Journal of Industrial Microbiology, 4 (1989) 419–428
Elsevier

SIM 00196

Enzymatic characterization of *Bacilli* from food packaging paper and board machines

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Received 16 August 1988
Accepted 14 January 1989

Key words: Bacillus; Paper and board machines; Starch degrading enzymes; Cellulase; Proteases; Slimicides; Food packaging

SUMMARY

Aerobic spore-forming bacteria were found dominant in the microflora of food packaging paper and board. Twenty-five strains of bacteria belonging to the genus *Bacillus* were isolated from these paper and board machines, papermaking chemicals, and final products of papermaking. Nineteen strains were analyzed for production of α-amylase, α-glucosidase, glucoamylase, pullulanase, β-glucanase, carboxymethyl cellulase, and caseinase, and also for resistance towards industrial biocides. pH and temperature optima for the activity of the enzymes were determined. All strains were found to produce one or more of the enzymes studied. The amylolytic enzymes of most strains had high temperature optima for activity. Vegetative cells of all strains were found very resistant towards the different commercial slimicides used in paper and board mills. This property together with the ability to survive through the dry end of the machine to the final board and paper, and the production of enzymes degrading papermaking chemicals makes these bacteria potentially harmful in paper and board mills.

INTRODUCTION

Microorganisms can disturb the manufacture of food packaging board and paper in many different ways. Firstly, the pulp (cellulose) itself as well as most of the papermaking chemicals are biodegradable and microbial contamination may thus impair their technical performance. For example starch-degrading microorganisms may hydrolyze the native, oxidized and derivatized starches that are used as sizing agents and as stabilizers of mineral pigment slurries and resin sizers. Casein is another frequently used stabilizer of paper making chemicals and similarly biohydrolyzable. Secondly, microbial infection of the papermaking chemicals may cause odor, taste, and undesirable coloring of the paper or board product [8]. Thirdly, the heating of the paperboard by the drying drums of the machine may be too short to kill the microorganisms inside

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the board or paper, resulting into microbially contaminated end product [3,8]. This lowers the hygienic quality of the product and is especially undesirable in the manufacture of aseptic food packaging materials.

Paper machine is an open system and cannot be operated aseptically. The use of biocides is in most countries regulated in food quality papermaking to a level too low to be generally desinfecting. Therefore additional selective action is needed towards the microorganisms that cause the problems. The microbial ecosystem of paper machines is poorly known. The white water and some papermaking chemicals may contain 10^3 to 10^9 viable bacteria per ml [8,10,26]. We examined four paper and board machines at three different mills and found high densities of both Gram-negative and Gram-positive bacteria. The dominant Gram-positive bacteria were members of the genus *Bacillus*. Several members of this genus are known to excrete proteases and starch-degrading enzymes [21]. *Bacilli* also form heat resistant spores and are among the most resistant bacteria against agents used for sterilizing packaging materials before aseptic filling [3]. They therefore are potential troublemakers at the paper and board machines. We isolated and identified 25 different strains of *Bacillus* from paper and board machines, additives used, and the final products. In this paper we describe these *Bacilli* and their production of proteolytic and amylolytic enzymes, the activity of these enzymes at the various pH and temperatures prevailing in the mill, and the relative sensitivites to industrially used biocides.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and their origins are listed in Table I. Bacteria were grown (unless otherwise stated) at 28°C on starch-nutrient medium containing per litre: 3 g of beef extract, 5 g of yeast extract, 5 g of peptone, 10 g of potato starch, and 5 g of NaCl. Agar plates contained 1% of agar. For enzyme assays the bacteria were grown shaking in liquid medium.

