

Antimicrobial Action of Hydrolyzed Chitosan against Spoilage Yeasts and Lactic Acid Bacteria of Fermented Vegetables

TONY SAVARD,^{1*} CAROLE BEAULIEU,² ISABELLE BOUCHER,³ AND CLAUDE P. CHAMPAGNE¹

¹Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600 Casavant Boulevard W., Saint-Hyacinthe, Québec, Canada J2S 8E3; and ²Department of Biology and ³ISM Biopolymer Inc., Faculty of Sciences, University of Sherbrooke, Sherbrooke, Québec, Canada J1K 2R1

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ABSTRACT

The antimicrobial properties of various chitosan-lactate polymers (ranging from 0.5 to 1.2 MDa in molecular weight) against two yeasts isolated from fermented vegetables and against three lactic acid bacteria from a mixed starter for sauerkraut on methylene blue agar (MBA) and in vegetable juice medium (VJM) were investigated. Chitosan-lactate reduced the growth of all microorganisms in solid (MBA) as well as in liquid (VJM) medium. In MBA, a concentration of 5 g/liter was needed to inhibit the growth of *Saccharomyces bayanus*, while 1 g/liter was sufficient to inhibit the growth of *Saccharomyces unisporus*. Lactic acid bacteria were also inhibited in this range of concentrations. The low-molecular-weight chitosan-lactate DP3 (0.5 kDa) was most efficient in solid medium (MBA), and inhibitory activities decreased with increasing hydrolysate lengths. In liquid medium (VJM), 0.5 g of chitosan-lactate per liter reduced the growth rates for both yeasts, but 10 g/liter was insufficient to prevent yeast growth. Intermediate-molecular-weight chitosan-lactate (5 kDa) was more efficient than chitosan of low molecular weight. Native chitosan (1.2 MDa) showed no inhibition in either medium. Microscopic examination of *S. unisporus* Y-42 after treatment with chitosan-lactate DP25 showed agglutination of a refractive substance on the entire cell wall, suggesting an interaction between chitosan and the cell wall. When chitosanase was added to the culture media containing chitosan-lactate, refractive substances could not be observed.

The shelf life of unpasteurized fermented vegetables is influenced principally by the presence of fermentative yeasts (2). The growth of yeasts occurs during storage when sugar catabolism by lactic acid bacteria (LAB) is incomplete (6). The main negative effect of secondary fermentation is the production of CO₂ by yeasts, which causes the swelling of packages or bloater damage in cucumbers (6). Chemical agents such as sorbate or propionate could be added to vegetables after fermentation to control spoilage yeasts, but some consumers demand products that do not contain these chemical substances.

Chitosan, a deacetylated derivative of chitin, exhibits antimicrobial activity against some strains of bacteria (25), filamentous fungi (1, 4, 10, 17), and yeasts (5, 17, 21). Minimum inhibitory concentrations of chitosan vary as a function of target organism, media composition, pH, temperature, and the molecular weight of the polymer. Concentrations as low as 0.02 g/liter have shown inhibitory activity against some plant pathogenic fungi in liquid media (1, 10), but concentrations as high as 10 g/liter have been necessary to inhibit the growth of some fungal strains (20). Little has been shown with regard to the inhibitory activity of chitosan in food products. Concentrations of 5 g/liter had no effect on yeast growth in complex media like chickpea dip (16). Medium thus has a strong influence on the inhibitory properties of chitosan, but no data are available on its effectiveness in fermented vegetables.

The inhibitory activity of low-molecular-weight (low-MW) chitosan has been reported to be greater than that of high-MW chitosan against bacteria, while native chitosan has been shown to be more effective against *Fusarium* spp. (22). In another study, Kendra and Hadwiger (10) reported that the shortest chitosan oligomer that exhibited maximum antifungal activity against *Fusarium solani* was the heptamer. For many studies, the MW is not given, and more data are required on the effects of chitosan MW and the degree of polymerization on the inhibition of microorganisms.

