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Optimization of the Chiral Inversion of 2-Phenylpropionic Acid by *Verticillium lecanii*

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Abstract

Previous studies have demonstrated that *Verticillium lecanii* might be used as a microbial model of the inversion of 2-arylpropionic acids in man. This paper describes the optimization of the inversion process in respect of culture medium, pH, cell density and substrate concentration.

The study demonstrates that optimum inversion occurs in Sørensen's phosphate buffer at pH 5·5. The extent and rate of inversion were also shown to be dependent on substrate concentration and cell density.

This study will form the basis of the development of a microbial model of the metabolism of 2-arylpropionic acids which might be suitable for the in-vitro screening of new compounds in this class.

Metabolic studies on new and existing drugs are frequently hindered by difficulties associated with the chemical synthesis of metabolites, or potential metabolites, for use as analytical reference standards. Similarly, the use of animal models poses problems in respect both of the isolation of small quantities of metabolites and of the relevance of the animal system to the clinical situation in man. It has been suggested that micro-organisms could mimic many of the biotransformations observed in mammals and that the metabolic products could be isolated in relatively large quantities (Smith & Rosazza 1975). The 2-arylpropionic acid non-steroidal anti-inflammatory drugs show stereoselectivity in action, protein binding and metabolism. Several members of this group have been shown to undergo metabolic chiral inversion from their inactive, or weakly active, *R* enantiomers to their active *S* antipodes (Hutt & Caldwell 1983; Caldwell et al 1988). An extensive screening programme by our group, using ibuprofen, has previously demonstrated that the fungus *Verticillium lecanii* is able to mimic metabolic processes observed in man (Kooloobandi et al 1988). A metabolite was isolated and identified as 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid by NMR and mass spectroscopy (Hutt et al 1993), a major metabolite of ibuprofen in man (Mills et al 1973). The metabolite was found to be predominantly of the *S* configuration, indicating that chiral inversion of ibuprofen or the hydroxy metabolite, or both, had occurred. In an effort to understand this process, 2-phenylpropionic acid was used as a model compound and it was found that inversion of the *R* enantiomer to its *S* antipode occurred with no loss of substrate (Hutt et al 1993).

Investigations into the effect of environmental parameters on the growth of *V. lecanii* and its metabolism of ibuprofen, in an attempt to optimize the system and elucidate the possible control mechanisms, revealed a lag-phase of up to 12 h after inoculation of the second-stage cultures, followed by up to 4 days of rapid log-phase growth (Hanlon et al 1994). The optimum growth conditions were found to be pH 7·0, an incubation temperature of 24°C and a culture volume of up to one tenth of the incubation flask (Hanlon et al 1994). The metabolism of ibuprofen in growing cultures was also found to have a lag-phase, with little metabolism occurring until the cells were in stationary phase. This lag period was also observed when substrate was added to the culture after growth had finished and when non-growing washed concentrated cell suspensions in buffer were used. The metabolic rate was also shown to be dependent on both pH and temperature, but in a complex way not related the conditions for optimum cell growth (Hanlon et al 1994).

There have been many reports of alterations in physical and chemical incubation conditions affecting the products of fermentation and biotransformation reactions both qualitatively and quantitatively (Smith & Rosazza 1975; Goodhue 1982; Clark et al 1985). It has also been shown that the optimum conditions for cell growth are not necessarily optimum for the desired biotransformation reaction, and thus the two processes are commonly separated and optimized individually (Goodhue 1982). This paper describes investigations into the optimization of the chiral inversion of 2-phenylpropionic acid by *V. lecanii* in respect of the use of growing or non-growing cultures, media composition, pH, substrate concentration and cell density.

Materials and Methods

Microorganism, media and chemicals

V. lecanii (IMI 32287) was a gift from Dr J. Hanson (University of Sussex, UK) and was maintained on refrigerated (4°C) agar slants of malt-extract agar obtained from Oxoid (Basingstoke, Hampshire, UK), prepared and sterilized according to the manufacturer's instructions. The culture was transferred to a fresh slant every six months to maintain viability. Racemic *R,S*-2-phenylpropionic acid, the pure enantiomers *R*- and *S*-2-phenylpropionic acid (97% stated purity) and HPLC-grade dimethylsulphoxide (DMSO) were purchased from Aldrich, Gillingham, Dorset, UK. Disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride were purchased from BDH, Poole, Dorset, UK. Yeast extract and mycological peptone were purchased from Oxoid.

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Water was double distilled and passed through a 0·2-μm filter (Purite, Thame, Oxfordshire, UK) before use. The growth medium used in these studies (DP-dextrose peptone medium) consisted of: glucose, 20 g; bacteriological peptone (Oxoid L37), 5 g; yeast extract (Oxoid L21), 5 g; NaCl, 5 g; dibasic potassium phosphate, 5 g; distilled water to 1 L and the pH adjusted as required with concentrated HCl.

