CLEANSING CONTAMINATED PITCHING YEAST WITH NISIN

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Received 21 August 1986

The ability of solutions of nisin to cleanse pitching yeast of contaminating gram-positive lactic bacteria (at levels of about 1% by cell numbers) was compared to that of three different acid-washing procedures: ammonium persulphate, acidified ammonium persulphate or phosphoric acid. 1000 international units ml^{-1} of nisin killed all the cells of more sensitive bacterial strains in less than 30 minutes, equivalent to the killing ability of the acid-washing procedures, and more than 99·9% of cells of a more resistant strain in 2 hours. Washing contaminated pitching yeast with nisin has several advantages over acid-washing methods. It has no effect on yeast viability and vitality, and, in subsequent fermentations, the yeast shows unchanged flocculation characteristics and fermentative performance.

Key words: *Nisin, pitching yeast, acid-washing, lactic acid bacteria.*

INTRODUCTION

It is well recognised that a contaminated pitching yeast is the most important reservoir of bacterial infection in a brewery or distillery.[1,2,7,9] Contaminants in fermentations usually come from the pitching yeast rather than the unpitched wort. The most frequently found bacteria are strains of the gram negative genus *Hafnia* (or *Obesumbacterium*)[8] and of the gram-positive genera *Lactobacillus* and *Pediococcus*.[1,2] In distilleries, strains of *Leuconostoc* are also common.[9] By the time contaminating bacteria are detected microscopically they can constitute more than 1%, by cell numbers of the pitching yeast. This level is considered unacceptable as it can lead to production of unwanted flavour components during subsequent fermentations, or may inhibit yeast activity.[1,7,8]

Pitching yeast contaminated with bacteria can be dealt with in one of three ways:[7] (i) the contaminated yeast is rejected and replaced with freshly-propagated yeast. This is the ideal method but is not always possible as many breweries do not possess the facilities for yeast propagation. (ii) Yeast is imported from another brewery. This imported yeast, however, may itself be infected, thus introducing fresh contaminants to the brewery. (iii) The pitching yeast is treated to remove the bacteria. This treatment usually involves washing the yeast with a dilute solution of acid (tartaric, phosphoric or sulphuric)[7] or acidified ammonium persulphate.[3] Acid-washing, however, does have disadvantages: after treatment the yeast becomes loosely dispersed with no tendency to settle out, its ability to ferment and flocculate in the fermentation immediately following treatment is altered, and there can be a substantial decrease in the yeast's viability.[7,14]

Washing yeast with antibiotics such as polymyxin, neomycin, and penicillin has been tried but not generally adopted. This is due to them not being active against a wide range of brewery contaminants, and also because their use could lead to a build-up of antibiotic-resistant strains of bacteria.[4,7,12]

Nisin is a potent antibacterial agent active against many gram-positive organisms. In a recent survey, it was shown to be very active against lactic acid bacteria isolated from brewing sources.[11] Also, when added to brewing fermentations, it has no effect on the growth and fermentative ability of brewing yeasts.[10] The aim of the present work was to see if contaminated pitching yeast could be washed with nisin as a milder alternative to acid washing.

MATERIALS AND METHODS

Strains.—Three strains of bacteria from the Brewing Research Foundation's collection of beer-spoilage organisms (BSO) were used in this work: two were of the genus *Lactobacillus* (BSO 343 and 375) and one of the genus *Pediococcus* (BSO 75). All three strains were of undetermined species, were obtained originally from British breweries, and shown to be either killed, or their ability to grow inhibited, by nisin.[10,11] Working cultures of these strains were grown and maintained in liquid cultures (10 ml) of MRS-broth (Oxoid).

Brewing yeasts were obtained from the National Collection of Yeast Cultures (Food Research Institute, Colney Lane, Norwich) and maintained in YM-broth (Difco).

