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The antifungal properties of chitosan in laboratory media and apple juice

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Abstract

The antimicrobial properties of chitosan glutamate, a derivative of chitin, were investigated in laboratory media and apple juice against 15 yeasts and moulds associated with food spoilage in order to assess the potential for using chitosan as a natural food preservative. Of the seven strains of filamentous fungi studied, chitosan reduced the growth rate of *Mucor racemosus* at 1 g/l whilst concentrations of 5 g/l were required to completely prevent growth of three strains of *Byssochlamys* spp. on agar plates incubated at 25°C for 3 weeks. Three strains of filamentous fungi were resistant to the antifungal effects of chitosan at 10 g/l. The presence of chitosan in apple juice (pH 3.4) at levels ranging from 0.1 to 5 g/l inhibited growth at 25°C of all eight spoilage yeasts examined in this study. The initial effect of chitosan in apple juice was biocidal with viable numbers reduced by up to 3 log cycles. Following an extended lag phase, some strains recovered and resumed growth to levels similar to those observed in unsupplemented apple juice. The most sensitive strain was an isolate of *Zygosaccharomyces bailii* obtained from a spoiled carbonated beverage; this yeast was completely inactivated by chitosan at 0.1 and 0.4 g/l for 32 days of storage at 25°C. The most resistant strain was *Saccharomycodes ludwigii*, an isolate from spoiled cider: a level of addition of 5 g/l of chitosan was required to inactivate this strain and to maintain yeast-free conditions in apple juice for 14 days at 25°C. Growth inhibition and inactivation of filamentous moulds and yeasts, respectively, was concentration-, pH- and temperature-dependent. It was concluded that chitosan was worthy of further study as a natural preservative for foods prone to fungal spoilage.

Keywords: Chitosan; Antifungal; Apple juice; Spoilage yeast

1. Introduction

Chitin, a β-(1,4)-D-linked polymer of N-acetylglucosamine, is a common constituent of crustacean and arthropod cell walls and is extracted commercially from shellfish wastes (Skjak-Braek et al., 1989; Goosen, 1997). Chitin makes up to 45% of the cell wall of *Aspergillus niger* and *Mucor rouxii* and 20% of the cell wall of *Penicillium notatum*; however, it is present at only around 3% in *Saccharomyces cerevisiae* (Arcidiacono and Kaplan, 1992; Knorr, 1984). It has been estimated that chitin

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is synthesised in nature at a level of up to 10^9–10^{10} tonnes per year (Peter, 1997).

Chitosan, a deacetylated derivative of chitin, is found in the cell walls of some fungi (*Basidiomycetes* spp.) and is produced from chitin by alkali treatment. Most commercial chitosans have a degree of deacetylation that is greater than 70% and a molecular weight ranging between 100 000 and 1.2 million Da (Li et al., 1997; Onsoyen and Skaugrud, 1990). Chitosan is polycationic at $pH < 6$ and interacts readily with negatively charged substances such as proteins, anionic polysaccharides (alginate, carrgeenan), fatty acids, bile acids and phospholipids due to the high density of amino groups present in the polymer (Knorr, 1984; Muzzarelli, 1996). Perhaps less predictably, chitosan also selectively chelates metal ions such as iron, copper, cadmium and magnesium. Chitosan has attracted much research attention in the last 20 years as a potentially important renewable resource that is both non-toxic and biodegradable (Goosen, 1997; Muzzarelli et al., 1997; Skjak-Braek et al., 1989).

One of the earliest applications of chitosan has been in chelation of harmful metal ions such as lead, mercury and uranium out of industrial wastewaters (reviewed by Li et al., 1997; Onsoyen and Skaugrud, 1990) and in the removal of suspended solids from food processing wastes (reviewed by Knorr, 1984). The coagulating ability of chitosan has been harnessed to remove solids, unwanted dyes and acid substances from fruit juices effectively (Li et al., 1997). Chitosan and its depolymerised derivatives are reportedly used in personal care products such as hair conditioners and facial creams to impart moisturising properties (Hirano, 1989; Muzzarelli and De Vincenzi, 1997). Recently, the hypocholesteraemic properties of chitosan in the diet have been demonstrated in laboratory rats and in a limited number of human volunteers in short-term trials (Muzzarelli, 1996; Muzzarelli and De Vincenzi, 1997).

