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ORIGINAL PAPER

Influence of growth conditions on the nisin production of bioengineered *Lactococcus lactis* strains

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Abstract Nisin production of three bioengineered strains, (LAC338, LAC339 and LAC340) with immunity (*nisFEG*) and/or regulation (*nisRK*) genes of nisin biosynthesis on plasmids in the *Lactococcus lactis* LL27 nisin producer, was evaluated under pH-controlled and pH-uncontrolled batch fermentations. Optimization studies showed that fructose and yeast extract yielded the highest nisin activity. The strains LAC338, LAC339, and LAC340 produced 24, 45, and 44% more nisin, respectively, than wild-type *L. lactis* LL27 after 12-h incubation. However, sharp decreases in the yield of nisin were observed at the late phase of fermentation with LAC339 and LL27 in contrast to LAC340 and LAC338 strains for which the high level of nisin could be maintained longer. Obviously, increasing the copy number of the regulation genes together with immunity genes in the nisin producers retarded the loss of nisin in the late phase of the fermentation.

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Introduction

Nisin is an antimicrobial peptide which belongs to the type I lantibiotics and is produced by some strains of *Lactococcus lactis* and *Streptococcus uberis* [6, 28]. Nisin has strong antimicrobial activity against certain gram-positive bacteria. It occurs naturally in various dairy products and has been unwittingly consumed by humans without any ill effect. Nisin is widely used as a preservative in the food industry and has potential for use in health care products such as toothpaste and skin care products [10]. A cluster of 11 genes is involved in the complex biosynthesis of nisin and transcriptionally arranged in two nisin inducible *nisABTCIPRK* and *nisFEG* operons having, in addition, constitutive promoters in front of *nisI* and *nisR*. The gene cluster include the structural gene (*nisA,* or natural variants *nisZ*, *nisQ*, *bacF* and *nisU*); genes involved in post-translational modifications (*nisB, nisC*), transport (*nisT*) and extracellular precursor processing (*nisP*); genes encoding immunity to the producer strain (*nisI, nisFEG*); and the regulatory genes, which belong to the class of two-component auto-regulatory system consisting of a response regulator (*nisR*) and a histidine kinase (*nisK*) [14, 23]. Nisin induces via signal transduction through NisRK the expression of the biosynthesis genes and the immunity system in which NisI intercepts nisin and NisFEG is an ABC transporter that expels nisin into the medium [14, 21, 23]. In view of the widespread use of this bacteriocin, an important factor to consider for its application is the cost of nisin production. Therefore, many aspects have been studied extensively in order to improve the production rate and productivity [2, 6, 26].

Genetic strategies offer an alternative approach to enhance the production of nisin and other lantibiotics. Overexpression of the nisin immunity genes from plasmids transformed to the nisin producer improved the nisin resistance of the producer strain and lead to a slight improvement in the rate and level of nisin production [11]. Also in another study, provision of additional copies of the regulatory (*nisRK*) and immunity (*nisFEG*) genes with high copy number plasmid resulted in 1.5–1.7-fold greater yields of nisin [4]. In *Bacillus subtilis* ATCC 6633, additional copies of subtilin self-protection (immunity) genes *spaIFEG* have been integrated into the genome of the producer strain which enhanced the subtilin tolerance level and concomitantly, the subtilin yield [8]. Furthermore, the bioengineered derivatives of the lantibiotic lacticin 3147 were overproduced when additional copies of genes encoding the biosynthetic/production machinery and the regulator *ltnR* were present [5]. However, the enhancement of the bacteriocin productions has been identified in the overnight cultures in all of the studies mentioned above. Consequently, whether these genetic manipulations could be efficient in fermentation medium is far from clear.

It is well known that nisin production at fermentation systems is influenced by many factors such as type and the level of carbon, nitrogen and phosphate sources, pH and temperature [2, 6, 7, 15, 20]. Moreover, nisin production is also affected and limited by the challenging characteristics such as produced metabolites, adsorption of nisin onto the producer cells and enzymatic degradation by proteases [7]. On the other hand, nisin production abilities of the producer strains can also differ [12]. Therefore, to date nisin production ability of many *L. lactis* strains was enhanced with optimization under several environmental conditions [3, 6, 13, 15, 18].

The purpose of this study was to optimize the fermentation conditions and to evaluate the effect of increasing the copies of immunity and regulation genes on nisin production. Results showed that increasing the copies of immunity genes together with regulation genes has considerable advantage on the late phase of fermentation, whereas increasing regulation or immunity genes separately could not maintain high production ability in the strains.

