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ORIGINAL PAPER

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Selective antimicrobial action of chitosan against spoilage yeasts in mixed culture fermentations

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Abstract The effect of chitosan on *Saccharomyces cerevisiae* (the yeast that carries out alcohol fermentation), *Brettanomyces bruxellensis* and *Brettanomyces intermedius* (contaminants of alcohol fermentations), was investigated. The effect of chitosan was tested on each yeast, as well as on mixed cultivations of *S. cerevisiae* + *B. bruxellensis* and *S. cerevisiae* + *B. intermedius*. Chitosan enhanced the lag period of both strains of *Brettanomyces* (80 h for *B. bruxellensis* and 170 h for *B. intermedius* with 6 and 2 g/l chitosan, respectively). The growth rate of *S. cerevisiae* was inversely proportional to the chitosan concentration; the former was 50% when 6 g/l polysaccharide was used. Moreover, in mixed cultivations of *S. cerevisiae* and *Brettanomyces* strains, it was found that both *B. bruxellensis* and *B. intermedius* failed to grow while growth of *S. cerevisiae* was not affected (using 3 and 6 g/l chitosan, respectively). An interesting collateral result was that the presence of chitosan accelerated the consumption of glucose in the mixed cultivations (60 h instead of 120 h).

Keywords Chitosan · Mixed cultivation · *Brettanomyces bruxellensis* · *Brettanomyces intermedius* · *Saccharomyces cerevisiae*

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Introduction

Chitosan, the deacetylated derivative of chitin, is an important bioactive polymer because of the high density of amino groups present. It is considered a natural regulator in plant-fungal pathogen interactions, where it increases crop yields. For the past 10 years, the antimicrobial activity of chitosan in agricultural and medical fields, as well as its effectiveness against foodborne pathogens in the food industry, has been investigated. In this context, *Salmonella typhimurium*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Escherichia coli*, have been successfully inhibited [19]. Additionally, chitosan exhibits antifungal activity [1]; chitosan glutamate was also shown to be an effective preservative against yeast spoilage in apple juice [15].

Yeasts of the genus *Brettanomyces* have long been recognised as contaminants of both industrial alcohol fermentation and production, and of stored fermented beverages [3,4,6,7,9,11], producing bad flavours such as mousy taint and horse sweat taste [9]. Moreover, this genus of yeast is also able to grow under aerobiosis, as has been found in industrial alcohol fermentation [6,7]. These properties increase the chance of finding *Brettanomyces* either during production or storage steps. The increasing problems of wine spoilage have given rise to new research on these kinds of microorganisms. Indeed, the literature reports mainly taxonomic and ecological studies [8, 18], but very little data are available on metabolism [16, 20] or the kinetics of fermentation [11]. To the authors' knowledge, there is no information pertaining to this yeast's growth control by chitosan. The objective of the present study was to investigate the effect of chitosan on *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *Brettanomyces intermedius*, both separately and in mixed cultivations, for further application in the control of *Brettanomyces* in alcohol fermentation.

Materials and methods

Materials

Chitosan from crab shells, supplied by Sigma (St. Louis, Mo.) (deacetylation 91%), was ground and sieved through mesh 40. The powder was sterilised separately at 121°C for 15 min before being added to cultures. All other ingredients used were supplied by Baker (Xalostoc, Edo. de Mex, Mexico), except yeast extract and agar, which were supplied by Bioxon (Cuautitlan, Edo. de Mex, Mexico).

Strains

B. bruxellensis 6037 and *B. intermedius* 4025 were isolated from a contaminated alcohol fermentation in a distillery plant in France, and were identified by the IHEM (Institute of Hygiene and Epidemiology-Mycology) in Brussels. These strains were kindly provided by P. Strehaiano (Laboratory Bioreactors, ENSIACET, INP, France). *S. cerevisiae* ITV1 was obtained from the collection of the Institute Technological of Veracruz. The strains were maintained on agar slants (glucose 10 g/l, yeast extract 10 g/l, agar-agar 20 g/l), incubated at 30°C and stored at 4°C.