Much of the interest in the antimicrobial properties of chitosan has focused on its possible role in plant protection (Gooday, 1991, 1994, 1997). Minimum Inhibitory Concentrations (MICs) as low as 0.075 g/l for particulate chitosan and 0.018 g/l for soluble chitosan against some plant pathogenic fungi in liquid growth media have been reported (Allan and Hadwiger, 1979; Kendra and Hadwiger, 1989). However, other authors have found that levels as high as 10 g/l were necessary to inhibit growth of some fungal strains (Stossel and Leuba, 1984). Chitosan at a concentration of 1 g/l has been reported to reduce growth by up to 50% on agar plates at 25°C of several phytopathogenic fungi important in post-harvest spoilage of fruit and vegetables, including *Botrytis cinerea* (Hirano, 1997). The use of acidic chitosan solutions (containing 0.1% Tween as wetting agent) as disinfecting dips for fresh strawberries inoculated with *B. cinerea* conidia and stored at 13°C has been reported to reduce spoilage to a similar extent as treatment with the conventional chemical fungicide iprodione (Ghaouth et al., 1991).

Relatively little work has been reported on the antagonistic properties of chitosan against microorganisms important in foods. Furthermore, many researchers have targeted bacteria rather than fungi as target organisms for chitosan. For example, Sudarshan et al. (1992) tested the sensitivity of nine bacteria, including *Salmonella typhimurium*, to chitosan glutamate and chitosan lactate (both at 2 g/l) in phosphate buffer (pH 5.8) at 32°C and reported inactivation ranges of between one and five log cycles (depending on the organism) within 1 h of exposure. Both chitosan salts were similarly bactericidal against Gram-positive and Gram-negative organisms, indicating non-specific action. Papineau et al. (1991) have reported greater sensitivity of *S. cerevisiae* compared with *E. coli* or *Staph. aureus* in the presence of 1 g/l chitosan lactate in distilled water at 37°C. However, the biocidal properties of chitosan in relatively 'clean' systems such as distilled water and buffers are a poor indication of likely performance in complex food systems where interactions with other components may modulate the activity of chitosan, as well as of other food preservatives that may be present.

Very few attempts have been made to date to assess the antimicrobial properties of chitosan in real foods. Darmadji and Izumimoto (1994) investigated the effect of chitosan (type of salt not specified by authors) on the development of spoilage in minced beef patties stored at 30°C for 2 days and at 4°C for 10 days. At the higher storage temperature, a reduction of one to two log cycles of total bacteria, pseudomonads, Staphylococci, coliforms, Gram-negative bacteria and Micrococci was observed in the presence of 1% chitosan; at the lower storage

temperature, similar reductions in spoilage flora were reported after 10 days. Fang et al. (1994) have investigated the use of chitosan as an antimicrobial agent against mould spoilage in candied kumquat. The authors reported that a concentration of 6 g/l of chitosan was required to maintain a mould-free shelf life of 65 days when the sugar concentration in the syrup was reduced from the traditional 65° Brix to 61.9° Brix at pH 4 (Fang et al., 1994). Chitosan is reportedly used as a preservative in solid foods in Japan in products such as kamaboko, noodles, soy sauce, Chinese cabbage and sardines; however, many of these reports are lacking in detail so that the conditions/formulations would be difficult to replicate and verify (Li et al., 1997; Hirano, 1997).

The objective of this study was to investigate the antifungal properties of chitosan against 15 food-associated yeasts and moulds in laboratory media and an acidic beverage in order to assess the potential for using chitosan as a natural preservative in foods prone to spoilage by yeasts and moulds.

2. Materials and methods

2.1. Materials

Chitosan glutamate (trade name Seacure 110) was obtained from Pronova (Drammen, Norway). This chitosan preparation contained 42% glutamate and had a deacetylation range of 75–85% (manufacturer's data). All growth media and diluents were obtained from Oxoid (Basingstoke, UK). All other chemicals were from Sigma Chemical Co. unless otherwise indicated. Clear, UHT-treated, shelf-stable apple juice containing no added preservatives and packed in laminated, 1 litre cartons was purchased from a local retailer. The juice was transferred aseptically from its packaging to appropriate culturing vessels without further sterilisation.

2.2. Microbial strains and culture conditions

A total of 15 microorganisms (8 yeasts and 7 filamentous fungi) associated with food spoilage were assembled for this study.

Four strains of filamentous fungi were obtained from the International Mycological Institute (Egham, Surrey, UK) and included *Aspergillus flavus* IMI 242687, *Cladosporium cladosporioides* IMI 274019, *Mucor racemosus* IMI 017313 and *Penicillium aurantiogriseum* IMI 297953. Three strains of *Byssochlamys* spp, isolated from baby food (BF), green coffee beans (GCB) and soybeans (SB), respectively, were kindly donated by Nestec Ltd., Switzerland.

