Innovative approaches to nisin production

Burcu Özel[1] , Ömer Şimşek[2], Mustafa Akçelik[3], Per E. J. Saris[4]

[1]: University of Pamukkale, Çal Vocational High School, Department of Food Processing, Denizli, Turkey

[2]: University of Pamukkale, Department of Food Engineering, Denizli, Turkey

[3]: University of Ankara, Department of Biology, Ankara, Turkey

[4]: University of Helsinki, Department of Microbiology, Helsinki, Finland

Corresponding Author:

Ömer Şimşek, PhD

University of Pamukkale, Department of Food Engineering

Denizli, Turkey, 20017

Tel: +90 258 296 30 15

Fax: +90 258 296 32 62

Email: omers@pau.edu.tr

Abstract

Nisin is a bacteriocin which is produced by *Lactococcus lactis* and approved by FDA to be utilized as GRAS status food additive. Nisin has antimicrobial activity against *Listeria*, *Clostridium, Staphylococcus* and *Bacillus* species or spores. Also it some circumtances, it has an immune modulator role and a selective cytotoxic effect against cancer cells. However, it is notable that the high production cost of nisin is an important issue which restricts its intensively use. The major reason is the low nisin production yield of producer strains. In recent years, the production of nisin has been significantly improved by genetic modifications in nisin producer strains or also innovative applications in fermentation conditions. Recently, 15400 IU/mL nisin production has been achieved in *L. lactis* cells by genetic modifications with eliminating the factors that affect nisin biosynthesis or by increasing the density of the producing strains in the fermentation medium. In this review, the innovative approaches, which were applied in cell and fermentation systems where nisin production is increased, are comparatively discussed and interpreted regarding developing industrial nisin production.

Keywords: Nisin, innovative system, fermentation, bacteriocin

1. Introduction

Nisin is a bacteriocin of the type I lantibiotic group, which is produced by *Lactococcus lactis* (Hurst, 1981). This bacteriocin has a wide antimicrobial activity against the spores of Gram-positive bacteria, as well as especially the food pathogens *Listeria monocytogenes*, *Staphylococcus aureus, Clostridium* and *Bacillus* species. Nisin can show an inhibition effect on Gram-negative pathogen species (such as *Escherichia coli* and *Salmonella*) when used together with applications that disrupt the cell wall such as EDTA, thermal treatment and freezing (Belfiore et al. 2007). Nisin is a cationic, hydrophobic, heat-resistant peptide with a

molecular weight of 3500 Da. In the structure of this molecule, there are the dehydroalanine (DHA), dehydrobutyrine (DHB), lanthionine and β–methylantionine amino acids that are scarce in nature and that are connected to each other by thioether bridges. To date, five variants have been characterized as follows: nisin A (Gross and Morell 1971), nisin Z (Graeffe et al. 1991; Mulders et al. 1991), nisin Q (Zendo et al. 2003), nisin U (Wirawan et al. 2006) and nisin F (Kwaadsteniet et al. 2008). Nisin A, Z and U producers have been isolated from milk and dairy products (Gross and Morell 1971, Graeffe et al. 1991; Mulders et al. 1991), nisin Q producer from river water (Zendo et al. 2003) and nisin F producer from catfish (Kwaadsteniet et al. 2008). Of these variants, only nisin U is produced by a bacteria (*Streptococcus uberis*) other than the *L. lactis* strains.

Nisin, as it has a wide antimicrobial spectrum and as it is decomposed by the digestive enzymes, has been defined and documented (E234) by the FDA (Food and Drug Administration) as a GRAS agent (Generally Recognized As Safe) and has been allowed to be used in food systems (Luck, 1995). Nisin was used in the 1950s to solve the problem arose from *Clostridium tyrobutyricum* in cheese. Currently, commercial nisin preparations are being produced in powder form of 2,5% purity and having $40x10^6$ IU (International Unite) biological activity in one gram (Tramer and Fowler, 1964). Today, nisin is used in the production of various cheese, ready-made soups and canned foods.

The current industrial nisin production is carried out in batch fermentation systems by growing the nisin A producing *L. lactis* cells in supplemented whey or milk medium, and the product is subsequently partially purified. However, the high cost of industrial nisin production is an important issue that restricts widespread use of this bacteriocin in food systems due to the low production yield of the producer cells, the feedback inhibition factors depending on fermentation conditions and the sensitivity of the producers to nisin. Therefore many studies

have focused on the elimination of the factors that restrict the amount of production since the high commercial value of nisin. These studies have demonstrated that high amount of nisin production is closely related with the biomass amount as well as the genetic properties of the producer cells, the fermentation conditions and the fermentation metabolites which cause feedback inhibitions. In this respect, many innovative systems have been developed by the researchers to eliminate these restrictions such as, metabolic regulation at producers aiming the increase biomass and relatively nisin, high producer construct which could overcome the restrictions and fermentation optimization in term of pH, temperature, substrate and dissolved oxygen concentrations (Kong and Lu, 2014; Simsek, 2014; Zhu et al. 2015; Papagianni ve Avramidis, 2012; Hao et al. 2017; Ni et al. 2017; Liu et al. 2017; Zhang et al. 2014; Zhang et al. 2016; Kordikanlioglu et al. 2015; Zheng et al. 2015; Jiang et al. 2015; Ariana ve Hamedi, 2017).

In this review, the innovative approaches applied in cell and fermentation systems where nisin production was increased, were comparatively discussed and were interpreted concerning development of industrial nisin production. Firstly, the current commercial nisin production was evaluated, then the studies aiming for increasing the nisin production in cells and the applications in fermentation conditions were subsequently disscussed. Thus, a perspective to improve industrial nisin production was tried to be established in this paper.

