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ORIGINAL RESEARCH PAPER

Aeration and fermentation strategies on nisin production

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Abstract
Objectives To optimize nisin production in *Lactococcus lactis* by using different aeration and fermentation strategies.
Results The nisin titer and specific nisin production rate reached maximum values of 11,900 IU/ml and 4110 IU/g/h, respectively, in aerobic batch fermentation with glucose as C source. These values were higher than in anaerobic batch fermentation (10,700 IU/ml and 3260 IU/g/h, respectively). The maximum specific nisin production rates appeared earlier in aerobic batch fermentation, which suggests that nisin production is stimulated by aeration. Different fermentation strategies were compared: maximum nisin production (15,400 IU/ml) was achieved with fed-batch fermentation with a variable rate of feeding under aerobic conditions.
Conclusion Nisin production can be stimulated by aeration, which goes against the typical conditions involving strict anaerobiosis.

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Keywords Aeration · Batch fermentation · Fed batch fermentation · Glucose · *Lactococcus lactis* · Nisin

Introduction

Nisin, which belongs to a group of bacteriocin called lantibiotics, is effective against a wide range of Gram-positive bacteria and most heat-resistant spores in foods and beverages. Nisin has not induced widespread biological resistance after being used as a food preservative for more than four decades worldwide, which makes its biosynthesis, production and application of interest. Nisin is produced by culturing lactic acid bacteria, such as *Lactococcus lactis* subsp. *lactis*, and its production is affected by the nutrient composition of media, pH and aeration of the culture and the fermentation strategy (Arauz et al. 2009).

Aeration is an important factor affecting the growth of *L. lactis* and nisin production. *L. lactis* has been studied mainly under anaerobic conditions because it does not have catalase, which is widely distributed among aerobic bacteria (Lv et al. 2005 and references therein). However, there are some reports for culturing *L. lactis* under aerobic conditions (Arauz et al. 2012 and references therein). Aerotolerance is related to the ability to induce superoxide dismutase and NADH oxidase (Jiang and Bommarius 2004). Aerobic culturing results in an altered redox state and can cause

increased NADH oxidase activity (Duwat et al. 2001). The aerotolerance of *L. lactis* is associated with different metabolic pathways that give different product yields (Rezaïki et al. 2004 and reference therein).

Different fermentation strategies significantly affect nisin production. Examples of culturing *L. lactis* with batch and fed-batch fermentation have been reported (Arauz et al. 2009 and references therein). Good results were obtained when the fermenters were fed with the substrate at a variable rate rather than at a constant rate (Ghalfi et al. 2007).

The carbon source affects the growth of *L. lactis* and nisin biosynthesis. *L. lactis* has an efficient phosphoenolpyruvate-dependent phosphotransferase system for sucrose uptake, transport and metabolism (Vuyst and Vandamme 1992) and there is a genetic link between sucrose metabolism and nisin production (Steele and Mckay 1986). As a result, sucrose is commonly used as the carbon source for culturing *L. lactis* to produce nisin (Vuyst and Vandamme 1992; Lv et al. 2005; Wu et al. 2009), and glucose seems to be less studied in this respect. Glucose has been used, however, to produce nisin (Papagianni et al. 2007). The relationship between specific glucose uptake rate and nisin production by *L. lactis* was investigated in aerobic batch and fed-batch cultures in which the concentration of glucose was maintained as constant. Glucose concentration is thus an important parameter for nisin production in a glucostat fermentation.

To investigate how aeration and fermentation strategies affect nisin production with glucose as the carbon source, *L. lactis* was cultured using different fermentation strategies under anaerobic or aerobic conditions. The reported fermentation strategies include batch fermentation and fed-batch fermentation at variable rate of feeding were compared in this work.

Materials and methods

Bacterial strains and media

The nisin-producing strain *L. lactis* subsp. *lactis* ATCC11454 was mutated with diethylsulfate in our laboratory to obtain a high yielding strain *L. lactis* subsp. *lactis* LD2. *L. lactis* subsp. *lactis* LD2 (*L. lactis* LD2) was grown on the medium containing 23 g glucose/l, 24.1 g yeast extract/l, 28.6 g peptone/l, 5.9 g NaCl/l, 10 g KH_2PO_4/l and 0.2 g $MgSO_4 \cdot 7H_2O$/l. *Micrococcus flavus* NCIB 8166, which was used as the indicator strain in the nisin bioactivity assay, was grown on medium containing 10 g tryptone/l, 5 g yeast extract/l and 10 g NaCl/l. All chemicals were of analytical grade.

