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Inhibition of bacilli in industrial starches by nisin

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The properties of *Bacillus coagulans* and of other bacilli that contaminate paper and paperboard manufacturing processes were investigated under simulated industrial conditions. Nisin (0.05 to 0.125 μg ml^{-1}) blocked growth of indigenous bacilli that contaminate sizing starches. *B. coagulans* starch isolates, *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus* grew at $\geq$50°C in industrial starch and produced α-glucosidase and cyclodextrins. The industrial isolates and reference strains of *B. amyloliquefaciens, B. cereus, B. coagulans, B. flexus, B. licheniformis, B. pumilus, B. sporothermodurans, B. stearothermophilus* and *Alicyclobacillus acidoterrestris* were inhibited by $\leq$0.125 μg of nisin on agar. *B. coagulans* and *B. stearothermophilus* were similarly inhibited by $\leq$0.025 μg of nisin ml^{-1} and by 3 μg of the biocide DBNPA ml^{-1} in industrial starch. *B. licheniformis* and *B. amyloliquefaciens* strains were less sensitive. About 40% of nisin added to starch was retained after cooking. Fifty percent of the nisin remained active after 11 h of storage at 60°C. The results show that nisin has potential as a preservative for modified industrial starches. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 107–114.

Keywords: *Bacillus*; starch; biodeterioration; paper mill; nisin; thermotolerant

Introduction

Starches are bulk raw materials for the manufacture of food, paper, textiles, pharmaceuticals, and adhesives. The paper industry in North America uses over 2 million tons per year of modified starches for surface sizing, as wet-end additive and in coating [17,20]. In Finland the annual use of starch is 250 000 tons by the paper industry and 20 000 tons by the food industry.

Microbially deteriorated sizing starch impairs the printing quality of office and journal papers and endangers the hygienic quality of food-packaging paper and board [27]. Although enzymatic liquefaction of starch has been studied intensively [5,9,26,43], the microbiological events leading to hydrolysis and spoilage of industrial starches and their prevention are poorly understood [11,27,30,34]. There are few published papers on the microbial species capable of spoiling starch in a paper machine environment [23,40–42]. The preservation strategies using biocides to prevent spoilage are therefore chosen by trial and error.

Biocides are toxic by definition [35] and may be sensitising [8,10]. Surface-sizing starch is spread on paper or board at the dry end of the machine, representing the immediate interface to consumers' skin or to food in types of paper having no polyethylene extrusion coating. Understanding of microbial starch spoilage and the antimicrobial sensitivities of the spoilage organisms will facilitate the development of consumer-friendly starch-preservation strategies.

This paper describes aerobic spoilage microorganisms in industrial starches and their abatement using nisin.

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Materials and methods

Bacterial strains, inoculation and media

Aerobic starch-degrading isolates were picked from nutrient agar (LAB M, Bury, England) or from starch nutrient agar (nutrient agar with 0.2 wt.% of soluble starch, Merck, Darmstadt, Germany) incubated at 28 or 50°C. Origins of isolates and reference strains used in this study are listed in Table 1.

The bacterial density of the cultures was measured with a laser nephelometer (Minineph™, The Binding Site, Birmingham, UK) calibrated using Polybead® polystyrene 3-μm (*B. cereus*) or 1-μm (other bacilli) microspheres (PolySciences, Washington). Industrial starch medium contained chemically oxidised surface-sizing potato starch (20 g l^{-1}), yeast extract (0.1 g l^{-1}), Na_2HPO_4 (16 mg l^{-1}), malic, citric and lactic acids (50 mg l^{-1} each) and Tween 80 (0.1 vol.%), pH 6.1. All chemicals used were obtained from local sources and are of analytical quality unless otherwise stated.

Inhibition assays

The stock solution of commercial nisin (Sigma, St. Louis, MO), 50 μg (2000 IU) active substance ml^{-1} in 0.02 M HCl with 0.2% Tween 80, was stored at −20°C in the dark and neutralised with one volume of 0.02 M NaOH before dilution with water for use. 2,2-Dibromo-3-nitrilopropionamide (DBNPA) was used as the tradeware Daracide 7819 (Betz-Dearborn, Helsinki, Finland) containing 9% to 12% w/vol of the active substance.

The resazurin reduction assay [1] was performed on a microtiter plate (96 wells) with 330 μl of inoculated (10^6 cells ml^{-1}) industrial starch medium and 20 μl of resazurin (sodium salt, 0.05 mg ml^{-1} in water, ICN Biomedicals, Ohio) per well supplemented with nisin, DBNPA, EDTA, or nil as indicated. The plate was incubated stationary at 45°C and the fluorescence of the emerging semireduced form of resazurin (resorufin) was recorded at intervals of 15 min using the kinetic fluorometer Fluoroskan Ascent (Labsystems, Helsinki, Finland) and the filter pair 544/590 nm.

