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Antagonistic activity of *Staphylococcus* siderophores and chemical biocides against *Bacillus subtilis* in a paper-machine environment

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The antagonistic potential of nonpathogenic *Staphylococcus* strains against *Bacillus subtilis* wild and type strains were studied under conditions simulating a paper- and board-machine environment. The antimicrobial activity was measured by growth inhibition in an automated turbidimeter. The antagonistic potential was compared with that of generally used chemical biocides in a paper- and board-machine environment. The siderophore-containing extracts of *Staphylococcus* strains significantly inhibited vegetative growth of *B. subtilis* and delayed the germination of spores both in synthetic and in white-water media. The mill strains were more resistant than type strain against *Staphylococcus* siderophores and against chemical biocides. The *Staphylococcus* siderophore-containing extracts did not interfere with the bacteriostatic effect of chemical biocides, but no synergy was detected. The results indicate the potential for application of *Staphylococcus* siderophore-containing extracts as biocontrol agents in paper- and boardmachine environment.

Keywords: *Bacillus*; siderophores; antimicrobial; biocides; white water; paper- and boardmachine

Introduction

The safety of consumers, the packaging material and the environment are important considerations for food-quality packaging board and paper. The cleanliness of packaging paper and board is dependent on the quality of raw materials used, the pulping and bleaching process and hygiene at the paper and board machine. Biofilms formed on machine surfaces may seed microbes into the product, resulting in a deterioration of its hygienic quality. Biofilm and slime in paper and board machines also cause paper breaks, holes, spots, discoloration and unpleasant odours in the product as well as microbially induced corrosion, leading to significant losses in profit [3–5,15,18,19].

At present mainly chemical biocides are used to control microbial growth and biofilm formation on paper and board machines. Many effective applications have been developed, and treatment with chemical biocides is constantly being improved. The diverse microflora in mills, however, as well as environmental aspects and human safety set great demands for effective microbiocides. Bacteria growing as a biofilm are more resistant to biocides than are planktonic bacteria, and it is possible that the use of biocides will favour biofilm bacteria over unattached organisms [11,35].

The paper machine is an open system that cannot be operated aseptically. The white water and some papermaking chemicals may contain 10^3–10^9 microorganisms per ml. The organisms usually dominant in paper products are sporeforming aerobic bacteria of the genera *Bacillus* and *Paenibacillus* [24,32–34], which are also present in liquid packaging paper and board. Bacilli and paenibacilli form heat-resistant spores, which explains their survival during the drying phase of machine operation, and results in end products with a certain microbial load. Too high a load is not desirable in the manufacture of aseptic food-packaging materials. Several members of the genus *Bacillus* also excrete potential food-degrading enzymes, eg proteases and lipolytic, cellulolytic and starch-degrading enzymes [24,33–34].

Since almost all aerobically growing bacteria require iron for growth, chemical complexing has been utilized in biocide formulations to control bacterial growth in paper- and board-machines [28]. Synthetic chemical iron complexing agents, however, have environmentally undesirable properties such as aquatic toxicity and environmental persistence. One option for biocontrol could be to use biological chelating agents such as siderophores. Siderophores are defined as organic iron-specific chelators that are produced by many fungi, moulds and bacteria for ensuring their iron availability. Studies have shown that siderophores have particularly high affinity for iron when compared with inorganic chelates that are less specific [22,28,31]. The mode of action can also include antibiosis, and growth inhibition can be based on the interaction of siderophores and other types of antibiosis. Staphyloferrins A and B, siderophores from *Staphylococcus hyicus*, have been isolated and characterized [10,13,16,20]. In addition to staphyloferrins A and B *Staphylococcus* strains produce other uncharacterized siderophores [17]. Nonpathogenic *Staphylococcus* starter cultures are siderophore producers [25]. *Staphylococcus* strains used as starter cultures in fermented meat products

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have a GRAS (Generally Regarded As Safe) status, which opens the potential for using these strains and their antimicrobial agents in biocontrol in sensitive areas such as food packaging.

The present study aims to illustrate the potential of *Staphylococcus* siderophores against the vegetative growth and spore germination of both *Bacillus subtilis* type and paper mill strains isolated from liquid packaging board. The experiments were performed in white-water medium and compared with the effects of generally used chemical biocides.

