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NISIN AND BREWING

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Nisin, an internationally accepted food preservative has been shown to inhibit the growth of almost all Gram-positive beer-spoilage bacteria investigated. The initial effects on these bacteria appear to be upon the cell membrane. Nisin has little effect on most Gram-negative bacteria and has no effect upon the growth and fermentation properties of brewing yeasts. Nisin activity survives kieselguhr filtration, fining and pasteurisation and has no effect on beer shelf life. Nisin has potential applications in preventing spoilage of worts or beers by Gram-positive bacteria (particularly lactic acid bacteria).

Key words: *Nisin, food preservative, beer spoilage organism, lactic acid bacteria, brewing yeast.*

Beer Spoilage Bacteria

Beer is a hostile environment for bacterial growth and consequently spoilage of beers is limited to only a few species. Serious spoilage problems can arise, however, as witnessed by the abundance of literature in brewing journals on the subject of identification, monitoring and control of bacterial contamination (for reviews see references 12, 14, 15, 25).

Wort temperatures rarely fall below those of the mash on their way to the coppers, but if, for some reason, they drop to below 50°C thermophilic bacteria soon appear. These can develop at a rapid rate, acidifying the wort and producing diacetyl. They are, however, usually hop sensitive and do not develop in hopped wort or beer.

If cooled wort is stored before pitching, Gram-negative coliform bacteria can grow but these are not usually a problem if wort is pitched immediately as most are unable to proliferate in fermenting wort. The exception to this are bacteria of the genus *Obesumbacterium (Hafnia)* which can grow in wort at about pH 5.0 producing a characteristic parsnip smell. No growth occurs in the later stages of fermentation (i.e. at lower pH's) but they can survive in the recovered yeast and be carried into the next fermentation.

Gram-positive lactic acid bacteria of the genera *Lactobacillus* and *Pediococcus* are the most prevalent contaminants in fermentations. Lactobacilli can grow throughout fermentations and may well be passed to the next fermentation with the yeast. Beers from contaminated fermentations show excess turbidity and acidity and carry off-flavours, such as those caused by diacetyl and 2,3-butanediol. Pediococci are usually found in breweries practising bottom-fermentation. They grow mainly in the yeast layer at the bottom of the fermenter after the primary fermentation has ceased. *Pediococcus* contamination gives the so-called "sarcina-sickness", beers become turbid, with a granular sediment, have excess acidity and enhanced levels of diacetyl. Some strains also produce "rope" (a complex polysaccharide slime) in the beer.

Gram-negative acetic acid bacteria are not affected by hop substances and can tolerate acid conditions, growing over a wide pH range. They are ubiquitous and will quickly infect any worts or beers open to the air. They convert ethanol into acetic acid and produce ropiness or a thick, viscous surface pellicle. These bacteria require aerobic conditions for growth and, with the current emphasis on sealed fermentation vessels, are nowadays less of a problem in breweries than in the past. They are, however, still a major problem in trade.

Gram-negative *Zymomonas* infections are comparatively rare, which is fortunate for these organisms can spoil beer in only a few hours. They develop rapidly in cask beers producing H_2S and acetaldehyde.

Control of Infection

Any means by which the level of bacterial contamination of beers can be reduced or eliminated would, obviously, be of interest to the brewing industry. The idea of using natural agents to inhibit microbial growth is not new. Some beers appear to be inherently more resistant to bacterial contamination.[7] This has been tentatively attributed to metabolites produced by the yeast strain during fermentation, the identification of which could lead to their use as antibacterial agents.[7] The preservation of packaged beers with antimicrobial compounds was first described some years ago.[32] The use of yeast killer factors (or zymocins) active against the wild yeast encountered in brewing has also been considered,[38] and the genetic construction of zymocin-producing brewing strains has been achieved.[9,37]

Until recently, bacteriocins, which are usually low molecular weight (less than 10 000 daltons) polypeptide anti-bacterial agents, have not been considered for use in brewing. One reason for this is that most bacteriocins have a very narrow range of activity.[27] This would mean finding different bacteriocins for each type of contaminant encountered in breweries.