Table 1 The bacterial strains used and their starch depolymerization abilities

Code	Isolated from	Ref./source	Depolymerization of starch	Cyclodextrin α-production (7 d)	α-glucosidase production	T (°C) used
Paper industry isolates						
B. amyloliquefaciens TSP55	Food packaging board	this paper	+		+	50
B. cereus TSP6	Food packaging board	Pirttijärvi *et al*, 1999 [25]	–	–		37
B. cereus TSP2	Food packaging board	Pirttijärvi *et al*, 1999 [25]	+	+		37
B. cereus Cal1	Board machine	Pirttijärvi *et al*, 1999 [25]	+	+		37
B. coagulans S11	Surface-sizing starch, corn	this paper	+	+	+	50
B. coagulans S22	Surface-sizing starch, corn	this paper	+	+	+	50
B. coagulans S29	Surface-sizing starch, corn	this paper	+	+	+	50
B. coagulans S35	Surface-sizing starch, corn	this paper	+	+	+	50
B. coagulans SB2b-50-1	Surface-sizing starch, potato	this paper	+	+	+	50
B. flexus TSP3b	Food-packaging board	this paper	+	+		37
B. licheniformis TSP29a	Food packaging board	Salkinoja-Salonen *et al*, 1999 [37]	+	+[a]	+	50
B. pumilus TSP66	Food-packaging board	this paper	–	–		37
Other strains						
B. amyloliquefaciens DSM7^T	Soil	DSMZ	+	+		50
B. cereus DSM31^T	type strain	DSMZ	+	+		37
B. cereus F4810/72	Emetic food poisoning	Turnbull *et al*, 1979 [39]	–	–		37
B. cereus EE121	Emetic food poisoning	Pirttijärvi *et al*, 1999 [25]	–	–		37
B. cereus F5881/94	Emetic food poisoning	Pirttijärvi *et al*, 1999 [25]	–	–		37
B. cereus NS88	Live Norway spruce	Hallaksela *et al*, 1991 [12]	–	–		37
B. cereus NS117	Live Norway spruce	Hallaksela *et al*, 1991 [12]	–	–		37
B. cereus L9	Whey syrup	Pirttijärvi *et al*, 1998 [24]	–	–		37
B. coagulans DSM1^T	Evaporated milk	DSMZ	+	+	+	50
B. flexus DSM1320^T	type strain	DSMZ	+	+		37
B. licheniformis DSM13^T	type strain	DSMZ	+	+[a]	+	50
B. pumilus DSM27^T	type strain	DSMZ	–	–		37
B. sporothermodurans DSM10599^T	UHT-milk	DSMZ	–	–		37
B. stearothermophilus var. *calidolactis* DSM1550	Evaporated milk	DSMZ	+	+	+	55
A. acidoterrestris DSM3922^T	Garden soil	DSMZ				50

[a]Read after 3 days.

For the kinetic turbidometric assay a 100-well microtiter plate was filled as above except that no resazurin was used. Optical density was measured at 45°C using Bioscreen C (Labsystems) and a wide-band filter (420–580 nm). The plate was shaken before each measurement, once an hour.

The inocula were grown and the agar diffusion assay was carried out at 37 or at 50°C on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) pH 7.0, except for *A. acidoterrestris*, which was grown on potato dextrose agar (PDA, Difco), pH 3.5. An overnight culture of the test bacterium, 10^6 cells in 3.5 ml semisolid agar with 0.1 vol.% Tween 80, was spread on agar plates (90 mm) with 0.1 vol.% Tween 80. Nisin solution (5 μl) was placed on the inoculated agar surface and the plates were read after 24 h at 37 or at 50°C.

Analytical protocols

Whole-cell fatty acid analysis was performed as described by Väisänen *et al* [41] except that the biomass was grown at 50°C. Sherlock Microbial Identification System with the aerobic TSBA library version 3.90 (MIDI, Newark, DE) was used to identify the fatty acids. Partial 16S rRNA gene sequences comprising 488 nucleotide positions at the 5′-end of the gene were determined as described previously [31].

ATP content was measured using the ATP biomass kit (1243-118, BioOrbit, Turku, Finland) and a BioOrbit 1253 luminometer. Thermotolerant viable counting was done on plate count agar (PCA, Biokar Diagnostics, Beauvais, France) at 50°C; plates were read after 2 days.

To measure the α-glucosidase activity of the *Bacillus* strains, industrial starch medium was inoculated with 10^6 bacteria ml^{-1} and incubated for 18 h at 37 or at 50°C. α-Glucosidase activity of the cultures was measured using methylumbelliferyl-α-glucoside (MUF; Sigma) as substrate (1.4 mM final concentration). The culture (50 μl) and the MUF-substrate stock (10 μl) were dispensed in each well of the 96-well microtiter plate and incubated at 55°C. After 15 min pH was adjusted to 8.9 by adding 10 μl of 1 M Tris–HCl buffer to each well, and the fluorescence was measured using the filter pair 355/460 nm in the Fluoroskan Ascent. For the blank the fluorescence was measured without incubation.