Seven yeasts isolated from spoiled beverages were kindly donated by Pepsico (Valhalla, New York) and included: three strains of *Saccharomyces cerevisiae* ('28' from juice-based beverage; '3085' from juice; and 'SD' isolated in San Diego from Pepsi premix); two strains of *Zygosaccharomyces bailii* ('906' from carbonated beverage and 'HP' from Hawaiian Punch); *S. exiguus* 391 from tea; and *Schizosaccharomyces pombe* from juice. *Saccharomycodes ludwigii*, an isolate from spoiled cider, was a gift from Prof Ron Board (University of Bath, UK).

Stock cultures of yeasts and moulds were grown on Malt Extract Agar (MEA) and Malt Extract Broth (MEB) at 25°C for 3 days, followed by storage at 4°C. Fresh inocula for antimicrobial assessment were prepared by growing the yeast strains in apple juice for 2 days and the moulds on MEA for 2 weeks at 25°C.

2.3. Evaluation of antimicrobial activity

The effect of chitosan glutamate on growth of filamentous fungi was studied using agar plates. A drop of fungal spore suspension was inoculated onto the centre of a MEA plate containing chitosan glutamate at a level of 0, 1, 5 and 10 g/l. Since the chitosan glutamate used in this study contained 42% glutamate (manufacturer's data), the effect of glutamic acid alone (added at a level of 0.42 g/l) was also investigated. The pH of the agar was either left unadjusted at 5.4 or was adjusted to 5.2 or 4.5 with HCl, as required. The plates were prepared in triplicate and incubated at 25°C for up to 3 weeks. Initially, the germination time (lag phase) and the number of days taken for the mould to cover the entire surface of the agar plate (or reach its maximum diameter) were recorded for the seven fungal strains under study. Subsequently, more detailed radial measurements were made to monitor the growth of *M. racemosus* in the presence of 0, 0.1, 0.5, 1 and 2 g/l chitosan glutamate. Each fungal colony, prepared in triplicate, was measured in mm using calipers at three points across the colony

diameter. The latter method was also used to assess the effect of autoclaving 2g/l chitosan glutamate in MEA at 121°C for 10, 15, 20, 25 and 30 min on the antimicrobial properties of chitosan against *M. racemosus*.

The effect of chitosan glutamate on spoilage yeasts was evaluated using viable counting. Screw-capped Duran bottles of 250 ml capacity were filled with 45 ml of apple juice and 5 ml of sterile water (control) or 5 ml of a chitosan glutamate stock solution, and were inoculated with 0.1 ml of an overnight culture (prepared in apple juice) of each of the spoilage yeasts. The inoculated apple juice was incubated at 25°C. Samples were taken periodically up to 32 days, diluted appropriately using Maximum Recovery Diluent (a commercial diluent containing peptone and sodium chloride, Oxoid, Basingstoke, UK) and plated out in 20 μl aliquots on MEA in which 50% of the water used in preparation had been replaced with apple juice.

3. Results

3.1. Antimicrobial activity of chitosan against filamentous fungi

Of the seven strains of filamentous fungi studied, three strains (*A. flavus*, *C. cladosporioides* and *P. aurantiogriseum)* showed no discernible sensitivity (in terms of germination time and number of days to complete growth) when exposed to chitosan-supplemented agar (up to 10 g/l) at 25°C for up to 3 weeks. By contrast, *M. racemosus* and 3 strains of *Byssochlamys* spp inoculated on agar plates containing 5 and 10 g/l of chitosan glutamate failed to grow at 25°C within 3 weeks at which point the experiment was terminated. At lower levels of addition of chitosan glutamate (1 g/l), the germination time (i.e. number of days for first onset of growth) of *M. racemosus* was unchanged (1–2 days) by comparison with the controls containing no chitosan; however, completion of growth took longer (8 days) than on the control plates (6 days) indicating an inhibitory effect on growth. In the case of the three strains of *Byssochlamys* spp, neither the germination time nor the time to complete growth were affected by the presence of 1 g/l of chitosan glutamate in the agar. It was concluded that the Minimum Inhibitory Concentration (MIC) for chitosan glutamate against *Byssochlamys* spp. and *M. racemosus* was between 1 and 5 g/l chitosan.

More detailed studies of the effect of chitosan glutamate on growth of *M. racemosus* were carried out using radial growth measurements over a period of 6 days at 25°C. As shown in Fig. 1, the first onset of growth (1–2 days) was not affected by the presence of 0.1, 0.5 and 1 g/l chitosan glutamate by comparison with the control plates containing no additions. However, the presence of 1 g/l chitosan glutamate inhibited the rate of growth of the mould over a period of 6 days (Fig. 1).