2. Commerical Production of Nisin

Commercial nisin has been produced in batch fermentation systems at industrial scale. Whole milk or skim milk is sterilized after the casein or para-casein fractions separated by enzymatically or acidification and subsequently the whey is used as a substrate to grow the producer *L. lactis* in large scale reactor systems. The fermentation conditions were kept under

promoting conditions whereas the pH is 6.0 and temperature is 30ºC. In the optimum production conditions, nisin producer *L. lactis* cells show common bacterial growth curve where the highest nisin activity is measured after 8 h and reached maximum at 12 h of fermentation. However, suprisingly nisin activity dramatically decreased at the following hours of fermentation. Figure 1 shows the repsentative cell growth and relavant nisin production versus to time scale. After fermentation, the foaming procedure is applied to the fermentate to separate and partially to concentrate the produced nisin. Firstly, the fermentate pH is reduced to 4.50 that enables the precipitation of milk protein such as casein and serum proteins. Then, the fluid is taken into the system which contains circulating vertical tubes. To ensure that the nisin-containing liquid is foamed, 0.1% Tween is added; ventilating from the bottom, the foam formed at the top is collected. At the last stage, sodium chloride and acetone are used to separate nisin and the precipitate is dried. Thus, commercial nisin preparation that has a purity of 2.5% and an activity of $40x10^6$ IU in 1 gram is obtained (Patent, US2935503 A).

The main aim at nisin production is to increase the amount of active producing cells in fermentation to achieve high nisin production yield. On the other hand, at nisin production, the lactic acid produced by the *L. lactis* resulted feedback inhibition on the producer cells. Accumulation of the lactate concentration in the medium accelerates protein denaturation in the cells, it also causes the *L. lactis* cells to spend more energy to be able to tolerate the unfavourable fermentation condition. These adversities lead to the disruption of active nisin production phase in the *L. lactis* cells and even to a decrease in cell density. Another factor that inhibits nisin production is a high concentration of nisin produced in the fermentation. The high amount of nisin that is produced by *L. lactis* cells in turns inhbited the itself, although these producer cells have a limiting or resistance level against nisin concentration (Kim et al. 1998; Ra et al. 1996; Kim et al. 1998).

3. Innovative Nisin Production Systems

The innovative studies, such as constructing producer strains giving high yield through genetic manipulations in the producing cells to minimizing the factors affecting nisin production and re-routing the metabolic pathways in producing cells or ensuring special fermentation conditions are the main targets of the studies which aim increasing the nisin production. The innovative approaches applied have provided higher nisin production at various levels. In Table 1 recently high nisin production innovative sytems are listed.

3.1. Recombinant Nisin Producers

Characterization the molecular mechanism of nisin biosynthesis and metabolic regulation on species level have opened the way to genetic regulation efforts to increase the yield in nisin production. Within this context, the genetic manipulations have focused on the problems that restrict nisin production, including i) the high nisin concentration in the medium, ii) high lactate accumulation and iii) low biomass formation.

The active nisin production is coded by the 14 kb site of the conjugative nisin-saccharose transposon that carries 11 genes (*nisA/Z/Q BTCIPRKFEG*). This gene cluster includes two operons (*nisA/Z/Q BTCIPRK* and *nisFEG*) and is transcribed as three different mRNA molecules (*nisA/Z/Q, nisBTCIPRK* and *nisFEG*). Of the genes in this site, *nisA/Z/Q* encodes for the synthesis of the pre-peptide*; nisB,C* encodes for the modification of the pre-peptide after translation; *nisP* encodes for the protease that allows the formation of the nisin peptide from pre-nisin; *nisT* encodes for the transfer of the prenisin molecule; *nisF,E,G* encodes for the resistance of the producing cell to nisin; *nisR,K* encodes for the transcriptional regulation of nisin production. The regulation of nisin production is ensured by a dual regulation system comprising histidine kinase (NisK) and its regulator (NisR) and nisin, in turn, induces the

136 transcription of the biosynthesis genes (Engelke et al., 1992; Kuipers et at., 1995; Ra et al.,
137 1996).

138 Increasing the copy number of the key genes in nisin biosynthesis directly contributed to the
139 increase of nisin production. Likewise, in the studies, increasing the copy number of the
140 regulation and resistance genes (*nisRK*, *nisFEG*) involved in nisin biosynthesis in producing
141 cells significantly improved nisin production (Kim et al., 1998; Cheigh et al. 2002; Simsek et
142 al., 2009a,b; Ni et al. 2017). Although nisin is functional in its own biosynthesis as a regulator,
143 nisin producing *L. lactis* cells are adeversely affected by nisin. Particularly in the later times of
144 fermentation, high nisin accumulation in the growing medium causes disruption on the cell
145 membranes of the producer *L. lactis* strains. Although various proteins have been produced in
146 *L. lactis* cells against the nisin are produced, the producers have different level of resistance
147 against nisin. Thus, increasing the expression of nisin resistance genes (*nisI, nisF,nisE* and
148 *nisG*) in producer *L. lactis* cells enhanced the resistance against nisin. Because, as elucidated,
149 there is a nisin tolerance limit at each producer cells. A relevant study reported that by
150 transferring *nisI* genes on a vector plasmid to the wild type producer strain and ensuring the
151 expression of these genes resulted 20% increase in the amount of nisin production (Kim et al.
152 1998). Using the *nisI* gene led the way to increase the copy number of the other genes in the
153 operon at the producer cells. In a study conducted on this basis, it was attempted to increase
154 nisin Z production in an *L. lactis* subsp. *lactis* 164 strain by cloning the essential gene (*nisZ*),
155 regulations genes (*nisR*, *nisK*) and the resistance genes (*nisF*, *nisE*, *nisG*). Nisin activity which
156 was 16,000 AU ml^{-1} in the control strain was improved to 25,000 AU ml^{-1} by increasing the
157 copy number of the regulatory genes. The transcription of the *nisZ* gene when the *nisR* and *nisK*
158 genes were highly expressed and resulted favorable improvements (Cheigh et al. 2002). In
159 another study, the copy number of the nisin regulation and resistance genes (*nisRKFEG*) in the
160 *L. lactis* LL27 strain was increased simultaneously which gained 45% increase in nisin

161 production compared to that of the wild type strain. In the same study, in the batch fermentation
162 system of the recombinant strain, in which the copy number of the *nisRKFEG* genes were
163 simultaneously increased, the dramatic losses experienced in the nisin activity of the control
164 strain in the late hours of the fermentation was prevented (Simsek et al. 2009a). In nisin
165 production in continuous nisin fermentation, increasing the copy number of the regulation and
166 resistance genes simultaneously has allowed working at high dilution ratios (0.29 h^{-1}) of the
167 producer strain. Thus, a significant increase was ensured in specific nisin production amount
168 (Simsek et al. 2009b).