Isolation of bacteria

Bacteria were isolated as follows: The sample was homogenized in sterile water (1 g/5 ml for slimes and papermaking chemicals, 1 g/100 ml for paper and board) and pasteurized at 80°C in water bath for 10 min. Both before and after pasteurizaton homogenized samples were spread on starch-nutrient agar, tryptone glucose yeast agar (TGY, plate count agar, [9]), and half-strength trypticase soy agar [15]. The plates were incubated at room temperature for a few days. Pure cultures were obtained with repeated streaks on respective media. Since all isolated spore-forming strains grew on starch-nutrient agar, this medium was used for subsequent cultivations.

Viable count assays

Pasteurized and non-pasteurized homogenized paper and board samples were assayed on plate count agar by the pour plate method [9,24].

Identification of bacteria

The bacteria were identified by morphological and physiological characteristics determined according to Bergey's Manual of Systematic Bacteriology [5].

Enzyme assays

Buffers used for enzyme assays were as follows (unless otherwise stated): pH 4.6: 0.1 M sodium acetate, pH 6.8: 0.05 M Tris-acetate, and pH 9.4: 0.1 M glycine NaOH.

Hydrolysis of starch. Ability of strains to hydrolyze starch was tested on agar plates using Lugol's iodine solution to detect undegraded starch [14].

α-amylase. Activity of α-amylase was determined from supernatants of three days old liquid cultures by measuring the reduction in iodine-staining capacity of starch [22]. The measurements were made with potato starch (Merck; Soluble starch) as substrate at 30°C, 45°C, 60°C, and 70°C with a reaction time of 10 min. One unit (U) of enzyme activity was defined as the amount of enzyme which produces 10% reduction in the intensity of blue colour of iodine complex of 0.5 mg starch in 10 ml in 10 min under the conditions used.

Table 1

Paper and board machine bacteria and their origin

Strain	Identified as	Site of isolation[a]
E47	*B. brevis*	Pink slime from wire wet end of machine 1
K8	*B. cereus*	Slime from top wire (hard wood kraft pulp) of machine 4
K9	*B. cereus*	Same as K8
E45	*B. circulans*	High cationic potato sizing starch slurry, 55–65°C, pH 4.8
E85	*B. circulans*	Liquid packaging board made on machine 1
S97	*B. circulans*	Grease-proof pergamin paper of Ca-sulphite pulp
E2	*B. licheniformis*	Pink slime from wire of machine 1
E3	*B. licheniformis*	White slime from suction roll of machine 1
E4	*B. licheniformis*	Green slime from wire of machine 3
E5	*B. licheniformis*	Pink slime from wire dry end of machine 1
E16	*B. licheniformis*	Slime from wire of machine 2
E46	*B. licheniformis*	Pink slime from wire wet end of machine 1
E53	*B. licheniformis*	Same as E16
E54	*B. licheniformis*	Same as E16
K7	*B. licheniformis*	Slime from middle wire (ground soft wood) of machine 4
K17	*B. licheniformis*	Same as K7
E6	*B. megaterium*	Slime from wire wet end of machine 1
K2	*B. pumilus*	Sizing resin, 30°C, pH 6.0
S96	*B. pumilus*	Same as S97
E100	*B. subtilis*	Surface sizing starch, 55–65°C, pH 6.5
E101	*B. subtilis*	Same as E100
E102	*B. subtilis*	Same as E100
K1	*B. subtilis*	Same as K2
S98	*B. subtilis*	Same as S97
E1	*B. thuringiensis*	White slime from wire wet end of machine 1

[a] Machine 1: Alkaline machine for bleached kraft pulp liquid packaging board (60% hard wood), 39–47°C, pH 9.0 in head box. Machine 2: Machine making liquid packaging board from bleached kraft pulp (70% hard wood), 35–41°C, pH 5.4 in head box, pH 4.4 in wire water. Machine 3: Machine making mineral coated kraft bleached board for fast food packaging (>90% hard wood), 39–46°C, pH 8.6 in head box. Machine 4: 3-wire board machine using mechanical pulp in middle wire (31°C, pH 4.7) and kraft pulp on top (30°C, pH 3.8) and bottom, (>90% soft wood).

