Chapter Seven

7. Analysis of an antifungal product from lactic acid bacteria: characterisation and attempted purification

7.1 Introduction

In order to understand the nature of the antifungal activity, it is necessary to discover whether a discrete chemical entity is responsible, and then to characterise it as far as possible. To do this, the effect of enzymes on the activity was investigated, and then attempts were made to separate any agent from the producer cells, and to purify and concentrate it.

7.2 Enzyme susceptibility of antifungal product

The effect of enzymes on the antifungal activity of the test organisms identified in Section 5 was investigated by using a modification of the standard assay for antifungal activity (Section 3.5.2). The enzymes to be tested (shown in Table 7.1) were incorporated into the second agar layer, comprised of SSM inoculated with yeast.

No attempt was made to standardise the activity of the enzymes used in the assay, because the activity given by Sigma for these enzymes is for standard substrates under standard conditions, and so it is unlikely to be a fair guide to the enzyme activity under test conditions. As shown in Table 7.1, antifungal activity was only affected by lipase I, a crude extract from wheat germ. This effect was very dramatic, as can be seen from Figure 7.1, with the addition of lipase I resulting in indicator yeast growth which is indistinguishable from the growth over the control organism, MTD/1. This effect does not seem to be an indirect effect on yeast or lactobacilli growth because, when the test and indicator organisms were grown as isolated colonies on SSM containing this amount of lipase I, no effect was seen on either colony or cell morphology.

Although an effect is seen only with lipase I, this does not, unfortunately, give much information as to the nature of the agent responsible for the antifungal effect. This is because lipase I is an impure product. According to Sigma, it contains considerable acid phosphatase activity (0.75 U/mg). When tested for its effect on peroxides, it showed no catalase activity (Section 3.5.7), but it did show very strong (unquantifiable) peroxidase activity (Section 3.5.8). Analysis of the protein constituents by SDS-PAGE (Section 3.9.4) showed at least 20 components (Figure 7.2). The lack of effect observed with other enzymes leaves open the strong possibility that some other, unidentified, effect (enzymatic or otherwise) is responsible for the loss of antifungal activity.

Table 7.1. Effect of enzymes on the antifungal activity expressed by the test organisms (M0042, M0050, LPC-22). The indicator organism was *S. exiguus*. Activity is the activity given by Sigma for the enzymes under standard (*i.e.* not test) conditions. Solid enzyme preparations were added at 5 mg/ml. Liquid preparations were added at concentrations designed to give approximately commensurate total activity.

Enzyme	**Activity IU/ml**	**Effect**
Proteases:		
Pronase E	28	None
Papain	8.5	None
Pepsin	2,750	None
Carboxypeptidase A	29	None
Trypsin	9,350	None
Lipase I	38	Abolished antifungal activity
Lipase II	160	None
Lipase VII	4,700	None
Lipase XI	17,000	None
Esterase	100	None
Acid Phosphatase	3	None
Catalase	9,400	None
α-Amylase	145	None
Ribonuclease	186	None

Key:

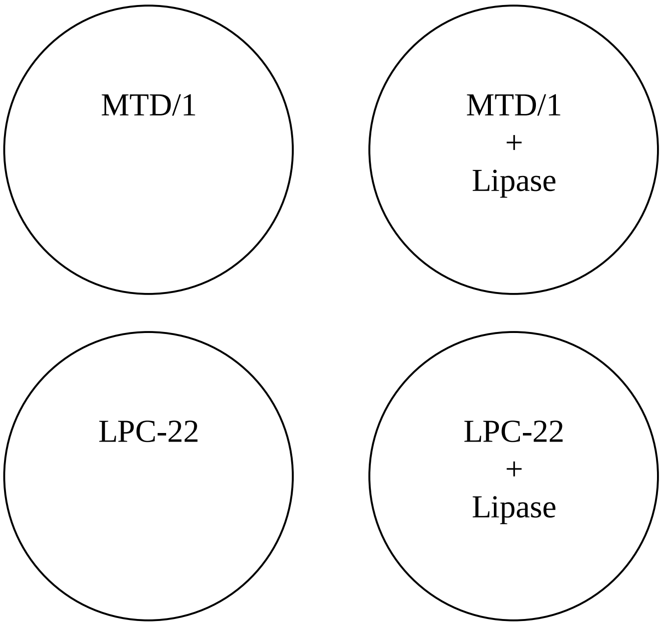

Figure 7.1. The effect of lipase I on the antifungal activity of LPC-22. The test was a modification of the standard screening protocol (Section 3.5.2), in which lipase I was added to the two Petri dishes on the right, at a concentration of 5 mg/ml of the SSM layer.

Lipase I MW markers

208

144

87

44

Approximate MW

32

18

Figure 7.2. SDS-PAGE of lipase I.

7.3 Activity of sterile culture supernatant

The results of the initial screen (Section 5) suggest that the putative antifungal activity is diffusable (because the test and indicator strains are separated in the agar medium). If this is the case, then the cell free supernatant of a culture of the producer strain(s) should repress growth when compared to the cell free supernatant from the control strain (MTD/1).

