Characterization of Pyrogallol Production from Gallic acid by Enterobacter spp.

Manish Soni\textsuperscript{a}, Kanti Prakash Sharma\textsuperscript{b} and P. J. John\textsuperscript{a}\textsuperscript{*}

\textsuperscript{a}Centre for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur, India
\textsuperscript{b}Department of Biotechnology, FASC, Mody Institute of Technology and Science, Lakshmanagarh, Sikar, Rajasthan, Iran

ABSTRACT

A bacterial strain, isolated from soil, and identified as Enterobacter spp., showed an inducible gallic acid decarboxylase activity producing pyrogallol from gallic acid. The aim of this study was to optimize the culture conditions for pyrogallol production in Enterobacter spp. induced by gallic acid. Bacteria were grown in carbon deficient medium (CDM) using 0.2% gallic acid as a carbon source. The maximal production of pyrogallol was observed when the bacterium was cultured at 30°C for 20 hrs in a medium containing 0.2% gallic acid, 30 mM phosphate buffer at pH 6.6, 0.4% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and 0.05% MgSO\textsubscript{4}. The other parameters optimized were incubation temperature, agitation speed and Inoculum size. Gallic acid degradation and pyrogallol formation was determined by UPLC.

Keywords: Enterobacter spp.; GAD Activity (Gallic acid decarboxylase Activity); Pyrogallol; UPLC (Ultra Performance Liquid Chromatography).

INTRODUCTION

Pyrogallol (1,2,3-trihydroxy benzene), a polyphenol has been exploited in a variety of industrial sector, for example, as a developer in photography, to make colloidal solutions of metals, as a mordant for wool, for staining leather, in process engraving, in the manufacture of various dyes, and in the dyeing of fur, hair, etc. In analytical chemistry it is used as a reagent for antimony and bismuth. It is an active reducer for gold, silver and mercury salts. In gas analysis it is used for absorption of oxygen [1,2]. Pyrogallol is the end product in ruminal bacteria like Selenomonas spp. and Streptococcus spp. [1,3,4,]. Chemically, pyrogallol is produced using gallic acid in presence of 6 N HCl [5] which additionally results in formation of huge amount of salts. So, biological conversion of gallic acid using gallic acid decarboxylase enzyme activity exhibited by microorganisms has been exploited. In addition to it, the specificity and eco-friendly nature add values to the biological conversion.

Tannase enzyme initiates tannic acid degradation to gallic acid. Gallic acid decarboxylases catalyze the second step in the degradation of the polyphenol tannic acid [6, 7], the decarboxylation of gallic acid to pyrogallol (Fig. 1). A few species had been identified exhibiting gallic acid decarboxylases activity [2,8, 9, 10, 11, 12, 13, 14,].

Some bacterial species identified are unique in having both tannase and gallic acid decarboxylases activity like Streptococcus galollitycicus [15] Lonepinella koalarum [16], Lactobacillus plantarum, L. paraplantarum, and L. pentosus [17], Pantoea agglomerans [5].

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A bacterium belonging to the Enterobacter spp. which had been previously isolated in our Lab. [18] could grow in the presence of gallic acid and accumulate pyrogallol. Enterobacter was found to have both tannase activity[18] and gallic acid decarboxylase activity.

Despite its vivid biotechnological and industrial applications, studies on gallic acid decarboxylases and microbial production of pyrogallol have been very few.

So, keeping in mind the industrial application of the enzyme, the present study was carried out. UPLC was used to detect substrate and product formed by the GAD enzyme. Culture conditions were optimized for maximal production of pyrogallol using Gallic acid as a substrate using biochemical assays.

**MATERIALS AND METHODS**

2.1 Chemicals:
All chemicals unless specified otherwise were obtained from Sigma Chemical, USA, and Merck and were of certified reagent grade. Media for growth and buffers were purchased from Hi-media, Mumbai, India.

2.2 Microorganism and growth conditions:
A bacterium isolated in the laboratory previously [18] and identified as Enterobacter spp. was used in this study. The culture was maintained on gallic acid agar slants stored at 4°C and sub cultured in regular intervals of three weeks. For each experiment a single colony was grown at 30°C for overnight in Luria Bertani (LB) Media and 5% of this culture was resuspended in Carbon Deficient media (CDM or production medium) supplemented with 0.2% gallic acid as carbon source incubated at 30°C for 20 h to for further assays. The CDM includes 30 mM phosphate buffer pH 6.6, 0.06% MgSO4, 0.4% (NH4)2SO4, and 0.2% gallic acid.

2.3 Optimization of culture conditions for pyrogallol production by spp. Enterobacter induced by Gallic acid.
The production medium (CDM) was optimized for the following parameters taking each parameter in triplicate. Each parameter was optimized by keeping other parameters constant and varying one each time.