The mechanism associated with the inhibition activity of chitosan has not been clearly identified, even though it has been established that the antifungal activity of chitosan is greater at low pH values (8, 17). Since chitosan has a pK_a of 6.3, it interacts with negatively charged substances at pHs of <6.0. Chelating activity on metal ions and on acids has been reported by Li et al. (12). Other hypotheses on the mechanism of action of chitosan include membrane disturbance induced by the leakage of proteinaceous and UV-absorbing materials of some filamentous fungi (5, 11, 13) and “suffocation” of yeast cells caused by the formation of an impervious layer on the cellular surface (15, 23).

The main objective of this study was to investigate the antifungal and antibacterial properties of hydrolyzed and native chitosan-lactate against two spoilage yeasts and three LAB used in a lactic starter for vegetable fermentation. Next, the interaction between chitosan and microbial cells was studied at a microscopic level.

* Author for correspondence. Tel: (450) 773-1105; Fax: (450) 773-8461; E-mail: savardt@em.agr.ca.

TABLE 1. Chemical properties of chitosan^a

DP	MW
Native chitosan (no polymerization)	1.2 MDa
DP101	10 kDa
DP42	7 kDa
DP25	4 kDa
DP8	1.3 kDa
DP3	0.5 kDa

^a Degree of polymerization (DP) and molecular weight (MW) were obtained from the manufacturer (ISM Biopolymer). Hydrolysates of chitosan were obtained by enzymatic hydrolysis with chitosanase of *Streptomyces* sp.

MATERIALS AND METHODS

Soluble chitosan. Hydrolysates and native chitosan-lactate were obtained from ISM Biopolymer Inc. (Sherbrooke, Quebec, Canada). This 40-g/liter chitosan preparation contained 2.5% food grade DL lactic acid (88%) and had a deacetylation level of 90% (manufacturer's data) (pH 5.5). Native and hydrolyzed chitosan-lactates of different molecular weights were tested with chitosan concentrations of 0.5, 1, 2, 5, and 10 g/liter. The mean MWs for different degrees of polymerization (DP) are presented in Table 1. All samples were sterilized by filtration through 0.22 μ m nitrocellulose membranes (Millipore, Milford, Mass.).

Microbial strains. Microbial strains used in this study included three species of LAB used as sauerkraut starter (BLAC 1, Caldwell BioFermentation Canada Inc., Compton, Quebec, Canada) and two spoilage yeasts. The LAB were *Lactobacillus plantarum* NK 312 (Lallemand, Saint-Simon, France), *Pediococcus acidilactici* AFERM 772 (Quest, Lachine, Quebec, Canada), and *Leuconostoc mesenteroides* BLAC (CRDA, Saint Hyacinthe, Quebec, Canada). Yeast strains were isolated from spoiled fermented vegetables by Savard et al. (18) and identified as *Saccharomyces unisporus* Y-42 and *Saccharomyces bayanus* Y-43. LAB strains were maintained on deMan, Rogosa, and Sharpe agar (BDH, Montreal, Quebec, Canada) at pH 5.6, and yeast strains were maintained on YM agar (yeast and malt agar also known as yeasts and molds agar; pH 3.5; BDH) acidified with 5 N HCl.

Antimicrobial activity against LAB and spoilage yeasts in agar-based medium. Antimicrobial activity on methylene blue agar (MBA) was first examined as described by Walker et al. (24). MBA was distributed in aliquots of 15 ml and then autoclaved at 121 °C for 15 min and cooled to 45 °C before the addition of LAB or yeasts at a final cell number of 1×10^5 per petri dish. Target cells were seeded into the molten MBA, mixed gently, and then poured into petri dishes. Five-millimeter-diameter punctures were made with a stainless steel punch in the solidified agar to obtain wells.

Sterile native and hydrolyzed chitosan solutions (50 μ l) at concentrations of 0.5, 1, 2, 5, and 10 g/liter were distributed into 5-mm-diameter wells. Lactate (2.5%) was used as a negative control. On MBA, test organisms grew as a background lawn, and inhibitory activity was indicated by a clear zone surrounding the well, which could itself be surrounded by blue-stained-line (dead) colonies if fungicidal or bactericidal activity was present. Plates were incubated at 30 °C for 72 h and kept at 4 °C for 2 weeks. Three independent assays were performed in duplicate, and the diameters of inhibition (clear) zones delimited by the blue line were measured in millimeters.