7.3.1 Well diffusion assay of culture supernatant

The twelve test LAB identified in the first phase of the screen (Section 5.2) were assayed for activity in their culture supernatant. The supernatant was obtained from cultures of the test LAB after 8, 15, 24 and 32 hours (Section 3.7.1) and tested in a well diffusion assay (Section 3.5.3), using *S. exiguus* as the indicator strain, but no activity was observed in the supernatant obtained at any time point.

7.3.2 Sealed plate method for detecting volatile antifungal activity

The method is a modification of the screening protocol previously described, and it is detailed in Section 3.5.5. The principal variation is that the second layer, containing the indicator yeast (*S. exiguus*), was not overlaid onto the first layer, but placed into the base of a separate Petri dish. This was then inverted and placed over the dish containing the test strains (LPC-22, M0042, M0050, MTD/1). The plates were sealed with parafilm and incubated at 30°C until yeast growth could be observed on the surface of the control dish. No inhibition of yeast growth was observed on any of the dishes sealed with the test strains when compared with MTD/1 or a LAB free control.

7.3.3 Serial culture of test LAB and *S. exiguus*

The lack of antifungal activity of the culture supernatant in a well diffusion assay suggests that any such activity in the supernatant is small. A more sensitive technique for testing antifungal activity is to grow the test and the indicator organisms in serial culture. That is, grow the test culture in the desired medium, remove the bacterial cells, then add the indicator yeast and observe the cell growth.

The first technique used was simply to grow the test lactobacilli to stationary phase in either SGM or SSM. The lactobacilli were removed, and the sterile culture supernatant was inoculated with *S. exiguus*. Growth was measured as the change in optical density under either aerobic or anaerobic conditions. (Figure 7.3).

The results showed that the growth of *S. exiguus* is always slowest in the supernatant from MTD/1. This disappointing result probably reflects the metabolic efficiency and acid tolerance of MTD/1 which would result in, at the end of the culture period, a greater loss of nutrients and energy from cultures of this organism, as well as a higher production of toxic organic acids.