Nisin.—Two grades were used: Nisaplin (Commercial preparation), 1000 I.U.mg^{-1} (international units mg^{-1})[13,15] was obtained as a gift from Aplin and Barrett Ltd., Trowbridge, Wiltshire, and nisin, 40,000 I.U.mg^{-1}, from Koch-light Ltd.

Preparation of yeast slurries.—Slurries of brewing yeasts were prepared for assay using a method similar to that of Bruch *et al.*[3] Initially, strains were propagated aerobically in hopped wort (1·040 original gravity) at 25°C, and then pitched (inoculated) at a rate of 2·5 g yeast wet weight litre^{-1} (equivalent to an initial concentrations of *ca.* 10^7 cells ml^{-1} .into 1·5 litre batches of hopped wort in 2 litre glass vessels. Fermentations were performed at 20°C, under a constant flow of oxygen-free nitrogen (40 ml. min^{-1}) and with continuous stirring (160 rev. min^{-1}). At the end of fermentation, after 3–4 days, the vessels were removed to a cold room (0–4°C) and left for a further 3 days to allow the yeast to settle, after which time excess beer was decanted and the yeast from each fermentor slurried into a final volume of 150 mls. The yeast cell concentration in this slurry was *ca.* 10^9 cells ml^{-1}.

50 ml samples of the slurries were transferred into sterile 250 ml conical flasks. Bacteria, from 10 ml MRS-broth cultures grown for 24 h at 25°C without shaking were added, when required, to give a contamination level of approximately 1% by cell numbers (i.e. *ca.* 10^7 bacterial cells ml^{-1}).

Yeast washing.—Yeast slurries were washed with nisin by the addition of 50 ml of 0·1 M citrate-phosphate buffer pH 5·0 containing twice the final desired concentration of either nisin or Nisaplin. These suspensions were then stirred gently and continuously for 2 h at room temperature. Samples were taken at different times and assayed for the viability of both yeast and bacteria (see below). Citrate-phosphate buffer containing no nisin was added to yeast slurries to provide untreated controls.

Slurries were 'acid-washed' by one of three different methods:

i) phosphoric acid (3 M) was added dropwise, and with continuous gentle stirring, to the slurry to give a pH of 2·2. Sterile deionised water was then added to give a final volume of 100 ml.
ii) 50 ml of 1·5% (w/v) ammonium persulphate solution was added to the slurry (final concentration = 0·75% (w/v).[3]

