Killer Yeasts – Cause of Stuck Fermentations in a Wine Cellar

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Sluggish fermentations in five fermenters in a wine cellar were investigated. Methylene blue-stains of yeast suspensions revealed that approximately 90% of the total yeast population in each of the fermenters were dead. The viable cells in each fermenter were killer yeasts. Polyacrylamide gel electrophoresis of total soluble cell proteins showed that the same killer yeast occurred in each of the five fermenters. The effect of killer yeast on viability and fermentation activity of the wine yeast was studied in an enriched grape juice medium at 20°C and 30°C. Death rate of the wine yeast was considerably higher in the presence of the killer yeast and fermentations were retarded at both temperatures. The killer yeast induced flocculation of the non-flocculent wine yeast.

Protracted or stuck fermentations cause serious problems in the wine industry. The phenomenon leads to longer fermentation times and high residual fermentable sugars in dry wines. These factors and the inferior quality of the final product result in financial losses to wineries.

Some sluggish fermentations appear to be associated with musts deficient in oxygen and nutrients, low fermentation temperatures, compounds toxic to yeast, yeast strains, and even variation in climate, soil type and cultivation, soil moisture, fertilizer practices and grape varieties (Agenbach, 1977; Bell, Ough & Kliewer, 1979; Geneix, Lafon-Lafourcade & Ribéau-Gayon, 1983; Tromp, 1981; Ingledew & Kunkee, 1985). There is no agreement on how to avoid stuck fermentations (Blackburn, 1984). Since production of wine is brought about by yeast-mediated fermentation of grape must, protracted or stuck fermentations can only be ascribed to yeast metabolism slowing down and eventually stopping.

Killer yeasts are known to occur in wineries (Naumov *et al.*, 1973; Shimizu *et al.*, 1985). These yeasts secrete a proteinaceous killer toxin lethal to susceptible or sensitive strains of the same species. Neutral strains exist that do not produce a toxin and are not sensitive to the killer factor. Killer strains are immune to their own toxin (Bevan & Makower, 1963). However, so-called K/S killer strains are sensitive to toxins produced by certain other killer strains (Woods, Ross & Hendry, 1975).

Killer yeasts possess two major types of double stranded RNA (dsRNA), the L and M genomes, that are separately encapsidated in virus-like particles. The M-genome codes for the toxin and immunity to this polypeptide (Mitchell & Bevan, 1983). Based on the properties of the killer toxin, killer yeasts are classified into at least 11 groups (K1-K11), three of which (K1, K2 and K3) are specific to *Saccharomyces cerevisiae*. The K1 killers were first described by Bevan & Makower (1963). The K1 toxin is sensitive to high temperatures, proteases and the optimum pH for the production and stability lies between pH 4,6-4,8 (Woods & Bevan, 1968). K1 killers are therefore not important in fermenting grape must as their toxin is inactive at low pH. However, the K2 killer toxin is stable at pH 2,8-4,8 (Shimizu *et al.*, 1985).

K2 killers have been isolated from wine (Naumov & Naumova, 1973; Naumov *et al.*, 1973), beer (Maule & Thomas, 1973; Rogers & Bevan, 1978) and laboratory yeast strains (Young & Yagiu, 1978). Not much is known about the K3 killer toxin. Killer types K4-K11 occur among other genera and species viz; *Candida, Cryptococcus, Debaryomyces, Hansenula, Kluyveromyces, Pichia, Saccharomyces* (non *cerevisiae*), *Ustilago* and *Torulopsis* (Young & Yagiu, 1978). Therefore the K2 killer yeasts and possibly the K3 killers pose a threat to the wine industry since their toxins are lethal to sensitive wine yeasts in grape must fermentations.

In this study we report on the occurrence and properties of K2 killer yeasts isolated from five stuck fermentations in a wine cellar. The effect of the killers on wine yeast grape juice fermentations is discussed. Our findings indicate that these killer yeasts were responsible for the stuck fermentations.

EXPERIMENTAL PROCEDURES

Viability of Yeast in Stuck Fermentations:

The methylene blue-staining technique was used (Anon, 1971).