Materials and methods

Bacterial strains

Siderophores were produced by *Staphylococcus carnosus* (VTT E-94525), *Staphylococcus* spp (VTT E-94553, VTT E-94554, VTT E-94555) strains which originated from meat and by *Staphylococcus xylosus* commercial starter (DD34 Christian Hansen, VTT E-96660). *Staphylococcus* strains were from the VTT Culture Collection and were maintained as lyophilized cells (except *S. xylosus*). *Bacillus subtilis* board mill isolates (MC-1 from liquid packaging board, AA from kraftliner) and *B. subtilis* type strain (ATCC 6051) were used as target organisms. The wild strains from the culture collection of the University of Helsinki, Department of Applied Chemistry and Microbiology, were stored at −196°C and maintained during the experiments on Trypticase Soy Agar (TSA without glucose; BBL, Cockeysville, MD, USA) slopes at 4°C.

Production and detection of Staphylococcus siderophores

The *Staphylococcus* strains were grown in semisynthetic minimal medium according to Raaska and Mattila-Sandholm [25]. All glassware was acid-washed in 6 M HCl for 24 h before use. The minimal medium was deferrated by Chelex 100 resin [9] to a residual content of 2.4–2.9 μM Fe as determined by atomic absorption spectrometry using the flame technique. The *Staphylococcus* inocula were grown in deferrated minimal medium for 24 h at 37°C with shaking (150–170 rpm). For siderophore production the *Staphylococcus* strains were grown in flask culture (25 ml per 100 ml) aerobically (150–170 rpm) for 2 days at 37°C after which the cells were removed by centrifugation and culture supernatants filtered through an Ultrafee-MC filter (PLGC filter, nominal molecular weight limit 10 000; Millipore, Tokyo, Japan) to remove proteins, pooled, neutralized and stored at −25°C for subsequent assays. Siderophores were detected in culture supernatants according to the method of Alexander and Zuberer [1] by chrome azurol S reagent (Fluka Chemicals, Buchs, Switzerland) from samples properly diluted in MES buffer (2-[N-morpholino]ethanesulphonic acid; Sigma, St Louis, MO, USA) after 24 h incubation at room temperature using a Multiscan MCC microplate reader (Labsystems Oy, Helsinki, Finland) at 690 nm [26]. The siderophore production (Δ OD 690 nm) of *Staphylococcus* strains measured by chrome azurol S reagent varied 3.4–4.1/10^{10} CFU ml^{-1}.

Chemical biocides

The commercial biocides used were Daracide® 7849 (e.a. 10% methylenebisthiocyanate), Daracide® 7819 (e.a. 12% 2,2-dibromo-3-nitrilopropionamide), Daracide® 856 (e.a. 1.53% 5-chloro-2-methyl-4-isothiazolin-3-one, 0.57% 2-methyl-4-isothiazolin-3-one), Daracide® 7848 (e.a. 1.4% 5-chloro-2-methyl-4-isothiazolin-3-one, 0.5% 2-methyl-4-isothiazolin-3-one, 7.5% glutaraldehyde), dazomet (e.a. 3,5-dimethyltetrahydro-2-thio-1,3,5-thiadiazine, Dr Ehrenstorfer GmbH), 2-mercaptobenzothiazolin (Aldrich M3,30–2). Daracide® biocides were manufactured by WR Grace Oy (Helsinki, Finland). Stock solutions of biocides (0.1%) were made in ultrapure water and filter-sterilized (0.22 μm; Millipore, Molsheim, France) before use. Chemical biocides were used at concentrations of 1 and 10 ppm of the effective substance in antimicrobial assays.

Testing of antimicrobial activity

White water used in testing of antimicrobial activity originated from a board machine producing packaging board of bleached and unbleached kraft pulp. The white water was stored at −25°C, filtered (Whatmann 40, ash-free, Maidstone, UK), buffered and supplemented with nutrients before use. The buffers used were 0.1 M MES pH 5.5, 0.1 M PIPES (piperazine-N,N′-bis-2- ethanesulphonic acid, Na_2-salt; Sigma, St Louis, MO, USA) pH 7.0 and 0.1 M BIZINE (N,N-bis(2-hydroxyethyl)-glycine; Sigma) pH 8.5.