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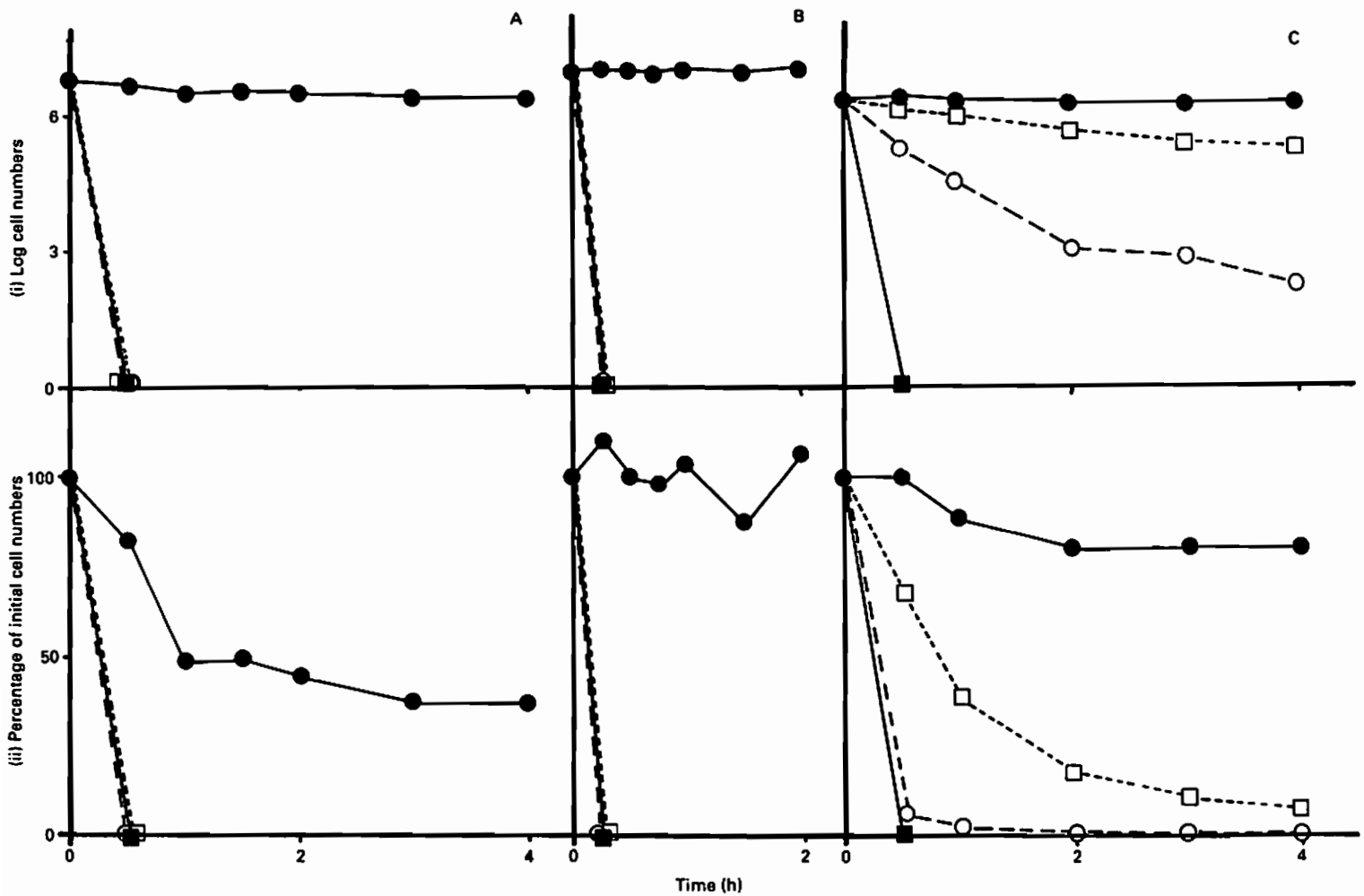

Fig. 1. Comparison of the effects with time of nisin-washing and three methods of acid washing on the viability of gram-positive bacterial contaminants in slurries of pitching yeast (NCYC 1236). A—BSO 375; B—BSO 75; C—BSO 343. ●—untreated; □—washed with 100 I.U. ml^{-1} nisin; ○—washed with 1000 I.U. ml^{-1} nisin; ■—all three acid-washing procedures (phosphoric acid, ammonium persulphate and acidified ammonium persulphate).