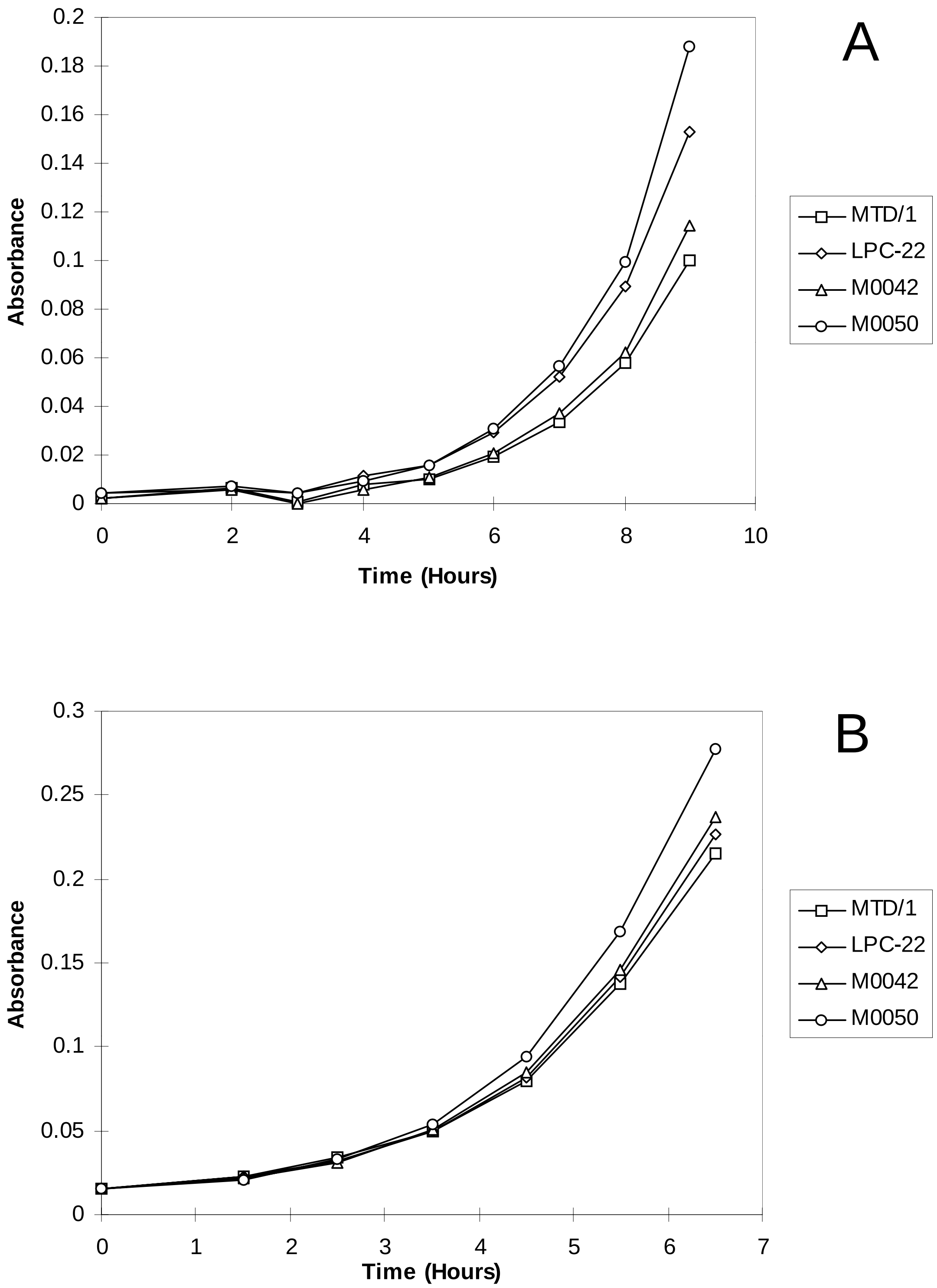

Figure 7.3. Growth of *S. exiguus* in the supernatant from LAB cultures. **(A)** LAB medium was SGM, *S. exiguus* growth was anaerobic, *n*=4. **(B)** LAB medium was SGM, *S. exiguus* growth was aerobic, *n*=4.

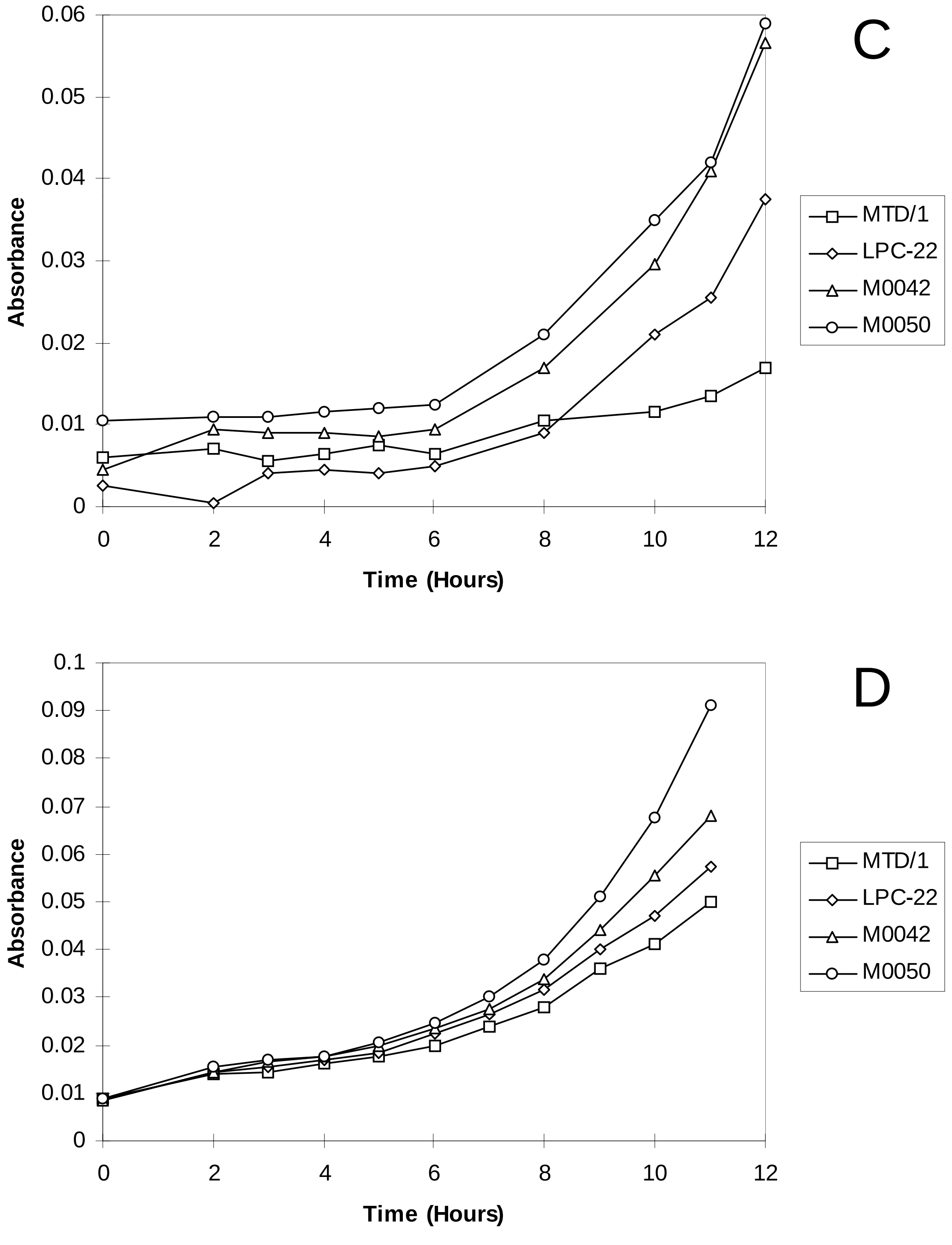

Figure 7.3(Cont.). Growth of *S. exiguus* in the supernatant from LAB cultures. **(C)** LAB medium was SSM, *S. exiguus* growth was anaerobic, *n*=4. **(D)** LAB medium was SSM, *S. exiguus* growth was aerobic, *n*=4.

To control for this, a more exacting series of studies were undertaken. Because of their time consuming nature, only one strain, LPC-22, was tested against MTD/1. LPC-22 was chosen because it was shown in the preceding tests and elsewhere (Section 6.2) to be the most potent of the organisms screened in Section 5. The objective was to produce cultures of LPC-22 and MTD/1 in which an equivalent degree of bacterial metabolism had occurred.

