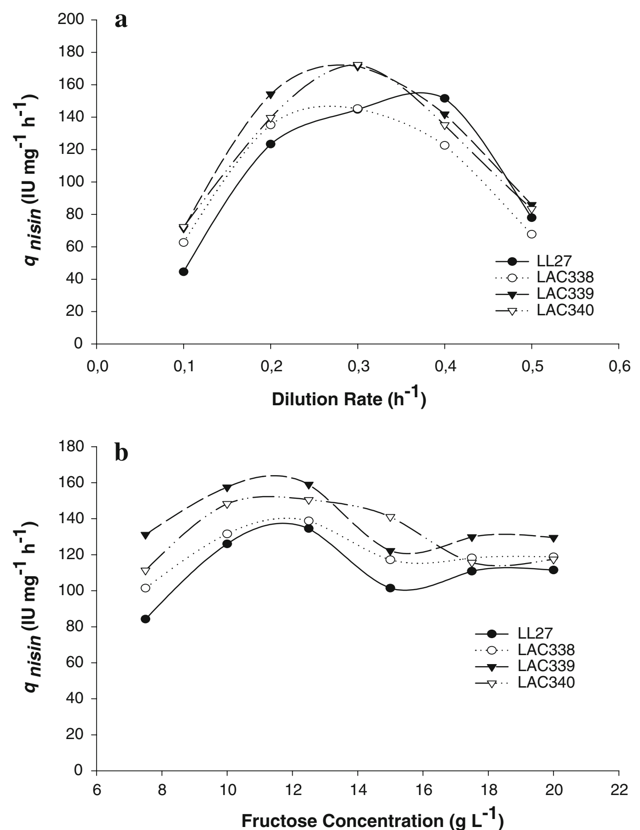

Fig. 1 Specific nisin productivity of LL27 and bioengineered strains at different dilution rates (**a**) and fructose concentrations (**b**)

The effect of fructose concentrations on nisin production in continuous fermentation

The 0.2 h^{-1} dilution rate was used to study fructose concentrations due to its efficiency at volumetric nisin production of bioengineered strains. The biomass formation increased up to 12.5 g l^{-1} fructose, but decreases in biomass formation were observed at the higher fructose concentrations (15, 17.5, and 20 g l^{-1}). The highest biomass of all strains was formed at 12.5 g l^{-1} fructose concentration. The fructose consumption of the cells decreased with increased fructose concentration, whereas the initial concentrations of 7.5 and 10 g l^{-1} were consumed completely. The decrease in biomass formation with increasing fructose concentration clearly showed that high fructose concentrations had inhibitory effects on the strains (Table 2).

Wild-type strain LL27 and its recombinant strains showed similar nisin production patterns at the tested fructose concentrations. Nisin production levels of the cells increased with fructose concentrations of 7.5–12.5 g l^{-1}. However, at the higher fructose concentrations, slight decreases to 1,070 IU ml^{-1} for LL27, to 1,200 IU ml^{-1} for LAC338, to 1,340 IU ml^{-1} for LAC339, and to 1,130 IU ml^{-1} for LAC340 were observed. Thereby the highest nisin production of all strains occurred at 12.5 g L^{-1} fructose concentration. In addition, high values of nisin production yield per milligram biomass, nisin productivity, and the specific nisin production rates also indicated that 12.5 g L^{-1} of fructose concentration is optimal for all strains. There were no significant differences in plasmid instability in the strains for all tested fructose concentrations (Table 2).

All recombinant strains produced higher amounts of nisin than the wild-type strain LL27 at all tested fructose concentrations. No significant differences in nisin production related to the fructose concentration could be detected among the recombinant strains. However, only strain LAC340, which included the extra regulation genes with the immunity *nisFEG* operon, maintained its high nisin production rate up to 15 g l^{-1} fructose concentration (Fig. 1b).