Preparation of buffers

Sørensen's phosphate buffer was prepared using Na_2HPO_4 (0·067 M) and KH_2PO_4 (0·067 M) to produce six replicate buffered cultures at the required pH. McIlvaine's citrate-phosphate buffer was prepared from appropriate mixtures of citric acid (0·1 M) and Na_2HPO_4 (0·2 M) to produce six replicate buffered cultures at the required pH.

General incubation conditions

Growth of microorganism. Week-old slants of *V. lecanii* were flooded with sterile DP medium and the surface growth removed by gentle agitation under aseptic conditions. This suspension was then used to inoculate 25-mL samples of dextrose peptone medium (pH 6·0) in 100-mL foil-capped Erlenmeyer flasks, previously sterilized in an autoclave at 115°C for 30 min. The flasks were incubated at 24 ± 0·5°C in an orbital shaker (Infors HT; Infors, Crewe, UK) operating at 150 rev min^{-1} for 48–72 h. Samples (1 mL) of these first-stage cultures were then used to inoculate second-stage cultures (100 mL) which were incubated as described for the required time periods.

Growing cultures. The second-stage DP cultures were incubated for 24 h after which 250 μL of a 100 mg mL^{-1} solution of *R,S*-2-phenylpropionic acid in DMSO was added to give a final concentration of 250 μg mL^{-1}. Samples were removed at regular time-intervals into 12-mL polypropylene centrifuge tubes, centrifuged at 2000 *g* for 5 min to remove the cells, and the supernatant liquid decanted into a second, clean tube and stored at −20°C until required for analysis.

Non-growing cultures. Ninety-six hour second-stage DP cultures were harvested by centrifugation at 1000 *g*; the pellet of cells was washed with sterile distilled water three times to remove traces of the growth medium and the combined cell pellets from 200 mL DP medium were resuspended in 100 mL of sterile buffer, forming a concentrated cell suspension of approximately 2 g dry weight/100 mL. *R,S*-2-Phenylpropionic acid was then added as described above to give a final concentration of 250 μg mL^{-1}. The flasks were then returned to the incubator and samples were removed at regular time-intervals as described above.

For both sets of incubations, control flasks were also prepared without addition of the substrate to the cultures or containing 250 μg mL^{-1} *R,S*-2-phenylpropionic acid in 100 mL of media in the absence of *V. lecanii*.

Measurement of cell density

Cell density was measured as the dry weight of the entire biomass of the incubation cultures as the cells are not dispersed uniformly in the incubation flasks. The dry-cell weight thus represents the total cell mass present and does not take into account material formed and lost by cell lysis or provide any indication of the viability of the cell population. The medium was evaporated by placing the flasks in an oven at 70°C and stable dry-weight readings were obtained after drying for five days. The results are presented in Table 1.

Table 1. Cell density of *Verticillium lecanii* expressed as dry-cell weight.

Number of 2nd-stage growth flasks resuspended in buffer	Cell density (mg mL^{-1})
0·5	5·2
1·0	11·2
2·0	20·3
3·0	29·1
4·0	42·8

Effect of cell density on chiral inversion of 2-phenylpropionic acid by V. lecanii

Cells were re-suspended in Sørensen's phosphate buffer (pH 6·0; 100 mL) to give a final cell density 0·5, 1, 2, 3 and 4 times that of the original DP cultures harvested (Table 1). A solution of *R,S*-2-phenylpropionic acid in DMSO (100 mg mL^{-1}; 250 μL) was added to give a final concentration of 250 μg mL^{-1}. The flasks were incubated as described and samples removed at the required time intervals. Two control flasks were also prepared, one without the addition of the substrate and another containing 250 μg mL^{-1} *R,S*-2-phenylpropionic acid in 100 mL buffer in the absence of *V. lecanii* and sampled in the same way as the experimental flasks.

Effect of substrate concentration on the chiral inversion of 2-phenylpropionic acid by V. lecanii

Cells were re-suspended in Sørensen's phosphate buffer (pH 6·0) to give a final cell density twice that of the original DP cultures harvested. Samples (1000, 750, 500 or 100 μL) of a solution of *R,S*-2-phenylpropionic acid in DMSO (100 mg mL^{-1}) were added to each flask to give final concentrations of 1000, 750, 500 and 100 μg mL^{-1} respectively, and the pH of each flask was adjusted to 6·0 by addition of HCl (5 M) or NaOH (5 M) as required. The flasks were then incubated as described and samples removed at the required time intervals. Single control flasks of each substrate concentration in 100 mL buffer in the absence of *V. lecanii* were prepared, incubated and sampled in the same way.

Enantiomeric analysis of 2-phenylpropionic acid

The enantiomeric composition of 2-phenylpropionic acid in the incubation extracts was determined by derivatization of the acids with *S*-(−)-α-methylbenzylamine to yield pairs of the corresponding diastereoisomeric amides using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 1-hydroxybenzatriazole as coupling agents as described previously (Thomason et al 1994).