The first technique used was to grow the test organisms to a fixed OD_{600} before removing the bacteria and inoculating with yeast (Section 3.6.1). This approach was hampered by the fact that MTD/1 will autoflocculate in SGM at an OD_{600} above 0.6 and so, above this value, OD_{600} is not likely to be an accurate reflection of microbial biomass. There were also practical difficulties (without online OD measurement) in establishing the exact moment that the desired OD_{600} was reached. The results that were obtained using this technique are shown in Table 7.2. At low test bacterial OD_{600}, the subsequent yeast growth is slower in the supernatant from MTD/1, but at a higher OD_{600} (0.6), growth is slower in the supernatant from LPC-22.

The second approach simply involved growing the two test organisms for a specific time (Section 3.6.2) after which, for comparison with the previous dataset, the OD_{600} was measured. The results of this technique are shown in Table 7.3. It can be seen that, when the test LAB are grown to mid to late log phase, the subsequent yeast growth is lower in the supernatant from LPC-22. That is, over a short but fixed periodof LAB growth, the supernatant from LPC-22 is more inhibitory. This can be compared with the results shown in Figure 7.3, where growth of the LAB for 48 hours – to stationary phase – resulted in the supernatant from MTD/1 being more inhibitory. The inhibition seen in supernatant from log-phase cultures of LPC-22 may be due to the putative antifungal agent produced, or it may indicate that, in the short term, LPC-22 uses nutrients faster than MTD/1.

Table 7.2. Relative growth of *S. exiguus* (at 30°C) in the supernatant from cultures in SGM of LPC-22 and MTD/1. LAB cultures were grown to a fixed OD_{600}.

OD of LAB culture before filter sterilisation	Number of replicates	Yeast growth in supernatant from LPC-22 after 7 hours (relative to MTD/1 control)
0.1	2	112%
0.3	2	100%
0.6	2	90%

Table 7.3. Relative growth of *S. exiguus* (at 30°C) in the supernatant from cultures in SGM of LPC-22 and MTD/1. LAB cultures were grown for a fixed time.

Age of culture (hours)	OD_{600} of MTD/1	OD_{600} of LPC-22	Number of replicates	Yeast growth in supernatant from LPC-22 after 7 hours (relative to MTD/1 control)
14	0.90	1.0	2	91%
24	1.45	1.7	5	92%*

*Significantly different from MTD/1 control (p<0.05)

To control for this, two indicators of bacterial metabolism, pH and OD_{600}, were tested for their effect on subsequent yeast growth. These two indicators were used since the OD_{600} may be expected to provide a measure of the total microbial biomass formed, and the pH may be expected to provide a measure of the total metabolic activity (since both strains are homofermentative [Section 6.1], both will synthesise lactic acid as a sole metabolic end product from hexose fermentation [Section 1.3]).

Firstly, the pH and OD_{600} of cultures of MTD/1 and LPC-22 was followed (Section 3.6.3), and the results are shown in Figure 7.4. where it can be seen that MTD/1 causes a greater reduction in pH than does LPC-22. The effects on OD_{600} are substantially different, with MTD/1 cultures initially producing a higher OD_{600} but, after 15 hours, LPC-22 cultures producing a higher OD_{600}. This is an artefact caused by the autoflocculation of the MTD/1 cultures and, because of this, the best indicator of the sum total of bacterial metabolism within these cultures is probably the culture pH.

A set of cultures (six each of MTD/1 and LPC-22) were grown for fixed time periods designed to give a good range of final pH and OD_{600} (Section 3.6.2), and the relationship between the pH or OD_{600} of these cultures and the subsequent yeast growth in the supernatant (after 6 hours) is shown in Figure 7.5.

The correlation between the pH or OD_{600} of the test LAB cultures and the subsequent yeast growth was, surprisingly, found to be very low. It is also not statistically significant ($p>0.1$, Students t-Test). However the linear regression would seem to indicate that, the lower the pH and the higher the OD_{600} of both the LAB cultures (*i.e.* the more bacterial metabolism that has occurred in these cultures), the slower the subsequent yeast growth. This suggests that the inhibition seen in the supernatant from LPC-22 cultures after a given period of incubation is not due to greater bacterial metabolism since, at any given time point, the pH in LPC-22 cultures is higher than that in MTD/1. This effect can be seen in the linear regression lines (Figure 7.5) from which it can be seen that, at any given pH or OD_{600} of the LAB cultures, the subsequent yeast growth is lower in the supernatant from LPC-22.

Because the linear regression was not significant, the effect of pH or OD_{600} on subsequent yeast growth had to be ignored in any more rigorous comparison of the two strains, and the data from the six trials were pooled into a single dataset. If the inverse relationship between pH and subsequent yeast growth is a true one then, since the cultures of MTD/1 produce a lower pH than those of LPC-22, this pooling of the data may be expected to bias the results in such a way as to suggest that the supernatant from MTD/1 cultures is more inhibitory.

