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Production and Characterization of Phenylalanine Ammonia-lyase from *Rhodotorula aurantiaca* K-505

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Abstract

Optimal cultivation conditions for the production of phenylalanine ammonia-lyase(PAL) from *Rhodotorula aurantiaca* K-505 were selected, and the kinetic parameters of the produced PAL were determined. The most suitable carbon and nitrogen sources were glucose and tryptone, respectively. The strain expressed PAL constitutively when using the optimized semi-complex media. High cell density culture could be critical for maximal production of PAL since the PAL biosynthesis was growth associated. Maximum PAL activity was observed at initial pH 6.0. Although the cell growth was not markedly affected by temperature between 22 and 28°C, the cells yielded the maximum PAL activity when cultivated at 22°C. The maximum activity for the deamination of L-phenylalanine to trans-cinnamic acid was observed around pH 8.8. The PAL activity gave the maximum at 45°C, and greatly decreased at higher than 50°C. Activation energy(E_a) calculated from Arrhenius equation was 6.28kcal/mol in the range of 22°C to 40°C. A Hanes-Woolf plot showed that the enzyme reaction follows Michaelis-Menten equation, whose K_M and V_{max} values were 4.65×10^{-3}M and 0.89μ mol/mg-min respectively.

Key words: Phenylalanine ammonia-lyase(PAL), *Rhodotorula aurantiaca*, phenylalanine, culture conditions, kinetic parameters

INTRODUCTION

Phenylalanine ammonia-lyase(PAL, EC. 4.3.1.5) catalyses the non-oxidative deamination of L-phenylalanine to form trans-cinnamic acid and ammonia. The enzyme plays a major role in the catabolism of L-phenylalanine. It is ubiquitous in plants as the initial enzyme in biosynthetic pathways for the formation of many phenylpropanoid compounds including lignin, flavonoids and other phenolic derivatives(1). The enzyme is implicated in a number of functions such as lignification, biochemical changes in diseased conditions and injury(2), and the quantitative analysis of serum phenylalanine(3).

In recent, the production of L-phenylalanine has been received a considerable attention because of its increasing demand of L-phenylalanine as a precursor to the dipeptide sweetener, aspartame. Traditionally, L-phenylalanine has been produced by chemical synthesis and cellular fermentation. However, the advantage of stereospecific product makes the enzymatic production favorable. It is possible by reversing the enzyme reaction with trans-cinnamic acid and ammonia(4).

The enzyme has been found in various higher plants (5), yeast(6,7), and streptomyces(8). There has been very few report on PAL from *Rhodotorula aurantiaca*. Some strains of yeasts and fungi including *R. aurantiaca* were screened for the synthesis of high levels of L-phenylalanine by Kupletskaya and Dol'nikova(9).

In yeasts, PAL is known to be an inducible enzyme, but induction of PAL activity is affected by various culture conditions including composition of nutrients, temperature, and pH. In the present study, the effects of various cultivation conditions on the production of PAL in *R. aurantiaca* and kinetic properties for the enzyme reaction were investigated.

MATERIALS AND METHODS

Microorganism

Rhodotorula aurantiaca K-505 was isolated from deciduous tree and identified by Korea Research Institute of Bioscience and Biotechnology. The cells were main-

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tained on malt agar slants at 4°C and transferred every month.

Medium and culture conditions

Seed cultures were carried out in 250ml Erlenmeyer flasks filled with culture media of 50ml. The seed culture medium contained 1% glucose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract. The initial pH was adjusted to 6.0 by 1N NaOH. The cultures were incubated at 22°C for 48h on a reciprocating shaker. The basal culture medium for the production of PAL was composed of 0.5% glucose, 1% tryptone, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.01% $CaCl_2$, and 0.05% NaCl. A 100ml portion of the basal medium in a 500ml flask was autoclaved at 121°C for 15 min, inoculated with the seed culture(2%), and then incubated at 22°C.

