Properties of a Wine Yeast Antagonist, *Saccharomyces cerevisiae* T206. A Review

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Regardless of the type of killer yeast, winemaking may be adversely affected by a single type of killer yeast. In this review we present the properties of a single K_2 strain, *Saccharomyces cerevisiae* T206, which was isolated from a stuck fermentation in a South African winery. This zymotidal strain has demonstrated its potential as a wine yeast antagonist and may be differentiated from other NCYC killer strains of *S. cerevisiae* on the basis of CHEF karyotyping and mycoviral RNA separations. Resolution of its genomic DNA into 13 chromosome bands, ranging from 0.2 to 2.2 Mb, has been reported. The resident viral-like particle in strain T206 yields pancreatic RNase-sensitive L and M double-stranded RNA species of 5.1 and 2.0 kb, respectively. The latter can be cured using cycloheximide to produce a K^-R^- derivative. In microscale vinifications the K_2 toxin of strain T206 demonstrates a lethal effect on sensitive mesophilic wine yeasts over a narrow pH range of 3.2 to 3.5, disrupting the cell wall structure and perturbing cytoplasmic membranes. Contrary to known fermentation trends, the challenged fermentations are neither stuck nor protracted as strain T206 competes for substrates and induces over 65% cell death in enriched Hanepoot grape juice media. Mucoid secretions of mesophilic wine yeasts, induced by nutrient limitation, appear to restrict the K_2 killer effect. However, the supplementation of 1 to 100 ppm bovine submaxillary gland mucin in nutrient-enriched grape juice media also depresses the K_2 killer effect of strain T206 in mesophilic wine yeast starter culture strain, *S. cerevisiae* VIN7. Preliminary results suggest that mucoid secretions either affect the level of toxin production by strain T206 or block the cognate K_2 receptor on the cell wall of challanged yeasts.

INTRODUCTION

Killer (zymocidal) strains of *Saccharomyces cerevisiae* are commonly encountered in winery ecosystems (Starmer *et al.*, 1987; Van Vuuren & Jacobs, 1992). Such K^+R^+ yeasts are known to secrete a proteinaceous mycoviral toxin (killer toxin or zymocin) which is lethal to K^-R^- sensitive strains of the same species (Heard & Fleet, 1987; Starmer *et al.*, 1987; Bussey, 1991). The so-called neutral stains (K^-R^+) do not secrete any killer toxin, but they are resistant to the zymocins of other *S. cerevisiae* strains (Wingfield *et al.*, 1990). Based on the properties of the toxin, killer yeasts have been classified into at least 11 groups (K_1 – K_{11}), five of which (K_1, K_2, K_3, K_{28} and K_3GR1) are specific to *S. cerevisiae* (Young & Yagiu, 1978; Extremera *et al.*, 1982; Pfeiffer & Radler, 1982). Killer yeasts are generally immune to their own toxin; however, since wine fermentations are conducted within a non-sterile grape must environment, there exists the potential for ubiquitous wild killer yeasts to interfere with the fermentation. The enological importance of detecting killer yeasts, regardless of the genus or species to which they belong, relates to their potential ability to dominate natural, spontaneous wine fermentations or those wine fermentations initially inoculated with a sensitive wine yeast. In either scenario the domination of the fermentation by the killer strain has been reported to delay the onset of the fermentation and cause sluggish or "stuck" fermentations (Van Vuuren & Wingfield, 1986; Radler & Knoll, 1988; Longo *et al.*, 1990; Carrau *et al.*, 1993), thereby contributing to a lower turnover of tankage (Jacobs & Van Vuuren, 1991). Killer-strain-dominated fermentations have also been reported to give wines an unfavourable organoleptic profile, evident in high volatile acidity, H_2S production and off-flavours caused by fusel oils, acetaldehyde and lactate as well as reduced ethanol yields. These factors can result in serious financial losses to the wine industry (Benda, 1985; Van Vuuren & Jacobs, 1992).

Van Vuuren & Wingfield (1986) first described stuck or protracted fermentations in a South African wine cellar which were induced by the K_2 killer yeast, *S. cerevisiae* T206. Noting that considerable heterogenecity existed among killer yeasts with respect to their ability to kill different sensitive strains (Jacobs & Van Vuuren, 1991), Franken *et al.* (1998) re-investigated the killer property of strain T206 using a sensitive mesophilic wine yeast in a simulated challenge experiment. Contrary to known fermentation trends (Van Vuuren & Wingfield, 1986; Longo *et al.*, 1990; Carrau *et al.*, 1993), the challenged fermentations were neither stuck nor protracted, although over 70% of the mixed cell population was killed in 72 hours.

In this article we present the fermentation properties of *S. cere-*

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visiae T206 in microscale vinifications using enriched and dilute Hanepoot grape juice samples. Emphasis is given to simulated challenges involving strain T206 and mesophilic wine yeasts using both enriched and nutrient-depleted media. Characteristics of the K_2 killer effect are reported using electron microscopy. In addition, the differentiation of *S. cerevisiae* T206 from other yeasts on the basis of nucleic acid constitution is also presented. Previous reviews on killer yeasts (Bussey, 1991; Jacobs & Van Vuuren, 1991) concentrated on the K_1 killer strain of *S.cerevisiae,* which appeared to be the dominant spoilage yeast in certain wineries. Given the equal potential that K_2 killer strains may also interfere with the winemaking process, we find it pertinent to present in this review the properties of such a yeast in comparison with those of other types. In addition, this review is the first to collate the properties of a South African K_2 killer strain of *S. cerevisiae.*