169 In industrial nisin production, the pH that decreases due to the production of lactic acid by *L.*
170 *lactis* is neutralized by adding alkali to establish suitable physiological conditions for the cells
171 in question. However, the lactate that accumulates in the medium at progressive hours of the
172 fermentation inhibits cell proliferation, and consequently, there are losses in nisin production.
173 Examining the nisin production graphs in batch systems, it is seen that nisin activity seriously
174 decreases towards the end of the fermentation. It is discussed that in such a case, the cells
175 undergo autolysis and release proteases and thus the nisin activity decreases. One of the first
176 steps taken to solve this identified problem is cloning and expressing the pyruvate
177 decarboxylase (PDC) and alcohol dehydrogenase (ADH) genes to direct the carbohydrate
178 metabolism in *L. lactis* cells to ethanol production. As Wardani et al. (2006) pointed out nisin
179 production in the cells in which the metabolic pathway is converted to heterofermentative has
180 increased 1.7 times.

181 One of the objectives of increasing nisin production is improving the tolerance of the cells to
182 acidic conditions in the fermentation medium. In a study within this context, it was ensured that
183 the asparagine synthetase (*asn*H) gene was expressed more to increase the ratio of D-Asp
184 amidation in the cell wall composition of the *L. lactis* F44 strain. The tolerance of the

recombinant *L. lactis* F44A strain obtained was improved at a significant level, and consequently, it was determined that the F44A strain could produce more nisin in the fermentation media compared to the wild type strain. In both batch and fed-batch fermentation systems, 2884 and 3405 IU ml^{-1} nisin were produced at wild type F44 strain where as the nisin production was enhanced to 3876 and 5346 IU ml^{-1} nisin was achieved respectively at the acid-tolerant *L. lactis* F44A strain respectively (Hao et al. 2017). In another study with the paralel hypothesis, 17 genes giving acidic tolerance were transferred to *L. lacits* F44 sustaining the optimum pH value in the cell, Among these genes, the *hdeAB*, *Idh* and *murG* resulted the highest amount of nisin production at recombinant *L. lactis* cells (5560 IU ml^{-1}) (Zhang et al. 2016). These results are also the evidences showing the adverse effect of the lactate on nisin production.

The nisin production amount is closely dependent on the biomass yielded in the fermentation. Hence, the presence of active producer cells in the system promoted nisin production. Therefore, many studies aimed to increase the physiological wellness or the number of the active nisin-producing cells under fermentation conditions. Especially, increasing the energetic level of the producers is one of the most basic approach. For example, Papagianni and Avramidis (2012) provided the cells to be produced more energy with some manipulations promoting the oxidative respiration in the nisin producer, thus accelerating cell division and metabolism under fermentation conditions. In this study, Papagianni and Avramidis succeded to clone the *aox1* gene from *Aspergillus niger* to the nisin producer *L. lactis* ATCC11454 and used it in a fermentation system consisted 90% dissolved oxygen and 10 g l^{-1} glucose concentration. Accordingly, the biomass and nisin production of *L. lactis* ATCC11454 was 3.2 g l^{-1} and 5900 IU ml^{-1} while the *aox1* gene including *L. lactis* ATCC11454 produced 5.8 g l^{-1} and 7900 IU ml^{-1} biomass and nisin respectively. This enhancement revealed the necessity to express the *pfk* gene encoding the phosphofructokinase (pfk) together with the *aox1* gene in *L.*

lactis ATCC11454. Thereby, the *aox1* gene was further cloned to the *L. lactis* ATCC11454 together with the *pfk13* ve *pkaC* genes responsible for the phosphofructokinase and AMP protein kinase and it was demonstrated that the biomass and nisin production of the recombinants including the *pfk13-pkaC-aox1* genes in a fed-batch fermentation system containing hemin reached 7.5 g L^{-1} and 14000 IU mL^{-1} the highest nisin activity ever reported (Papagianni and Avramidis, 2012). In another similar study, the 8-phosphofructokinase *pfk* gene was expressed in the *L. lactis* N8 strain and 20% more nisin yield was obtained in the recombinant strain after 10 hours of fermentation (Zhu et al. 2015).

Well organized and optimized fermentation systems enable to produce high amount of nisin. Thus increase at nisin production and yield was been hypothesized for continuous fermentation if the nisin producer *L. lactis* cells could be kept in reactor against dilution. In this respect, the gene encoding the chitin binding domain of chitinase, one of the enzyme cleaving the chitin which is the abundant polysaccharide in nature, was successfuly cloned and expressed in nisin producer *L. lactis* N8. Subsequently, the chitin which the cells be intact was used in the continuous fermentation system of the study to prevent the cell outflow. In the system, the nisin producers that can adhere to chitin, remain in the continuous fermentation system (CICON-FER), and a nisin production over 10000 IU mL^{-1} was able to be produced with 0.9 h^{-1} dilution rate (Simsek, 2014).