Materials and methods

Bacterial strains, plasmids and growth conditions

Strains used in this study are described in Table 1. The plasmid vector used in the cloning experiments was pLEB124 [21] with an *erm* gene and P45 promoter followed by multiple restriction sites (Table 1). *E. coli* strains were grown in Luria-Bertani broth at 37°C with shaking whereas *L. lactis* strains were cultivated without aeration at 30°C in M17 broth containing 0.5% (w/v) glucose (GM17), sucrose (SM17) or fructose (FM17). When needed, media were supplemented with antibiotics in the following concentrations: Erythromycin 200 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *L. lactis.*

DNA and RNA manipulations

Plasmid DNA was isolated from *E. coli* TG1 using the EZNA spin plasmid preparation kit (Omega, USA) and from *L. lactis* strains as described by O'Sullivan and Klaenhammer [17]. Extraction of chromosomal DNA from *L. lactis* LL27 was performed with EZNA bacterial DNA extraction kit (Omega, USA). Restriction enzymes and other DNA modifying enzymes from various sources were used according to the manufacturer's recommendations. PCR fragments were purified with the EZNA purification kit (Omega, USA). Standard molecular cloning techniques were performed as described by Sambrook et al. [24]. Electro-transformation of *L. lactis* LL27 was performed according to Holo and Nes [9]. For isolation of *L. lactis* total RNA, EZNA RNeasy mini kit (Omega, USA) was used according to the manufacturer's procedure. The RT-PCR reactions were done according to the Ra and Saris [22] with using downstream and upstream primers given in Table 1.

Influence of culture media on nisin production

To test the effect of carbon sources on nisin production by *L. lactis* LL27 and bioengineered strains each carbon source (glucose, sucrose, lactose, fructose, and maltose) was added to M17 broth (Oxoid) at 0.5% level. To determine the influence of different nitrogen sources on nisin production, each nitrogen source (yeast extract, beef extract, tryptone, casein hydrolysate, malt extract and soy peptone) was added to a Basal Medium at 1.0% level. The Basal Medium (BM) (fructose, 5 g L^{-1}; ascorbic acid, 0.5 g L^{-1}; $MgSO_4$, 0.25 g L^{-1}; disodium hydrogen phosphate, 19 g L^{-1}) was based on M17 medium in which all nitrogen sources (soytone, 5 g L^{-1}; beef extract, 5 g L^{-1}; yeast extract, 2.5 g L^{-1}; tryptone, 5 g L^{-1}) were eliminated. In experiments to determine the influence of phosphate concentration, different concentrations of disodium hydrogen phosphate (Merck) were used in the modified Basal Medium (mBM), which was including 10 g L^{-1} yeast extract additionally to BM composition. Each trial was carried out in flask culture at 30°C for 12 h with 100-rpm agitation.

Table 1 Strains, plasmids and oligonucleotide primers used in this study

Strains, plasmids or primers	Relevant properties	Reference
Strains		
Escherichia coli TG1	Transformation host	[24]
Lactococcus lactis LL27	Wild-type nisin A producer	[1]
L. lactis LAC338	LL27 carrying plasmid pLEB672, Erm^r	This study
L. lactis LAC339	LL27 carrying plasmid pLEB673, Erm^r	This study
L. lactis LAC340	LL27 carrying plasmid pLEB674, Erm^r	This study
Micrococcus luteus NCIBM8166	Nisin sensitive indicator	[26]
Plasmids		
pLEB124	4.5 kb, gram-positive cloning vector, Erm^r	[21]
pLEB672	6.5 kb, Erm^r, including *nisFEG* genes	This study
pLEB673	6.2 kb, Erm^r, including *nisRK* genes	This study
pLEB674	9.6 kb, Erm^r, including *nisRKFEG* genes	This study
Primers		
NisF-fw- *Hin*dIII	AGATACAAGCTTGGGCCCTAAAGTGAGGAAATATAATGCAGGTA	This study
NisG-rw-*Sal*I	AGATTCGTCGACTTCCCGGGAGGTTAAAATGCACTTTATATGTCTATC	This study
NisR-fw-*Hin*dIII	TATCATAAGCTTAATCGGAGGTAAAGTGGTGTATA	This study
NisK-rw-*Apa*I	AGATAGGGCCCTTCAGAAACAAAAAAAGTAATCCTTAGA	This study
NisG-rw-*Apa*I	AGATTCGGGCCCAGGTTAAAATGCACTTTATATGTCTATC	This study
P45-RT-PCR-Up	TAGAATAGTGAAAAAAATTAACTTAAGT	This study
NisR-RT-PCR-Down	AATATAGTCATCCCCACCAATA	This study
NisF-RT-PCR-Down	AATATAGTCATCCCCACCAATA	This study

Primers were designed from the sequences with the GeneBank accession numbers X68307 and U17255. Restriction enzyme sites used for cloning of the amplified fragments are underlined

Effect of temperature and pH on nisin production

The temperature effect on nisin production was determined in flasks containing mBM, (fructose, 5 g L^{-1}; yeast extract, 10 g L^{-1}; ascorbic acid, 0.5 g L^{-1}; $MgSO_4$, 0.25 g L^{-1}; disodium hydrogen phosphate, 19 g L^{-1}) which was maintained at different temperatures (25, 30 and 37°C) after 12 h. The influence of pH on the level of nisin production was studied in a 3-L fermentor (Minifors, Switzerland). An overnight culture was inoculated at 1% level into 1.5 L of mBM. The fermentor was operated with slow agitation (100 rpm) at 30°C without aeration. The pH of the culture broth was adjusted initially to predetermined values (pH 5.5, 6.0, 6.5) and maintained constantly during fermentation by automatic addition of 5-M NaOH solution. The 20 ml of samples were aseptically withdrawn from the fermentor at 1-h intervals and tested for cell density, pH, and nisin activity. All cultivations were performed in triplicate.

