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Effect of glycerol, polyethylene glycol and glutaraldehyde on stability of phenylalanine ammonia-lyase activity in yeast

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SUMMARY

The polyhydric alcohols, glycerol and sorbitol, significantly increased the rate of L-phenylalanine production from trans-cinnamic acid using whole cells of *Rhodotorula rubra*. Chloride ions and oxygen prevented the stimulatory effect of the polyhydric alcohols. Furthermore, the severe inhibition of the biotransformation by high trans-cinnamic acid concentrations was alleviated in the presence of glycerol and sorbitol. The rate of conversion could be manipulated still further, even with high trans-cinnamic acid concentrations, by elevating the reaction pH to 12 in the presence of polyhydric alcohol. When cells were also treated first with glutaraldehyde (0.1% v/v) and then polyethylene glycol (15% v/v), although neither compound stimulated the actual rate of bioconversion, the reaction was markedly stabilised and gave a 73% yield after 28 days of continuous operation.

INTRODUCTION

Phenylalanine ammonia-lyase (PAL) from yeast has been identified as a major route to L-phenylalanine production [8,14]. The commercial importance of the latter amino acid has increased dramatically since the advent of the popular dipeptide sweetener, aspartame. Although chemical synthesis and direct fermentation have traditionally been used to produce L-phenylalanine [12], recent interest has been in the application of biotransformation methods to increase titres and recovery of the optically pure product [3]. Phenylalanine biotransformation processes have now been established using substrates such as trans-cinnamic acid, phenylpyruvic acid, acetamidocinnamic acid, benzyl-hydantoin and *N*-acetyl-DL-phenylalanine [1,2,9,11,16]. One of the better known bioconversion routes is the reverse PAL reaction using trans-cinnamic acid, ammonia and the PAL enzyme from *Rhodotorula,* a process which has now been successfully scaled up by Genex Corporation [7]. However, problems associated with the stability and reuseability of the PAL catalyst greatly impair the overall productivity of this process.

In this laboratory we have undertaken an extensive programme aimed at manipulating the yeast

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PAL process to establish a superior economic route to L-phenylalanine production [4–6].

In this paper we report some novel methods for increasing the productivity and stability of the PAL catalyst under continuous reaction conditions. Such stabilities have never been observed before with PAL from any source, including that from yeast.

MATERIALS AND METHODS

Strain and growth conditions

Isolate SPA10 is a strain of *Rhodotorula rubra* which was isolated in this laboratory from contaminated soil [5]. It has been deposited, under restricted access, in the ATCC Culture Collection, Rockville, MD, U.S.A. The yeast was cultivated in a medium containing ($g \cdot l^{-1}$): yeast extract 10, peptone 10, NaCl 5, L-isoleucine 0.5, and L-phenylalanine 5; 10-litre volumes were used in a 15 litre fermenter (LH 2000 series) with controlled oxygen supplies (above 40% saturation), constant pH at 5.5 and constant temperature at 30°C. The culture was monitored for synthesis of PAL, as described previously [5], and after approximately 18 h growth, at peak PAL activity, the culture was sparged with nitrogen gas and aeration was switched off. The temperature was decreased to 10°C and cells were harvested immediately.

Preparation of cell suspensions

Cells were harvested by centrifugation at 4°C for 10 min at 10000 × *g* and washed twice in 100 mM phosphate buffer, pH 7.0. The cells were resuspended in phosphate buffer and stored under anaerobic conditions at 4°C until use. Prior to reaction, cells were centrifuged at 4°C and resuspended in 200 mM borate buffer, pH 9.5–10.3, to a final concentration of 200 mg cell dry weight per ml.

Glutaraldehyde fixation

Cells were incubated to a final concentration of 15 mg cell dry weight per ml of glutaraldehyde solution (final concentrations 0.01–10% v/v). Mixtures (final volumes of 200 ml) were gently stirred and sparged with nitrogen for 1 h at 30°C. Cells were then washed for 1 h at 30°C, and then washed twice in 200 ml distilled water prior to reaction with PEG-containing substrate mixtures.