Analytical methods

The cell growth was measured by UV/VIS spectrophotometer(HP 8452A diode array) at 660nm. Dry cell weight was calculated from a calibration curve with measured optical density. The activity of PAL was measured by means of the method described by Kalghatgi and Rao (10) with some modification. After centrifugal separation at 12,500rpm for 5min at 4°C, the cells were washed twice with ice-cold distilled water and immediately used as a source of enzyme activity assay and enzyme characterization on kinetic parameters. The standard assay mixture contained 2.5ml of 25mM L-phenylalanine, 2.5ml of 50mM tris(hydroxymethyl) amminomethane-hydrochloride buffer(pH 8.8), 0.01% cetylpyridinium chloride, and 1ml of cell suspension in a final volume of 6.0ml. The reaction was carried out 30°C for 30min, and then terminated with 0.5ml of 6N HCl. The formation of trans-cinnamic acid was determined by following the increase in absorbance at 278nm. One unit of PAL activity corresponded to the amount of the enzyme that catalyzed the formation of trans-cinnamic acid at a rate of 1μmol/min under the standard assay condition. The specific activity was expressed in terms of unit per g-dry weight of the cell.

RESULTS AND DISCUSSION

Culture conditions for the induction of PAL activity

Various parameters such as composition of nutrients, pH, and temperature were investigated with *Rhodotorula aurantiaca* K-505 to establish the optimum culture conditions for the formation of PAL.

Effect of carbon sources

The effect of carbon sources on the formation of the enzyme was investigated in shake flask studies, as shown in Table 1. *Rhodotorula aurantiaca* was grown on the PAL production medium containing different amounts of glucose, fructose, or sucrose as a major carbon source. Among the tested carbon sources, the highest specific activity of PAL was obtained with 0.5% glucose. Although the cell growth was increased when more than 1.0% of the carbon source was added, the PAL activity was decreased. It indicated catabolite repression by glucose, which was similar result of Orndorff et al.(11). Accordingly, 0.5% glucose was used as the main carbon source for subsequent experiments.

Effect of nitrogen sources

To choose the most favorable primary nitrogen source, media containing various nitrogenous compounds were compared at a concentration of 1%. As shown in Table 2, inorganic compounds, except for ammonium sulfate,

Table 1. Effects of carbon sources on the cell growth and PAL activity at 30h[1)]

Carbon source	Concentration (%)	Cell growth (g-dry cell/L)	Specific PAL activity (U/g-dry cell)
Glucose	0.5	4.61	540
	1.0	5.23	210
	1.5	5.41	190
Fructose	0.5	3.95	310
	1.0	4.38	260
	1.5	4.72	240
Sucrose	0.5	5.26	350
	1.0	5.94	220
	1.5	6.06	200

[1)]Nitrogen source: tryptone(1%)

Table 2. Effects of nitrogen sources on the PAL activity[1)]

Nitrogen source	Cell growth (g-dry cell/L)	Specific PAL activity (U/g-dry cell)
Peptone	4.02	46
Yeast extract	4.42	28
Malt extract	1.26	ND[2)]
Tryptone	4.54	550
$(NH_4)_2SO_4$	3.54	510
NH_4Cl	3.01	ND
$(NH_4)_2HPO_4$	3.72	ND

[1)]At 30h, carbon source: glucose(0.5%)
[2)]Not detected

were wholly ineffective on the biosynthesis of PAL. Tryptone and ammonium sulfate gave the best results for the enzyme activity. On the other hand, most organic compounds were more effective than inorganic compounds. In particular, tryptone has an important role for the formation of PAL in *R. aurantiaca*. However, the combinations of tryptone and other nutrients such as ammonium sulfate, yeast extract, and peptone, little affected on the PAL activity(data not shown). Tryptone was finally selected as the nitrogen source for the PAL production.

Effect of amino acids

In general various amino acids, in particular hydrophobic aromatic amino acids were reported to induce PAL(12). The effects of various amino acids on the PAL induction were examined, and the results are shown in Table 3. The data demonstrated that various amino acids, even though the physiological inducer L-phenylalanine, induce PAL to levels equivalent to those observed in the absence of any inducer. It is probably due to the use of rich nitrogen source such as tryptone. In particular, L-phenylalanine, which has been known as an inducer of PAL synthesis in *Rhizoctonia solani*(13), also had no effect on the increase of the PAL activity. Moreover, the addition of L-phenylalanine more than 0.1% found to be inhibitory on the cell growth as well as the PAL induction(Fig. 1). In a separate experiment, the cells were hardly grown and the PAL activity was not detected when L-phenylalanine was used as a sole source of nitrogen(data not shown). Like many *Rhodotorula* species, this strain hardly grows on aromatic substrates, and aromatic amino acids as a sole nitrogen source are inhibitory(14). Contrary to the result of Marusich et al.(14) in *R. glutinis*, L-phenylalanine had no effect on PAL induction in *R. aurantiaca* used in this study. L-isoleucine, which has been known as stabilizer of the PAL activity(15), had no effect on sustaining the PAL activity.