Nisin production at pH-controlled batch fermentation

A 1.5-L (working volume) stirred bioreactor (Minifors, Switzerland) equipped with instrumentation for measurement and control of temperature (30°C), pH (6.0, by addition of NaOH) and agitation speed (100 rpm) was used in batch fermentations. The fermenter was filled to 1.5 L with the fermentation medium (FM) (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}; disodium hydrogen phosphate, 25 g L^{-1}) and inoculated with 1% of a 16-h *L. lactis* cultures grown in FM at 30°C. At each interval 20-ml samples were withdrawn from the fermentor to determine the nisin activity, cell mass, and consumed fructose amount.

The stability of the recombinant plasmids during the fermentation was analyzed by measuring the proportion of erythromycin colonies obtained from samples. Twenty colonies from the FM17 agar plates of each sample were inoculated onto FM17 agar containing also 5 μg ml^{-1} erythromycin. The stability of recombinant plasmids was calculated by comparing the number of cells growing on the erythromycin including FM17 agar plates to the number of colonies initially inoculated.

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 blank. 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; the supernatant was removed and 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 weighted and the CDW calculated.

Nisin titer was measured by the method of Tramer and Fowler [27]. 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* NCIBM 8166. A standard curve (50–600 International Units 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 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 pH-controlled batch fermentation.

Statistical analysis

Student's *t* test and one-way analysis of variance (ANOVA) along with the Tukey and Hsu MCB (multiple comparisons with the best) comparisons were carried out using the MINITAB 14.0 (Minitab Inc. State College, PA) to determine the differences of the nisin production of each bioengineered and control strains.

Results and discussion

Generation of strains with increased nisin regulation and immunity genes

Three different recombinant strains were generated harboring not only immunity and regulation genes independently, but also immunity and regulation genes together, to enhance the ability of nisin production of *L. lactis* LL27. Immunity (*nisFEG*), regulation (*nisRK*) and immunity/regulation (*nisRKFEG*) fragments which were 2.5, 2.3 and 5.1 kb in size, respectively, were amplified with the primers pairs *NisF-fw-Hin*dIII/*NisG-rw-Sal*I, *NisR-fw-Hin*d*III/NisK-rw-ApaI* and *NisR-fw-Hin*d*III/NisG-rw-ApaI*, respectively (Table 1), and cloned downstream of the constitutive lactococcal promoter P45 present in the shuttle vector pLEB124. *NisFEG* and *nisRK* genes were cloned without the *nisF* and *nisR* promoters. However, the fragment containing *nisRKFEG* had the native *nisF* promoter between the *nisRK* and *nisFEG* genes. The resulting plasmids pLEB672, pLEB673 and pLEB674 were transformed into *L. lactis* LL27 yielding strains LAC338, LAC339, and LAC340. Comparison of the plasmid profile of these strains with LL27 showed that they had an extra plasmid with a size corresponding to the transformed (Fig. 1a). Successful amplification by RT-PCR using transcripts from strains