α-glucosidase. Activity of α-glucosidase was measured both from culture supernatants and from cell suspensions after sonication (0.12 g wet cells per 5 ml of medium) of 1–2 days old cultures by the *p*-nitrophenyl-α-D-glucopyranoside (Sigma) procedure [25] at 30°C, 45°C, 60°C, and 70°C. pH 6.8 buffer was 0.067 M potassium phosphate. One unit (U) of enzyme activity was defined as the amount of enzyme which hydrolyzes 1 μmol *p*-nitrophenyl-α-D-glycopyranoside per minute under the conditions used.

Glucoamylase. Glucoamylase activity of 2–3 days old culture supernatants was measured by the amount of glucose formed from starch at 30°C at pH 4.6, and at pH 6.8 (0.067 M potasssium phosphate buffer) in 60 minutes [4]. The glucose was measured with the UV-test of Boehringer Mannheim (No. 716 251) . One unit of enzyme activity was defined as the amount of enzyme which produces μmol of glucose per min under the conditions used.

Pullulanase. Pullulanase was screened for on test plates with nutrient agar containing 0.1% pullulan from *Aureobasidium pullulans* (Sigma). After 2–3 days of growth the plates were flooded with cold ethanol, kept and read after 6 h to overnight at +4°C [19]. Production of pullulanase is seen as a clear halo around the colony.

Pullulanase activity of supernatants of 2–3 days old cultures was assayed for with 3,5-dinitrosalisylic acid (DNS) method with pullulan as substrate [20] at 30°C, 45°C, 60°C, and 70°C with reaction time of 30 min. One unit (U) of enzyme activity was defined as the amount of enzyme which releases 1 μmol of glucose equivalents per minute under the conditions used.

β-glucanase. β-glucanase was detected with the staining method of Cantwell and McConnell [2] by using 0.4% (w/v) barley β-glucan (Sigma) as substrate in nutrient agar.

Carboxymethyl cellulase (CMCase). CMCase was screened for using the plate assay of Cantwell and McConnell [2] with 1% (w/v) carboxymethyl cellulose (CMC) in nutrient agar as substrate.

CMCase activity was determined from supernatants of two days old cultures grown in nutrient broth with filter paper (1 g^{-1}) as inducer. The activity was measured using the DNS method with CMC as substrate at pH 4.8 (0.05 M sodium citrate buffer) and at pH 7.0 (0.018 M citric acid, 0.165 M sodium phosphate buffer) at 50°C with reaction time of 3 h [16]. One unit (U) of enzyme activity was defined as the amount of enzyme which releases 1 μmol of glucose equivalents per min under the conditions used.

Caseinase. Caseinase was screened for using milk agar plates [5]. Caseinase activity of supernatants of three days old cultures grown in nutrient broth was determined using casein (Hammarsten; Serva) as substrate [23] at 30°C, 45°C, 60°C, and 70°C at pH 7.4 (0.05 M Tris·HCl buffer). One unit (U) of enzyme activity was defined as the amount of enzyme which releases an equivalent of 1 μmol tyrosine per minute under the conditions used. The effect of inhibitors ethylenediaminetetraacetic acid (EDTA, 1 mM) and phenylmethylsulphonylfluoride (PMSF, 0.1 mM) on caseinase activity was determined according to Markkanen and Bailey [17].

Sensitivity to chemical slimicides

Minimal inhibitory concentrations (MIC) were determined as follows: Overnight broth culture was pipetted onto microtiter plates, from which inocula were replicated on nutrient agar plates (pH 7) containing different concentrations (1–1000 μl l^{-1}) of slimicides. The plates were incubated at 28°C and read after 18 h.

