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Rhodotorula glutinis: STRAIN ENRICHMENT AND EVALUATION OF PHENYLALANINE AMMONIA LYASE

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The enrichment of a *Rhodotorula glutinis* strain and the determination of its phenylalanine ammonia lyase (E.C.4.3.1.5 – PAL) activity and attempts to measure peroxidase (E.C.1.11.1.7) activity included conventional mycological procedures along with chemical and microscopic examination. Sabouraud-dextrose medium was found to be the most suitable for cell growth, but cells grown on yeast-extract medium exhibited optimal enzyme activity. Growth and PAL activity were measured in yeast cells grown in yeast-extract broth medium for 24-27 h. The appearance of a reddish pink color associated with the yeast cells coincided with the appearance of appreciable PAL activity. The maximum PAL activity and biomass of yeast obtained in the yeast extract medium ranged from 33 to 35 units/mg dry cells and 7.5 to 8.0 g dry cells/L, respectively. In addition to phenylalanine, *Rhodotorula* PAL also used phenylalanine methyl-ester as a substrate. No peroxidase activity was found in these *R. glutinis* cells.

L'enrichissement de la souche de *Rhodotorula glutinis* et la détermination de l'activité de la phénylalanine ammoniac-lyase (E.C.4.3.1.5 – PAL) chez cette souche, de même que les tentatives de mesure de l'activité de la peroxydase (E.C.1.11.1.7), ont compris l'utilisation de procédures mycologiques traditionnelles ainsi que des examens microscopiques et chimiques. Nous avons constaté que la gélose Sabouraud au dextrose est le meilleur milieu pour assurer la croissance cellulaire, mais que l'activité enzymatique est optimale dans les cellules cultivées sur un milieu à base d'extrait de levure. Nous avons mesuré la croissance de cellules de levure cultivées dans un bouillon à base d'extrait de levure pendant 24 à 27 heures et nous avons mesuré l'activité de la PAL dans ces mêmes cellules. L'apparition d'une couleur rose rougeâtre associée aux cellules de levure a coïncidé avec le début d'une période d'activité notable de la PAL. L'activité maximale de la PAL obtenue dans le milieu à base d'extrait de levure a varié de 33 à 35 unités par mg de cellules sèches, tandis que la biomasse de levure maximale obtenue dans le même milieu a varié de 7,5 à 8,0 g de cellules sèches par litre. En plus de la phénylalanine, la PAL de *Rhodotorula* a utilisé l'ester méthylique de la phénylalanine comme substrat. Aucune activité de la peroxydase n'a été observée dans ces cellules de *R. glutinis*.

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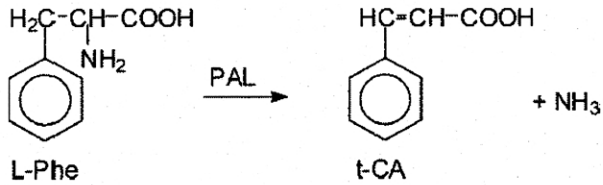
INTRODUCTION

The red basidiomycetes yeast *Rhodotorula* contains a variety of enzymes including phenylalanine ammonia lyase (Koukol & Conn 1961), epoxide hydrolase (Kronenburg et al. 1999), and invertase (Rubio et al. 2002). 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).



The enzyme is widely distributed in higher plants (Koukol & Conn 1961, Jahnen & Hahlbrock 1988, Whetten & Sederoff 1992), some fungi (Kalghatgi & 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, however, absent in true bacteria and animal tissues.

PAL is one of the few non-hydrolytic enzymes that have several commercial applications e.g. treatment of certain mouse neoplastic tumors (Fritz et al. 1976), the quantitative analysis of serum L-Phe in patients with phenylketonuria (Watanabe et al. 1992, Ambrus et al. 1978, Hsia & Holtzmann 1973) and production of L-Phe (Wall & D'Cunha 2006, Yamada et al. 1981, Evans et al. 1986a, b, Hamilton et al. 1985, Evans et al. 1987, Malcolm & Huei-Hsuing 1985, Nelson 1976). Despite its widespread distribution, PAL from *Rhodotorula* yeast has been used exclusively for commercial purposes because of this yeast's high PAL levels and nonfastidious requirements for growth and PAL synthesis (Orndorff et al. 1988, Evans et al. 1987). Peroxidases (E.C.1.11.1.7) are a class of heme-containing enzymes that can be used to reduce hydrogen peroxide while oxidizing a second substrate (Veitch 2004).

Availability of rich enzyme sources is a prerequisite for a biocatalysis in commercial applications. The focus of this paper is to report the results of a study of *Rhodotorula glutinis* strain enrichment and the evaluation of an enzyme of considerable commercial significance: PAL and attempts to detect the presence of peroxidase in this yeast.

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

L-phenylalanine (L-Phe), L-phenylalanine methyl ester (L-PM), trans-cinnamic acid (t-CA), trans-cinnamyl methyl ester (t-CM), cetyl pyridinium chloride (CPC), tris, guaiacol, hydrogen peroxide, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were obtained from Fisher Scientific (Fairlawn, NJ, USA). Malt extract, Sabouraud-dextrose medium, yeast extract, peptone and agar were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England). Commonly used chemicals and reagents of highest analytical grade were purchased from commercial sources and used without further treatment.