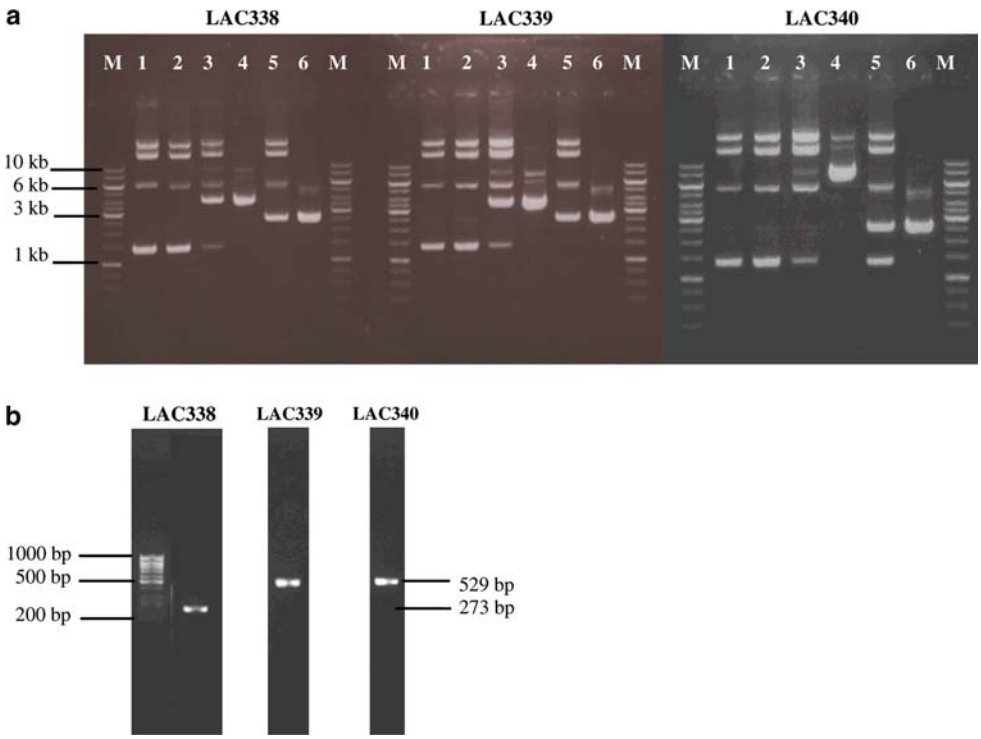

Fig 1 The plasmid profiles (**a**) and RT-PCR analysis of transcripts initiated from P45 promoter (**b**) of *L. lactis* LL27 and bioengineered strains. *1 L. lactis* LL27 wild type, *2* empty electroporated *L. lactis* LL27, *3* bioengineered strain, *4* purified recombinant plasmid, *5 L. lactis* LAC341, *6* vector plasmid pLEB124, *M* 1-kb molecular marker (Fermentas). The transcripts, involving transcription region of P45 promoter and the part of the following cloned first gene (*nisF* or *nisR*) were targeted in the RT-PCR. Negative control LL27 did not give any relevant band at the expected sizes

LAC338, LAC339, and LAC340 as template and a primer pair (one in P45 and the other in the target gene) showed that the *nisRK*, *nisFEG*, and *nisRKFEG* genes in the plasmids were successfully transcribed excluding the *nisFEG* genes in pLEB674 due to the native *nisF* promoter located downstream of the *nisK* terminator (Fig. 1b).

Strains LL27 and LAC341 produced 520 (±114) IU ml^{-1}, whereas LAC338, LAC339 and LAC340 produced 650 (±78), 750 (±72) and 745 (±89) IU ml^{-1} nisin per CDW (mg ml^{-1}), respectively, suggesting that additional *nisRK*, *nisFEG* and *nisRKFEG* transcription from the plasmids improved significantly ($P < 0.01$) the nisin production ability of wild-type LL27 by 20, 44, and 43%. This observation correlates with recent results showing that increased expression of lantibiotic immunity and regulation genes is accompanied with increased lantibiotic productivity [4, 5, 8, 11]. However, there are no studies showing results on lantibiotic production when expression of regulation and immunity genes are increased together in the host nisin producer. In this respect LAC340 is novel, as it most probably overexpresses the *nisRK* and *nisFEG* genes due to an increasing copy number of these regulation and immunity genes.

The effect of culture media on nisin production of LL27 and bioengineered strains

Maximum nisin production was obtained by using fructose followed by sucrose, glucose, maltose, and lactose, respectively (Fig. 2a). Increasing the copy number of immunity and regulation genes in the producer strains did not affect the sugar fermentation ability. For all bioengineered strains, lactose yielded the smallest increase in nisin production compared to the other carbon sources. Carbon source selection has been reported as critical control step in nisin production because of its effects on the cell growth and nisin biosynthesis [3, 6, 15]. For example, sucrose and lactose were determined as an efficient carbon source for nisin production in strains *L. lactis* NIZO 22186 [20], *L. lactis* ATCC11454 [19] and *L. lactis* A164 [3], while xylose was the most efficient carbon source in strain *L. lactis* IO-1 [15]. Our results indicated that fructose is the most efficient carbon source in nisin production of LL27.

Different concentrations of fructose (0.5, 1, 1.5, 2, and 3%) were used to determine which fructose concentration yields the highest concentration of nisin at the end of the fermentation. One percent of fructose resulted in the highest nisin production ($P < 0.05$) in all strains and slight decreases were obtained above this concentration (Table 2a). The cell mass of strains at 1% of fructose concentration was higher than any of the other concentrations. The low pH of the spent medium (in average pH 4.25) for all strains indicated that reduction in nisin production is probably because