Culture medium and conditions

The basic fermentation medium was made up of glucose 30 g/l, $(NH_4)SO_4$ 2.5 g/l, KH_2PO_4 5.0 g/l, $MgSO_4{\cdot}7H_2O$ 0.4 g/l and yeast extract 1.0 g/l. The pH was adjusted to 4 using 10% (v/v) orthophosphoric acid. For mixed cultures, the glucose was increased to 50 g/l. The culture medium was then sterilised at 121°C for 15 min. The experiments were carried out in 150 ml medium for one-strain cultures (in 250 ml Erlenmeyer flasks) and 300 ml for mixed cultivations (in 500 ml Erlenmeyer flasks), both being inoculated with $3{\times}10^5$ log phase cells/ml from a preculture growing at 30°C in liquid medium. Cell cultivation was carried out at 30°C with agitation at 250 rpm. Samples were taken every 3 h. All experiments were performed in triplicate.

Effect of chitosan on yeast growth

The yeasts were inoculated separately and in mixed cultures (*S. cerevisiae* + *B. bruxellensis* and *S. cerevisiae* + *B. intermedius*) in liquid medium. For one-strain culture, sterilised powdered chitosan was added to give final concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g/l. The concentration of chitosan used subsequently in mixed cultivations was determined based on the sensitivity of each strain. The glucose consumption rate (r) was defined as $r = ds/dt$ (g l^{-1} h^{-1}), s = substrate (glucose).

Statistical assay

The results obtained were analysed by ANOVA using MINITAB, and a comparison of means by a Tukey's test was applied in cases of significant difference ($P \leq 0.05$).

Analytical techniques

Growth was followed by cell counts using a haematocytometer (Thoma cell). The percentage of viable cells was measured using methylene blue staining [2]. *Brettanomyces* strains were differentiated from *S. cerevisiae* in mixed cultures by cell shape, the former having a longer shape compared to the oval shape of the latter. Cell counts were monitored every 2 or 3 h until the end of the experiment. Experimental error was estimated to be 8% [10]. Glucose concentration was determined by the Miller method using 3,5-dinitrosalisylic acid [12] on cell-free supernatants centrifuged at 10,000 *g*, 10 min, 20°C, (centrifuge: 5810R Eppendorf). After the assay, absorbance was measured at 540 nm in a Bio-Rad Smart-Spec 3000 spectrophotometer.

Results and discussion

Effect of chitosan on *S. cerevisiae*

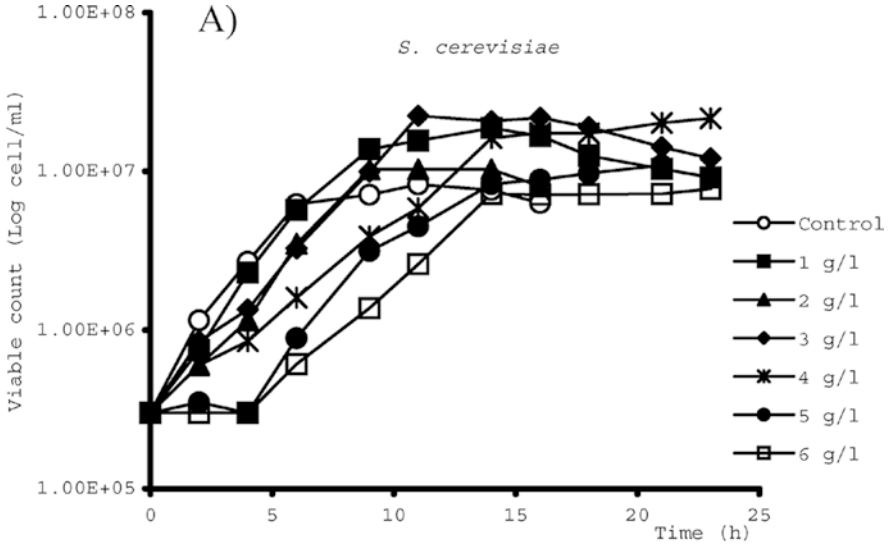
Cell growth of *S. cerevisiae* increased by approximately 1 log cycle, compared to the control, within 8 h at concentrations of 1–4 g/l chitosan. However, with 5 and 6 g/l, the growth pattern was similar to that of the control but in 15 h due to a lengthening of the lag period (Fig. 1A). In general, there was a reduction in growth rate in all cases (data not shown), indicating that chitosan has an influence on the length of the lag phase of *S. cerevisiae* (Table 1). According to Ohtakara et al. [13], chitosan can be hydrolysed by chitosanases and chitinases. The small increase in the cellular concentration was probably due to some hydrolysis of chitosan used as a nutrient at concentrations under 4 g/l. Above this concentration (5 and 6 g/l), it is possible that the polymer could be adsorbed by the cell wall. Allan and Hadwiger [1] suggested that the presence of chitin in fungal cell walls confers resistance to the antimicrobial action of chitosan. Roller and Covill [14] observed that high amounts of chitosan were necessary to alter the growth of *S. cerevisiae*.

