Appl Microbiol Biotechnol (1987) 26:305—312

Applied and Microbiology Biotechnology

Biotransformation of phenylpyruvic acid to L-phenylalanine using a strain of *Pseudomonas fluorescens* ATCC 11250 with high transaminase activity

Christopher Thomas Evans*, Wendy Peterson, Christin Choma, and Masanaru Misawa

Allelix Inc., 6850 Goreway Drive, Mississauga, Ontario, Canada L4V 1P1

Abstract. The rate of L-phenylalanine production from phenylpyruvic acid by whole cells of *Pseudomonas fluorescens* strain ATCC 11250 was greater than 3 $g \cdot l^{-1}$ h^{-1}. Synthesis of transaminase was constitutive but activity was greatest in medium containing D- or L-phenylalanine as sole nitrogen source. Maximum conversion was observed at 34—40° C and at alkaline pH, with over six times initial rate of conversion at pH 12 than at pH 5. The optimum catalyst (cell) concentration was between 10—20 mg ml^{-1} dry weight. The initial rate of conversion was directly proportional to phenylpyruvate concentration, up to 4%, but the conversion yield steadily decreased between 2% and 4% substrate concentration. The rate of conversion, as expected, increased as the concentration of glutamate increased. Whole cells were still capable of over 63% conversion after 40 days providing reactions were supplemented with pyridoxal phosphate. Immobilisation of cells in calcium alginate and operation of a packed bed bioreactor enabled the continuous production of L-phenylalanine in concentrations greater than 15 $g \cdot l^{-1}$ after 60 days operation.

Introduction

During the last six years, interest in the synthesis of the aromatic amino acid, L-phenylalanine, has risen quite dramatically, due to the increasing importance of the dipeptide sweetener Aspartame (Klausner 1985).

Substrates such as benzaldehyde, glycine and acetic anhydride have been traditionally used to produce L-phenylalanine via chemical routes, and direct fermentation methods, using Coryneform bacteria and *Escherichia coli*, have also been well documented (Tsuchida et al. 1975; Choi and Tribe 1982). Although conventional fermentative strain improvement has been an ongoing process (Hagino and Nakayama 1974; Park et al. 1984; Hwang et al. 1985), recent emphasis has been on the use of bioconversion technology to satisfy the demands for high yields of an optically pure product. One of the better known biotransformations is the conversion of trans-cinnamic acid using phenylalanine ammonia-lyase from *Rhodotorula* yeasts (Yamada et al. 1981; Hamilton et al. 1985; Evans et al. 1986a, b). Bioconversion processes have also been described using acetamidocinnamic acid as substrate (Nakamichi et al. 1984) and various microbial acylases and a novel phenylalanine dehydrogenase (Kitahara and Asai 1983; Ziehr et al. 1984). However, the oldest bioconversion method for production of L-phenylalanine is certainly that utilising the aromatic aminotransferases to aminate phenylpyruvic acid (Sakurai 1956; Asai et al. 1960).

The conversion of phenylpyruvic acid to L-phenylalanine has been investigated by many workers (Kitai et al. 1962; Hummel et al. 1984; Bulot and Cooney 1985) and the aminotransferase system has consequently become the subject of a series of competitive patent applications (Fusee 1984; Rozzell 1985; Primrose 1984; Lawlis et al. 1985; Wood and Calton 1985). However, from a survey of the journal and patent literature to date, there have been very few reports of phenylpyruvate transformations to L-phenylalanine in titres greater than 12 $g \cdot l^{-1}$ and little information regarding the catalytic properties of aminotransfer-

* *Present address:* Genzyme (UK) Ltd., Springfield Mill, Maidstone, Kent ME14 2LE, England

Offprint requests to: C. T. Evans (at his present address)

ase-containing cells, maximum titres, conversion rates and catalyst stability over extended periods of continuous reaction.

Subsequent to the preparation of this manuscript, some of the latter points were addressed in a publication describing the phenylpyruvate conversion process using whole cells of *E. coli* (Calton et al. 1986). In this paper, however, we describe some novel results from the characterisation of a *Pseudomonas*-catalysed phenylpyruvate transformation and demonstrate its suitability for commercial production of L-phenylalanine.

Materials and methods

Strains. All identified microorganisms were obtained from the ATCC Culture Collection, Rockville, USA. Strains 5BH-5, SP6-4PP, IB10, IB4, SP19 and *S. cerevisiae AH22* were all natural wild type strains isolated in this laboratory.

