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Manganese (Mn^{2+})-Dependent Storage Stabilization of *Rhodotorula glutinis* Phenylalanine Ammonia-lyase Activity

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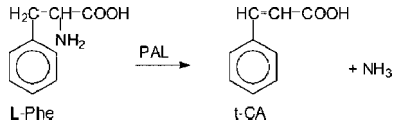
Phenylalanine ammonia-lyase (PAL; E C 4.3.1.5) reverse reaction has been exploited for the commercial production of optically pure L-phenylalanine from *trans*-cinnamic acid. Optimal conditions for the growth and PAL activity of *Rhodotorula glutinis* cells and an improved method for the synthesis of L-phenylalanine have been reported. A major problem encountered during these studies was rapid loss of PAL activity during storage of the yeast cells, which were therefore unsuitable for long-term and repeated use. Enhancement of enzyme stability in the presence of various additives including polyhydric compounds and metal ions is described. Whole cells retained nearly 85% of the original enzyme activity for at least 12 weeks when a low concentration of Mn^{2+} (0.01%) was included in the storage buffer medium (50 mM Tris-HCl, pH 8.8). In contrast, <3.0% activity was present in the control within 4 weeks. Mn^{2+}-dependent stabilization of PAL was also observed with an isolated enzyme preparation (73% retention in activity for 12 weeks) obtained by ultrasonication of *R. glutinis* whole cells. The data suggest that Mn^{2+} ions may be responsible for the specific stabilization of a more active conformation of the enzyme. In addition, enzyme stability as a function of temperature was studied, and the optimal temperature for maximal activity retention was 0–2 °C. The effects of various additives on the induction of PAL have also been examined. These results could have direct implications in studies on activity, inhibition, and reaction mechanism of this biotechnologically important enzyme.

KEYWORDS: *Rhodotorula glutinis* yeast cells; phenylalanine ammonia-lyase; Mn^{2+}-dependent storage stability; enzyme induction; GC-MS determination of PAL reverse activity

INTRODUCTION

Phenylalanine ammonia lyase (EC 4.3.1.5, PAL) is the first enzyme of the phenyl propanoid metabolic pathway, a secondary metabolic pathway operative in higher plants (*1*). The enzyme is present in higher plants (*1–3*), some fungi (*4, 5*), yeasts (*6–8*), and a single prokaryote, *Streptomyces* (*9*). However, it is absent in true bacteria and animal tissues. The enzyme is soluble and cytoplasmic in origin in the majority of cases. PAL catalyzes the spontaneous nonoxidative deamination of L-phenylalanine (L-Phe) to form *trans*-cinnamic acid (*t*-CA) and ammonia (*1*).



Commercially, *Rhodotorula* yeast has been the principal source of PAL because of its high levels of PAL and nonfastidious requirements for growth and PAL synthesis (*8, 10*). PAL reaction is of great interest in commercial applications including treatment of certain mouse neoplastic tumors (*11*), quantifying serum L-Phe (*12*), treatment of phenylketonuria (*13*), and preparation of low-phenylalanine diets (*14*). The reverse of the PAL physiological reaction has been utilized for the synthesis of L-Phe and its methyl ester (*15–18*).

We have recently reported optimal conditions for the maximal growth and PAL activity of *R. glutinis* yeast cells and an improved method for the synthesis of L-Phe using PAL reverse reaction (*19*). Although the yeast cells could be used as a rich enzyme source, especially for effective transformation of *t*-CA to L-phe (>85% conversion), PAL activity declined rapidly during storage. The present study was undertaken to explore the possibility of stabilizing the biocatalyst so that it could be repeatedly used over the long term. This study also included induction of PAL enzyme during the growth of *R. glutinis* yeast cells. In this paper, we also describe the modification of a gas

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chromatography–mass spectrometry (GC-MS) method for the rapid and sensitive determination of PAL reverse reaction of *R. glutinis*.

Enzyme stabilization is an extremely important aspect of biocatalysis in commercial applications to reduce production costs. The main objective of our study is focused on Mn^{2+}-dependent stabilization of PAL, which has not been reported before. In addition to further information on stability of PAL (a tetrameric protein), these data may be significant in predicting metal ion stabilization of other multimeric enzymes and proteins.

MATERIALS AND METHODS

Microorganism. The yeast strain *R. glutinis* RE4607095D used in this study, procured from Oxoid Inc. (Nepean, ON, Canada), 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 bacteriological agar were obtained from Oxoid Ltd. (Basingstoke, Hampshire, U.K.). Sabouraud dextrose agar was procured from Acumedia Manufacturers, Inc. (Baltimore, MD). L-Phe, L-tyrosine (L-Tyr), L-isoleucine (L-Ile), L-alanine (L-Ala), L-aspartic acid (L-Asp), L-tryptophan (L-Trp), L-serine (L-Ser), L-glutamic acid (L-Glu), *t*-CA, Tris, *n*-butanol, trifluoroacetic anhydride, cetyl pyridinium chloride (CPC), glycerol, sorbitol, and sucrose were purchased from Fisher Scientific (Fair Lawn, NJ). Polyethylene imine (PEI), polyethylene glycol (PEG), ethylene glycol, ATP, pyridoxal phosphate, and glutaraldehyde were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). Commercially available inorganic chemicals including metal ion salts of the highest analytical grade were used without any further treatment.

Culture Conditions. *R. glutinis* cells from Sabouraud dextrose agar plates were grown in a culture medium containing 1.0% yeast extract, 1.0% peptone, and 0.5% NaCl at 30 °C on an orbital shaker (150 rpm). Cells were harvested in the late log phase (24–27 h) when maximal PAL activity was observed. Cell amounts are reported on a dry weight basis.