Biotransformation and analyses

Substrate solution and cell suspension were normally mixed to give between 30 and 100 $mg \cdot ml^{-1}$ cell dry weight (catalyst) in 4.3 M NH_4OH, adjusted to pH 10 with HCl or H_2SO_4 as appropriate, and trans-cinnamic acid between 10 and 40 $mg \cdot ml^{-1}$. Reaction mixtures were incubated in 100 ml conical flasks at 30°C with shaking at 132 rpm. L-Phenylalanine and trans-cinnamic acid were measured as described previously [5]. All reagents were of analytical grade (AR).

RESULTS

Effect of polyhydric alcohols on L-phenylalanine production

Polyhydric alcohols successfully stabilised PAL enzyme in this strain of *R. rubra* during several consecutive reactions with trans-cinnamic acid [4]. In the present work the effect of PAL-stabilising agents on the production of L-phenylalanine from trans-cinnamic acid was examined under O_2-sparging/chloride-containing and N_2-sparging/chloride-deficient reaction conditions. Under the former conditions, only slight stimulatory effects were observed and conversion yields of approximately 40–50% were obtained (Fig. 1A). When oxygen and chloride ions were omitted from reactions, a marked increase in conversion rate was observed. The addition of sulphate ions (as ammonium sulphate) to O_2/chloride-containing reactions did not stimulate the conversion rate. Both glycerol and sorbitol resulted in a 1.7-fold increase in L-phenylalanine production with conversion yields up to 88% (Fig. 1B).

Alleviation of PAL substrate inhibition by polyhydric alcohols

The reverse PAL reaction is inhibited by its substrate, trans-cinnamic acid [6,10,14]. The effect of

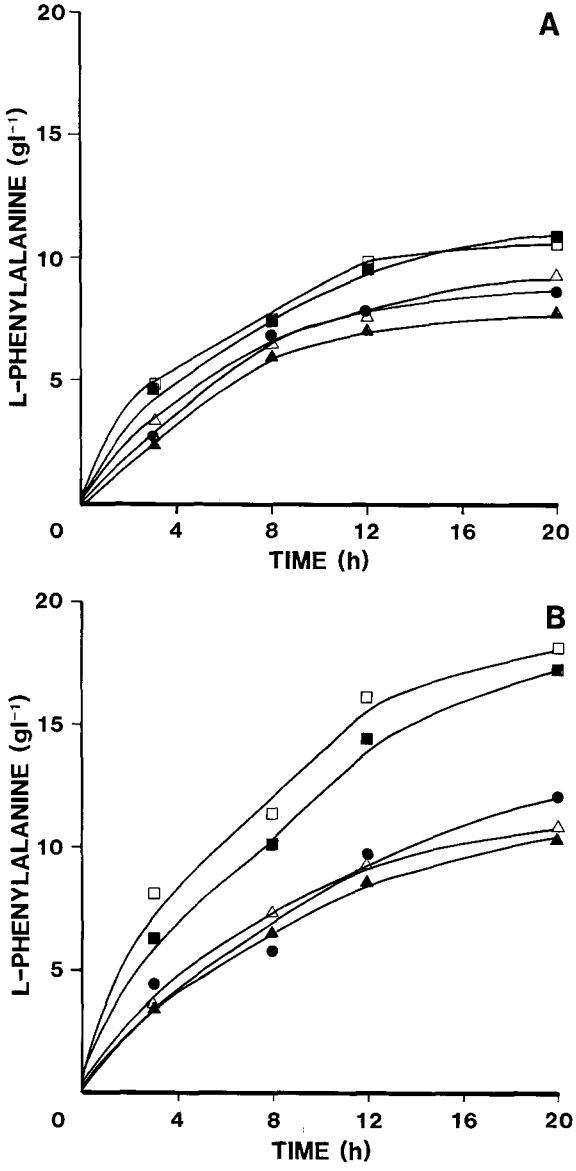

Fig. 1. Production of L-phenylalanine from trans-cinnamate in the presence of stabilising agents. A fully induced cell suspension of *R. rubra* SPA10 was reacted with the following components in a final volume of 20 ml: 39 mg · ml^{-1} cells, 20 mg · ml^{-1} trans-cinnamate, 4.3 M NH_4OH pH 10, and stabilisers. The reaction flasks were sparged with pure nitrogen and oxygen gas and sealed prior to incubation. Flasks were sparged and resealed after removal of samples. (A) HCl + oxygen. (B) H_2SO_4 + nitrogen. ●, control; ■, glycerol 2.0 M; □, sorbitol 2.0 M; ▲, glutaraldehyde 0.2%; △, polyethylene glycol 15%.