RESULTS

Fig. 1 shows the proportion of pasteurization surviving bacteria of the total aerobic viable bacterial count of different types of food packaging paper and board. It is seen that in most samples >50% of the total colony count were resistant to pasteurization. In several samples the proportion of pasteurization surviving bacteria exceeded 100% indicating heat activation of spores during pasteurization. At lower bacterial counts the effect of experimental error is considerable due to inhomogeneity of the sample slurries. Since the contribution of pasteurization survivers is essential, we considered it important to study spore-forming bacteria present in paper and board mill environment. We isolated a great number of strains from paper and board machines, papermaking chemicals, and end products of papermaking. Twenty-five of such aerobic spore-forming bacteria were taken into further study. All of these strains were identified as species

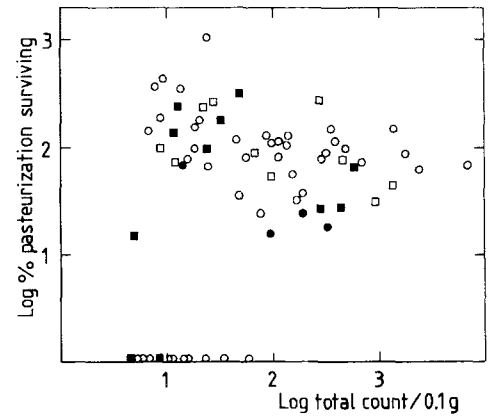

Fig. 1. Pasteurization surviving bacteria of the total aerobic heterotrophic plate count of food packaging paper and board samples. The percentage of pasteurization surviving bacteria is plotted over the non-pasteurized total count of the same samples. The paper and board samples were: ○: board made on machine 1 (see Table 1); □: board made on machine 3; ●: board made on machine 4; ■: grease-proof pergamin paper.

Table 2

Hydrolysis of glucose polymers and protein by the *Bacilli* from paper and board machines tested by plate assays

Bacterial strain	Hydrolysis[a] of				
	Starch	*Pullulan*	*β-glucan*	*CM-cellulose*	*Casein*
B. brevis E47	−	−	−	−	+
B. cereus K8	+	+	−	+	+
B. circulans E45	(+)	−	−	+	−
B. circulans E85	+	−	+	+	−
B. circulans S97	+	−	−	+	−
B. licheniformis E3	+	−	−	+	+
B. licheniformis E16	+	−	−	+	+
B. licheniformis E46	+	−	−	+	+
B. licheniformis E53	+	−	−	+	+
B. licheniformis E54	+	−	−	+	+
B. licheniformis K7	+	−	−	+	+
B. licheniformis K17	+	−	−	+	+
B. megaterium E6	+	+	−	−	+
B. pumilus K2	−	−	+	+	+
B. pumilus S96	−	−	+	+	+
B. subtilis E100	+	−	+	+	+
B. subtilis E101	+	−	+	+	+
B. subtilis S98	+	−	+	+	+
B. thuringiensis E1	+	−	−	−	+

[a] +: clearing zone around the colony; (+): clearing only underneath the colony; −: no clearing around or underneath the colony.

of the genus *Bacillus* with physiological and biochemical tests (Väisänen and Salkinoja-Salonen: Use of phage typing and fatty acid analysis or the identification of *Bacilli* isolated from food packaging paper and board machines; System, Appl. Microbiol., in press). These strains and their sites of origin are presented in Table 1.

We studied the production of the hydrolytic enzymes that could cause problems in paper and board making. Table 2 displays the hydrolytic activity of these strains towards glucose polymers and protein. It shows that most of the strains hydrolyzed starch, carboxy methyl cellulose (CMC), and casein. A few strains also degraded β-glucan and two strains hydrolyzed pullulan.

The temperature and pH optima of the hydrolytic activities of these enzymes are shown in Table 3. Comparison of the results in Table 2 to those in Table 3 shows that while almost all *Bacilli* hydrolyzed starch in plate assay, only *B. subtilis* strains, *B. thuringiensis*, *B. circulans* E85 and S97, and *B. licheniformis* E3 produced measurable amounts of extracellular α-amylase. The optimum pH for amylase activity was around 6.8 for all strains. The temperature optima were around 60°C to 70°C, except for *B. thuringiensis* and *B. circulans* E85 amylases, which had temperature optima of 30–45°C and 45–60°C, respectively.

