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Purification and characterization of gallic acid decarboxylase from *Enterobacter spp.* isolated from a region in Rajasthan, India

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Abstract

Gallic acid decarboxylase is a gallate dependent enzyme, which catalyses gallic acid to pyrogallol. Pyrogallol has a number of industrial applications. The enzyme produced by *Enterobacter spp.*isolated from a soil sample collected from a region in Rajasthan, India was purified using DEAE cellulose ion exchange chromatography and Sephadex G-50 gel filtration chromatography. *Enterobacter spp.* was grown in shake flask broth culture in presence of gallic acid as a substrate to induce gallic acid decarboxylase enzyme. Molecular weight of gallic acid decarboxylase was 57 kDa as determined on SDS PAGE. Enzyme was purified to a fold of 15.32 and a yield of 14.48%. Purified gallic acid decarboxylase had maximum activity at a pH and temperature of 7.0 and 35° C respectively. In presence of Mg^{2+} but not any other metal ions, enzyme showed more activity. The decline in activity was observed by adding detergents.

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Introduction

Enzymes are among the most important products obtained for human needs through plants, animals and microbial source. Nowadays, enzymes are important as they find their application in every sector of day to day life, for example milk, pulp, leather pharmaceuticals etc.

Tannic acid, a polyphenol is catalysed enzymatically by tannase to gallic acid and gallic acid decarboxylase further catalyzes gallic acid to py-rogallol (Haslam et al., 1961; Brune and Schink, 1992). Pyrogallol (1,2,3-trihydroxy benzene), has been exploited in a variety of industrial sector, for example, photography, metallurgy, garment industries, leather staining, dying and painting .It has been exploited as an reductant of gold, silver and mercury salts. It is an absorbent of oxygen in its analysis (Yoshida et al., 1982; Kumar et al., 1999). Pyrogallol and several of its analogs nhibit Al-2 (Autoinducer- 2) mediated quorum sensing in *V. harveyi* and are target potent therapeutics (Nanting et al., 2008). Gallic acid decarboxylase activity had been identified in a few species (Grant and Patel, 1969; Yoshida et al., 1982; Yoshida and Yamada, 1985; Gupta et al., 1986; Samain et al., 1986; Krumholz et al., 1987; Nakajima et al., 1992; Haddock and Ferry, 1993). Some bacterial species had been reported to have both tannase and gallic acid decarboxylase activity like *Streptococcus gallolyticus* (Osawa et al., 1995a), *Lonepinella koalarum* (Osawa et al., 1995b), *Lactobacillus plantarum*, *L. paraplantarum*, and *L. pentosus* (Kar et al., 2003) and *Pantoea agglomerans* (Zeida et al., 1998).

A bacterium belonging to the *Enterobacter spp.* had been isolated from soil sample collected from a region in Rajasthan, India (Sharma and John, 2011) that exhibited both tannase activity (Sharma & John 2011) and gallic acid decarboxylase activity (Soni et al., 2012). The presence of GAD activity was confirmed by vanillin assay followed by UPLC method (Soni et al., 2012). Earlier reports on purification of gallic acid decarboxylase have been very few (Nakazima et al., 1992; Zeida et al., 1998). In this report, we describe the purification and characterization of gallic acid decarboxylase from *Enterobacter spp.*

Materials and methods

All chemicals were purchased from Sigma Chemical, USA, and Merck and were of analytical grade. Media for growth and buffers were obtained from Hi-media, Mumbai, India. DEAE cellulose was purchased from GE healthcare, USA and Sephadex G-50 was supplied by Pharmacia Fine Chemicals, Sweden.

Microorganism and culture conditions

Enterobacter spp. had been isolated in the laboratory previously (Sharma and John, 2011) and was used for further in this study. Bacteria were grown in carbon deficient medium (CDM) using 0.2% gallic acid as a carbon source. The culture was maintained on gallic acid agar slants stored at 4ºC and sub cultured in regular intervals of three weeks.
Biomass at large scale was obtained by inoculating 5% of an overnight grown single colony in Luria Bertani (LB) Media to one liter LB media. The cells were grown at 30°C for 20 hours on a shaker. Cells were pelleted by centrifugation at 6,000 rpm at 4°C for 10 minutes. The cells were washed with 30 mM phosphate buffer (pH 6.5) (buffer A) with 1 mM dithiothreitol and 50 mM.

