PROC. N.S. INST. SCI. (2006)
Volume 43, Part 2, pp. 219-225

2005 NSIS Undergraduate Student Prize Winning Paper in the Life Sciences

GROWTH AND PHENYLALANINE AMMONIA LYASE ACTIVITY OF *Rhodotorula glutinis*: OPTIMIZATION OF CONDITIONS FOR THE SYNTHESIS OF L-PHENYLALANINE

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A procedure is described for the synthesis of L-phenylalanine from trans-cinnamic acid and ammonia utilizing the reverse reaction of phenylalanine ammonia lyase (PAL) from *Rhodotorula glutinis*. Optimal conditions determined for yeast growth and PAL production were pH 6, 1% inoculum, 30°C for 26-28 h. Optimization of conditions for maximal phenylalanine production included incubation time (16 -18 h), temperature (30°C), pH (9.0), selected concentrations of substrates: 0.1M *trans*-cinnamic acid and 4.0M NH_4OH and 0.1M Tris-HCl. Under these conditions about 85% conversion of substrate to product was obtained.

Nous décrivons une procédure pour la synthèse de L-phénylalanine à partir de l'acide cinnamique (trans-) et d'ammoniac au moyen de la réaction inverse de la phénylalanine ammonia lyase (PAL) de *Rhodotorula glutinis*. Les conditions optimales pour la croissance de la levure et la production de PAL étaient les suivantes : inoculum à 1 %, pH 6, milieu à 30°C, pendant 26 à 28 h. L'optimisation des conditions pour la production maximale de phénylalanine a nécessité une période d'incubation (de 16 à 18h), une température de 30°C, un pH de 9,0 et des concentrations données de substrats (0,1M d'acide cinnamique (*trans*-), 4,0M de NH_4OH et 0,1M de Tris-HCl). Dans ces conditions, environ 85 % du substrat a été converti en produit.

INTRODUCTION

The synthesis of L-Phe in optically pure form is an important reaction with clinical and industrial significance. This essential amino acid has several applications including its use as a dietary supplement in intravenous infusions (Trevan et al. 1987) and as a precursor of the artificial sweetener, aspartame (Oyama et al. 1987). Phenylalanine ammonia lyase (E.C.4.3.1.5 - PAL) catalyzes the spontaneous non-oxidative deamination of L-phenylalanine (L-Phe) to *trans*-cinnamic acid (t-CA) and ammonia (Koukol & Conn 1961).

This enzyme is widely distributed in higher plants (Koukol & Conn 1961, Jahnen & Hahlbrock 1988, Whetten & Sederoff 1992), some fungi (Kalghatgi

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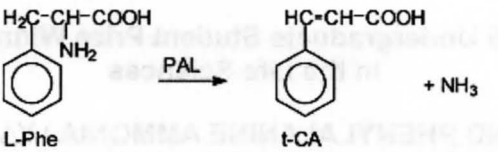

& Subba Rao 1975, Sikora & Marzluff 1982), yeasts (Ogata & Uchiyama 1967, Marusich et al. 1981, Orndorff et al. 1988) and in a single prokaryote, *Streptomyces* (Bezanson et al. 1970). It is absent from true bacteria and animal tissues.

PAL is the first and key enzyme of the phenyl propanoid sequence, a secondary metabolic pathway mainly involved in production of precursors of lignin in higher plants (Koukol & Conn 1961). In microorganisms, it has a catabolic role, allowing them to utilize L-Phe as a sole source of carbon (Marusich et al. 1981). Among the microorganisms, PAL occurs abundantly in yeast, especially in the red yeast basidiomycetes family, *Rhodotorula* (Marusich et al. 1981, Orndorff et al. 1988, Fritz et al. 1976). Other PAL producing yeasts are *Sporobolomyces roseus and Sporidiobolus pararoseus* (Ogata & Uchiyama 1967, Watanabe et al. 1992).

PAL is one of the few nonhydrolytic enzymes that have several commercial applications (Fritz et al. 1976, Watanabe et al. 1992, Ambrus et al. 1978, Hsia & Holtzmann 1973, Yamada et al. 1981, Evans et al. 1986a, b, Hamilton et al. 1985, Evans et al. 1987, Finkelman & Yang 1985, Nelson 1976). Commercially *Rhodotorula* yeast has been used exclusively for production of PAL because of its high PAL levels, nonfastidious requirements for growth and PAL synthesis (Orndorff et al. 1988, Evans et al. 1987). Purified *Rhodotorula* PAL is effective in the treatment of certain mouse neoplastic tumors (Fritz et al. 1976), the quantitative analysis of serum L-Phe in monitoring patients with phenylketonuria (Watanabe et al. 1992, Ambrus et al. 1978) and also in the preparation of low phenylalanine diets (Yamada et al. 1981).