Yeast strains:

A *Sacch. cerevisiae* strain from Geisenheim, used to inoculate two of the stuck wine fermentations, was obtained from Stellenbosch Farmers' Winery (Pty) Ltd. Killer yeasts were isolated from five commercial wine fermentations (T205, T206, T214, T234 and T243) which ceased to ferment before sugars had been depleted. Samples taken from tanks where stuck fermentations occurred were streaked onto yeast extract malt extract (YM) agar plates and incubated at 26°C for 48 hours. Single colonies were purified by successive streaking on YM agar. Stock cultures were made on YM agar plates and kept at 4°C.

Seeded agar phenotype test:

Low-pH agar medium was prepared by dissolving 6 g Bacto yeast extract, 12 g, Bacto peptone, 12 g dextrose

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and 12 g Bacto agar in 533 ml distilled H_2O. After sterilization 67 ml phosphate citrate buffer (pH 4,5) and methylene blue (20 mg in 5 ml of H_2O) were added aseptically. One millilitre of Geisenheim yeast actively growing in YM medium was spread onto previously dried low-pH agar plates. Yeast strains isolated from stuck fermentations were cultured in YM broth at 26°C. After 18 hours they were spotted on agar plates previously spread with Geisenheim yeast. The plates were incubated at 26°C for 48 hours.

Characterization of yeast strains by poly-acrylamide gel electrophoresis of total soluble cell proteins:

Culture conditions: Individual yeast strains were inoculated in 30 ml YM broth in 250 ml Erlenmyer flasks and incubated at 30°C for 10 hours on a rotary shaker. Each liquid culture was transferred to 120 ml of YM broth in a 250 ml Erlenmyer flask and incubated for a further 9 hours while shaking. The 150 ml culture was finally inoculated into 850 ml YM broth in a 1*l* Erlenmyer flask and incubated (stationary) for 15 hours.

Preparation of cell-free extracts: Yeast cells were harvested and washed once by centrifugation in 0,01 M phosphate buffer pH 7,0 and twice in 3,2 mM Tris-HCI buffer pH 7,0. All Tris-HCI buffers were made up in bidistilled water. Five millilitres of 6,4 mM Tris-HCI buffer pH 8,4 containing 0,001% deoxyribonuclease (Sigma Chemical Company, St. Louis, U.S.A.) and 5 ml of 0,5 mm diameter glass beads were added to approximately 4 g (wet weight) yeast cells. Cells were disrupted in an Edmund Bühler cell mill (Edmund Bühler, Tübingen, West Germany) for 10 minutes. Intact cells and debris were removed by centrifugation (4°C, 15 000 x g, 15 minutes) in a Beckman J2-21 centrifuge. Approximately 4 ml of turbid supernatant was centrifuged for 1 hour at 4°C (80 000 x g) in a Beckman L8-55M ultracentrifuge. Two millilitres of this extract was centrifuged for a further 4 hours at 4°C and 80 000 x g. Protein extracts were stored at -18°C. The protein concentration was determined by the Folin-Lowry method (Plummer, 1971) and adjusted to a concentration of 12 mg/m*l* with 6,4 mM Tris-HCI buffer pH 8,4. Bovine serum albumin (Nutritional Biochemicals Corporation) was used as the standard.

Polyacrylamide gel electrophoresis: The method described by Kersters & De Ley (1975) was used with some modifications. Instead of distilled water, a 5 mm layer of n-butanol was carefully applied on top of the acrylamide gel with an Agla micrometer syringe (Wellcome Reagents Limited, Wellcome Research Laboratories, Beckenham, England).

Electrophoresis was performed in a GE-2/4 Gel Electrophoresis Apparatus (Pharmacia Fine Chemicals, Sweden). The electrode buffer (0,064 M Tris-HCI, pH 8,7) was circulated from the lower to the upper electrode chamber and kept at 8-9°C by water circulating in a glass coil from an Endocal refrigerated bath (Neslab Instruments Inc., Portsmouth, U.S.A.). Each protein extract was run at least three times.