Table 1 shows the effect of two concentrations of chitosan glutamate (1 and 2 g/l), one concentration (0.42 g/l) of glutamic acid (as a control for 1g/l chitosan glutamate) at two temperatures (18° and 25°C) and two pH values (5.2 and 4.5) on growth of the mould. The results (Table 1) suggest that glutamic acid alone may have stimulated growth of the mould slightly but the effect was small. By contrast, chitosan glutamate reduced the rate of growth of *M. racemosus* at all temperatures and pH values tested. Furthermore, the antimicrobial activity of chitosan appeared to be more pronounced at the lower pH and temperatures tested with a reduction in growth rate of nearly 75% occurring in the presence of 2 g/l of chitosan glutamate at 18°C and pH 4.5.

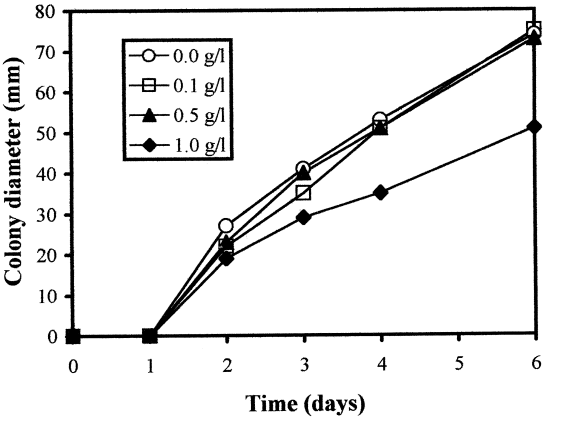


Fig. 1. Growth of *Mucor racemosus* on Malt Extract Agar at 25°C and pH 5.2 in the presence of 0 (○), 0.1 (□), 0.5 (▲) and 1.0 (♦) g/l of chitosan glutamate. Each point represents the mean of nine measurements of colony diameter +/− 2 mm (three determinations × three colonies).

Table 1
The effect of chitosan glutamate (1 and 2 g/l) in Malt Extract Agar on the growth rate (mm/day) of *Mucor racemosus* at two temperatures and two pH values[a]

Growth conditions		Radial growth rate (mm/day±1)				% Reduction in growth rate due to chitosan glutamate	
Temp.	pH	Control, no additions	Glutamic acid added 0.42 g/l	Chitosan glutamate added			
				1 g/l	2 g/l	1 g/l	2 g/l
25°C	5.2	10.3	10.5	8.6	n.d.	17	n.d.
	4.5	9.5	9.8	7.6	n.d.	20	n.d.
18°C	5.2	8.5	9.1	6.7	4.0	21	53
	4.5	7.5	7.8	5.6	2.0	25	73

n.d. = not determined
N.B. Mean colony diameters at each time point were determined on the basis of nine measurements + / − 2 mm (three measurements for each of three colonies). For further details see Fig. 1 and Materials and Methods.

Since heat and acid may cause degradation of some large polymers and it has been suggested that the antimicrobial activity of chitosan may be dependent on high molecular weight (Skjak-Braek et al., 1989), the effect of autoclaving for different periods of time on the antimicrobial activity of chitosan against *M. racemosus* was investigated. Radial growth at pH 4.5 and 18°C was observed to vary between 2.0 and 3.5 mm/day on agar supplemented with 2 g/l chitosan glutamate irrespective of the length of time exposed to 121°C (10, 15, 20, 25 or 30 min). These growth rates were very similar to those reported in Table 1 under the same conditions of temperature and pH. Therefore, the results suggested that the antimicrobial activity of chitosan was not affected by the process of autoclaving in laboratory media for up to 30 min.

During the course of this study, it was noted that after 2 days of growth at 25°C on MEA supplemented with 1 g/l chitosan, *M.racemosus* formed compact colonies with vertically-growing aerial mycelia whilst the same strain grown on unsupplemented agar formed broad colonies closely adhering to the surface of the agar.

3.2. Antimicrobial activity of chitosan against spoilage yeasts

Figs. 2–5 show that the presence of chitosan glutamate in apple juice (pH 3.4) at levels ranging from 0.1 to 5 g/l inhibited growth at 25°C of all eight spoilage yeasts examined in this study. The initial effect on all the yeasts was biocidal with viable numbers reduced by up to three log cycles within the first 5 h of exposure, and in some cases,

