

Chapter Ten

10. Discussion and future prospects

The aim of this work was to identify a lactic acid bacterium which would protect silage from aerobic spoilage, estimated to cost UK agriculture around £110 million per annum (Woolford, 1990). It was shown in Section 4 that these losses may be expected to rise since, in England and Wales, there is a trend towards the production of HDM silage. Aerobic spoilage of HDM silage was considered to be a particular problem by 1 in 3 farms that made it, and by 1 in 3 farms that use inoculants. Therefore, the development of an inoculant that would minimise these losses can be expected to be economically useful.

As the first step towards this goal, a large number of bacterial strains were screened, as detailed in Section 5. As a result, three strains (LPC-22, M0042, and M0050) were identified that were consistently able to inhibit at least two different species of yeast typically found in silage. The screening protocol was designed to be both sensitive and repeatable, as well as pragmatic and representative of natural conditions. However it proved to be inconsistent at times, for reasons which could not be identified. For example, amongst the strains that were re-tested (Table 5.5), the activity towards *C. lambica* was considerably reduced. LPC-22 was also unable to consistently inhibit *C. famata* (compare Table 5.6 and Table 6.3). Activity towards *Acetobacter* sp. was also not found using the agar screening protocol however, using liquid methods, some effect was seen to be exerted by all the test strains (Figure 6.2(G)). Part of this effect may have been due to subtle variations in the starter cultures, since different results were observed depending on whether the starter cultures were prepared from frozen stocks or from stocks on agar plates (Table 6.3.).

In Section 6, the three strains that had been provisionally identified as having an antifungal effect were investigated more rigorously. It was first shown that any antifungal effect was unlikely to be a result of the overproduction of lactic acid, and then it was shown that the test organisms identified in the screening work were probably homofermentative species (either *L. plantarum* or *L. pentosus*), and so production of

acetic acid was also unlikely to be the cause of the antifungal effect. The activity of one of the organisms (LPC-22) towards a range of potential spoilage organisms was investigated (Section 6.2), and it was found that its range of activity was quite limited. Given the low reliability of the method used, this observation of a limited target range may be more apparent than real. The antifungal effect was investigated using a variety of different methods, including three methods based on agar plate assays – which all failed to provide positive results indicating, most probably, the relative insensitivity of these techniques. A more sensitive method for investigating the antifungal effect proved to be the liquid co-culture of the test strains and the target yeast. Here it was shown that the desired effect, the maintenance of a low pH, could be most successfully achieved by LPC-22, which was found to maintain a low pH in the presence of *S. exiguus*, *S. cerevisiae* and, surprisingly, *Acetobacter* sp. The ability to inhibit *Acetobacter* sp. is important, firstly because suggests that the antagonistic effects exerted by LPC-22 are not specifically antifungal, and secondly because *Acetobacter* spp. can be important spoilage organisms.

Attempts to elucidate the chemical nature of the active agent met with mixed success (Section 7). The active agent proved to be susceptible to the action of wheat germ lipase (lipase I), but not to other lipases, or to proteases, amylase, RNase, or catalase. The effect of lipase I on the antifungal activity effect towards *S. exiguus* was seen with all three strains selected in the original screening work (Section 7.1). The effect of lipase I is not dependent on the fungal species, as shown with LPC-22 (Table 6.3). The limited susceptibility to enzymatic degradation suggests that the active agent (or agents) is a low molecular weight non-oligomeric compound, and thus dissimilar to the bacteriocins which are the most widely studied LAB antimicrobials, but the complexity of the lipase I mixture precludes any definitive statement regarding active moieties. The antifungal activity of LPC-22 was demonstrated in the absence of cells: when compared with the supernatant from MTD/1, a small inhibition (~10%) was observed when *S. exiguus* was grown in the supernatant from cultures of LPC-22. This observation suggests the antifungal activity is caused by a discrete chemical entity. No effect was seen when the supernatant was analysed in a well-diffusion assay but, given the relative insensitivity of this technique (al-Hiti & Gilbert, 1982), this does not seem surprising. No activity was

observed in the test for a volatile agent, which would suggest that the active agent is not volatile (or that the technique is, again, insensitive).