NUCLEIC ACID CONSTITUTION AND STRAIN DIFFERENTIATION

Chromosomes

Electrophoretic karyotyping may be used circumspectly in "fingerprinting" *S. cerevisiae* strains as some of them tend to show dynamic changes in chromosome number and size owing to meiotic recombination (Bidenne *et al.*, 1992; Longo & Vezinhet, 1993) or segregation of "plasmid minichromosomes" from intact chromosomes (Kaback, 1989). Franken *et al.* (1998) presented the chromosome banding pattern of strain T206 using a combination of the embedded-agarose procedure of Carle & Olsen (1985) and the CHEF technique (Van der Westhuizen & Pretorius, 1992). The genomic DNA of strain T206 was resolved into 13 chromosome bands, ranging from approximately 0.2 to 2.2 Mb, the smallest and largest chromosomes corresponding to marker chromosomes I and XII of *S. cerevisiae* strain YNN 295 (Fig. 1). Strain T206 could be differentiated from the wine yeast, *S. cerevisiae* CSIR Y217; on the basis of doublet bands corresponding to the 0.58 and 0.46 Mb markers of strain YNN 295 (Fig. 1, lanes 1 and 2). Strain Y217 produced only a single chromosome band in the 0.46 Mb position (Fig. 1, lane 5). Strain T206 could also be differentiated from the K_2 killer *S. cerevisiae* NCYC 738 and the K_1 killer yeast, *S. cerevisiae* NCYC 190 on the basis of electrophoretic karyotypes (Fig. 1, lanes 2-4).

Mycoviral RNA

K_2 killer strains of *S. cerevisiae* are known to harbour virus-like particles (VLPs) which contain two major types of double-stranded ribonucleic acid (dsRNA), designated the L and M species (Bostian & Tipper, 1984). The LdsRNA genome codes for an RNA-dependent RNA polymerase and capsid protein for both genomes, whereas the MdsRNA generally encodes the proteinaceous killer toxin and immunity against this compound. In *S. cerevisiae* T206 VLPs of a hexagonal structure of approximately 30-40 nm have been isolated and viewed by transmission electron microscopy (TEM) and the double-stranded L and M mycoviral RNA species were estimated to be 5.1 and 1.5 kb, respectively (Van Vuuren & Wingfield, 1986). Franken *et al.* (1998) reported that the size of the LdsRNA genome was found to be reproducible and stable at 5.1 kb. However, the M species of RNA was found to be approximately 2.0 kb, 33% larger than originally reported. The RNA genomes of the K_2 killer, *S. cerevisiae* NCYC 738, and K_1 killer, *S. cerevisiae* NCYC 190, were found to be similar in size to those of strain T206 (Fig. 2). In that study all RNA species, including a high concentration of low molecular species (< 0.83 kb), were found to be susceptible to pancreatic RNase digestion but resistant to DNase I action as expected (Fig. 2). Size variation of the MdsRNA genome is known to occur in *S. cerevisiae* diploids when K_1 killer strains possessing the M_1 genomic species of ca. 1.8 kb are mated with K_2 killer cells containing the M_2 RNA genome of 1.5 kb. The heterozygous diploids lose the M_2 genome and retain the larger M_1 species, which appears to compete more effectively for replication factors (Sommer & Wickner, 1984). Other mechanisms which could contribute to size variation in MdsRNA genomes include the duplication of genes due to polymerase errors and the incorporation of foreign sequences during replication (Sommer & Wickner, 1984; Wingfield *et al.*, 1990).

Cycloheximide curing of strain T206 yields a K^-R^+ trait when testing the derivative against its killer progenitor T206 on methylene blue-yeast extract agar, buffered with citric phosphate (Heard

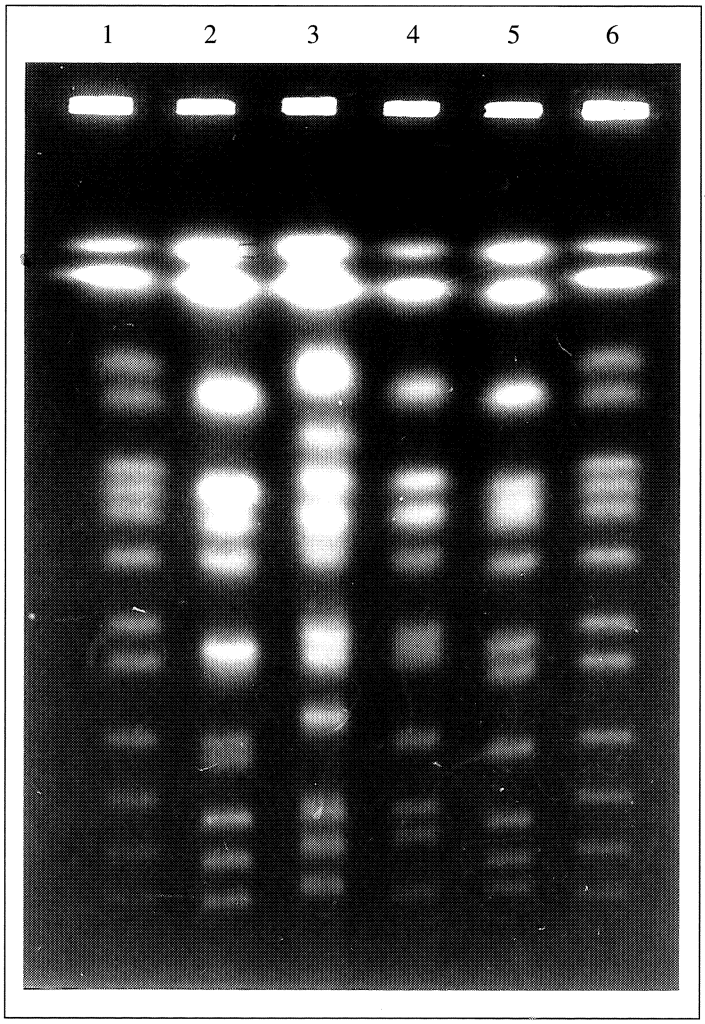

FIGURE 1

Electroforetic karyotyping of killer yeast strains. Contour clamped homogenous electric field (CHEF) banding patterns of chromosomal DNA of *S. cerevisiae* strains: Lane 1, YNN 295 (marker); lane 2, T206; lane 3, NCYC 738; lane 4, NCYC 190; lane 5, CSIR Y217; and lane 6, marker strain (repeated) (Franken *et al.*, 1998).