In the another innovative study, to increase nisin Z production in the *L. lactis* YF11 strain, genome shuffling was applied using repeated protoplast fusion method. In genome shuffling, ultraviolet radiation and diethyl sulfate mutagenesis were used for template line production. After 4 rounds of shuffling, an F44 strain that can tolerate both high glucose (8% to 15% w/v) and high nisin concentrations (5000-14000 IU ml^{-1}) was obtained. This recombinant nisin producer produced 2.4 fold more (4023 IU ml^{-1}) nisin in the fed-batch fermentation system

234 comparing to the YF11 control strain. The findings showed that the transcription levels of nisZ
235 and nisI, the structural genes of nisin, in the F44 were higher (48% and 130% respectively)
236 compared to those of the control strain (Zhang et al. 2014).

237 **3.2. Modified Nisin Fermentation Systems**

238 Nisin production was first carried out in batch systems, then, fed-batch fermentation systems
239 were used. Relevant studies have demonstrated that the factors limiting nisin production are: i)
240 inhibition of producers by high substrate concentration, ii) lactate repression on producers, iii)
241 nisin feedback inhibition on producers, and iv) nisin degradation by the proteases released from
242 the cells. To eliminate these disadvantages, various innovative modifications have been made
243 in fermentation systems which have contributed to some extend to the improvement of nisin
244 production (e.g., de Vuyst 1992; Pongtharangkul et al. 2006; Simsek et al. 2009).

245 One of the fermentation trials carried out for nisin production was cycling the active nisin
246 producer cells that cultivated in the batch system into the fresh substrate. This application
247 sustained the active physiological state of the logarithmic phase cells in the batch system
248 maintaining the high nisin production characteristics. Bertrand et al. (2001) transferred 10^{11}
249 CFU g^{-1} *L. lactis* subsp. *lactis* biovar. *diacetylactis* cells, immobilized on k-carrageenan/legume
250 gum onto a fresh medium every one hour and achieved 8200 IU ml^{-1} total nisin activity and
251 5730 IU ml^{-1} h^{-1} volumetric nisin activity. However, since the cells were stabilized in solid
252 phase, the substrate access and also the high nisin concentration exposed to cells were the
253 adverse factors. In another similar study, to increase the specific nisin production, *L. lactis* N8
254 and LAC48 strains were grown in the batch system and every 30, 60 and 120 min cells were
255 seperated and suspended in the fresh substrate. Thus, nisin production was sustained with
256 continuously in active phase. Although high nisin productivity was attained in 60 min cycles,

the cellular stability could be maintained more in 30 min cycles. However the cell stability decreased within 120 min at nisin producers significantly after 5^{th} cycle. In this study, 60 min cycle was offered continuously produce nisin with high productivity (Şimşek et al. 2009).

The high substrate concentrations in the fermentation medium adversely affect the nisin producers. Therefore, fed-batch fermentation systems are recommended in nisin production instead of batch fermentation systems. However, some studies to prevent the high substrate inhibition in fed-batch fermentation systems are still carried out. Malvido et al. (2016), re-alkalized the medium in line according to the pH reduction rate of the cells in the fermentor and glucose was added depending on the amount of spent NaOH. In this fermentation system, glucose was used by monitoring the activity of the *L. lactis* CECT539 cells. As a result, it was reported that use of 400 g L^{-1} glucose in fed-batch fermentation system, where the medium was re-alkalined increased the production and this system was established an alternative for economic nisin production.

Nisin production is closely associated with the number and the physiological stability of the producing cells in the fermentor medium. It is notable that particularly a high number of active producing cells in the fermentor significantly increase the nisin production. It is known that bacterial activity is related with the energetic level. Therefore, nisin producer *L. lactis* cells were promoted to oxidative respiration in fermentor systems inducing heterofermentative instead of homofermentative metabolism which resulted the cells have high energy by activating the ETS system. Figure 2 is showing heterofermentative metabolism of a *L. lactis* when the aerobic fermentation is applied in the presence of hemin. Additionally, this reroute of metabolism also resulted higher biomass after fermentation. Because acetone, diacetyl and ethanol were produced instead of lactic acid which avoided lactate acumulation. Thereby lactate inhibition could be also eliminated by heterofermentative metabolism.

Respiration of facultative anaerobic *L.lactis* cells is only possible by the activation of cytochromes in the fermentation media where hemin and hemin-menachinon are present (Bryan-jones et al. 1969; Sijpestejn 1970; Whittenbury et al. 1978; Lechardeur et al. 2004; Brooijmans et al. 2009; Pedersen et al. 2012). However, since lactic acid bacteria do not have the enzymes to carry out hemin biosynthesis, adding hemin into the medium is necessary to start respiration in these strains.

There is only one type of cytochrome oxidase (CydAB) enzyme in all lactic acid bacteria. This enzyme complex can function in media containing oxygen and contributes to the oxygen tolerance of the bacterial cell (Rezaiki et al. 2004). The cytochromes inducing the proton motive force by being final proton acceptor in membranes warrant the cell to be able to respire in media even with low oxygen concentration (Brooijmans et al. 2009). *L. lactis* that is known for its fermentative metabolism can respire under aerobic conditions in the presence of hemin since it has the *cyd*AB gene. Bolotin et al. (1999) reported that the *cydA* gene is present in the genome of the *L. lactis* IL1403 strain responsible for the respiration encoded by cytochrome bd oxidase. During respiration, the *cydA* gene play an active role in the electron transfer system and enable ATP production with transferring the electrons to the oxygen.

In the fed-batch fermentation system, where the glucose, hemin and dissolved oxygen concentrations were optimized and the *L. lactis* N8 induced for respiration was used, nisin production was 3.1 times more than the control group fermented without hemin and a maximum nisin production of 5410 IU ml^{-1} was achieved. It was reported that this increase in nisin production was attributed to the increase in cell biomass within the energetic level in the producing strain and to the minimizing the feedback inhibition by the lactate accumulated in the medium (Kordikanlioglu et al. 2015).