All strains except *B. brevis*, *B. circulans* E45, *B. licheniformis* E46, E53, E54, and *B. pumilus* S96 produced cellbound but not extracellular α-glucosidase activity. The strains produced enzymes with high temperature optima, around 60°C or 70°C for all except *B. licheniformis* K17 α-glucosidase, which had the optimum temperature of 30°C. *B. licheniformis* E3 and E16 enzymes showed alkaline pH optima, and *B. licheniformis* K17 and *B. pumilus* K2 enzymes showed acidic pH optima, all others being optimally active at neutral pH.

B. cereus K8 and *B. megaterium* E6 degraded pul-

lulan on plates, but no strain showed pullulanase activity in liquid medium. None of the paper and board mill *Bacilli* produced glucoamylase (activity was <0.01 U in a volume of culture supernatant corresponding to one mg dry cells).

Most of the strains could break β-glucosidic linkages. We found CMCase activity in all strains except *B. brevis, B. licheniformis* E53, *B. megaterium, B. pumilus* K2, and *B. thuringiensis* (in plate assay only *B. brevis, B. megaterium,* and *B. thuringiensis* were negative), although the activities were very low. *B. pumilus, B. subtilis,* and *B. circulans* E85 degraded also barley β-glucan, which has β-1,3-linkages in addition to β-1,4-linkages.

Results in Tables 2 and 3 show that casein hydrolysis was a common feature of the paper and board mill *Bacilli;* only *B. circulans* strains did not produce caseinase. Table 3 shows the response of the proteases to inhibitors, EDTA which inhibits metalloproteases, and PMSF which inhibits serine proteases. Proteases of *B. licheniformis* E3, E16, E46, and E54, and *B. pumilus* K2 and S96 were strongly inhibited by PMSF, and thus these strains produced only serine protease. Protease of *B. subtilis* E101 was inhibited only by EDTA, and thus this strain produced metalloprotease. Proteases of *B. brevis, B. licheniformis* K17, *B. megaterium,* and *B. subtilis* S98 were inhibited by both EDTA and PMSF, and might thus be of the metalloserine protease type [21]. *B. subtilis* E100, *B. cereus* K8, and *B. thuringiensis* E1 proteases were not or only slightly inhibited by PMSF or EDTA, suggesting that these strains produced a fourth type of protease. The optimum temperature for caseinase activity varied from 30°C to 60°C, being as a whole lower than for α-amylase and α-glucosidase activities.

Table 4 shows the sensitivity of the strains to commercial slimicides used in paper and board mills. It is seen that the response of all strains was similar towards each of the 14 different slimicides with 11 different active substances. The resistance patterns of paper and board mill *Bacilli* were found to be similar to those of the type strains of the corresponding species (data not included in Table 4). Methylenebisthiocyanate, 8-hydroxyquinoline acetate, and 1,2-benzisothiazolin-3-one most effectively inhibited growth of these *Bacillus* strains at pH 7. Different commercial formulations with the same active component (slimicides 4a and 4b; 8a, 8b, and 8c, respectively) did not differ in their effect on the *Bacilli.*

DISCUSSION

Bacilli were found to predominate the aerobic flora of different types of paper and board. The presence of viable *Bacilli* in food quality paper and board mill is not only a hygienic problem but the members of the genus *Bacillus* are also known as producers of a wide spectrum of extracellular enzymes, the most important of which are amylolytic enzymes and proteases [21]. We studied the production of several hydrolytic enzymes by 19 paper and board mill *Bacilli,* and found that one or more of the enzymes were produced by each of these strains.