Secretion of PAL Enzyme during Incubation. Ten milliliter aliquots of a 27 h grown culture were centrifuged (7500*g* for 15.0 min). The enzyme activities of the whole cells and supernatant fluid were determined independently to check for secretion of PAL during the growth incubation period.

Crude Enzyme Isolation. *R. glutinis* whole cells suspended in Tris-HCl buffer (50 mM, pH 8.8) were subjected to the following treatments: (a) intermittent ultrasonication (50 Hz/ sec) in a Branson 1510 ultrasonic bath for an hour in ice, (b) CPC (0.001%) detergent permeabilization for an hour, and (c) synergistic ultrasonication and CPC detergent solubilization treatment. After the respective treatment, the cell extracts were centrifuged at 7500*g* for 15.0 min using a Damon IEC HN-SII centrifuge. PAL activity of the supernatant fluids and cell debris residue was monitored at the end of each treatment. Aliquots of supernatant fluid corresponding to 10.0 mg of *R. glutinis* cells were used for enzyme assays.

PAL Forward Assay. The PAL forward activity of *R. glutinis* was measured by a modification of a method reported earlier: spectrophotometric determination of *t*-CA formation from L-phe at an absorbance of 290 nm (*17*). The reaction mixture (5.0 mL) containing 50 mM Tris-HCl buffer, pH 8.8, 37.5 mM L-Phe, 0.005% CPC, and 10.0 mg *R. glutinis* cells was incubated at 30 °C for 10.0 min. Inactivating the enzyme with concentrated HCl and separating the cells by centrifugation then terminated the reaction. The absorbance of the clear supernatant fluid was measured at 290 nm. The reaction mixture in which substrate was added after termination of the reaction served as the blank. The enzyme activity is expressed as units; 1 unit of enzyme is defined as the amount required to convert 1 nmol of L-Phe per minute per milligram of dry cells at room temperature.

PAL Reverse Assay. The reaction mixture was made up of 5 mL with 0.1 M Tris-HCl buffer, pH 9.0, containing 0.1 M *t*-CA, 4.0 M NH_4OH, 0.005% CPC, and 400.0 mg of *R. glutinis* cells. Samples containing cells boiled at 100 °C for 15 min served as controls. The reaction mixtures were incubated at 30 °C with shaking (200 rpm) on an orbital shaker incubator. The reaction was allowed to proceed overnight to obtain an appreciable amount of product, L-Phe. PAL reverse reaction product was then identified by paper chromatography and quantified by spectrophotometric determination at 520 nm, using a method reported earlier (*19*).

Stabilization of PAL Activity during Storage. Stability studies included analyzing the PAL forward reaction using both *R. glutinis* whole cells as the enzyme source and the crude enzyme extract obtained by ultrasonication treatment of the yeast cells.

The influence of the presence of various compounds on the stability of PAL activity of *R. glutinis* during storage at 0–2 °C was investigated. The effect of different temperatures (−20, 0–2, 25, 30, and 37 °C) and various concentrations of Mn^{2+} (0.0025−0.05%) on the stability of *R. glutinis* PAL during storage was studied separately. Cells were grown in a yeast extract medium (27 h, 30 °C, and 150 rpm), washed twice with 0.9% NaCl, harvested, suspended in 10 mL of 50 mM Tris-HCl buffer, pH 8.8, and stored at 0–2 °C. Each of the compounds used for enzyme stabilization was added to the storage buffer medium prior to adjustment of the pH to 8.8. Cells stored in storage buffer without any additive served as a control. PAL activity of the stored cells was monitored periodically every 2 weeks for a total of 12 weeks.

The influence of selected metal ion additives (Mn^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and Li^{+}) and temperature (from –20 to 37 °C) on the stability of PAL isolated from *R. glutinis* whole cells was studied in a similar manner. Aliquots of supernatant fluid (obtained from sonicated cell extracts) were mixed with buffers containing the respective additives.

Induction of PAL Activity. Various additives were incorporated into the yeast extract growth medium to determine their effect on induction of PAL activity. The following additives were tested: L-Ala, L-Asp, *t*-CA, L-Ile, L-Phe, L-Ser, L-Trp, and L-Tyr. Cells were then grown for 27 h at 30 °C on an orbital shaker. Cells grown in a medium without any of the above additives served as control. The effect of different concentrations of L-Phe on the induction of PAL activity was studied separately.

Gas Chromatography–Mass Spectrometric (GC-MS) Determination of PAL Reverse Reaction. Initially PAL reverse reaction product, L-Phe, was identified and quantified by using a paper chromatography−visible spectrophotometric method developed in our laboratory earlier (*19*). Because paper chromatography is an out-dated and time-consuming method, a modified GC-MS method based on a protocol reported by Deng and Deng (*21*) was optimized for determining L-Phe formed as a result of PAL reverse reaction.

Samples included PAL reverse reaction control (*R. glutinis* cells boiled for 15 min) and experimental and commercial L-Phe and *t*-CA standards.

Derivatization. One hundred microliters of each sample was evaporated under a stream of N_2 at 40 °C, and the residue was reacted with 100 μL of *n*-butanol at 150 °C for 60 min. The solvent was evaporated to dryness under a stream of N_2 at 40 °C, and the butyl derivatives were reacted with 100 μL of a mixture of trifluoroacetic anhydride and acetonitrile (1:1 v/v) at 100 °C for 30 min. Finally, the derivatized samples were evaporated to dryness under a stream of N_2 at 40 °C and redissolved in 100 μL of methanol.

Separation. Analytes were separated using an Agilent Technologies Inc. HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) inserted in a Hewlett-Packard GC-MS in 70 eV EI mode. One microliter of derivatized standards (L-Phe and *t*-CA) and 3 μL of control and experimental were injected in splitless mode.