The antimicrobial activity of *Staphylococcus* culture supernatants against wild and type strains of *B. subtilis* was studied with an automated turbidometer Bioscreen® (Labsystems Oy, Helsinki, Finland) [29,30]. The analyser measures microbial growth by vertical pathway and the optical changes in liquid medium are correlated with microbial counts in the samples. The optical measurements (wide-band filter) conducted at a fixed schedule throughout the run are recorded in the memory of a desk-top computer and a kinetic follow-up of the run provides a growth curve that can be analyzed by the software. The area under the growth curve was used as a measure of microbial growth, and the reduction of area (%) was used to express the growth inhibition in the presence of the antimicrobials. *B. subtilis* strains were grown in Modified King's Medium B containing per litre: 20 g of protease peptone, 10 g of glycerol, 0.3 g of K_2HPO_4, 1.5 g of $MgSO_4$, 1 g of PIPES Na_2-salt and 10 g of glucose at 37°C for 24 h with shaking (120 rpm). The growth of *B. subtilis* strains in white water supplemented with 10% trypticase soy broth (TSB without glucose; BBL) or 0.3% soluble starch (May & Baker, Dagenham, UK) was studied by dispensing 30 μl of a 10^{-1} dilution (10^6 CFU ml^{-1}) from overnight cultures to microtitre plate wells with 270 μl of white-water media and incubated at 45°C for 24 h. *Bacillus* spores were produced in Modified King's Medium B supplemented with 10 mg of $MnSO_4 \cdot H_2O$ L^{-1} at 37°C for 3 days. The cultures were heated at 80°C for 10 min in a water bath and rapidly cooled to room temperature with cold water. Antimicrobial activity was studied by dispensing to microtitre plate wells 30 μl of a 10^{-1} dilution of indicator strain (10^6 CFU ml^{-1}) or spores (10^4 CFU ml^{-1}) with 60 μl of the substance to be studied and 210 μl of the test medium. In the control sample wells, the antimicrobial agent was replaced by an

equal volume of simultaneously treated minimal medium. Effects of the antimicrobials were studied in Modified King's Medium B or in white-water medium supplemented with 0.3% starch or 10% TSB at 45°C for 24 h. All determinations were performed with four replicates and results were expressed as mean values.

Results

Antagonistic activity of Staphylococcus siderophores

The growth of *B. subtilis* wild strain was partially (17–46%) prevented at 45°C by the crude siderophore-containing extracts of *Staphylococcus* strains tested in Modified King's Medium B (Figure 1a). The strains differed in their efficacy against *B. subtilis* wild strain; *Staphylococcus* sp strains E-94553 and E-94525 inhibited 25–46% and *S. xylosus* VTT E-96660 <20%. The pH of the test medium had little effect on the efficacy of the *Staphylococcus* extracts, except for strain E-94553 which was more effective in neutral and alkaline pH than in an acid environment.

To simulate conditions prevailing at the board machine the test was repeated in white-water medium and at a temperature of 45°C which is similar to that at the machine wet end. The growth of *B. subtilis* was poor in buffered (pH 7) white water, and therefore the medium was supplemented with TSB or soluble starch. The antimicrobial activity of *Staphylococcus* siderophore-containing extracts was clear in the starch-supplemented white-water medium (Figure 1b). The growth of *B. subtilis* type strain was inhibited by all extracts 58–67%; the wild strain (MC-1) was more resistant towards three *Staphylococcus* siderophore extracts but similarly sensitive (60% growth inhibition) towards *S. xylosus* extract.

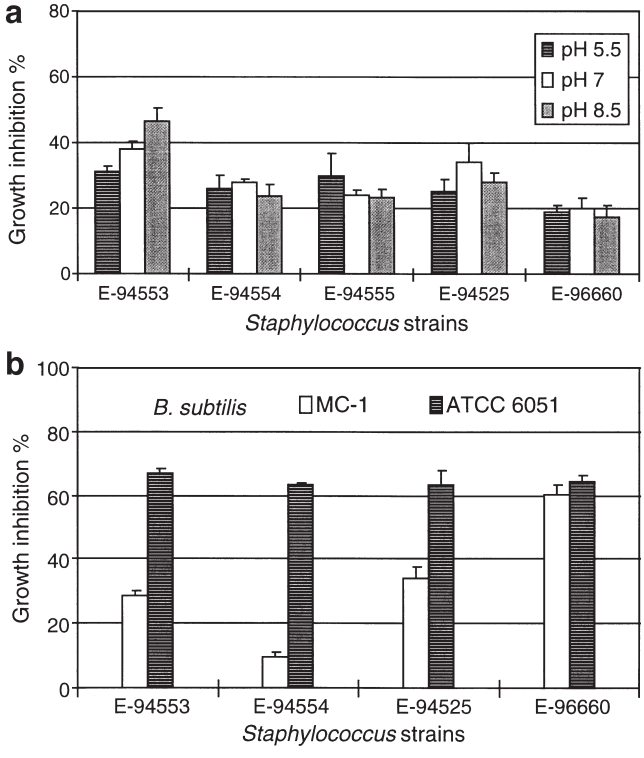

Figure 1 (a) Antagonistic activity of *Staphylococcus* crude siderophore extracts against *B. subtilis* MC-1 in Modified King's Medium B at 45°C within 24 h at different pH. Growth inhibition was expressed as the percentage of growth area decrease in the presence of the siderophore extract compared with the control. (b) Antagonistic activity of *Staphylococcus* crude siderophore extracts against *B. subtilis* wild (MC-1) and type (ATCC 6051) strains in buffered (pH 7) white-water medium (0.3% starch) at 45°C within 24 h.