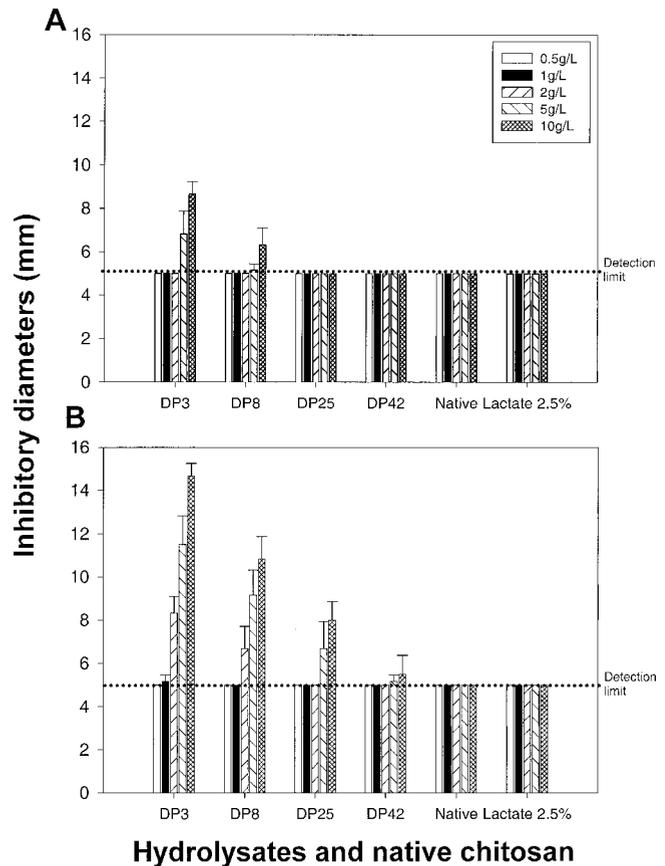


FIGURE 1. Inhibitory diameters of hydrolysates and native chitosan-lactate at concentrations of 0.5, 1, 2, 5, and 10 g/liter on MBA agar (pH 4.4) for (A) *S. bayanus* Y-43 and (B) *S. unisporus* Y-42. Results are given as the means of three trials in triplicate. Error bars represent the standard deviation. The detection limit corresponds to the well diameter.

Antimicrobial activity against LAB and spoilage yeasts in liquid media. Data obtained with agar-based medium were compared with those obtained with vegetable juice medium (VJM) by automated spectrophotometry with a Bioscreen apparatus (Lab-systems, Helsinki, Finland). The VJM was composed of 61% carrot juice, 12% cabbage juice, 3% onion juice, and 24% brine containing 20 g of sea salt per liter and was prepared according to Gardner et al. (7). The initial pH of the blend was 6.34. To determine the inhibitory potential of chitosan in fermented vegetables, VJM was acidified at pH 3.8 with lactic acid (85%). VJM was supplemented with hydrolyzed chitosan-lactate at 0.5, 1, 2, 5, and 10 g/liter and distributed in a 200-well microplate. Wells were inoculated with test strains in triplicate with 0.1% (vol/vol) of a 72-h yeast preculture (YM broth, 30 °C for 72 h). For each sample, control experiments without chitosan were carried out in triplicate, and the mean value for blanks (chitosan without yeasts) was subtracted from the mean value obtained for the test wells. Experiments were conducted for three independent assays in triplicate. The microplates were incubated at 26 °C for 28 h, and the optical density at 600 nm was measured every 15 min. The Bioscreen unit is designed to shake the microplates and set intervals, and in this study the microplates were shaken for 20 s before and after optical density readings were taken.

