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EFFECT OF VARIOUS NUTRIENT SOURCES ON NISIN PRODUCTION BY *LACTOCOCCUS LACTIS* SUBSP. LACTIS SM526

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Abstract *Lactococcus lactis* subsp., *lactis* strain SM526 isolated from a commercial sauerkraut fermentation was shown to produce a lantibiotic(nisin). The effects of different sources and their concentrations of carbon, phosphorus and nitrogen on SM526 growth and nisin production were investigated. Nisin biosynthesis seemed to be regulated by the sources of carbon, nitrogen and phosphate. Sucrose and KH_2PO_4 were found to be the most appropriate carbon or phosphorus sources, respectively. Nisin behaved as a primary metabolite, its formation took place during the active growth phase and totally stopped when cells entered the stationary phase. High nisin yields were also obtained using mixed media supplemented with soy peptone or yeast extract as nitrogen source. Nisin exhibited a bactericidal mode of action to the indicator strain—— *Lactobacillus plantarum*. Fig.3, Tab 5, Ref 14

Keywords *Lactococcus lactis* subsp. *Lactis*; nisin; primary metabolite; bactericidal
CLC Q939.118.06

乳酸乳球菌 SM526 产生乳链菌肽的营养调控

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摘 要 自酸泡菜中分离并鉴定了一株产生乳链菌肽的菌株 – 乳酸乳球菌(*Lactococcus lactis* subsp. *lactis*) SM526. 研究了不同种类与浓度的碳源、氮源和磷源对该菌产生乳链菌肽的影响,结果表明乳链菌肽的生物合成受碳源、氮、磷源的调控,蔗糖和磷酸二氢钾分别为其最佳碳源和磷源,大豆蛋白胨和酵母粉为其理想氮源. 利用分批培养研究乳链菌肽合成的代谢动力学则表明,乳链菌肽在 SM526 菌株的指数生长期产生,到指数生长期末期达到高峰,进入稳定期逐渐停止产生,表现出一种初级代谢动力学特征. 以植物乳杆菌(*Lactobacillus plantarum*)作指示菌株则表明乳链菌肽对细菌的作用方式是杀菌. 图 3 表 5 参 14

关键词 乳酸乳球菌;乳链菌肽;初级代谢;杀菌
CLC Q939.118.06

Lactic acid bacteria (LAB) are widely used for the fermentation and preservation of many kinds of food, such as milk, meat and vegetable[1]. The antimicrobial compounds produced by these bacteria play an essential role in ensuring the safety and extending the shelf-life of these products. Increasing consumers' demands for 'natural' and 'additive-free' products have led to greater interest in the application of natural inhibitory substances as food preservatives which could replace or reduce the use of chemical additives. LAB bacteriocins are biologically active proteins or protein complexes that act bactericidally against Gram positive bacteria, usually closely related to the producer strain. Bacteriocins have interesting

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application potential since bacteriocinogenic LAB used as starter cultures or bacteriocins used as food additives may be useful in controlling food spoilage and food borne pathogenic microorganisms[2]. The antimicrobial peptide nisin, as well as subtilin, epidermin and Pep5, belongs to the rapidly expanding family of lantibiotics[3~6], a group of ribosomally synthesized and posttranslationally modified peptide antibiotics which are characterized by the occurrence of the sulfur containing amino acid lanthionine and β-methyl lanthionine. The biosynthesis of nisin and other lantibiotics is thought to take place by successive enzyme-catalyzed modifications of the ribosomally synthesized precursor peptide. These specific modifications of precursor nisin include: dehydration of serine and threonine residues, resulting in dehydroalanine and dehydrobutyrine residues, respectively; the addition of free thiol groups of cysteine residues to the double bonds of dehydrated residues, resulting in *meso*-lanthionine and β-methyl lanthionine residues; cleavage of the leader peptide from the mature precursor nisin after secretion into the medium[2]. Nisin is the only lantibiotic intentionally used as a preservative in foods to date and has a well established history of safe use in foods with a constantly growing range of future application opportunities[7]. It is commercially produced by microbial fermentation. Several attempts to increase nisin yields by genetic manipulation prove to be unsuccessful. A better understanding of the nisin biosynthetic process and of its metabolic regulation seems to be a prerequisite for achieving improved nisin yields. In the present work we first describe the isolation and identification of a nisin-producing strain, *Lactococcus lactis* subsp. *lactis* SM526 and then the influence of various carbon, phosphorus and nitrogen sources and their concentrations on *L. lactis* subsp. *lactis* SM526 growth and nisin production.