Starch depolymerization was tested on agar containing 15 g soluble potato starch (Sigma S-4251), 2.5 g $NaNO_3$, 1 g K_2HPO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g NaCl, 1 mg $FeCl_3$ and 10 g agar per liter, pH 7. The plates were incubated for 7 days at 37, 50 or 55°C and cooled to +4°C. Depolymerization of starch was visible as a clearing zone around the colony and cyclodextrin

Table 2 Inhibition zones of selected starch-deteriorating bacilli toward nisin

	Codes	Inhibition zone to nisin (μg in 5 μl)			
		0.125	0.0125	0.00625	0.0025
B. amyloliquefaciens	TSP55	+	–	–	–
B. amyloliquefaciens	DSM7^{T}	+	+	+	–
B. cereus	EE121, F5881/94, TSP6, TSP2, Cal1, NS88, L9	+	–	–	–
B. cereus	F4810/72[a], NS117[a], DSM31^{Ta}	–	–	–	–
B. coagulans	DSM1^{T}, S11, S22, S29, S35, SB2b-50-1	+	+	+	–
B. flexus	TSP3b, DSM1320^{Tb}	+	+	+	+
B. licheniformis	TSP29	+	+	–	–
B. licheniformis	DSM13^{Ta}	–	–	–	–
B. pumilus	TSP66, DSM27^{T}	+	–	–	–
B. sporothermodurans	DSM10599^{T}	+	+	+	–
A. acidoterrestris	DSM3922^{Tb}	+	+	+	+

The tests were done on BHI agar, pH 5.8, or on PDA, pH 3.5 (*A. acidoterrestris*). Five microliter of diluted nisin was spotted on the inoculated agar surface. The induced inhibition zone was read after 24 h at the temperature indicated in Table 1. No inhibition zone was observed with $<1.25\times10^{-5}$ μg nisin.
(+) Inhibition zone (3–30 mm) present.
(–) Inhibition zone <3 mm.
[a]Transiently (<24 h) inhibited by 0.125 μg nisin.
[b]Sensitive to 1.25×10^{-4} μg nisin.

accumulation as a zone of white precipitate inside the clearing zone (van der Veen and Dijkhuizen, personal communication).

Nisin activity in industrial starch medium (pH 5.5) was bioassayed as described by Wahlström and Saris [44] using *L. lactis* subsp. *lactis* LAC182. Starch was sampled in 5 mM HCl (1:1), stored (<24 h) at room temperature in the dark and analysed for residual nisin. A standard curve was prepared using commercial nisin (Sigma) in industrial starch medium.

Results

Twenty-six thermophilic starch-degrading isolates of *Bacillus coagulans* were isolated from cooked surface-sizing starches, sampled from 10 storage tanks and feed tanks in paper mills. They grew well at 50°C and poorly at 28°C. The isolates were identified as *B. coagulans* based on the whole-cell fatty acid compositions compared to the type strain (all strains grown at 50°C). The partial 16S rRNA gene sequences (488 nucleotides) determined for the strains S11, S29 and SB2b-50-1 shared 100% similarity with that determined for *B. coagulans* type strain DSM1^{T}.

Depolymerization of starch by selected *B. coagulans* strains and other bacilli relevant to the hygiene of food-grade paperboard is shown in Table 1. All strains that depolymerized starch produced cyclodextrins. *B. coagulans*, *B. licheniformis, B. stearothermophilus*, and *B. amyloliquefaciens* reference strains and papermaking-related isolates grew well at 50°C and most of them also had α-glucosidase activity.

Table 2 records the presence or absence of inhibition zones by nisin of the studied bacilli from paper-industry-related sources, food and food-poisoning incidents. Starch isolates of *B. coagulans* (BHI agar, pH 5.8), *B. coagulans* type strain, *B. flexus, B. sporothermodurans* and *A. acidoterrestris* (PDA, pH 3.5) were very sensitive to nisin whereas *B. cereus*, *B. pumilus*,