Oven Temperature Program. An initial temperature of 50 °C for 2 min was increased to 300 at 15 °C/min and maintained at 300 °C for 10 min. Helium carrier gas at a flow rate of 1 mL/min was used, and the detector was set at a temperature of 280 °C. The qualitative analysis was performed under full-scan acquisition mode within 50–550 amu range, and quantification was carried out under scan mode: *m*/*z* 50–300.

Data Analysis. The values reported are the mean of at least three independent determinations.

Table 1. Optimal Parameters[a] for the Assay of *R. glutinis* Yeast PAL

parameter	range/parameter tested	optimal value/parameter
cell amount	2.0–40.0 mg	10.0 mg
concentration of L-Phe	10.0–75 mM	37.5 mM
time	5.0–60.0 min	10.0 min
temperature	25, 30, 37 °C	30 °C
concentration of CPC	0.001−0.05%	0.005%
50 mM buffer, pH 7.5	borate, phosphate, Tris-HCl	Tris-HCl
pH	7.0–10.0	8.8

[a] Under these optimized conditions, PAL activity of *R. glutinis* cells was 34.0 units. Cells grown in a yeast extract medium for 27 h at 30°C were used for determining PAL activity. One enzyme unit is defined as nanomoles of L-Phe transformed per minute per milligram of dry cells.

Table 2. PAL Activity[a] of Enzyme Isolated from *R. glutinis* Whole Cells

treatment	PAL activity (units)
control (untreated cells)	34.0
ultrasonication	30.3
0.001% CPC	16.0
ultrasonication + CPC	30.5

[a] PAL activity is reported in terms of nanomoles of L-Phe transformed per minute per milligram of dry cells. The details of each treatment procedure for extracting the enzyme from *R. glutinis* yeast whole cells are discussed under Materials and Methods.

RESULTS

Optimal Parameters for Assay of *R. gluitinis* PAL. The various parameters that were optimized to obtain maximal PAL activity in *R. glutinis* whole cells are presented in **Table 1**. The enzyme activity was proportional with time and the amount of cells in the range selected for routine assays. The optimal concentration of the substrate, L-Phe, was found to be 37.5 mM, which is about 18 times the K_m value of 2.1 mM reported for PAL from *Rhodotorula* (*22*). The amount of cells and concentration of L-Phe exceeding the optimal values (10 mg and 37.5 mM, respectively) did not further increase PAL activity of cells significantly. The presence of a low concentration (0.005% v/v) of detergent, CPC, in the assay mixture was necessary to permeabilize the yeast cells to substrate and products. Omitting the detergent from the assay mixture gave only 50% of the PAL activity obtained in its presence. CPC concentrations of >0.1% were found to be inhibitory to PAL.

Secretion of PAL Enzyme during Incubation. The PAL activity of the supernatant fluid (0.068 unit) obtained after the centrifugation of the cells from the culture was negligible compared to that of the whole yeast cells (34.0 units). This ruled out the secretion of the enzyme by the yeast cells into the medium during growth. Therefore, it can be concluded that there is no rupture of cells during incubation due to osmotic factors.

PAL Activity of Isolated Enzyme. The PAL activity of the enzyme extracts obtained at the end of each isolation treatment is shown in **Table 2**. It is evident that ultrasonication was an effective method of PAL extraction (about 89% enzyme activity was recovered in the supernatant fluid). Because the presence of 0.001% CPC along with ultrasonication did not show a significant effect in enhancing enzyme recovery, enzyme extracted by ultrasonication treatment alone was used for stability studies. The cell debris residue obtained on centrifugation of the sonicated cell suspension was discarded as it had very low PAL activity (<5% activity of the original whole cells).

Stability of *R. glutinis* PAL Activity during Storage. The PAL enzyme has several applications (*11–14*) including the synthesis of L-Phe (*15–17*) and its methyl ester (*18*); therefore, a source of stable PAL is of considerable significance. With this aim, an effort was made to stabilize PAL by eliminating/retarding the factors that could be possibly contributing to enzyme inactivation. As these inactivation mechanisms are poorly understood, conventionally used methods of protein stabilization were employed.

(a) PAL Stabilization by Exogenous Compounds. From the data presented in **Table 3**, it is clear that among the additives tested for their effect on the stability of PAL, only polyhydric alcohols (25% glycerol, 5% ethylene glycol, and 1% PEG) gave significant reduction in enzyme inactivation during storage. Appreciable PAL activity (about 30–35%) was retained for about 10–12 weeks in the presence of glycerol and PEG.

It is evident from **Table 4**, which summarizes the effect of different metal ions on the storage stability of PAL, that both Mn^{2+} and Mg^{2+} had influenced a remarkable stabilizing effect on PAL and that appreciable enzyme activity was retained on at least 12 weeks of storage at 0–2 °C. The retention of PAL activity in the presence of Mn^{2+} and Mg^{2+} at the end of 12 weeks was 85 and 65%, respectively. Mn^{2+} gave very encouraging results; therefore, the concentration dependency of this divalent cation for PAL stabilization was explored further. PAL activity could be extended to about 10 weeks in the presence of Zn^{2+} and Li^{+}.

(b) Effect of Mn^{2+} Concentration and Temperature. The optimal concentration of Mn^{2+} required for obtaining maximum stabilization of PAL was determined. At an optimal Mn^{2+} concentration of 0.01%, *R. glutinis* cells retained nearly 85–90% of the original PAL activity after 12 weeks of storage (**Figure 1**). A higher concentration (>0.01%) of Mn^{2+} did not make a significant difference in enzyme stability. It can be seen from **Figure 2** that cells retained maximum PAL activity (about 85% for 12 weeks) when stored at 0–2 °C. Cells stored at –20 °C retained nearly 80% of the original enzyme activity after 12 weeks of storage. However, a progressive decline in enzyme activity was observed when cells were stored at 25 °C or higher temperatures.