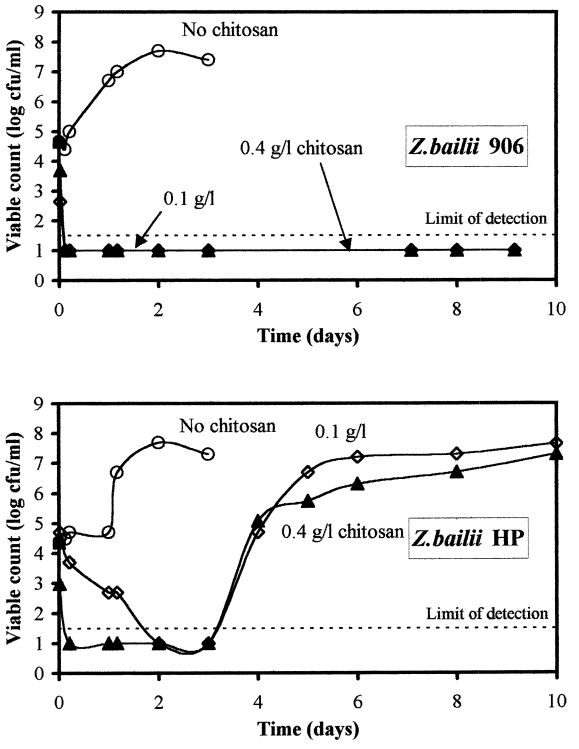
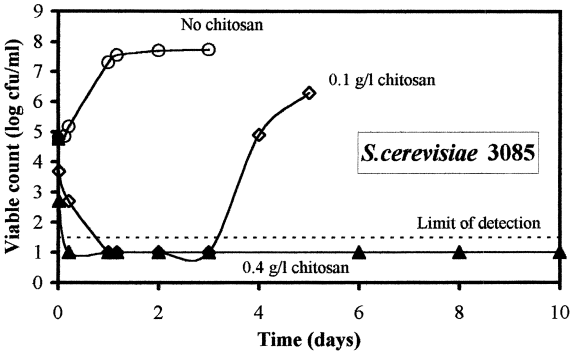
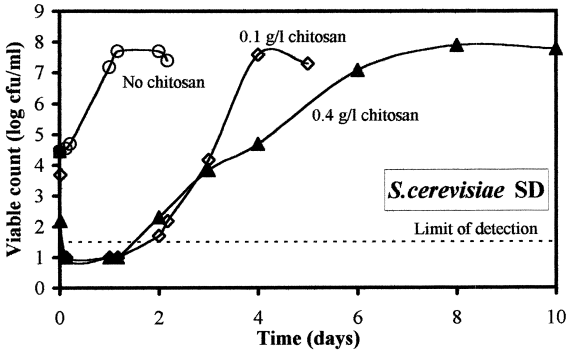


Fig. 2. Fate of two spoilage strains of *Zygosaccharomyces bailii* (HP and 906) in apple juice (pH 3.4) at 25°C and in the presence of 0 (○), 0.1 (◇) and 0.4 (▲) g/l of chitosan glutamate. Dashed line represents the limit of detection for the agar plate assay.





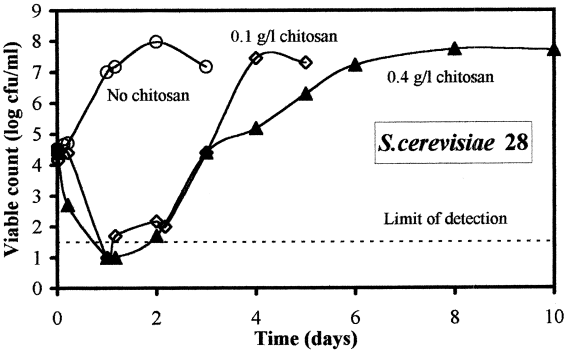
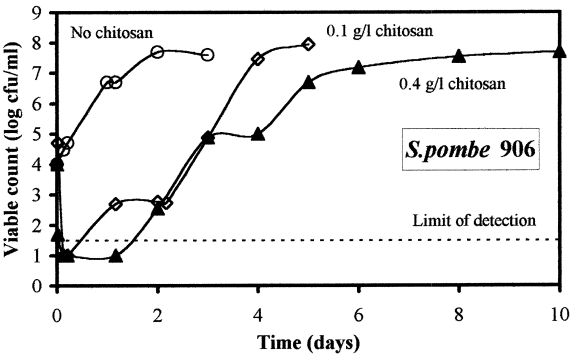


Fig. 3. Fate of three spoilage strains of *Saccharomyces cerevisiae* (3085, SD and 28) in apple juice (pH 3.4) at 25°C and in the presence of 0 (○), 0.1 (◊) and 0.4 (▲) g/l of chitosan glutamate. Dashed line represents the limit of detection for the agar plate assay.

within 30 min. In all cases, viable numbers within the first 24 h of exposure were reduced to below the limit of detection of the plate counting technique. Following an extended lag phase, some strains recovered and resumed growth to levels similar to those observed in the control flasks.