Media. Pseudomonas B_1 medium contained ($g \cdot l^{-1}$): glucose 15, NH_4Cl 3, KH_2PO_4 6, Na_2HPO_4 2, $MgSO_4$ 0.5, yeast extract 5, L-phenylalanine 2, $CaCl_2 \cdot 2H_2O$ 0.005, $FeCl_3 \cdot 6H_2O$ 0.008, $ZnSO_4 \cdot 7H_2O$ 0.0001, pH 7.0. Basal medium contained KH_2PO_4, $MgSO_4$, $CaCl_2$, $FeCl_3$ and $ZnSO_4$ at the concentrations shown above. L-Phenylalanine (C-source) enrichment medium contained ($g \cdot l^{-1}$): basal medium, yeast extract 0.2, NH_4Cl 3.0 and L-phenylalanine 15. L-Phenylalanine (N-source) enrichment medium contained ($g \cdot l^{-1}$): basal medium, yeast extract 0.2, glucose 15 and L-phenylalanine 2.5. Phenylpyruvate enrichment medium contained ($g \cdot l^{-1}$): basal medium, yeast extract 0.5, L-glutamate 1.2 and phenylpyruvate 7.5. All media were adjusted to pH 7.0 with HCl and sterilised by filtration.

Isolation of microorganisms. Strains were isolated from soil, sewage, mud, leaves, trees and various contaminated soils from industrial effluents using the soil percolator techniques described previously (Evans et al. 1986a).

Culture conditions. Strains were cultivated in shake flasks at 30° C and usually harvested after 24–28 h growth (refer to legends for specific details). Cells for alginate slurry and immobilisation studies were cultivated in 10 l. *Pseudomonas* B_1 medium in fully instrumented 14 l fermenters. (LH Engineering 2000 series) with dissolved oxygen maintained above 40% saturation, pH 7.0±0.1 and temperature 30° C±0.1° C. Cells were harvested after 18 h growth using ultrafiltration and the washed cells stored on ice at 4° C until use.

Phenylpyruvate-transaminase activity. Assays contained, in a final volume of 5 ml; 30 mg ml^{-1} cell dry weight, 0.2 M Tris-HCl pH 7.5, 12 mg ml^{-1} phenylpyruvic acid, 30 mg ml^{-1} aspartic acid, 0.05% cetylpyridinium chloride, and 0.1 mM pyridoxal phosphate. Reactions were incubated at 30° C for 18 h with shaking at 162 rpm and the production of L-phenylalanine measured. The same assay was used with cell-free extracts, prepared from sonicated cells.

Preparation of alginate-immobilised cells. A fresh cell suspension was prepared in 0.2 M Tris-HCl pH 7.0 and 20% glycerol containing 5% (w/v) *Pseudomonas fluorescens* strain ATCC 11250 cells. One volume of suspension (600 ml) was mixed with an equal volume of 4% (w/v) sodium alginate and the mixture shaken vigorously at 25° C for 10 min to evenly disperse the cells. The mixture was extruded through a hypodermic needle (0.5 mm) into 3 l of 0.2 M $CaCl_2$ at 25° C. The resulting beads varied in diameter from 1.5 to 2.0 mm and were allowed to stabilise at 25° C for 1 h. Beads were collected, washed in H_2O and stored at 4° C in 100 mM $CaCl_2$ until use. Beads were hardened by incubating at 25° C in 2 l 0.1% (v/v) glutaraldehyde pH 7.5 for 15 min. The hardened beads were washed free of excess glutaraldehyde and stored at 4° C in 100 mM $CaCl_2$.

Operation of packed bed immobilised cell reactor. Hardened beads were packed into a glass column (20×3.5 cm) with 110 ml working volume and retained by a scintered glass disc filter. The column was operated as a packed bed at 37° C and substrate feedstock consisting of 2% phenylpyruvate, 3.8% glutamate or 3.5% aspartate in 0.2 M borate pH 11.2, 20 mM $CaCl_2$ and 0.1 mM pyridoxal phosphate was pumped through the column at a rate of 0.25 ml h^{-1}.

Phenylpyruvate transformation. Details of reactor volume and concentration of cells and reaction constituents are provided in the legends.

Analysis. Phenylpyruvate was estimated as its enol-borate complex at A_{300} (Fujioka et al. 1970). L-Phenylalanine was estimated by paper chromatography and cell dry weight measured according to Evans et al. (1986a). Protein was estimated according to Bradford (1976). All chemicals and reagents were analytical grade.

Results

Isolation of phenylpyruvate-transforming microorganisms

Three types of enrichment were employed in order to isolate as wide a variety of microorganisms as possible. Selection was made on the basis of the largest colonies growing on the various agar media after 4 days at 30° C. As expected, large numbers of isolates were obtained on media containing L-phenylalanine as sole carbon or nitrogen source and most were capable of producing some L-phenylalanine under the transformation-screening conditions employed.