(c) Effect of Metal Ions and Temperature on Stabilization of Isolated PAL. The effect of selected metal ions on stabilization of PAL isolated from *R. glutinis* whole cells is shown in **Table 5**. The protective effects of Mn^{2+} and Mg^{2+} on isolated PAL were similar to their effects on PAL-containing cells. In the presence of 0.01% of Mn^{2+} and Mg^{2+}, the isolated enzyme could retain 73 and 58% activity, respectively, for at least 12 weeks. The optimal temperature for maximal retention of activity of isolated enzyme upon storage was also 0–2 °C. At this temperature, the extracted PAL preparation retained 70–75% activity for about 12 weeks.

Induction of *R. glutinis* PAL Activity. The effect of *t*-CA and individual amino acids including L-Phe on PAL enzyme during fermentation was assessed in shake flask studies (**Table 6**). L-Phe, L-Tyr, and L-Ile gave significant enhancement of PAL activity, whereas *t*-CA was found to be a strong inhibitor. Addition of 0.05% L-Phe to fermentation medium resulted in a nearly 4-fold increase in enzyme activity (**Figure 3**). Higher concentrations of L-Phe (>0.05%) gave negligible increases in PAL activity. Although L-Tyr at a concentration of 0.05% showed inducive effects on PAL activity of the yeast cells, a decrease in enzyme activity was observed when the concentration was increased beyond 0.25% (data not shown). Inhibition of PAL activity was observed with the use of *t*-CA at a concentration as low as 0.025%.

Determination of *R. glutinis* PAL Reverse Reaction. An improved method for the synthesis of L-Phe, its identification, and quantification by paper chromatography was developed in

Table 3. Effect of Various Compounds[a] on Storage Stability of *R. glutinis* PAL

compound	PAL activity on storage (units) after					
	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
control (no additive)	10.3	2.9	0.1			
1.0 mMATP	10.4	3.2	2.0	0.6		
0.5 mM pyridoxal phosphate	10.4	3.2	1.9	0.5		
1.0% L-isoleucine	10.5	2.9	0.2	0.1		
25.0% glycerol	33.4	25.7	21.4	17.5	12.6	4.0
5.0% ethylene glycol	32.7	20.4	16.7	8.2	2.1	
1.0% polyethylene glycol	30.2	26.8	21.3	16.7	6.9	1.8
10.0 mM glutamic acid	10.3	2.9	0.2	0.1		
1.0 M sorbitol	10.7	3.7	2.1	0.6		
0.1 M sucrose	21.1	14.2	6.1	2.7	0.5	
1.0% polyethyleneimine	9.5	2.4	0.9	0.2		
0.5% glutaraldehyde	9.8	2.8	1.8	0.5		

[a] Each of these compounds was added to the storage buffer medium prior to adjustment of the pH to 8.8. Original PAL activity of *R. glutinis* cells was 34.0 units. PAL activity is reported in terms of units (nanomoles of L-phe transformed per minute per milligram of dry cells). Cells were stored at a refrigerated temperature (0–2 °C).

Table 4. Effect of Various Metal Ions[a] on Storage Stability of *R. glutinis* PAL

metal ion (0.01%)	PAL activity on storage (units) after					
	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
control (no metal ion)	10.3	2.9	0.1			
Na^{+}	8.6	2.5	1.1	0.4		
K^{+}	10.3	4.2	1.1			
Li^{+}	11.7	10.8	6.6	3.6	1.5	
Zn^{2+}	32.4	28.9	16.0	7.1	2.1	
Mg^{2+}	33.5	31.9	30.6	27.2	24.5	22.1
Mn^{2+}	33.6	33.4	32.9	32.0	30.6	28.7
Ca^{2+}	31.7	18.1	8.4	2.0		
Co^{2+}	30.9	9.4	5.2	1.9		
Cu^{2+}	15.7	3.9				
Fe^{2+}	34.0	9.9	4.2	1.7		

[a] Each of these metal ions was added to the storage buffer medium prior to adjustment of the pH to 8.8. Original PAL activity of *R. glutinis* cells was 34.0 units. PAL activity is reported in terms of units (nanomoles of L-Phe transformed per minute per milligram of dry cells). Cells were stored at refrigerated temperature (0–2 °C).

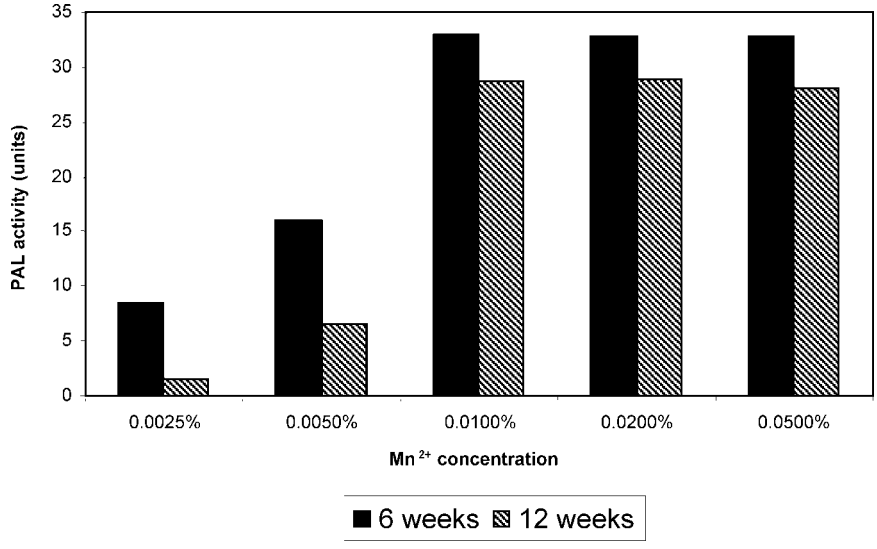

Figure 1. Effect of Mn^{2+} concentration on the storage stability of *R. glutinis* PAL. Mn^{2+} was added to the storage buffer medium prior to adjustment of the pH to 8.8. The details of the storage buffer are mentioned under Materials and Methods. Original PAL activity of *R. glutinis* cells was 34.0 units. PAL activity is reported in terms of units (nanomoles of L-Phe transformed per minute per milligram of dry cells). Cells were stored at refrigerated temperature (0–2 °C).