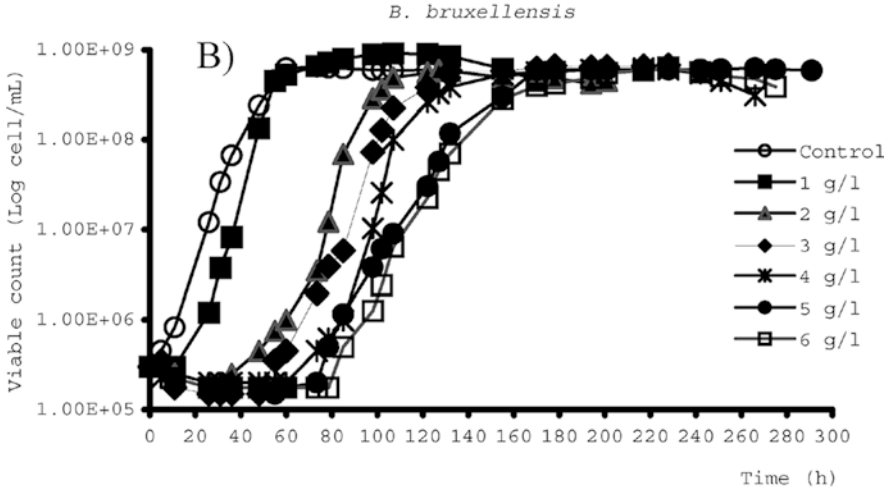
Effect of chitosan on the growth of *B. bruxellensis*

The effect of chitosan on *B. bruxellensis* was quite different to that observed in *S. cerevisiae*. In all cases the presence of chitosan above the 1 g/l level resulted in a longer lag period (Fig. 1B, Table 1). In contrast, the *S. cerevisiae* growth rate did not decrease, and neither did the maximum cell concentration, which was similar to the control, although this was reached at different times (60 h for the control and 170 h for 6 g/l chitosan). This suggests that chitosan has a fungistatic effect on *B. bruxellensis*. The glucose consumption rate decreased as chitosan increased. This confirms that chitosan has a negative effect on the growth of *B. bruxellensis*. Sensitivity of this organism to the antimicrobial action of chitosan has not been reported before.

Effect of chitosan on the growth of *B. intermedius*

This yeast was more sensitive to chitosan than *B. bruxellensis*. Only 0.5 g/l chitosan was enough to delay the lag period until 30 h. When 2 g/l were used, the lag period was prolonged to 170 h (Fig. 1C, Table 1). At concentrations of 3, 4, 5 and 6 g/l chitosan, the yeast failed to grow until 300 hours of cultivation. A higher