However, in the latter reaction, only a few isolates were capable of generating more than 7.5 $g \cdot l^{-1}$ L-phenylalanine which was used as the criteria for selecting strains with good conversion potential. Interestingly, the greatest frequency of phenylpyruvate transformants was isolated from soil percolators enriched with phenylpyruvate/glutamate medium. No strains were isolated which could grow on phenylpyruvate as sole carbon source in minimal medium, but the biomass levels observed in enrichment medium containing

glutamate, as well as phenylpyruvate, indicated that the latter carbon skeleton was certainly being used for growth (results not shown).

Comparison of phenylpyruvate conversion to L-phenylalanine by various microorganisms

Table 1 shows the variation in phenylpyruvate transformation by a wide range of microorganisms. Six of the natural isolates, which were included by way of comparison, were found to be superior producers of L-phenylalanine compared to the majority of culture strains. The four species of *Pseudomonas* were clearly the best phenylpyruvate transformants and isolates SP6-4PP, IB10, IB4 and IB8 were all identified as belonging to the genus *Pseudomonas*, using Bergey's Manual for Determinative Bacteriology (7th edition). Isolate IB10 was shown to be a strain of *P. fluorescens* and isolate SP19 as belonging to the genus *Alcaligenes*.

Table 1. Comparison of phenylpyruvate conversion to L-phenylalanine by various microorganisms

Microorganism		L-Phenylalanine (mg ml^{-1})
Saccharomyces cerevisiae AH22		5.2
Candida utilis 42402		4.8
Rhodosporidium toruloides 26194		1.7
Rhodotorula glutinis 20147		4.2
Candida boidinii 18810		3.6
Hansenula polymorpha 26012		2.7
Agrobacterium radiobacter 6467		5.4
Alcaligenes faecalis 25094		0.9
Alcaligenes eutrophus 17697		2.9
Brevibacterium lactofermentum 13819		3.5
Brevibacterium flavum 21889		1.6
Brevibacterium ammonigenes 6872		0.2
Citrobacter freundii 10787		2.6
Corynebacterium glutamicum 21675		5.4
Escherichia coli 23785		6.9
Pseudomonas putida 15175		8.5
Pseudomonas olevorans 8062		10.3
Pseudomonas denitrificans 13867		8.0
Pseudomonas fluorescens 11250		10.6
5BH-5	Natural soil isolates	5.9
SP6-4PP		12.6
IB10		13.9
IB4		10.8
IB8		6.8
SP19		14.7

All strains were grown in *Pseudomonas* B1 medium for 24 h. Aliquots (5 ml) of cell suspension (65 mg c.d. wt. per ml) were mixed with 5 ml of reaction mixture containing 30 mg ml^{-1} phenylpyruvate, 50 mg ml^{-1} aspartate, cetylpyridinium chloride 0.05%, in 0.2 M Tris-HCl pH 7.8. Reactions were run for 18 h at 30° C

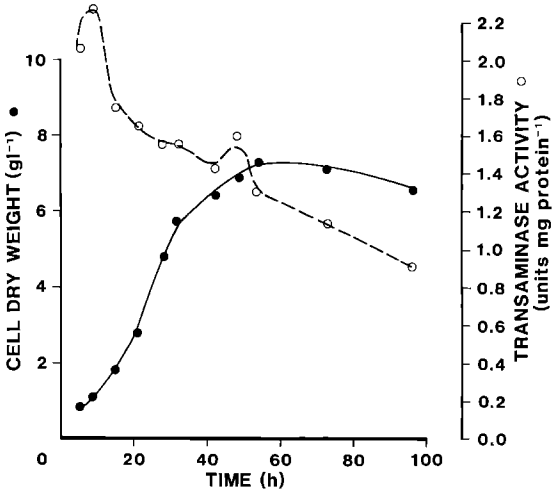

Fig. 1. Production of phenylpyruvate-transaminase during batch culture of *P. fluorescens* 11250. Aliquots (20 ml) of exponential culture were removed and used to prepare cell-free extracts as described in methods. Transaminase activity is expressed as μmoles L-phenylalanine formed per mg protein per hour. ○ Transaminase activity; ●, cell dry weight

In order to compare the true bioconversion potential of some of the most promising strains, cells were reacted with 1.5% w/v phenylpyruvate and 2.8% aspartate and the transformation monitored for 24 h. Both the natural isolates, SP19 and B10, gave excellent conversion yields of 98% and 87%, respectively, and with rates of conversion (volumetric productivity) which were approximately 1 mg ml^{-1} h^{-1}. However, the strain, *P. fluorescens* 11250, resulted in a remarkable initial rate of conversion which, between 1 and 4 h reaction time, exceeded 3 mg ml^{-1} h^{-1}. The final conversion yield was approx. 70% but the reaction appeared to be complete within 7 h.

Thus, although isolates B10 and SP19 were clearly useful strains for achieving high titres of L-phenylalanine in the first instance, it was decided that strain ATCC No. 11250 warranted further investigation because of its unusually high conversion rate even in the unoptimised state. The rest of the data reported in this paper refer exclusively to *P. fluorescens* strain ATCC 11250.