Photography and normalization of photographs: Methods described by Kersters & De Ley (1975) were used. However, Ilford, Ilfospeed photographic paper (grade 1,1 m) was used.

Isolation of virus-like particles:

Virus-like particles (VLPs) were isolated using a modification of the method described by Adler, Wood & Bozarth (1976). Yeast cells were cultured in 100 ml of modified CM broth (4% glucose, 0,5% yeast extract and 0,3% peptone) at 26°C. After 24 hours the yeast suspension was added to 1 litre of CM broth. After 3 days cells were harvested by centrifugation at 10 000 x g for 20 minutes. To every 4 g (wet weight) of cells, 5 ml of buffer (0,03 M sodium phosphate (pH 7,5) and 0,15 M sodium chloride) and 5 ml of glass beads (0,5 mm diameter) were added. The yeast cells were homogenized in an Edmund Bühler homogenizer (Edmund Bühler, Tübingen, West Germany) for 10 minutes. The homogenate was centrifuged at 12 000 x g for 30 minutes and polyethylene glycol added to the supernatant (final concentration 4%). The suspension was allowed to stand on ice for 2 hours and then centrifuged (27 000 x g) for 30 minutes. The pellet was resuspended in the same buffer and the VLPs were harvested by high-speed centrifugation through a 5 ml underlayer of cesium chloride (0,4 g/m*l*) for 2 hours at 4°C (163 000 x g). Pellets were resuspended in 0,15 M ammonium acetate (pH 7,5) or processed further for double-stranded RNA isolation.

Electron microscopic examination of virus-like particles:

Drops of viral suspension were placed on carbon coated "Collodion flexible" grids, washed with water, negatively stained with 2% sodium phosphotungstate, and examined in a Hitachi H300 electron microscope.

Isolation of double-stranded RNA:

Double-stranded RNA (dsRNA) was isolated from the VLPs. The VLP pellet was resuspended in 50 mM Tris-HCI (pH 8,0) and 10 mM $MgCl_2$ and contaminating nucleic acids were removed by digesting with DNase I (final concentration 200 μg/m*l*) and RNase A (final concentration 200 μg/m*l*) for 30 minutes at 37°C. The VLPs were further purified by a second cesium chloride centrifugation and the resulting pellet resuspended in 20 mM Tris-HCI (pH 8,0) 10 mM EDTA and 0,5% SDS. Proteinase K was added (final concentration 200 μg/m*l*) and allowed to digest for 1 hour at 37°C. The dsRNA was extracted twice with phenol, once with chloroform:isoamyl alcohol (24:1), the aqueous layer removed and 1/10 volumes of 3 M sodium acetate and 2,5 volumes of ethanol added. The precipitate was pelleted by centrifugation for 30 minutes at 15 000 x g, the pellet washed with 70% ethanol and the dsRNA was dissolved in 10 mM Tris-HCI (pH 8,0) and 1 mM EDTA for electrophoresis. Electrophoresis of dsRNA in 1% Agarose gels was done using the method of Bolivar & Backman (1979) with 0,5 μg/m*l* ethidium bromide added to the gel and running buffer (89 mM Tris borate, 89 mM boric acid and 8 mM EDTA).

Effect of killer yeasts on fermentation:

Culture procedures: The *Sacch. cerevisiae* strain from Geisenheim and the killer yeast isolated from stuck fermentation T206 were each inoculated into 10 m*l* YM-broth in 50 m*l* Erlenmyer flasks and incubated at 26°C for 20 hours on a rotary shaker. Each culture was added to 100 m*l* commercial grape juice (Monis) previously diluted with water (1:1) and enriched with 0,5% yeast

extract (Merck), 0,05% di-ammonium hydrogen-phosphate and 0,0005% (v/v) Tween 80 (DGM medium). The 250 m*l* Erlenmyer flasks were incubated on a rotary shaker at 26°C for 24 hours. Each yeast was cultured in duplicate. The cells were harvested by centrifugation (10 000 rpm for 15 minutes) under sterile conditions. The Geisenheim yeast was resuspended in 200 m*l* sterile DGM broth and the killer yeast (T206) in 30 m*l* DGM broth. Yeast counts were done using the plate count method and YM agar.