1 MATERIALS AND METHODS

1.1 Bacteria and culture conditions

Strains used as indicator organisms for nisin screening were obtained from the culture collection maintained by the Institute of Microbiology, the Chinese Academy of Sciences. Nisin-producing *L. lactis* subsp. *lactis* ATCC11454 and ATCC7962 used as control strains were obtained from American Type Culture Collection, Rockville, Md. All cultures were stored at -70℃ in their appropriate growth medium with 160 g/L glycerol. Frozen stock cultures were streaked onto agar, and an isolated colony was transferred to M17 broth (Difco), with 5 g/L glucose (M17G), MRS (Difco), or Trypticase soy (TS) broth (BBL Microbiology Systems) before each experiment. When solid medium was required, 15 g/L agar (Difco) was added to the broth. Cultures were incubated at 32℃ (lactic acid bacteria) or 37℃ (non-lactic acid bacteria), unless otherwise indicated.

1.2 Strain isolation and characterization

Lactic acid bacteria were isolated from a local sauerkraut-manufacturing plant. Brine from tanks of sauerkraut which had been fermenting for 4 ~ 10 d was used to inoculate MRS and APT (Difco) agar slants, which containing 0.2 g/L sodium azide. Growth from MRS and APT slants was used to streak MRS or APT agar plates. Duplicate colonies of each morphological type arising on the plates were selected and purified on MRS agar. Lactococci were taxonomically identified to species level according to criteria given in Bergey's Manual of Systematic Bacteriology[8].

1.3 Identification of bacteriocin-producing strains

Bacteriocin production was determined by a modification of the agar spot test described by Fleming *et al*[9]. Overnight cultures were spotted (2 μL) onto TS agar plates. The cultures were incubated for 24 h before being overlaid with 3 mL of agar (7.5 g/L) inoculated with 3 μL of an overnight culture of the indicator strain. After further incubation for 24 h, colonies were examined for clear zones of inhibition surrounding them.

1.4 Bacteriocin assay and characterization

Bacteriocin production was assayed by a variation of the critical dilution method[10]. Culture supernatant (200 μL) was heated in a boiling-water bath for 5 min and cooled rapidly on ice. Serial two-fold dilutions of the heated supernatants were made in 0.02 mol L^{-1} HCl, and 10 μL of each dilution was spotted onto fresh, duplicate indicator lawns. The highest dilution yielding a clear zone of inhibition was defined as containing one arbitrary unit (A.U.) mL^{-1}. Indicator lawns were

prepared by overlaying agar plates with 3 mL of 7.5 g/L agar inoculated with 3 μL of an overnight culture of *Micrococcus flavus MG101*. *Cultures were incubated for 24 h*.

Bacteriocins were characterized with respect to proteinase sensitivity. Stock solutions of trypsin, protease K and α-chymotrypsin (Sigma) were dissolved (1 mg mL^{-1}) in 0.1 mol L^{-1} potassium phosphate buffer (pH 6.5), filter sterilized with a 0.22 μm syringe filter (Millipore Corp.), and stored at -20℃. Enzyme solution (10 μL) was added to 75 μL of bacteriocin preparation, and the mixture was incubated at 37℃ for 2 h. Heat-inactivated enzymes (100℃ for 10 min) served as controls.