Sensitivity of Bacillus strains towards chemical biocides

Six different effective substances of chemical biocides generally used in paper machines were tested against *B. subtilis* at concentrations of 1 and 10 ppm of active substance, which are concentrations used in the paper industry to control microbial growth. The biocides effectively inhibited growth of *B. subtilis* MC-1 at a concentration of 10 ppm except 2-mercaptobenzothiazol (MBTA) which was less effective (Figure 2). At 1 ppm only the combined isothiazolin-3-ones were effective. The sensitivities of *B. subtilis* wild and type strains to 2,2-dibromo-3-nitrilopropionamide (DBNPA) combined with *Staphylococcus* siderophores are compared in Table 1. The results show that the combined use gave no additive effect but also that the antimicrobials were not significantly antagonized by each other.

Antagonistic activity of Staphylococcus siderophores against Bacillus spores

The antagonistic activity of *Staphylococcus* siderophore-containing extracts against *B. subtilis* spores was measured and compared to that of glutaraldehyde (GA; Figure 3). The spores of the type strain were more sensitive to the siderophore extracts and also to GA (1 ppm) than the spores of two board mill strains (Figure 3). The germination and outgrowth of spores of the type strain were inhibited 75–90%, whereas spores of the wild strains were inhibited 25–75% depending on the origin of the siderophore extract. The *Staphylococcus* siderophore-containing extracts were active against *B. subtilis* wild strain spores both in acidic and alkaline environments although the activity was enhanced under acidic and neutral conditions (Figure 4). The germination of *Bacillus* spores was delayed when cultured in white-water medium supplemented with siderophore extract or GA.

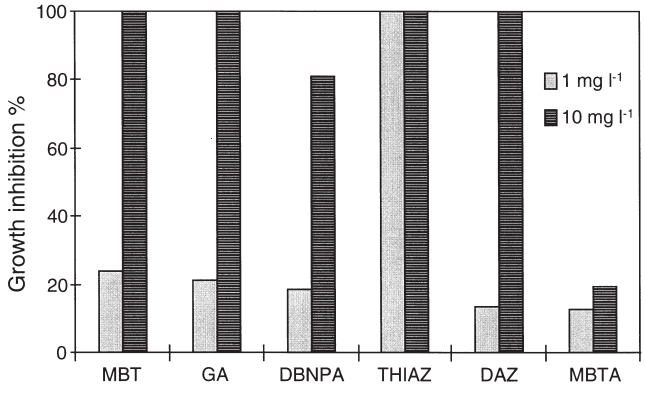

Figure 2 Growth inhibition of *B. subtilis* MC-1 by generally used commercial biocides in buffered (pH 7) white-water medium (0.3% starch) at 45°C within 24 h. Biocides: MBT = methylene bisthiocyanate, GA = glutaraldehyde, DBNPA = 2,2-dibromo-3-nitrilopropionamide, THIAZ = 5-chloro-2-methyl-4-isothiazolin-3-one, DAZ = dazomet, MBTA = 2-mercaptobenzothiazol.

Table 1 Combined effect of 2,2-dibromo-3-nitrilopropionamide and *Staphylococcus* siderophore-containing extracts against *B. subtilis* MC-1 and the type strain in white-water medium (0.3% starch, pH 7) at 45°C within 24 h. The results are expressed as a percentage of growth inhibition ±SD