Electron microscopy. Samples were examined after four treatments at two pH values (3.8 and 6.0) to evaluate the effect of chitosan as a function of pH. *S. unisporus* Y-42 and *S. bayanus*

Y-43 were preincubated at 30°C for 48 h in YM broth (control). Intermediate hydrolyzed chitosan (DP25) was added at a concentration of 2 g/liter, and samples were taken after 1 and 5 h of incubation for the first and second treatments, respectively. Chitosanase (ISM Biopolymer) was mixed with chitosan-lactate DP25 in the third treatment to confirm the nature of the aggregate, and the results of all treatments were compared with those of the control treatment (without chitosan and chitosanase).

After incubation, samples were centrifuged at $4,000 \times g$ for 15 min at 4°C, and yeast cells were embedded in 4% softened agar (pH 6.0 or 3.8, 45°C; bacteriological agar, Difco Laboratories, Detroit, Mich.) The remaining gel was cut into small pieces of about 1 mm³. Samples were then fixed in 2% glutaraldehyde-cacodylate buffer (0.1 M, pH 7.3) for 2.5 h at room temperature, rinsed several times in the same buffer for 1.5 h, and postfixed overnight in 2% osmium tetroxide-water at 4°C. Afterward, samples were rinsed three times within 1.5 h, dehydrated in a graded ethanol series (30, 50, 70, and 100%), infiltrated with Spurr resin, and polymerized for 24 h at 60°C. Thin sections were obtained, stained with uracyl acetate and lead citrate, and observed on a transmission electron microscope at 80 kV (Philips model 420, Eindhoven, The Netherlands).

RESULTS AND DISCUSSION

Antimicrobial activity of chitosan-lactate in agar-based medium. The antimicrobial activities of native and hydrolyzed chitosan-lactate on MBA agar were assessed first. Figures 1 and 2 illustrate the level of inhibition for *S. bayanus* Y-43 (Fig. 1A), *S. unisporus* Y-42 (Fig. 1B), and LAB (Fig. 2). The highly degraded chitosan (DP3) showed the greatest inhibitory activity for all microorganisms. Inhibition decreased with increasing molecular weight. Trimers (DP3) were the most potent inhibitor, followed by octamers (DP8). Intermediate-MW chitosans (DP25 and DP42) showed little activity, and no growth inhibition could be observed with polymers of up to 10 kDa. However, the inhibitory activities of the different oligomers varied as a function of concentration and target organism. No growth inhibition was observed with concentrations of 0.5, 1, and 2 g/liter for *S. bayanus* Y-43, which needed a minimum concentration of 5 g/liter to show inhibition (Fig. 1A). For *S. unisporus* Y-42, a similar inhibitory pattern was observed, even though this strain was more sensitive to chitosan hydrolysates ($P < 0.002$, *t* test). Inhibition was also observed for LAB with an MIC of 2 g/liter, and *L. plantarum* was the most resistant of the three strains (Fig. 2). There was no inhibitory activity observed under control conditions with only lactic acid at 2.5%.

On the basis of these results, the inhibitory activity of chitosan-lactate was greater with low- and intermediate-MW hydrolysates and was directly proportional to the concentration added to the MBA. Inhibitory activity was lost with hydrolysates between DP8 and DP42, depending on the target organism. These data are consistent with those obtained by Hirano and Nagao (9), who observed that low-MW chitosan was more inhibitory than intermediate- and higher-MW chitosan in agar systems, although the MWs for their study were not specified. Therefore, the fact that LAB were also affected by chitosan indicates that chitosan-