Growth and aminotransferase activity during batch culture

As shown in Fig. 1, activity of the aminotransferase, which catalyses the phenylpyruvate conversion to L-phenylalanine, was highest in very early exponential phase and probably reflects the necessary constitutive synthesis of such a primary en-

Table 2. Effect of growth medium formulation on production of phenylpyruvate transaminase from *P. fluorescens* 11250

Carbon	Nitrogen	Cell dry weight g l^{-1}	Relative activity %
Glucose	NH_4Cl	2.8	100
Glycerol	NH_4Cl	3.0	105
Fructose	NH_4Cl	2.5	112
Sucrose	NH_4Cl	2.5	91
Peptone	NH_4Cl	2.9	72
Yeast Extract	NH_4Cl	3.1	59
Glucose	L-Aspartate	2.9	121
Glucose	L-Glutamate	3.0	114
Glucose	L-Phenylalanine	2.4	134
Glucose	D-Phenylalanine	2.0	150
Glucose	L-Tyrosine	2.5	129
Glucose	L-Tryptophan	2.5	94
L-Phenylalanine	—	3.2	147
L-Alanine	—	2.9	82
L-Aspartate	—	3.5	100
L-Glutamate	—	3.7	81
L-Histidine	—	2.4	74
L-Leucine	—	3.1	75
L-Asparagine	—	3.9	100
Glycerol	L-Phenylalanine	3.5	138
Glycerol	L-Asparagine	3.9	112
Glycerol	D-Phenylalanine	1.8	163

Media contained basal medium with 1% carbon sources, except where amino acids were used and these were 1.5%, nitrogen sources were all 0.4%. Samples were removed after 24 h growth. The activity of phenylpyruvate transaminase in *Pseudomonas* medium B_1 was 1.8 μmoles phenylalanine formed per mg·protein per hour (100% activity).

zyme essential to central metabolism in the cells. Although activity diminished throughout the rest of growth, cells in midexponential phase growing for 24 h still possessed over 75% of the maximum activity originally observed. To facilitate cell generation for subsequent experiments, flasks were normally harvested after 24 h growth where the activity was varied between 1.65—1.98 μmoles per mg protein per hour. The constitutive synthesis of the phenylalanine-aminotransferase was confirmed by growing of *P. fluorescens* 11250 in 22 different media, see Table 2. The activity was lowest, at 72% of the control activity, in peptone/NH_4Cl medium and highest, at 63% greater than the control, in glycerol/D-phenylalanine medium. The activity was generally higher in cells grown in all media containing D- or L-phenylalanine, irrespective of carbon or nitrogen source.

Table 3. Influence of reaction components on phenylpyruvate conversion by whole cells of *P. fluorescens* 11250

Reaction mixture	L-Phenylalanine (mg ml^{-1})
Complete at 30° C	17.5
− Pyridoxal phosphate	18.5
− Cetylpyridinium chloride	17.5
− Aspartate	0.8
+ Pyridoxal phosphate (10 mM)	19.2
+ Cetylpyridinium chloride (0.5%)	21.6
Aspartate (6.5%)	22.3
Glutamate (6.5)	18.9
Borate (0.2 M)	19.0
Cells (8.9 mg ml^{-1})	17.5
Cells (89.0 mg ml^{-1})	10.2
Complete at 37° C	21.4

Complete reactions were carried out for 24 h at 30° C in a 1.0 ml volume containing 8.9 mg ml^{-1} cell dry weight, 22.0 mg ml^{-1} phenylpyruvic acid, 40 mg ml^{-1} aspartic acid, 0.2 M glycine buffer pH 10

Optimisation of reaction components for phenylpyruvate bioconversion

Table 3 shows that neither cell permeabilising agent (cetylpyridinium chloride) nor cofactor (pyridoxal phosphate) were essential for L-phenylalanine production. The elimination of the amino donor, aspartate, resulted in a 96% reduction in product formation; the low concentration of L-phenylalanine detected probably originated from mobilisation of endogenous amino acid pools to facilitate the reaction.

Similarly, the use of excess cetylpyridinium chloride resulted in an extra 4 mg ml^{-1} L-phenylalanine produced, indicating that end product feedback inhibition of the cell permease could be occuring above 17 mg ml^{-1} of the product. Maximum conversion yield (100%) occured when the amino donor concentration was greatly elevated, illustrating the very high titres possible when the *Pseudomonas*-catalysed process. The fact that a low cell concentration facilitated a better conversion further illustrated the potential of this process.