Maximal pyrogallol production was determined for incubation period (4 h-24 h), substrate conc. (1% -5%), buffer system and buffer concentration, pH (4.5-6.5), nitrogen source, metal ions and concentration, temperature (25°C-45°C), agitation speed(50rpm – 200rpm), and inoculum size (1%-5%) v/v.

2.4 Analytical methods
Gallic acid and pyrogallol were analyzed with a Waters Acquity UPLC equipped with a BEH C18 column (1.7um 2.1 x150mm); 10 mM KH2PO4-H3PO4 (pH 2.8)-acetonitrile (99:1, vol/vol) a flow rate of 0.3 ml/min was the eluent, and the eluate was monitored using PDA e detector at 254nm. Authentic gallic acid and pyrogallol were used for calibration. The retention times of gallic acid and pyrogallol were 2.428 and 2.918 min, respectively. The column was maintained at 40°C and the samples at 15°C and the Empower pro Software was used for analysis.
2.5 Biochemical Assays

**Pyrogallol Estimation and gallic acid decarboxylase Assay**

Pyrogallol and Gallic acid decarboxylase activity were estimated spectrophotometrically using vanillin assay [19] with certain modifications [20].

To a 1.0 ml of sample, 2.0 ml of 1% vanillin in 70 % (v/v) sulphuric acid was added and the color developed after 18 min was read at 500 nm against a reagent blank. The concentration of pyrogallol was determined from a standard graph plotted with authentic pyrogallol.

To estimate gallic acid decarboxylase activity, the reaction mixture containing 2.0 ml of 0.05 M Acetate buffer pH 5.0 and 1.0 ml of the fermented broth was incubated at 30°C for 10 min and the reaction was stopped with 2.0 ml of 1% vanillin in 70% (v/v) Sulphuric acid. A control was run where enzyme was added after the addition of vanillin and the absorbance was recorded at 500 nm after 18 min of incubation and pyrogallol produced was estimated. Gallic acid decarboxylase activity was defined as the amount of enzyme that catalyzed the formation of 1µmol of pyrogallol per minute.

**RESULTS AND DISCUSSION**

3.1. Screening of spp. *Enterobacter* for pyrogallol production

*Enterobacter* spp. was screened for gallic acid decarboxylase enzyme on CDAM amended with gallic acid as substrate. The appearance of colonies and the formation of zone of clearance provided preliminary confirmation for the presence of gallic acid decarboxylase enzyme in the isolate.

3.2. Characterization of gallic acid degradation by UPLC

A HPLC method given by Zieda [5] was slightly modified for UPLC analysis. UPLC chromatogram (Fig 2, (a, b, c, d)) shows the time course of gallic acid degradation and production of pyrogallol at different time intervals by bacteria. The maximum amount of pyrogallol production was at 20 hours of sample incubation (Fig.2 (d)).
Fig. 2 Chromatograms of extracts from cultures of *Enterobacter* spp. grown in CDM medium at beginning (a), after 8 hours (b), after 16 hours (c) and 20 hours of incubation (d). Gallic acid and pyrogallol showed a retention time of 2.428 and 2.918 min, respectively.

Biochemical assay also showed maximum activity at 20 hours of incubation (Fig. 3). Quantitatively, the amount of pyrogallol at various time period was 10.54 ug, 13.33 ug, 13.58 ug, 15.81 ug at 4, 8, 16 and 20 hours. Quantification of pyrogallol produced was done by using a standard curve plotted for various concentration of standard pyrogallol injected.
3.3. Optimization of culture conditions for pyrogallol production by *Enterobacter* spp. using Biochemical assay.

3.3.1. Time course of study

The time course of pyrogallol production by *Enterobacter* spp. was monitored. The maximum gallic acid decarboxylase enzyme activity was observed at 20 hours culture 0.193 U/ml and the maximum pyrogallol production 190 µg/ml released at 20 hour’s culture (Fig. 3).

Yoshida [14] observed maximum pyrogallol production in *Citrobacter* spp. at 48 hours of incubation. Zeida [5] reported that *P. agglomerans* produced high amount of gallic acid decarboxylase in enriched media after 24 hours under reciprocal shaking conditions.

3.3.2. Effect of different concentration of gallic acid and phosphate buffer on extracellular protein and gallic acid decarboxylase activity

Five different concentrations of gallic acid as a carbon source - 0.1%, 0.2%, 0.3%, 0.4%, 0.5% were tested for gallic acid decarboxylase production in *Enterobacter* spp. Among them 0.2% supported a maximum gallic acid decarboxylase activity of 0.156 U/ml with a highest extracellular protein 62.45 µg/ml (Fig. 4).

![Fig.4. Effect of different concentration of gallic acid on GAD activity](image)

The production of gallic acid decarboxylase enzyme diminished with enhanced concentration of gallic acid as carbon source. 0.2% gallic acid supported good growth and gallic acid decarboxylase production. Zeida [5] reported that cultivation of *P. agglomerans* at 3 g/l gallic acid resulted in a better enzyme activity.