Purification of gallic acid decarboxylase

Gallic acid decarboxylase was purified as per the method given by Zeida et.al (1998) with certain modifications. The purification steps were performed at 4°C. Resting cell suspension was subjected to sonication in an ultrasonicator for 5 minutes with a pulse of 20 seconds on and 7 minutes off and centrifuged at 13,000 rpm for 45 minutes at 4ºC. The supernatant was used a source of crude enzyme. Ammonium sulphate precipitation was used to concentrate the crude enzyme. The fraction of 40-60% saturation with maximum enzyme activity was loaded onto a DEAE-cellulose column (1.5 by 12 cm) equilibrated with buffer A. The elution of bound

proteins was done by gradient mode with increasing concentration of NaCl (0 to 0.5 M) at a flow rate 5 ml/ min. The eluted fractions were checked for gallic acid decarboxylase activity and active fractions were pooled and stored at 4°C. Pooled samples were loaded on Sephadex G-50 column (bed volume 60 ml). The protein elution was done with buffer A with 1 mM dithiothreitol and 50 mM $Na_2S_2O_3$ and 100 mM NaCl at a flow rate of 0.75ml/min.

12 % SDS- PAGE was run to determine the molecular weight of purified enzyme (Laemmli et al. 1970). Protein concentration was determined by Bradford's method (1976) with bovine serum albumin (BSA) as standard.

Decarboxylase assay

Gallic acid decarboxylase activity was estimated spectrophotometrically using vanillin assay (Swain and Goldstein, 1964) with certain modifications (Srivastava and Kar, 2007). Briefly, the reaction mixture containing 2.0 ml of 50 mM Acetate buffer pH 5.0 and purified enzyme was incubated at 30°C for 10 min and 2.0 ml of 1% vanillin in 70% (v/v) sulphuric acid was used to terminate the reaction. A control was run where enzyme was added after the addition of vanillin and the absorbance at 500 nm was recorded after 18 min of incubation and pyrogallol produced was estimated.

Enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1µmol of pyrogallol per minute.

Effect of pH and temperature on purified enzyme activity

30 mM phosphate buffer with pH variations of 5.5 to 8 was used to determine pH optima of the purified enzyme. To determine the optimum temperature, the temperature range used was from 25°C to 60°C.

Effect of metal ions and additives on stability of purified enzyme

Effect of metal ion such as Ca^{2+}, Mg^{2+} , Mn^{+2}, Ni^{+2}, Fe^{+2} and Zn^{2+} and inhibitors namely SDS, Tween 20, Triton X-100 and EDTA were also studied on enzyme activity using 1mM of metal ions and inhibitors.

Substrate specificity of gallic acid decarboxylase

The substrate specificity of purified enzyme was checked by vannilin assay and pyrogallol produced was estimated. 1mM of each substarte was used in the reaction mixture. The substrates were: benzoic acid, anthranilic acid, protocatechuic acid, 3, 5-dihydroxybenzoic acid, gentisic acid, and gallic acid. The Michaelis-Menten (Km) constant and maximum reaction rate (Vmax) of the purified enzyme was determined based on equation of Lineweaver-Burk.

Results and discussion

Purification of enzyme

The enzyme GAD was purified from its native conditions. Ammonium sulphate precipitation at a fraction of 40-60% showed maximum enzyme activity. The enzyme was purified with a yield of 14.48% and fold purification of 15.32 (Table 1) using various purification procedures. Gallic acid decarboxylase of *Enterobacter sp.* was eluted as a single peak after Sephadex G-50 gel filtration. The molecular mass of gallic acid decarboxylase was found to be 57 kDa on 12% SDS PAGE (Figure.1).

The pH optima of the purified GAD were between 6.5 and 7.5 and maximally active at pH 7 (Figure.2). Below pH 5.5 and above pH 8, the enzyme lost more than 80% of activity. Previously purified GAD was stable at pH range of 6 and 6.6 (Grant and Patel, 1969; Yoshida et al., 1982; Nakazima et al., 1992; Zeida et al., 1998). The optimum pH was in accordance with other bacterial decarboxylase (Grant and Patel, 1969; Hsu et al., 1990; Liu et al., 2007; He et al., 1995).