Effect of transformation pH and temperature

The initial rate of phenylpyruvic conversion, measured over 2.5 h, was only slightly greater at alkaline pH 7.8 than acidic pH 5, whilst at pH 10 the rate of L-phenylalanine production increased dramatically to approx. 2.8 mg ml^{-1} h^{-1} (Fig. 2). Increasing the reaction pH still further to 11 and 12 resulted in very rapid initial conversions (6 mg ml^{-1} in 30 min) which decreased to a rate of approx 3 mg ml^{-1} h^{-1}.

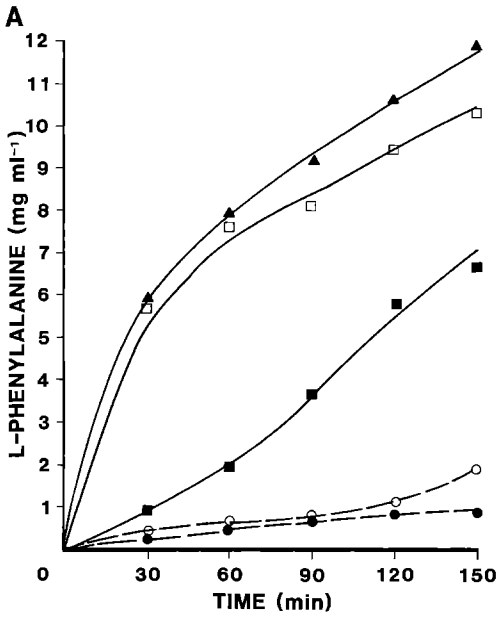

Fig. 2. Effect of pH on L-phenylalanine production from phenylpyruvic acid using *Pseudomonas fluorescens* 11250. Reaction mixtures contained 5 ml cells (42 mg ml^{-1} cell dry weight) and 5 ml of a solution containing 3.6% phenylpyruvic acid, 5% aspartic acid and 0.005% cetylpyridinium chloride in either phosphate or borate buffer. ● pH 5; ○ pH 7.8; ■ pH 10; □ pH 11; △ pH 12

The conversion yield after 2.5 h was shown to increase with temperature and at 37° C was almost double that observed at 30° C (results not shown).

Effect of catalyst concentration

Unlike that of most biotransformations, the optimum catalyst concentration using *Pseudomonas* cells was relatively low, see Fig. 3. Reactions containing 1% (w/v) dry cells resulted in over a 97% conversion yield whilst higher cell concentrations were not only wasted, but also proved detrimental to accumulation of high product titres.

Effect of phenylpyruvate and glutamate concentration

The initial transformation rate was shown to be directly proportional to the phenylpyruvate concentration up to about 4% (w/v) substrate.

However, the final conversion yield decreased steadily between 2% and 4% substrate concentrations. The observation of decreasing final conversion yield was attributed to end product inhibition by the L-phenylalanine produced. Thus, although faster conversion rates were observed with addition of high substrate concentrations, the product titres never exceeded 16 mg ml^{-1} (data not shown). As expected, the rate and yield of the conversion were also directly proportional to the concentration of amino donor employed. Using a ratio of 1:3, substrate to amino donor, almost a 90% conversion was achieved in 3 h.

Stability of P. fluorescens 11250 cells for phenylpyruvate conversion

It was found that cells incubated in water and a variety of buffers at 200 mM pH 9.8 were unable to catalyse any phenylpyruvate conversion after 12 days at 37° C (results not shown). It was observed (from thin layer chromatography and HPLC) that increasing amounts of amino acids, and other ninhydrin-positive materials, were produced from the cells during prolonged incubation, probably due to cell lysis. Incubation of cells in 0.8% alginate suspension (no calcium added) prevented any obvious release of ninhydrin-positive material and cells were found to be still capable of producing L-phenylalanine after 40 days at 37° C, see Fig. 4. Cells assayed in the absence of pyridoxal phosphate after 5 days incubation appeared as active as cells assayed with the cofactor indicating that the endogenous cofactor was prob-

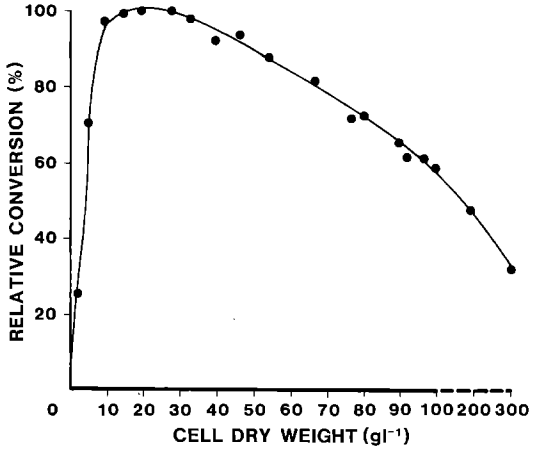

Fig. 3. Effect of cell concentration on phenylpyruvate conversion to L-phenylalanine. Incubations contained, in 12 ml, an aliquot of fresh cell suspension and a final concentration of phenylpyruvate 20 $g \cdot l^{-1}$ aspartate 38 $g \cdot l^{-1}$ and cetylpyridinium chloride 0.05% in 0.1 M borate buffer pH 8.5. The results are expressed as relative conversion yields, where the maximum conversion (100%) produced was 18.5 $g \cdot l^{-1}$ L-phenylalanine after 24 h