Optimization of continuous nisin production using response surface methodology

Response surface methodology (RSM) was used to determine the optimum dilution rates and fructose concentrations

Table 2 Effect of fructose concentration on nisin production of LL27 and bioengineered strains during continuous cultures

	Fructose concentration (g l^{-1})					
	7.5	10.0	12.5	15.0	17.5	20.0
LL27						
Biomass (g l^{-1})	2.2	2.7	2.8	2.8	1.9	1.9
Fructose consumption (g l^{-1})	7.5	9.9	10.9	9.9	8.0	8.0
Nisin production (IU ml^{-1})	910	1,720	1,890	1,400	1,070	1,070
Nisin productivity (IU ml^{-1} h^{-1})	182	344	378	280	214	214
$Y_{N/X}$ (IU mg^{-1})	414	637	675	500	563	563
LAC338						
Biomass (g l^{-1})	2.1	2.6	2.8	2.6	2.0	2.0
Fructose consumption (g l^{-1})	7.5	9.9	11.2	10.1	8.2	8.5
Nisin production (IU ml^{-1})	1,070	1,730	1,950	1,500	1,200	1,200
Nisin productivity (IU ml^{-1} h^{-1})	214	346	390	300	240	240
$Y_{N/X}$ (IU mg^{-1})	510	665	696	577	600	600
Plasmid instability (%)	7.7	3.2	6.3	5.0	9.5	6.4
LAC339						
Biomass (g l^{-1})	2.2	2.7	2.8	2.8	2.1	2.1
Fructose consumption (g l^{-1})	7.5	9.9	11.4	10.7	9.1	8.6
Nisin production (IU ml^{-1})	1,430	2,150	2,250	1,680	1,370	1,340
Nisin productivity (IU ml^{-1} h^{-1})	286	430	450	336	274	268
$Y_{N/X}$ (IU mg^{-1})	650	796	804	600	652	638
Plasmid instability (%)	4.5	3.2	5.0	6.4	9.5	9.8
LAC340						
Biomass (g l^{-1})	2.1	2.6	2.8	2.8	2.1	2.0
Fructose consumption (g l^{-1})	7.5	9.9	11.0	10.0	8.6	8.8
Nisin production (IU ml^{-1})	1,180	1,950	2,130	1,940	1,220	1,130
Nisin productivity (IU ml^{-1} h^{-1})	236	390	426	388	244	226
$Y_{N/X}$ (IU mg^{-1})	562	750	761	693	581	565
Plasmid instability (%)	7.7	6.1	10.4	7.61	7.5	10.7

Reported data are means of duplicate cultures

$Y_{N/X}$ Nisin production levels per biomass

for nisin productivity of LL27 and recombinant strains. Nisin productivity values of each strain obtained in the continuous fermentation system at dilution rates from 0.1 to 0.4 h^{-1} and fructose concentrations from 7.5 to 15.0 g l^{-1} were used in face-centered design for the model analysis. Variance (ANOVA) and R^2 values for LL27 and recombinant strains (LAC338, LAC339, and LAC340) were above 0.98, indicating that the model fitted well and explained 98.0% of the variability in nisin productivity. Furthermore, the F test for regression was significant at a level of 5% ($P < 0.05$), adequately explaining the variation observed in nisin productivity with the designed levels of factors. Also the lack of fit was not found to be significant at the 5% level for all strains, indicating that the experimental data obtained fitted well with the model. These statistical results also showed that the model can satisfactorily explain the effects of dilution rates and fructose concentrations on nisin productivity of LL27 and recombinant strains in continuous fermentation system. By applying multiple regression analysis on the experimental data, the following second-order polynomial equations were found to explain nisin productivity in each of the strains.

$$Y_{\mathrm{LL27}} = -1636.34 + 4731.38X_1 + 233.1X_2 - 8261.25X_1^2 - 9.75X_2^2 \quad (1)$$

$$Y_{\mathrm{LAC338}} = -1394.35 + 4543.82X_1 + 199.40X_2 - 8368.30X_1^2 - 8.30X_2^2 \quad (2)$$

$$Y_{\mathrm{LAC339}} = -1611.53 + 5750.20X_1 + 231.53X_2 - 10757.40X_1^2 - 10.01X_2^2 \quad (3)$$

$$Y_{\mathrm{LAC340}} = -1397.19 + 4785.74X_1 + 195.39X_2 - 8696.82X_1^2 - 7.81X_2^2 \quad (4)$$

where X_1 and X_2 are the dilution rates and fructose concentrations, respectively.