The average yeast growth at different times in these trials is shown in Figure 7.6, and the difference between the growth in the supernatants at each measurement time point was compared (Students t-Test). It can be seen that growth in the supernatant from LPC-22 is about 10% lower than that in the supernatant from the MTD/1 control. This concurs with the results shown in Table 7.2 and Table 7.3 and it suggests that, although LPC-22 cultures have a lower total metabolism after any given incubation period, the inhibition of subsequent *S. exiguus* growth in the bacteria free supernatant is greater.

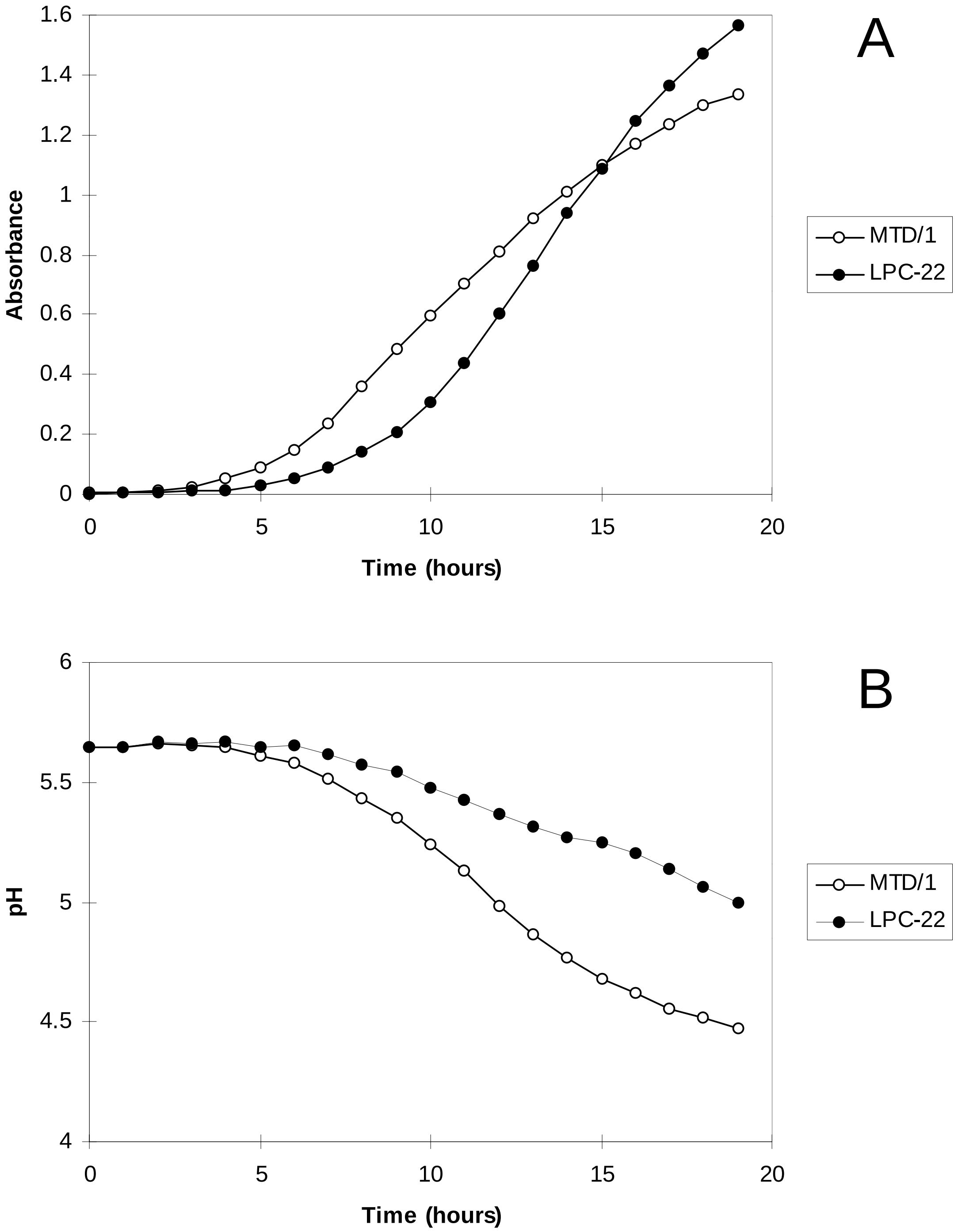

Figure 7.4. Changes in OD (A) and pH (B) in cultures of the test LAB in SGM, *n*=3