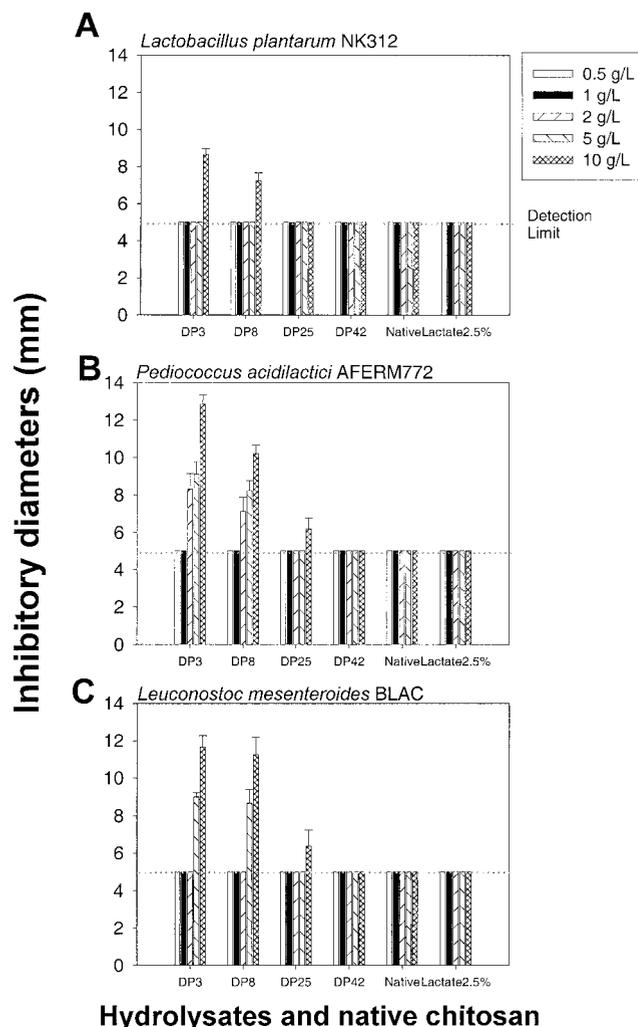


FIGURE 2. Inhibitory diameters of hydrolysates and native chitosan-lactate at concentrations of 0.5, 1, 2, 5, and 10 g/liter in MBA agar (pH 4.4) for (A) *L. plantarum* NK312, (B) *P. acidilactici* AFERM 772, and (C) *Leuconostoc mesenteroides* BLAC. Results are given as the means of three trials in triplicate. Error bars represent the standard deviation. The detection limit corresponds to the well diameter.

lactate should not be added at the beginning of vegetable fermentation. However, the MBA agar test was carried out at pH 4.5, and it remains to be determined if a similar inhibition pattern would occur at the higher pH levels encountered at the beginning of vegetable fermentation.

Antimicrobial activity of chitosan-lactate in VJM.

The antimicrobial activities of native and hydrolyzed chitosan-lactate in liquid medium were assessed only on yeast strains, since at pH 3.8 (used with VJM), the growth of the LAB was critically inhibited and no increase in optical density values could be obtained during the 28-h recording period (data not shown). VJM was used as a complex medium representative of conditions encountered following lactic fermentation. Figure 3 shows the growth curves of *S. bayanus* Y-43 in the presence of (A) DP3, (B) DP8, (C) DP25, and (D) DP42 at different concentrations. In contrast to data obtained for agar-based medium, all hydrolysates were inhibitory, and hydrolysates of intermediate MW be-