Of the Buffer System tested, phosphate buffer gave a maximum gallic acid decarboxylase activity. The maximum enzyme activity was 0.137 U/ml and protien content of 54.67 µg/ml at concentration of 30mM (Fig. 5).

3.3.3. Effect of different pH and temperature on extracellular protein and gallic acid decarboxylase activity

It was evident that the pH significantly influenced the extracellular protein content and gallic acid decarboxylase activity in *Enterobacter* spp. The bacteria released a maximum protein content of 52.45 µg/ml at pH 6.6 and gallic acid decarboxylase activity of 0.131 U/ml (Fig 6).

The pH of the culture medium is critical to the growth, polyphenolic enzymes production and degradation of products. The optimum pH of gallic acid decarboxylase production, as reported in many bacteria, falls between 5- 6.5. In the present study, pH 6.6 was found suitable for the maximum growth and gallic acid decarboxylase production by *Enterobacter* spp.
Enterobacter spp. grown at different temperature – 25°C, 30°C, 35°C, 40°C and 45°C released maximum extracellular protein content of 42.42 µg/ml and maximum gallic acid decarboxylase production of 0.106 U/ml at 30°C (Fig. 7).
3.3.4. Effect of various metal ions on gallic acid decarboxylase production

Iron (Fe$^{2+}$), Copper (Cu$^{2+}$), Magnesium (Mg$^{2+}$), Cobalt (Co$^{2+}$), Manganese (Mn$^{2+}$), Potassium (K$^{+}$) and Zinc (Zn$^{2+}$) ions were tested for extracellular protein and gallic acid decarboxylase production in Enterobacter spp. Among them, Magnesium ion supported the maximum gallic acid decarboxylase production of 0.175 U/ml and maximum extracellular protein content of 70.29 µg/ml (Fig 8).

Of the nine different concentrations - 0.01% to 0.09% of magnesium ion tested, 0.06% supported the maximum gallic acid decarboxylase production of 0.164 U/ml and maximum extracellular protein content of 65.75 µg/ml (Fig 9).

Yoshida [14] obtained maximum decarboxylase activity in Citrobacter spp. when MgSO$_4$ was used at a concentration of .05%. MgSO$_4$ also proved to be a promising inducer for decarboxylase production at 0.5M MgSO$_4$ by Citrobacter freundii TB3 [21]. Zeida [5] used 0.5 g of MgSO$_4$. 7 H$_2$O, and 0.01 g of FeSO$_4$. 7 H$_2$O per liter to induce decarboxylase activity in P. agglomerans T71.
3.3.5. Effect of different nitrogen sources on extracellular protein and gallic acid decarboxylase activity

Three different nitrogen sources - ammonium nitrate, ammonium chloride, and ammonium sulphate were tested for extracellular protein and gallic acid decarboxylase production in *Enterobacter* spp. Among them, ammonium sulphate supported the maximum gallic acid decarboxylase activity. The maximum enzyme activity of 0.101 U/ml and protein released 43.11 µg/ml was at a concentration of 0.04% (NH$_4$)$_2$SO$_4$ (Fig 10).

The most widely used nitrogen sources for bacterial decarboxylase enzyme production are KCl, NH$_4$Cl, NaCl. In the present study, ammonium sulphate favored high decarboxylase production in *Enterobacter* spp.
3.3.6. Effect of Agitation Speed and inoculums size of culture on gallic acid decarboxylase production

At 150 rpm, maximum amount of pyrogallol was produced at 37.92 µg/ml and gave maximum of 0.095 U/ml gallic acid decarboxylase activity (Fig. 11).

![Fig.11. Effect of Agitation Speed on GAD production](image1)

Inocula of various size – 1%, 2%, 3%, 4%, 5% and 6% were tested to inoculate culture flasks containing 100 ml of production medium. At 5% of inocula supported the maximum gallic acid decarboxylase production of 0.057 U/ml and maximum extra cellular protein content of 22.64 µg/ml (Fig 12).

![Fig.12. Effect of inoculums size on GAD production](image2)
CONCLUSION

In view of the results obtained, it is concluded that the isolate was induced by 0.2% gallic acid as a substrate. The optimization of various cultural and nutritional parameters for the maximum production of pyrogallol from spp. *Enterobacter* showed that the enzyme production by this isolate is governed by parameters such as pH of the production medium and other nutritional and physical parameters. Also the UPLC analytical method was a robust method to detect the substrate and product in a time efficient manner, as both compounds were detected in a run time of only six minutes. In future we are interested to scale up the production of pyrogallol by using various recombinant engineering techniques, also test the ability of this enzyme as an anticancer target. Moreover, due to vivid industrial applications of the enzyme, this strain seems to be a prospective organism for further biotechnological exploitation.

Acknowledgement

Financial assistance by ICMR, New Delhi is thankfully acknowledged. CIF, Biotech Centre, UDSC, New Delhi, is acknowledged for providing UPLC facilities.

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