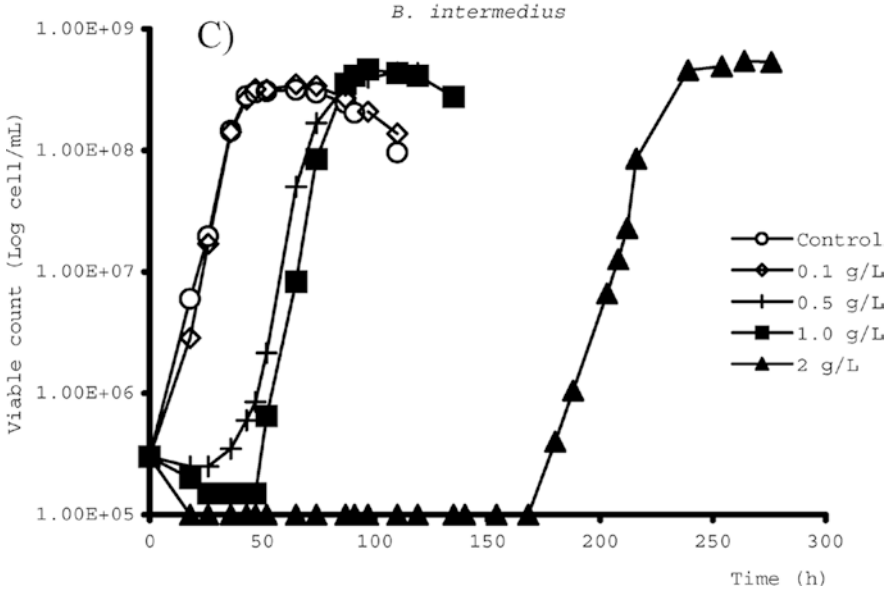


Fig. 1A–C Growth of yeasts in the presence of various concentrations of chitosan. **A** *Saccharomyces cerevisiae*, **B** *Brettanomyces bruxellensis*, **C** *Brettanomyces intermedius*

Table 1 Effect of chitosan on the length of phase lag of *Saccharomyces cerevisiae* and *Brettanomyces* spp

Chitosan (g/l)	Length of phase lag (h)		
	S. cerevisiae	*B. intermedius*	*B. bruxellensis*
0	0	0	0
0.1	nd[a]	0	nd
0.5	nd	30	nd
1	0	45	11
2	2	170	30
3	0	ng[b]	50
4	2	ng	50
5	4	ng	80
6	4	ng	80

[a]Not determined
[b]No growth

chitosan concentration increased the lag period and decreased the specific growth rate and glucose consumption rate. This strain probably does not have the ability to produce chitinase or chitosanase, and therefore chitosan degradation is more difficult.

Mixed cultivation of *S. cerevisiae* and *B. intermedius*

In mixed cultures of *S. cerevisiae* and *B. intermedius*, *S. cerevisiae* exhibited diauxic growth, possibly due to competition for the substrate by *B. intermedius* (Fig. 2A). Only after *S. cerevisiae* was in the death period at 80 h did *B. intermedius* start to grow. The time required for total glucose depletion was 120 h (Table 3). *B. intermedius* may use the small amount of glucose at 80 h to begin growth but this declines at 120 h when glucose has been consumed. When 2 g/l chitosan were added, a biphasic plot for *S. cerevisiae* was observed (Fig. 2B). *B. intermedius* remained in the medium for approximately 60 h, but then the population declined drastically. This phenomenon may be related to the amount of chitosan added and to competition for glucose by *S. cerevisiae*. With 3 g/l chitosan, *B. intermedius* failed to grow (Fig. 2C). In general, for mixed cultures with or without chitosan, the cell cycle of *S. cerevisiae* was lengthened to between 60 and 200 h (Fig. 2), compared to 24 h for *S. cerevisiae* alone, perhaps because *S. cerevisiae* is using the lysed cells of *B. intermedius*, and can also be fed on hydrolysed chitosan. Glucose consumption was much faster in the presence of chitosan (Table 2). The total time for glucose depletion in mixed cultures without chitosan was 120 h, while with 2 and 3 g/l chitosan glucose was depleted at 70 and 60 h, respectively (Table 3). It is not clear why this acceleration of glucose consumption exists. Shaidi et al. [17] pointed out the efficiency of small amounts of chitin (0.2%) in reducing the time for ethanol production using sugar cane molasses from 72 to 30 h, indicating an acceleration in substrate consumption. Chitosan probably functions in a manner similar to chitin.