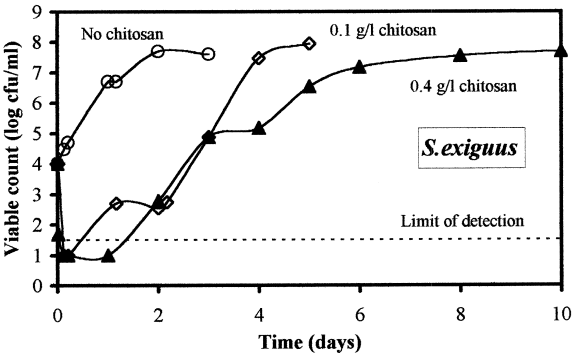


Fig. 4. Fate of *Schizosaccharomyces pombe* and *Saccharomyces exiguus* in apple juice (pH 3.4) at 25°C and in the presence of 0 (○), 0.1 (◊) and 0.4 (▲) g/l of chitosan glutamate. Dashed line represents the limit of detection for the agar plate assay.

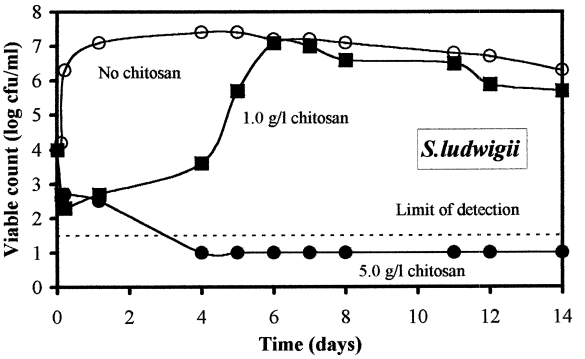


Fig. 5. Fate of *Saccharomycodes ludwigii* in apple juice (pH 3.4) at 25°C and in the presence of 0 (○), 1.0 (■) and 5.0 (●) g/l of chitosan glutamate. Dashed line represents the limit of detection for the agar plate assay.

As shown in Fig. 2, the most sensitive strain was *Z. bailii* 906. This yeast was completely inactivated by chitosan glutamate at 0.1 g/l for 9 days. After 32 days at 25°C, no viable yeast was detected in either of the two chitosan-containing flasks, at which point the experiment was terminated (results after Day 9 not shown in Fig. 2). Similarly, *S. cerevisiae* 3085 was completely inactivated by 0.4 g/l chitosan glutamate for 10 days (Fig. 3) and again, no resumption of growth was observed after 32 days (results after Day 10 not shown in Fig. 3). However, in the presence of 0.1 g/l of chitosan glutamate, *S. cerevisiae* 3085 recovered and resumed growth after a lag phase of 3 days, compared with a lag phase of 5 h in the control flask (Fig. 3). *Z. bailii* HP (Fig. 2), *S. cerevisiae* SD and 28 (Fig. 3), *S. pombe* and *S. exiguus* (Fig. 4) were less sensitive still with lag phases of about 2–3 days at both 0.1 and 0.4 g/l of chitosan glutamate. It was noted that at the higher level of chitosan addition (0.4 g/l), some yeasts grew in a biphasic mode (Fig. 4).

A survival curve for *S. ludwigii*, a particularly recalcitrant spoilage yeast of cider, in the presence of 1 g/l and 5 g/l chitosan in apple juice stored at 25°C is shown in Fig. 5. In the presence of 1 g/l chitosan, there was a reduction in viable count of nearly two log cycles but growth resumed after a lag phase of 4 days (compared with less than 1 day in the control). Chitosan at a concentration of 5 g/l inactivated and prevented re-growth of *S. ludwigii* in apple juice for 14 days at 25°C (Fig. 5). Whereas most of the strains of yeast examined in this study were sensitive to chitosan levels as low as 0.1 g/l, higher concentrations (1 g/l and above) were required to inactivate and/or inhibit growth of *S. ludwigii*.

Preliminary investigations on the morphological effect of chitosan glutamate on *Z bailii* 906, the most sensitive of the strains tested in this study, were carried out using differential scanning microscopy (results not shown). The yeast was exposed to two concentrations of chitosan (5 and 0.5 g/l) at room temperature and the changes in morphology over a period of 60 min were recorded using a video camera attached to the microscope. In the presence of 5 g/l chitosan, the yeast showed evidence of a pitting effect and plasmolysis within 45 s of exposure to chitosan and these effects remained unchanged over the next 60 min. In the presence of 0.5 g/l chitosan, a similar reaction was observed but after 10 min a small proportion of the yeasts appeared to deplasmolyse and regain their normal appearance. Therefore, it appeared that at lower concentrations the antifungal effects of chitosan were reversible.