Although L-Phe can be produced by chemical synthesis (Herbst & Shemin 1955), fermentation (Trevan et al.1987) or by enzymatic bioconversion of precursors (Yamada et al. 1981, Evans et al. 1986a, b, Hamilton et al. 1985, Evans et al. 1987, Finkelman & Yang 1985, Nelson 1976), the great demand for high yields of the optically pure L-isomer, has focused attention on the stereospecific production of L-Phe by biochemical methods. In addition, among the bioconversions, the use of PAL for the production of L-Phe has received maximum attention because of the low price of the raw feed stock, trans-cinnamic acid (t-CA) (Evans et al. 1986a, b, Hamilton et al. 1985, Evans et al. 1987, Finkelman & Yang 1985, Nelson 1976).

Our study has been conducted to extend the earlier work of Yamada et al. (1981) and Evans et al. (1987) on the synthesis of L-Phe from t-CA by PAL containing *Rhodotorula* yeast cells in an attempt to improve the yields of the product obtained by these authors.

MATERIALS AND METHODS

Microorganism

The yeast strain *Rhodotorula glutinis*, RE4607095D used in this study, procured from Oxoid Inc. (Nepean, Ontario) was maintained by weekly transfers on 3.0% agar plates and slants containing 1.0% peptone, 1.0% yeast extract, and 0.5% NaCl.

Chemicals

Yeast extract, peptone and agar were obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK). L-Phe and Tris buffer were purchased from Fisher Scientific (Fairlawn, NJ, USA) and trans-cinnamic acid was obtained from Acros Organics (Morris Plains, NJ, USA). Commercially available reagents of highest analytical grade were used without further treatment.

Physiological manipulation of *Rhodotorula glutinis*

The effects of the following parameters on the growth and PAL activity of the *Rhodotorula glutinis* yeast were determined. (a) Medium composition 1) the minimal salts medium contained 1.0% $(NH_4)_2SO_4$, 0.5% KH_2PO_4, 0.05% $MgSO_4$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, 0.001% NaCl, and 2.0% glucose or sorbitol in distilled water 2) the standard yeast extract medium contained 1.0% yeast extract, 1.0% peptone and 0.5% NaCl in distilled water; (b) pH (5-9); (c) size of inoculum (0.2-10%); (d) temperature during growth (30-42°C) and (e) time of harvest of cells (0-54 h) grown on an orbital shaker (160 rpm). The cells harvested by centrifugation at 7,500 x g for 15 min at room temperature were preserved at 0-2°C. Cell amounts used are reported on a dry weight basis.

PAL forward assay

The PAL forward activity of *Rhodotorula* yeast whole cells was monitored spectrophotometrically by the formation of t-CA from L-Phe at an absorbance of 290nm (Herbst & Shemin 1955, Evans et al. 1986b). The reaction mixture (5.0 mL) containing 50 mM Tris–HCl buffer, pH 8.5, 37.5 mM L-Phe and 10.0 mg of *R. glutinis* whole cells was incubated at 30°C for 10.0 min. The reaction was stopped by inactivating the enzyme with concentrated HCl and separating the cells by centrifugation at 7,500 x g for 15 min. The absorbance of the clear supernatant fluid was measured at 290 nm; the reaction mixture to which the substrate was added after termination of the reaction served as the blank. The enzyme activity is expressed in units; one unit of enzyme is defined as the amount required to transform 1 nmole of L-Phe/min/mg dry cells at room temperature.

PAL reversal assay

The reaction mixture consisted of 5 ml 0.1M Tris-HCl buffer, pH 9.0, containing 0.1M t-CA, 4.0M NH_4OH and 400 mg yeast whole cells. Samples containing boiled cells (100°C, 15 min.) served as controls. The reaction mixtures were incubated at 30°C with shaking (200 rpm). The reaction was

allowed to proceed overnight to obtain a measurable amount of the product, L-Phe. L-Phe was identified by paper chromatography; chromatograms were developed with 1-butanol: acetic acid: water (4:1:5, upper phase) for 2-3 h, air-dried for 30 min, and sprayed with 0.2% ninhydrin in ethanol. The chromatograms were heated at 105°C for 5 min. to reveal coloured spots, which were eluted with 0.5% $Cu(NO_3)_2$ in methanol for 20 min. in the dark, and the concentrations of eluted L-Phe was measured spectrophotometrically at 520 nm.

Data analysis

The values reported are the means of at least three separate determinations.

RESULTS

The optimal conditions for the growth and PAL activity of *Rhodotorula glutinis* are shown in Table 1. Under these conditions the PAL activity and biomass of *Rhodotorula glutinis* RE4607095D whole cells ranged from 17-19 units/mg dry cells and 3.5-4.0g dry cells/L in the standard yeast extract medium to 10-11 units/mg dry cells and 2.5g dry cells/L produced in minimal salts medium.

Table 1. Growth and PAL Activity - Optimal Conditions

Parameter	Range/Parameter Tested
Medium Composition	Minimal Salts or Yeast Extract Medium
pH	5.0 - 9.0
Size of Inoculum (%)	0.2 - 10.0
Temperature (°C)	15, 28, 30, 37, 42
Time (h)	0 - 54

L-phe formation during the PAL reversal reaction was proportional to the cell amounts up to 400mg. Maximal L-Phe accumulated after 16-18 hours of incubation (Fig 1).