Fermentations: Fermentation trials were conducted in two Multigen Fermenters (New Brunswick Scientific, Edison, N.J., U.S.A.) using sterile enriched grape juice broth (DGM broth pH 3,2) as substrate. Both fermenters were inoculated with 70 ml Geisenheim yeast suspension. Subsequently, the one fermenter was inoculated with 7 m*l* of killer yeast (strain T206) suspension. The killer yeast population in the DGM broth was 2 x 10^5 cells/m*l* and that of the Geisenheim yeast 1 x 10^8 cells/m*l*. The final volume in both fermenters was 1552 m*l*. The fermentation temperature was controlled at 20°C by an Endocal refrigerated circulating water bath (Neslab Instruments Inc., Portsmouth, U.S.A.). The experiment was repeated at 30°C. The viability of the yeast suspension was monitored daily by using the methylene blue-staining technique and the specific gravity of the fermenting grape juice was measured.

RESULTS AND DISCUSSION

Microscopic examination of methylene blue-stained yeast cells in the five stuck commercial fermentations revealed that approximately 90% of the total yeast population in each of the fermenters were dead. The dead yeast cells were much smaller than the viable cells and their shape was different (Fig. 1). Yeast cells killed by by the killer factor are usually shrunken (Bussey, 1974). Furthermore, results obtained with the seeded agar phenotype test revealed that the Geisenheim wine yeast was sensitive to the killer toxin and that the viable yeast cells in all five stuck fermentations were killer yeasts (Fig. 2).

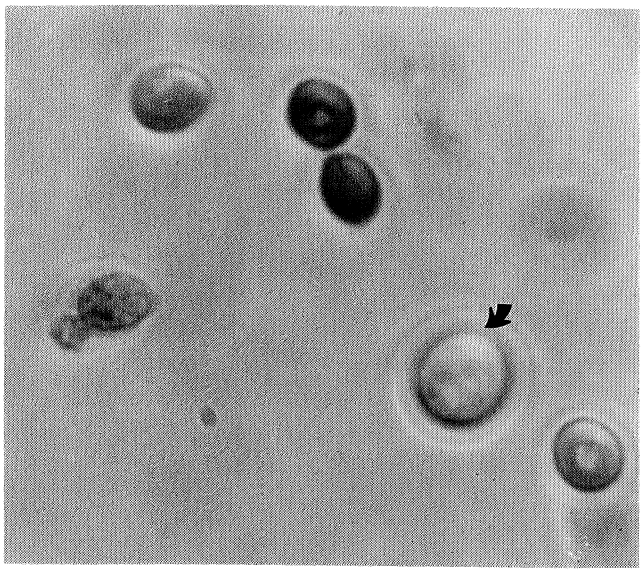

FIG. 1

Photomicrograph of methylene blue-stained wine yeast cells from fermenter T206. The stain is concentrated in dead yeast cells. Viable yeast cells were subsequently shown to be killer yeasts.

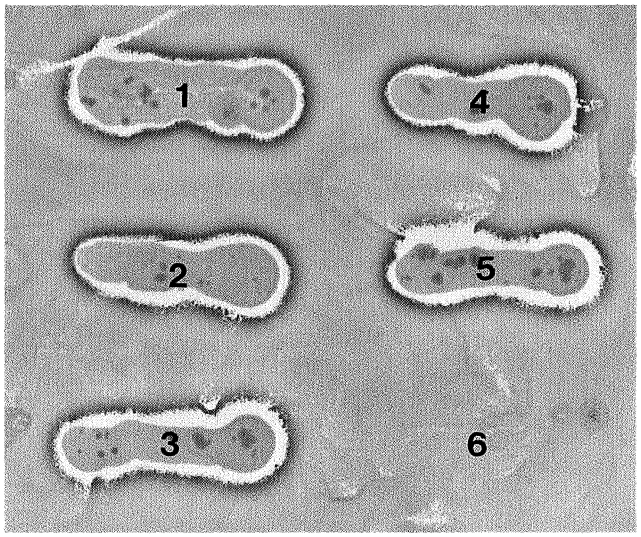

FIG. 2

Samples of killer yeasts isolated from fermenters T205, T206, T214, T234, T243 (1–5) and a neutral strain (6) inoculated onto a methylene blue-containing agar medium previously spread with Geisenheim yeast. Dark zones indicate dead Geisenheim yeast cells.