Those few bacteriocins which exhibit a wider spectrum of activity are from Gram-positive strains of bacteria.* Indeed, some are active against almost all other Gram-positive bacterial strains.[27,33] One of these Gram-positive bacteriocins, nisin, is produced commercially for use by the dairy and canning industries.[13]

(*Note: There is one exception to this general rule. Recently, strains of *Zymomonas* have been found which produce an activity similar to that of bacteriocins, which is active not only against other *Zymomonas* strains but also against other Gram-negative bacteria such as enterobacteria.[31] Little is known about this activity, but it may prove useful for controlling spoilage by Gram-negative bacteria.)

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Nisin: Sensitivity of Bacteria

Nisin is produced by strains of *Streptococcus lactis*, an organism commonly used in the dairy industry. It is a polypeptide containing 34 amino acids with a molecular size of 3510 daltons.[8] It has five internal disulphide bridges making it very stable to heat under acid conditions[5] and is most active at low pH's.

Nisin has been recognised for more than 40 years. It is internationally accepted as a food preservative and is specifically approved for use in cheese, canned foods and clotted cream in the U.K.[1,13,36]

The activity of nisin against beer-spoilage bacteria has been determined using a well-test method.[23] Petri-dishes containing agar medium seeded with the strain to be assayed were prepared. Wells were cut into the agar and filled with different concentrations of nisin solution. The plates were incubated and resistance or sensitivity to nisin was assessed by the absence or presence of zones of growth inhibition around the wells (Fig. 1).

In the original survey,[23] 149 strains of bacteria were examined, 117 Gram-positive and 32 Gram-negative, of which 92% of the former and only 3 strains of the latter (all *Flavobacter* species) were sensitive to 100 units of nisin. (1 unit of nisin is defined in terms of inhibition of growth of *Streptococcus agalactiae*. The activity of 1 μg of pure nisin is 40 units). The survey has been expanded since by the inclusion of a further 19 strains (Table I): three strains (two *Lactobacilli* and one *Leuconostoc*) isolated from ciders were found to be very sensitive, seven strains of *Lactobacillus* obtained from a whisky distillery were all sensitive, but to differing degrees: three gram-positive strains of unknown genus isolated from mash-tun last-runnings were very sensitive: of five strains of *Hafnia* and one of *Gluconobacter* tested, only one of the *Hafnia* strains showed a weak sensitivity. In total, 93% of Gram-positive strains and 10% of Gram-negative strains showed some degree of sensitivity to nisin. Using much higher nisin concentrations (1000 units), the remaining Gram-positive strains were inhibited to some extent.[21] Nisin had no effect on any of 12 brewing yeast strains that were tested (8 ale and 4 lager).[23]

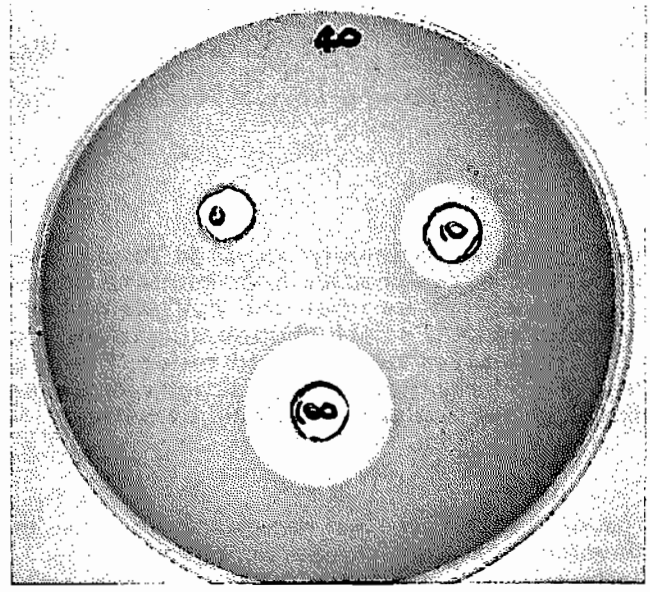

Fig. 1. Well Test Assay of Nisin.
Plate spread with a culture of *Lactobacillus brevis* and wells filled with 0,10 and 100 units of Nisin.