Regression analysis (Table 3) of the experimental data showed that dilution rates and fructose concentrations had positive linear effects on nisin productivity. Of the two factors tested, the high coefficient value for the dilution rate indicated that this variable has a higher impact on the

Table 3 Results of regression and ANOVA analysis for nisin productivity of LL27 and bioengineered strains

Term	LL27			LAC338			LAC339			LAC340		
	Coeff.	*T*	*P*	Coeff.	*T*	*P*	Coeff.	*T*	*P*	Coeff.	*T*	*P*
Constant	418.5	34.7	0.01	412.0	40.0	0.01	481.7	41.5	0.01	464.9	106.4	0.01
D	90.1	7.5	0.01	54.0	5.3	0.01	55.7	4.7	0.01	65.6	15.0	0.01
F	51.3	4.3	0.05	47.9	4.7	0.01	23.8	2.0	0.03	73.5	16.9	0.01
$D \times D$	−185.9	−10.2	0.01	−188.3	−12.2	0.01	−242.0	−13.6	0.01	−195.7	−29.7	0.01
$F \times F$	−137.2	−7.6	0.01	−116.6	−7.5	0.01	−140.7	−7.9	0.01	−109.9	−16.7	0.01
	$R^2 = 0.98$, $F = 49.1$			$R^2 = 0.98$, $F = 53.0$			$R^2 = 0.99$, $F = 55.7$			$R^2 = 0.99$, $F = 365.2$		

D Dilution, *F* fructose, $D \times D$ dilution × dilution, $F \times F$ fructose × fructose