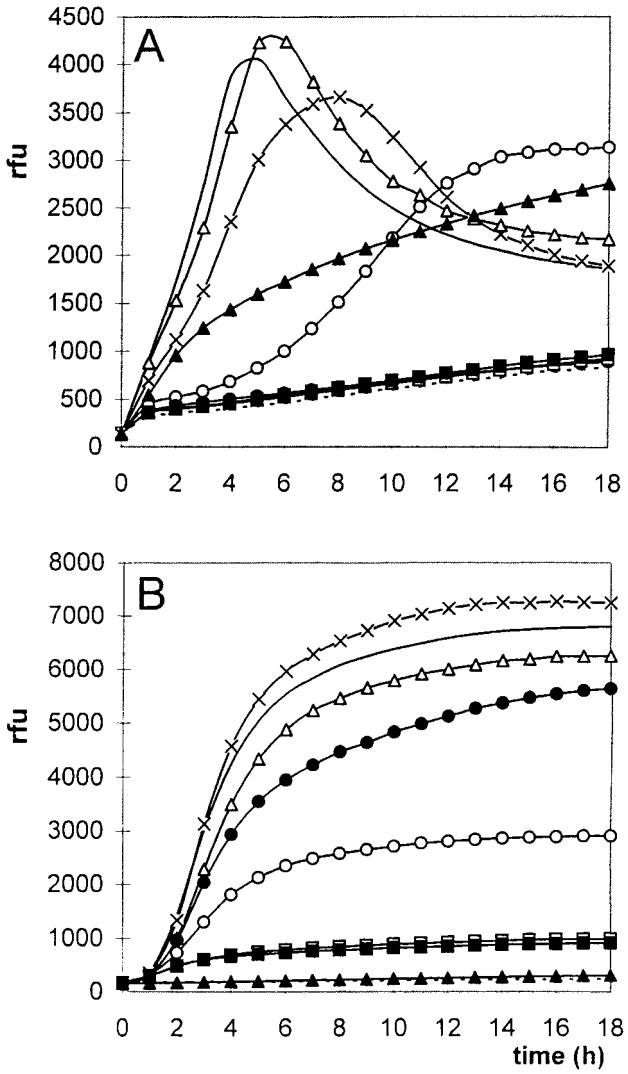

Figure 1 The metabolic inhibition by nisin, by 2,2-dibromo-3-nitrilopropionamide (DBNPA), and by EDTA of *B. coagulans* S11 (A) and of *B. stearothermophilus* DSM1550 (B). Cooked 2% industrial starch medium (pH 6.1) was inoculated with 10^{6} cells ml^{-1} and incubated without shaking at 45°C. The curves represent the time course of resazurin reduction as averages of four parallel measurements. Reduction of resazurin into resorufin was recorded by its fluorescence at 590 nm. The fluorescence curves obtained after treatments with 0.125 or with 0.5 μg of nisin ml^{-1} (not shown), with or without EDTA, were similar to those with 0.025 μg of nisin ml^{-1}. (–) No added inhibitor; (○) 0.0025 μg nisin ml^{-1}; (●) 0.0025 μg nisin ml^{-1} + 2 μg EDTA ml^{-1}; (□) 0.025 μg nisin ml^{-1}; (■) 0.025 μg nisin ml^{-1} + 2 μg EDTA ml^{-1}; (△) 0.5 μg DBNPA ml^{-1}; (▲) 3 μg DBNPA ml^{-1}; (×) 2 μg EDTA ml^{-1}; (···) no inoculum.

Table 3 Metabolic delay of selected starch-degrading bacilli by nisin, DBNPA, and EDTA in cooked 2% industrial starch medium using resazurin reduction as an indicator (cf., Figure 1)

Added inhibitor (μg ml^{-1})	Addition of EDTA (2 μg ml^{-1})	Postponement of emergence of the fluorescence peak; hours ± SD					
		B. coagulans				*B. licheniformis* TSP29a	*B. amyloliquefaciens* TSP55
		DSM1^T	S11	S29	SB2b-50-1		
None	+	−5.8±0.2	3.0±2.1	−1.0±0.0	−2.2±0.1	0.0±0.1	0.0±0.0
Nisin							
0.0025	−	5.3±0.7	12.8±0.9	10.5±1.3	>11	1.8±0.1	
0.0025	+	>11	>13.5	11±3.8	>11	1.8±0.4	
0.025	−	>11	>13.5		>11	3.5±0.3	3.8±0.8
0.025	+	>11	>13.5	>13	>11	3.5±0.8	4.9±0.8
0.125	−	>11	>13.5	>13	>11	7.5±1.6	8.8±1.8
0.125	+	>11	>13.5	>13	>11	9.9±1.2	10.7±3.3
0.5	−	>11	>13.5	>13	>11	>15.5	>16
0.5	+	>11	>13.5	>13	>11	>15.5	>16
2	−						>16
2	+						>16
DBNPA							
0.5	−	9.1±1.1	0.9±0.4	0.7±0.0	−0.6±0.1	0.1±0.0	0.2±0.2
3	−	>11	>13.5	2.1±0.0	>11	11.6±7.7	>16

Fluorescence peaks of *B. amyloliquefaciens* TSP55, *B. licheniformis* TSP29a and *B. coagulans* strains S11, S29, SB2b-50-1 and DSM1^T emerged 2, 2.5, 4.5, 5, 7 and 7 h after inoculation, respectively, when no inhibitor was present. The table indicates the delay in the emergence of the fluorescence peak caused by the presence of the inhibitor compared to the absence of the inhibitor. The test was performed in inoculated industrial starch medium (10^6 cells ml^{-1}, pH 6.1, 45°C). Averages and deviations were calculated from four parallel experiments.

B. licheniformis, and *B. amyloliquefaciens* were less sensitive. Three industrial contaminant strains of *B. cereus* and the type strain of *B. licheniformis* were only inhibited transiently by nisin.