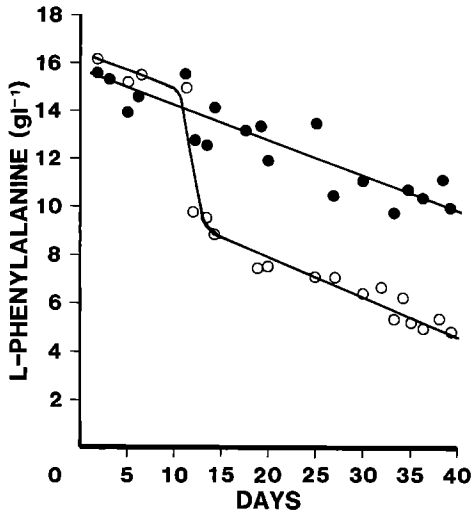

Fig. 4. Activity of phenylpyruvate transaminase in whole cells of *P. fluorescens* 11250 incubated in alginate suspension. Fresh cells were mixed with 0.8% alginate to give a final concentration of cells at 17.5 $g \cdot l^{-1}$ dry weight, 0.5% alginate and 0.5 M borate buffer pH 9.8. The above cell slurry (200 ml) was incubated at 37° C with shaking and samples (1 ml) removed periodically for determination of catalyst activity using 2% phenylpyruvate, 4% glutamate in 0.1 M borate pH 11.2 with and without 50 mM pyridoxal phosphate. ● with pyridoxal phosphate; ○ without pyridoxal phosphate

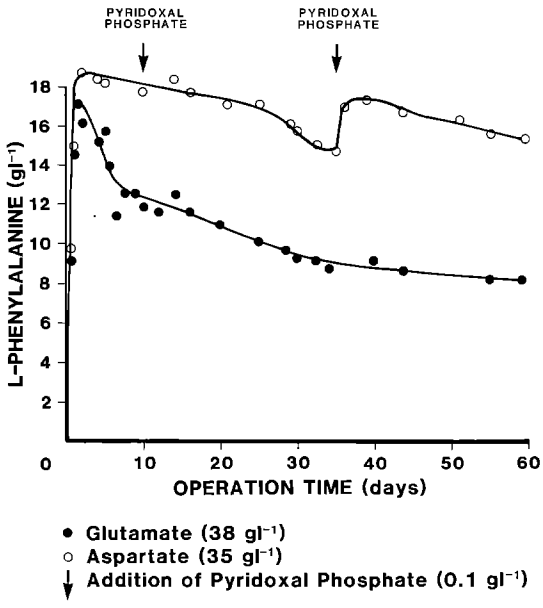

Fig. 5. Continuous production of L-phenylalanine by *P. fluorescens* 11250 immobilized in alginate beads. A substrate feedstock consisting of 2% phenylpyruvate, 3.8% glutamate or 3.5% aspartate in 0.2 M borate pH 11.2 and 0.1 mM pyridoxal phosphate was passed through the column of beads under the conditions of operation described in the methods. Fresh pyridoxal phosphate was added to the reactor containing 3.5% aspartic acid at the intervals indicated

ably tightly associated with the enzyme. However, after 40 days incubation the exclusion of cofactor from reactions resulted in a 50% reduction in L-phenylalanine production, presumably because of dissociation of the cofactor from the enzyme and its ultimate loss from the cells altogether.

Production of L-phenylalanine by immobilised cells of P. fluorescens strain ATCC 11250

Cells were entrapped in calcium alginate beads and reacted with substrate mixture which was slowly pumped through the packed bed.

The cells were initially allowed to react until L-phenylalanine had accumulated to a concentration greater than 17 $g \cdot l^{-1}$, then the flow rate was adjusted to try and maintain this titre in the effluent stream. The flow rate was subsequently adjusted several times during the 60 days operation. It can be seen from Fig. 5 that aspartate was a superior amino donor to glutamate. After 10 days of continuous operation, the aspartate-containing column was still accumulating L-phenylalanine above 17 $g \cdot l^{-1}$ whilst the glutamate column had increased significantly to below 12 $g \cdot l^{-1}$.

In this particular experiment, the aspartate column was supplemented with extra pyridoxal phosphate at intervals and this appeared to have pronounced effect after 35 days. Although data is not shown here, the aspartate-containing column was successfully operated, with product titres above 7.5 $g \cdot l^{-1}$, for a further 30 days. Although not determined in this experiment, it is highly likely that the addition of extra pyridoxal phosphate to the glutamate-containing column would have had a similar stimulatory effect as shown with the aspartate column.