Culture conditions

L. lactis LD2 was grown in a bioreactor (Biostat ED-ES10, B. Braun Biotech International) with working volume of 10 l.

For batch fermentation, the bioreactor was inoculated with 3 % (v/v) of the 12 h culture of *L. lactis* LD2. The pH was maintained at 6.8 by automatically adding 5 M NaOH, and the fermenter was maintained at 37 °C. For anaerobic conditions, the medium was flushed with N_2. For aerobic culture, aerobic conditions were ensured by sparging the reactor with air to ensure that the dissolved O_2 tension was 30 % of saturation.

For fed-batch fermentations with variable rates of feeding, fermentations were initiated as a batch culture with glucose initially at 6 g. Glucose at 500 g/l was pumped into the bioreactor when the initial glucose was almost exhausted. Glucose was fed into the bioreactor to keep its concentration at 0.2 g/l during the whole fermentation process.

Analytical methods

Biomass was measured as the OD_{600} value which was converted to cell dry weight using a standard curve. Glucose was determined using a glucose oxidase/peroxidase method. The nisin titer was measured by the method of Tramer and Fowler (1964).

Results and discussion

Anaerobic batch fermentation

The curve of *L. lactis* growth, nisin production and glucose consumption in anaerobic batch culture is shown in Fig. 1. Glucose consumption was slow in anaerobic batch fermentation and its concentration decreased as a function of time but was not completely used up. A similar result was observed by Ghalfi et al. (2007).

The nisin titer increased with cell growth and reached a maximum (10,700 IU/ml) at 15 h under

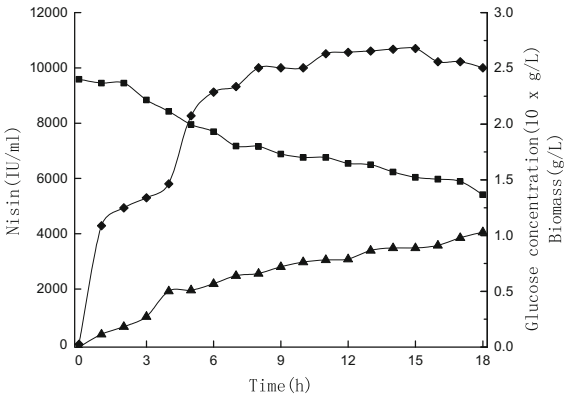

Fig. 1 Nisin production and cell growth of *L. lactis* LD2 cultured with batch fermentation under anaerobic conditions. Cultivation was carried out in a 10 L bioreactor. Initial glucose concentration was 23 g/l. Temperature and pH were maintained at 37 °C and 6.8, respectively. Cell dry weight *filled triangle*; residual glucose *filled square*; nisin titer *filled diamond*

anaerobic conditions. The nisin titer then dropped, probably because nisin was adsorbed to the cells or destroyed by non-specific proteolytic enzymes released during cell lysis (Lv et al. 2004a; Vuyst and Vandamme 1992).

The specific growth rate increased in the first 3 h, and the specific glucose consumption rate increased at the same time, with maximum values of 0.6 h^{-1} and 0.4 mmol/s/g, respectively at 3 h (Fig. 2). The fact that the specific glucose consumption rate follows the same trend as the specific growth rate suggests that glucose is the dominant factor for the growth of *L. lactis*.

Figure 2 shows that there is the lag between the maximum specific nisin production rate (3260 IU/g/h at 5 h) and the maximum specific growth rate. Nisin synthesis increases late in the exponential phase of batch fermentations (Arauz et al. 2009). The prenisin structural gene (*nisA*) is expressed early in the growth phase so the large increase of nisin production towards the end of the active growth phase could be the result of delayed formation of the necessary prenisin-modifying enzymes (Vuyst and Vandamme 1992). In addition, nisin production is dependent on a quorum-sensing mechanism. Besides its antimicrobial activity, nisin also acts as an extracellular peptide pheromone signal involved in regulation of its own biosynthesis. Nisin accumulates in culture broth and triggers a response in the target cells when its concentration reaches a certain threshold value. Production of nisin then keeps on increasing to reach maximal levels at the beginning of the stationary phase (Kleerebezem 2004).