The optimal temperature of purified GAD was 35°C (Figure. 3). Zeida et al. (1998) reported 50°C and Nakazima et al. (1992) 45° C as optimal temperature of the purified enzyme. A number of decarboxylases are stable upto 50° C.

Table. 1: Purification scheme for gallic acid decarboxylase from *Enterobacter spp.*

Purification Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Extract	984	5215	5.3	1	100
$(NH_4)_2SO_4$ (40-60%)	160	3924	24.4	4.6	75
Dialysis	43.1	1646	38.2	7.2	31.6
DEAE Cellulose	19.2	1292	67.3	12.7	24.8
Sephadex G-50	9.3	755.4	81.23	15.32	14.48

Table 2. Substrate specificity of gallic acid decarboxylase.

Substrate(1mM)	Relative activity (%of control)
Gallic acid	100
Gentisic acid	0
3, 5-Dihydroxybenzoic acid	12
Protocatechuic acid	59
Anthranilic acid	0
Benzoic acid	0

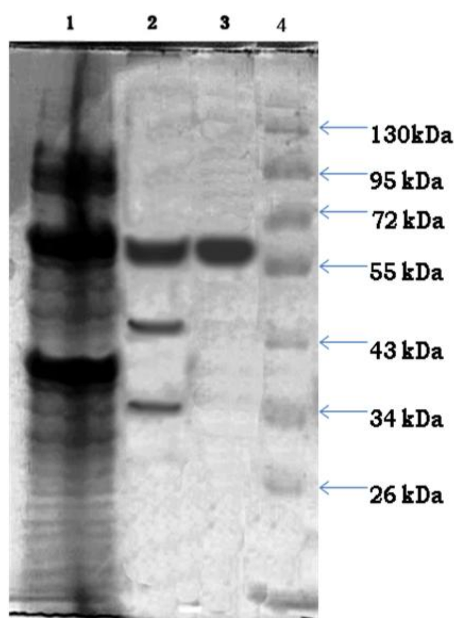

Fig. 1. SDS-PAGE of purified gallic acid decarboxylase from *Enterobacter spp.* Lane 1: crude cell extract, Lane 2: 50 ug of protein from DEAE ion exchange purification step, Lane 3: 10 µg of purified enzyme, Lane 4: Molecular markers.

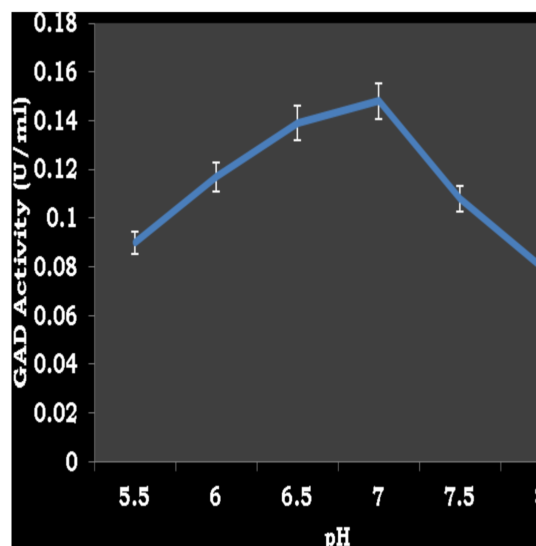

Fig. 2. Effect of pH on purified gallic acid decarboxylase activity.

Stability of enzyme

In crude extract the enzyme was stable stored at 4ºC for a week. However the purified enzyme lost its activity to less than 50% when stored at 4ºC in 9 days. But in presence of 1mM ascorbate as a stabilizing agent, the enzyme retained 87% of its activity till 10 days. Zeida et.al (1998) had reported that the activity of enzyme reduced to 76% even after addition of ascorbic acid within 3 hours.