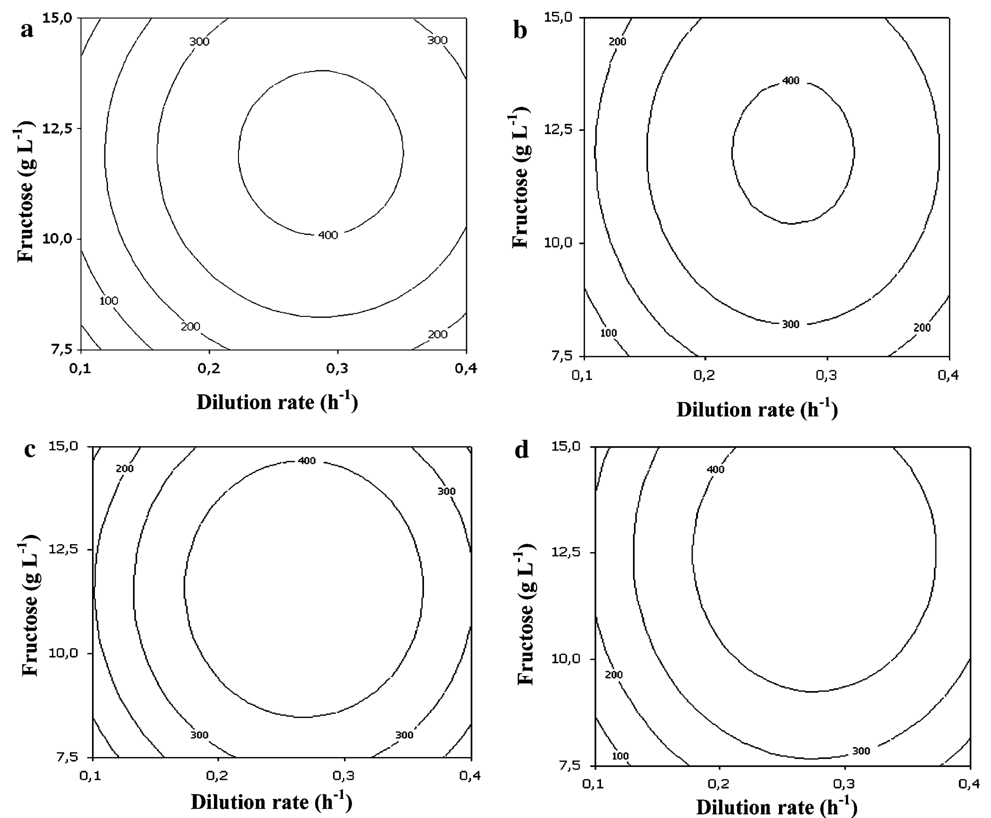

Fig. 2 Contour plots for nisin productivity of **a** LL27, **b** LAC338, **c** LAC339, and **d** LAC340 for paired dilution rates and fructose concentrations

nisin productivity of strains. Dilution rate and fructose concentration factors also showed significant negative quadratic effects on nisin productivity, indicating that nisin concentration increased as the level of these factors increased and decreased as their level increased above certain values. Interactions between dilution rates and fructose concentrations were not found to be significant. Therefore, this interaction coefficient was eliminated in the equations.

Figure 2a–d shows the contour plots of nisin productivity of the LL27 and recombinant strains for paired dilution rates and fructose concentrations. In this figure, the circles in the center denote the limit of the dilution rates and fructose concentrations for nisin productivity above 400 IU ml^{-1} h^{-1}. From the figure it is clear that nisin productivity levels above 400 IU ml^{-1} h^{-1} can be achieved at a wider range of dilution rates (0.18–0.35 h^{-1}) and fructose concentrations (8–15 g l^{-1}) with the strains LAC339 and LAC340 (Fig. 2c, d) than with LL27 and LAC338 (Fig. 2a, b). This indicates that additional regulation genes in the producer strain enable it to reach high productivity at a wide range of dilution rates and fructose concentrations.

In order to determine the maximum nisin concentration corresponding to the optimum level of dilution rates and fructose concentrations, second-order polynomial models were used to calculate the values of these variables. Fitting

of the experimental data to Eqs. 1–4 allowed determination of the levels of optimum dilution rates (0.29 h^{-1} for LL27, 0.27 h^{-1} for LAC338, 0.26 h^{-1} for LAC339, and 0.27 h^{-1} for LAC340) and fructose concentrations (11.95 g l^{-1} for LL27, 12.01 g l^{-1} for LAC338, 11.63 g l^{-1} for LAC339, and 12.50 g l^{-1} for LAC340). Furthermore, nisin productivities of LL27 and recombinant strains were determined as 434, 440, 496, and 483 IU ml^{-1} h^{-1}, respectively, under optimum conditions. These data supported the values obtained in continuous fermentation at different dilution rates and fructose concentrations.

Discussion

Production of bacteriocins using continuous fermentation technology is a relatively recent approach in bacteriocin research. Nisin production with different producer cells has been extensively reported in the literature [1, 5, 9, 16, 18, 19]. However, recombinant strains constructed for improving nisin production levels have not been used in continuous fermentation. In the current study, nisin formation was greatly influenced by the dilution rate of the medium. Nisin production of recombinant strains was found to be highest at a dilution rate between 0.2 and 0.3 h^{-1}, which is similar to results reported in previous studies using natural nisin producers [9, 16, 18, 19]. However, the maximum nisin activity in these studies could not be compared directly because of differences in carbon source and methodologies used for nisin bioassays.

In this study, low dilution rates ($D < 0.3$ h^{-1}) were found to be more efficient for nisin production for all recombinant strains. In particular, nisin productivity of all strains declined at dilution rates above 0.3 h^{-1} (Fig. 1a). There are two possibilities that could explain the differences in specific nisin productivity above and below the dilution rate of 0.3 h^{-1}. It has been emphasized that in continuous culture, specific production rates are related to the in vivo enzyme activities [5, 9, 18, 19]. Hence, loss of high specific nisin productivities in recombinant strains observed in the current study might be the result of insufficient post-translational enzyme activity owing to increased metabolic activity at higher dilution rates.