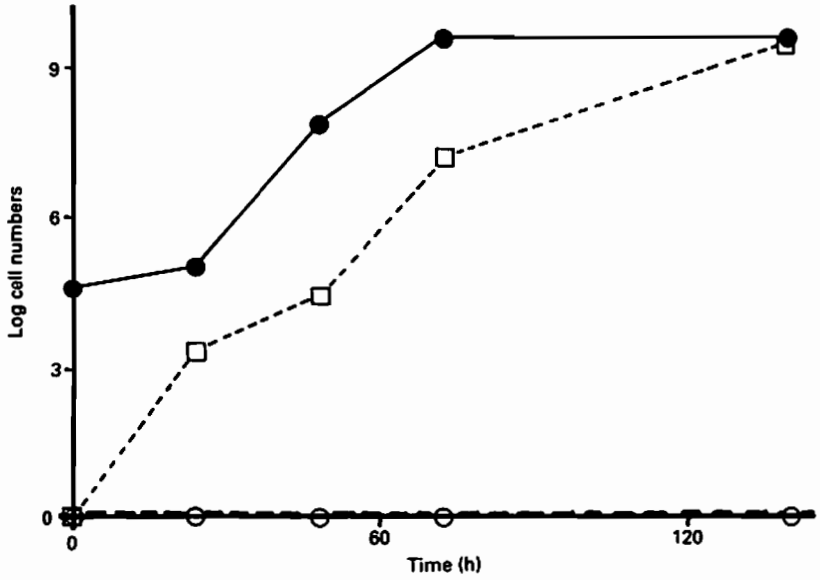

Fig. 2. The growth of bacteria (BSO 375) in MRS-broth+0·01% actidione following inoculation with contaminated slurries of pitching yeast (NCYC 1236). ●—unwashed; □—washed for 4 h with 100 I.U. ml^{-1} nisin; ○—washed for 4 h with 1000 I.U. ml^{-1} nisin.

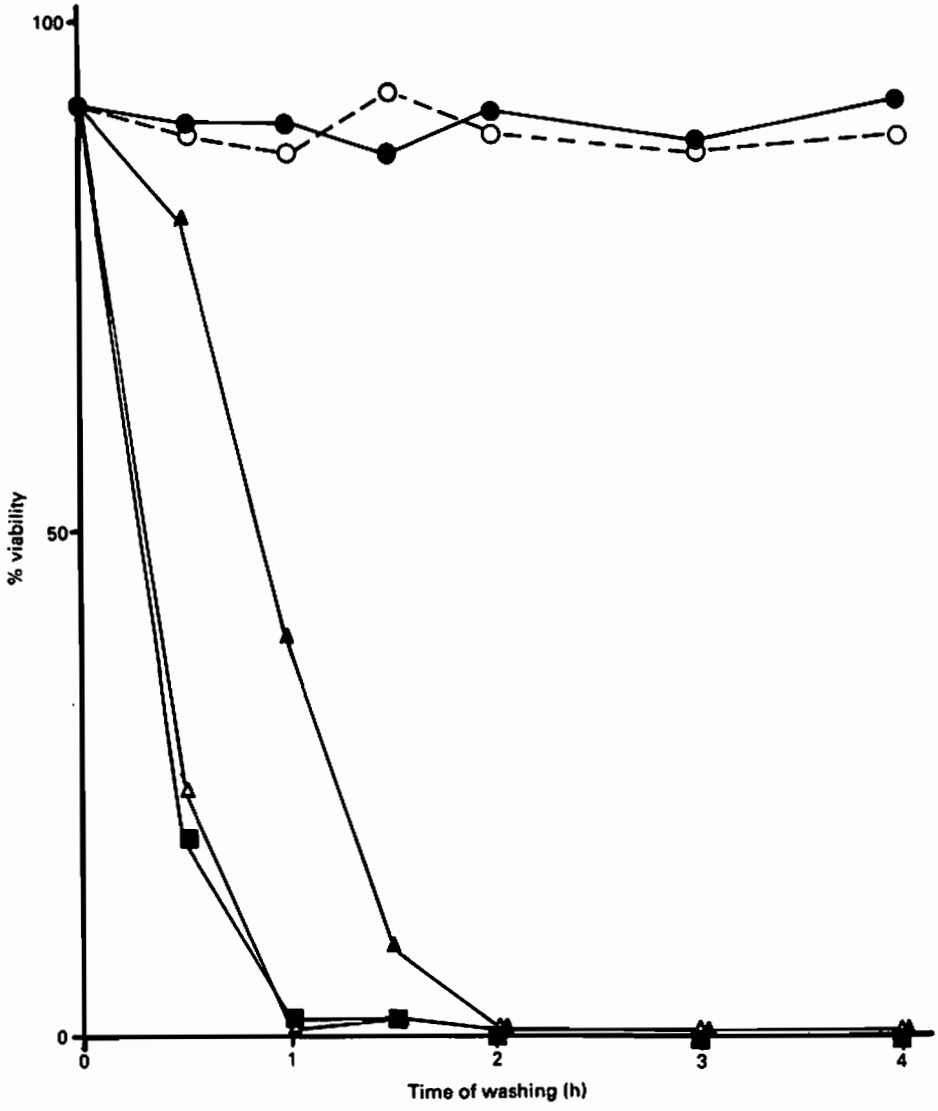

Fig. 3. Comparison of the effects of nisin-washing and three methods of acid-washing on the viability of brewing yeast (NCYC 1236). ●—untreated; ○—1000 I.U. ml^{-1} nisin; ■—ammonium persulphate; △—acidifed ammonium persulphate; ▲—phosphoric acid.