The internal standard (phenylacetic acid, 250 μg mL^{-1}) was added to the supernatant fluid from the incubation mixtures (2 mL). The pH of the mixture was adjusted to 1 by addition of HCl (5 M; 200 μL) and the whole extracted with toluene (2 × 2 mL). The toluene was removed by rotary evaporation and the residue dissolved in dichloromethane and

derivatized as described elsewhere (Thomason et al 1994). The samples were then analysed by an indirect reversed-phase HPLC assay using a Techsphere 5-μM ODS column and an isocratic mobile phase consisting of acetonitrile-NaH_2PO_4 (0·075 M) 45 : 55, v/v, containing sodium pentanesulphonate (0·005 M; ion-pair reagent) and H_3PO_4 (0·09% v/v); the flow rate was 1 mL min^{-1}.

Results

Investigations of chiral inversion by V. lecanii *in growing and non-growing cultures*

Chromatographic analysis of samples from the control incubation of *V. lecanii* in DP and buffer media in the absence of *R,S*-2-phenylpropionic acid showed no interfering peaks from the contents of the incubation flasks or derivatization reagents. Analysis of samples from the control incubation of *R,S*-2-phenylpropionic acid in the absence of *V. lecanii* showed no alteration in recovery or enantiomeric ratio, indicating that 2-phenylpropionic acid is enantiomerically stable under the experimental conditions used and that any observed change in the enantiomeric ratio in the other incubation flasks was a result of the presence of *V. lecanii*.

Comparison of chiral inversion in growing and non-growing cultures

Growing cultures. Incubations were prepared in DP medium (pH 6·0; n = 6) as described above; the results are shown in Fig. 1. Chiral inversion from *R*- to *S*-2-phenylpropionic acid occurred under these experimental conditions yet essentially quantitative substrate recovery was obtained over the period of the experiment (Fig. 2), thus demonstrating that the observed change in enantiomeric ratio is not a result of stereoselective metabolism of one enantiomer. The maximum rate of inversion was observed between 3 and 6 days incubation, with little inversion occurring before 3 days, during the period of active growth, and inversion was found to be essentially complete by 6 days. The pH of the contents of the incubation flasks was found to vary considerably during incubation (Table 2), falling slightly over the first three days during the period of active growth, then increasing rapidly between day 3 and day 15, when maximum inversion was occurring. The pH then levels out in the range 8·5 to 9·0 between days 15 and 21.

Non-growing cultures. Incubations were prepared in McIlvaine's citrate-phosphate buffer and Sørensen's phosphate buffer, both at pH 6·0; the results are shown in Fig. 1. Inversion was found to proceed much faster than in the corresponding incubations in growth medium, with the maximum rate of inversion occurring between day 1 and day 3 and the enantiomeric ratio starting to plateau between days 3 and 6. This corresponds well to the observations from incubation in DP medium, where inversion was found to occur after the period of active growth. The percentage of *S*-2-phenylpropionic acid present reached maxima of approximately 75% and 84% of the total 2-phenylpropionic acid recovered after three days incubation in citrate-phosphate buffer and phosphate buffer, respectively. This compares with a maximum of 65% *S*-2-phenylpropionic acid after six days incubation in DP medium. The pH of the citrate-phosphate-buffered incubation flasks was found to increase to a higher pH than in DP medium

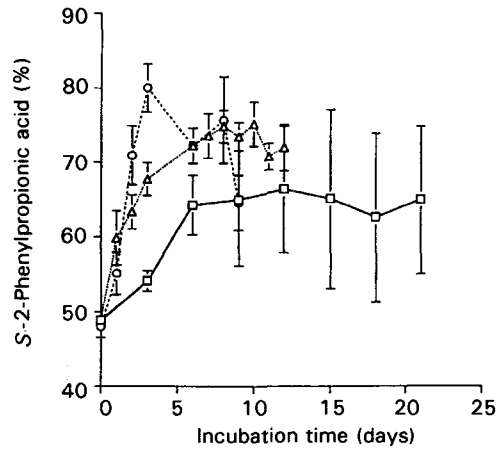

FIG. 1. Comparison of the rate of chiral inversion of *R,S*-2-phenylpropionic acid by *Verticillium lecanii* in growing and non-growing cultures. □ Dextrose-peptone growth medium, △ McIlvaine's citrate-phosphate buffer and ○ Sørensen's phosphate buffer. Mean ± s.d., n = 6.