Target strain	Biocide	Extract from *Staphylococcus*-strain				
		none	E-94553	E-94554	E-94525	E-96660
B. subtilis MC-1	0 ppm	0.00 ± 0.0	28.6 ± 1.5	9.63 ± 1.5	34.3 ± 3.3	60.5 ± 3.1
	1 ppm	3.44 ± 5.7	15.8 ± 2.6	10.7 ± 3.5	28.4 ± 5.7	63.1 ± 0.9
	10 ppm	96.1 ± 0.6	85.4 ± 1.3	88.4 ± 2.1	94.1 ± 1.3	92.9 ± 1.9
B. subtilis ATCC 6051	0 ppm	0.00 ± 0.00	66.7 ± 1.7	63.2 ± 0.7	63.1 ± 4.8	64.4 ± 1.9
	1 ppm	2.51 ± 9.6	62.6 ± 1.7	66.1 ± 3.6	64.7 ± 1.0	63.8 ± 0.9
	10 ppm	94.3 ± 2.3	93.2 ± 1.1	93.9 ± 1.0	96.6 ± 1.4	97.6 ± 1.4

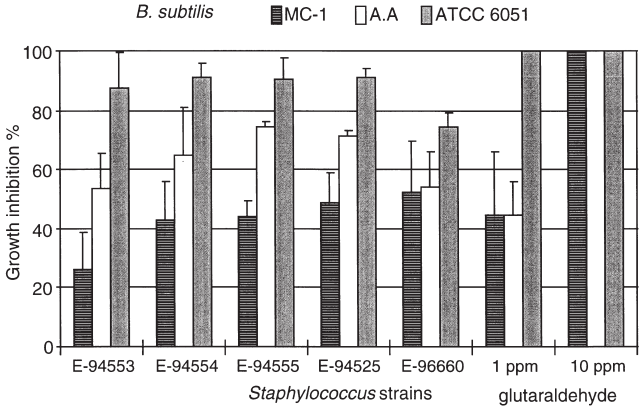

Figure 3 Inhibition of germination and early growth of *B. subtilis* spores by *Staphylococcus* siderophore crude extracts and glutaraldehyde in buffered (pH 7) white-water medium (0.3% starch) at 45°C within 24 h.

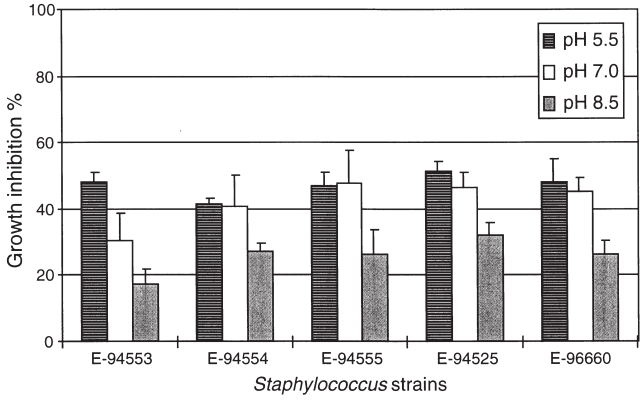

Figure 4 Effect of pH on the antagonistic activity of *Staphylococcus* siderophore crude extracts against *B. subtilis* MC-1 spores in white-water medium (10% TSB) at 45°C within 24 h.

Germination of spokes of the type strain was delayed up to 5.3 h and that of the mill strain for 1.3 h compared with the control with no siderophores. GA was very effective against the type strain, but almost as effective as siderophore extracts at a concentration of 1 ppm against the mill strain (Figure 5).

Discussion

Increased recycling of process water by paper and board mills has decreased wastewater discharges. This may

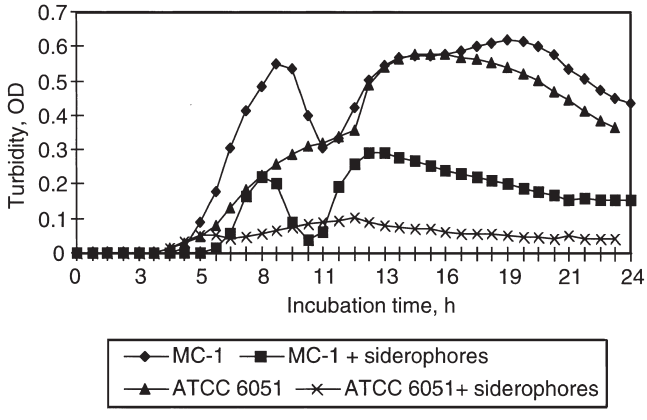

Figure 5 The germination and growth of *B. subtilis* spores treated with siderophore-containing extract of *Staphylococcus* sp VTT E-94553 strain in white water medium supplemented with 0.3% starch at 45°C within 24 h. Application of 10 ppm glutaraldehyde totally prevented growth of *B. subtilis* during the 24 h incubation. Application of 1 ppm of glutaraldehyde totally prevented the germination and growth of the type strain and delayed germination of the mill strain for 3 h.

increase nutrient concentration in process water and hence increase microbial problems and the demand for biocides, contrary to what consumers and environmental authorities have been asking for.