Fingerprinting of yeasts by protein electrophoresis is a valuable tool to identify individual strains (van Vuuren & van der Meer, In Press). The total soluble cell proteins of killer yeasts isolated from stuck fermentations as well as the Geisenheim yeast originally used in two of the tanks are presented in Figure 3. It is clear that the same killer yeast strain occurred in each of the five fermenters. Furthermore, the protein pattern of the killer yeast differs from that of the Geisenheim wine yeast which was used to inoculate two of the fermenters. The other three tanks were originally inoculated with *Sacch. cerevisiae* (Assmanshausen). However, this yeast strain was not available for investigation.

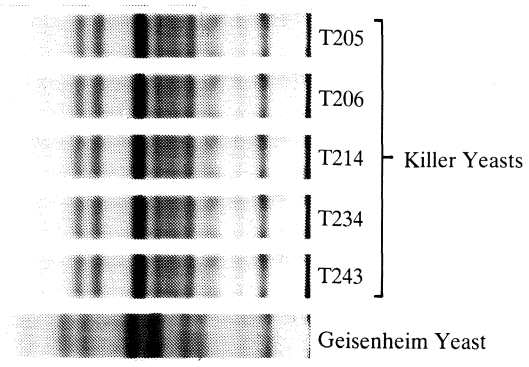

FIG. 3

Normalized gel photographs of total soluble cell proteins of five killer yeasts from different fermenters and the Geisenheim wine yeast.

Figure 4 is a micrograph of the VLPs isolated from killer yeast T206. The VLPs from K1 and K2 killer yeasts contain two major types of dsRNA: L and M dsRNA. Both types of dsRNA were isolated from VLPs obtained from the killer yeasts found in the stuck fermentations (Fig. 5). The Geisenheim yeast did not contain any VLPs. The M dsRNA is known to code for the production of the kill[er] toxin (Bostian & Tipper, 1984). However, the activity and stability at low pH va-

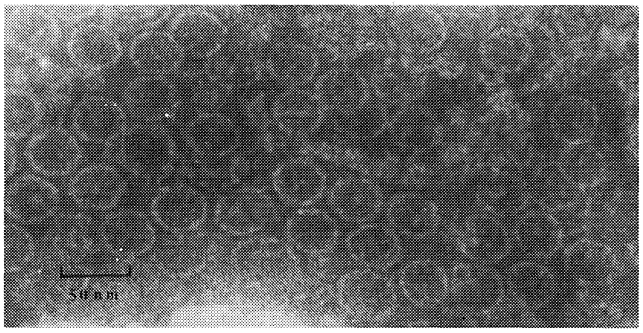

FIG. 4

Electron micrograph of virus-like particles from killer yeast T206.

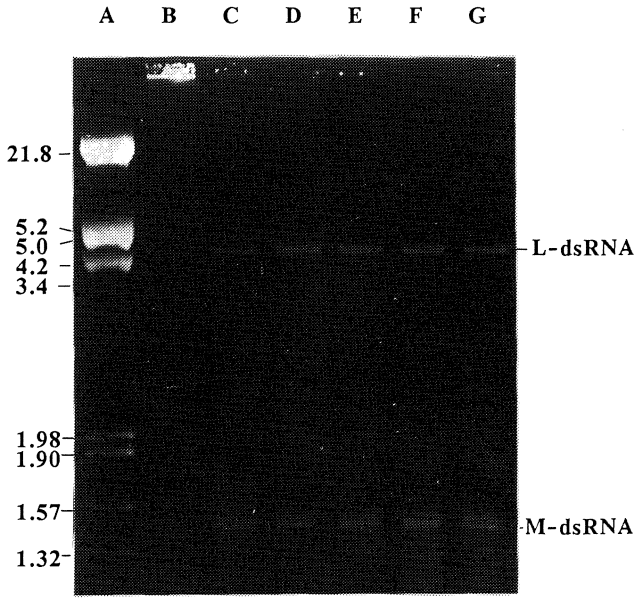

FIG. 5

Agarose gel of dsRNA isolated from killer yeasts (lane A, phage lambda DNA cut with Hind III and Eco RI. Fragment sizes are indicated in kilobases. Lane B, Geisenheim wine yeast; lanes C-G, killer yeasts T205, T206, T214, T234 and T243 respectively).