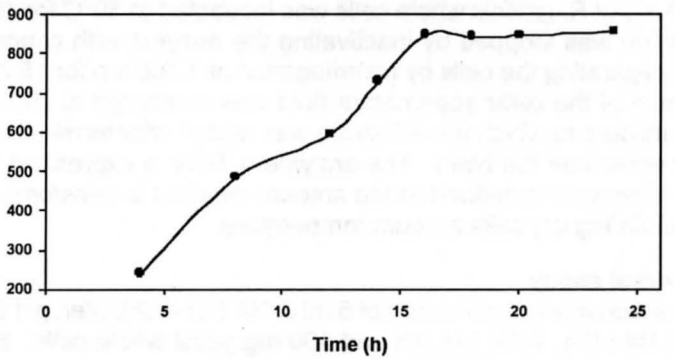

Fig 1 Effect of time on PAL reversal reaction.
Maximal L-Phe accumulated after 16-18 hours of incubation.

Optimal concentrations of t-CA and NH_4OH were 0.1-0.5M and 4.0-7.0M respectively. Higher concentrations of t-CA (>1.0M) were found to inhibit PAL reversal activity (Tables 2, 3).

Table 2. Effect of t-CA concentration on PAL

No.	Concentration of t-CA (M)	L-Phe (nmol h-1 mg-1 dry cells)
1	0.01	4
2	0.025	32
3	0.05	60
4	0.1	132
5	0.5	133
6	1	129
7	2	42

Table 3. Effect of NH_3 concentration of PAL

No.	Concentration of NH_3 (M)	L-Phe (L-Phe nmol h-1 mg-1 dry cells)
1	1.0	24
2	2.0	46
3	3.0	87
4	4.0	134
5	5.0	133
6	6.0	139
7	7.0	135

Among the various buffers (each at 0.1M concentration and pH 9.0) the yield of L-Phe in Tris-SO_4, borate, Tris-HCl, HEPES, and glycine was 105, 104, 133, 98, and 88 nmol/h/mg dry cells respectively. As 0.1M Tris-HCl buffer at pH 9.0 gave maximal conversions of t-CA to L-Phe it was used for all remaining studies (Fig 2).

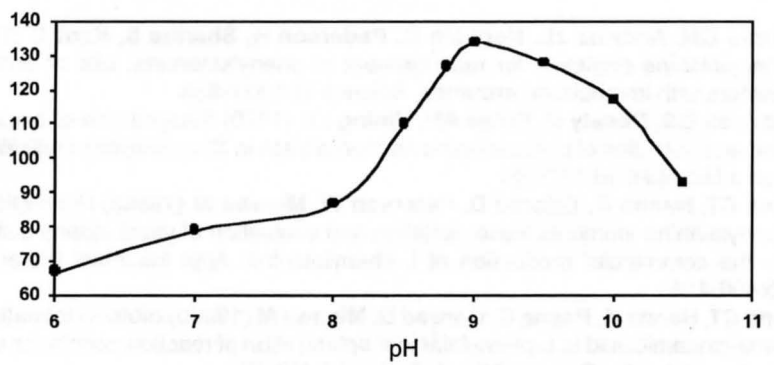

Fig 2 Effect of pH on PAL activity.
0.1M Tris-HCl buffer at pH 9.0 gave maximal conversion of t-CA to L-Phe.

The PAL reversal reaction carried out over a range of temperatures (25, 28, 30, 32, 37 and 42°C) gave yields of L-Phe at these temperatures of 87, 135, 138, 129, 98, and 73 nmol L-Phe/h/mg dry cells respectively. Under the optimized conditions for maximal L-Phe production (16 – 18 h, 30°C, 0.1M Tris-HCl, pH 9.0, 0.1M t-CA, 4.0M NH_4OH and 400mg dry cells *R. glutinis*) the reaction gave 850μmol L-Phe after 16 hours incubation at 30°C with shaking at 200rpm. This corresponds to an 85% substrate conversion.

DISCUSSION

The *Rhodotorula* PAL reversal reaction has been reported to be inhibited by its own substrate, t-CA (Evans et al. 1987). It is evident from table 2 that a higher concentration (>1.0M) of t-CA inhibited PAL reversal activity. Higher concentrations (>4.0M) of NH_3 (Table 3), however, did not make a significant difference with respect to inhibition of PAL reversal activity.

Genex Corporation has established a large-scale production process based on Rhodotorula cells for the synthesis of L-Phe (Finkelman & Yang 1985). Similarly, the authors of a US patent (Nelson et al. 1976) list a method of L-Phe production using *R. glutinis* PAL. Unfortunately, the details of the reaction, the conversion yield, and the concentration of L-Phe obtained are obscure. The product yield (85% substrate conversion) obtained in our study is appreciably higher than the 70% substrate conversion reported for L-Phe production by Yamada et al. (1981) and Evans et al. (1987). Our future studies are aimed at using L-Phe for the production of aspartame and the use of *R. glutinis* PAL in quantifying L-Phe in biological samples.

Acknowledgements. We thank Dr. Michael Tanchak and Mr. Paul MacDougall (Biology Department, Cape Breton University, Sydney, NS) for their kind cooperation.

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