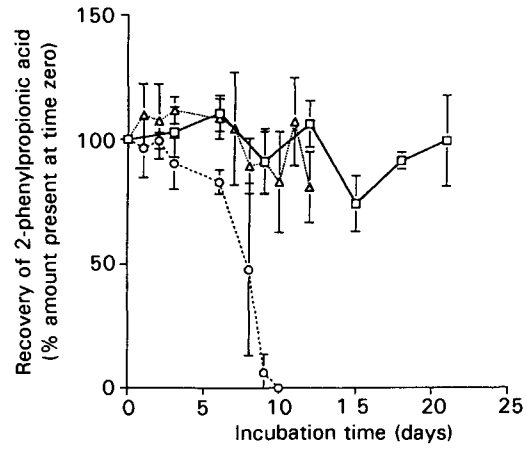

FIG. 2. Recovery data from incubations of *R,S*-2-phenylpropionic acid with *Verticillium lecanii* in growing and non-growing cultures expressed as a percentage of the initial amount present at time zero. □ Dextrose-peptone growth medium, △ McIlvaine's citrate-phosphate buffer and ○ Sørensen's phosphate buffer. Mean ± s.d., n = 6.

Table 2. Change of media pH in incubations of *Verticillium lecanii* in three different incubation media.

Incubation time (days)	pH Values DP medium	McIlvaine's citrate-phosphate buffer	Sørensen's phosphate buffer
0	5·78 ± 0·3	6·0 ± 0	6·0 ± 0
3	5·58 ± 0·03	7·33 ± 0·23	6·47 ± 0·13
6	7·47 ± 0·39	8·27 ± 0·2	6·95 ± 0·09
9	7·98 ± 0·07	8·38 ± 0·13	7·19 ± 0·11
12	8·32 ± 0·26	8·81 ± 0·12	ND

Values are expressed as mean ± s.d., n = 6. ND indicates that the pH was not measured because there was no substrate left after this time. DP medium = dextrose peptone medium.

whereas the pH of the phosphate-buffer incubation flasks was also found to increase during the course of the incubation but to a much lesser extent than in either growth medium or citrate-phosphate buffer (Table 2). The results also show

much less variation in the pH between replicate incubation flasks sampled at the same time in this media. Loss of substrate occurs in both the Sørensen's and McIlvaine's buffers, occurring after 18 days incubation in citrate-phosphate buffer and after only 6 days in phosphate buffer. This is a previously unreported metabolic biotransformation of 2-phenylpropionic acid to an unknown metabolite occurring after inversion has reached a plateau.

Comparison of chiral inversion of 2-phenylpropionic acid at different incubation pH in growing and non-growing cultures

Incubations were prepared at different pH values using the three incubation media used previously and time-course samples taken and analysed as described above to determine the pH optima for chiral inversion in each medium. The results are expressed as surface-response 3D graphs in Fig. 3. The maximum amount of inversion in growing cultures was found to occur at pH 5·5 (Fig. 3a) whereas pH 5 was found to be the optimum in citrate-phosphate buffer, although the amount of inversion at pH 5·5 was not significantly different (Fig. 3b). This contrasts with the results obtained for phosphate buffer where there was a distinct optimum at pH 6·0 (Fig. 3c).

Results for substrate recovery were similar to those obtained for the incubations undertaken at pH 6·0, above (Fig. 2). No substrate metabolism was observed in growing cultures whereas metabolism was observed for incubations in both Sørensen's and McIlvaine's buffers at most pH values. Although the results were not linear, it appears that the higher the initial pH, the faster the rate of metabolism in the non-growing cultures.

Incubation in non-growing cultures with the pH held constant at pH 6·0

The above results (Fig. 3) demonstrate that inversion of *R,S*-2-phenylpropionic acid might have some pH-dependency, but adequate control of pH could not be achieved in the different buffer systems investigated. Sørensen's phosphate buffer produced the least increase in pH with time, and does not contain a carbon source that could be used by the cells as a nutrient. Incubations were prepared containing *R,S*-2-phenylpropionic acid in non-growing cultures of *V. lecanii* in Sørensen's phosphate buffer at initial pH 6·0, with the pH artificially held constant by the addition of HCl daily in an attempt to achieve maximum rate and extent of inversion. A pH of 6·0 was chosen because the earlier studies had shown this to be the optimum pH for inversion (Fig. 3c). The rate of inversion was found to proceed slightly faster and to a greater extent than for the corresponding incubation without daily pH adjustment (Fig. 4). A loss of substrate was observed after 6 days incubation, indicating that the loss of substrate was not avoided by controlling the incubation pH, although it does appear to delay the onset of this metabolism.

Effect of cell density on the chiral inversion of 2-phenylpropionic acid

The above results demonstrate that the optimum rate of chiral inversion was obtained in non-growing cultures of *V. lecanii* incubated in Sørensen's phosphate buffer at pH 6·0. These incubation conditions were thus employed in the subsequent optimization investigations.