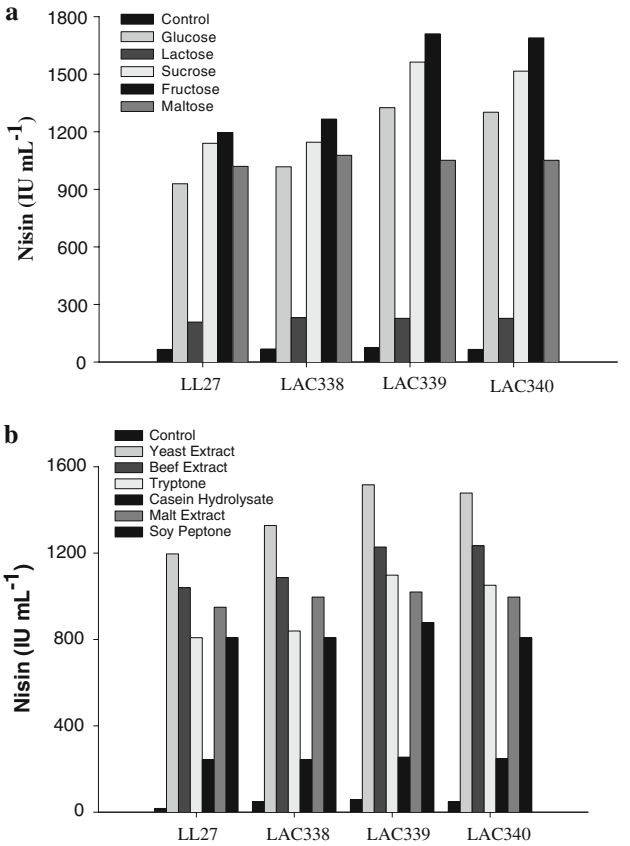

Fig 2 Effect of different carbon (**a**) and nitrogen sources (**b**) on nisin production by *L. lactis* LL27 and bioengineered strains. LAC338: extra *nisFEG* genes on plasmid, LAC339: extra *nisRK* genes on plasmid, LAC340: extra *nisRKFEG* genes on plasmid. M17 and BM were used as control mediums. Data are means of triplicates. Standard errors were less than 5.0% of the means

of end-product inhibition of lactic acid. In addition, different high initial sugar concentrations, e.g., more than 1% sucrose and 3% glucose, at the uncontrolled fermentations have been reported to cause catabolic suppression leading to lower growth and nisin production [6, 15]. The limiting effect of fructose on nisin production and cell growth at lower concentrations than glucose can be attributed to carbon type and strain specificity.

The maximum nisin activity for all strains was obtained by using yeast extract but all strains also produced nisin efficiently from beef extract. However, casein hydrolysate resulted in the lowest nisin activity among the tested nitrogen sources. Although recombinant strains produced more nisin than wild-type LL27 in presence of all nitrogen sources except casein hydrolysate and soy peptone, the highest increase in nisin production was obtained with yeast extract indicating that yeast extract is sufficient as a single nitrogen source for the recombinant strains (Fig. 2b). Nisin producer *L. lactis* strains require many organic and inorganic medium components to grow and to produce nisin. Among the

organic nitrogen sources, most likely yeast extract, beef extract and tryptone have been considered to be of vital importance because of their stimulation of cell growth [7].

The concentration of yeast extract induced the cell mass and nisin production of all tested strains. The biomass of wild-type LL27 and recombinant strains increased with additional yeast extract. For all of the yeast extract concentrations, LAC340 had higher cell density than the strains LAC338 and LAC339 (Table 2b). Nisin activities of all strains increased as a function of yeast extract, but after reaching the maximum activity additional yeast extract did not result in considerable changes in the nisin production. Therefore, while maximum nisin production titer was at 3% of yeast concentration for LL27 and LAC338 (1,480 and 1,550 IU ml^{-1}), LAC339 and LAC340 produced maximum nisin (1,900 IU ml^{-1}) at 5 and 4%, respectively (Table 2b).

For all of the strains, increasing initial phosphate concentration from 1.9 to 3%, increased the cell growth and nisin production rates. Higher initial concentrations of this phosphate source drastically decreased both biomass and produced nisin levels. The maximum nisin activity was obtained at 2.5% of phosphate concentration for LL27, LAC339, LAC340 and 3% for LAC338 which was not found significantly different ($P > 0.05$) (Table 2c). These results clearly indicate that cell yield and nisin production levels are stimulated by added phosphate. However, differences in nisin production for all used phosphate concentrations could not be detected between recombinant strains involving additional immunity and regulation genes suggesting that phosphate might not have any regulatory effect on nisin biosynthesis. On the other hand, it has been suggested that the role of phosphate in the nisin fermentation is due to its buffering capacity and stimulating formation of ATP leading to high energy charge of the cells [7]. However, phosphate could also have negative effects depending on the counter ion present in the medium which may cause cell lysis at higher concentrations [3, 7, 15].

Table 2 Effect of different fructose (a) yeast extract (b) and phosphate (c) concentrations on nisin production of *L. lactis* LL27 and bioengineered strains