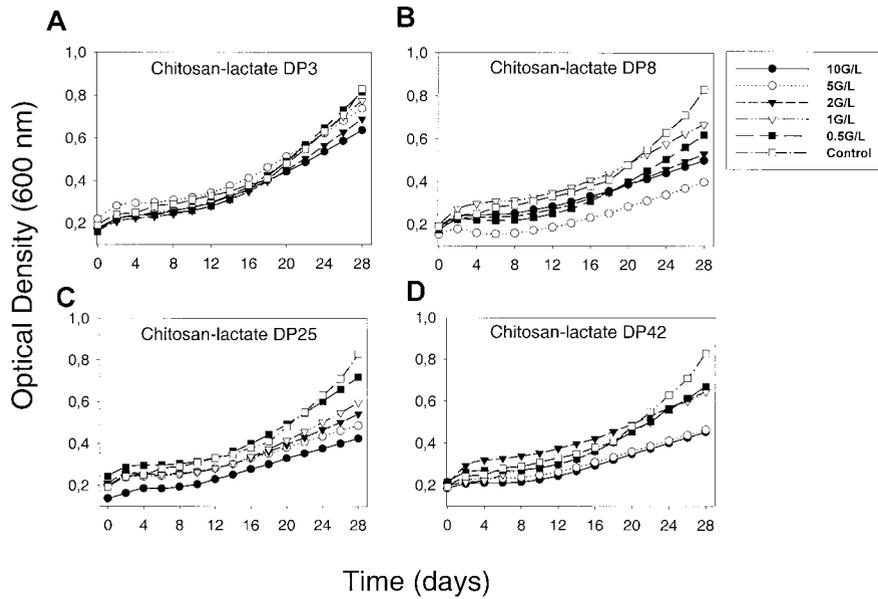


FIGURE 3. Growth curves by optical density (600 nm) for *S. bayanus* Y-43 in VJM acidified to pH 3.8 with 85% lactic acid. Curves represent a function of degree of polymerization of the chitosan hydrolysates and concentration (0.5 to 10 g/liter). The control samples contained 2.5% lactic acid without chitosan. Results are given as the means of three trials in triplicate. Error bars represent the standard deviation.

came the most potent inhibitors. With *S. bayanus* Y-43, all concentrations tested were effective against this yeast, but complete growth inhibition in VJM was not obtained with concentrations as high as 10 g/liter. Nevertheless, the inhibition was concentration dependent.

As shown in Figure 4, complete growth inhibition was seen for *S. unisporus* Y-42 (the most sensitive strain in agar-based medium) at a concentration of 0.5 g/liter except with the trimer (DP3), which did not reduce the growth of this yeast from the level observed for the control. Interestingly, we observed in a previous work that *S. unisporus* is more resistant to low pH and acid than is *S. bayanus* (18). This resistance is generally conferred by more effective proton pumping at the cell wall and a more acidic internal cytoplasmic pH (3). These characteristics are linked to anionic charges on the cell wall (26) and could explain the high sensitivity to the polycationic charge of chitosan-lactate.

The inhibition patterns for liquid medium (VJM) differed from those obtained for solid medium (MBA). Hydrolysates of low-MW chitosan-lactate were less toxic than

were hydrolysates of intermediate MW. These data are consistent with those obtained by Rhoades and Roller (16) and Roller and Covill (17), who found that the medium composition and the degree of hydrolysis influenced the inhibitory activity of hydrolysates. The hypothesis put forth by Rhoades and Roller (16) to explain the discrepancies observed between liquid and solid media was that the particulate nature of solid media restricted mass transfer of the relatively large polymeric chitosan molecules, which reduced the chance of contact with a microbial cell in the agar. It is also possible that the discrepancies were caused by a dilution factor related to the number of molecules in a static medium. Chitosan stock solutions are prepared with a concentration of 40 g/liter, and chitosan is then degraded with chitosanase. For the same initial number of molecules, the most hydrolyzed chitosan shows a higher relative number of molecules than does the native chitosan.

Scanning electronic microscopy. Figures 5 and 6 show transmission electron micrographs for *S. unisporus* Y-