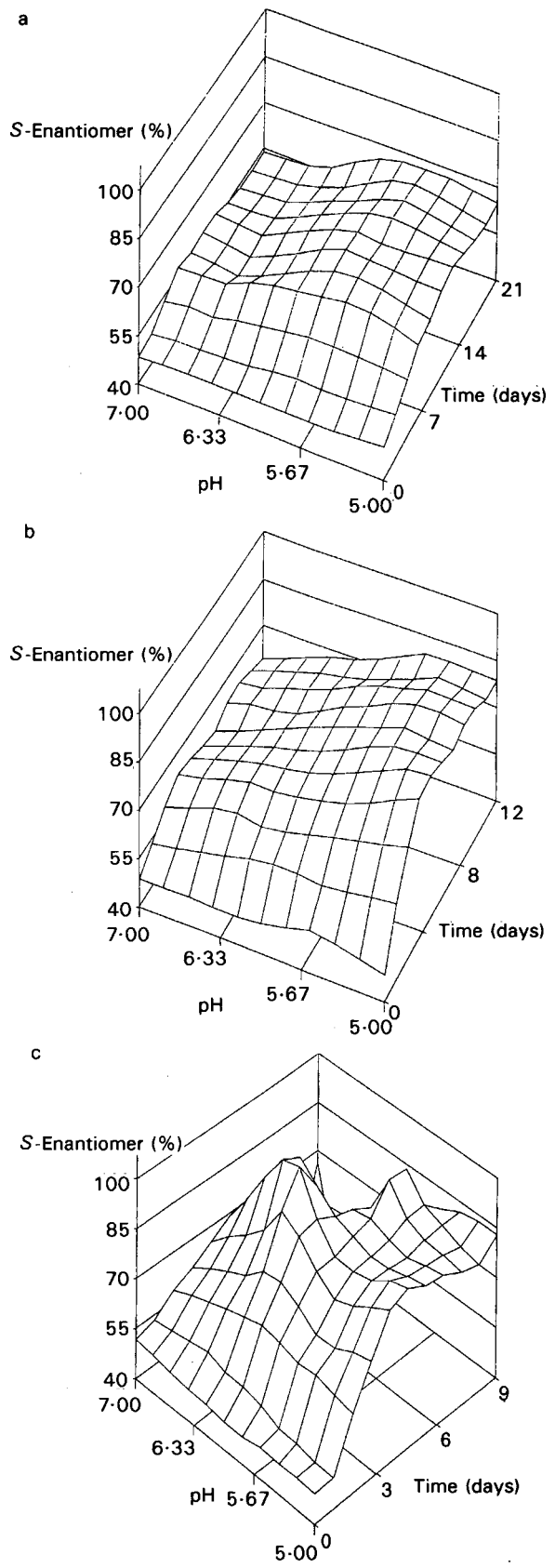

FIG. 3. Surface-response 3D graphs of the rate of chiral inversion of *R,S*-2-phenylpropionic acid by *Verticillium lecanii* in a growing cultures (a), McIlvaine's citrate-phosphate buffer (b) and Sørensen's phosphate buffer (c) at different initial media pH. Mean, n = 6.

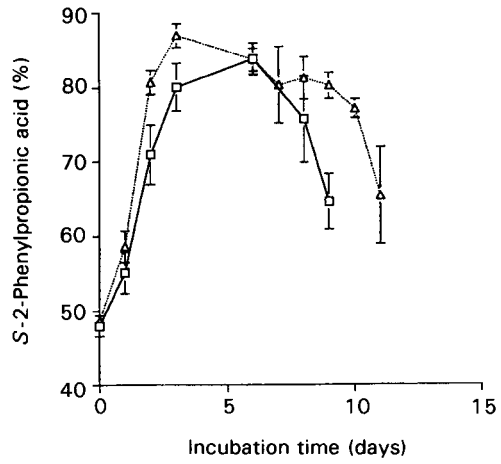

FIG. 4. Comparison of the rate of chiral inversion of *R,S*-2-phenylpropionic acid by *Verticillium lecanii* in non-growing cultures suspended in Sørensen's phosphate buffer at pH 6·0. □ Sørensen's phosphate buffer initial pH 6·0, △ Sørensen's phosphate buffer with pH adjusted to 6·0 daily. Mean ± s.d., n = 6.

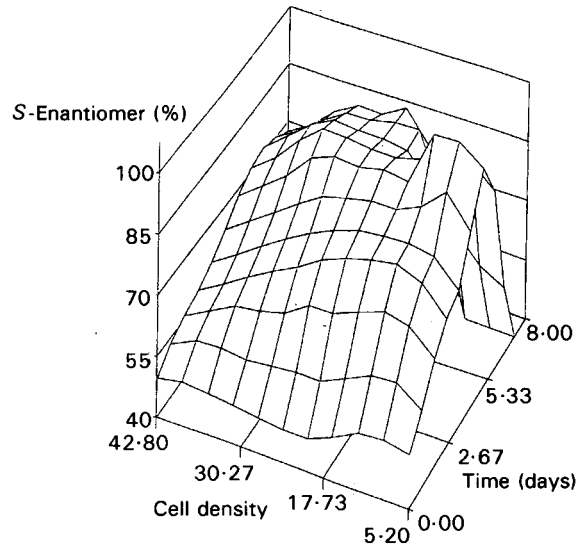

FIG. 5. Surface-response 3D graph of the rate of chiral inversion of *R,S*-2-phenylpropionic acid by *Verticillium lecanii* in non-growing cultures suspended in Sørensen's phosphate buffer, pH 6·0, at different cell densities. Mean, n = 6.