Nisin: Mode of Action

Until recently the mechanism of action of nisin has largely been unexplained, but evidence indicated that the prerequisite for nisin activity is adsorption to the affected organism.[11,26,34] Hirsch,[11] working with *Streptococcus agalactiae*, observed that the organism was killed in 10–15 minutes by 150 units ml^{-1} nisin, but if charcoal was added it took 20–30 minutes to obtain the same result. He proposed that a nisin-bacterium complex was formed that could be disrupted by charcoal. Ramsier[26] also found strong adsorption of nisin to vegetative cells of *Clostridium butyricum*. This was accompanied by leak-

TABLE I. The sensitivity to Nisin of brewing contaminants

	Genus	No. of strains tested	No. of resistant strains	No. of sensitive strains WS	S	VS	Total No. of sensitive strains
Gp	*Lactobacillas*	66	6	18	34	8	60
	Pediococcus	37	1	13	18	5	36
	Micrococcus	12	2	1	8	1	10
	Leuconostoc	9	0	2	6	1	9
	Streptococcus	3	0	1	2	0	3
	Unknown	3	0	0	0	3	3
Gn	*Acetobacter*	10	10	0	0	0	0
	Zymomonas	8	8	0	0	0	0
	Flavobacter	3	0	3	0	0	3
	Kluyvera	1	1	0	0	0	0
	Citrobacter	3	3	0	0	0	0
	Enterobacter	2	2	0	0	0	0
	Klebsiella	3	3	0	0	0	0
	Hafnia	7	6	1	0	0	1
	Gluconobacter	1	1	0	0	0	0

WS = 'weak sensitive'—an inhibition zone of 0.5 cm or less surrounding a well containing 100 units of Nisin.
S = 'sensitive'—an inhibition zone of 0.5–1.0 cm surrounding a well containing 100 units of Nisin.
VS = 'very sensitive'—an inhibition zone of greater than 1.0 cm surrounding a well containing 100 units of Nisin and/or inhibition zone of greater than 0.5 cm surrounding a well containing 10 units of Nisin.
Gp = Gram-positive.
Gn = Gram-negative.

age from the cells of ultraviolet absorbing materials and subsequent cell lysis. These changes were particularly evident during the logarithmic phase of growth. Also, the observation that anionic soaps neutralised the effects of nisin led him to conclude that nisin behaved like a cationic surface active detergent. Linnett and Strominger,[18] using an *in vitro* system, estimated that 40 μg ml^{-1} nisin caused a 50% inhibition of the synthesis of the major cell wall component peptidoglycan by enzyme systems from either *Bacillus stearothermophilus* or *Escherichia coli*. Similar results were reported by Reisinger *et al*,[28] and in addition they observed that the inhibition resulted from the formation of a complex between nisin and the lipid intermediate of the murein biosynthetic pathway. However, the concentration of nisin necessary to cause this inhibition (1600 units ml^{-1}) was about 1000 fold higher than the minimum inhibitory concentration for *B. stearothermophillus*. Thus, it is unlikely that the primary site of nisin action is via inhibition of peptidoglycan synthesis.

In our experiments, we found that when nisin was added to lactic acid bacteria (Lactobacilli and Pediococci) the cells started to clump together, and after 15–20 minutes the majority of cells were in the form of aggregates.[24] However, even after an hour no evidence of lysis was seen, in contrast to Staphylococci where nisin has been shown to interfere with the regulation of autolytic enzymes.[30] These rapid physical effects were also matched by a consistent loss of cell viability. At cell concentrations of about 6×10^4 cells ml^{-1}, 95% of cells of susceptible strains lost the ability to grow (in a plate count test and confirmed by vital staining) after only one minute in contact with nisin (100 units ml^{-1}), and none grew after a 10 minute contact period. Even with relatively low nisin concentrations (10 units ml^{-1}) less than 1% of the cells survived a 10 minute treatment.[24]

As nisin has such a rapid effect on cell viability it is very likely that the cell membrane is the primary site of action. Many antimicrobial compounds such as polymyxins, polyene macrolides, yeast killer factor (zymo-