Although we only studied mesophilic *Bacilli,* the carbohydrate degrading enzymes produced by these strains had high temperature optima for activity (50–70°C) except for two strains. This means that paper and board mill ecosystem favours bacteria the produce carbohydrate degrading enzymes with high temperature optima. The presence of such strains is dangerous for sizing starches, which are stored and pumped at high temperatures (55–65°C). As the results showed, most of the strains producing α-amylase with high temperature optimum originated from starches or final products. We have also observed that starch present in sizing materials is degraded.

In the literature temperature optima of 50–65°C have been described for α-amylases from mesophilic *Bacilli,* except for a *B. licheniformis* α-amylase, the optimum temperature for which has been reported 76°C [6].

Although α-glucosidase has been reported in *Bacilli* to occur extra- or intracellularly or both [6], we found in these paper and board mill strains only cellbound α-glucodidase activity, which also had high temperature optimum. In the literature such enzymes have been described in thermophiles only [6,11,25]. Two strains produced an α-glucosidase,

Table 3

Activity[a], and temperature and pH optima[b] of enzymes degrading glucose polymers and protein produced by the *Bacilli* from paper and board machines

Bacterial strain	Extracellular enzymes									Cellbound		
	α-amylase			Pullulanase	CM-cellulase[c]	Caseinase				α-glucosidase		
	Activity (U)	Optimum		Activity (U)	Activity (U × 10^3)	Activity (U)	Inhibition (%) by		Optimum T(°C)	Activity (U)	Optimum	
		T(°C)	pH				EDTA[d]	PMSF[d]			T(°C)	pH
B. brevis R47	<4			<0.1	<2	11	68	100	30	<1		
B. cereus K8	<4			<0.1	11	8	0	0	60	33	45–60	6.8
B. circulans E45	<4			<0.1	4	<2				<1		
B. circulans E85	93	45–60	4.6–6.8	<0.1	21	<2				13	60	6.8
B. circulans S97	136	60	6.8–9.4	<0.1	34	<2				5	60–70	6.8
B. licheniformis E3	24	60	6.8	<0.1	22	24	0	100	60	90	70	9.0[e]
B. licheniformis E16	<4			<0.1	16	14	0	75	60	38	70	9.0[e]
B. licheniformis E46	<4			<0.1	8	40	0	100	60	<1		
B. licheniformis E53	<4			<0.1	<2	<2				<1		
B. licheniformis E54	<4			<0.1	28	24	0	100	30–45	<1		
B. licheniformis K7	<4			<0.1	14	24	29	55	45–60	115	70	7.5[e]
B. licheniformis K17	<4			<0.1	14	20	100	100	30	5	30	4.6
B. megaterium E6	<4			<0.1	<2	9	62	100	45	12	70	6.8
B. pumilus K2	<4			<0.1	<2	23	0	71	30	35	45–60	5.0[f]
B. pumilus S96	<4			<0.1	4	40	0	100	45	<1		
B. subtilis E100	49	70	6.8	<0.1	7	16	18	16	30	45	70	6.8
B. subtilis E101	32	70	6.8	<0.1	6	17	57	0	30	10	70	6.8
B. subtilis S98	107	60	6.8–9.4	<0.1	66	33	100	85	30–45	43	70	6.8
B. thuringiensis E1	5	30–45	6.8	<0.1	<2	4	0	21	45–70	41	45–60	6.8

[a] Activity figures for extracellular enzymes show the activity measured under optimum conditions as units in a volume of culture supernatant corresponding to 1 mg dry cells. The dry weight concentration of bacteria was calculated as described by Koch [12]. Figures for cellbound α-glucosidase show the activity as units per mg dry cells.

[b] Temperature optima were determined at pH 6.8 (for caseinase at pH 7.4), and pH optima were determined at the appropriate optimum temperatures. The figures are approximate, because the measurements were made at only four different temperatures and three different pH values.

[c] CM-cellulase activity was similar at pH 4.8 to that at pH 7.0.