Effect of metal ions, detergents and chemical additives

As shown in Figure. 4, detergents and additives inhibited the activity of GAD enzyme. The study shows that in presence of detergents like Triton X 100 and Tween 20 and other additives the enzyme lost its activity. So, for maximal activity we need to remove detergents from the buffer and other chemicals used. A number of oxidants (K_2CrO_4, $(NH_4)_2S_2O_8$, H_2O_2) totally inhibits the enzyme activity (Zeida et al., 1998). Some other compounds partially inhibit enzyme activity (Zeida et al., 1998). Of the metals, only in presence of Mg^{++} the activity enhanced to 135% with respect to purified enzyme. And in presence of all other metal ions, no stimulation was observed. (Figure.5). In general, aromatic decarboxylase have no co factor requirement (Grant and Patel, 1969; Nakazawa and

Hayashi, 1978; Yoshida et al., 1982; Pujar and Ribbons, 1985; Samain et al., 1986; Krumholz et al., 1987; Jones, 1992; Nakazima et al., 1992; Haddock and Ferry, 1993; He and Wiegel, 1995; Santha et al., 1995; He and Wiegel, 1996; Santha et al., 1996). Zeida et al. (1998) had reported that Fe^{++} increased specific activity of the GAD enzyme in *P. agglomerans*. Also Brune and Schink (1992) report the requirement of Mg^{++} in *Pelobacter acidigallici*. We also found the same requirement of Mg^{++} for enhancing enzyme activity. In presence of Fe^{++} enzyme was only 68% active (Figure.5).

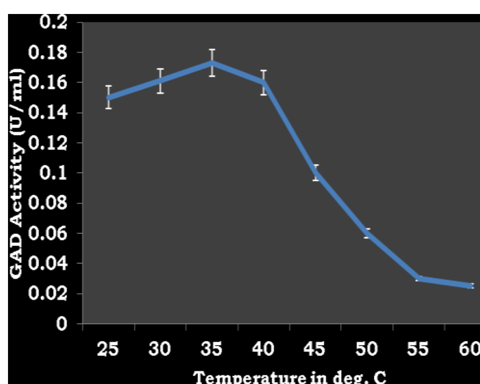

Fig. 3. Effect of temperature on purified gallic acid decarboxylase activity

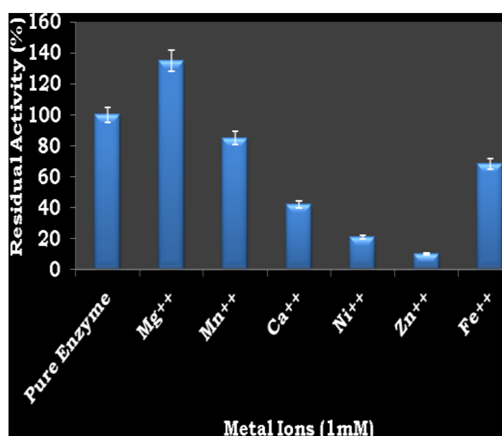

Fig. 4. Effect of Metal ions on purified gallic acid decarboxylase activity

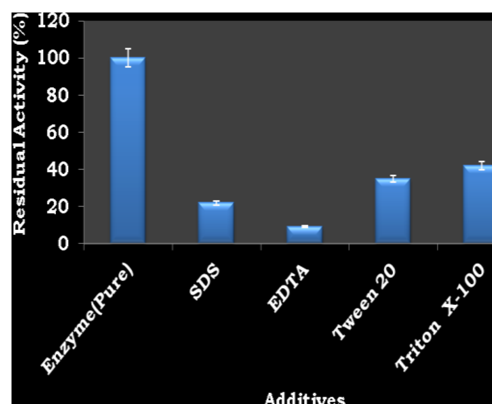

Fig. 5. Effect of detergents and Additives on purified gallic acid decarboxylase activity