1.5 Mode of action

Various concentrations of nisin were added to the indicator organisms ($n = 10^7$ mL^{-1}) in 0.05 mol L^{-1} sodium phosphate buffer (pH 6.8) and incubated at 30℃. At appropriate time intervals, the number of viable bacteria was determined by dilution and plating on TS agar plates.

1.6 Nisin Fermentation

Nisin was produced by *L. lactis* subsp. *lactis* SM526. Stock cultures were maintained at 4℃ in litmus milk medium[11] and transferred every three months. A vegetative culture was cultivated for 16 h at 32℃ in GM17 broth (M17 [Difco] medium with 5 g/L glucose). The initial pH was adjusted to 6.8. Cells were harvested and used as the inoculum. A complex medium was used for nisin production. It contained the following ingredients (ρ/ g L^{-1}): sucrose, 10; peptone (Oxoid), 10; yeast extract (Oxoid), 10; KH_2PO_4, 10; NaCl, 2; $MgSO_4.7H_2O$, 0.2. The initial pH of the medium was adjusted to 6.8. Nisin activity was detected by a bioassay with *M. flavus* MG101[12].

Fermentations were run in Erlenmeyer flasks (1000 mL) containing 700 mL production medium and in a 5-litre fermenter filled with 4 litres of medium to ensure microaerophilic conditions. A fresh culture of actively growing cells was always used as the inoculum; the inoculum size was always 1% (φ). The fermenter was operated at 32℃ with slow agitation (50 r min^{-1}) continuously provided to keep the fermentation broth homogeneous. The pH was measured (uncontrolled pH fermentations) or controlled continuously by automatic addition of 10 mol L^{-1} NaOH.

1.7 Growth determination

Growth was measured by cell dry weight (CDW) determinations, samples were centrifuged (10 000 × g, 15 min) and washed three times with saline (8.5 g/L) and dried at 105℃ to constant weight.

2 RESULTS AND DISCUSSION

2.1 Isolation and identification of bacteriocin-producing strains

For a total of 446 isolates, 9 strains produced antimicrobial compounds which inhibited one or more of the indicator organisms (*Bacillus cereus*, *Clostridium botulinum*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides*). One isolate, SM526, produces a compound which inhibited all four indicator strains; it was therefore selected for further characterization. SM526 was isolated from a commercial sauerkraut tank which was on the early stages (d 6) of fermentation with temperature 22℃, ρ(NaCl) = 10 g/L, pH 3.8, and a titratable acidity of ρ(lactic acid) 16 g/L.

SM526 exhibited growth characteristics similar to that of the control strain *L. lactis* subsp. *lactis* ATCC11454 and fermented N-acetylglucosamine, amygdalin, L-arabinose, arbutin, cellobiose, esculin, fructose, galactose, gentibiose, gluconate, glucose, lactose, maltose, mannose, ribose, salicin, sucrose, trehalose, and *D*-xylose. The strain was able to deaminate arginine and grow at 10℃, pH 9.2, and in the presence of 40 g/L NaCl, but was unable to grow at 4℃, 45℃, pH 9.6 or in 65 g/L NaCl. No gas was produced from glucose. x(G + C) of the chromosomal DNA of SM526 was determined to be 38.5%. On the basis of these data, SM526 was tentatively identified as *Lactococcus lactis* subsp. *Lactis*.