[d] EDTA: ethylenediaminotetraacetic acid, 1 mM; PMSF; phenylmethylsulphonyl-fluoride, 0.1 mM. Inhibition was measured at 30°C.

[e] Activity was measured at pH values 4.6, 6.0, 6.8, 7.5, 9.0 and 9.4.

[f] Activity was measured at pH values 4.0, 4.6, 5.0, 5.5, 6.8 and 9.4.

Table 4

Sensitivity of board and paper mill *Bacilli* to slimicides

Bacterial strain	Resistance[a] to commercial slimicides[b]													
	1	2	3	4a	4b	5	6	7	8a	8b	8c	9	10	11
B. brevis E47	++	+++	+	+	+	+	++	+++	+	++	++	+++	++	+
B. cereus K8	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	+	+
B. circulans E45	++	+++	++	+	+	+	++	+++	++	++	++	+++	++	+
B. circulans E85	+	+++	+	+	+	+	++	+++	++	++	++	+++	+	−
B. circulans S97	++	+++	+	+	+	+	++	+++	++	++	+	+++	++	+
B. licheniformis E2	++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. licheniformis E3	+++	+++	++	+	+	+	++	+++	++	++	++	+++	++	+
B. licheniformis E16	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. licheniformis E46	++	+++	+	+	+	+	++	+++	++	++	++	+++	++	+
B. licheniformis E53	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. licheniformis E54	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. licheniformis K7	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. licheniformis K17	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. megaterium E6	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	+	+
B. pumilus K2	+++	+++	++	+	++	+	+++	+++	++	++	++	+++	++	+
B. pumilus S96	+++	+++	++	+	+	+	+++	+++	+++	++	++	+++	++	+
B. subtilis E100	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. subtilis E101	++	+++	++	+	+	+	++	+++	+++	++	++	+++	++	+
B. subtilis E102	+++	+++	++	+	+	+	++	+++	++	++	++	+++	++	+
B. subtilis K1	++	+++	+	+	+	+	+++	+++	++	++	++	+++	++	+
B. subtilis S98	++	+++	++	+	+	+	+++	+++	++	++	++	+++	++	+
B. thuringiensis E1	+++	+++	++	+	+	+	+++	+++	++	++	++	+++	+	+

[a] −: MIC < 1 μl l^{-1}; +: MIC = 1–20 μl l^{-1}; ++: MIC = 20–200 μl l^{-1}; +++: MIC = 200–1000 μl l^{-1}.

[b] The active components of the commercial slimicides were as follows: 1: Disodium cyanodithioimidocarbonate, 12.7% Potassium methyldithiocarbamate, 17.5%; 2: 2,2-dibromo-3-nitrilopropionamide, 12%; 3: 1,2-dibromo-2,4-dicyanobutane, 40%; 4a: Methylenebisthiocyanate, 10%; 4b: Methylenebisthiocyanate, 10%; 5: 8-hydroxyquinoline acetate, 42%; 6: 3,5-dimethyl-1,3,5(2H)-tetrahydrothiadiazine-2-thione, 20%; 7: *o*-phenylphenol sodium salt, 30%; 8a: 5-chloro-2-methyl-4-isothiazolin-3-one, 1.5% Methyl-4-isothiazolin-3-one, 0.6%; 8b: 5-chloro-2-methyl-4-isothiazolin-3-one, 1.5% Methyl-4-isothiazolin-3-one, 0.6%; 8c: 5-chloro-2-methyl-4-isothiazolin-3-one, 1.5% Methyl-4-isothiazolin-3-one, 0.6%; 9: 5-oxo-3,4-dichloro-1,2-dithiol, 2.5%; 10: 1,4-bisbromoacetoxy-2-butene, 48%; 11: 1,2-benzisothiazolin-3-one, 33%.

which was both alkaline and had high temperature optimum, a kind which to our knowledge has not been reported before. One of these two came from machine with an alkaline head box (pH 9.0).