Table 3. Effects of amino acids on the cell growth and PAL activity[1)]

Amino acids[2)]	Cell growth (g-dry cell/L)	Specific PAL activity (U/g-dry cell)
L-phenylalanine	4.56	500
L-isoleucine	3.95	490
L-tyrosine	4.73	470
L-leucine	3.50	160
L-methionine	0.70	ND[3)]
L-tryptophan	4.46	480
None	4.61	540

[1)]At 30h, carbon source: glucose(0.5%), nitrogen source: tryptone(1%)
[2)]Amino acid concentrations: 0.5%
[3)]Not detected

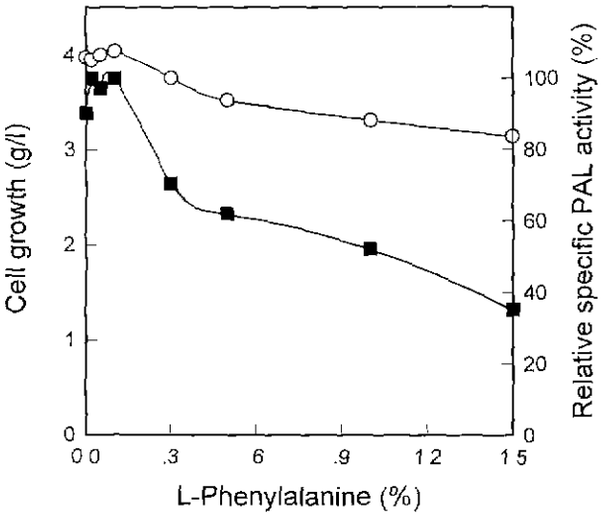

Fig. 1. Effects of L-phenylalanine concentration on the cell growth and specific PAL activity at 24h.
Symbols: (○) cell growth; (■) PAL activity.

Effect of pH and temperature

The effects of the initial pH on the cell growth and PAL activity are shown in Fig. 2. The final pHs after 24h-cultivation were measured in the experiments at different initial pHs, and the degree of pH change was not significantly different(data not shown). Maximum PAL activity was observed at pH 6.0 and there was 80% of the maximum PAL activity at pH 7.0. However, the PAL activity was greatly decreased below pH 6.0 or above

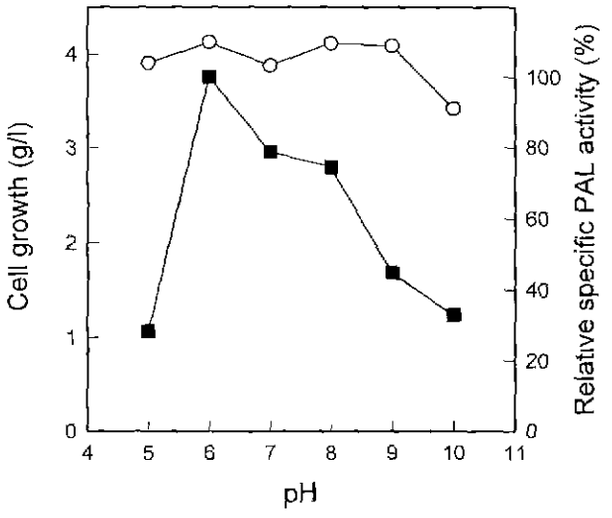

Fig. 2. Effects of pH on the cell growth and specific PAL activity at 24h.
Symbols: (○) cell growth; (■) PAL activity.

