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MUTAGENIZED PHENYLALANINE AMMONIA-LYASE FROM YEAST

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Phenylalanine ammonia-lyase (PAL) from yeast has been a major route to the production of L-phenylalanine (L-Phe) [1, 2]. L-Phe biotransformation processes have been established using trans-cinnamic acid, ammonia, and PAL enzyme from *Rhodotorula* [3].

L-Phe is an aromatic ammonia acid with physiological activity. It is one of the essential amino acids in humans. L-Phe is widely used in the food industry, for health protection, and in pharmaceuticals.

A wild-type PAL-containing strain of yeast has been isolated and characterized in the laboratory. The aim of this study was to generate yeast mutants with increased PAL activity to provide more efficient catalysts for the conversion of *trans*-cinnamic acid to L-Phe. In this paper we report the screening and properties of various mutants of PAL-containing yeast.

After treatment of the original strain SA1 with 8-MOP (8-methoxypsoralen), it was irradiated with UV light to determine the kill curve.

As shown in Fig. 1, the strain was rapidly killed with less than 10% survivors after 2 min of exposure.

L-Tyr and L-Phe were used to isolate the mutants. Table 1 shows the result of selection. Mutant PS115 exhibited PAL activity significantly above that of the original strain SA1.

8-MOP, a kind of photosensitive reagent, can increase strain sensitivity to UV light. In combination with UV light, 8-MOP can induce mutagenesis and is mainly used in treating some skin diseases [4]. 8-MOP used in treating yeast has not been reported before. In the study, the use of 8-MOP to induce mutagenesis was an attempt. L-Phe, the substrate of PAL, can induce the synthesies of the enzyme. L-Tyr is an analog of L-Phe and is also a poor substrate for PAL. It has been shown that the affinity of yeast PAL enzyme for L-Tyr is low (high Km Value), and rapid growth on this amino acid can be accommodated by synthesizing more PAL enzyme [2]. A previous report [5] revealed that the mutant exhibited 1.5 times the PAL activity of the original strain with UV irradiation (without 8-MOP). The result of this study showed that the use of the 8-MOP, L-Tyr, and L-Phe selection regime for screening mutants of high PAL enzyme activity was very effective.

Upon successive subculture, the mutant PS115 showed consistently higher PAL activities than the original strain. The properties of mutant PS115 were investigated.

Figure 2 shows the PAL induction profiles of the mutant PS115 as compared with the original strain. At peak PAL synthesis, the original strain SA1 produced 31.97 mU/mL culture, while the mutant PS115 generated over 125.32 mU/mL culture. In mutant PS115, a rapid increase in the synthesis of PAL enzyme was immediately followed by a rapid inactivation period, indicating that the PAL degrading proteolytic enzyme was still active.

Strain and Media. *Rhodosporidium paludigenum*, SA1 was provided by the food fermentation institute of Guangxi University.

PAL-induction (PAL-i) medium: 5g/L L-Phe, 10° Be malt extract.

Minimal medium (g/L):$(NH_4)_2SO_4$ 20; K_2HPO_4 1; $MgSO_4$ 0.2; $FeSO_4$ 0.06; $CaCl_2$ 0.02.

Mutagenesis. The yeast was cultivated in 200 mL of PAL-i medium at 30°C for 48 h with shaking at 160 rpm. Cells were removed and diluted in 0.98% (w/v) NaCl to approximately 5×10^3 cells/mL. An aliquot (10 mL) of suspension was mixed with 1 mL 8-MOP (dissolved in alcohol) and cultivated for 1 h; then the aliquot was poured into the base of a sterile Petri dish. The dish was exposed to UV irradiation (15 W) for a period of 2 min. Aliquots (100 μL) were removed every 30 s and immediately spread on PAL-i agar plates to determine the survival frequency. The plates were incubated at 30°C in darkness for 4 days before counting colonies.

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TABLE 1. PAL Enzyme Activity against Mutant Yeast Colonies and the Original Strain

Strain	PS74	PS110	PS114	PS115	PS120	SA1
$A_{290\ nm}$*	0.318	0.451	0.443	0.455	0.384	0.127
PAL activity (mU/mL)	77.39	109.94	108.04	110.72	93.60	30.57

*Mutants were isolated using L-Tyr and L-Phe selection regime. As Evans et al. reported, *trans*-cinnamic acid has the greatest absorbance at 290 nm; it is the basis of PAL enzyme activity. According to the standard curve of *trans*-cinnamic acid, we can estimate its formation. Enzyme activity was calculated according to the definition of PAL activity.