lues and high temperatures of the K1 and K2 toxins differ markedly. Optimum production and stability of the K1 killer factor lie within the narrow pH range of 4,6-4,8 (Woods & Bevan, 1968). In contrast, the K2 killer factor is stable at pH 2,8-4,8 (Shimizu *et al.*, 1985). These differences imply a different toxin structure which in turn means that the M2 dsRNA that codes for the production of the K2 toxin is also different. We are currently investigating the dsRNAs isolated from the K2 killer yeast T206.

The effect of the killer yeast on the viability and fermentation activity of the Geisenheim yeast strain at 20°C and 30°C is presented in Figures 6 and 7 respectively. At both temperatures the killer yeast decreased the viability of the wine yeast and the rate of fermentation. The death rate of the yeast cells in the presence of the killer yeast was significantly higher. For example, in the presence of the killer yeast only 30% of the total population remained viable after 3 days at 20°C. However, in the control fermentation, 97% of the yeast cells remained viable. At 30°C, mortality of yeast cells both in the presence and absence of the killer yeast was higher than at 20°C. Viability of the Geisenheim yeast in the presence of killer yeast could not be monitored after

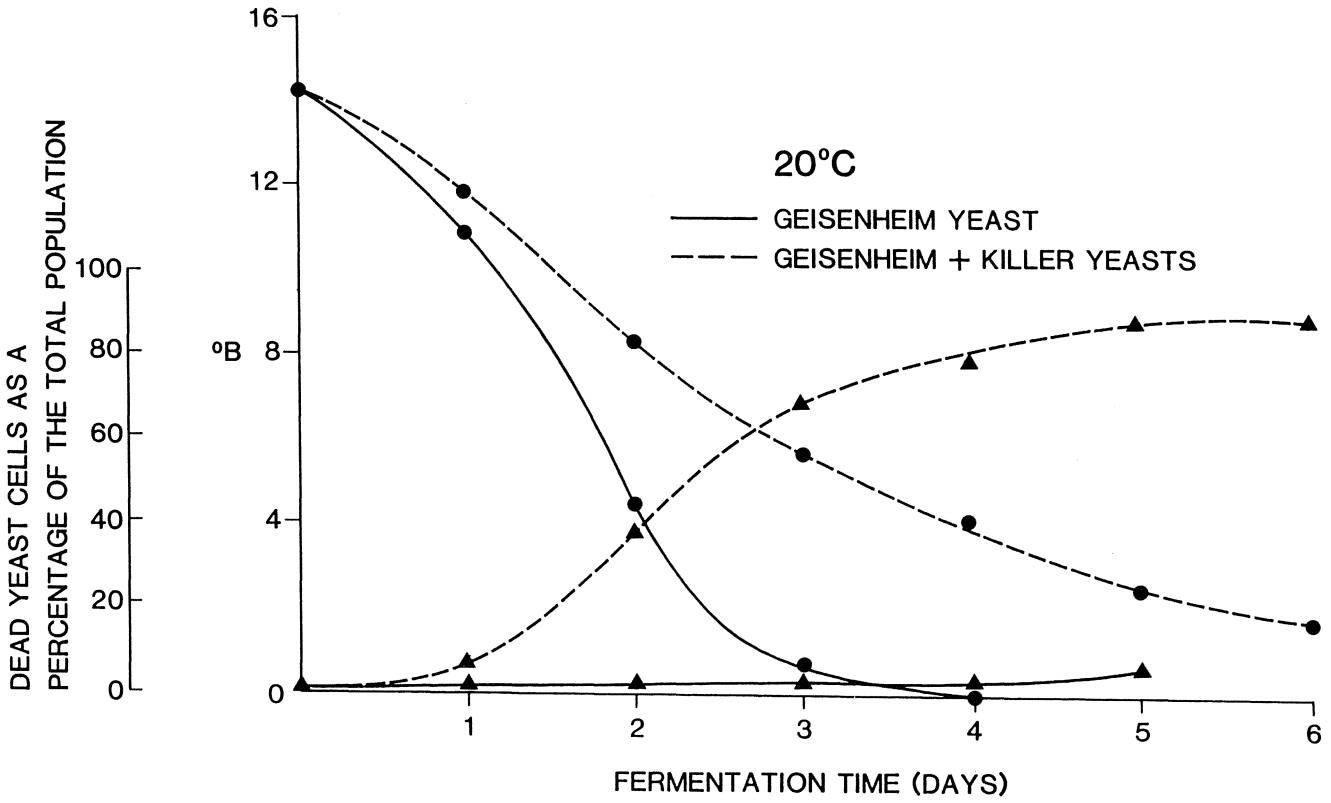

FIG. 6

The effect of killer yeast on the fermentation rate (●) and viability (▲) of wine yeast during fermentation at 20°C.

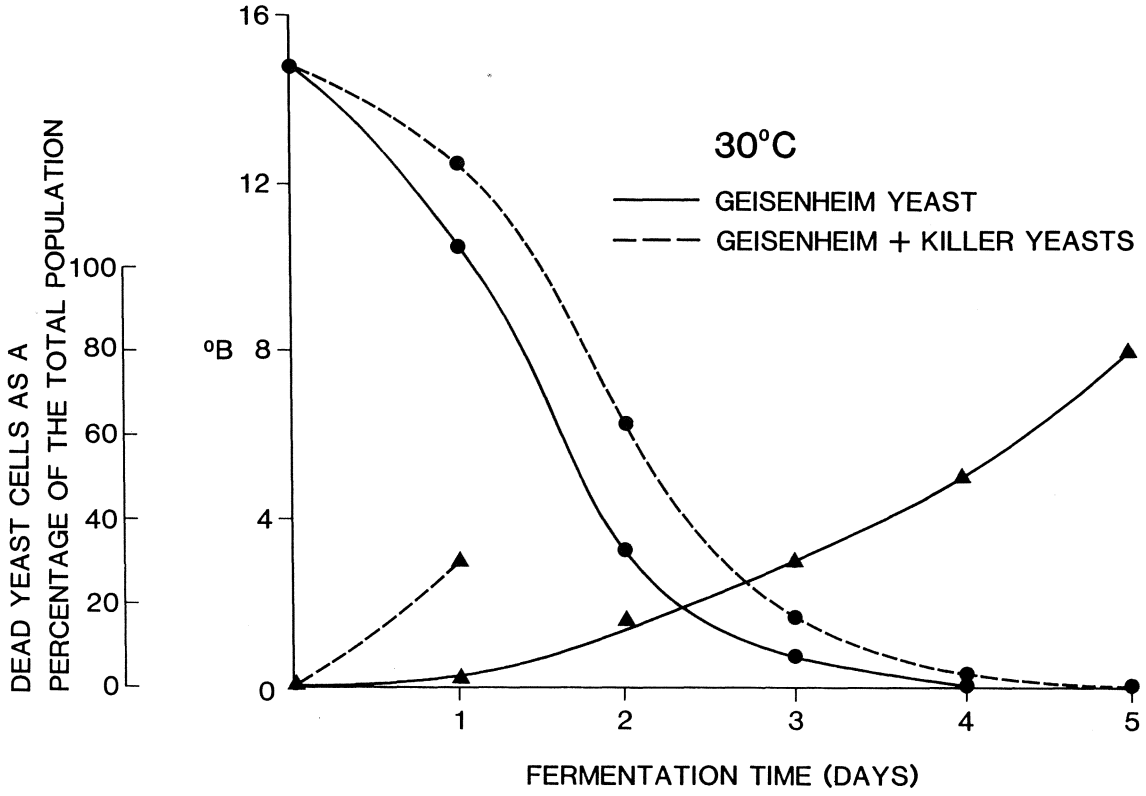

FIG. 7

The effect of killer yeast on the fermentation rate (•) and viability (▲) of wine yeast during fermentation at 30°C.

one day due to extensive flocculation and clump formation.