Nisin also has antimicrobial activity on itself. Although there are immunity proteins and various proteases in the cell wall to protect from nisin, the high concentration accumulated in the fermentation seriously affect the producer strains. Therefore, one of the issues on which researchers focus on is to online separation of nisin from the fermentation system. Indeed, this success will also allow the nisin to be concentrated and pure. The foam fractionation method is an innovative method for obtaining nisin and similar surfactant compounds from media at low cost and high concentration. As reported in many studies, aeration of the fermentation medium aiming to optimize the percentage of dissolved oxygen in the medium has a stimulating effect on nisin production (Amiali et al. 1998; Cabo et al. 2001; Kordikanlioglu et al. 2015). In a study examining the effectiveness of the foam fractionation method to improve nisin production in the fermentation medium, the fermentation medium was subjected to foam fractionation with sterile air inlet at flow rate of 30 ml min^{-1} while the producer cell was in exponential growth phase. In this relevant study, maximum total nisin activity was measured as 4657 IU ml^{-1}. This strategy resulted in a 36.2% increase in the total nisin activity measured in the control group fermentation performed with the conventional batch system (Zheng et al. 2015). The aerotolerance of microorganisms is related to superoxide dismutase enzyme activity and the ability to induce NADH. Since *L. lactis* species are catalase negative, fermentation media is generally anaerobic. In fact, the aeration of the fermentation medium has an effect on promoting the growth and nisin production of *L. lactis* cells (Jiang et al. 2015; Kordikanliglu et al. 2015). In a study, which the relation between the aeration of the fermentation media and nisin production was determined, 10700 IU ml^{-1} nisin amount was produced in the anaerobic condition, where the production amount was 15400 IU ml^{-1} in the aerobic condition (Jiang et al. 2015).

There are also different ways of reducing the accumulation of lactate that adversely affect nisin production in *L.lactis*. Extraction of lactate in the medium using solvent or neutralization by

alkalisation can be given as examples. Another innovative approach, unlike all these, is the mixed culture fermentation technique. The microorganism to be selected as an adjuvant to *L. lactis* in the mixed culture fermentation technique should meet certain criteria such as i) it should not have the ability to use the main carbon source, ii) must have the ability to use lactate, an inhibitor metabolite produced by *L. lactis,* iii) do not adversely affect the nisin produced by *L. lactis*, iv) increase the production of the desired target product, stimulate it. *Yarrowia lipolytica* that consumes lactate as a substrate source and the lactate producer *L. lactis* were simultaneously cultured in a fermentation medium that contains molasses and 50% increase was achieved in this mixed culture fermentation in nisin production compared to the control group where only *L. lactis* was used. While the nisin concentration in the control group was 170 mg l^{-1}, it was 270 mg l^{-1} in the mixed culture (Ariana and Hamedi, 2017).

4. Conclusion

Nisin is the most widely known and applied lantibiotic and is the first and only bacteriocin allowed for use in food. Widespread use of nisin in biomedical applications, its therapeutic characteristic and as well as its production at industrial scale are the justifications for many studies to focus on to enhace its production and thereby reduce the cost. To date, many recombinant strains have been developed with different metabolic regulation to increase yields in producer strains. In non-molecular innovative approaches, the alternative ways have been developed to improve the adaptation capabilities of native strains to the conditions of the medium and such studies have been accelerated. It is inevitable that future research on nisin might be the fermentations used together with such innovative systems. When the innovative systems addressed above are upscaled to industrial scales, the factors that have limited nisin use will be tolerated to a great extent.

352 **5. References**

353 Amiali, M.N., Lacroix, C., Simard, R.E. (1998). High nisin Z production by *Lactococcus lactis*
354 UL719 in whey permeate with aeration. *World J Microb Biotechnol*., 14:887–894.

355 Arianaa, M. ve Hamedi, J. (2017). Enhanced production of nisin by co-culture of *Lactococcus*
356 *lactis sub sp. lactis* and *Yarrowia lipolytica* in molasses based medium. *Journal of*
357 *Biotechnology*, 256:21–26.

358 Belfiore, C., Castellano, P. and Vignolo, G. (2007). Reduction of *Escherichia coli* population
359 following treatment with bacteriocins from lactic acid bacteria and chelators. *Food Microbiol*.
360 24:223–229.

361 Bertrand, N., Fliss, I., Lacroix, C. (2001). High nisin-Z production during repeated-cycle batch
362 cultures in supplemented whey permeate using immobilized *Lactococcus lactis* UL719, *Int.*
363 *Dairy Jour*., 11 (12): 953-960.

364 Bolotin, A., Mauger, S., Malarme, K., Ehrlich, S.D., Sorokin, A. (1999). Low redundancy
365 sequencing of the entire *Lactococcus lactis* IL1403 genome, *Ant. Van Leeuwen*., 76:27-76.

366 Brooijmans, R.J., Smit, B., Santos, F., Riel, J.V., Vos, W., Hugenholtz, J. (2009). Heme and
367 menaquinone induced electron transport in lactic acid bacteria. *Microbiol Cell Fact*. 8:1475–
368 1486.

369 Bryan-Jones, D.G., Whittenbury, R. (1969). Haematin-dependent oxidative phosphorylation in
370 *Streptococcus faecalis*, *J. Gen. Microbiol*., 58:247-60.

371 Cabo, M.L., Murado, M.A., Gonzalez, M.P., Vazquez, J.A., Pastoriza, L. (2001). An empirical
372 model for describing the effects of nitrogen sources on nisin production. *Lett Appl Microbiol*.
373 33(6):425-9.

374 Cheigh, C.I., Choi, H.J., Park, H., Kim, S., Kook, M., Kim, T., Hwang, J., Pyun, Y. (2002).
375 Influence of growth conditions on the production of a nisin-like bacteriocin by *Lactococcus*
376 *lactis subsp. lactis* A164 isolated from kimchi, *J. Biotechnol*., 95:225-235.

377 de Vuyst, L., Vandamme, E.J. (1992). Influence of the carbon source on nisin production in
378 *Lactococcus lactis subsp. lactis* batch fermentations, *J. Gen. Microbiol*., 138:571-578.