Two of the paper and board mill *Bacilli* produced also pullulanase. Such has been described earlier in various *Bacilli* [19] and some other bacteria [6]. Glucoamylase was not produced by any of the strains. This enzyme is rare in bacteria, but has been shown in *Clostridium acetobutylicum* [4].

Most of the *Bacilli* produced enzymes degrading β-glucosidic linkages. This was expected according to the literature [1,13,18]. This property makes these bacteria potentially dangerous organisms in paper mill wire water, because this water is used as diluent when pulp slurries are pumped and stored before introduction into the head box.

It was important to study production of caseinase, because casein is used as a stabilizing agent for resin sizer, neutral sizer, and mineral pigment slurries. If these papermaking chemicals are contaminated by caseinase producing *Bacilli*, this can lead to coagulation and precipitation in storage tanks containing these materials. Most of the strains turned out to be caseinase producers, which is very common for *Bacilli*. Protease types produced by these strains were serine or metalloproteases as described for other *Bacilli*, too [7]. Some strains produced a third type of protease, which was inhibited by neither divalent metal complexing agent nor serine inhibitor. Such a type of protease might be the acidic type, rare in bacteria, but reported in *B. cereus* [7]. We did not determine the pH optima.

To our knowledge the present data is the first report on the sensitivity pattern of *Bacilli* against biocides and preservatives used in industry. *Bacillus* spores can be expected to have a high biocide resistance, but the results presented in this study showed, that also vegatative growth of paper and board mill *Bacilli* as well as *Bacillus* type strains tolerated many different biocides. The normal dosing into paper and board mill white water, starches, and resins is 2–10 $\mu l\ l^{-1}$ and the overall use of most slimicides for food quality paper and board is restricted to one kg per ton of paper or board. Our study thus shows that of the 11 active ingredients studied here only methylenebisthiocyanate, 8-hydroxyquinoline acetate, and 1,2-benzisothiazolin-3-one were effective enough to be of practical value.

When enzyme production in different species is considered, one *B. licheniformis* strain excreted none of the hydrolytic enzymes in measurable amounts, although in plate assay it was positive for degradation of starch, CM-cellulose, and casein. *B. brevis* produced only caseinase, also in plate assay, and one *B. circulans* produced only CM-cellulase in small amount; it hydrolyzed starch in plate assay, though decolorization of starch was seen only underneath the colonies. On the other hand *B. subtilis* strains produced all enzymes that were looked for, except pullulanase.

One *B. licheniformis* strain, isolated from a wood grinding plant, was an exception to the heat resistance: optimum temperature for α-glucosidase and caseinase activities was 30°C, when with other *B. licheniformis* strains it was 45–60°C for caseinase and 70°C for α-glucosidase.

As a whole there was no relation between the temperature and pH optima of the enzyme activities and the temperature and pH of the sites of origin of respective bacteria. There also was no correlation between the enzyme temperature optima and maximum growth temperatures of the bacteria (Väisänen and Salkinoja-Salonen: Use of phage typing and fatty acid analysis for the identification of *Bacilli* isolated from food packaging paper and board machines; System. Appl. Microbiol., in press).

Ten out of twenty-five strains isolated were *B. licheniformis*. However, no preferences for certain species could be seen in the different sites of isolation. Neither was there correlation between the site of isolation and the spectrum of enzymes produced by the corresponding species.

As a conclusion, the formation of heat-resistent endospores, the resistance to biocides, and the production of enzymes degrading papermaking chemicals makes these organisms potentially harmful in paper and board mills.

ACKNOWLEDGEMENTS

We thank Dr. Ilkka Palva for the gifts of pullulan and pullulanase enzyme for use as reference, and for helpful discussions. We also thank Riitta Boeck for help in the laboratory and Helmi Savolainen for media preparation. This work was supported by the Foundation of Finnish Sugar Co.

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