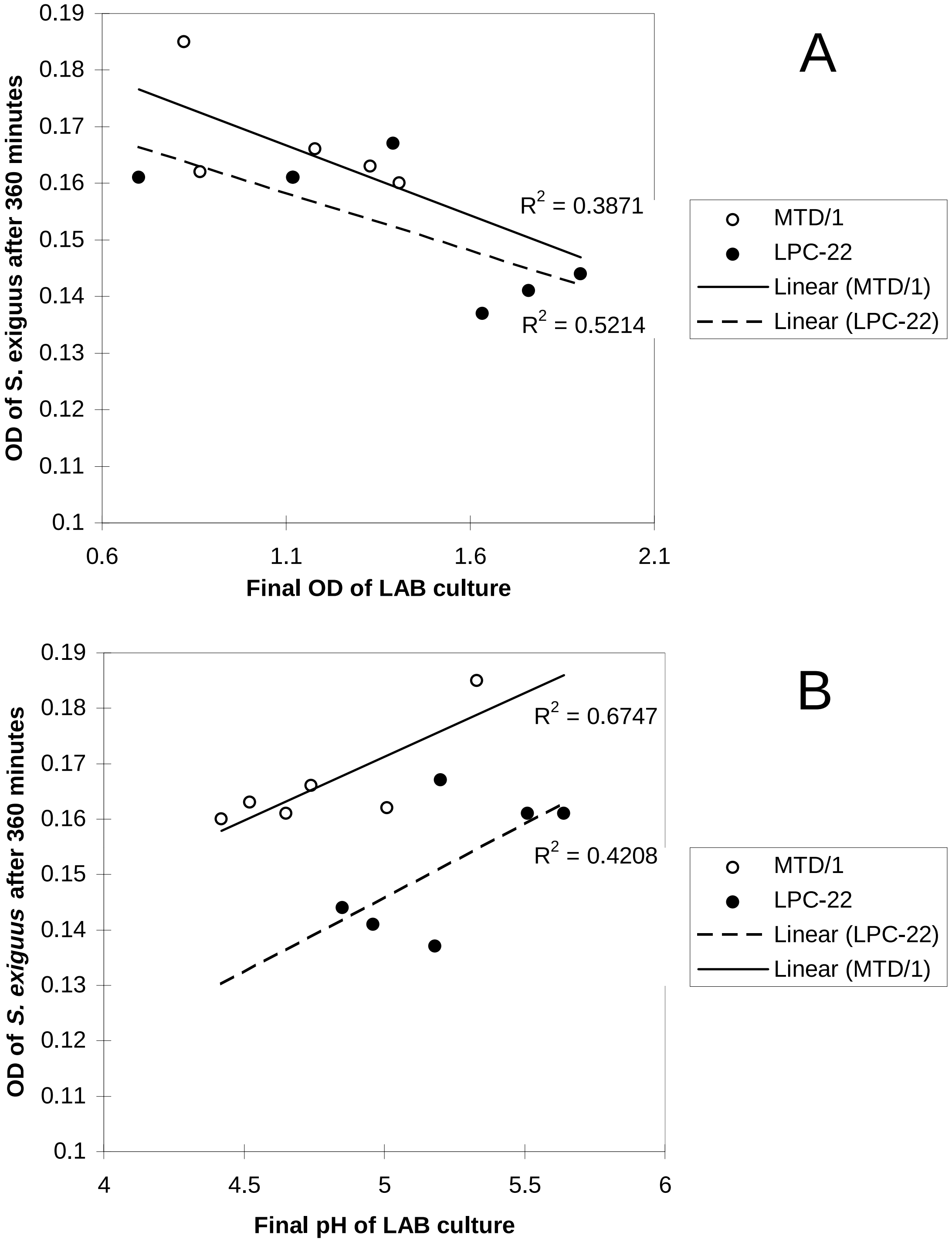

Figure 7.5. Growth of *S. exiguus* in the filter sterilised supernatant from cultures of MTD/1 and LPC-22 in SGM. **(A)** Relationship between OD of LAB culture and subsequent yeast growth, *n*=6. **(B)** Relationship between pH of LAB culture and subsequent yeast growth, *n*=6