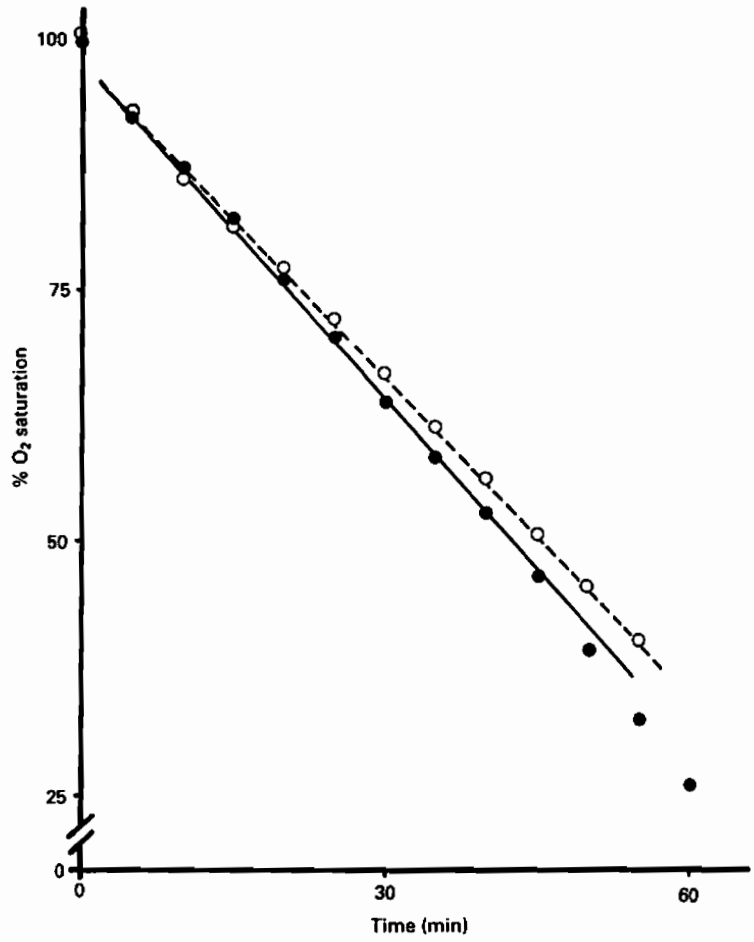

Fig. 4. The rate of oxygen uptake by a sample of nisin-washed yeast (NCYC 1236) compared to that of an unwashed sample of the same strain. ●—unwashed yeast; ○—washed for 4 h with 1000 I.U. ml^{-1} nisin.

iii) 50 ml of 1·5% (w/v) ammonium persulphate was added as above and the pH adjusted to 2·8 by the dropwise addition, with gentle stirring, of 2·0 M sulphuric acid (only 4–5 drops were required).[3]

These flasks were stirred for 2 h at room temperature and samples were taken at various times for viability determinations.

Analyses.—The numbers of surviving bacteria in samples taken from the yeast slurries were estimated by plating aliquots of serially-diluted samples onto MRS-agar (Oxoid) plates and counting the number of colonies that grew after incubation at 25°C for 2–4 days. Cycloheximide (0·01%, w/v) was included in the medium to inhibit yeast growth.