our laboratory earlier (*19*). An optimized GC-MS method for the determination of *R. glutinis* PAL reverse reaction is reported in the current study. **Figure 4** shows the chromatograms of the standards (L-Phe and *t*-CA) and PAL reverse reaction samples (control and experimental); the mass spectra of the derivatized standards are shown in **Figure 5**. The retention times of derivatized L-Phe and *t*-CA standards were found to be 12.75 and 12.60 min, respectively. It is evident from the chromatogram of control that the substrate, *t*-CA, was unreacted because the enzyme was inactivated by boiling. The experimental gave distinct peaks for L-Phe (formed as a result of PAL reverse reaction) and unreacted *t*-CA at retention times of 12.75 and 12.60 min, respectively. These retention times coincided very well with the retention times of L-Phe and *t*-CA standards.

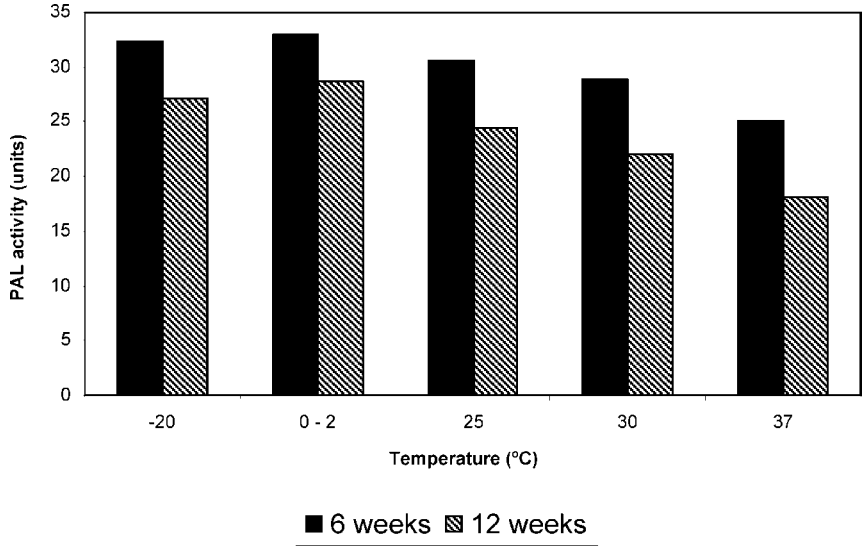

Figure 2. Effect of temperature on the storage stability of *R. glutinis* PAL. 0.01% Mn^{2+} was added to the storage buffer medium prior to adjustment of the pH to 8.8. Original PAL activity of *R. glutinis* cells was 34.0 units. PAL activity is reported in terms of units (nanomoles of L-Phe transformed per minute per milligram of dry cells).

Table 5. Effect of Various Metal Ions[a] on Storage Stability of Isolated PAL

	PAL activity on storage (units) after					
metal ion (0.01%)	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
control (no metal ion)	5.4	1.0				
Li^{+}	9.4	7.0	3.4			
Mn^{2+}	29.5	29.0	27.8	26.5	24.9	22.1
Mg^{2+}	29.1	27.3	24.7	22.6	21.2	17.4
Zn^{2+}	25.2	17.9	8.2	2.6		
Ca^{2+}	20.5	15.7	5.2	1.3		

[a] Each of these metal ions was added to the storage buffer medium prior to adjustment of the pH to 8.8. Aliquots of supernatant fluid containing isolated PAL were added to the storage buffer medium. Original activity of isolated enzyme was 30.3 units. PAL activity is reported in terms of units (nanomoles of L-Phe transformed per minute per milligram of dry cells). Storage buffer medium containing extracted enzyme was stored at refrigerated temperature (0 –2 °C).

Table 6. Effect of Various Compounds[a] on Induction of *R. glutinis* PAL

compound (0.05%)	PAL activity (units)
control (no additive)	34.0
L-alanine	37.3
L-aspartic acid	31.6
trans-cinnamic acid	7.18
L-isoleucine	116.5
L-phenylalanine	128.5
L-serine	35.4
L-tryptophan	42.5
L-tyrosine	101.9

[a] Each of these additives was added to the fermentation medium prior to inoculation with *R. glutinis* seed culture. The cells were assayed for PAL activity after 27 h of growth in the yeast extract medium. The details of the medium are mentioned under Materials and Methods. PAL activity is reported in terms of units (nanomoles of L-Phe transformed per minute milligram of dry cells).

DISCUSSION

The availability of a rich and stable enzyme source is a prerequisite for a biocatalyst in commercial applications. In addition to a number of potential applications (*11–14*), PAL mediates the stereospecific formation of optically pure L-Phe (*15–17*) and L-phenylalanine methyl ester (*18*). However, there have been very few reports on stabilization (*10*) and induction (*8*). The present study confirms and further extends the earlier work on *Rhodotorula* PAL stabilization, induction, and determination.