Incubations were prepared using the optimum incubation conditions described above but with different cell densities (Table 1). The results from the analysis of the time-course samples taken are shown in Fig. 5. Chiral inversion of *R,S*-2-phenylpropionic acid was found to have occurred at all the cell densities investigated, the maximum extent of inversion was observed at a cell density of 29·1 mg mL^{-1}; this produced a change in the enantiomeric ratio from $S:R = 50:50$ at day zero to approximately 85 : 15 in 6 days, although inversion at this cell density was found to proceed more slowly than at a cell density of 20·3 mg mL^{-1}. Inversion in the very thick suspension at a cell density of 42·8 mg mL^{-1} was found to be slower and to proceed to a lesser extent than in the thinner cultures. Incubation at a cell density of 11·2 mg mL^{-1} produced about the same rate and extent of inversion as incubations using a cell density of 20·3 mg mL^{-1} but loss of substrate was found to occur earlier. Incubation at a cell density of 5·2 mg mL^{-1} was found to produce rapid inversion, but to a lesser extent than incubations at a cell density of 20·3 mg mL^{-1}, followed by rapid loss of substrate. From these results, all subsequent investigations were performed using a cell density of 20·3 mg mL^{-1}.

The pH of the contents of the flasks at cell densities of 5·2 and 11·2 mg mL^{-1} were found to rise to a much lesser extent than incubations at the higher cell densities. This provides evidence that the rise in pH is because of breakdown of the cells into alkaline products; there is a greater quantity of cells at the higher cell densities and so more breakdown products are produced.

Effect of substrate concentration on the chiral inversion of 2-phenylpropionic acid

Incubations were prepared using the optimum incubation conditions in respect of media composition, pH and cell density, but with different concentrations of *R,S*-2-phenylpropionic acid. Chiral inversion was found to occur at all the substrate concentrations investigated (Fig. 6). The maximum rate and extent of inversion were obtained with a substrate concentration of 100 μg mL^{-1}, which produced a change in the enantiomeric ratio from $S:R = 50:50$ at day 0 to approximately 85 : 15 by day 3. An enantiomeric ratio of approximately 80 : 20 was produced by day 3 at 500 μg mL^{-1}, and by day 6 at 750 and 1000 μg mL^{-1}. These results do not take into account the metabolism of the substrate which was found to occur in non-growing cultures. Loss of substrate appeared to be most rapid at a substrate concentration of 500 μg mL^{-1}, which occurred after 3 days incubation, whereas the loss of substrate at 750 and 1000 μg mL^{-1} began after the same length of incubation but took longer to complete. Using a substrate concentration of 100 and 250 μg mL^{-1}, some substrate metabolism was observed after incubation for 3 days but in both cases, greater than 90% of the initial amount of substrate remained after incubation for 6 days compared with 20, 80 and 80% for 500, 750 and 1000 μg mL^{-1}, respectively. A plot of reaction rate against substrate concentration and the corresponding Lineweaver-Burk plot for the various concentrations investigated (data not shown) demonstrated that the points corresponding to a substrate concentration of 1000 μg mL^{-1} do not fit the straight-line relationship and that the rate of inversion is lower than is predicted by this model. This suggests that some degree of toxicity or enzyme inhibition has occurred at this high substrate concentration. If this point is removed from the plot, a good straight-line relationship between 1/V and 1/[S] is achieved which gives values for the Michaelis constant (K_M) of 12·7 mM and for V_{max} as $2{\cdot}59 \times 10^{-8}$ mol s^{-1}. These results indicate that the substrate concentrations used were all considerably less than the value of K_M, and so the enzyme system should not be saturated with substrate. Also, the inversion reaction in this system appears to proceed at a considerably lower rate than the theoretical maximum this model predicts. It should, however, be noted that this model relates to single-enzyme systems at low substrate concentrations and that the inversion phenomenon under investigation probably involves several enzymes and might also heavily depend on partitioning of the substrate into the cells and subsequent distribution to the enzyme active sites.