Metabolic inhibition by nisin and by DBNPA, a commonly used biocide, was studied in thermotolerant starch-degrading strains of *Bacillus*. Figure 1 shows the metabolic sensitivities of an industrial starch contaminant, *B. coagulans* S11 (Figure 1A), and of *B. stearothermophilus* DSM1550 (Figure 1B) in industrial starch medium. With no added inhibitor, *B. coagulans* S11 showed a fluorescence peak, indicating reduction of resazurin, 4.5 h after inoculation with 10^6 cells ml^{-1}, a usual level of contamination in deteriorating starches in the process lines. Three micrograms of DBNPA ml^{-1} was required to attenuate the metabolism of *B. coagulans* S11 for >18 h. The same effect was achieved with 0.0025 μg of nisin ml^{-1} when 2 μg of EDTA ml^{-1} was present. In the absence of EDTA, 0.025 μg of nisin ml^{-1} was needed. EDTA alone (2 μg ml^{-1}) postponed emergence of the fluorescence peak of *B. coagulans* S11 for 3 h. Treatments with ≥0.025 μg nisin ml^{-1}, with or without EDTA, completely blocked the metabolism of *B. coagulans* S11 and of *B. stearothermophilus* DSM1550.

The sensitivities of the metabolism of *B. coagulans* S11 (Figure 1A) and five other *Bacillus* strains to nisin and DBNPA in industrial starch medium are compiled in Table 3. It shows that 0.0025 to 0.025 μg nisin ml^{-1} blocked the metabolism of all *B. coagulans* strains during the measurement period of 18 h, whereas *B. licheniformis* and *B. amyloliquefaciens* were less sensitive. The industrial *B. coagulans* isolates were as sensitive as the type strain. The data in Table 3 and Figure 1 thus show that 0.0025 to 0.025 μg nisin ml^{-1} were equally or more effective than 3 μg of DBNPA ml^{-1} against all *B. coagulans* and *B. stearothermophilus* strains. Industrial contaminant strains of *B. licheniformis* and *B. amyloliquefaciens* were equally sensitive to 0.5 μg of nisin ml^{-1} and to 3 μg of DBNPA ml^{-1}.

Figure 2 shows growth curves of *B. coagulans* S11 in industrial starch medium with or without added inhibitors. The observed increment of optical density in this medium was small, 0.1 O.D. units in 18 h, indicating that the medium, which is low

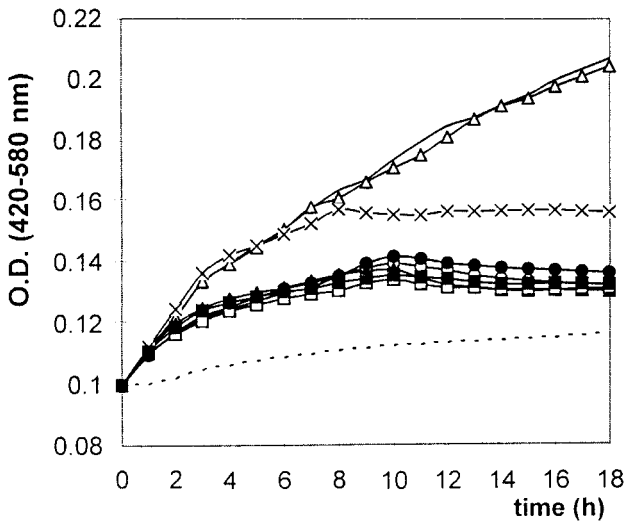

Figure 2 Growth inhibition of starch-degrading *B. coagulans* S11 by nisin, DBNPA and EDTA. The figure shows the time course of optical density increase of microtiter plate cultures of the strain S11 in cooked 2% industrial starch (pH 6.1) at 45°C as averages of five parallel wells. Turbidity curves obtained after treatments with 0.125 or with 0.5 μg nisin ml^{-1} (not shown), with and without EDTA, were similar to those with 0.025 μg of nisin ml^{-1}. Symbols are as in Figure 1.

Table 4 Growth inhibition by nisin, DBNPA, and EDTA of selected bacilli in cooked 2% industrial starch medium (inoculum 10^6 cells ml^{-1}, pH 6.1, 45°C)

Added inhibitor ($\mu g\ ml^{-1}$)	Addition of EDTA ($2\ \mu g\ ml^{-1}$)	Inhibition %±SD					
		B. coagulans				*B. licheniformis* TSP29a	*B. amyloliquefaciens* TSP55
		DSM1^T	S11	S29	SB2b-50-1		
None	+	no inhibition	33±4	12±3	25±1	no inhibition	no inhibition
Nisin							
0.0025	–	3±8	65±3	72±5	62±4	6±5	
0.0025	+	35±9	61±4	40±2	56±7	3±3	
0.025	–	42±5	70±3	87±9	64±5	6±4	15±6
0.025	+	53±3	66±5	73±20	62±3	22±4	no inhibition
0.125	–	57±3	70±1	88±3	65±1	38±7	60±6
0.125	+	55±5	69±1	84±1	66±3	42±9	57±12
0.5	–	57±3	64±3	80±2	52±4	66±8	89±3
0.5	+	60±5	65±3	83±2	53±3	63±4	81±11
2	–						81±5
2	+						85±6
DBNPA							
0.5	–	26±8	8±4	13±5	6±7	0±3	1±2
3	–	50±1	66±5	29±10	60±7	80±5	81±2