The other hypothesis to explain decreased specific nisin productivities for those strains could be the instabilities of recombinant plasmids. In fact, it has been shown that plasmid instability is a limiting factor during continuous cultures as observed frequently with recombinant cells [4, 11, 13]. Additionally, expression of a gene in recombinant cells was reported to be proportional to the plasmid copy number [21]. Huang et al. [11] showed that the decreased pediocin production in a free cell continuous fermentation system at high dilution rates apparently resulted from a corresponding decrease in plasmid copy number. These reductions in both plasmid stability and plasmid copy number in cells during continuous fermentation have been attributed to the compulsion of cells to divide at a high rate [4, 11, 13, 21]. Differences in specific nisin productivity in the current study could be particularly related to the number of the plasmid copy rather than to the plasmid instability, since the instabilities of the relevant plasmids at 0.4 and 0.5 h^{-1} dilution rates were lower than the instabilities reported by others [4, 11], although they were found to be significantly lower ($P < 0.05$) at these dilution rates than at lower dilutions rates. Separately, high stabilities of the plasmids incorporating immunity genes at tested dilution rates indicated that those genes have a considerable effect on increasing the stability compared to regulation genes.

Fructose was selected previously [23] for yielding high nisin productivity of wild-type LL27 and its recombinant strains. In the current study, at a 0.2 h^{-1} dilution rate, it was determined that strains produced their maximum nisin amount by consuming on average 4.48 g fructose per biomass unit per hour, which is in the range of data obtained using other carbon sources [16, 18, 22]. However, higher specific nisin productivities with the recombinant strains than wild-type LL27 for all tested fructose concentrations proved that, in continuous culture, fructose concentration had no effect on the nisin production of recombinant strains (Fig. 1b). In fact it is also evident that cell growth decreased at certain concentrations of fructose.

RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors, and searching optimum conditions for desirable responses. To date, several studies optimizing medium composition for nisin production in batch and fed-batch fermentation systems with this method have been reported [14, 15]. However, this is the first report that optimized and modelled nisin productivity of recombinant strains in a continuous culture with RSM. The model generated in this study satisfied all the necessary arguments for its use in optimization. By fitting the experimental data to a second-order polynomial equation, optimum levels of nisin productivity for the recombinant strains were determined. Contour plot figures of the model equations fitted to the data notably showed that high nisin productivity (>400 IU ml^{-1} h^{-1}) of the strains with additional regulation genes (LAC339 and LAC340) could be achieved at wide ranges of dilution rates and fructose concentrations (Fig. 2c, d). There is evidence that nisin regulates its own production by induction that specifically interacts with histidine kinase present in the cell wall [12, 20]. Therefore, nisin present in the fermentation environment triggers the regulation genes that enable high productivity at wide dilution rates and fructose concentrations. Thus, it was estimated that under the optimum conditions, LAC339

could produce 14% more nisin while using 3% less medium compared to the control strain LL27. These results suggest that over-expressing nisin regulation genes rather than immunity genes in the producer strain is beneficial for high nisin production in continuous fermentation. In contrast, immunity genes were found to be important in batch fermentation [23].

Conclusions

Nisin production of bioengineered strains incorporating additional immunity and regulation genes was determined in a continuous fermentation system for the first time in this study. Basically, results showed that nisin productivities could be improved further with continuous culture. Nevertheless, immobilization fermentation techniques should be attempted to obtain higher nisin productivity using the bioengineered strains as such fermentation could keep plasmids and strains highly stable [4, 11, 13]. Hence, high dilution rates would be possible for bioengineered strains that could proportionally increase nisin productivity.

Nisin producer strains incorporating additional regulation genes provided a significant contribution to the continuous fermentation. In particular, strictly stabilizing the dilution rate or fructose concentration might not be feasible at industrial scales. Therefore, over-expressing regulation genes could enable cells to be in a high nisin productivity state even with variations in dilution rate and fructose concentration. In this sense, strain LAC339 has great potential to be used in such systems.

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