2.2 Bacteriocin characterization

SM526 produced a bacteriocin capable of inhibiting a broad range of Gram-positive bacteria, including *Bacillus subtilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium botulinum*, *Staphylococcus aureus*, *Staphylococcu albus*, *Streptococcus cremoris*, *Micrococcus flavus*, *Lactobacillus casei*, *Lactobacillus plantarum and Leuconostoc mesenteroides*. The

strain was resistant to nisin concentrations of at least 10 μg mL^{-1} (i.e. 400 IU mL^{-1}), and the bacteriocin it produced did not inhibit nisin-producing *L. lactis* subsp. *lactis* ATCC 11454 or ATCC 7962. Bacteriocin activity was stable to boiling for at least 10 min at pH 2.0, resistant to trypsin digestion, and sensitive to digestion by α-chymotrypsin and protease K. These characteristics are typical of nisin[3] and indicated that the bacteriocin produced by SM526 was nisin. To determine whether nisin was bactericidal or bacteriostatic, the indicator organisms were exposed to various concentrations of nisin. The number of surviving indicator bacteria was determined by plate counting. The results are summarized in Fig. 1, which shows that nisin exhibited a bactericidal mode of action.

2.3 Suitability of different carbon sources

In the first set of experiments, a large number of carbon sources were screened for their suitability to promote growth and give high nisin yields (Table 1). SM526 was found to assimilate all the tested carbon sources except for arabinose. Also sorbose was a rather poor carbon source for growth. A pronounced lag of growth was observed with xylose, galactose, lactose and trehalose (data not shown), which probably indicates the necessity for induction of enzymes specifically concerned with catabolism of these carbon sources. All other carbon sources tested were assimilated well by the bacterium. Despite good assimilation, growth on glucose, maltose and sucrose also led to accumulation of higher amounts of nisin. It is especially noted that sucrose, although a disaccharide, was a better carbon source than the corresponding monomers glucose and fructose (Table 1). Sucrose is rapidly utilized by nisin-producing *L. lactis* strains, probably because they possess a very efficient phosphoenolpyruvate-dependent phosphotransferase system for its uptake, transport and metabolism[13]. Further studies used sucrose as the carbon source.

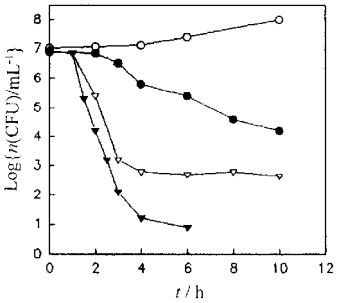

Fig. 1　The killing of *Lactobacillus plantarum* ML6 by various concentrations of nisin. The concentrations of nisin were: 0 (○), 2 (●), 20 (▽) and 200 (▼) μg/mL.

Table 1　Effects of various carbon sources on biomass and nisin production by *Lactococcus lactis* subsp., *lactis* SM526 at uncontrolled pH

Carbon source (ρ = 10 g/L)	Cell DW (ρ/g L^{-1})	pH$_f$	Nisin yield (ρ/mg L^{-1})	Carbon source (ρ = 10 g/L)	Cell DW (ρ/g L^{-1})	pH$_f$	Nisin yield (ρ/mg L^{-1})
Glucose	1.026	4.14	28.10	Arabinose	0.086	6.58	0.13
Mannose	1.089	4.18	23.52	Sucrose	1.298	4.12	33.74
Fructose	0.808	4.48	18.13	Maltose	1.106	4.20	29.38
Galactose	0.875	4.45	20.74	Trehalose	0.552	5.32	11.27
Sorbose	0.284	6.25	3.70	Cellobiose	1.122	4.26	24.16
Xylose	0.417	6.04	5.68	Lactose	0.950	4.36	22.93

2.4 Nisin production in batch fermentations

Nisin production by SM526 was studied in batch fermentation process using the complex medium described above. A fermentation profile of microbial growth and nisin production at uncontrolled pH is shown in Fig. 2. Nisin production appeared to parallel that of biomass. The highest nisin yield was reached at the end of the exponential phase, and corresponded with maximal biomass. So nisin was produced in the active growth phase, and its production fully stopped when cells entered the stationary phase. These properties differentiate nisin production from that of secondary metabolites[14]. However, because of a decrease in pH, owing to lactic acid accumulation, final high biomass and nisin levels could not be obtained. The low pH had an adverse effect on the growth of SM526. So fermentations were studied at controlled pH. Fig. 3 shows a batch fermentation profile of cell growth and nisin production at a controlled pH of 6.8. Neutralization of lactic acid by continuous addition of 10 mol L^{-1} NaOH increased the final biomass yield as well as the nisin yield. The nisin yield increased almost proportionally with growth and got its maximum at the end of the exponential growth phase, again corresponding with maximal biomass.