Inhibition is expressed as the decrease (%) of the area (arbitrary units) below the turbidity curves (18 h) compared to noninhibited controls (cf. Figure 2). Averages and deviations were calculated from five parallel measurements.

in nutrients and already contained an unknown amount and type of biocide supplied by the starch manufacturer, supported only slow growth ($\mu<0.5\ h^{-1}$). The presence of $\geq$0.0025 μg nisin ml^{-1} blocked growth. Results of tests similar to those presented in Figure 2 were obtained also for other industrial contaminant strains and for reference strains of *B. coagulans, B. licheniformis* and *B. amyloliquefaciens*, as summarised in Table 4. *B. stearothermophilus* DSM1550 did not grow measurably under the conditions used. The areas beneath the growth curves of four *B. coagulans* strains were effectively diminished by 0.0025 to 0.025 μg nisin ml^{-1}, compared to uninhibited controls. *B. licheniformis* and *B. amyloliquefaciens* strains were inhibited by $\geq$0.125 μg nisin ml^{-1}. Inhibition by nisin of the *B. coagulans* type strain but not of the other strains was enhanced by 2 μg EDTA ml^{-1}.

The effects of nisin and DBNPA on the ATP contents and on cultivability of the contaminant bacilli were measured after 18 h incubation. Nisin (0.0025 to 0.025 $\mu g\ ml^{-1}$) suppressed viable counts (Table 5) of the tested strains of *B. coagulans* and of *B. stearothermophilus*. For *B. licheniformis* and *B. amyloliquefaciens* strains, $\geq$0.5 μg nisin ml^{-1} was required to obtain the same level of inhibition as by 3 μg of DBNPA ml^{-1}. Industrial contaminant *B.*

Table 5 Viable counts of the *Bacillus* cultures (PCA 2 days, 50°C) 18 h after inoculation in industrial starch medium

Added inhibitor ($\mu g\ ml^{-1}$)	Addition of EDTA ($2\ \mu g\ ml^{-1}$)	log (cfu ml^{-1})						
		B. coagulans				*B. licheniformis* TSP29a	*B. amyloliquefaciens* TSP55	*B. stearothermophilus* DSM1550
		DSM1^T	S11	S29	SB2b-50-1			
Nisin								
0	–	7.2	7.7	7.8	7.8	7.4	7.6	4.0
0	+	4.5	6.9	7.2	7.3	7.7	7.4	3.9
0.0025	–	7.1	4.0	7.1	6.7	7.7		2.3
0.0025	+	3.1		7.3	7.0	7.8		3.2
0.025	–	6.8	3.9	1.7	4.4	7.3		2.8
0.025	+	4.4	3.9	4.5	2.0	7.8	>7	2.8
0.125	–	4.3	4.0	4.1	<1	7.6	7.0	2.7
0.125	+	4.6	3.9	4.0	<1	7.5	6.3	2.7
0.5	–		3.8	4.0	1.7	6.9	>7	<1
0.5	+	<1	3.9	4.0	1.3	5.0	>7	<1
2	–						2.7	
2	+						>3	
DBNPA								
0.5	–	6.7	7.4	7.7	7.6	7.7	1.4	4.0
3	–	<1	3.9	7.6	1.0	5.3	1.6	2.7

For experimental details see Table 4.

coagulans cultures (strains S11, S29, SB2b-50-1) lost >99% of ATP when 0.025 μg nisin ml^{-1} was present. Paperboard contaminant *B. licheniformis* TSP 29a and *B. amyloliquefaciens* TSP55 lost 88% and 91% of ATP, respectively, when 0.5 μg nisin ml^{-1} was present. Nisin sensitivity of the strains measured by ATP assay thus supported results obtained by the fluorometric resazurin reduction test. This was expected as both methods reflect effects on energy metabolism.

The conclusion from the results presented above is that the starch-degrading thermotolerant bacilli were effectively inhibited by nisin under conditions simulating those prevailing in the paper industry. To see the effect of nisin on a mixed culture situation such as is likely to occur in the industry, cooked surface-sizing starches from storage tanks (80°C) and from feed tanks (46–65°C) were sampled (Table 6). These contained the biocides used by the mills at the time of sampling (information on the effective substances or concentrations not available). Three (A, B, C) of the 10 samples, taken during 4 months, contained 50–400 cfu of thermotolerant (50°C) bacteria ml^{-1}. Nisin (0.05 or 0.125 μg ml^{-1}) effectively suppressed the thermotolerant colony counts of these during 24 h of incubation in the laboratory (Table 6). This is sufficient for industrial application, as the storage time of cooked starches at the mills is usually only a few hours.

Because the biocides used to preserve surface-sizing starches in paper mills gradually lose their activity, they are repeatedly dosed, i.e., into the starch slurry, into the storage tank of the cooked starch, and into the feed tank. To determine the stability of nisin, industrial starch medium containing 0.025 μg nisin ml^{-1} was "cooked" (5 min, 90°C), stored at 60 or 80°C and the residual nisin activity measured. Calibration showed that the accuracy of the assay in industrial starch was optimal at assay dilutions of 0.001 to 0.01 IU of nisin ml^{-1} (Figure 3A). The persistence of active nisin in starch during cooking and storage at 60 or 80°C was measured. About 40% of the added nisin (1 IU ml^{-1}) remained active after cooking, 50% of that remained active after 11 h of storage at 60°C, and somewhat less after storage at 80°C (Figure 3B).