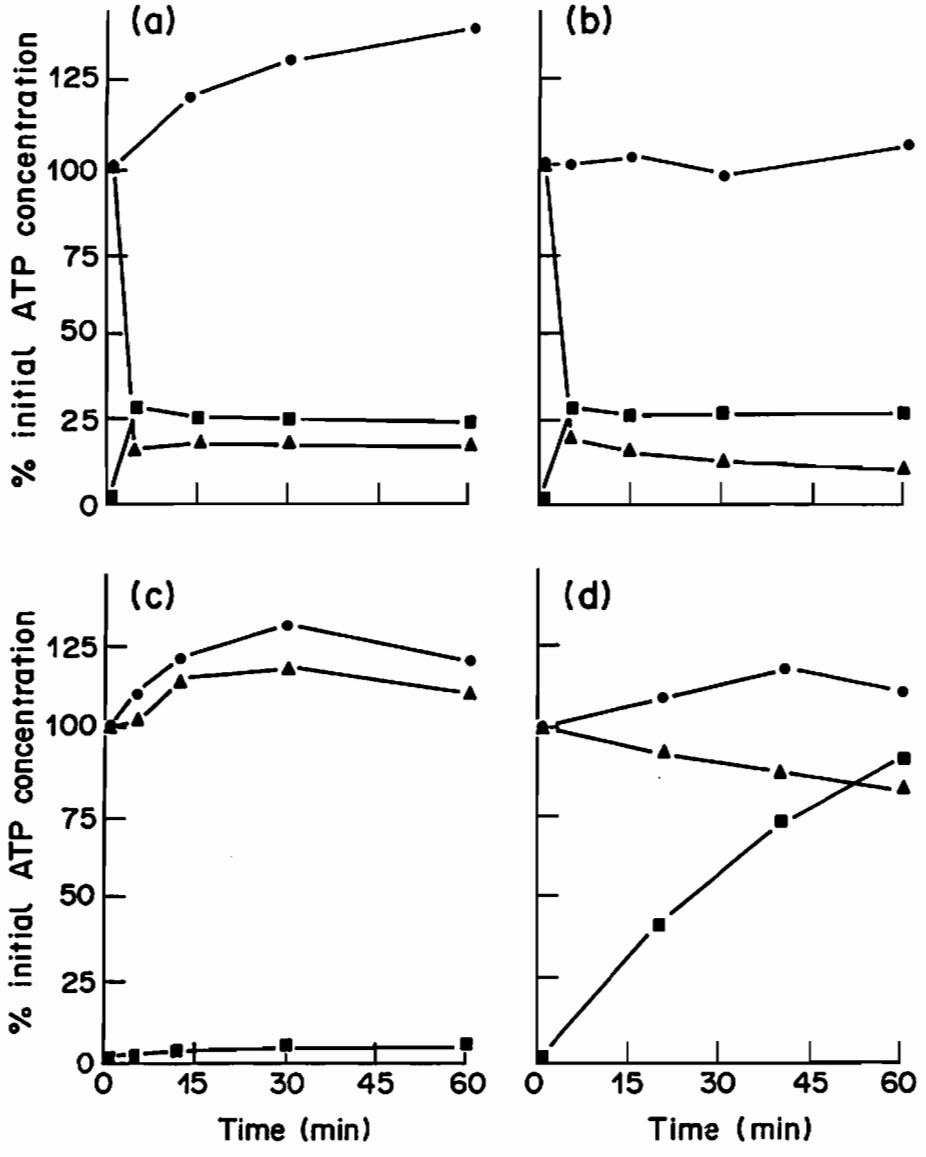

Fig. 2. Time Course of ATP release from bacteria incubated with 100 units ml^{-1} of nisin.
(a) *Lactobacillus brevis* BS028, (b) *Pediococcus* sp. BS075, (c) *Acetobacter aceti* BS01, (d) *Obesumbacterium proteus* NCIB 8770.
●: Intracellular ATP, untreated cells
▲: Intracellular ATP, cells treated with nisin
■: Extracellular ATP, cells treated with nisin.