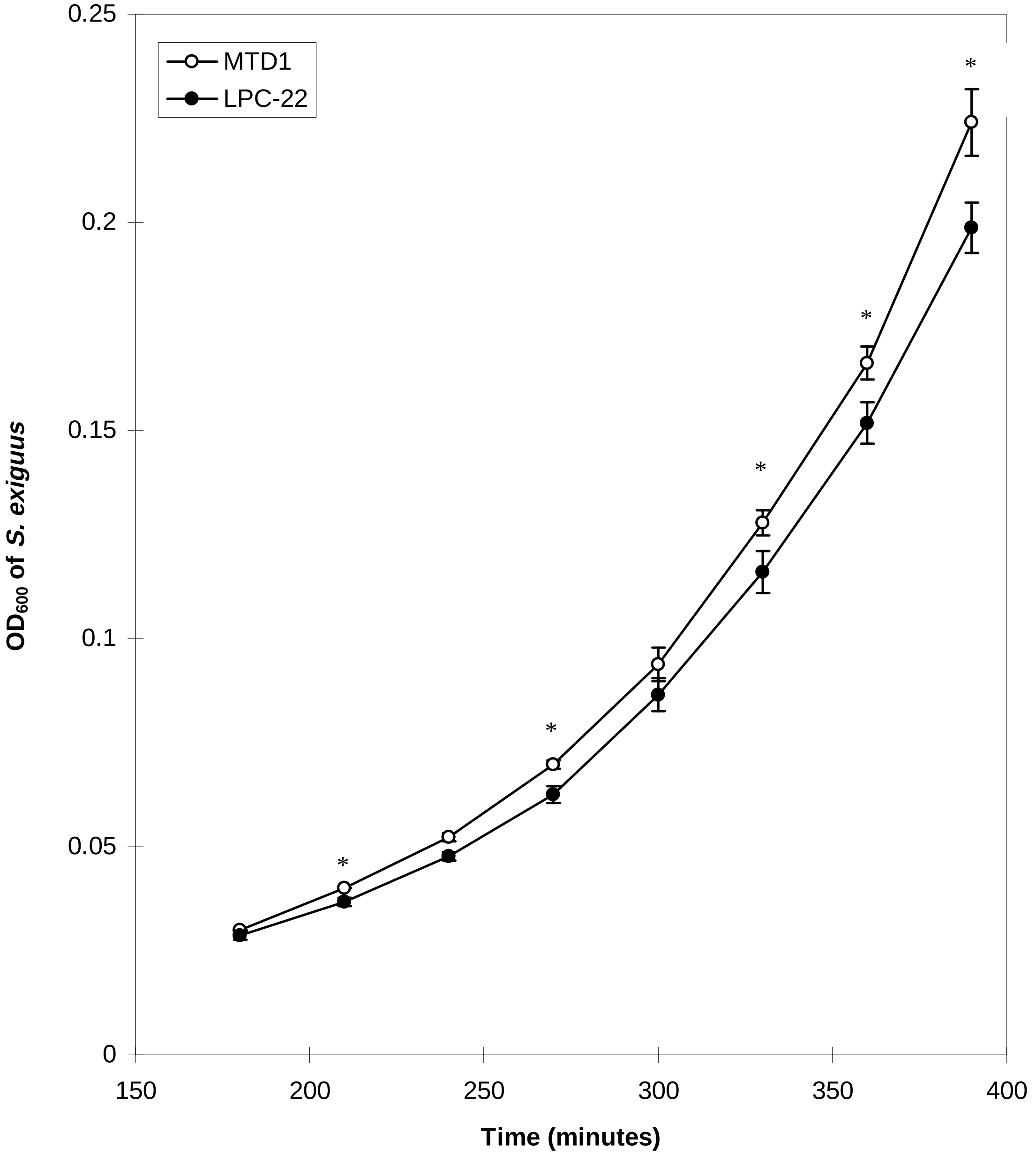

Figure 7.6. Change in the OD_{600} of aerobic *S. exiguus* cultures grown at 30°C in the filter-sterilised supernatant of either MTD/1 or LPC-22.

Error bars indicate SEM (*n*=6)

*Significantly different ($p<0.05$)

7.4 Extraction and concentration of antifungal product from cultures of lactic acid bacteria

7.4.1 Freeze drying

A starter culture of the test strains (MTD/1 and LPC-22) was inoculated into SGM (500 ml in a screw-capped bottle). Cells were removed by filtration (0.45 µm), and 100 ml of the supernatant was frozen by dropwise addition to liquid nitrogen. The nitrogen was strained off and the supernatant dried over a three day period in a freeze dryer. The yield was approximately 45 g/l of supernatant.

The freeze-dried material was reconstituted in sterile water at concentrations of 100, 250, 500 and 1,000 g/l, and tested in a well diffusion assay against *S. exiguus* on SGM and SSM (Section 3.5.3).

No inhibition was observed on any of the plates. On plates containing the highest concentration of supernatant (1,000 g/l), some stimulation of growth was observed.

7.4.2 Solvent extraction

With the technique given in Sections 3.7.3 and 3.7.4, four solvents were used in an attempt to concentrate the antifungal effect produced by LPC-22 in agar medium. The agar cultures were prepared by following the established screening protocol on which the effect had first been observed, but with the absence of indicator yeast in the second (SSM) layer. Three immiscible solvents were used (butanol, ethyl acetate, diethyl ether), and one miscible solvent (methanol), and the resultant extracts were tested in a well diffusion assay (Section 3.5.3).

The extracts made using the two most polar solvents, ethyl acetate and diethyl ether, failed to give a positive result in a well diffusion assay. The extracts obtained with methanol and butanol both gave a positive result, producing a large (~20 mm) zone of inhibition after 18 hours (the earliest time that yeast growth was apparent), gradually fading over the ensuing 36 hours. This antifungal effect was observed in extracts from both LPC-22 and the control organism MTD/1, and it was also seen when a control extraction was made from sterile agar. When the incubation period of the cultures was shortened from 48 to 24 hours per stage, no effect on the extracted activity was observed. The initial extractions were made from agar cultures which contained no yeast, but the incorporation of a yeast (*S. exiguus*) into the second (SSM) layer had no effect on the observed activity. The activity was also not susceptible to lipase I (5 mg/ml). Collectively, these results suggest that the antifungal activity in these extracts is not due to LAB metabolilsm.

7.4.3 Precipitation

An attempt was made to produce an active precipitate by acid precipitation from an agar extract (Section 3.7.5), and ammonium sulphate precipitation from liquid culture supernatant (Section 3.7.6). The extracts were tested in a well-diffusion assay (Section 3.5.3). No activity was observed.

7.4.4 Molecular sieving

Attempts to concentrate the active principle by size fractionation used two techniques, high pressure ultrafiltration and dialysis.

The dialysis techniques used are described in Section 3.7.7. Native and acidified agar extracts were dialysed against distilled water, and the retentate was concentrated by freeze drying, and then tested in a well-diffusion assay (Section 3.5.3). The resultant preparation was inactive. Native and acidified agar extracts were also dialysed against a solution of PEG 20,000, buffered with Na_2HPO_4, but this method was not effective in concentrating them.