A compilation of PAL activity of different *Rhodotorula* species from earlier work reveals that the enzyme activity varies in the range of 2.0–80.0 units/mg of dry cells (*8, 10–12, 15*). Although not the highest PAL producer, the value of 34.0 units obtained in the present study (**Table 1**) is higher than most values reported before. No significant amount of PAL secretion by *R. glutinis* has been shown to occur, although a related fungus, *Neurospora crassa*, is known to secrete this enzyme during growth (*5*). The absence of secretion of enzyme in the growth medium is advantageous in preventing loss of available enzyme. Ultrasonication treatment of *R. glutinis* whole cells was very effective in enzyme isolation, and we were able to extract about 90% of the cell PAL activity.

The use of stabilizing additives is a common method of maintaining enzyme activity (*10, 25, 26*). Additives are known to stabilize enzymes by either binding to the active form, shifting the equilibrium to that form, or forming a more stable conformation, thereby preventing denaturation (*25, 26*). In the present study (**Table 3**), it was found that polyhydric alcohols (25% glycerol, 5% ethylene glycol, and 1% PEG) gave appreciable protection to PAL and prolonged the PAL activity of stored cells to about 10 weeks. The most pronounced influence was observed with glycerol, and cells retained nearly 37% activity for 10 weeks. On the basis of a study involving the interaction of glycerol with a number of purified model proteins in aqueous solutions, Gekko and Timasheff (*26*) have concluded that the conformation of protein is stabilized due to preferential hydration of protein in the presence of glycerol. Although mere extrapolation of the results of the data obtained with purified proteins on complex cellular systems is not strictly valid, the polyhydric alcohols tested here somehow maybe

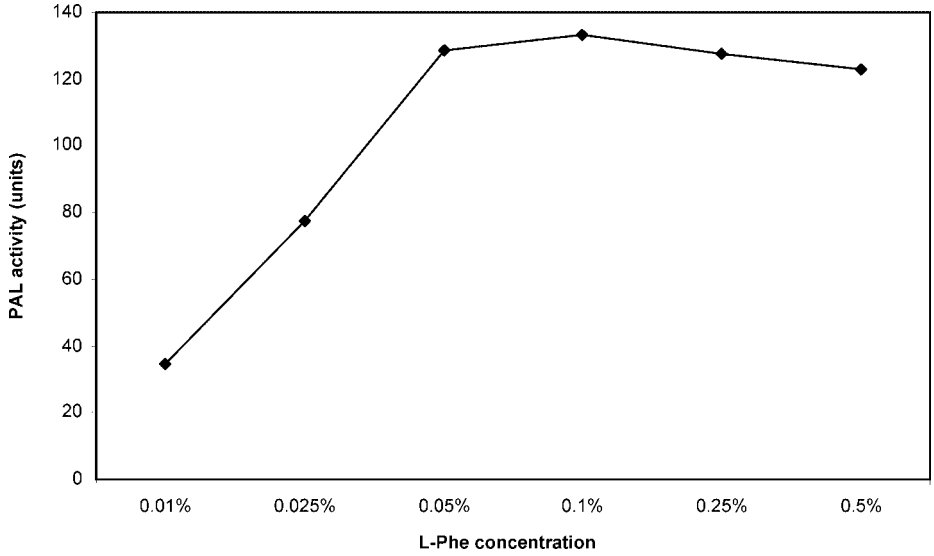

Figure 3. Effect of phenylalanine concentration on the induction of *R. glutinis* PAL. L-Phe was added to the yeast extract fermentation medium prior to inoculation with *R. glutinis* seed culture. The details of the fermentation medium are mentioned under Materials and Methods. The cells were assayed for PAL activity after 27 h of growth in the yeast extract medium. Original PAL activity of *R. glutinis* cells (cells grown in the absence of an additive) was 34.0 units. PAL activity is reported in terms of units (nanomoles of L-Phe transformed per minute per milligram of dry cells).