%	Strains											
	LL27			LAC338			LAC339			LAC340		
	pH	CDW (mg m^{-1})	Nisin (IU ml^{-1})	pH	CDW (mg ml^{-1})	Nisin (IU ml^{-1})	pH	CDW (mg ml^{-1})	Nisin (IU ml^{-1})	pH	CDW (mg ml^{-1})	Nisin (IU ml^{-1})
a												
M17	6.65	0.20	70a	6.67	0.19	70a	6.65	0.19	80a	6.65	0.19	80a
0.5	5.22	1.86	1,170b	5.27	1.80	1,290b	5.23	1.80	1,520b	5.22	1.79	1,480b
1	4.82	2.30	1,330c	4.87	2.30	1,480c	4.85	2.32	1,900c	4.85	2.31	1,620c
1.5	4.25	2.28	1,330c	4.26	2.26	1,480c	4.26	2.26	1,850c	4.25	2.30	1,550b
2	4.24	2.28	1,330c	4.26	2.25	1,330b	4.27	2.23	1,760d	4.26	2.24	1,550b
3	4.25	2.25	1,270b	4.27	2.23	1,330b	4.28	2.22	1,160e	4.28	2.21	1,550b
b												
BM	6.64	0.05	50a	6.61	0.08	50a	6.62	0.07	50a	6.61	0.07	50a
1	5.29	1.81	1,190b	5.29	1.74	1,290b	5.29	1.75	1,520b	5.31	1.78	1,460b
2	5.12	2.01	1,330c	5.14	1.98	1,480c	5.13	2.02	1,760c	5.13	2.00	1,620c
3	5.06	2.19	1,480d	5.08	2.10	1,550c	5.07	2.15	1,850c	5.06	2.15	1,620c
4	5.02	2.21	1,480d	5.03	2.11	1,480c	5.02	2.15	1,850c	5.03	2.20	1,900d
5	4.98	2.22	1,390d	4.99	2.15	1,550c	4.99	2.17	1,900c	4.98	2.23	1,850d
6	4.96	2.22	1,480d	4.97	2.20	1,550c	4.97	2.20	1,900c	4.97	2.23	1,850d
c												
mBM	5.31	1.81	1,190a	5.35	1.73	1,290a	5.29	1.75	1,520a	5.31	1.76	1,480a
2.5	5.77	1.87	1,270b	5.78	1.76	1,290b	5.78	1.77	1,740b	5.77	1.78	1,620b
3	5.79	1.93	1,270b	5.79	1.86	1,380b	5.78	1.86	1,620b	5.77	1.86	1,620b
3.5	6.02	1.33	330c	6.04	1.11	360c	6.04	1.16	400c	6.03	1.16	600c
4	6.14	1.02	330c	6.14	0.93	360c	6.14	0.97	400c	6.12	0.97	400d
4.5	6.22	0.76	110d	6.23	0.65	150d	6.23	0.77	200d	6.23	0.76	360e
5	6.28	0.74	110d	6.32	0.63	150d	6.30	0.76	200d	6.32	0.76	200e

M17, BM, and mBM were used as controls. Data are means of triplicates. Different letters shown in the nisin production columns represent the statistical significance ($P < 0.05$). Standard errors were less than 5.0% of the means

The effect of cultivation temperatures on nisin production of LL27 and bioengineered strains

The wild type and the recombinant strains showed similar behaviors in terms of cell growth at each of the tested temperatures. Cell growth of all strains was lower at 25 than at 30 and 37°C in which cells were able to grow to the same level. However, the nisin activities obtained at 30°C were higher ($P < 0.05$) than those at other temperatures (data not shown) explaining why 30°C was chosen as cultivation temperature for all other fermentations.

The effect of pH on the nisin production of LL27 and bioengineered strains

To determine the optimal pH for optimal production of nisin at LL27 and recombinant strains, pH-controlled fermentation was carried out at various pH values. Specific nisin production of all strains was found similar until the 12th hour of fermentation ($P < 0.05$). However, at the late phase of the fermentation involving 18th and 24th hours, significantly higher specific nisin production was determined at pH 6.0, where pH 5.5 yielded lower specific nisin production than other two values (Fig 3a–d) ($P < 0.05$). Additionally, high specific production was possible to be maintained longer at pH 6.0 compared to pH 6.5 and 5.5. All these results indicated that the optimal pH for nisin production for all of the bioengineered strains is pH 6.0, which is also optimal for LL27. Interestingly, the bioengineered strains involving extra *nisFEG* genes showed less decrease of specific nisin production during the last 15 h at all tested pH values suggesting that the adhered nisin molecules on the cells were potentially released by the high activity of NisFEG proteins in those strains (Fig. 3b, d). The optimal pH for nisin production has previously been found to vary between pH 5.5 and 6.8 depending on the strains of nisin producer and culture media [15, 16]. Nisin Z production with *L. lactis* IO-1 was optimal at pH 6.0 in