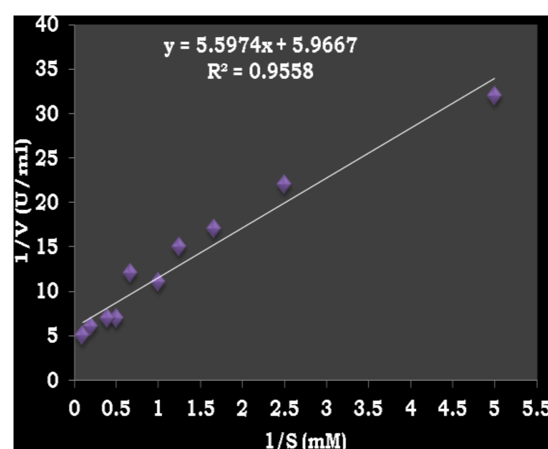

Fig. 6. Vmax and Km using double reciprocal plot

Substrate specificity and K_m

The enzyme could decarboxylate only Protocatechuic acid (59 %) and 3, 5-dihydroxybenzoic acid (12 %) of the five other benzoic acid derivatives checked for the substrate specificity. Protocatechuic acid and 3, 5-dihydroxybenzoic acid had 59 % and 12% enzyme activity relative to control (gallic acid) (Table 2). The result is consistent with previous reports (Yoshida and Yamada, 1985. Other derivatives were not able to activate enzyme carryout decarboxylation of the substrate. K_m and V_{max} of the purified enzyme were 0.934 mM and 0.167 U/ml respectively (Figure.6). K_m was approx. same as reported earlier but V_{max} was much lower (Zeida et al., 1998).Yoshida et al. (1982) had reported 3.3 mM as K_m of GAD in *Citrobacter sp.*

Conclusions

Based on the results and discussion, it can be concluded that: the enzyme was purified using DEAE cellulose ion exchange chromatography and Sephadex G-50 gel filtration. The purified enzyme was maximally active at pH of 7.0 and the enzyme was active in the pH range of 6 to 8. The molecular weight of purified enzyme was 57 kDa. Pyrogallol has a number of industrial applications, so the purified enzyme from locale bacteria isolate *Enterobacter spp* needs to be exploited at large scale using various molecular biology and engineering techniques.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Bradford MM.1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72,** 248–254.

Brune A, Schink B.1992. Phloroglucinol pathway in the strictly anaerobic *Pelobacter acidigallici*: fermentation of trihydroxybenzenes to acetate via triacetic acid. Archives of Microbiology **157,** 417–424.

Grant DJW, Patel JC.1969.The non-oxidative decarboxylation of p-hydroxybenzoic acid, gentisic acid, protocatechuic acid and gallic acid by *Klebsiella aerogenes* (*Aerobacter aerogenes*). Antonie Leeuwenhoek **35,** 325– 343.

Gupta JK, Jebsen C, Keifel H.1986. Sinapic acid degradation by yeast *Rhodotorula glutinis*. Journal of General Microbiology **132,** 2793–2799.

Haddock JD, Ferry JG. 1993. Initial steps in the anaerobic degradation of 3, 4, 5-trihydroxybenzoate by *Eubacterium oxidoreductans*: characterization of mutants and role of 1, 2, 3, 5-tetrahydroxybenzene. Journal of Bacteriology **175,** 669–673.

Haslam E, Haworth RD, Jones K, Rogers HJ. 1961. Gallotannins. Part I. Introduction: and the fractionation of tannase. Journal of the Chemical Society 1829–1835.

He Z, Wiegel J. 1995. Purification and characterization of an oxygensensitive reversible 4-hydroxybenzoate decarboxylase from *Clostridium hydroxybenzoicum*. European Journal of Biochemistry **229,** 77–82.

He Z, Wiegel J. 1996. Purification and characterization of an oxygensensitive, reversible 3, 4-dihydroxybenzoate decarboxylase from *Clostridium hydroxybenzoicum*. Journal of Bacteriology **178,** 3539–3543.

Hsu TD, Lux MF, Drake HL. 1990. Expression of an aromaticdependent decarboxylase which provides growth-essential CO2 equivalents for the acetogenic (wood) pathway of *Clostridium thermoaceticum*. Journal of Bacteriology **172,** 5901–5907.

Jones M.E. 1992. Orotidylate decarboxylase of yeast and man. Current Topics in Cellular Regulation **33,** 331–342.

Kar B, Banerjee R, Bhattacharyya BC. 2003. Effects of additives on the behavioural properties of tannin acyl hydrolase. Process Biochemistry **38,** 1285–1293.

Krumholz LR, Crawford RL, Hemling ME, Bryant MP.1987. Metabolism of gallate and phloroglucinol in *Eubacterium oxidoreductans* via 3-hydroxy-5-oxohexanoate. Journal of Bacteriology. **169,** 1886–1890.