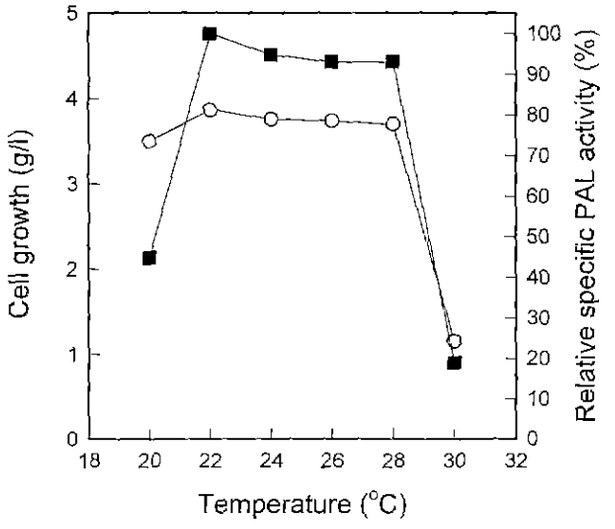

Fig. 3. Effects of incubation temperature on the cell growth and specific PAL activity at 24h.
Symbols: (○) cell growth; (■) PAL activity.

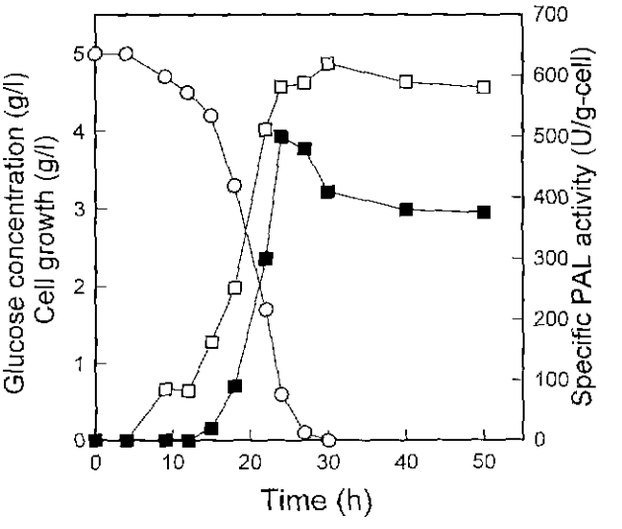

Fig. 4. Time courses of the yeast cultivation for the production of PAL.
Symbols: (○) residual glucose concentration; (□) cell growth; (■) PAL activity.

pH 8.0. Different temperatures from 20 to 30°C were tested to determine the influence of cultivation temperature on the PAL biosynthesis, as shown in Fig. 3. The cells were hardly grown at 30°C. Although the cell growth was not markedly affected by temperature between 22 and 28°C, the cells yielded the maximum PAL activity when cultivated at 22°C. The specific activity of PAL was significantly low at 20°C and 30°C.

Time courses of yeast cultivation for PAL production

Fig. 4 shows the time courses of the cell growth and the enzyme biosynthesis. The PAL activity was detectable after 18h of cultivation, reached a maximum around 27h to 30h of incubation(late log-phase of growth), and declined when the yeast cells reached stationary phase. However, sudden decrease of the PAL activity was not observed, and more than 70% of the maximum PAL activity was maintained up to 50h. In complex media, PAL activity in *R. glutinis* appeared during stationary growth, since catabolism of L-phenylalanine is required only under carbon- or nitrogen-limited conditions(14). This strain expressed PAL constitutively when using the optimized semi-complex media containing tryptone. The PAL activity expressed by the strain in the present study was exactly dependent on the cell concentration and thus growth-associated. Therefore, high cell density culture will be critical for maximal production of PAL. This point is a desirable characteristic because the regulation of PAL synthesis is complex and occurs at the transcriptional level in yeast cells.

In conclusion, *Rhodotorula aurantiaca* K-505 clearly possesses several desirable characteristics as a production strain. *R. aurantiaca* K-505 produces significantly higher specific PAL activity than do other yeast species(4,15). Additionally, the strain expressed PAL constitutively when using the optimized semi-complex media.

Optimal reaction conditions of the enzyme and kinetic parameters

Optimal pH and temperature for PAL-catalyzed reaction

The effect of pH on the specific PAL activity in the enzyme reaction was investigated under the standard assay conditions using different buffers according to pH. As shown in Fig. 5(a), the maximum activity for the deamination of L-phenylalanine to trans-cinnamic acid was observed at pH 8.8, and 90% of the maximum activity was maintained at pH 7.8 to 9.3. However, the specific activity was significantly decreased under pH 7.0 or over pH 10. The temperature dependence on the enzyme reaction was investigated. As shown in Fig. 5(b), the specific PAL activity gave the maximum at 45°C and greatly decreased at 50°C and above.