Attempts to concentrate the antifungal activity largely failed (Section 7.3). Freeze drying produced an inactive concentrate, possibly indicating that the agent is volatile. Solvent extraction produced a toxic extract, but this effect could be achieved when extracting from sterile medium, and so is probably due to a medium component. Solvent extraction has been used successfully in the extraction of antifungal agents from *Bacillus subtilis*, such as chaetomacin (Tautorus & Townsley, 1984), mycosubtilin (Walton & Woodruff, 1949), and aspergillus factor (Michener & Snell, 1949). The lack of a successful result here suggests that the agent from LPC-22 is either not lipophilic, or that it is volatile.

Precipitation with either ammonium sulphate or inorganic acids has also been successfully used to concentrate antimicrobial substances from culture supernatants (for example Rossall, 1989, Lebbadi *et al.*, 1994). The antimicrobials purified using this technique tend to be high molecular weight proteinaceous compounds (with molecular weights of several thousand kD or more), and the lack of observed activity in a precipitate of LPC-22 culture supernatant suggests that the active agent is either not a peptide, or that it is a very small one.

An active extract was also not reproducibly retained using size fractionation techniques, which suggests the agent is of low molecular weight. These negative results from the variety of attempts to concentrate the active agent would be compatible with the idea that it is a small compound that is, to some degree, volatile (a highly volatile compound is unlikely because the test for such agents [Section 7.2.2] proved negative). Just such compounds have been described by Niku-Paavola *et al.* (1996) as products from *L. plantarum* VTT E-78076 (Section 1.7). These compounds exert antifungal activity, detectable in a well diffusion assay of the culture supernatant at pH 4.0, towards the mycelial fungus *Fusarium avenaceum*. The inability to detect such activity in the supernatant from LPC-22 may indicate that the active agent is different, or it may reflect the different assay conditions (pH 5 rather than pH 4, *S. exiguus* as the indicator rather than *F. avenaceum*, and a different medium composition).

The ultimate purpose of the antifungal screening work was to discover an LAB capable of preventing the aerobic spoilage of silage, and for this purpose the study described in Section 8 was undertaken. Unfortunately, there was such variability within the treatment groups was such that an objective assessment of the preservative effect could not be made. This observed variability can be partly ascribed to the inherent variability of natural processes involving a multitude of species, but similar work conducted by Ecosyl Products Ltd is often more consistent. The problems encountered in this work may therefore be methodological – in that the maintenance of homogeneity was made difficult by difficulties encountered in defrosting the maize samples, or that the addition of small amounts of spoiled maize samples resulted in a heterogeneous distribution of spoilage organisms. In addition, it is well known that experimental consistency is a function of operator experience, and it is likely that a lack of experience with the technique used may have contributed to the intratreatment variability.

It is for these reasons that work conducted subsequently by Ecosyl Products Ltd, in Israel, comparing the preservative capacities of LPC-22 and MTD/1, is interesting. This work involved the ensilage of fresh whole-crop wheat in 1.5 litre laboratory-scale silos, without spiking with mouldy maize. The aerobic stability was measured in polystyrene boxes with temperature loggers, in a manner analogous to that described in Section 3.10, and, in addition, CO₂ production was measured. The results obtained are shown in Table 10.1.

Table 10.1. The aerobic stability of silage produced with either MTD/1 or LPC-22. The fodder was whole crop wheat, two measures were used to assess aerobic stability – (A), the time to increase 2°C above ambient, and (B), CO₂ production.

(A) Time to increase 2°C above ambient (note – recording was stopped at 120 hours).

	Untreated control	MTD/1	LPC-22
Replicate 1	104 hours	104 hours	>120 hours
Replicate 2	117 hours	101 hours	>120 hours
Replicate 3	>120 hours	102 hours	>120 hours

(B) CO₂ production (g/Kg DM)

	Untreated control	MTD/1	LPC-22
Replicate 1	94.1	27.1	2.6
Replicate 2	22.8	27.0	0.7
Replicate 3	8.4	28.4	1.9

The results are encouraging for the prospects of LPC-22 as a silage inoculant. Although statistical analysis is not possible due to the small sample size, subjective analysis suggests that LPC-22 was consistently able to increase temperature stability and reduce CO₂ production. These observations are tempered by the fact that LPC-22 was inoculated at a higher rate than MTD/1 (7×10^6 CFU/g for LPC-22, compared with 1×10^6 CFU/g for MTD/1), but the likelihood that this difference had a major impact on the fermentation process is remote.