To determine whether nisin-washed bacteria could grow in subsequent liquid cultures, samples of washed yeast slurry were taken, filtered through a 0·22 µm membrane, washed with MRS-broth, and resuspended in fresh MRS-broth. These were then inoculated at a rate equivalent to adding *ca.* 10^7 yeast cells ml^{-1} (equivalent to *ca.* 10^5 bacteria cells ml^{-1}) into 500 ml MRS-broth containing cycloheximide (0·01%, w/v). These cultures were incubated at 20°C under a constant flow of oxygen-free nitrogen (40 ml min^{-1}) and with constant stirring (160 rev. min^{-1}). Samples were taken periodically and assayed for bacterial growth using the plate counting method described above.

Yeast viability was estimated by staining with methylene blue.[6]

The 'vitality' of nisin-washed yeast was compared with that of the untreated yeast using a specific oxygen uptake method (ref. 5 and B. A. Searle and I. S. Daoud, personal communication). The yeast slurry (100 ml) was added to 1·5 l of air-saturated YM-broth equilibrated to, and maintained at, a constant 20°C. The rate of oxygen uptake from the medium by the yeast was then monitored by circulating medium from the vessel past an oxygen probe (Orbisphere-2120 attached to an Orbisphere-2957 oxygen meter).

Fermentations using nisin-washed yeasts, and unwashed control yeasts were performed as described above, except that the pitching rate was 20 ml of yeast slurry l^{-1} of medium. Samples were taken throughout the fermentations and the specific gravities were determined using a density meter (Paar, DMA 45).

Results and Discussion

Effect of Nisin-washing on bacteria

Contaminated pitching yeast can be cleaned by washing with any of a variety of acidic solutions. For washing with nisin preparations to be considered as an alternative procedure, it must be shown that the ability of nisin to kill contaminating bacteria is on a comparable level to those of the different acid-washing methods. Yeast slurries were contaminated deliberately with lactic acid bacteria (three different strains) to a level of about 1% by cell numbers. This represents a heavy contamination, and, if this yeast was used for pitching, it would give rise to levels of about 10^5 bacterial cells ml^{-1} in the wort. Three different acidic solutions (phosphoric acid, ammonium persulphate and acidified ammonium persulphate: see Materials and Methods) were used to wash contaminated yeast slurries. With each treatment all the