& Fleet, 1987). A lawn of the cured derivative did not yield a ring of darkly stained dead cells or a zone of growth inhibition (Fig. 3b) when challenged on this medium by streaks of T206 K^+R^+ (Fig. 3a). The K^- trait of the derivative, against K^-R^- mesophilic wine yeasts, was confirmed using the same medium (data not shown). Neutral K_2 strains of *S. cerevisiae*, which are cured of the MdsRNA genome, are expected to be non-producers of the viral toxin and generally sensitive to it. Indeed, this is valid if the MdsRNA genome encoded both the toxin and immunity against it, as documented in the literature (Wingfield *et al.*, 1990). Curing of the MdsRNA genome in strain T206 manifests in a K^-R^+ trait, suggesting that the immunity against the toxin may not be entirely cytoplasmically encoded. The origin of resistance in this derivative against its killer progenitor has yet to be investigated.

K_2 TOXIN ACTIVITY AND CELL DAMAGE

Properties and activity of the K_2 toxin

Very little is currently known about the chemical composition of the K_2 toxin of strain T206. A comparison of protein profiles of the killer strain with those of the killer-cured derivative revealed a protein band of approximately 20 kD, believed to be the putative killer toxin (Wingfield *et al.*, 1990). In microscale vinifications Franken *et al.* (1998) demonstrated toxin activity in a narrow pH range of 3.2 to 3.5 at 25°C when *S. cerevisiae* CSIR Y217 K^-R^-, a mesophilic wine yeast, was challenged by strain T206 in the early logarithmic phase of growth at a killer:sensitive (k/s) cell ratio of 1:100. The minimum variation in pH during 96 h of fermentation was attributed to the buffer capacity of $(NH_4)_2HPO_4$ used to enrich a Hanepoot grape juice medium with nitrogen. Other researchers found the K_2 killer toxin to be stable over a wide pH range of 2.8 to 4.8 (Rogers & Bevan, 1978) and the optimum pH for killer activity was reported to be between 4.2 and 4.4 (Pfeiffer & Radler, 1984).

These are reasons to believe that the extent of killing in a challenge experiment involving killer and sensitive yeasts might be influenced by the ratio of the different cells. Heard & Fleet (1987) studied the incidence of killer yeasts in Australian wineries and found that the killer effect was not apparent when the ratio of