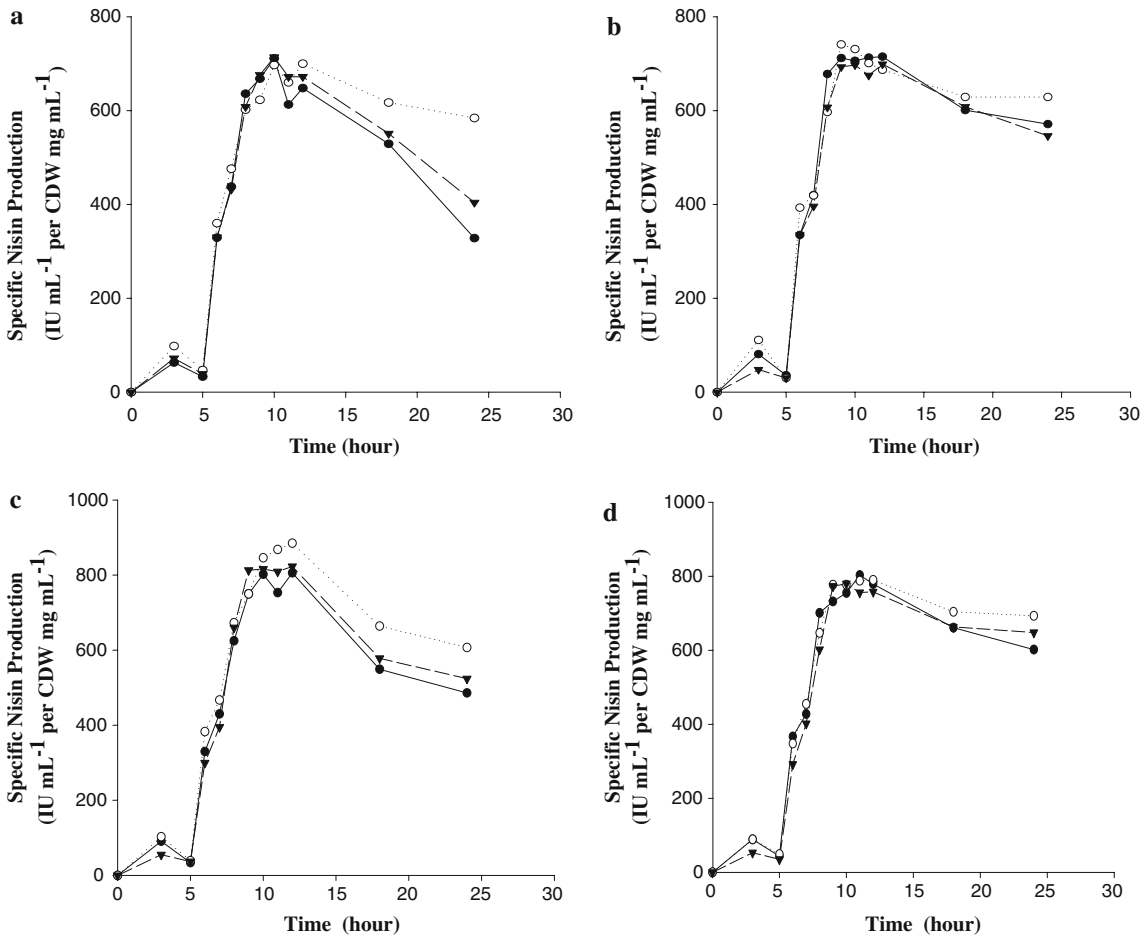

Fig. 3 Specific nisin production of *L. lactis* LL27 and bioengineered strains in mBM controlled at different pH values. **a** *L. lactis* LL27, **b** *L. lactis* LAC338, **c** *L. lactis* LAC339, **d** *L. lactis* LA C340. *Line with filled circles* pH 5.5, *line with open circles* pH 6.0, *line with filled triangles* pH 6.5. Standard errors were less than 5.0% of the mean

xylose media but at pH 5.5 in glucose media [15]. In this study, pH 6.0 was found optimal for nisin production of wild-type strain LL27 and bioengineered strains.

Nisin production of bioengineered strains at pH-controlled batch fermentation

Since the efficient type and level of carbon, nitrogen, phosphate and incubation temperature and pH values were determined for each strain, a pH-controlled batch fermentations were carried out with optimum parameters without erythromycin, in order to improve nisin production and to observe the efficiency of the bioengineered strains. Figure 4 presents the nisin batch fermentation profile of LL27 and bioengineered strains. Exponential growth took place during a period of about 3–6 h for all of the strains. Maximum cell density was determined after 8 h for all strains except LAC338 which reached its maximum at 10 h. At the remaining of fermentation after initial exponential phase, a linear cell growth was observed in the strains LL27, LAC338 and LAC340, whereas a slight decrease was determined for LAC339. Ninety percent of initial fructose concentration (10 g l^{-1}) was consumed by both strains after 10 h of fermentation and it was lower than the detectable value at 12 h (Fig. 4). Nisin production appeared to parallel that of cell growth profile. Maximum nisin activity was reached after 9 h for all strains except LAC338 which was parallel to cell growth. LAC340 including additional immunity and regulation genes together had the highest nisin activity (2,880 IU ml^{-1}) which was followed by LAC339 (2,730 IU ml^{-1}). LAC338 showed negligible increase in nisin production when compared with wild-type LL27 (Fig. 4). After reaching the maximum activity peak value, the flat maximum was maintained about 4 or 5 h and then sharp decreases occurred with LAC339 and LL27 lowering the nisin titre. When LAC340 and LAC338 were used in the batch fermentation system, the level of nisin maintained high up to 24 h and thereafter gradually decreased. In earlier studies, it has been reported that nisin is produced during the growth phase and a more or less sharp decrease occurs in activity at the end of growth [3, 6, 26]. The high nisin activity of LAC340 did not decrease sharply indicating that increasing the regulation and immunity genes together prevented the dramatic decrease in nisin activity which occurred with the other strains. It is likely that LanFEG