Preparation of media

Four different media were used in these studies: 1. Malt extract medium (malt extract media powder dissolved in distilled water to give a final concentration of 5.0%) 2. Sabouraud-dextrose medium (Sabouraud-dextrose media powder dissolved in distilled water to give a final concentration of 6.5%), 3. Yeast extract medium (1.0% peptone, 1.0% yeast extract, and 0.5% NaCl) and 4. Minimal salts medium (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). For the preparation of slants and plates, 3.0% agar was used. Minimal salts medium prepared with distilled water was the only one of these used in broth form. All glassware and media used in enrichment of the strain were sterilized by steam sterilization, 15 psi and 121 °C for 15 minutes.

Pure cultures on different growth media

R. glutinis yeast cells on culti-loop as obtained from the suppliers were transferred to agar slants containing malt extract medium, Sabouraud-dextrose medium, and yeast extract medium. Cells from three-day old slants were streaked on agar plates of each of the three media. After four days incubation at 37°C, morphological (pigmentation, consistency, opacity, etc.) and microscopic examination of individual colonies appearing on each of the three media was carried out. Cells from plates were inoculated in the broth media containing 1) the malt extract, 2) the Sabouraud-dextrose medium and 3) the yeast extract and minimal salts medium. Growth and PAL activity were monitored after the yeast cells were grown in broth media for 24-27 h.

PAL forward assay

PAL forward activity of *Rhodotorula* yeast whole cells was monitored spectrophotometrically by following 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 50.0 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 terminated by inactivating the enzyme with concentrated HCl and separating the cells by centrifugation. 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 by defining one unit of enzyme as the amount required to transform 1.0 nmole of L-Phe/min/mg dry cells at room temperature.

PAL assay using L-phenylalanine methyl ester

The PAL reaction was performed using 37.5 mM L-PM in a 5.0 ml reaction mixture as the substrate instead of L-Phe. The product formed was identified by paper chromatography as follows. At the end of the 10.0 min. PAL incubation period, *R. glutinis* cells were removed by centrifugation (7,500 x g for 5 min). Fifty µl of the clear supernatant fluid along with appropriate standards was spotted on Whatman No.3 paper and developed in a descending system using the organic phase of benzene: acetic acid: water (2:2:1) as the solvent. After the solvent front had traveled 80-85% distance on the paper, the run was stopped and the paper was air-dried. The developed spots in the samples, visible by ultraviolet light, were identified by comparing Rf values with that for the authentic standard. The product of the reaction was quantified by measuring the absorbance of the clear supernatant fluid at 290 nm.

Determination of cell biomass

Growth of *R. glutinis* cells in each of the four media used in this study was compared by measuring the cell biomass. Thirty ml of cultured cells (grown for 24-27 h at room temperature) was centrifuged at 5000 x g for 10 min. The dry weights (45-50°C) of the cell residues were determined until consistent values were obtained. Cell biomass is reported in terms of g dry weight/L.

Peroxidase assay

The *R. glutinis* cells were examined for peroxidase activities by measuring the oxidation of guaicol at 436nm (Veitch 2004). The reaction mixture (3.18 ml) contained 3.0 ml, 50.0 mM phosphate buffer pH 7.0, 30.0 µl H_2O_2, 50.0 µl guaicol and 100.0 µl enzyme. Initially cells suspended in phosphate buffer were used as the enzyme source and in a later trial, cells suspended in 10.0 ml phosphate buffer containing 100.0 µl cetyl pyridinium chloride were sonicated disrupting all whole cells (using Branson 1510 ultrasonic bath for two 15 minute periods). A reaction mixture excluding the enzyme served as the control.

Data analysis

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

RESULTS

Rhodotorula yeasts are known to contain high levels of PAL and have non-fastidious requirements for growth and PAL synthesis (Orndorff et al. 1988, Evans et al. 1987). Cells of the *Rhodotorula glutinis* commercial strain were enriched by conventional mycological procedures which included plating of cells on agar medium, selection of colonies followed by culturing them in liquid growth medium. Colonies appeared pink, opaque, circular and had a smooth consistency. Colonies of the yeast cells grown on malt extract medium and Sabouraud-dextrose medium had a glistening texture, while those on yeast extract medium had a dull texture. The colony margin was entire with a slightly raised, convex elevation. Microscopic examination of individual cells revealed gram positive, elliptical cells with an occasionally visible nucleus. Budding was conspicuous in some cells. Cells did not show any appendages such as cilia or flagella. Growth was most abundant on Sabouraud-dextrose agar (Table 1).