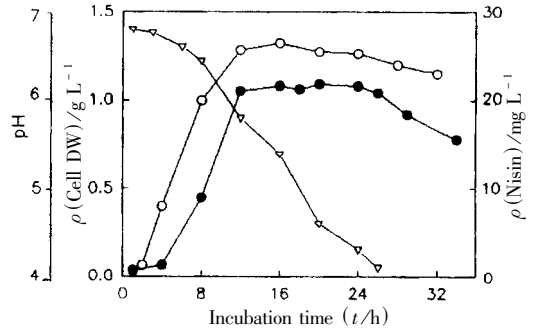

Fig.2 Batch fermentation profile of biomass and nisin production by *L. lactis* subsp., Lactis SM526 at uncontrolled pH. Cell DW, ○; nisin yield, ●; pH, ▽

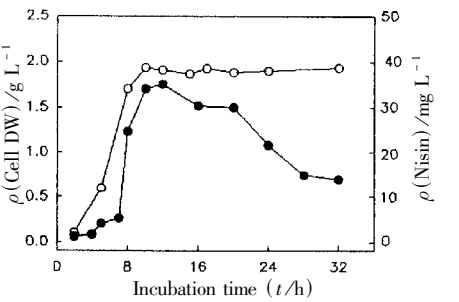

Fig.3 Batch fermentation profile of biomass and nisin production by *L. lactis* subsp., Lactis SM526 at controlled pH 6.8. Cell DW, ○; nisin yield, ●.

2.5 Effect of carbohydrate concentration

Since nisin production seems to be proportionally related to biomass yield, especially under pH-controlled conditions, achieving a high biomass may be a prerequisite for high nisin production. In order to improve nisin yield further by generating higher cell densities, higher concentrations of the carbon source (sucrose) were tested in fermentations run at a controlled pH of 6.8. The results are shown in Table 2. Higher concentrations of sucrose yielded a higher biomass, but did not increase the nisin yield concomitantly. At a level of 120 mmol L^{-1}, the biomass and nisin production amounted to 2.347 g Cell DW L^{-1} and 54.92 mg nisin L^{-1}, respectively. For high nisin yields, a critical amount of biomass seems to be a very important parameter. However, with sucrose concentrations higher than 180 mmol L^{-1}, both biomass and nisin yields decreased. At a level of 240 mmol L^{-1} sucrose, growth of SM526 was severely inhibited.

Table 2 Effect of initial sucrose concentration on biomass and nisin production by *L. lactis* subsp., lactis SM526 at controlled pH 6.8

Initial sucrose (c/mmol L^{-1})	Cell DW (ρ/g L^{-1})	Nisin yield (ρ/mg L^{-1})	Initial sucrose (c/mmol L^{-1})	Cell DW (ρ/g L^{-1})	Nisin yield (ρ/mg L^{-1})
30	1.192	31.53	150	1.658	39.72
60	1.514	38.13	180	0.773	21.64
90	2.086	49.24	210	0.428	13.84
120	2.347	54.92	240	0.098	1.02

2.6 Suitability of different phosphorus sources

Different phosphorus sources were screened for their suitability to promote growth and give high nisin yields by using the complex medium supplemented with 20 g/L sucrose and 10 g/L of an inorganic phosphorus source. The results are shown in Table 3. All the phosphorus sources tested were found to stimulate biomass and nisin production levels effectively, most probably as a result of creating favorable pH conditions caused by the buffering capacity of the phosphate source used. Among them, KH_2PO_4 was the most appropriate phosphorus source and gave the highest biomass and nisin production levels. It is especially noted that phosphate sources, containing ammonium as counter ion, results in lower biomass and nisin production levels.