379 Engelke, G., Gutowski-Eckel, Z., Hammelman, M., Entian, K-D. (1992). Biosynthesis of the
380 lantibiotic nisin genomic organization and membrane localization of the NisB protein. *Appl*
381 *Environ Microbiol*, 58: 3730-3743.

382 Graeffe, T., Rintala, H., Paulin, L. ve Saris, P. (1991). A natural nisin variant. In nisin and novel
383 lantibiotics, *Sci. Publishers*, 260-268, (1991).

384 Gross, E., Morell, J.L. (1971). The structure of nisin. *J. Am. Chem. Soc*. 93; 4634-4635.

385 Hao, P., Liang, D., Cao, L., Qiao, B., Wu, H., Caiyin, Q., Zhu, H., Qiao, J. (2017). Promoting
386 acid resistance and nisin yield of *Lactococcus lactis* F44 by genetically increasing D-Asp
387 amidation level inside cell Wall. *Appl Microbiol Biotechnol*, 101:6137–6153

388 Hurst, A. (1981). Nisin. *Adv. Appl. Microbiol*. 27:85–123.

389 Jiang, L., Liu, Y., Yan, G., Cui, Y., Cheng, Q., Zhang, Z., Meng, Q., Teng, L., Ren, X. (2015).
390 Aeration and fermentation strategies on nisin production, *Biotechnol Lett*., 37:2039–2045.

391 Kim, W.S., Hall, R.J., Dunn, N.W. (1998). Improving nisin production by increasing
392 immunity/resistance genes in the producer organisim *Lactococcus lactis*, *Appl. Microbiol*.,
393 50(4):429-433.

394 Kong W, Lu T. (2014). Cloning and optimization of a nisin biosynthesis pathway for
395 bacteriocin harvest. *Biotechnol.* 18;3(7):439-45.

396 Kordikanlioglu, B., Simsek, O., Saris P.E. (2015). Nisin production of *Lactococcus lactis* N8
397 with hemin-stimulated cell respiration in fed-batch fermentation system. *Biotechnol Prog*.
398 31(3):678-85.

399 Kuipers, O.P., Beerthuyzen, M.M., de Ruyter, P.G.G.A., Luesink, E.J., de Vos, W.M. (1995).
400 Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *J Biol Chem*,
401 270: 27299-27304.

402 Kwaadsteniet, M., Doeschate, K. ve Dicks, L.M.T. (2008). Characterization of the structural
403 gene encoding nisin F, a new lantibiotic produced by a *Lactococcus lactis subsp. lactis* isolate
404 from freshwater catfish (Clarias gariepinus), *Appl. Environ. Microbiol*., 74, 547-549.

405 Lechardeur, D., Cesselin, B., Fernandez, A., Lamberet, G., Garrigues, C., Pedersen, M., Lv,
406 W., Cong, W., Cai, Z. (2004). Nisin production by *Lactococcus lactis subsp. lactis* under
407 nutritional limitation in fed-batch culture, *Biotechnol. Lett*., 26:235-238.

408 Liu, J., Maa, Z., Zhua, H., Caiyina, Q., Lianga, D., Wua, H., Huangd, X., Qiaoa, J. (2017).
409 Improving xylose utilization of defatted rice bran for nisin production by overexpression of a
410 xylose transcriptional regulator in *Lactococcus lactis*, *Bioresource Tech*., 238:690-697.

411 Luck, E., Jager, M. (1995). Nisin Antimicrobial, *Food Additives*, 27, 208-213.

412 Malvido, C., González, A., Guerra, P. (2016). Nisin production in realkalized fed-batch
413 cultures in whey with feeding with lactose- or glucose-containing substrates. *Appl Microbiol*
414 *Biotechnol*. 100(18):7899-908.

415 Mulders, J.W.M., Boerrigter, I.J., Rollema, H.S., Siezen, R.J. ve de Vos, W.M. (1991).
416 Identification and characterization of the lantibiotic nisin Z, a natural nisin variant, *Eur. J.*
417 *Biochem*., 201, 581-584.

418 Ni, Z., Zhang, X., Liu, F., Wang, M., Hao, R., Ling, P., Zhu, X. (2017). Effect of Co-
419 overexpression of nisin key genes on nisin production improvement in *Lactococcus lactis* LS01,
420 *Probiotics & Antimicro. Prot*., Baskıda.

421 Papagianni, M., Avramidis, N. (2012). Engineering the central pathways in *Lactococcus lactis*:
422 functional expression of the phosphofructokinase (pfk) and alternative oxidase(aox1) genes
423 from *Aspergillus niger* in *Lactococcus lactis* facilitates improvedcarbon conversion rates under
424 oxidizing conditions, *Enzyme Microb. Technol*.,51(3):125–130.

425 Patent, US2935503 A. (1960). United States Patent Office, Production of nisin, Patented May
426 3.

427 Pedersen, M., Gaudu, P., Lechardeur, D., Petit, M. ve Gruss, A. (2012). Aerobic respiration
428 metabolism in lactic acid bacteria and uses in biotechnology, *Annu. Rev. Food Science*
429 *Technology*, 3, 37-58.

430 Pongtharangkul, T. ve Demirci, A. (2006). Evaluation of culture medium for nisin production
431 in a repeated-batch biofilm reactor, *Biotechnol. Prog*., 22, 217-224.

Ra, R., Qiao, M., Immonen, T., Pujana, I., Saris, P.E.J. (1996). Genes responsible for nisin synthesis, regulation and immunity form a regulon of two operons and a re induced by nisin in *Lactococcus lactis* N8. *Microbiol*, 142: 1281-1288.

Rezaiki, L., Cesselin, B., Yamamato, Y., Vido, K., van West, E., Gaudu, P. ve Gruss, A. (2004). Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis, Mol. Biology*, 53 (5): 1331-1342.

Sijpestejn, A.K. (1970). Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*, *Ant. van Leeuwen*., 36:335-48.