4. Discussion

4.1. The effect of chitosan on filamentous fungi

Of the seven strains of filamentous fungi tested in this study, *M. racemosus*, a rapidly-growing Zygomycete, and three strains of *Byssochlamys* spp. (belonging to the Ascomycetes group) were inhibited by chitosan glutamate at levels near or just above 1 g/l. By contrast, the fungi in the Deuteromycete group, *A. flavus*, *C. cladosporioides* and *P. aurantiogriseum* were resistant to chitosan at the maximum concentrations tested in this study (10 g/l). Following a study on the sensitivity of 46 fungal strains to particulate chitosan in laboratory media, it has been suggested by Allan and Hadwiger (1979) that the presence of chitosan within the cell walls of some fungi rendered those strains (including *Mucor* spp.) more resistant to the antimicrobial action of externally-added chitosan. The results of our study showing inhibition of growth of *M racemosus* at 1 g/l of chitosan do not support Allan and Hadwiger's proposition.

Although the germination time of *M. racemosus* was not affected by chitosan, the rate of subsequent radial growth on agar was reduced in the presence of 1 g/l chitosan glutamate, as shown in Fig. 1. A similar observation was made by Ghaouth et al. (1991) in their work with fresh strawberries dipped in 1% chitosan solutions and inoculated with *B. cinerea*. These authors have noted that the early signs of mould development on fruit occurred after 8 days of storage at 13°C regardless of type of treatment received; however, after 21 days, the extent of mould growth on chitosan-coated berries, as well as on berries treated with the more conventional chemical fungicide iprodione, was substantially reduced compared with untreated berries (Ghaouth et al., 1991).

The qualitative differences in colony morphology between *M. racemosus* grown with and without

chitosan in the agar medium suggest that the organism grew away from the agar surface in order to avoid direct contact with the toxic effects of chitosan. On the basis of this observation, it can be proposed that the antimicrobial activity of chitosan against filamentous fungi may be improved by ensuring better contact between the chitosan and the target organism; however, this may be difficult to achieve given the inherent mode of growth of fungi. The extent of mycelium penetration within the agar containing chitosan was not investigated in this study.

4.2. The effect of chitosan on spoilage yeasts

The results of this study have shown that chitosan glutamate is an effective antimicrobial agent at very low levels of addition against yeasts associated with spoiled beverages. Seven of the eight yeast strains tested in this study were inactivated by levels of chitosan as low as 0.1 g/l although some species showed evidence of subsequent recovery and growth during prolonged incubation in apple juice at 25°C. Higher concentrations (0.4 g/l) of chitosan were necessary to prevent recovery of some of the yeast strains such as *S. cerevisiae* 3085 (Fig. 3). In general, chitosan concentrations needed to inactivate or inhibit growth of *S. cerevisiae* strains in this study were lower than those reported previously by Allan and Hadwiger (1979). It is possible that the differences in results were due to the use of particulate (as opposed to soluble) chitosan in the earlier study. *S. ludwigii*, an isolate from spoiled cider, was the most resistant of the strains investigated in the present work and required at least 1 g/l of chitosan for reversible inactivation and 5 g/l for irreversible inactivation for 2 weeks (Fig. 5).

It was noted that in the presence of 0.4 g/l of chitosan glutamate, some yeasts grew in a biphasic mode (Figs. 3 and 4). A biphasic mode of growth, also known as diauxie, is frequently an indication of sequential utilisation of substrates. Two classical examples of diauxie occur when species of *Saccharomyces* switch from using glucose to ethanol (Deak and Beuchat, 1996; Lewis et al., 1993) and when *E. coli* change from using glucose to lactose (Pirt, 1975; Roseman, 1972). The plateau in the growth curve occurs when the first substrate is exhausted and a new enzyme system for breakdown of the second substrate is induced. Diauxic growth has also been observed in *E. coli* when grown in media adjusted to A_w 0.986 with 0.42 M sodium chloride (24.6 g/l) and it has been suggested that such growth may be an indication of metabolic adaptation by the organism in response to environmental stress (Roller and Anagnostopoulos, 1982).

4.3. The effect of pH on chitosan activity

Above a pH of 5.5, the solubility of hydrated chitosan salts is significantly diminished (Skjak-Braek et al., 1989) and it has been suggested by Popper and Knorr (1990) that antimicrobial activity was directly proportional to the amount of chitosan in solution. In this study, chitosan glutamate was tested at pH levels well below 5.5 (pH 5.2 and 4.5 for moulds and pH 3.4 for yeasts); therefore, it is unlikely that the greater sensitivity of yeasts compared with the filamentous fungi could be explained by greater chitosan solubility alone. Nevertheless, the work reported here has shown that the inhibitory properties of chitosan against *M. racemosus* were greater at pH 4.5 than at pH 5.2 (Table 1). It is possible that chitosan glutamate may be more antimicrobial at reduced pH values due to a combination of factors including increased solubility.