The present paper focused on the antagonistic potential of nonpathogenic *Staphylococcus* strains against *B. subtilis* paper mill strains. The *Staphylococcus* strains used were starters from meat fermentation. *B. subtilis* was chosen as the model target organism because it is one of the important contaminants in paper and board machines and has significant heat resistance and amylolytic activity [33]. The effects of *Staphylococcus* siderophore crude extracts were studied at 45°C in Modified King's Medium B, which is generally used as a test medium for siderophore activity [21], and in white-water medium which simulates the growth environment at the board machine. The inhibitory effects of siderophores were compared with those of chemical biocides generally used in paper making.

The antimicrobial effects of several of the presently used chemical biocides for slime control are believed to reside in irreversible binding of cations essential for microbial growth; eg methylenebisthiocyanate (MBT) and the thiols (eg MBTA) as well as EDTA, often used as an auxiliary biocide, are believed to deprive microbes of iron and calcium [8,23]. Many of the synthetic chemical biocides are

not readily degraded or inactivated by the gut of warm-blooded animals or fish and therefore are inherently toxic to man and animals. Recently, methods directed at controlling microbial slimes also include the addition of microbes or enzymes in the process waters [12,14,36].

Many microbes excrete effective complexing agents, siderophores, to scavenge iron. Meat starter cultures of *Staphylococcus* are known to produce such siderophores and yet to be completely safe for human consumption. It is therefore assumed that *Staphylococcus* siderophores are also safe in the environment. The present paper shows that *Staphylococcus* siderophore extracts inhibited the growth of *B. subtilis* board mill and type strains at 45°C both in complex rich medium as well as in white-water medium, the highest inhibitions being observed in low-nutritious white-water medium where the inhibition percentage of mill strain attained 60%. The iron concentration, one of the most important controlling factors of siderophore activity in white-water medium was very low (0.02 mg L^{-1}) compared with in the Modified King's Medium B (0.44 mg L^{-1}), indicating more effective activity of *Staphylococcus* siderophores [25]. The culture supernatants of *Staphylococcus* strains were ultrafiltered before use to remove proteins from the samples, however, antimicrobial agents other than siderophores may have been contained in the crude extracts of the cultures used in the present study. Interestingly, the mill strains were generally less sensitive than the type strain both towards *Staphylococcus* siderophore-containing extracts and towards conventional chemical biocides, indicating the importance of using genuine strains isolated from the application problem area when assessing the effectivities of various antimicrobials.

Bacillus species isolated from the paper process or products produce hydrolytic enzymes attacking raw materials of paper and board, for example starch and hemicellulose, proteins, lipids and fats [24,33]. The siderophore crude extracts studied showed inhibitory activity towards amylolytic board mill strains of *B. subtilis*, comparable or better than some of the presently used chemical biocides in the paper industry. Staphylococcal siderophore extracts may have the potential for protection of industrial starches against colonization by amylolytic *B. subtilis*.

The crude siderophore extracts also delayed the germination and outgrowth of *B. subtilis* spores. Since the spores most likely germinate and propagate during the pulping and storage phases of broke [34], which then seeds the machine chest and white water, siderophores may offer one option for attenuating the flow of *Bacillus* species. The most important property of GA is its sporicidal efficacy, which is favoured with increased pH [23,27]. The activities of *Staphylococcus* siderophores against the germination and outgrowth of *B. subtilis* spores were enhanced at neutral and acidic conditions.

Gram-positive microorganisms including several *Bacillus* species are sensitive to nisin and a range of other conventional bacteriocins [2,7]. Furthermore, many *Bacillus* species are susceptible to EDTA and other nonspecific chelators [28]. The chelating agents remove magnesium and calcium ions from the cell membrane, disturbing the cell integrity, whereas siderophores bind iron specifically and their activity is receptor-bound [6,37]. The combined use of *Staphylococcus* siderophore-containing extracts and DBNPA showed that neither interfered with the activity of the other, which would be important for the combined use of chemical biocides and antimicrobials in the wet end of the paper or board machine. Synergism, however, has been detected between nisin and *Staphylococcus* siderophore-containing extracts [31]. The results clearly show that *Staphylococcus* siderophore-containing extracts have potential for application as biocontrol agents. Hence, it would be important to further study the antimicrobial activities of siderophores alone or in combination with biocide activities in real paper or board machine environments and to determine the economic and environmental benefits of siderophore application.

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