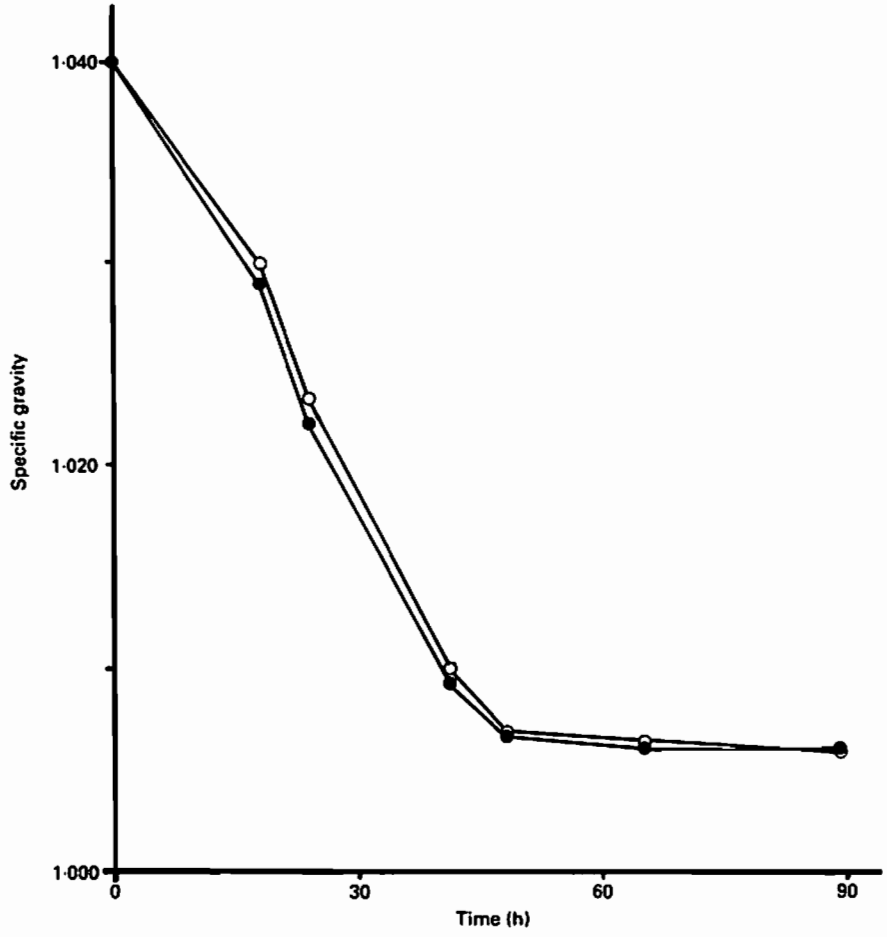

Fig. 5. The fermentative performance of a sample of nisin-washed yeast (NCYC 1342) compared to that of an unwashed sample of the same strain. ●—unwashed yeast; ○—washed for 4 h with 1000 I.U. ml^{-1} nisin.

contaminating bacteria were killed within 30 minutes, irrespective of which strain was used (Fig. 1). When contaminated yeast slurries were washed with either of two concentrations of nisin (100 or 1000 I.U. ml^{-1}), the results varied with bacterial strain. A nisin concentration of 1000 I.U. ml^{-1} killed, within 30 minutes of treatment, all the cells of the sensitive strains BSO 375 (Fig. 1a) and BSO 75 (Fig. 1b), but not of the more resistant strain BSO 343 (Fig. 1c). With this last strain the number of viable cells was reduced from $2{\cdot}5 \times 10^6$ ml^{-1} to about 1200 ml after 2 h treatment, a reduction of more than 99·9%. When a lower concentration (100 I.U. ml^{-1}) of nisin was used fewer cells of the strain BSO·343 were killed. After 4 h treatment there were still about $1{\cdot}9 \times 10^5$ cells ml^{-1}, representing a drop in viability of about 92% (Fig. 1c). This lower nisin concentration killed almost all cells of BSO 375 and BSO 75 within 30 minutes, but even after 4 h treatment an occasional colony was found to have grown on the assay plates, equivalent to less than one surviving contaminating cell ml^{-1}.

The results for these experiments were similar whether pure nisin or the commercial, unpurified grade (Nisaplin) was used. Therefore, all further experiments were performed using only the latter grade.

Significant numbers of cells of the strain BSO 343 survived a nisin-washing treatment, and it is highly likely that these could grow and cause problems in a subsequent fermentation (1200 surviving cells ml^{-1} in a yeast slurry would give in the region of 12 cells ml^{-1} in the fermentation, while $1{\cdot}9 \times 10^5$ cells ml^{-1} would give 1900 cells ml^{-1}). The ability of those cells of BSO 375 that survived nisin washing to grow in subsequent cultures was determined and the results are shown in Fig. 2. After about 140 h of incubation the number of cells had increased from less than 1 ml^{-1} to almost 10^{10} ml^{-1}, a similar level to that when unwashed contaminated yeast slurries were used. Under fermentation conditions, in wort in the presence of actively-growing and fermenting yeast, the rate of bacterial growth would be much slower than that observed here. These results do show, however, that there is a potential for those cells which survive nisin treatment to grow and cause contamination problems in fermentations. As a confirmation that 1000 I.U. ml^{-1} of nisin killed all the bacterial contaminants, yeast slurries washed with this concentration showed no evidence of bacterial cell growth under similar conditions (Fig. 2).