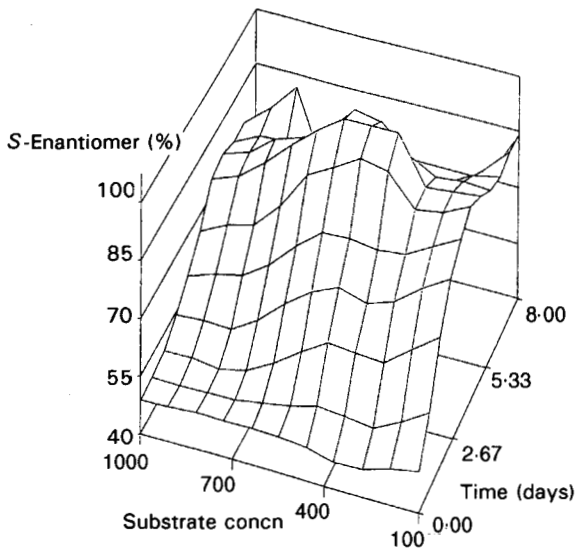

FIG. 6. Surface-response 3D graph of the rate of chiral inversion of *R,S*-2-phenylpropionic acid by *Verticillium lecanii* in non-growing cultures suspended in Sørensen's phosphate buffer, pH 6·0, at different substrate concentrations. Mean, n = 6.

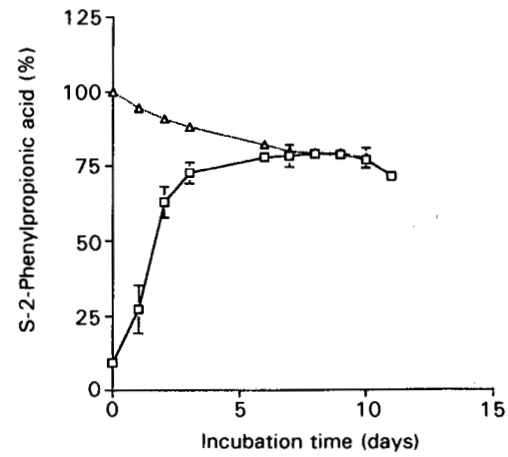

FIG. 7. Comparison of the rate of chiral inversion of pure enantiomers of 2-phenylpropionic acid by *Verticillium lecanii* in non-growing cultures suspended in Sørensen's phosphate buffer, pH 6·0. □ *R*-2-phenylpropionic acid, △ *S*-2-phenylpropionic acid. Mean ± s.d., n = 6.

Incubation of V. lecanii *with pure enantiomers of 2-phenylpropionic acid*

Incubations were prepared using the optimized conditions obtained from the investigations (Sørensen's phosphate buffer, pH 6·0; cell density 20·3 mg mL^{-1}) using pure enantiomers at half the optimum substrate concentration of the racemic substrate (125 μg mL^{-1}). As expected, *R*-2-phenylpropionic acid was shown to be inverted to the *S* enantiomer but interestingly, it was found that *S*-2-phenylpropionic acid was inverted to the *R* enantiomer, albeit at a considerably slower rate (Fig. 7). This bidirectional chiral inversion has not been observed in the growing cultures used previously (Hanlon et al 1994).

Discussion

Previous investigations into the chiral inversion of 2-arylpropionic acids by *V. lecanii* have centred around the optimization of growth conditions for the fungus (Hanlon et al 1994). As these conditions might not be optimum for the chiral inversion of the 2-arylpropionic acids (Goodhue 1982), investigations were undertaken to optimize the rate and the extent of inversion of the model compound 2-phenylpropionic acid. The previous investigations demonstrated that chiral inversion seems to occur in DP medium after the period of active growth (Hanlon et al 1994) and thus chiral inversion of 2-phenylpropionic acid in growing and non-growing cultures were initially compared followed by the optimization of medium pH, cell density and substrate concentration in the incubation.

Results from the comparison of the use of growing and non-growing cultures demonstrated that non-growing cultures resulted in a faster rate of chiral inversion, which proceeded to a greater extent, and that the inversion did not show any lag compared with that of the growing cultures (Fig. 1). The rate of inversion and the enantiomeric ratio would be expected to be greater in non-growing cultures because of the greater cell density present, but the absence of a lag-phase was interesting. The lag-phase observed using growing cultures might indicate that the enzyme systems responsible for *R,S*-2-phenylpropionic acid inversion are not synthesized or activated until the nutrient supply in the growth medium is exhausted and the cells actively attempt to utilize other carbon sources available to them. These results thus suggest that inversion is not the result of the normal growth process of the cells, and is not dependent on the presence of an energy source in the incubation medium.

Of the two buffer systems investigated, Sørensen's phosphate buffer resulted in the most rapid inversion of *R,S*-2-phenylpropionic acid and the greatest yield of *S*-2-phenylpropionic acid. Even at the optimum pH, however, the amount of the *S* enantiomer present never exceeded 85% of the total 2-phenylpropionic acid recovered. This might be explained by the unexpected result obtained from incubations using pure enantiomers in Sørensen's phosphate buffer (Fig. 7); bidirectional inversion occurs under these conditions, but has not been observed using growth media (Hanlon et al 1994). It would appear that some kind of equilibrium is set up such that the amount of *S*-2-phenylpropionic acid does not exceed the 85% observed. Such bidirectional inversion is uncommon in mammalian systems.