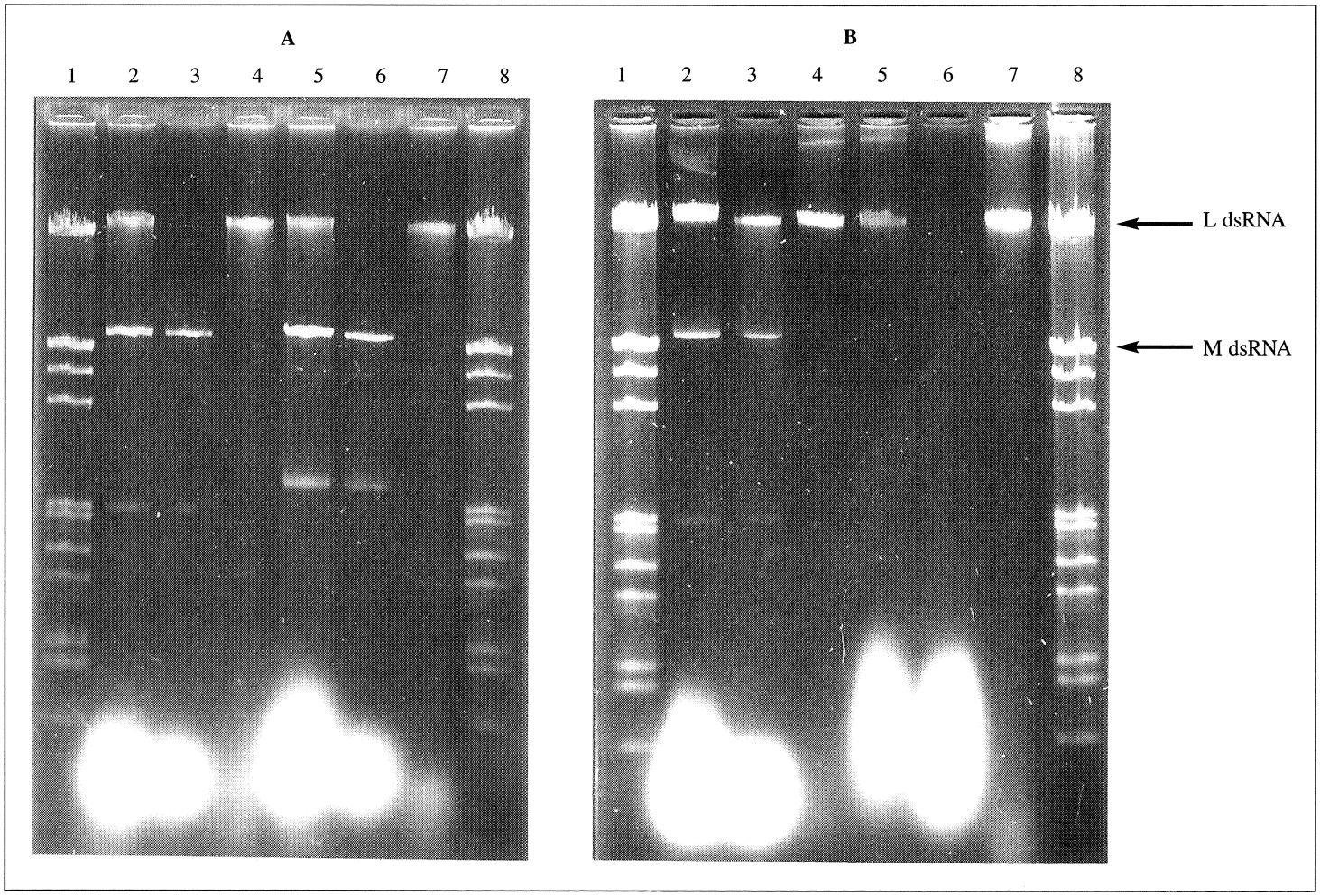

FIGURE 2

Agarose gel (1%) of L and M mycoviral RNA from killer yeasts. (A): Lanes 1 and 8, DNA marker III (*Eco*RI-*Hin*dIII digest of λ DNA) (Boehringer Mannheim, FRG); lanes 2-4, RNA of strain NCYC 738 undigested, DNase I and RNase-digested, respectively; lanes 5-7, RNA of strain NCYC 190 undigested, DNase 1 and RNase-digested, respectively. (B): Lanes 1 and 8, DNA marker III; lanes 2-4, RNA of strain T206 undigested, DNase I and RNase-digested, respectively; lanes 5-7, RNA of strain CSIR Y217K^-R^- undigested, DNase I and RNase digestion, respectively (Franken *et al.*, 1998).

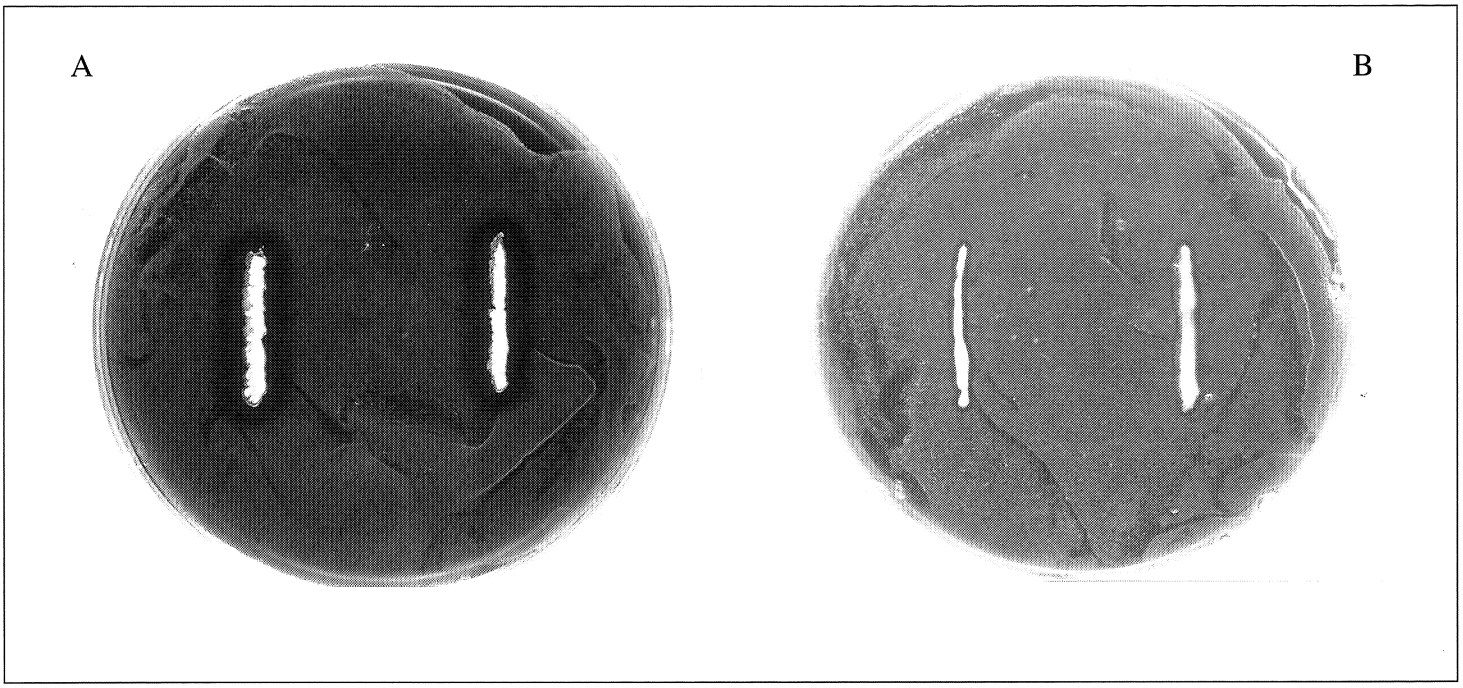

FIGURE 3

Methylene blue agar plate assays of killer activities. (A) The killer activity of strain T206 K^+R^+ demonstrated by a zone of clearing and ring of darkly stained dead cells of *S. cerevisiae* Y217 K^-R^-. (B): Resistance against the K_2 killer effect of strain T206 K^+R^+ by its derivative, *S. cerevisiae* T206 K^-R^+ (Vadasz, 2000a).

killer to sensitive cells (k/s ratio) at the commencement of the fermentation was as high as 1:7. However, pronounced killer activity was reported when the k/s ratio was approximately 1:1. Carrau *et al.* (1993) recorded 82% cell death when a k/s ratio of 1:100 was used during inoculation. In contrast, they also reported cell death of 22 and 21% when k/s ratios of 1:1 and 1:10 000 were employed, respectively.