Aerobic batch fermentation

Growth of *L. lactis*, nisin production and glucose consumption in aerobic batch culture are shown in Fig. 3. Glucose decreased rapidly to 7.7 g/l in the first 9 h, and had been completely consumed at 18 h. The

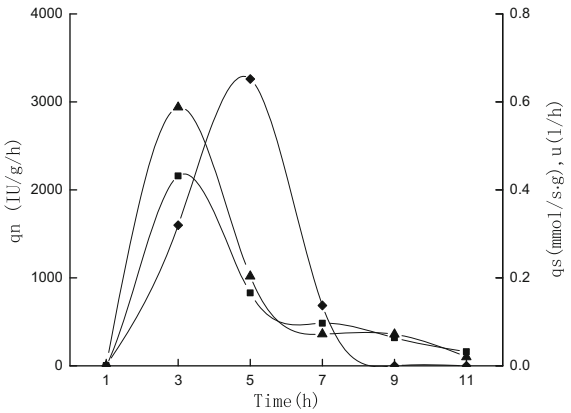

Fig. 2 Specific nisin production rates, specific growth rates and specific glucose consumption rates with batch fermentation under anaerobic conditions. Specific nisin production rate *filled diamond*; specific growth rate *filled triangle*; specific glucose consumption rate *filled square*

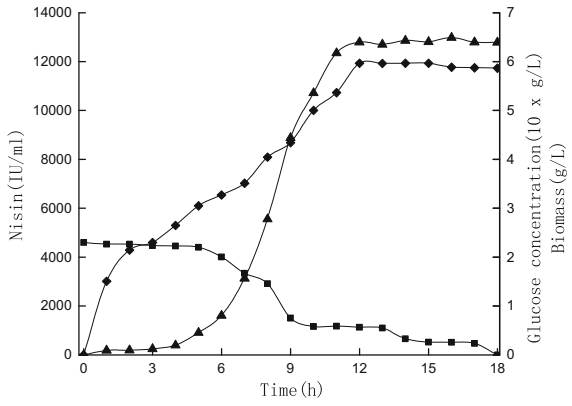

Fig. 3 Nisin production and cell growth of *L. lactis* LD2 cultured with batch fermentation under aerobic conditions. Cultivation was carried out in a 10 l bioreactor. Initial glucose concentration was 23 g/l. Temperature, pH and dissolved oxygen were maintained at 37 °C, 6.8 and 30 %, respectively. Cell dry weight *filled triangle*; residual glucose *filled square*; nisin titer *filled diamond*

nisin titer increased with cell growth, and reached its maximum titer (11,932 IU/ml) at 12 h. The maximum biomass and nisin titer in aerobic batch fermentation were higher than in anaerobic batch fermentation. The results are in general agreement with Papagianni et al. (2007) work: biomass production and specific nisin production rates were higher under moderate aeration level compared to anaerobic condition.

The specific growth rate increased in the first 5 h, and the specific glucose consumption rate increased simultaneously with maximum values of 0.7 h^{-1} and 0.2 mmol/s/g, respectively, at 5 h (Fig. 4). The specific nisin production rate increased in the first 3 h, reaching a maximum of 4110 IU/g/h (higher than 3260 IU/g/h in anaerobic batch culture). Although the discrepancies could be ascribed to variations in the strains, the results of the present study point to glucose giving higher specific growth rates compared with sucrose (Lv et al. 2005). Glucose also gave a higher nisin titer than sucrose in the work of Chandrapati and O'Sullivan (1998). The growth of *L. lactis* on glucose results in higher activities of the key glycolytic enzymes phosphofructokinase (pfk), pyruvate kinase (pyk), and l-lactate dehydrogenase (Ldh), compared with those obtained on sucrose or fructose (Luesink et al. 1998).

We have shown that aeration significantly stimulates nisin production by *L. lactis*, leading to an increased specific nisin production rate in aerobic fermentation. The specific nisin production at 60 % (23.4 AU/10^7 c.f.u.) or 90 % (24.9 AU/10^7 c.f.u.) initial air saturation was 8-fold higher than that under anaerobic condition (3.1 AU/10^7 c.f.u.) with *Lactococcus lactis* UL719 in the work of Amiali et al. (1998). It is clear, therefore, that the best conditions for nisin production are not the standard ones, which involve strict anaerobiosis.