Another unusual result obtained using non-growing cultures was the metabolism of the substrate after incubation for 18 and 6 days in McIlvaine's citrate-phosphate buffer and Sørensen's phosphate buffer, respectively. The metabolism of ibuprofen by growing cultures of *V. lecanii* has been demonstrated previously, but no metabolism of the model compound 2-phenylpropionic acid was observed (Hanlon et al 1994). No extra peaks which might correspond to metabolites appear on the HPLC chromatograms for the buffer incubations, indicating that the biotransformation probably leads to the removal of the carboxylic acid functional group from the molecule, which thus loses its ability to derivatize to form an amide. Any non-derivatized product would then probably be much more polar than the diastereoisomeric amides, and elute under the large solvent front. Such a loss could be effected by reduction of the carboxylic acid group to the corresponding aldehyde and then alcohol, or by oxidation processes leading to gross breakdown

of structure and utilization of the 2-phenylpropionic acid by the cells as a carbon source.

Loss of substrate might be linked to starvation of the cells. The exhaustion of nutrients in the medium could lead to the induction or activation of new enzyme systems to metabolize any available carbon source as a supply of energy. Washing the cells and re-suspending them in buffer will essentially remove all traces of growth medium, and so will induce starvation more rapidly than the exhaustion of glucose in growing cultures. Also, growth in DP medium might cease because of exhaustion of the carbon source while other nutrients essential for growth such as nitrogen and phosphate sources might still be present. Washing and re-suspension in buffer will remove these nutrients also, which might trigger a change in cell metabolism and enzyme synthesis not occurring in DP medium.

The optimum pH values for inversion in the three systems investigated were not identical but were between 5 and 6. Because the pK_a of 2-phenylpropionic acid is approximately 4·5, at these pH values, the compound will be predominantly in the ionized form. Whether this facilitates the uptake of 2-phenylpropionic acid remains to be proven, although the internal pH of the cells is unlikely to affected by the pH of the external buffer. The results obtained from incubations using Sørensen's phosphate buffer kept constant at pH 6 through the incubation (Fig. 4) suggest that the uptake of the substrate might be pH-dependent. The use of Sørensen's phosphate buffer resulted in the greatest rate and extent of chiral inversion observed, which could be because it has the best buffering capacity of the three media investigated, thus resulting in the most consistently rapid uptake of substrate during the course of the incubation.

The results obtained from varying the cell density in the incubations show no distinct pattern. It would be expected that the greater the number of cells present the greater would be the amount of enzymes present to act on the same amount of substrate. This was not found to be so—the greatest extent of chiral inversion was observed at a cell concentration of 29·1 mg mL^{-1}, which was not the highest used, and the fastest rate of inversion was observed at a cell concentration of 11·2 and 20·3 mg mL^{-1}. The results suggest that the number of cells present is not an index of the rate and the extent of chiral inversion observed. Other factors such as aeration, contact of the substrate with the surface of the cells and substrate partition into the cells might be important. At higher cell densities, the cultures are very thick and as a result the aeration is not as effective as at the lower densities. The thickness of the culture might also result in the substrate not being distributed uniformly through the medium, thus reducing the surface area of the cell in contact with the substrate. The consequence of these conditions might result in the slower rate of inversion observed but also might explain the greater extent of inversion observed. The more rapid inversion at the lower cell density might be because of the greater surface area of the cell available for substrate partition into the cells, whereas the lesser extent of inversion might be because of the lower amount of cells, and thus inverting enzymes, present. The difference in available cell surface area and difficulties in adequate agitation and equilibration might also account for the lesser rate and extent of inversion and greater variability seen at the higher cell densities.

Varying the substrate concentration in the incubations resulted in only relatively small changes in the extent of inversion in comparison with the results for the effects of varying the cell density. Although chiral inversion was found to occur at all the substrate concentrations investigated, it was found to proceed at a slower rate, in terms of enantiomeric ratio, at the highest substrate concentration. Similar results were also obtained when the data were subjected to statistical analysis, which suggested that significant inhibition occurred at this concentration. This might be because of inhibition of the enzyme system by substrate or product, or both, or might be because high concentration of 2-phenylpropionic acid is toxic to *V. lecanii*. High concentrations of ibuprofen have been shown to be toxic to growing cultures of *V. lecanii* so this might well be so here.

These studies demonstrate an advantage of using nutrient-depleted cultures for the study of the microbial chiral inversion of 2-phenylpropionic acid. The optimization of these culture conditions will facilitate the ultimate development of an in-vitro microbial screen for the preliminary assessment of the potential of novel profens to undergo microbial inversion in-vivo.

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