Mixed cultivation of *S. cerevisiae* and *B. bruxellensis*

In the absence of chitosan, growth of *B. bruxellensis* and *B. intermedius* in mixed cultures was generally different. *B. bruxellensis* did not grow, but it remained viable for at least 300 h (Fig. 3A). *B. bruxellensis* was more sensitive to the presence of *S. cerevisiae* than was *B. intermedius*. Glucose was totally consumed at 200 h (Table 3), and at this point a minor increase in *B. bruxellensis* cell count was observed (Fig. 3A). *B. bruxellensis* may feed on dead *S. cerevisiae* cells and/or on chitosan hydrolysed by *S. cerevisiae*. Glucose consumption increased as the concentration of chitosan increased (Table 3). The biphasic growth of *S. cerevisiae* could be due to the consumption of glucose and some chitosan hydrolysed, or to its acclimatisation to the medium with chitosan and *B. bruxellensis*. Delia et al. [5] carried out mixed cultivation of *S. cerevisiae* and *B. bruxellensis*, but biphasic growth was not observed, perhaps due to strain differences and to the absence of chitosan. When 2 g/l chitosan was added, an active growth phase was observed after a lag period of 100 h, when the growth of *S. cerevisiae* declined and glucose had been consumed (Fig. 3B). This suggests that *B. bruxellensis* can be fed by lysed *S. cerevisiae* cells or chitosan hydrolysed by *S. cerevisiae* or *B. bruxellensis*. With 3 g/l chitosan, *S. cerevisiae* showed biphasic growth, but it persisted longer when 2 g/l chitosan was added (Fig. 3C). *S. cerevisiae* continued to grow until 200 h after glucose had been exhausted, indicating that hydrolysed chitosan molecules may be used.

A level of 6 g/l chitosan was required to control *B. bruxellensis* growth on the one hand, but promoted excellent growth of *S. cerevisiae* on the other (Fig. 3D). The latter grew with a slight biphasic curve, suggesting that substrate competition with *B. bruxellensis* was low, leaving *S. cerevisiae* free to grow. *B. bruxellensis* persisted for only 25 h. Glucose consumption time was shorter than in previous experiments with chitosan (Table 2). In all cases (2, 3 and 6 g/l) the glucose consumption rate increased proportional to the chitosan concentration. Development of *S. cerevisiae* was better at higher chitosan levels, while *Brettanomyces* grew better at chitosan concentrations lower than 3 g/l. Chitosan, therefore, had a positive effect on *S. cerevisiae* growth and a negative effect on *B. bruxellensis*. In conclusion, the addition of chitosan could be a very good alternative for controlling growth of yeast pollutants of the genera *Brettanomyces* during alcohol fermentation without significantly affecting growth of *S. cerevisiae*, and also for increasing the glucose consumption rate, which could stimulate ethanol production. It would be interesting to carry out further experiments on the chitosanase activity of these yeasts to determine if chitosan degradation occurs. If it does, regulation mechanisms of chitosanase synthesis should be studied, as different concentrations of chitosan may inhibit it. Electron microscopy studies are in progress to elucidate the inhibition mechanism of chitosan in *Brettanomyces* strains.