The control fermentation at 20°C was complete after 4 days, whereas fermentation in the presence of the killer yeast was incomplete even after 6 days. Although the rate of fermentation in the presence of the killer yeast was slower at 30°C, it was completed one day after the control fermentation. The killer yeast probably grew more rapidly at 30°C than at 20°C and might have completed the fermentation. However, even at 30°C the effect of the killer toxin on the viability of the wine yeast and its fermentation activities was obvious.

The killer yeast cell concentration will affect the course of a wine fermentation inoculated with a sensitive yeast strain. Low fermentation temperatures (12-15°C) and high ethanol concentrations (>5%) will inhibit growth of killer yeast cells which may be present at low concentrations, still sufficient to kill the wine yeast, resulting in a stuck fermentation. Higher initial concentrations of killer yeast will lead to death of the wine yeast and a sluggish fermentation that could still be completed by the killer yeast.

Flocculation has been reported in yeasts used in the manufacture of beer but not in wine fermentation. Flocculation refers to the ability of yeast to aggregate spontaneously and form flocs which sediment in the culture during the stationary phase of growth (Stewart & Russell, 1981). During fermentation the Geisenheim yeast flocculated only in the presence of the killer yeast. Microscopic examination of the grape juice medium revealed that flocculation of both strains occurred and only a few cells remained in suspension. Even vigorous agitation for 15 minutes did not resuspend the cells. Flocculation was observed when the killer yeast was cultured separately under identical conditions in DGM broth. The adhesion of one cell to another is frequently mediated by salt bridges in which Ca^{2+} ions play an important role (Taylor & Orton, 1975). It has been speculated that other molecules such as polypeptides might be involved in bridge formation (Stewart, Russel & Garrison, 1975). Results from this study indicate that the toxin produced by the killer yeast mediated flocculation of the non-flocculent wine yeast strain. It would be interesting to know how many flocculating yeast strains produce killer toxins.

The use of killer yeasts to eliminate undesirable yeasts in fermenting grape must has been advocated (Hara *et al.*, 1981; Seki, Choi & Ryu, 1985) and such strains are commercially available. However, the toxin produced by *Sacch. cerevisiae* is only lethal to sensitive and K/S strains. Neutral and killer strains will therefore not be eliminated. Futhermore, reports on the ability of killer yeasts to kill other yeasts species or genera are conflicting. Bostian & Tipper (1984) found that killer yeasts secrete toxins which kill sensitive cells of the same species and frequently those of other yeast species and genera. However, according to Mitchell & Bevan (1983) killer toxin produced by *Sacch. cerevisiae* is lethal to other strains of the same species. Young & Yagiu (1978) determined that non-*Saccharomyces* strains, with the exception of *Torulopsis glabrata* (NCYC 388), were not killed by *Saccharomyces* spp. Furthermore, Hara *et al.* (1981) reported that a hybrid killer wine yeast only killed *Saccharomyces* yeast in grape must.

The ability of killer *Sacch. cerevisiae* wine yeasts to eliminate yeasts of other genera and species thus seems limited. Seki, Choi & Ryu (1985) suggested the construction of a wine yeast harbouring multiple killer factors. However, cytoplasmic dsRNA is known to occur only in *Saccharomyces* spp. and *Ustilago maydis* (Koltin & Day, 1976) and the genetic construction of such a killer yeast strain seems doubtful.

We believe that the use of killer yeasts to eliminate foreign yeasts in commercial wineries has limited application. In fact, we consider it a dangerous practice as contamination of the wine cellar with killers is inevitable and subsequent use of sensitive strains may result in protracted or stuck fermentations. We suggest that neutral yeast strains be used to inoculate grape must to overcome sluggish fermentations due to killer toxins.

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