cins), colicins and Gram-positive bacteriocins "attack" the cell membrane by altering its permeability. In many cases this results in leakage of low molecular weight intracellular metabolites and ions, such as leucine, glycine, ATP or potassium ions.[11,10,4,16,33] By monitoring both leakage of ATP (an energy source unique to living cells, which is rapidly destroyed as cells die) into the extracellular medium and the intracellular ATP content of cells, it is possible to examine both effects on membrane permeability and on cell viability. Our experiments revealed that when nisin was added to lactic acid bacteria the intracellular levels of ATP rapidly fell. With susceptible strains, 100 units ml^{-1} of nisin produced a 70% drop within one minute. These changes in intracellular ATP levels were reflected in a simultaneous appearance of ATP, and no doubt other low molecular weight materials, in the extracellular medium (Fig. 2(a) and 2(b)). However, the sum of intra- and extracellular ATP levels did not account for the ATP level prior to addition of the bacteriocin. This is possibly due to leakage of ATPases into the extracellular medium and/or their intracellular action on gaining access to substrate from which they are normally separated. In addition, as the production of ATP is dependent upon a pH gradient across the cell membrane, a nisin-induced alteration of membrane permeability may reduce or remove this gradient, which, with an accompanying leakage of ADP, could prevent regeneration of the ATP that is continually being utilised in metabolic processes. Hence, overall it appears that the initial effects of nisin are at the level of the cell membrane.

Many bacteriocins and zymocins act in a two stage process. Firstly, they adsorb to receptors in the cell wall or membrane and then, after a period of 10–30 minutes, they exert a lethal effect on the cell.[4,16,33] In order to kill a cell a certain number of molecules of antimicrobial compound are required to bind to receptors. The action of nisin on lactic acid bacteria, like the bacteriocins lactostrepcin and staphylococcin,[39] does not exhibit this two step process. However, lactostrepcin and staphylococcin show activity against energy depleted cells, whereas nisin, like the bacteriocin PepS from *Staphylococcus epidermidis*, appears to be more effective against energised cells.[17,29,30]

There have been few reports of nisin affecting Gram-negative bacterial cells. However, cells of *Escherichia coli* do exhibit sensitivity to nisin when the outer membrane is bypassed by osmotic shock or by formation of cytoplasmic membrane vesicles.[17] On examination of the influence of nisin on Gram-negative beer spoilage organisms it was found that nisin, at levels up to 100 units ml^{-1}, had no effect on *Zymomonas* spp. A slight effect was seen with the acetic bacteria, *Acetobacter aceti* (Fig. 2(c)) and *Gluconobacter oxydans*, both of which released a little ATP into the extracellular medium. The effects on *Hafnia* spp. (*Obesumbacterium* spp.) were more pronounced. All six strains of *Obesumbacterium proteus* tested continually released ATP, which accumulated in the medium, but intracellular levels were only reduced by 10–30% (Fig. 2(d)). In view of the importance of *Obesumbacterium proteus* as a spoilage organism these results warrant further investigation.

Application of Nisin to the Brewing Process

There are many areas within the brewing industry where nisin could be used to combat contamination by Gram-positive bacteria:

1. *Worts/Last-runnings*—Nisin could be added to worts if they need to be stored before boiling, or if the temperature falls below 50°C, to discourage the appearance of thermophilic Gram-positive bacteria. When added to wort, more than 30% of the initial levels of nisin activity survived a wort-boil of 60 minutes. Therefore, if nisin is added to help preserve unboiled wort, it would also provide some protection against infection at later stages in the brewing process. Nisin could also be added to last runnings prior to storage for use in a subsequent mash.
2. *Fermentations*—Nisin could be added to fermentations as an insurance, to prevent the growth of any contaminating lactic acid bacteria. In experiments where nisin was added at a concentration of 100 units ml^{-1} (the upper limit of the levels recommended for commercial use[20]) to fermentations deliberately contaminated with lactic acid bacteria, it was found to be very effective at controlling bacterial proliferation.[21] At levels of about 10^5 bacterial cells ml^{-1} (*ca.* 1% contamination by cell numbers) over 99.9% of sensitive Lactobacilli, and over 95% of sensitive Pediococci, cells were killed. Moreover, although cells of a nisin-resistant strain of *Lactobacillus* were not killed by nisin during the fermentation their growth was inhibited. At the end of the fermentation the bacterial concentration was unchanged from that at the beginning. The cell numbers, viability and fermentative performance of the 9 different brewing yeasts were unaffected by the presence of nisin (Table II).