Table 3 Effect of various phosphorus sources on biomass and nisin production by *L. lactis* subsp., *lactis* SM526 at uncontrolled pH

Phosphorus source (ρ = 10 g/L)	Cell DW (ρ/g L^{-1})	pH_f	Nisin yield (ρ/mg L^{-1})	Phosphorus source (ρ = 10 g/L)	Cell DW (ρ/g L^{-1})	pH_f	Nisin yield (ρ/mg L^{-1})
NaH_2PO_4	1.294	4.58	26.65	K_2HPO_4	0.898	4.34	27.65
Na_2HPO_4	0.869	4.12	24.56	$NH_4H_2PO_4$	0.725	4.40	16.08
Na_3PO_4	0.912	4.16	23.75	$(NH_4)_2HPO_4$	0.593	4.76	14.22
KH_2PO_4	1.402	4.28	37.58				

2.7 Effect of KH_2PO_4 concentration

Table 4 shows the effect of initial concentration of KH_2PO_4 on growth and nisin production by SM526. Increasing initial KH_2PO_4 concentrations drastically improved the nisin production levels. For instance, increasing initial phosphate

concentrations from 0 to 200 mmol L^{-1} KH_2PO_4 increased the nisin production levels to reach a maximum (ρ = 51.56 mg/L) at a level of 200 mmol L^{-1} KH_2PO_4. Here, increasing initial phosphate concentrations exerted a double effect, creating favorable pH conditions and particularly stimulating nisin production. However, c_{in} (> 250 mmol L^{-1}) of this phosphate source also drastically decreased both biomass and nisin production levels.

Table 4　Effect of initial KH_2PO_4 concentration on biomass and nisin yield at uncontrolled pH

K_2HPO_4 (c/mmol L^{-1})	Cell DW (ρ/g L^{-1})	pH_f	Nisin yield (ρ/mg L^{-1})	K_2HPO_4 (c/mmol L^{-1})	Cell DW (ρ/g L^{-1})	pH_f	Nisin yield (ρ/mg L^{-1})
25	0.680	4.17	24.33	200	1.547	4.32	51.56
50	0.894	4.23	33.50	250	1.297	4.77	39.60
100	1.262	4.20	44.95	300	0.262	6.02	4.38
150	1.485	4.45	47.08	350	0.106	6.50	1.04

2.8 Influence of nitrogen source

The influence of different nitrogen sources was tested on growth and nisin production by SM526, using the complex medium supplemented with 20 g/L sucrose and 10 g/L of the nitrogen source under investigation. The results are presented in Table 5. High biomass and nisin production yields were achieved in media containing yeast extract, meat extract and peptones. Casein hydrolysate, corn meal extract and malt extract were not good organic nitrogen sources. The highest nisin production level was obtained with soy peptone.

Table 5　Effect of various organic nitrogen sources on biomass and nisin yield at uncrotrolled pH

Nitrogen source (ρ = 10 g/L)	Cell DW (ρ/g L^{-1})	pH_f	Nisin yield (ρ/mg L^{-1})	Nitrogen source (ρ = 10 g/L)	Cell DW (ρ/g L^{-1})	pH_f	Nisin yield (ρ/mg L^{-1})
Casein hydrolysate	0.142	6.30	4.70	Peptone	1.050	4.28	26.14
Corn meal extract	0.106	6.52	2.52	Soy peptone	1.162	4.14	31.12
Cotton seed meal	0.798	4.85	18.20	Tryptone	0.920	4.31	34.06
Malt extract	0.546	4.64	11.23	Yeast extract	1.098	4.22	29.74
Meat extract	1.092	4.20	25.73				

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