Effects of nisin-washing on brewing yeast.—The above results indicate that washing with nisin could be used as a replacement for acid-washing when the contaminants are gram-positive bacteria. Nisin would be of little use, though, if it had a similar detrimental effect on the brewing yeast. Therefore, the effect of nisin and the three acid-washing techniques on the viability of cells in yeast slurries was examined. The results using the ale strain NCYC 1236 and the methylene blue viability assays are presented in Fig. 3. Similar results were obtained using four other brewing yeasts (2 ale—NCYC 1062 and NCYC 1245, and 2 lager—NCYC 1324 and NCYC 1342).

When slurries were washed with ammonium persulphate or acidified ammonium persulphate solutions the yeast viability decreased from over 90% to about 1–2% within an hour, and after 3 h no viable cells were found. A less marked decrease in viability was seen when phosphoric acid was used as the washing agent, but there were still only 1–2% of the cells viable after 2 h of treatment. Washing with a solution of nisin (1000 I.U. ml^{-1}), however, had no effect on the yeast viability. After 4 h treatment the number of viable cells did

not differ significantly from that of the untreated yeast. Previous experiments, in which the numbers of viable yeast cells in fermentations did not differ in the presence of nisin from those in its absence,[10] suggests that pitching yeast could be stored for times considerably longer than 4 h under a solution of nisin without affecting viability.

The above results would suggest that nisin has no effect on brewing yeast. An unchanged viability, however, does not necessarily mean that all characteristics of the yeast are unaffected by nisin. Therefore, the 'vitality' (i.e. the ability to take up O_2 from an air-saturated medium) of a nisin-treated sample of a brewing yeast (NCYC 1236) was compared with that of an untreated sample. The results are given in Fig. 4. The rate of oxygen uptake for nisin-treated yeast was 1·08% min^{-1} and that of the untreated yeast 1·14% min^{-1}. The difference between these two rates falls within the limits of the between samples error of about $\pm 5\%$ found when using this technique (B. A. Searle, personal communication). Furthermore, changes in the physiological state of a yeast would be expected to show a much more pronounced effect on vitality.[5] Therefore, it can be concluded that nisin-washing has no significant effect on yeast activity.

As a further test, the fermentative performance of a sample of nisin-washed yeast (in this case NCYC 1324) in a fermentation was compared with that of an untreated sample. Fig. 5 shows that there was no difference between the fermentative performance of the two samples. Also, at the end of fermentation, when the stirring was discontinued the yeast in both vessels flocculated and settled out in a similar manner. This result was not surprising as it has been shown previously that the addition of nisin to a wort fermentation has no effect on yeast growth and fermentative performance.[10]

Conclusions

Nisin can be used to cleanse contaminated pitching yeast when the contaminants are gram-positive, lactic acid bacteria. The ability of nisin to kill contaminating bacteria is comparable to that of acid treatments but nisin has several advantages. It has no effect on yeast viability, vitality, flocculation characteristics or fermentative performance. As nisin is very stable at low pH's the yeast could be stored in its presence, thus obviating the need for a distinct washing procedure. Yeast could be recovered from a fermentation and stored immediately under a solution of nisin until it is required for repitching, when, as it has no affect on fermentations[10], it could be used without further processing. This would have the double advantage of killing gram-positive contaminating bacteria and preventing further contamination arising during storage. In contrast if acid-washed yeast is not used immediately the pH must be raised to maintain viability, leaving the yeast open to reinfection. If nisin at a concentration of 1000 I.U. ml^{-1} was used to wash yeast and the yeast was added directly to the fermentation, the final beer would contain about 5 I.U. ml^{-1} of nisin. Under current legislation this would be acceptable for bottled and canned products but not for cask-conditioned beers. For these latter products the yeast would have to be separated from the nisin solution by filtration or centrifugation just prior to use.

However, the lack of any deleterious effects on yeast caused by nisin must mean that when contaminating organisms in samples of pitching yeast are known to be gram-positive, nisin washing appears to be far preferable to the alternative, potentially harmful acid washing procedures.

Acknowledgements. The author wishes to thank Dr M. J. Waites and Dr J. R. M. Hammond for helpful discussion and the Director of the Brewing Research Foundation for permission to publish this paper.

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