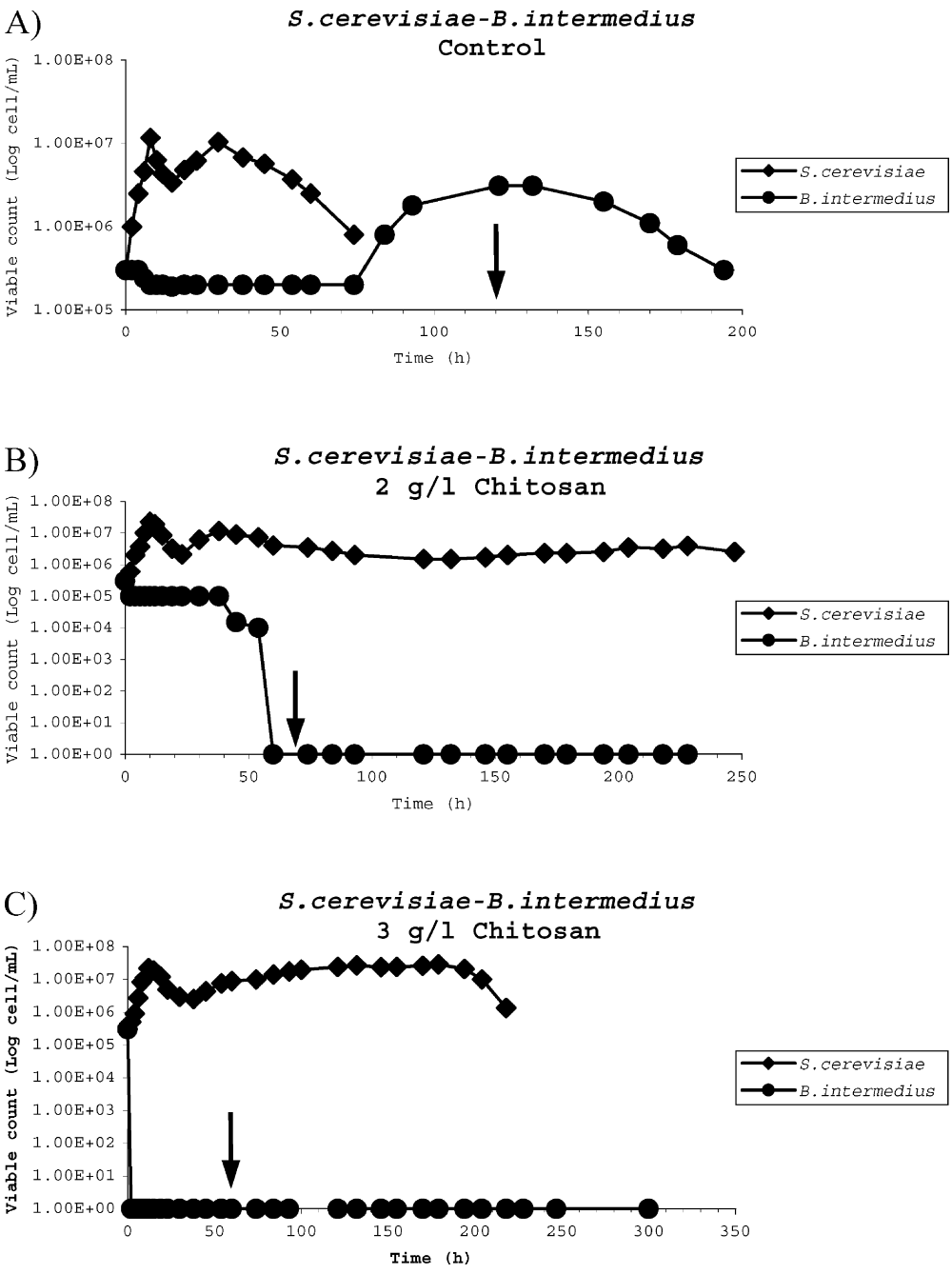


Fig. 2A–C Effect of chitosan on mixed cultures *S. cerevisiae* + *B. intermedius*. *Arrows* indicate total time of glucose depletion. **A** Control, **B** 2 g/l, **C** 3 g/l

Table 2 Effect of chitosan on the glucose consumption rate (r) in mixed cultivations of *S. cerevisiae* + *B. intermedius*

Chitosan (g/l)	r (g l^{-1} h^{-1})	
	S. cerevisiae + *B. intermedius*	*S. cerevisiae* + *B. bruxellensis*
0	0.416 ± 0.090^{a}*	0.250 ± 0.040^{a}
2	0.714 ± 0.050^{b}	0.588 ± 0.040^{b}
3	0.909 ± 0.140^{c}	0.667 ± 0.020^{b}
6	nd**	0.833 ± 0.120^{c}

*Tukey's test, $P = 0.05$, same letter indicates no significant difference
**Not determined

Table 3 Effect of chitosan on time of glucose depletion in mixed cultivations of S. *cerevisiae* + *B. bruxellensis* and *S. cerevisiae* + *B. intermedius*

Chitosan (g/l)	Time (h) *S. cerevisiae* + *B. intermedius*	Time (h) *S. cerevisiae* + *B. bruxellensis*
0	120	200
2	70	85
3	60	75
6	ng[a]	60