Cell damage

The nature of cell damage, induced by the K_2 killer toxin of strain T206, was evaluated by scanning (SEM) and transmission (TEM) electron miscroscopy (Franken *et al.*, 1998; Vadasz *et al.*, 2000a). Mesophilic wine yeasts, such as *S. cerevisiae* CSIR Y217 and VIN7, which were challenged by strain T206 in grape juice fermentations, showed rippled cell surfaces, characterised by "cracks" and pores (Figs. 4a, b and c). It is generally accepted that cytosolic efflux and cell death is the result of the initial interaction of yeast killer toxins to specific cell wall receptors (Hutchins & Bussey, 1983; Bussey, 1991), followed by the formation (Martinac *et al.*, 1990) or activation of endogenous ion channels in the plasma membrane of target cells (Ahmed *et al.*, 1999). One such example of the latter is the potassium selective ion channel, TOK1, which is expressed in the plasma membrane of *S. cerevisiae* strains. While the plasma membrane of target cells is implicated in cytosolic efflux, there is little information concerning the structural integrity of the cell wall and that of cytoplasmic membranes (Fig. 5a) in the event of a killer yeast challenge (Fig. 5b). Vadasz *et al.* (2000a) concluded from their studies that the K_2 toxin of *S. cerevisiae* T206 could involve interaction with a specific cell wall receptor, followed by its ability to disrupt cell wall structure (Fig. 5d) and perturb cytosolic membranes (Fig. 5b). In that study toxin-damaged cells showed a crenulated plasma membrane, generating "pinocytotic"-type vesicles (Fig. 5c) or endosomes possibly associated with the internalisation of toxin-receptor complexes (Figs. 6a and b). However, they also speculated that plasma membrane-associated vesicles may originate from golgi cisternae and these could be linked to the exocytosis of secretory compounds to the periplasmic space. A lobular nucleus and poorly defined mitochondrial cristae were amongst other features reported in the toxin-challenged cells (Fig. 5). The findings presented by Vadasz *et al.* (2000a) indicate that the ruptured cell wall provided the efflux outlet, consequently leading to the loss of turgidity (Fig. 4a) and the retraction of an intact plasma membrane from the periplasmic space, coupled with irregular folding (Fig. 5c). The possible activation of specific plasma membrane ion channels by the K_2 toxin of *S. cerevisiae* T206 remains to be investigated.

MICROSCALE VINIFICATIONS AND THE K_2 KILLER EFFECT

It is true that certain stuck or protracted wine fermentations have been influenced by different killer yeasts. Besides the killer yeast, it is also known that such fermentations may be influenced by a variety of factors such as oxygen levels, grape solids and nutrient content, as well as fermentation temperatures, toxic compounds, the wine yeast strain, grape varieties and growth conditions in vineyards (Ingledew & Kunkee, 1985; Backhus *et al.*, 2001). In many cases where stuck fermentations have been reported, both killer and wine yeasts were strains of *S. cerevisiae*. Given that killer yeasts are generally resilient to their own toxin, and utilise