Table I

Effect of glycerol and sorbitol on substrate inhibition of PAL bioconversion

Reactions contained, in 10 ml, 34 g · l^{-1} cells, 4.3 M NH_4OH adjusted to pH 10.5 with conc. H_2SO_4, 2.5 M polyhydric alcohol and the concentrations of trans-cinnamic acid as shown. Reactions were run at 30°C for 8 h. Rates of L-phenylalanine production were expressed as percent of maximum uninhibited rates (0.85 g · l^{-1} · h^{-1} control; 1.25 g · l^{-1} · h^{-1} glycerol; 1.43 g · l^{-1} · h^{-1} sorbitol). ND = not determined.

Trans-cinnamic acid (g · l^{-1})	Rates of L-phenylalanine production		
	control	glycerol	sorbitol
5	100	100	100
10	100	ND	ND
20	59	100	100
30	41	ND	89
40	31	83	74
50	25	74	ND
70	0	52	41

adding polyhydric alcohol to reactions containing increasing amounts of substrate is shown in Table 1. In control reactons, over 50% inhibition was observed with 3% trans-cinnamic acid, while less than 10% inhibition was seen in the presence of sorbitol. Substrate concentrations could be increased to 7% before a 50% inhibition in the rate of conversion occurred.

Effect of pH and glycerol on substrate inhibition

In the course of this work it was discovered that the substrate inhibition effect was influenced by pH. At pH 10, the rate of phenylalanine production from 3% trans-cinnamate was approximately half that from 1% substrate, whilst at pH 12 the reverse was observed (Fig. 2).

When glycerol was added to reaction mixtures containing 3% substrate, the rate of phenylalanine production increased as the pH was elevated. At pH 12 the rate of conversion with 3% trans-cinnamate and glycerol was 4-times that observed with 1% substrate and no stabiliser addition (Fig. 2).

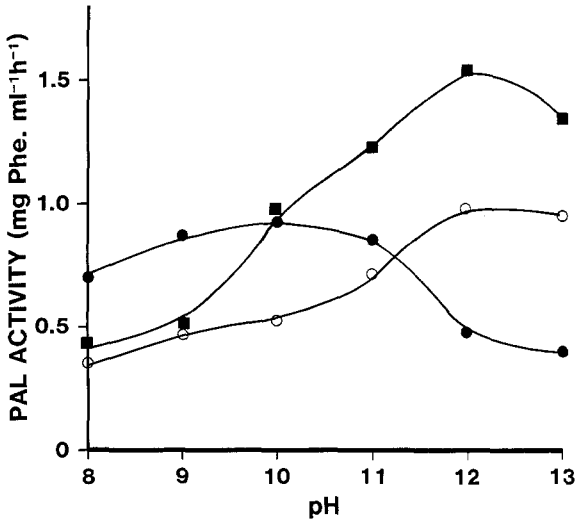

Fig. 2. Effect of pH on PAL activity in different trans-cinnamate concentrations. Reactions contained, in a final volume of 5 ml, 28 mg · ml^{-1} cell dry weight, 4.3 M NH_4OH adjusted to the different pH values with conc. H_2SO_4. Reactions were run in triplicate and stopped after 8 h. The average PAL activities (±0.12 units) are shown. ●, 1% trans-cinnamate; ○, 3% trans-cinnamate; ■, 3% trans-cinnamate + 2 M glycerol.

Reuseability of PAL catalyst by treatment with polyethylene glycol and glutaraldehyde

The PAL catalyst in the presence of sorbitol can be reused [4]. Table 2 shows the stabilisation of PAL achieved by optimising a mixture of glutaraldehyde and polyethylene glycol (PEG) treatments. Even in the presence of low PEG, fixation in 0.1% glutaraldehyde enabled the conversion to continue with a 73% yield of L-phenylalanine after 28 consecutive runs. The use of glutaraldehyde concentrations greater than 0.1% inhibited PAL activity.