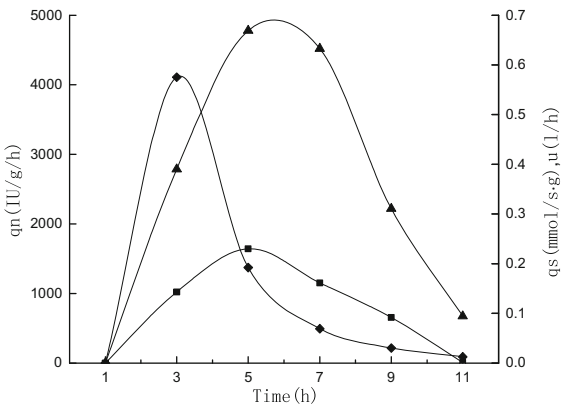

Fig. 4 Specific nisin production rates, specific growth rates and specific glucose consumption rates with batch fermentation under aerobic conditions. Specific nisin production rate *filled diamond*; specific growth rate *filled triangle*; specific glucose consumption rate *filled square*

Aeration not only promotes the specific nisin production rate but also brings forward the peak time of the specific nisin production rate. The specific reasons why may require the elucidation of the biosynthetic pathway of nisin production. It can be hypothesized that the aeration stimulates the formation of prenisin and prenisin-modifying enzymes, or decreases threshold concentration of nisin as a pheromones at the beginning of the exponential growth phase, leading to a rapid increase of nisin production in the early exponential phase. Increased production of reactive oxygen species (H_2O_2 and hydroxyl radicals) may then occur and begin to attack prote. H_2O_2 can directly oxidize protein cysteinyl residues, thus inactivating enzymes. Hydroxyl radicals are strong oxidant agent that can cause strand breaks and a wide spectrum of base modifications in DNA (Piard and Desmazeaud 1991; Miyoshi et al. 2003). The complex biosynthesis of nisin is encoded by 11 genes and regulated by many proteins (Cheigh and Pyun 2005; Mierau and Kleerebezem 2005). H_2O_2 and hydroxyl radicals would attack proteins and DNA and as the formation of nisin is inhibited by O_2, this would cause the specific nisin production rate to decrease. At the same time, glucose, which is used quickly by the cells under aerobic conditions, prolongs the growth of cells, as the peak time of the specific growth rate appears late. This would lead to the peak value of specific nisin production rate appearing earlier than that of specific growth rate under aerobic conditions.

Fed-batch fermentation with a variable rate of feeding under anaerobic conditions (FVAE)

Fed-batch fermentation can eliminate substrate inhibition and control the substrate concentration at the desired level. The fermentation started with a low concentration of glucose (6 g/l) to avoid initial inhibition of *L. lactis*. Concentrated glucose was fed to the bioreactor during the course of the fermentation to ensure a limited supply of glucose. The feeding started when the initial glucose was almost completely

consumed at 10 h (Fig. 5). Glucose was supplemented at a variable rate to maintain a low level (0.2 g/l). The biomass increased steadily during the whole process of batch and fed-batch fermentation. However, there was no *L. lactis* growth in the anaerobic fed-batch fermentation. The nisin titer reached the maximum of 12,000 IU/ml with FVAE.

Fed-batch fermentation with a variable rate of feeding under aerobic conditions (FVA)

The curve of cell growth and nisin production with FVA is shown in Fig. 6. The glucose was consumed quickly (after 4 h) under aerobic conditions. When the glucose was almost completely consumed in the initial batch phase, the fed-batch was started, and the glucose concentration was kept at 0.2 g/l. The nisin titer increased and reached a maximum of 15,367 IU/ml, 28 % more than that in FVAE. Most bacteriocins are produced during the exponential growth phase which, in turn, corresponds to a high rate of biomass production, therefore bacteriocin production exhibits primary metabolite kinetics. According to the findings of Vuyst and Vandamme (1992), nisin biosynthesis

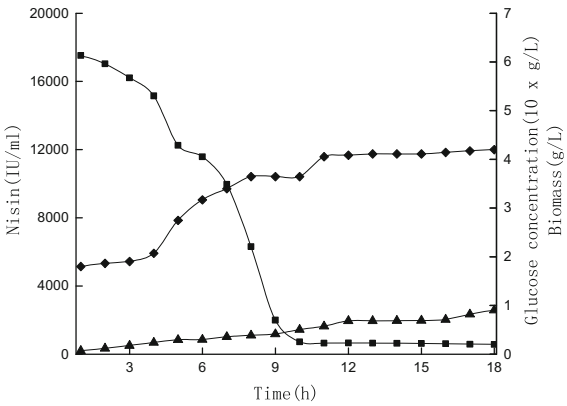

Fig. 5 Nisin production and cell growth of *L. lactis* LD2 cultured with fed-batch fermentation at a variable rate of feeding under anaerobic conditions. Cultivation was carried out in a 10 l bioreactor. Temperature and pH were maintained at 37 °C and 6.8, respectively. Initial glucose concentration was 6 g/l. The feeding glucose solution (500 g/l) was pumped into the bioreactor when the initial glucose was almost exhausted. The glucose was fed into bioreactor at variable rate of feeding to keep the residual glucose concentration at 0.2 g/l throughout the whole fermentation process. The nisin titer increased slowly, and reached a maximum of 12,000 IU/mL at the end. Cell dry weight *filled triangle*; residual glucose, *filled square*; nisin titer *filled diamond*