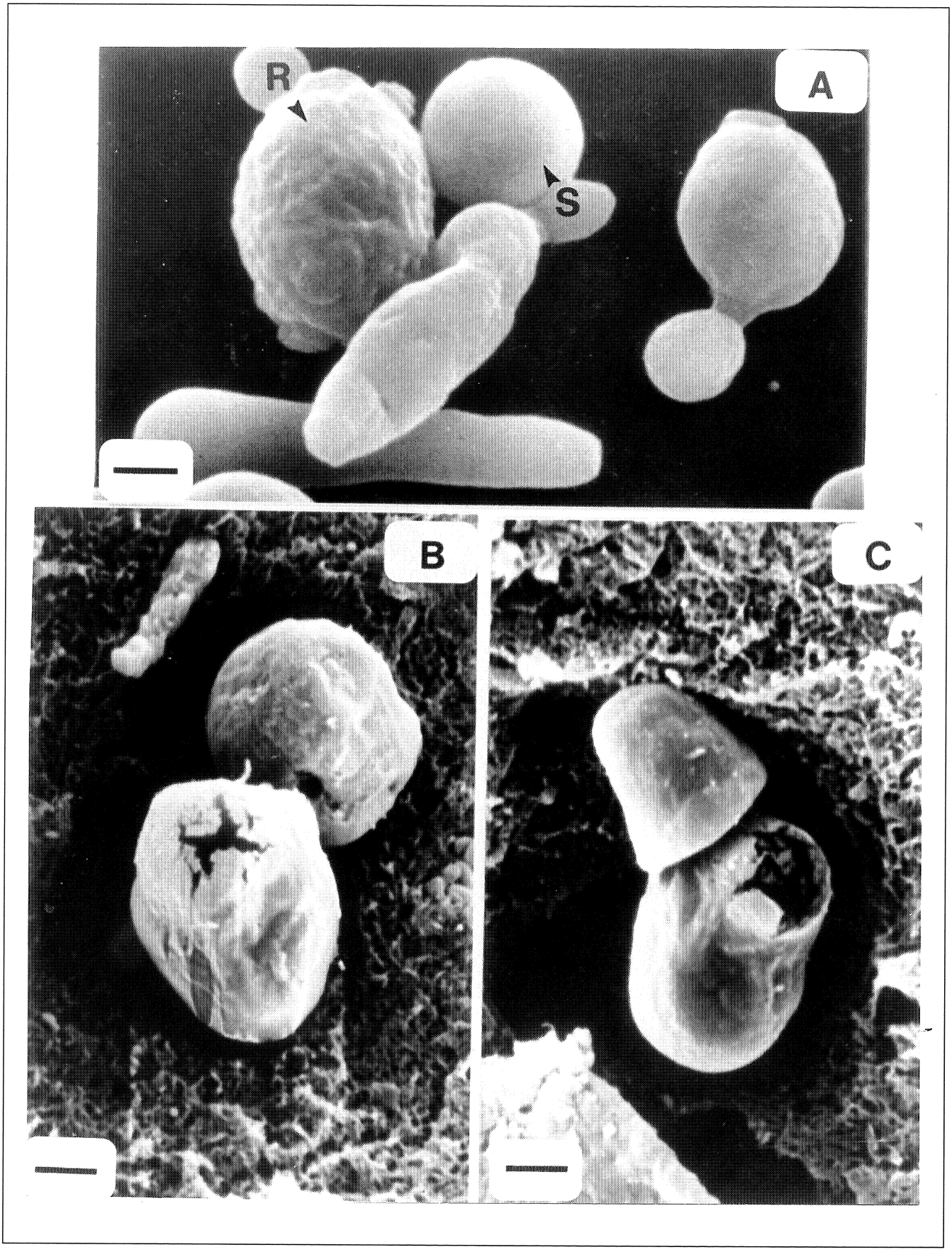

FIGURE 4

SEM images of yeast challenge experiments revealing smooth (S) undamaged cells, toxin-damaged rippled (R) cells (Panel A) and cells showing cracks (Panel B) and pores (Panel C). Bar scales represent 1 µm (Vadasz *et al.*, 2000a).

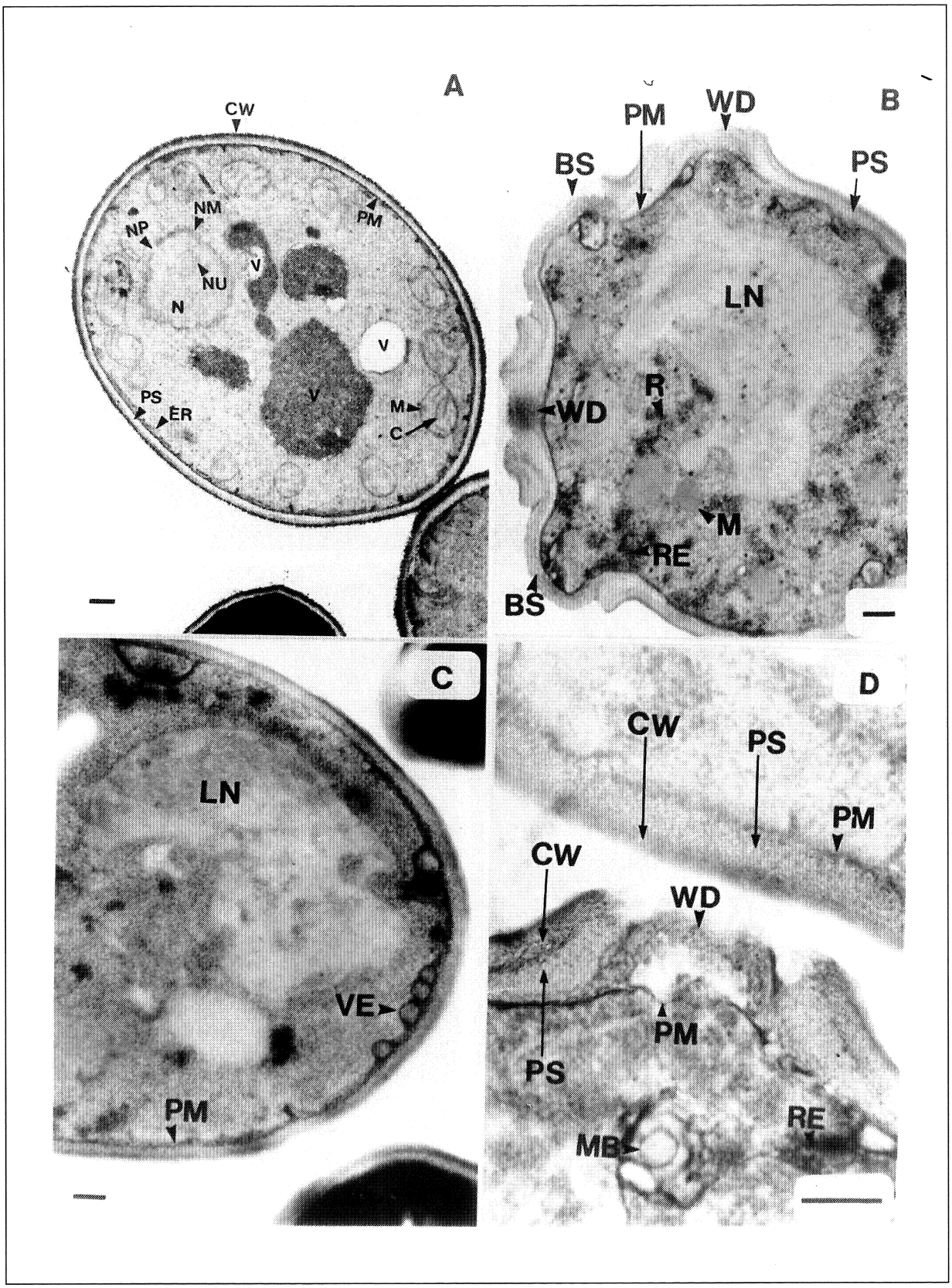

FIGURE 5

TEM images typical of undamaged (A) and toxin-damaged yeast cells (B) showing plasma membrane-associated vesicles (C) and disruption of the cell wall (D). Abbreviations: BS, bud scar; C, cristae; CW, cell wall; ER, endoplasmic reticulum; LN, lobular nucleus; M, mitochondrion; MB, microbody; N, nucleus; NM, nuclear membrane; NP, nuclearpore; NU, nucleolus; PM, plasma membrane; PS, periplasmic space; RE, rough endoplasmic reticulum; R, ribosomes; V, vacuole; VE, plasma membrane-associated vesicle; WD, cell wall damage. Bars represent 200 nm (Vadasz *et al.*, 2000c).