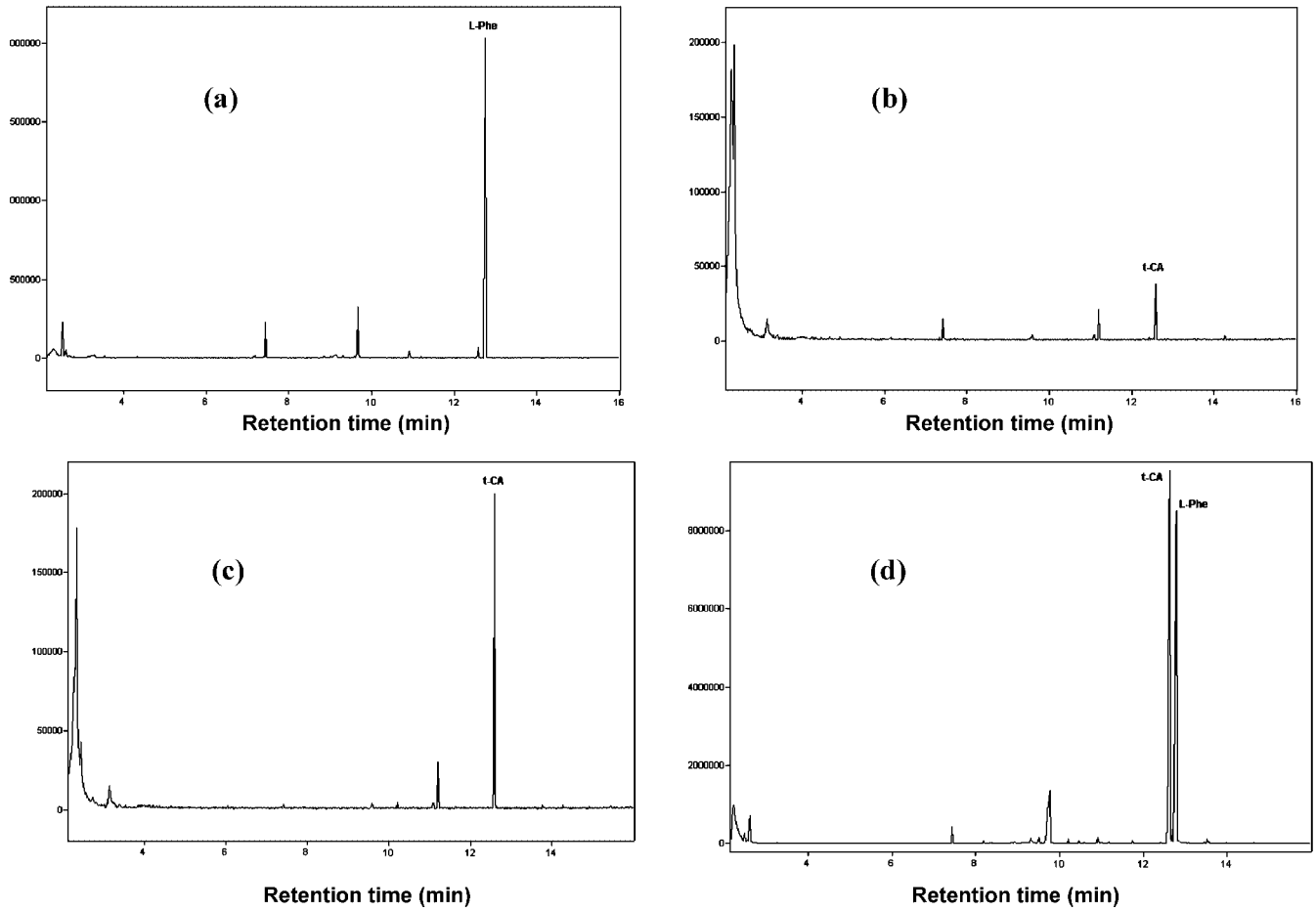

Figure 4. Chromatograms of (**a**) standard L-phenylalanine, (**b**) *trans*-cinnamic acid, (**c**) control, and (**d**) experimental. Retention time of derivatized standards was 12.75 (L-Phe) and 12.60 (*t*-CA) minutes, respectively. In the control, the substrate *t*-CA was not consumed because the enzyme was inactivated by boiling *R. glutinis* cells at 100 °C for 15 min. In the experimental, peaks for L-Phe formed as a result of PAL reverse reaction and unreacted *trans*-cinnamic acids were obtained at 12.75 and 12.60 min, respectively. These retention times are identical to the ones obtained with the standards.

stabilizing the PAL active conformation inside the cells in a similar manner. It is pertinent to mention here that L-Ile, which has been reported to have a stabilizing influence by retarding the decline of PAL activity during growth of cultures (*15*), did not have a pronounced effect on PAL stability during storage.