Table 1 Growth of *R. glutinis* cells in different broth media

Medium Tested	Dry Weight (g/L)	Standard Deviation
Minimal Salts	1.80	0.11
Yeast-extract	7.82	1.30
Malt-extract	8.97	0.92
Sabouraud-dextrose	*10.21	0.91

R. glutinis cells were harvested in the late log phase (24 – 27 h) of growth for each medium.

A comparison of PAL activity (Fig 1) of the yeast cells in these growth media showed that although Sabouraud-dextrose medium was the most suitable for growth, cells grown on yeast-extract medium exhibited optimal enzyme activity. It was consistently observed that appearance of a reddish pink color during growth coincided with the appearance of PAL activity. The role (if any) of PAL in pigment formation was not investigated further.

Although the yeast enzyme could be shown to use L-PM as a substrate, PAL activity was only about 50% of that obtained when L-Phe was used (Table 2). A chromatographic attempt to quantify the formation of trans-cinnamyl methyl ester (t-CM) from L-PM as a result of the PAL reaction did not produce conclusive results. However, t-CM could be quantified spectrophotometrically using the absorbance values obtained at 290 nm. PAL activity of the yeast cells was found to be 17.2 units. In this case, one

Table 2 PAL activity using different substrates

Substrate	PAL Activity	Standard Deviation
L-Phe	34.0	1.28
L-PM	17.2	1.35

The results reported refer to the PAL activity of cells grown in yeast extract medium.
PAL activity is reported in terms of nmoles of L-Phe or L-PM transformed/min/mg dry cells.

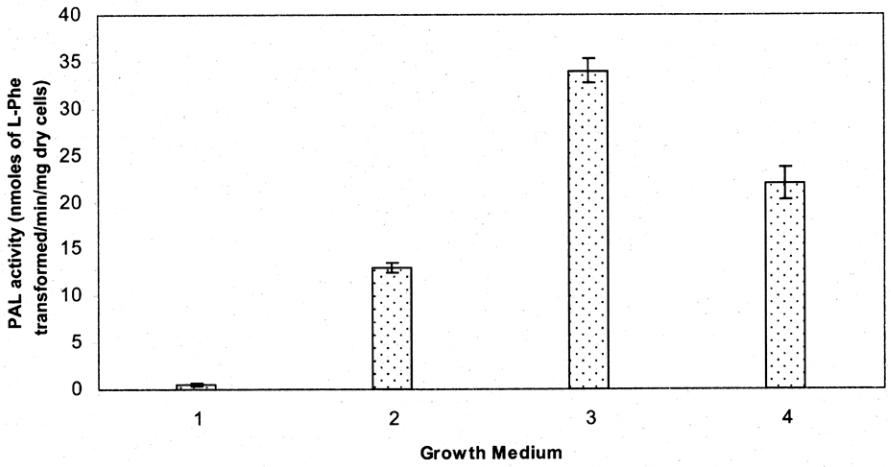


Fig 1 PAL activity of *Rhodotorula glutinis* cells on different growth media: 1. Minimal salts medium (1.0% $(NH_4)_2SO_4$, 0.5% KH_2PO_4, 0.05% $MgSO_4$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, 0.001% NaCl and 2.0% glucose), 2. Malt extract (5.0%), 3. Yeast extract (1.0% peptone, 1.0% yeast extract, and 0.5% NaCl), 4. Sabouraud-dextrose (6.5%).

unit is defined as the amount of enzyme required to transform 1.0 nmole of L-PM/min/mg dry cells at room temperature.

Recently, epoxide hydrolase (Kronenburg et al. 1999) and invertase (Rubio et al. 2002) activity have been demonstrated using *R. glutinis* whole cells. As a spin-off of the main study, the possibility of oxido-reductase (peroxidase) activity in this yeast was tested. The assay did not give any indication of the presence of peroxidase in *Rhodotorula* whole cells or when sonicated cells and CPC treated cells were used as the enzyme source.

DISCUSSION

Biotechnology is increasingly important in the chemical industry. Enzymes are efficient and highly specific catalysts found in living cells and the use of these biocatalysts has dramatically changed many of the chemical processes employed in the pharmaceutical, food processing and chemical manufacturing industries. An important aspect of the biotechnology industry is the enrichment of an organism that produces enzymes of interest and optimization of the conditions for enzyme production by the organism. Although *Rhodotorula* yeast is known to contain high levels of PAL (Evans et al. 1987), there are very few reports on the manipulation of the growth medium of *Rhodotorula* cells to improve enzyme activity (Orndorff et al. 1988). Our study extends earlier work using sonication and various detergents for enriching PAL activity (D'Cunha 2005).

The results obtained indicate that medium composition has a marked effect on growth of the cells and enzyme activity. It is evident from Table 1 that

Sabouraud-dextrose agar supported maximal growth, while yeast-extract medium gave optimal PAL activity (Fig 1). 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 dry cells (D'Cunha 1994). Although not the highest PAL producer, the value of 34.0 units/mg dry cells obtained in this study is higher than most values reported before (D'Cunha 1994). A unique observation was that formation of a pink pigment coincided with the appearance of PAL activity. The role of this pigment in PAL enzyme activity is not known and will be investigated further. The spin-off to detect peroxidase, an enzyme of commercial value, was negative.

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