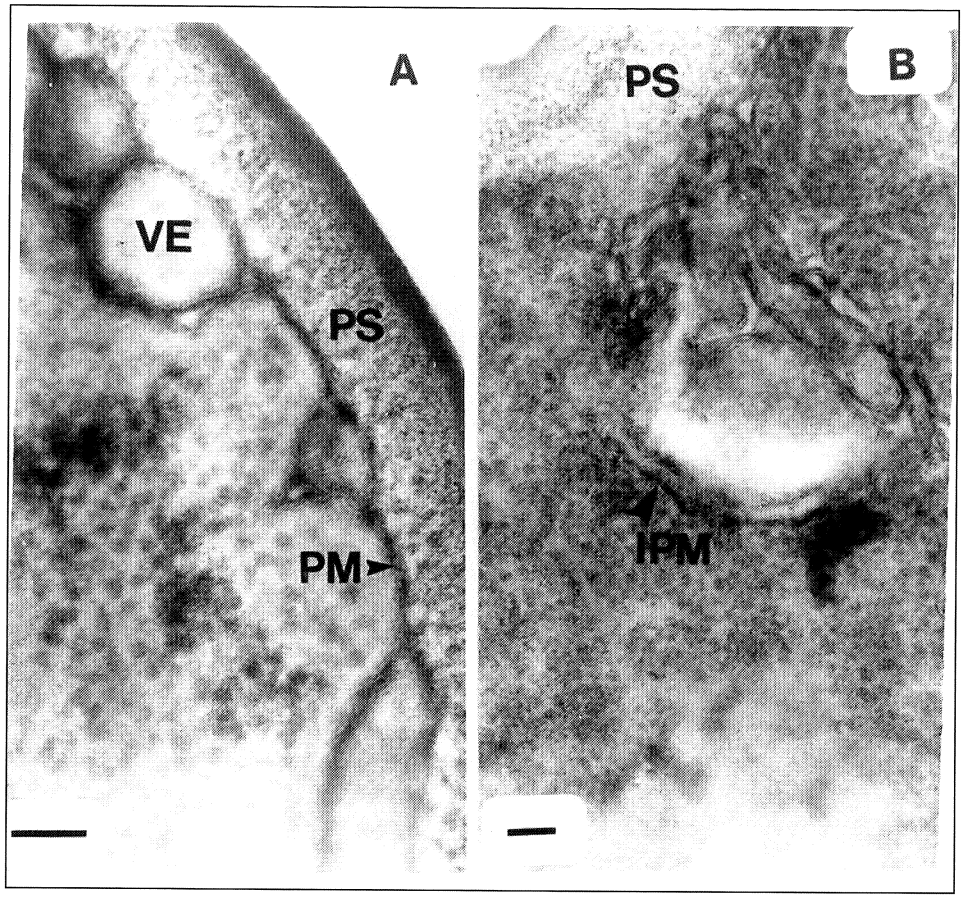

FIGURE 6

Plasma membrane-associated vesicle (VE) (A), possibly due to invagination (B) or fusion of golgi cisternae – originated vesicle. Abbreviations: PM, plasma membrane; PS, periplasmic space; IPM, invagination of plasma membrane or fusion of golgi cisternae – originated vesicle (Vadasz *et al.*, 2000a).

similar substrates as the desired wine yeasts, one questions the reason for a stuck fermentation. Dominant killer yeasts should be able to complete such fermentations; however, the flavour of the end product is less predictable. Indeed, Franken *et al.* (1998) demonstrated such trends when logarithmic phase cells of the mesophilic wine yeast, *S. cerevisiae* CSIR Y217 K^-R^-, were challenged by strain T206 K^+R^+ in a nitrogen-enriched Hanepoot grape juice medium at a killer:sensitive cell ratio of 1:100. Over 65% of the cell population was killed in 72 h; however, the killer yeast was able to produce similar yields of ethanol, acetic acid and glycerol compared with control (single) cultures. The fermentations were neither stuck nor protracted as the rate of production of these metabolites compared favourably with those of the unchallenged control experiments. Vadasz *et al.* (2000b) demonstrated similar fermentation trends using less-enriched 5% grape juice medium; however, the percentage of dead cells in killer-challenged cultures was barely 10 over 72 h. It is likely that nutrient depletion might influence lower levels of toxin production by strain T206, but this requires further investigation. Vadasz *et al.* (2000b) also showed that nutrient depletion induced mesophilic wine yeasts to produce mucoid secretions (Fig. 7) which could potentially mask the K_2 killer toxin receptor, thereby suppressing the killer effect.

In *S. cerevisiae* it is evident that the depletion of nutrients, such as grape sugar and nitrogen in growth media, can potentially activate flocculation or pseudohyphae formation through induction of the structural flocculation genes *FLO1*, *FLO5* and *FLO11/MUC1* (Lambrechts *et al.*, 1996; Gagiano *et al.*, 1999). In nutrient-limiting agar media it is presumed that yeasts anchor to such media with mucin-like proteins promoting adhesion or aggregation of cells, while pseudohyphae forage for the nutrients. More recently Reynolds & Fink (2001) have reported pseudohyphae formation and biofilm or mat growth patterns by *S. cerevisiae FLO11* strains on 0.3% semi-solid agar medium containing low glucose concentrations. In their study *S. cerevisiae Δflo11* strains were recalcitrant to producing pseudohyphae and mats or biofilms under such conditions.