Discussion

Although there have been numerous reports of aminotransferase-containing strains capable of transforming phenylpyruvic acid to L-phenylalanine, very few have presented data which could be considered suitable for eventual scale up to a realistic commercial process. In these authors' opinion, to establish economic and competitive technology for L-phenylalanine production, the bioconversion process must possess a highly efficient conversion yield, selectivity, product titre, product recovery and concentration and stability of the catalyst used. The relatively high cost of most

substrates for transformation to L-phenylalanine (i.e. ⩾$5.00/kilo) demands that bioreactors are operated at a substrate concentration above 2% (w/v), with a minimum of 85% (w/w) conversion yield, 99% selectivity and a catalyst lifetime of 3 months.

Few, if any, reports of microbial aromatic amino acid transamination reactions contain data which meet the latter set of process criteria and the majority of publications and patents filed are concerned with the biochemical and genetic manipulation of the aminotransferase from *E. coli* (Primrose 1984; Rozzell 1985; Wood and Calton 1985).

Most of the strains, including those from the ATCC culture collection, gave results similar to those reported by Asai et al. (1960) and Calton et al. (1986). *P. fluorescens* strain ATCC 11250 was chosen for all subsequent work on the basis of its remarkable initial rate of conversion. Although synthesis of the aromatic aminotransferase system was shown to be constitutive, higher rates of conversion were observed with cells grown in the presence of D- or L-phenylalanine. It is possible that two (or more) aromatic-amino acid aminotransferase exist *P. fluorescens* 11250, one of which is inducible, as reported in the case of *Brevibacterium linens 47* (Lee and Desmazeaud 1985).

The fact that whole cells of *P. fluorescens* 11250 were capable of achieving almost a 95% (w/w) conversion of a 2% (w/v) phenylpyruvate solution was also quite remarkable, considering that transamination reactions usually have an equilibrium constant of about 1.0, hence achieving little more than a 50% conversion (see Rozzell 1985). It is possible that the aromatic aminotransferase in strain 11250 is unusual with a very favourable equilibrium for L-phenylalanine synthesis, or that a very active oxaloacetate decarboxylase prevents accumulation of any keto by-product, so pulling the reaction to completion (Rozzell 1985). The effect of pH on the transformation appeared to confirm that strain 11250 did possess an unusual aminotransferase. The extremely rapid conversion rate at high alkaline pH illustrated the potential for biochemically manipulating this strain. The solubility of L-phenylalanine is greatly increased at such high pH's and enzymes catalysing the degradation of phenylalanine and the formation of by-products may be significantly inhibited at such high pH's. It is possible that the higher pH also increases activity of the oxaloacetate decarboxylase present or even accelerates the physical breakdown of oxaloacetate to pyruvate.

Furthermore, at the higher reaction pH the phenylpyruvate substrate will rapidly equilibrate between the keto and enol forms so facilitating the future use of very high concentrations of substrate (6 to 8%) with little inhibition of the reaction rate by the keto acid itself.

The observations that the conversion yield decreased as the substrate concentration was increased indicated that end product inhibition was probably occurring to some degree; this is illustrated further by the fact that the initial rate of conversion was seen to increase with substrate concentration. It was even more encouraging that only relatively low cell concentrations were required to achieve the high conversion yields observed, as this further improves the economics of such a process. The cells were shown to maintain their integrity and aminotransferase stability when immobilised and continuously reacted for prolonged periods at 37° C.

It is likely that a larger scale bioreactor would be operated at 30° C and, by extrapolation, its lifetime could be expected to greatly exceed the 60 days operation presented here. The titres of L-phenylalanine achieved using strain ATCC 11250 are well within the range to facilitate easy recovery and crystallisation of the product by conventional techniques. The L-phenylalanine product was shown to be exclusively the L-isomer, using Chiralpak WH column HPLC chromatography supplied by Daicel Chemicals Ltd., NY, USA. Selectivity was consistently ⩾99%. Mutants of strain ATCC 11250 isolated recently were capable of producing more than 33 $g \cdot l^{-1}$ L-phenylalanine. Costings based on these data (plus a source of phenylpyruvate less than $4.00/kilo) demonstrate the feasibility of producing L-phenylalanine for less than $18.00/kilo. Scale up of the *P. fluorescens* strain ATCC 11250 phenylpyruvate process will be reported at a later date.

Acknowledgements. We would like to thank D. Conrad and S. Theman-Slavec for their excellent technical assistance.