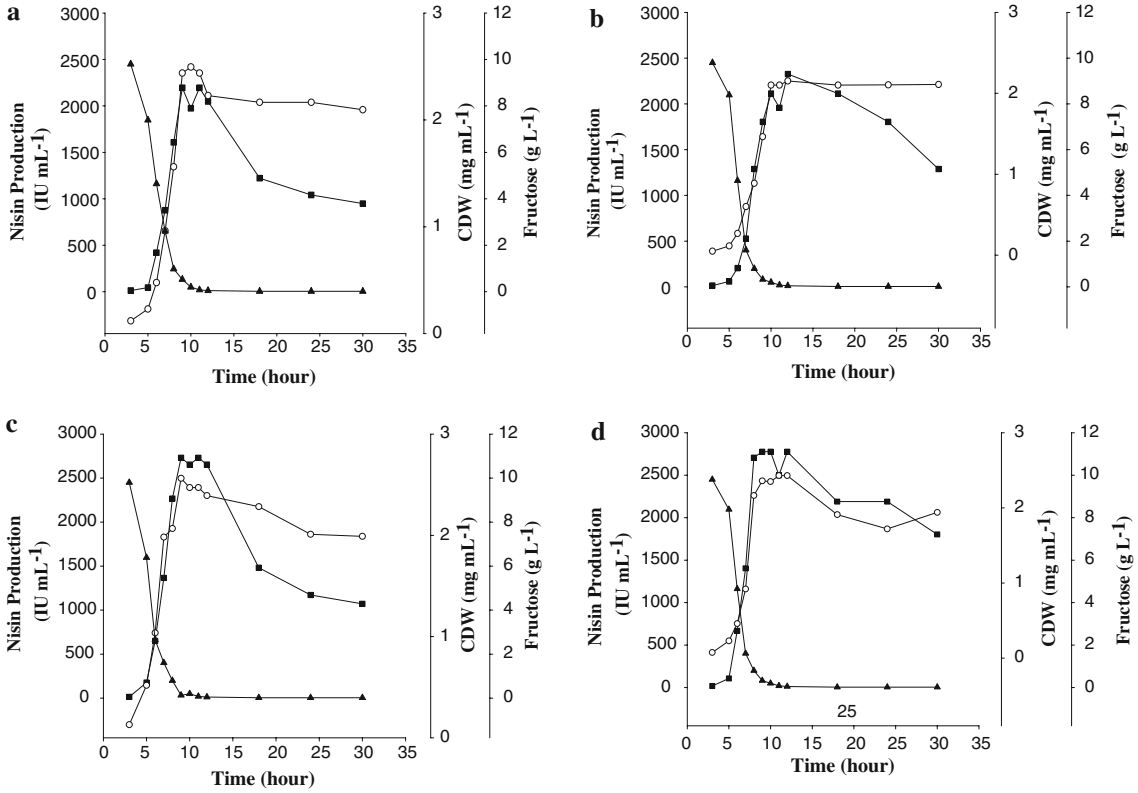

Fig 4 pH-controlled batch fermentation of *L. lactis* LL27 and bioengineered strains in FM. **a** *L. lactis* LL27, **b** *L. lactis* LAC338, **c** *L. lactis* LAC339, **d** *L. lactis* LAC340. *Line with filled squares* nisin activity (IU ml^{-1}), *line with open circles* cell dry weight (CDW) (mg ml^{-1}), *line with filled triangle* residual fructose (g l^{-1}). Data points are means of triplicates. Standard errors were less than 5.0% of the mean

decreases the quantity of cell-associated lantibiotic molecules by their extrusion from the membrane into the extracellular space [25], which would lead to an increase of the extracellular lantibiotic concentration. In addition, the potentially higher expression level of the NisFEG proteins in the LAC340 strain increased the resistance level to nisin (results not showed). This may lead to decreased cell lysis resulting in less release of intracellular peptidases/proteases and nisin degradation and therefore retarded loss of nisin with LAC340. All of the bioengineered strains produced more nisin than control strain LL27 although the selection marker of the recombinant plasmids erythromycin was not used. Results showed that all recombinant plasmids were stable from the beginning to end of fermentations (<8% lost the plasmids, data not shown).

Conclusions

In this study nisin production of three strains, overproducing the immunity genes *nisFEG*, the two-component regulatory genes *nisRK* or all of them, was studied and optimized. Strain LAC340 (overexpression of the *nisRK* and the *nisFEG* operon) improved nisin production ability almost to the same level as LAC339 (overexpression of the *nisRK* genes) However, LAC340 has significant advantage in the fermentation system because it is able to maintain high nisin activity in the late phase of the fermentation. Therefore, *L. lactis* LAC340 may be a candidate for use in industrial fermentation to achieve high nisin titers for non-food usages. In addition, this strain can efficiently produce nisin using fructose as carbon source. Fructose has not been previously described as optimal for any nisin producer. Fructose is one of the main carbon sources of fruit industry byproduct which is expected to be a useful carbon source for various kinds of fermentation. Therefore, using low cost raw materials including significant amount of fructose could reduce the cost of nisin production.

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