We have recently demonstrated in our laboratory that the killer effect of strain T206 on mesophilic wine yeasts can be strongly inhibited in a nitrogen-enriched Hanepoot grape juice medium when a supplement of either 1, 50 or 100 ppm bovine submaxillary gland mucin is added to the medium (Fig. 8). This result supports the assertion made earlier that mucin might mask the cognate K_2 killer toxin receptor; however, the effect of both nutrient status and mucin on the toxin production requires further investigation. The complexation and inactivation of free, secreted killer

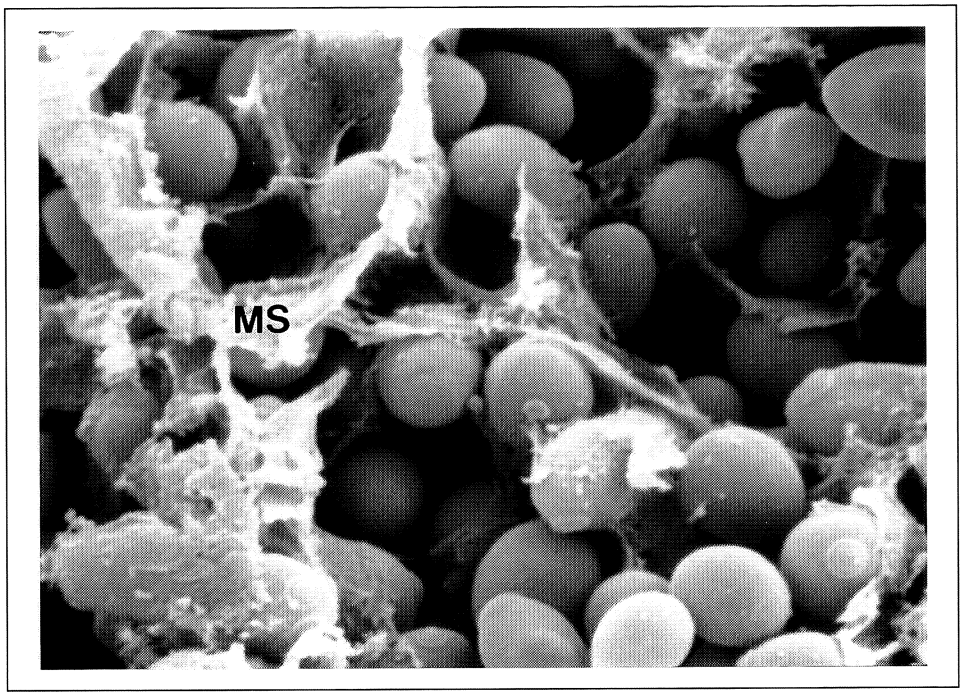

FIGURE 7

SEM image of the intermittent distribution of mucoid sheaths (ms) in liquid culture (Vadasz *et al.*, 2000b).

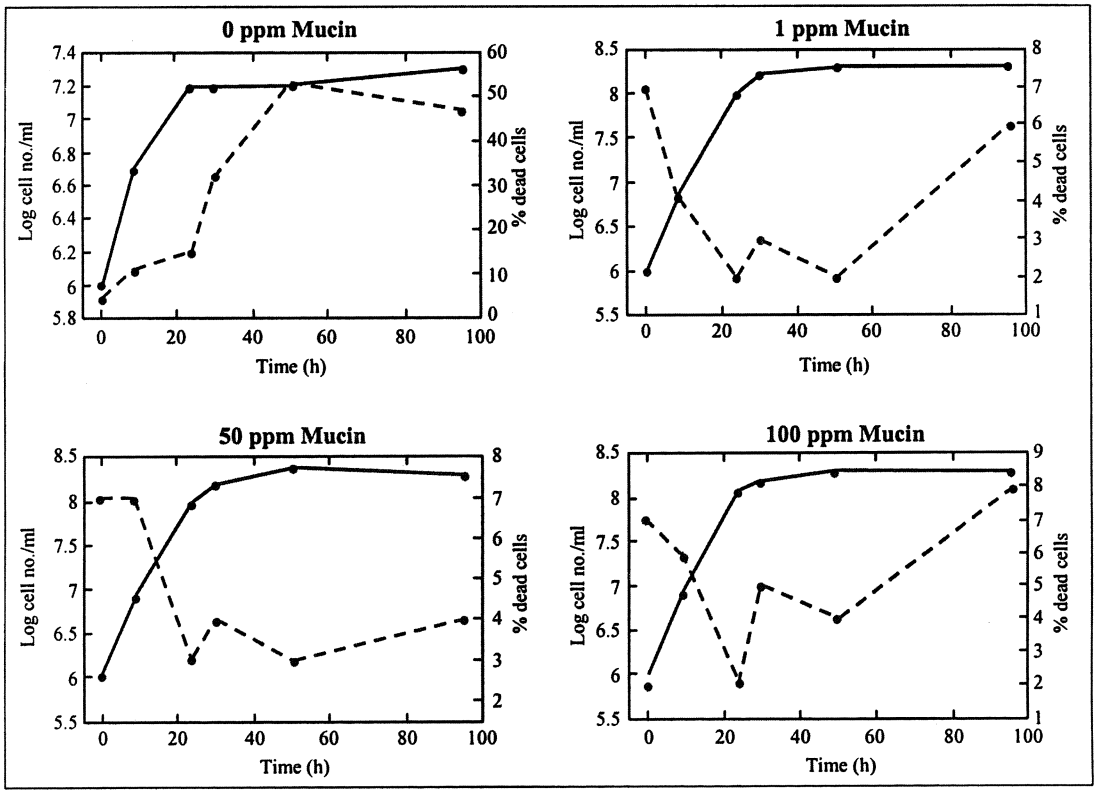

FIGURE 8

The effect of mucin supplementation on the killer activity of *S. cerevisiae* T206 against *S. cerevisiae* VIN7. The killer yeast was introduced at 9 h of growth of the sensitive yeast at a killer: sensitive cell ratio of 1:100 and grown in nitrogen-enriched Hanepoot grape juice medium as described (Franken *et al.*, 1998). The log cells/mL is indicated by the solid line and the percentage of dead cells by the dotted line.

toxin by mucin, prior to interaction with its cognate receptor, is another possibility.

Clearly, we have established that a single yeast strain (*S. cerevisiae* T206) has the potential to interfere with the wine fermentation process as demonstrated in simulated challenge experiments. Further research is therefore required on the *S. cerevisiae* T206 K_2 toxin as its interaction with sensitive yeast cells in grape juice mixtures is far from understood.

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