It was concluded that nisin, at levels recommended for commercial use, could be used to kill or inhibit the growth of almost all strains of lactic acid bacteria encountered in brewery fermentations at infection levels up to 10^5 cells ml^{-1}, without adversely affecting any of the characteristics of the brewing yeast. What is more, the addition of nisin to fermentations had no deleterious affect on the taste of the beer produced.[21]
3. *Yeast Washing*—It is well recognised that a contaminated pitching yeast is the most important reservoir of bacterial infection in a brewery or distillery.[2,3,12,19] Therefore, the possibility of using nisin to combat Gram-positive bacterial infections in pitching yeast samples is an attractive proposition, particularly when it is compared to alternative measures.[22] Currently the most common method of dealing with infected yeast in breweries involves washing the yeast with an acidic solution, usually tartaric, phosphoric or sulphuric acids, or acidified ammonium persulphate. This can have adverse effects on the yeast: it can become loosely dispersed with no tendency to settle out, its ability to ferment and flocculate in the subsequent fermentations can be altered, and the use of acid can lead to a decrease in yeast

TABLE II. The effect of nisin on the fermentation performance of brewing yeasts in hopped wort (O.G. 1.040) in E.B.C. tall-tube fermenters

Strain	Fermentation time (h) for specific gravity to decrease to 1.020		% Fermentation* performance in the presence of nisin
	No nisin	+ nisin	
Ale			
NCYC 240	36.5	36	102
NCYC 1062	39.5	38	101
NCYC 1134	42	41.5	97
NCYC 1236	35	34	100
NCYC 1245	36.5	36.5	99
Lager			
NCYC 1047	37.5	37	99
NCYC 1146	33	32	98
NCYC 1250	41	38.5	102
NCYC 1324	33.5	32.5	99

* The % fermentation performance in the presence of nisin was determined as: [degrees attenuated (starting gravity—final gravity) of the brewing strain in the presence of nisin/degrees attenuated in the absence of nisin] × 100.

viability.[12,35] It has been shown that washing contaminated pitching yeast with nisin has none of these disadvantages.[22] In yeast slurries containing different strains of bacteria at levels of about 1% by numbers, a concentration of 1000 units ml^{-1} of nisin killed more than 92% of the most resistant strain tested and 100% of the sensitive strain. At this concentration nisin had no effect on the viability of the yeast, its "vitality" (as determined using a specific oxygen uptake method[6]), flocculation characteristics or fermentative performance. It was concluded that yeast could be stored under a solution of nisin, obviating the need for a distinct washing procedure.[22]

4. *Plant Cleaning*—The majority of sensitive cells are killed within a minute of the addition of nisin.[24] This raises the option of using nisin as part of a cleaning cycle for production plant and equipment, such as in the form of a final cleansing rinse. This would only be possible, however, in those breweries which do not operate a caustic or alkali-based detergent cleaning system, as nisin is neither very active nor very stable at high pH values.[20]

5. *Reduced pasteurisation*—Among the beer-spoilage organisms, the Lactobacilli have the greatest resistance to heat. If nisin, which is heat stable at pasteurisation temperatures, was added to beers before pasteurisation it could provide an opportunity to reduce the time and temperature of treatment, energy costs and the risk of heat damage to flavour.

6. *Shelf-life*—Nisin could be used to increase the shelf-life of beers, particularly of unpasteurised cask- or bottled-conditioned beers. Nisin itself has no effect on shelf-life, as shown by adding the crude form of nisin, Nisaplin, to samples of a commercial lager which were then filtered, bottled and pasteurised. Immediately after addition, the nisin-treated sample developed a haze but this was easily removed by filtration through a sheet filter. The levels of nisin activity were unaffected by this filtration probably because the haze was caused by non-nisin components present in the crude preparation. After 35 weeks of forcing there was no difference between the haze of the control and nisin-treated beers clearly demonstrating the lack of effect of nisin on shelf-life. Moreover, the Rudin head retention values of both beers were very similar and there were still high levels of nisin activity remaining in the treated samples.

The effectiveness of nisin at controlling *Lactobacillus* infections in cask- or bottle-conditioned beers has been demonstrated by dosing such a beer with varying levels of lactobacilli and nisin and allowing the beers to condition normally in bottle. After 3 weeks storage at 23°C beers dosed with 3×10^4 bacteria ml^{-1} were heavily contaminated and spoiled in the absence of nisin. As little as 10 units ml^{-1} of nisin completely prevented the spoilage of beers dosed at the higher rate of 3×10^5 bacteria ml^{-1} even after 6 weeks storage.