Simsek, O., Akkoc, N., Con, A.H., Ozcelik, F., Saris, P.E.J. ve Akcelik, M. (2009). Continuous nisin production with bioengineered *Lactococcus lactis* strains, *J. of Ind. Microbiol. Biotechnol*., 36 (6):863-871.

Simsek, O., Con, A.H., Akkoc, N., Saris, P.E.J. ve Akcelik, M. (2009). Influence of growth conditions on the nisin production of bioengineered *Lactococcus lactis* strains, *J. of Ind. Microbiol. Biotechnol*., 36:481-490.

Simsek, O. (2014). Nisin production in a chitin-including continuous fermentation system with *Lactococcus lactis* displaying a cell wall chitin-binding domain, *J. Ind. Microbiol. Biotechnol*., 41:535-543.

Tramer, J. and Fowler, G. G. (1964). Estimation of nisin in foods. *J. Sci. Food Agric*. 15:522–528.

452 Wardani, A.K., Egawa, S., Nagahisa, K., Shimizu, H., Shioya, S. (2006). Robustness of cascade
453 pH and dissolved oxygen control in symbiotic nisin production process system of *Lactococcus*
454 *lactis* and *Kluyveromyces marxianus*. *J Biosci Bioeng*. 101(3):274-6.

455 Whittenbury, R. (1978). Biochemical characteristics of *Streptococcus* species, *Soc. Appl.*
456 *Bacteriol*. Symp. Ser., 7:51-69.

457 Wirawan, R.E., Klesse, N.A., Jack, R.W. ve Tagg, J.R. (2006). Molecular and genetic
458 characterisation of a novel nisin variant produced by *Streptococcus uberis*, *Appl. Environ.*
459 *Microbiol*., 72, 1148-1156.

460 Zendo, T., Fukao, M., Ueda, K., Higuchi, T., Nakayama, J. ve Sonomoto, K. (2003).
461 Identification of the lantibiotic nisin Q, a new natural nisin variant produced by *Lactococcus*
462 *lactis* 61-14 isolated from a river in Japan, *Biosci. Biotechnol. Biochem*., 67, 1616-1619,

463 Zhang, J., Caiyin, Q., Feng, W., Zhao, X., Qiao, B., Zhao, G., Qiao, J. (2016). Enhance nisin
464 yield via improving acid-tolerant capability of *Lactococcus lactis* F44. *Sci Rep*, 16;6:27973.

465 Zhang, Y.F., Liu, S.Y., Du, Y.H.,, Feng, W.J., Liu, J.H., Qiao, J.J. (2014). Genome shuffling
466 of *Lactococcus lactis subspecies lactis* YF11 for improving nisin Z production and comparative
467 analysis, *J Dairy Sci.* 97(5):2528-41.

468 Zheng, H., Zhang, D., Guo, K., Dong, K., Xu, D., Wu, Z. (2015). Online recovery of nisin
469 during fermentation coupling with foam fractionation, *Journal of Food Engineering*, 162 :25–
470 30.

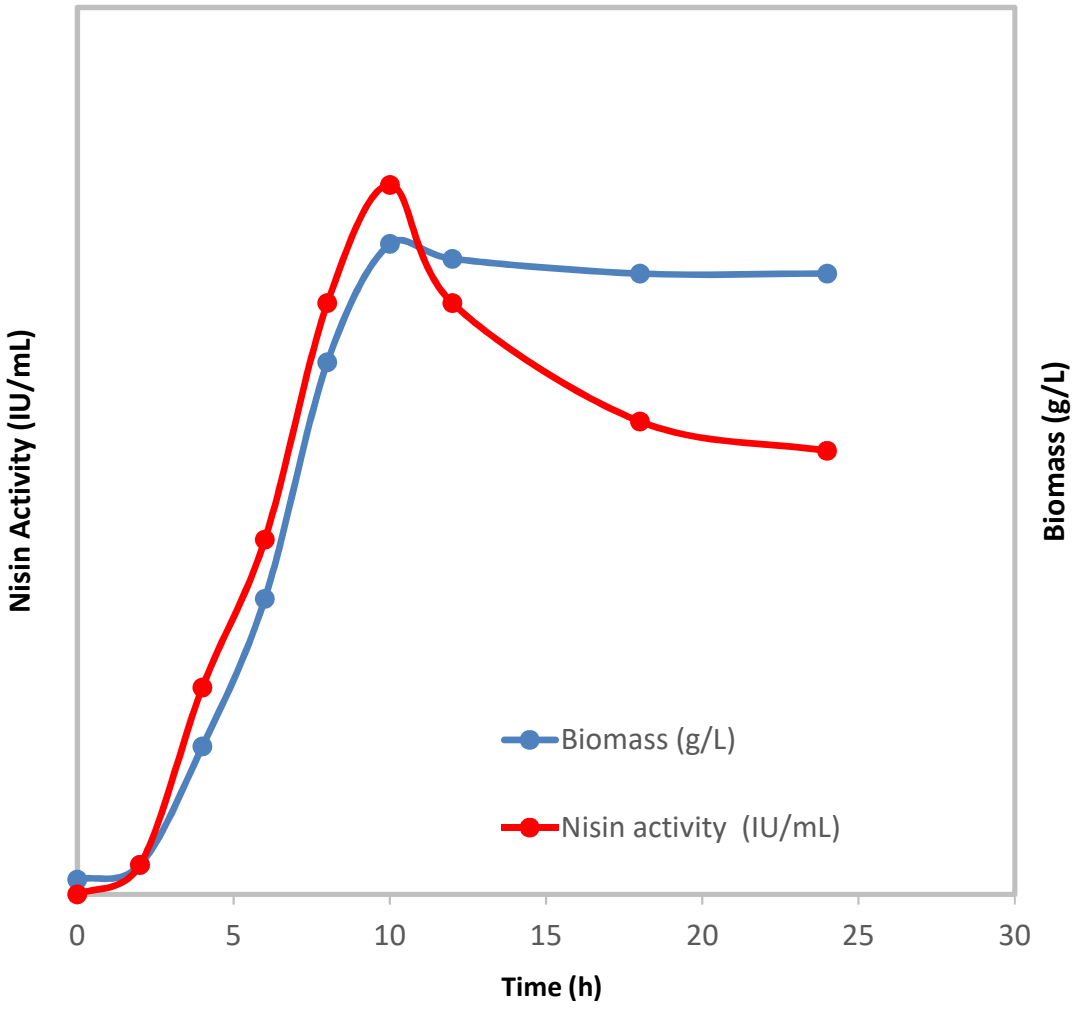
Zhu, D., Zhao, K., Xu,, H., Bai, Y., Zhang, X., Qiao, M. (2015). Effect of 6-phosphofructokinase gene-pfk overexpression on nisin production in *Lactococcus lactis* N8. *Wei Sheng Wu Xue Bao*. 4;55(4):440-7.