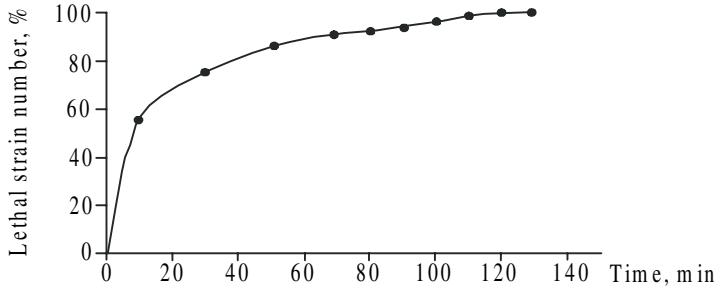

Fig. 1. UV kill curve of SA1.

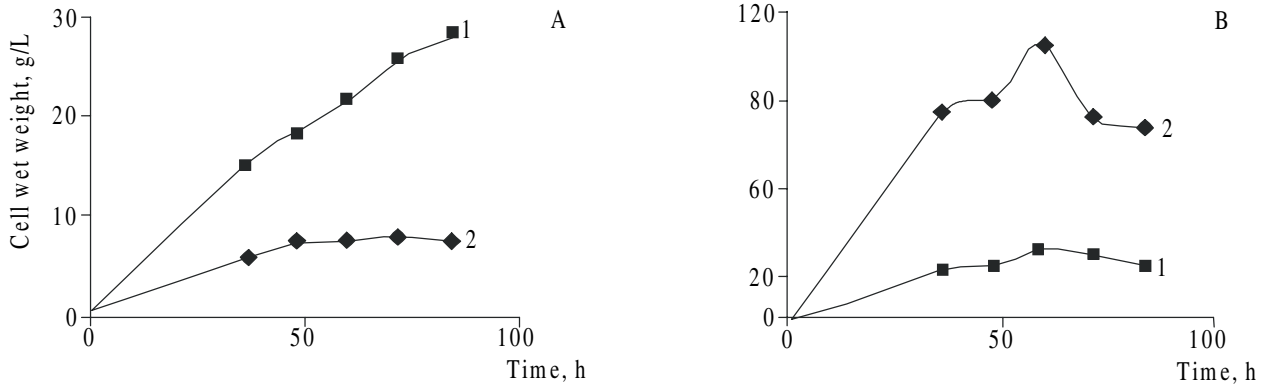

Fig. 2. Comparison of (A) growth rates and (B) PAL activities of mutant PS115 and original strain SA1. Strains were grown in 250 mL flasks with 100 mL of malt extract medium at 30° C and 160 rpm for 3days. The cultures were determined for cell weight and PAL activity.

Isolation of Analog-Resistant Mutants. Colonies from various points on the kill curve were picked after growth for 4 days in the dark. A second selection regime involved inoculation of mutagenized cells into the liquid medium (10 mL) containing 0.5% L-Phe and minimal medium. The cultures were grown for 3 days and then inoculated (4% inoculum) into minimal medium containing 0.2% L-Tyr. After growth for 3 days, the cells were spread on L-Tyr-containing agar plates, and the largest colonies were selected for screening. Colonies that exhibited resistance to analog were selected and inoculated into 100 ml of 10°Be malt extract medium and shaken at 30°C at 160 rpm for 72 h. Cells were harvested after 72 h of growth.

PAL Forward Assay. Cells were resuspended in 50 mM Tris-HCl buffer (pH 7.0) to a final concentration of 0.1g wet weight/mL. The cell suspension was sonicated (50 Hz/sec) for 10 min in ice. Then the suspension was centrifuged. Reaction mixtures containing 0.2 mL cell supernatant, 50 mM Tris-HCl buffer (pH 8.5), and 75 mM L-Phe were incubated at 30°C for 10 min. The reaction was terminated with 6M HCl. The reaction mixture without the substrate served as control. The production of *trans*-cinnamic acid was estimated by its specific absorbance at A_{290}nm, and PAL activity is expressed in units (U), where 1U represents the production of 1 μmol *trans*-cinnamic acid per min at 30°C [6].

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

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