Effects of temperature and substrate concentration on PAL-catalyzed reaction

The temperature dependence of reaction rate was plotted according to Arrhenius equation. The reaction conditions were those employed for the standard assay. From the linear regression of $\log V_{max}$ against 1/T(Fig. 6(a)),

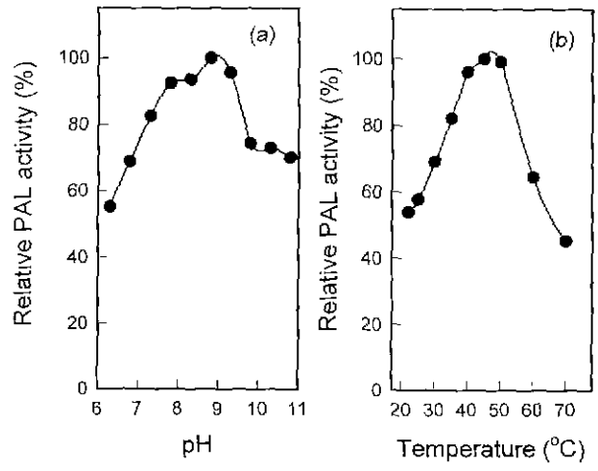

Fig. 5. Effects of (a) pH and (b) reaction temperature on the PAL-catalyzed reaction.
Used buffers: pH 6.3 to 7.8, potassium phosphate buffer; pH 8.3 to 9.3, tris-HCl buffer; pH 9.8 to 10.8, borax-NaOH buffer.

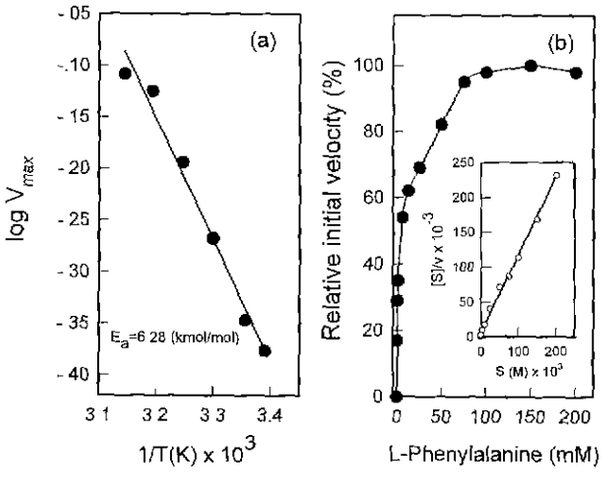

Fig. 6. (a) Arrhenius plot and (b) Hanes-Woolf plot of the PAL-catalyzed reaction for the calculation of activation energy and kinetic parameters.

the activation energy(E_a) was calculated as 6.28kcal/mol in the range of 22°C to 40°C. This result was lower than that of Havir and Hanson(16) whose results in potato tubers were 13.7kcal/mol in the range of 23°C to 30°C. The enzyme of lower activation energy makes it possible for substrate molecules with smaller energies to react.

To investigate the effect of substrate concentration in the reaction, L-phenylalanine concentration was varied at a range of 0.1~200mM, and the PAL activity was measured(Fig. 6(b)). Relationship between the enzyme reaction rate(v) and substrate concentration(S) was plotted by Hanes-Wolf plot with the linearized form of Michaelis-Meneten equation: $S/v=S/V_{max}+K_M/V_{max}$, where V_{max} and K_M are the maximum initial rate and Michaelis-Menten constant, respectively(17). The plot showed that the enzyme reaction follows Michaelis-Menten equation: $v=V_{max} \cdot S/(S+K_M)$. According to Hanes-Wolf plot, the K_M and V_{max} values were calculated as 4.65×10^{-3}M and 0.89μmol/mg-min, respectively. The K_M value was slightly greater than that of *Rhodotorula glutinis*(2), indicating lower affinity of enzyme molecules toward the substrate.

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