The other approach to the development of an anti-spoilage inoculant was discussed in Section 9. After demonstrating the aerobic production of acetate by *L. plantarum* MTD/1, methods were sought that would select a non-glucose-repressed mutant. The first hypothesis was that it might be possible to detect acetate production by one of the indirect consequences of acetate production, the ability to grow on a medium in which acetate production is the only viable energy producing pathway. However, growth on such a medium could not be evinced, and so it seems likely that energy production by this pathway is not sufficient to allow colony formation.

The second hypothesis tested was another indirect method: the detection of acetate production through one of the by-products of the acetate pathway, hydrogen peroxide. An assay was developed in which the glucose repressed production of peroxide by MTD/1 (although not the other lactobacilli examined) could be demonstrated and, after random mutagenesis, eight derepressed mutants were isolated. Unfortunately, acetate production by these mutants was no higher than that by MTD/1.

The reason for this was not discovered, but there are two possible explanations. First, the technique used assumes that peroxide and acetate production are inextricably linked. This would be the case if both pyruvate oxidase and acetate kinase are regulated by the same mechanism (*i.e.* if repression of the multi-enzyme pathway [Figure 1.5] is globally controlled). The literature evidence (Section 1.9.3) suggested that this would be the case. If the pathway is not globally controlled, peroxide production (with concomitant acetyl phosphate production) could occur without the dephosphorylation of acetyl phosphate. This inevitably means that acetyl phosphate must build up. The

consequences of this are not certain, but it may be expected that the pathway would be subjected to feedback inhibition, reducing peroxide production – and peroxide production was observed to be vigorous.

Second, it is feasible that another glucose repressed enzyme produces peroxide in *L. plantarum*. According to the literature, the only candidate is NADH oxidase, which the results of Götz *et al.* (1980) and Sedewitz *et al.* suggest is not glucose repressed. Both these results are somewhat doubtful (Section 1.9.3), because Götz *et al.* did not measure NADH oxidase directly, and because Sedewitz *et al.* did not demonstrate oxygen induction of NADH oxidase (unlike Tseng & Montville [1992]). However, the inability to demonstrate NADH oxidase activity in extracts from one of the mutants (Mut1) suggests that this explanation is not valid. For this reason, the most parsimonious explanation for the results in Section 9.3 may be that acetate kinase remains repressed in these mutants but, without further work, any explanation must remain ambiguous.

What are the implications of the work presented in this thesis for the development of silage inoculants capable of preventing aerobic spoilage? Of the two approaches developed the first, the discovery of a strain with improved antimicrobial activity, was the most successful. The laboratory screening produced unequivocal evidence that certain strains of lactobacilli were more inhibitory towards at least some spoilage organisms than was MTD/1. Trials in field conditions of the most efficacious, LPC-22, have given more equivocal results, and further trials are required before a definite statement can be made regarding the power of this strain to prevent aerobic spoilage.

The nature of the agent responsible for its antimicrobial effect must also give rise to caution – the results of Section 7 suggest that it may resemble the compounds found by Niku-Paavola *et al.* (1996) (Section 1.7), which possess an undefined degree of aspecific toxicity. Indeed it be hypothesised that, given the multi-specific nature of aerobic spoilage, it is likely that any chemical capable of preventing it must have a wide-ranging toxic effect. The consequence of this for silage manufacture should not be overlooked – a deleterious effect on animal performance may easily outweigh the benefits to be

gained from an improvement in aerobic stability. For this reason, even if LPC-22 does confer increased aerobic stability, the feed quality of the resulting silage must be closely examined.

The other approach investigated, the development of a derepressed producer of acetate, has fewer prospects of utility. It is evident that the control of acetate production is not simple, and there are no easy ways of relieving repression. A resolution to this problem must wait, at least, until further basic research has been conducted on the control of aerobic metabolism in lactic acid bacteria.

In conclusion, there is no certainty that a useful inoculant can be developed from the work presented in this thesis. However, the new knowledge which has been obtained has enabled one avenue of enquiry to be closed (at least temporarily), and has provided a positive direction for future work. The understanding of the range and scope of LAB antifungal activity has been increased, and there is a promising candidate (LPC-22) for future testing. Although much work remains to be done, the prospect of minimising aerobic spoilage by careful choice of inoculant has been improved by my work conducted at Cranfield Biotechnology Centre and Ecosyl Products Ltd over the past three years.