References

Asai T, Aida K, Oisha K (1960) Preparation of L-phenylalanine by bacterial transaminase. Hakko To Taisha 2:114—119

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Analyt Biochem 72:248—254

Bulot E, Cooney CL (1985) Selective production of phenylalanine from phenylpyruvic acid using growing cells of *Corynebacterium glutamicum*. Biotechnol Lett 7:93—98

Calton GJ, Wood L, Updike MH, Lantz L, Hamman JP (1986) The production of L-phenylalanine by polyazetidine immobilised microbes. Bio/Technol 4:317—320

Choi JJ, Tribe DE (1982) Continuous production of phenylalanine using an *Escherichia coli* regulatory mutant. Biotechnol Lett 4:223—228

Evans CT, Hanna K, Conrad D, Peterson W, Misawa M (1987a) Production of phenylalanine ammonia-lyase (PAL): isolation and evaluation of yeast strains suitable for commercial production of L-phenylalanine. Appl Microbiol Biotechnol 25:406—414

Evans CT, Hanna K, Payne C, Conrad D, Misawa M (1986b) Biotransformation of trans-cinnamic acid to L-phenylalanine: Optimisation of reaction conditions using whole yeast cells. Enzyme Microbial Technol (in print)

Fujioka M, Morino Y, Wada H (1970) Metabolism of phenylalanine. In: Methods In Enzymology, Vol. XVIIA. Tabor H, Tabor CW (eds) Academic Press, New York and London, pp 585—589

Fusee MC (1984) Production of L-amino acids from α-keto acids by fermentation. W. R. Grace & Co. UK Patent Application No. 8419142

Hagino H, Nakayama K (1974) L-phenylalanine production by analog-resistant mutants of *Corynebacterium glutamicum*. Agr Biol Chem 38:157—161

Hamilton BK, Hsiao H-Y, Swann W, Anderson D, Delente J (1985) Manufacture of L-amino acids with bioreactors. Trends in Biotechnology 3:64—68

Hummel W, Weiss N, Kula M-R (1984) Isolation and characterisation of a bacterium possessing L-phenylalanine dehydrogenase activity. Arch Microbiol 137:47—52

Hwang SO, Gil GH, Cho YJ, Kang KR, Lee JH, Bae JC (1985) The fermentation process for L-phenylalanine production using an auxotrophic regulatory mutant of *E. coli*. J Appl Microbiol Biotechnol 22:108—113

Kitahara T, Asai S (1983) Optical Resolution of D,L-phenylalanine with Acylase. Agric Biol Chem 47:991—996

Kitai A, Kitamura I, Miyachi N (1962) The formation of L-amino acids through the conjugated reaction system. I. Formation of L-alanine and L-phenylalanine by the alcohol dehydrogenase conjugated system. Hakko To Taisha 5:61—65

Klausner A (1985) Building for success in phenylalanine. Bio/Technology 3:301—307

Lawlis B, Rastetter W, Snedecor R (1985) Recombinant process for preparing L-amino acids, recombinant expression reactors and transformed microorganisms for use in the process. Genentech Inc., Eur Pat Applc No 84305532.8

Lee W-C, Desmazeaud MJ (1985) Partial purification and some properties of an aromatic-amino-acid and an aspartage amino-transferase in *Brevibacterium linens* 47. J Gen Microbiol 131:459—467

Nakamichi K, Nabe K, Yamada S, Tosa T, Chibata I (1984) L-phenylalanine formation from acetamidocinnamic acid by newly isolated bacteria. Appl Microbiol Biotechnol 19:100—105

Park SH, Hong KT, Yon SJ, Lee HJ, Bae HC (1984) L-phenylalanine production by auxotrophic regulatory mutants of *E. coli*. Korean J Chem Eng 1:65—69

Primrose SB (1984) The cloning the utilisation of aminotransferase genes. G. D. Searle & Co. Eur Pat Appl No 84100521.8

Rozzell DJ (1985) Production of L-amino acids by transamination. Genetics Institute Inc. Eur Pat Appl No 84110407.8

Sakurai S (1956) Enzymatic preparation of optically active essential amino acids. I. The preparation of L-phenylalanine. J Biochem 43:851—855

Tsuchida T, Matsui J, Enei J, Yoshinaga F (1975) L-phenylalanine by fermentation. US Patent 3,909,353

Wood L, Calton GJ (1985) Process and compositions for preparing phenylalanine. Purification Engineering Inc., Eur Pat Appl No 84304966.9

Yamada S, Nabe K, Izuo N, Nakamichi K, Chibata I (1981) Production of L-phenylalanine from *trans*-cinnamic acid with *Rhodotorula glutinis* containing L-phenylalanine ammonia-lyase activity. Appl Environ Microbiol 42:773—778

Ziehr H, Hummel W, Reichenbach H, Kula M-R (1984) Two enzymatic routes for the production of L-phenylalanine. In Proceedings of The Third European Congress on Biotechnology, Verlag Chemie, Weinheim 1:345—350

Received October 13, 1986/Revised March 16, 1987