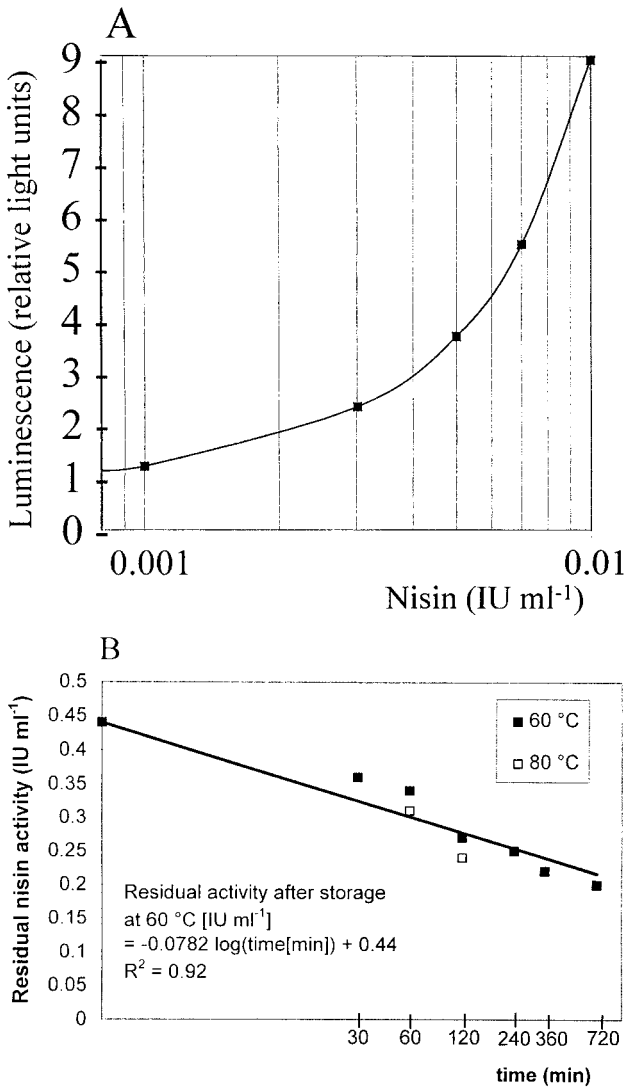

Figure 3 Assay and survival of nisin in cooked industrial starch. (A) Calibration curve for nisin obtained with *L. lactis* subsp. *lactis* LAC182 in industrial starch medium. The emitted luminescence by the strain LAC182 was measured after 3 h incubation with known amounts of nisin. 1 IU = 0.025 μg of active substance. (B) Residual nisin activity after storage at 60 or 80°C.

Table 6 Effects of nisin, DBNPA, and EDTA on the growth of indigenous bacteria in surface-sizing starches sampled from the feed tanks (46–65°C) of paperboard machines

Amendments in the laboratory	log (cfu ml^{-1})								
	Starch A		Starch B			Starch C		
	0 h	24 h	0 h	2.5 h	24 h	0 h	2.5 h	24 h
none	1.8	5.4	1.8	2	2.8	2.6	3	4.1
0.05 μg nisin ml^{-1}		n.t.[a]		1.1	<1		<1	<1
0.125 μg nisin ml^{-1}		<1		<1	<1		<1	<1
2 μg EDTA ml^{-1}		2.7		1.8	2.2		2.7	2.7
0.05 μg nisin ml^{-1} + 2 μg EDTA ml^{-1}		n.t.		1.5	<1		<1	<1
0.3 μg DBNPA ml^{-1}		6.0		1.9	3		n.t.	n.t.
3 μg DBNPA ml^{-1}		n.t.		1.5	3.7		2.7	2.6

Biocide-treated starches were sampled at the mills. The effective substances were routinely rotated at intervals of 2 to 4 weeks in the mills. Total thermotolerant colony counts (PCA 2 days, 50°C) were analysed in duplicate and the average is shown. The duplicates deviated ≤0.2 log units.
[a]Not tested.

Discussion

Bacterial contaminants are not evenly distributed inside paper and paperboard. Confocal laser scanning microscopy analyses have shown that the greatest number of microbes is located just underneath the polyethylene extrusion coating, and likely originated from surface-sizing starch [38]. The microbiological quality of surface-sizing starch is thus important for the hygienic quality of food-packaging paper and board.