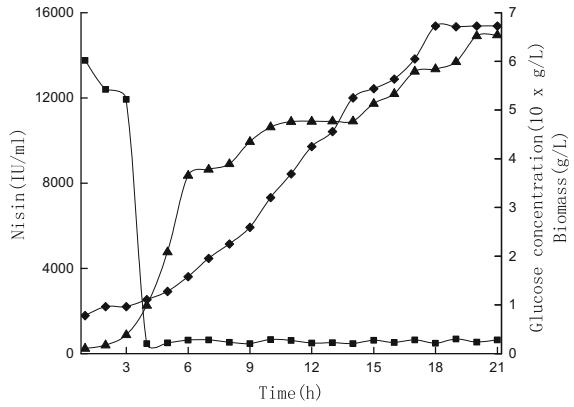

Fig. 6 Nisin production and cell growth of *L. lactis* LD2 cultured with fed-batch fermentation at a variable rate of feeding under aerobic conditions. Cultivation was carried out in a 10 l bioreactor. Temperature, pH and dissolved oxygen were maintained at 37 °C, 6.8 and 30 %, respectively. Initial glucose concentration was 6 g/l. The feeding glucose solution (500 g/l) was pumped into the bioreactor when the initial glucose was almost exhausted. The glucose was fed into bioreactor at variable rate of feeding to keep the residual glucose concentration at 0.2 g/l throughout the whole fermentation process. The cell logarithmic growth phase was prolonged until the 20 h fermentation phase. Cell dry weight *filled triangle*; residual glucose *filled square*; nisin titer *filled diamond*

takes place during the active growth phase and completely stops when cells enter the stationary phase. Maintaining the cell in continuous growth to avoid a stationary phase means that it is possible to achieve higher nisin production with a greater biomass or a longer exponential growth phase (Wu et al. 2009). The exponential growth phase was prolonged with FVA, which led to a higher nisin titer.

The maximum biomass and nisin titer in batch and fed-batch fermentations are compared in Table 1. It seems there are ceilings for the growth of *L. lactis* either under aerobic conditions (6.5 g/l) or anaerobic conditions (1.3 g/l) because of inhibition from lactic acid accumulating late in the fermentation broth (Lv et al. 2004b). The nisin titers under aerobic conditions (11,932 IU/ml in batch fermentation and 15,367 IU/ml in FVA, respectively) were higher than those under anaerobic conditions (10,700 and 12,000 IU/ml, respectively), demonstrating that aeration promotes the maximum of nisin titer in both batch and fed-batch fermentations. The higher nisin titer maybe comes from the higher cell concentration with aerobic culture. FVA achieved the maximum nisin production (15,367 IU/ml), compared with the other different fermentation strategies.

Table 1 Comparison of maximum biomass and yields of nisin with different fermentation strategies under anaerobic or aerobic fermentation conditions

Conditions	Initial glucose concentration (g/l)[a]	X_{max} (g/l)[b]	P_{nisin} (IU/ml)[c]	$Y_{N/X}$ (mg/g)[d]
Anaerobic batch culture	23	1.0	10,700	223
Aerobic batch culture	23	6.5	11,932	46
Fed-batch fermentation at variable rate of feeding under anaerobic conditions	6	0.9	12,000	250
Fed-batch fermentation at variable rate of feeding under aerobic conditions	6	6.5	15,367	59

[a] Initial glucose concentration with different fermentation strategies

[b] Xmax, maximum cell concentration

[c] PNisin, maximum nisin titer

[d] $Y_{N/X}$, correlation between nisin amount and cell concentration

It is expected that nisin production should be affected by aeration as O_2 tolerance of *L. lactis* is associated with different metabolic pathways which give different nisin yields. We have shown that nisin production is promoted under aerobic conditions which goes against the standard conditions involving strict anaerobiosis. Different fermentation strategies significantly affected nisin production, and gradual supplement of glucose prolonged the growth of *L. lactis*, which promoted nisin production, especially in the case of FVA. Glucose was shown to successfully culture *L. lactis* in this work, suggesting it would be a good option for the production of nisin in pilot scale fermentation.

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