488 **Table 1.** Recent innovative approaches resulting high nisin production.

Nisin Producers	***Nisin production***	***Innovative Approaches***	***Reference***
***L. lactis* ATCC11454**	7900 IU ml^{-1}	*aox1* gene was cloned and the rekombinant nisin producer was induced to oxidative respiration.	Papagianni et al. 2012
***L. lactis* ATCC11454**	14000 IU ml^{-1}	*aox*1gene was cloned together with *pfk*13 and *pka*C genes to increase the glikolitic activity as well as the oxidative respiration	Papagianni et al. 2012
***L. lactis* PLAC7**	10500 IU ml^{-1}	Chitin Binding Domain was cloned in producer *L. lactis* and this was used in continuous fermentation system in presence of chitin	Şimşek, 2014
***L. lactis* YF11**	4023 IU ml^{-1}	Genome shuffling was applied to improve nisin Z production of *L. lactis* ssp. *lactis* YF11 via recursive protoplast fusion.	Zhang et al. 2014
***L. lactis* N8**	5410 IU ml^{-1}	Fed-batch fermentation carried out with hemin under aerobic conditions.	Kordikanlioglu et al. 2015
***L. lactis* ATCC11454**	4657 IU ml^{-1}	Nisin was online recovered with foaming fractionation from the fermentation.	Zheng et al. 2015
***L. lactis* LD2**	15400 IU ml^{-1}	Nisin was produced in a aerated fed-batch fermentation system with a variable feeding rate.	Jiang et al. 2015
***L.lactis* F44**	5560 IU ml^{-1}	*hde*AB, *Idh* and *mur*F genes were cloned and expressed simultaneously to enhance the acidic tolerance of the producer *L. lactis.*	Zhang et al. 2016
***L. lactis* F44A**	5346 IU ml^{-1}	Acidic tolerance was enhanced by overexpression the asnH gene at nisin producer *L. lactis*	Hao et al. 2017
***L. lactis* UTMC106**	10800 IU ml^{-1}	This producer was used together with *Yarrowia lipolytica* ATCC18942 in fermentation system	Ariana and Hamedi, 2017

489

490



491

492 **Figure1**. Representative nisin production and biomass amount of nisin producer *L. lactis* in

493 batch fermentation systems.

494

495

496

497

498

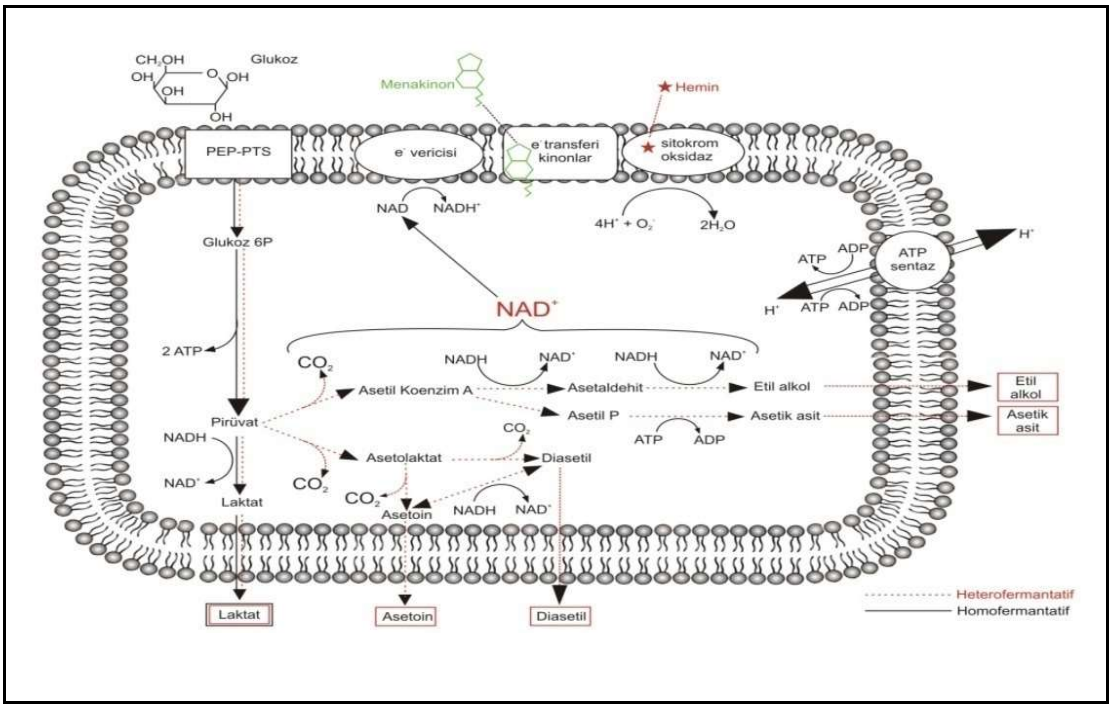


Figure 2. The metabolic pathway followed by the *L.lactis* bacteria induced for oxidative respiration.