Kumar RA, Gunasekaran P, Lakshmanan M. 1999. Biodegradation of tannic acid by *Citrobacter freundii* isolated from a tannery effluent. Journal of Basic Microbiology **39,** 161–168.

Laemmli UK. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Liu J, Zhang X, Zhou S, Tao P, Liu J. 2007. Purification and Characterization of a 4-Hydroxybenzoate Decarboxylase from *Chlamydophila pneumoniae* AR39. Current Microbiology **54**(2), 102-107.

Nakajima H, Otani C, Niimura T. 1992. Decarboxylation of gallate by cell-free extracts of Streptococcus faecalis and Klebsiella pneumoniae isolated from rat feces. Journal of the Food Hygienic Society of Japan **33,** 371–376.

Nakazawa T, Hayashi E. 1978. Phthalate and 4-hydroxyphthalate metabolism in Pseudomonas testosteroni: purification and properties of 4, 5-dihydroxyphthalate decarboxylase. Applied and Environmental Microbiology **36,** 264–269.

Nanting Ni, Choudhary G, Li M, Wang B. 2008. Pyrogallol and its analogs can antagonize bacterial quorum sensing in Vibrio harveyi. Bioorganic & Medicinal Chemistry Letters **18,** 1567-1572.

Osawa R, Fujisawa T, Sly Li. 1995a. *Streptococcus gallolyticus*, gallate degrading organisms formerly assigned to Streptococcus bovis. Systematic and Applied Microbiology **18,** 74–78.

Osawa R, Rainey F, Fujisawa T, Lang E, Busse HJ, Walsh TP, Stackebrandt E. 1995b. *Lonepinella koalarum*, a new tannin-protein complex degrading bacterium. Systematic and Applied Microbiology **18,** 368–373.

Pujar BG, Ribbons DW. 1985. Phthalate metabolism in *Pseudomonas fluorescens* PHK: purification and properties of 4, 5-dihydroxyphthalate decarboxylase. Applied and Environmental Microbiology **49,** 374–376.

Samain E, Albagnac G, Dubourguier HC. 1986. Initial steps of catabolism of trihydroxybenzenes in *Pelobacter acidigallici*. Archives of Microbiology **144,** 242–244.

Santha R, Savithri HS, Rao A, Vaidyanathan CS. 1995. 2, 3- Dihydroxybenzoic acid decarboxylase from *Aspergillus niger*. A novel decarboxylase. European Journal of Biochemistry **230,** 104–110.

Santha R, Savithri HS, Rao A, Vaidyanathan CS. 1996. Identification of the active site-peptide of 2, 3-dihydroxybenzoic acid decarboxylase from *Aspergillus oryzae*. Biochimica et Biophysica Acta. **1293,** 191–200.

Sharma KP, John PJ. 2011. Purification and characterization of tannase and tannase gene from *Enterobacter spp*. Process Biochemistry **46,** 240–244.

Soni M, Sharma KP, John PJ. 2012. Characterization of Pyrogallol Production from Gallic acid by *Enterobacter spp*. Journal of Microbiology and Biotechnology Research **2(2),** 327-336.

Srivastava A, Kar R. 2007. A Novel Agroresidue as a Substrate for Tannase and Pyrogallol Production by an *A.niger* Isolate. International Journal of Biotechnology and Biochemistry **3,** 205–212.

Swain T, Goldstein JL. 1964. The quantitative analysis of phenolic compounds. In: Pridham JB (ed) Methods in chemistry.Peragon. Oxford, p. 131-146.

Yoshida H, Tani Y, Yamada H. 1982. Isolation and identification of a pyrogallol producing bacterium from soil. Agricultural and Biological Chemistry **46,** 2539–2546.

Yoshida H, Yamada H. 1985. Microbial production of Pyrogallol through decarboxylation of gallate. Agricultural and Biological Chemistry **49,** 659–663.

Zeida M, Wieser M, Yoshida T, Sugio T, Nagasawa T. 1998. Purification and characterization of gallic acid decarboxylase from *Pantoea agglomerans* T71. Applied and Environmental Microbiology **64,** 4743–4747.