In this study we used several parallel methods to test the sensitivity of paper machine and starch contaminants to antimicrobials. The fluorometric resazurin–resorufin method

was superior to the other methods used. This method is targeted to detect metabolic activity, indicated by reduction of resazurin. Resazurin becomes fluorescent at the semireduced state, indicating accumulation of NADH inside cells. The fluorometric resazurin–resorufin test measures bacterial metabolic activity rather than growth, and was therefore suitable for food-industry applications [1,19]. Our results show that it was useful for detecting starch spoilage in the paper industry because it is possible to observe emergence of the fluorescence peak long before any significant increase in turbidity. Compared to plate counting, the fluorometric test was less laborious and shortened the time required for spoilage detection from several days to less than 1 day. ATP determination was about equal in sensitivity but more laborious.

Turbidometry has been used succesfully in inhibition studies of bacilli from paperboard in process waters [29] but its use for monitoring microbiological contamination in starch is seriously limited by the turbidity of most industrial starches. Similar problems were observed with paper industry mineral pigments [21]. The fluorometric resazurin reduction-based assay may also be an option for the microbiological quality control of other highly turbid raw materials like pulps, resin sizers, neutral sizers, and latexes.

The spoilers of starch in the paper industry are aerobic sporeforming bacteria [40,42]. Bacterial spores in starch survive cooking and storage at 60–80°C and may proliferate in the machine feed tank maintained at 45–65°C. *B. amyloliquefaciens*, *B. coagulans*, *B. licheniformis*, and *B. stearothermophilus* grow at 50–55°C and produce heat-stable starch-hydrolysing enzymes [9,28,40]. *B. circulans* and *B. subtilis* rarely grow at >50°C [28] but do produce heat-activable α-amylases and α-glucosidases with optimum temperatures of 50 to 90°C [9,40,43]. Formation of cyclodextrins from starch by cyclodextrin glycosyltransferases (CGTases) and their subsequent uptake and intracellular degradation by cyclodextrinases (CDases) is an alternative pathway to the combined actions of amylases and exo-glucosidases, especially among different bacilli [9,15,26,43].

B. coagulans was the most common contaminant in the surface-sizing starches analysed in this study. Cyclodextrins were produced by all starch depolymerizing isolates from paper industry-related materials, and also by the other tested strains belonging to the species *B. amyloliquefaciens*, *B. cereus*, *B. coagulans*, *B. flexus*, *B. licheniformis*, or *B. stearothermophilus*. The results indicate that cyclodextrin production is an important pathway for degradation of starch in the paper industry environment. α-Glucosidase activity was also commonly found.

Bacillus species are difficult to eliminate from the industrial process environment using biocides [36]. Nisin (E234) is an antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis*. It is authorised for food preservation in more than 50 countries [6] and it has been used to prevent spore outgrowth, e.g., in processed cheese, cheese spreads, canned vegetables and other heat-processed foods [6,7]. Heating sensitizes spores to nisin [6,7].

Nisin in concentrations of 0.0025–2.5 μg ml^{-1} (=0.1–100 IU ml^{-1}) is useful for inhibiting different bacilli [Refs. [2,16,32], this paper]. Spores of thermophilic bacteria such as *Bacillus stearothermophilus* and *B. coagulans* have been reported to be particularly sensitive [6,13,33]. Both vegetative cells and spores of bacilli are sensitive to nisin [7,32].

Our results show that 0.05–0.125 μg of nisin ml^{-1} (2–5 IU ml^{-1}) effectively preserved surface-sizing starches sampled from paperboard processes. Pure cultures of *B. coagulans* and *B. stearothermophilus* (industrial starch contaminants and reference strains) were even more sensitive. The concentration of nisin required to inhibit *B. licheniformis* and *B. amyloliquefaciens* in industrial starch (0.5 μg ml^{-1}) was close to that reported to be inhibitory to *B. licheniformis* spores in milk (25 IU ml^{-1}) [18]. Our results show that a major amount of nisin remained active under conditions simulating cooking and storage of preserved starch in the industry.

Although nisin inhibited all bacilli tested in the agar diffusion assay, *B. cereus* strains (type strain, industrial, environmental and food poisoning isolates) were among the least sensitive. Maintaining high temperature during storage of cooked starches (>50°C) is thus important for controlling growth of *B. cereus* and other mesophilic bacilli.

A. acidoterrestris and *B. sporothermodurans* are relatively new food contaminant organisms and have not yet been reported in packaging materials. *B. sporothermodurans* produces exceptionally heat-resistant endospores [22] and the acid-tolerant and heat-resistant *A. acidoterrestris* is a worldwide spoilage problem in pasteurized and heat-treated juices [4,14]. We show here that these species were also effectively inhibited by nisin, analogous to the findings for *A. acidoterrestris* in fruit juices [16].

Blackburn [3] observed that the inhibitory effect of nisin against vegetative Gram-positive bacteria at pH >5 was improved by the presence of 0.01–3 mM EDTA (=3–1000 μg ml^{-1}). We found synergism with 2 μg EDTA ml^{-1} under paper machine-simulating conditions with one *Bacillus* strain out of seven strains tested. Higher EDTA concentrations were not tested because they are not technically applicable in paper mills.

Our results show that nisin has potential for preservation of starch in industrial processes (Salkinoja-Salonen and Pirttijärvi, patent applied for).

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