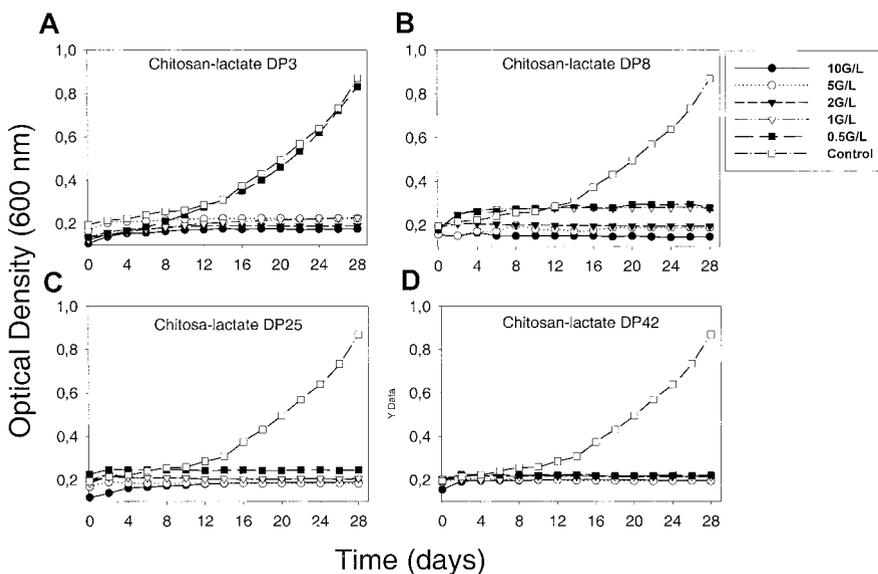


FIGURE 4. Growth curves by optical density (600 nm) for *S. unisporus* Y-42 in VJM acidified to pH 3.8 with 85% lactic acid. Curves represent a function of degree of polymerization of the chitosan hydrolysates and concentration (0.5 to 10 g/liter). The control samples contained 2.5% lactic acid without chitosan. Results are given as the means of three trials in triplicate. Error bars represent the standard deviation.

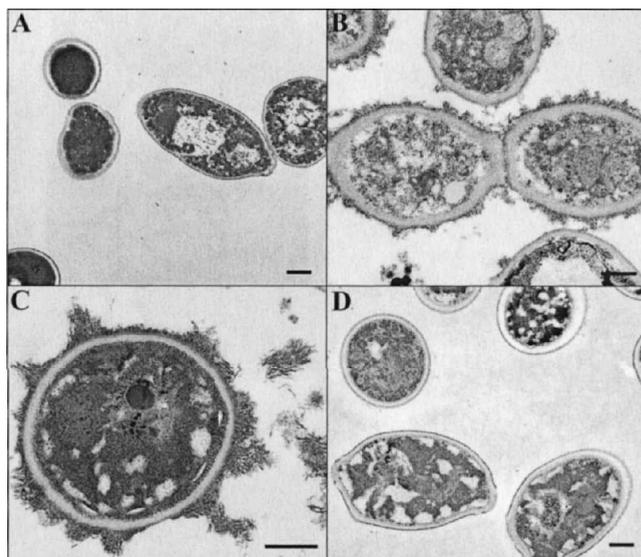


FIGURE 5. Transmission electron micrographs for *S. unisporus* Y-42 (A) immediately after exposure to chitosan-lactate DP25 at 2 g/liter in YM broth acidified with 3 N HCl at pH 4.0, (B) after 1 h, (C) after 5 h, and (D) after 1 h supplemented with chitosanase. Bar = 1 μ m.

42. Treatments with chitosan-lactate in YM broth at pHs of 3.8 and 6.0 were examined, but no differences could be observed between these treatments, probably because these pHs were below the pK_a of chitosan (6.34) (data not shown). For this reason, only micrographs for YM broth at pH 3.8 are presented. For *S. unisporus* Y-42, a thick sheath of refractive substances could be observed around the cell wall after 1 h of exposure to chitosan-lactate DP25 (Fig. 5B), and the thickness of the layer increased with the duration of exposure to chitosan-lactate (Fig. 5C). The adsorption pattern was irregular but often covered the entire cell wall surface. The material had the appearance of heaps of needles, as shown in Figure 6A. The placement of these heaps seemed to be linked at some specific sites on the cell wall (Fig. 6B). These refractive substances could be hydrolyzed by chitosanase. Furthermore, *S. bayanus* Y-43, the strain that was less sensitive to VJM chitosan hydrolysates (Fig. 3), did not exhibit this coating (Fig. 7).

Similar observations on the chitosan coating were made by Ueno et al. (23) with fluorescent microscopy for *Escherichia coli*, but no data have been available on yeasts. The results obtained for *S. unisporus* Y-42 reveal an irregular coating and support the hypothesis of "cell suffocation" proposed by Roller and Covill (17). This hypothesis is based on the ability of the reactive amino groups in chitosan to interact with a multitude of anionic groups on the yeast cell surface, thereby forming an impervious layer around the cell and causing a loss of active transport and a modification of membrane permeability that could become irreversible after a certain point. Therefore, this hypothesis does not exclude the possibility that chitosan causes a more direct disturbance of the membrane function through leakage of proteinaceous and UV-absorbing materials, as suggested by Leuba and Stossel (11) and Fang et al. (5).