Post-Fermentation Treatments and Nisin Activity

If nisin is to be added to fermentations and beers it is important to define the effects of post-fermentation treatments on its activity. It has been mentioned already that filtration through a sheet filter pad had no significant effect on the levels of nisin activity in a commercial lager. The effect of different filter aids on the activity of nisin in beer has also been determined in laboratory scale experiments. Filter beds were formed of four different filter aids: Standard Supercel, Hi-flow Supercel, Clarcel Dic-B and Perlite. Beer containing 100 units ml^{-1} of nisin was passed through these beds and the remaining activity measured. The results, expressed as a percentage of the original nisin levels, are shown in Table III. The levels of filter aid used (about ten times the levels used commercially) and the residence time ensured a good period of contact between nisin molecules and filter aid particles. In no case did the activity decrease by more than 8% showing that beer containing nisin could be filtered through any of these filter aids with no significant loss of antibacterial activity.

TABLE III. Effect of Filter Aids On Nisin Activity

Filter Aid	Recovered activity (%)
None	100
Standard Supercel	93
Hi-flow Supercel	96
Clarcel Dic-B	92
Perlite	98

TABLE IV. Effect of Finings on Nisin Activity

Finings	Recovered activity (%)
None	100
Isinglass F	93
Isinglass FF	91
Silicate	90
Alginate	90

The effect of four different types of finings, two different strength solutions of isinglass finings and both silicate and alginate auxiliary finings, on nisin activity in beer has also been examined. The finings were added to batches of beer containing nisin. After agitating gently for 24 h to give a long contact time between the nisin and finings particles, the beers were allowed to settle for a further 24 h. The beer was recovered and assayed for nisin activity. In no case did the finings remove more than about 10% of the activity (Table IV). Thus it should be possible to fine nisin-treated beers without a significant loss of activity.

Conclusions

Clearly, nisin is effective against a wide range of Gram-positive brewery spoilage organisms and could find application in a number of areas. The commercial grade of nisin, Nisaplin, is currently formulated for the dairy industry and reformulation may be required for some applications, particularly unfiltered beers.

Legal Aspects of Nisin Usage

Nisin has been used within the food industry for more than 35 years and is regarded as being non-toxic. In the United Kingdom there is no restriction on the amount of nisin that can be added to foods. The recommended maximum average daily intake of nisin would allow, at a level of 100 units ml^{-1} in beer, a 70 kg person to drink up to about 40 pints a day (assuming this was the sole source of nisin).

In the United Kingdom nisin is specifically approved for use in dairy products and canned foods. The definition of a "canned food" is a "food in a *hermetically sealed container* which has been sufficiently heat processed to destroy any *Clostridium botulinum* in that food or container or *which has a pH of less than 4.5*".[1,20] Beer normally has a pH of less than 4.5, and therefore, according to current legislation, nisin should be useable in bottled or canned beers or for the production of these.

The use of nisin in kegged beers would depend on whether these could be classed as hermetically sealed containers—and hence "canned foods".

As for the possibility of using solutions of nisin to wash or pretreat pitching yeast, Ministry of Agriculture, Fisheries and Food (MAFF) representatives consider that it could be used for this purpose provided that the residues are in sufficient to contravene the Materials in Food Regulations. Washing pitching yeast with 1000 units ml^{-1} of nisin would give about 5.5 units ml^{-1} in the final beer (assuming a pitching rate of 0.28 kg yeast slurry per hectolitre). As nisin does not adsorb to yeast cells it should be possible to recover the yeast from a nisin solution before pitching, and thus reduce the levels of nisin in the final beer to negligible amounts.

Cost of Nisin Usage

The current price of the commercial grade of nisin (Nisaplin) is £186 per kilogram (10^9 units). Adding nisin at 100 units ml^{-1} to fermentations or beers would, therefore, cost 186 pence for every hectolitre or 1.06 pence per pint. This would be considered too expensive for nisin to be used as an additive to all fermentations, but not if it is used as a remedial measure, to treat contaminated fermentations or beers. If it is used to wash or pretreat pitching yeast the costs are much less prohibitive. A typical cost for washing pitching yeast at 1000 units ml^{-1} would be 10.2 pence per hectolitre or 0.058 pence per pint and would not prevent the regular use of nisin.

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