4.4. The mode of antimicrobial action of chitosan

Several mechanisms for the antimicrobial action of chitosan have been proposed. For example, it has been suggested that chitosan may inhibit microbial growth by acting as a chelating agent rendering metals, trace elements or essential nutrients unavailable for the organism to grow at the normal rate (Skjak-Braek et al., 1989). The growth rates of fungal hyphae have been shown to be sensitive to all factors which influence intracellular Ca^{2+}, including variations in extracellular Ca^{2+} concentrations and the presence of Ca^{2+} transport inhibitors (Jackson and Heath, 1993). Therefore, it is conceivable that chitosan limits growth of filamentous fungi indirectly by making Ca^{2+} and other essential minerals and nutrients unavailable for growth. Such a mechanism would be consistent with the reduced growth rates of *M. racemosus* in the presence of chitosan observed in this study.

Several authors have proposed that the antimicro-

bial action of chitosan against filamentous fungi could be explained by a more direct disturbance of membrane function (Leuba and Stossel, 1986; Fang et al., 1994; Muzzarelli, 1996). Leuba and Stossel (1986) have reported that 5 and 10 g/l of particulate chitosan in phosphate buffer containing 1% maltose induced leakage of proteinaceous and UV-absorbing materials from the plant pathogen *Pythium paroecandrum*. Fang et al. (1994) have shown that 2 and 5 g/l of chitosan in 2.5% acetic acid induced leakage of proteinaceous and UV-absorbing materials from *A.niger*; furthermore, the extent of leakage was concentration-dependent. However, viable numbers were not determined in either of the two studies above and it is debatable whether results obtained in nutrient-deficient buffer systems are representative of chitosan action in complex laboratory media rich in trace elements and minerals.

It has been demonstrated by Feng et al. (1997) that removing iron from grape juice (pH 3.6) to a level below 5 ppb using proprietary chelating resins inhibited the growth of *S. cerevisiae* (unspecified strain) for at least 8 days at room temperature; re-introduction of the iron into the grape juice induced the yeast to grow normally again. Since chitosan has been reported to bind a range of heavy metals and trace elements (Onsoyen and Skaugrud, 1990; Skjak-Braek et al., 1989) it is possible that sequestration of iron or other essential minerals from apple juice may have caused the inhibition of yeast growth reported in our study. However, it is unlikely that nutrient deprivation alone could explain the rapid killing of yeasts shown in Figs. 2–5 and a more direct attack on the integrity of the outer cell wall, leading to a lethal lesion, is more plausible. It is also possible that the killing effect may be caused by the composition of the metals that are left in solution after chitosan has interacted with non-toxic minerals such as calcium.

The ability of chitosan to interact with and/or flocculate proteins is well-known and this property is exploited in the recovery of proteins from food processing wastes and in the immobilisation of enzymes such as glucose isomerase (Knorr, 1984; Skjak-Braek et al., 1989). However, the flocculating ability of chitosan is highly pH-dependent: the positive charge density and the hydrodynamic dimensions of the polymer increase as the pH decreases. At the pH of the apple juice used here (pH 3.4), most microbial proteins can be expected to be below their isoelectric point (pI) and so would carry a positive charge. Therefore, the theoretical likelihood of strong interaction between microbial proteins and chitosan at acidic pH values is low. Nevertheless, the possibility that weak interactions between chitosan and proteins occurred at the very acidic pH values used in this study cannot be ruled out completely.

Proteins are not the only constituents of fungal cell walls. The yeast cell wall has been reported to consist of a number of polymers including glucan (28.8%), mannan (31%), protein (13%), lipid (8.5%), and chitin and chitosan (2%) (Korn and Northcote, 1960). The outer layer of the cell wall consists of mannan polymers linked to proteins and has been shown to play an important role in the binding of heavy metal cations such as copper, cobalt and cadmium by the yeast cell (Brady et al., 1994). It is conceivable that the very reactive amino groups in chitosan have the ability to interact with a multitude of anionic groups on the yeast cell surface, thereby forming an impervious layer around the cell. Such a layer could be expected to prevent the transport of essential solutes, such as glucose (Ralston et al., 1964) and may also destabilise the cell wall beyond repair thereby causing severe leakage of cell constituents and ultimately cell death. This theory, however, remains to be proven.

4.5. Future work

The successful application of any novel antimicrobial agent in food preservation depends on a number of factors. Firstly, as confirmed in this study with chitosan, the antagonistic action is often directed against a select group of organisms and can vary considerably between genera, species, strains and even the same strains under different environmental conditions. As with traditional preservatives, target organisms may develop resistance or tolerance to the new antimicrobial agent upon prolonged exposure. Adequate control of microbial growth in foods using chitosan would require extensive additional knowledge of the factors that determine chitosan performance including the effects of pH, temperature, strain-specificity, other preservatives, food components, etc. Extensive further work on applications of chitosan in solid food systems would be required

before sufficient knowledge could be gained to make chitosan a more generally-accepted food preservative.

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