DISCUSSION

Two of the most serious problems associated with L-phenylalanine synthesis from trans-cinnamic acid are substrate inhibition of the conversion itself, and instability of the PAL catalyst. Recent work in our laboratory has examined the nature and extent

Table 2

Stabilisation of PAL-catalysed bioconversion by polyethylene glycol and glutaraldehyde

Reactions contained in 30 ml: 58 g · l^{-1} cells, 4.3 M NH_4OH pH 9.8 (adjusted with H_2SO_4), 10 g · l^{-1} trans-cinnamic acid and the concentration of PEG shown below. Cells were treated firstly with glutaraldehyde for 1 h (at the concentrations shown) prior to addition of reaction mixture. All reactions were sparged with nitrogen and incubated at 30°C. Cells were separated from mixtures after every 24 h and added to fresh PEG-containing reaction mixture devoid of glutaraldehyde. The L-phenylalanine concentration produced per 24 h is shown. ND = not determined.

PEG (% w/v)	Glutaraldehyde	Phenylalanine (g · l^{-1}) produced in run:					
		1	2	3	14	19	28
0	0	8.7	7.9	0.9	0.1	0	0
0.1	0	8.6	7.5	1.9	0.7	0	ND
1	0	9.2	8.7	3.6	2.4	1.2	0.9
15	0	7.9	8.2	4.3	1.7	0.9	ND
0.1	0.01	8.4	7.6	1.9	1.0	0	ND
0.1	0.1	8.4	8.9	7.6	4.0	1.2	ND
0.1	1	0.9	0	0	0	0	ND
0.1	10	2.9	0.4	0	0	0	ND
1.0	[0.01	ND	7.1	4.3	1.9	1.0	1.0
1.0	0.1	8.2	6.9	6.1	4.2	2.2	1.7
1.0	1	ND	0	0	0	0	ND
1.0	10	8.6	0.6	0	0	0	ND
15	0.01	9.3	8.2	6.3	3.1	1.2	ND
15	0.1	8.9	9.4	8.6	7.3	7.5	7.3
15	1	0.8	0	0	0	0	ND
15	10	0	0	0	0	0	ND

of PAL inactivation during L-phenylalanine production. This work highlighted several features of the process, namely inhibition or catalyst instability due to presence of O_2, chloride ions, high trans-cinnamic acid concentrations, high NH_4OH concentration or alkaline pH, as well as stimulation and stability caused by L-glutamic acid, D-glycerol, D-sorbitol, L-isoleucine, L-phenylalanine or reducing conditions [4–6].

Although substrate inhibition of PAL has been well documented [6,14], there have been no reports of methods alleviating this inhibition. The benefit of polyhydric alcohols in PAL-catalysed conversions is 3-fold, in providing an increased rate of reaction, higher conversion yield at high trans-cinnamic acid concentrations and prolonging catalyst lifetime under continuous reaction conditions. By manipulating the reaction pH in the presence of polyhydric alcohols, the productivity of the PAL conversion can be increased still further. Furthermore, an optimised glutaraldehyde and PEG combination confers remarkable stability upon PAL reactions. In the absence of glutaraldehyde 1% PEG provided greater stabilization than 15% PEG. At 0.1%, glutaraldehyde enhanced the stabilization and was more effective in combination with 15% PEG than 7% PEG. However, the stabilization could be increased further by manipulating reduced PEG concentrations (e.g. 5, 7, 10, 12%) in combination with even lower glutaraldehyde concentrations (e.g. 0.05–0.1%). Such combinations are currently being evaluated.

The mechanism of stabilization was not investigated. It is well known that polyhydric alcohols and polyethylene glycol stabilize enzymes in liquid preparations [15], and that when polyethylene glycol is covalently attached to the yeast PAL enzyme, the enzyme has increased resistance to proteolytic degradation [13]. The use of glutaraldehyde and PEG in electron microscopy is also well known. The initial fixation with glutaraldehyde could result in the destruction of the PAL-degrading protease [4] while polyhydric alcohols and PEG preserve integrity of the PAL itself resulting in both enhanced catalysis and stabilization.

The results shown here could be equated to a continuous biotransformation operating for 28 days with residual catalyst and fresh feedstock. The observations clearly indicate the potential for conferring sufficient catalyst stability to run an immobilised PAL process. The yields of L-phenylalanine observed with *R. rubra* cells under reduced chloride-free conditions are comparable, if not superior, to those reported for other yeast systems. Combining the latter methods with those involving polyhydric alcohols and glutaraldehyde, it is conceivable that a viable commercial process for L-phenylalanine production can be established.

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