The most extensive efforts made involved ultrafiltration, from which somtimes-contradictory results were obtained. Native and acidified extracts were concentrated by 500, 1,000 and 10,000 MWCO filters (Section 3.7.8), and a wide-ranging series of experiments was conducted over a six-month period in an attempt to produce consistent results. In the first few experiments, the retentate was frozen before analysis in a well-diffusion assay. This was because inexperience with the technique meant that the time taken to acheive a standard concentration could not be predicted. The time was, with hindsight, consistently around three days with the small filtration unit and a Spectrapor 1,000 MWCO filter to a concentration of 30%. This time varied with the filter (make, MWCO and when filters were re-used), size and make of the filtration unit, volume to be filtered (non-linearly), desired concentration (non-linearly) and acidity of the agar before extraction.

Initial filtration of the acidified extract, using a Spectrapor 1,000 MWCO filter, produced a inhibition zone around the extract from LPC-22. Repetition of this experiment with MTD/1 produced no inhibition. These filtrations were not run concurrently.

To run filtrations concurrently a pair of larger (400 ml) filter units with Amicon 1,000 MWCO filters had to be used. Unfortunately, because the extract from MTD/1 was filtered more rapidly, the retentates had to be frozen before simultaneous testing. When the retentates were tested the results were the opposite of that found previously, with only MTD/1 giving a zone of inhibition.

Because of the practical difficulties of concurrent filtration, subsequent experiments used the small unit. The extract of LPC-22 which gave a positive result in the first experiment, and had since been stored frozen, was filtered by a Spectrapor 1,000 MWCO filter, and the retentate frozen prior to analysis. This time, the retentate was inactive, as was the stored extract from MTD/1.

Filtration of a freshly prepared extract from LPC-22 using the small filter was repeated five times, and on three occasions a positive result was obtained. This active, neutralised retentate was frozen and then defrosted before retesting. It was found to now be inactive. This also occurred when the active, neutralised retentate was stored at 4°C for 24 hours, however the storage of non-neutralised retentate (pH 2) resulted in the maintenance of the activity. Filtration of a defrosted extract produced an inactive retentate.

Filtration of an MTD/1 extract was also positive, but some activity was still retained after storage of the neutralised retentate at 4°C for 48 h. When the retentate was stored at pH 2, the retained activity was much greater. Filtration of a defrosted extract produced an inactive retentate.

At this point, all the active retentates which had been produced using new Spectrapor filters. All the inactive retentates had used Amicon filters, or cleaned and reused Spectrapor filters.

Filtration of a freshly prepared extract from LPC-22 using a large filter and a 500 MWCO Spectrapor filter gave an active extract. The activity was destroyed by lipase I, esterase, catalase, and by heating (100°C, 10 minutes). The effect of catalase and esterase on activity was disturbing, since neither have any effect on activity when live cultures are tested (Table 7.1).

One of the steps in the extraction procedure may result in some contamination from hydrogen peroxide. This is the step, immediately before testing activity in the well diffusion assay, in which the pH is adjusted. For this step, the pH probe is sterilised in H_2O_2, before being rinsed in sterile water. To investigate the possibility that some H_2O_2 is being carried over, the extraction and concentration procedure was repeated without using H_2O_2 to sterilise the electrode.

Fresh extracts were made from LPC-22 agar cultures. Two were filtered first through 10,000 MWCO filters before filtration through a 500 MWCO filter. Two were filtered through a 500 MWCO filter alone. The retentates were adjusted to pH 5 using an electrode which had not been sterilised. None of the retentates were active.

Since catalase was able to abolish the activity in the active retentate, the possibility that the esterase and lipase preparations contained some peroxide-reducing activity was considered. Esterase and lipase I were tested for catalase and peroxidase activity (Sections 3.5.7 and 3.5.8). Esterase was found to possess considerable catalase activity (150 U/mg, compared with 500 U/mg for the Sigma catalase preparation). Lipase I contained no catalase activity, but it did contain a peroxidase activity in the presence of the agar extract retentate.

The inhibitory activity of H_2O_2 was tested in a well diffusion assay. A 10 mM solution in water gave a large (6 mm) zone of inhibition, which did not fade (unlike the inhibition caused by the active retentate preparations. H_2O_2 was then added to an inactive retentate preparations. It was found that 50 µl of the solution used to sterilise the electrode (giving a final concentration of 6.5 mM) produced a zone of inhibition (~4 mm). Again, this was unlike the zone of inhibition produced by active retentate preparations, in that the zone did not fade. Finally, the contamination event was simulated by soaking the electrode in the sterilisation solution, cursorily rinsing in water, then washing in some inactive retentate. The result was very slightly active (~1 mm zone of inhibition).

The lack of activity in extracts made without peroxide sterilisation may indicate that such sterilisation is responsible for the observed antifungal effect, although the antifungal activity was only observed inconsistently prior to the removal of this step. The high concentrations of peroxide needed to produce an antifungal effect, and the inability of deliberate peroxide contamination to replicate the observed antifungal effect exactly, may indicate that the antifungal effect which had previously been observed was not an artefact. No final conclusions can be drawn.