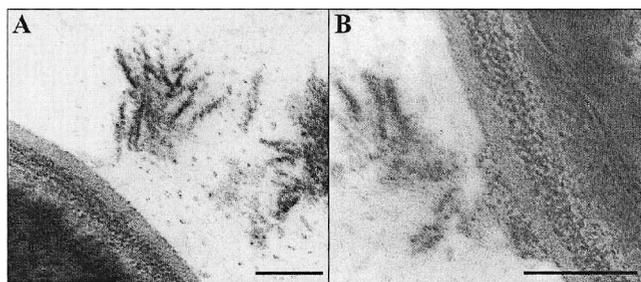


FIGURE 6. Transmission electron micrographs of *S. unisporus* Y-42 showing (A) the "heaps of needles" arrangement of chitosan hydrolysates and (B) the placement of these heaps at particular sites on the cell wall. Bar = 0.25 μ m.

The fact that no coating could be observed on *S. bayanus* Y-43, which also demonstrated a chitosan-lactate sensitivity in both solid and liquid media, suggests that the chitosan could have multiple mechanisms of action simultaneously that do not necessarily implicate the cell wall or the membrane. Onsoyen and Skaugrud (14) have reported that chitosan could bind a range of heavy metals and trace elements and have suggested that the antimicrobial activity of chitosan is principally linked to chelating properties conferred by the polycationic nature of chitosan (19). This hypothesis could apply to our results, but further investigation is needed to determine whether this is also the case for *S. bayanus* Y-43. Our results therefore suggest a multiple mechanism of action for chitosan.

CONCLUSIONS

Partly hydrolyzed chitosan is an interesting molecule that shows potential for use as a preservative in acidic foods such as fermented vegetables. In this study, we found that the antimicrobial activities of chitosan-lactate appear to be considerably influenced by the degree of hydrolysis, the target organism, and the composition of the media. For veg-

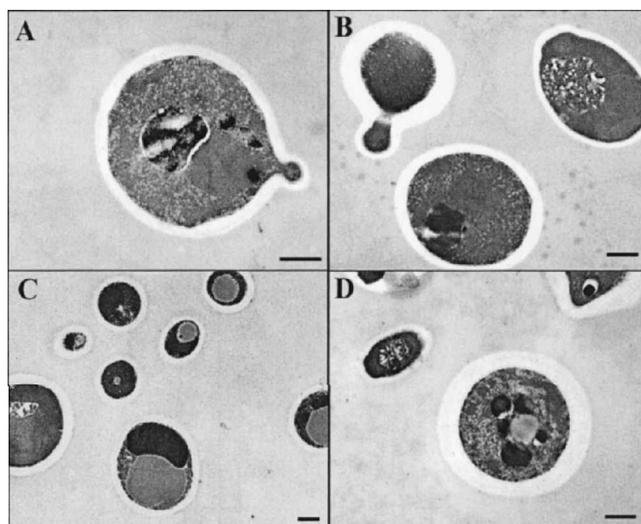


FIGURE 7. Transmission electron micrographs of *S. bayanus* Y-43 (A) immediately after exposure to chitosan-lactate DP25 at 2 g/liter in YM broth acidified with 3 N HCl at pH 4.0, (B) after 1 h, (C) after 5 h, and (D) after 1 h supplemented with chitosanase. Bar = 1 μ m.

etable fermentation, antimicrobial action against LAB may not permit the use of chitosan hydrolysates at the beginning of fermentation. However, the addition of chitosan before packaging would be an interesting alternative. Another possibility would be to use a mixture of different chitosan hydrolysates that could be incorporated into a static matrix like a polymer sheath in plastic bags, permitting the diffusion of chitosan hydrolysates into the product after packaging.

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