[a]No growth

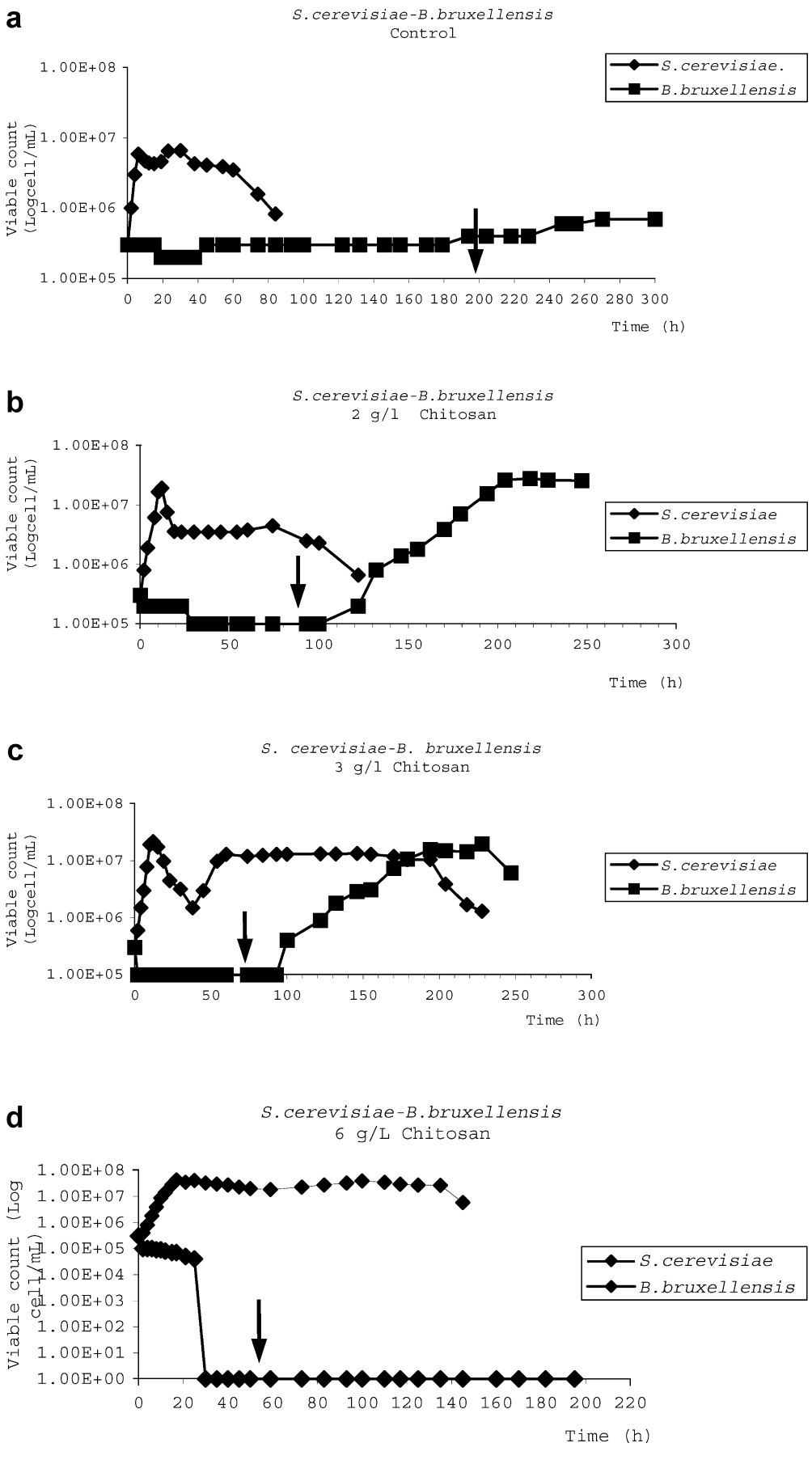


Fig. 3A–D Effect of chitosan on mixed cultures of *S. cerevisiae* + *B. bruxellensis*. Arrows point out total time of glucose depletion. **A** Control, **B** 2 g/l, **C** 3 g/I, **D** 6 g/l

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