The most significant and novel finding of the present study

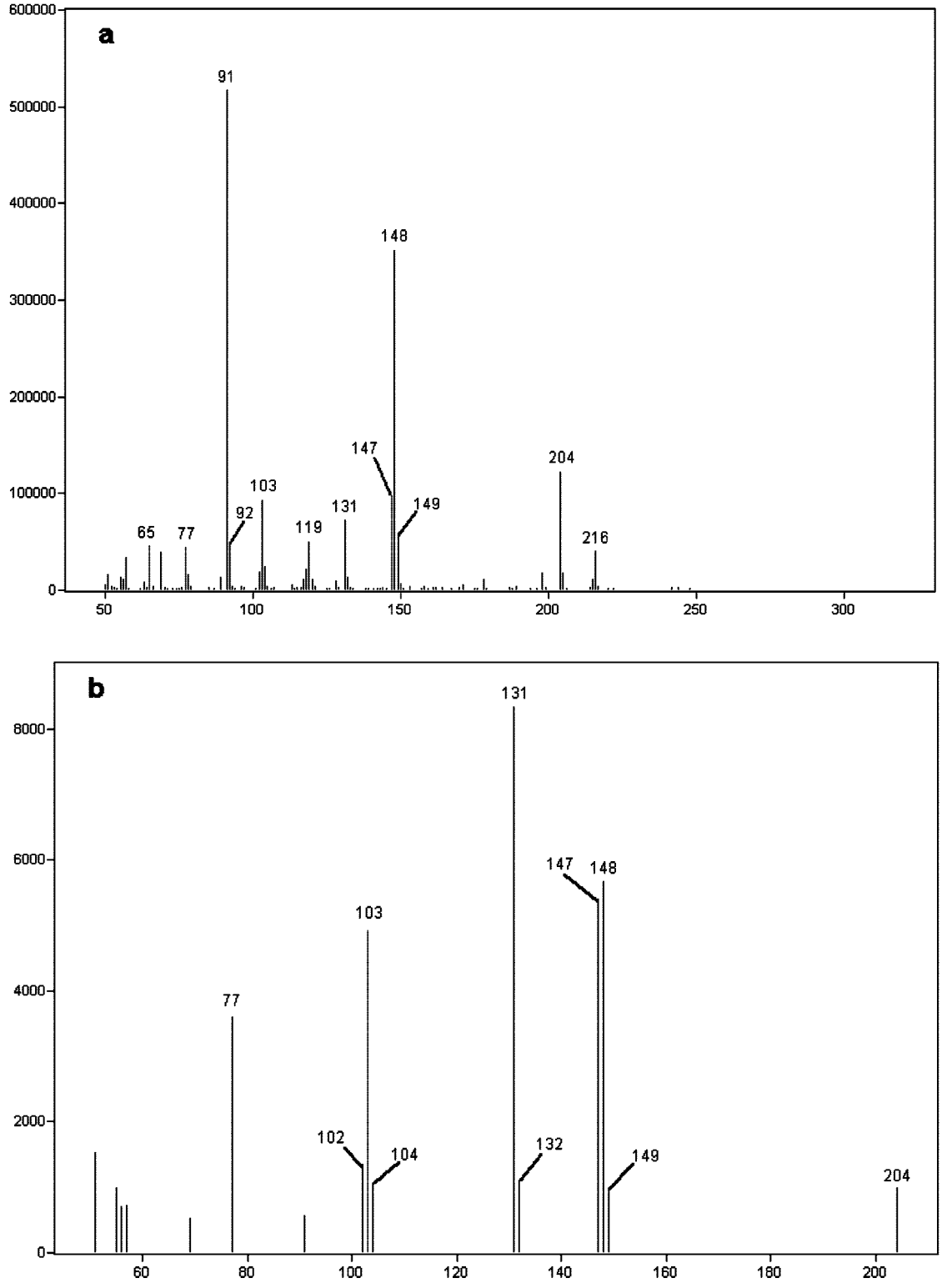

Figure 5. Mass spectra of derivatized standards (**a**) L-phenylalanine and (**b**) *trans*-cinnamic acid.

is the stabilization of PAL (contained in *R. glutinis* cells and in isolated form) during storage by a low concentration of Mn^{2+}. Salts containing Mn^{2+} are readily available and not expensive; therefore, these additives can be effectively used in protein stabilization procedures. Inclusion of 0.01% Mn^{2+} in the storage buffer medium resulted in retention of nearly 85% of the original enzyme activity in the case of PAL containing *R. glutinis* cells (**Figure 1**) and 73% with isolated PAL (**Table 5**) for a period of at least 12 weeks. In their study involving the bioconversion of *t*-CA to L-Phe, Evans et al. (*10*) have reported stabilization of *Rhodotorula* PAL with Mg^{2+} at a concentration of 2 M. It is difficult to interpret their data in light of the present findings because they have chosen a single and rather high (2 M) concentration of the metal ion to test retention of PAL activity. However, it is interesting to note that in the present study, a higher concentration of Mn^{2+} (>0.01%) did not make a significant difference in enzyme stability. It should also be mentioned that in addition to 85% retention of enzyme activity with 0.01% Mn^{2+}, about 65% of the original enzyme activity was retained for 12 weeks when 0.01% Mg^{2+} was present in

the storage buffer (**Table 4**). The role of Mg^{2+} in PAL stabilization reported earlier (*10*) is not known, and we have yet to investigate the possible biochemical mechanism of PAL stabilization by Mn^{2+}. It could be due to the specific stabilization of a more active conformation of the enzyme in the presence of these divalent metal ions. Another unique observation made during the investigation of the influence of metal ions on enzyme stabilization was that K^+, which is known to be cofactor for a number of enzymes, was not very effective in PAL stabilization, and activity was almost negligible in about 6 weeks. The rapid decline in PAL activity in the presence of Cu^{2+} could be possibly brought about by the heavy metal ion binding to essential SH group(s) on the enzyme protein, thereby distorting its biologically active form.

PAL is an inducible enzyme (*7, 8, 10, 15, 27*), and the results obtained from this study (**Table 6**) were in good agreement with earlier findings. Addition of 0.05% L-Phe in the fermentation medium resulted in a 3.8-fold enhancement of PAL activity of the yeast cells. The PAL activity of 128.5 units with L-Phe-induced cells is significantly higher than the values of 35, 50, and 79 units reported by Yamada et al. (*15*), Evans et al. (*10*), and Orndorff et al. (*8*), respectively. Inducible enzymes such as PAL show an increase in activity, as the inducer concentration is increased to a certain concentration when enzyme activity reaches a maximum. In the present study it was found that a higher concentration (>0.05%) of L-Phe did not show significant increase in PAL activity of the cells (**Figure 3**). L-Tyr, which is structurally similar to L-Phe, when present at a concentration of 0.05% in the fermentation medium, gave a 3-fold increase in PAL activity. However, a decrease in enzyme activity was observed when the concentration of L-Tyr was increased beyond 0.25% (data not shown). Because the role of L-Tyr in PAL induction is not known and was not investigated in this study, more work is needed in this direction. A report by Nakamichi et al. (*27*) suggested that other amino acids such as D- or L-isoleucine, D- or L-leucine, L-methionine, L-tryptophan, and L-tyrosine induced PAL activity in *R. glutinis*. Although we obtained a 3-fold enhancement in enzyme activity with 0.05% L-Ile, there was no significant increase in PAL activity in the presence of L-Ala, L-Asp, L-Ser, and L-Trp. It was found that the addition of *t*-CA (in a concentration as low as 0.025%) to growth media resulted in negligible PAL activity of the yeast cells. This is possibly because of the enzyme being inhibited by its end product.

PAL-catalyzed synthesis of L-Phe production using *Rhodotorula* cells has been reported before (*15–17*). However, details of determining L-Phe in these procedures are obscure. A simple, rapid, and highly sensitive GC-MS method with a high resolving power could prove to be attractive for the analysis of PAL reverse reaction, and it would be very useful for studies on the activity, inhibition, and reaction mechanism of this enzyme. The retention time of 12.75 min for L-Phe formed as a result of PAL reverse reaction (**Figure 4d**) is comparable to the retention time of 12.0 min for L-Phe reported by Deng and Deng (*21*) in their study involving the diagnosis of maple syrup urine disease. Unreacted *t*-CA remaining in the experimental sample (**Figure 4d**) gave a distinct peak with a retention time of 12.60 min. The minor peaks seen in the chromatograms of the samples (**Figure 5**) may be due to solvent impurities.

In this study we have demonstrated for the first time the feasibility of stabilizing PAL activity of *R. glutinis* cells and isolated PAL by using Mn^{2+} and induction of the enzyme during growth, and we have optimized the determination of PAL reverse reaction by GC-MS. Our future studies will be aimed at studying the mechanism of stabilization of *Rhodotorula* PAL by Mn^{2+} and Mg^{2+}.

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