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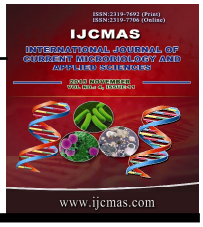


Original Research Article

Bioconversion of Naturally Occurring Tannins into a Value Added Pharmaceutical Intermediate Gallic Acid a New Approach

H.G. Shete* and M.P. Chitanand

P. G. Department of Microbiology, N.S.B. College, Nanded – 431601, India
**Corresponding author*

Keywords

Tannase, Gallic acid, Bioconversion, *Aspergillus niger*

A B S T R A C T

Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic compound, mainly used for the manufacturing of trimethoprim, an antibacterial substance. Gallic acid possesses antioxidant and anticancerous activity also. The global demand for gallic acid is more than eight thousand tons per year. Conventionally gallic acid is produced by acid hydrolysis of tannic acid but it has cost, yield and low purity disadvantages. Alternatively, gallic acid can be produced by the microbial hydrolysis of tannic acid by tannase (tannin-acylhydrolase EC 3.1.1.20), an inducible enzyme, that is secreted by microorganisms. Present project was designed to get Gallic acid using microbial isolates. 13 fungi and 2 bacteria were isolated from natural sources and confirmed as tannase producers. *Aspergillus niger* isolate which was proved efficient was used for gallic acid production. A two step bioconversion method was designed to get gallic acid from natural tannin rich sources. It was observed that 2% concentration of used and dried tea leaves could give 70mg gallic acid crystals per 6ml of substrate solution within 30 minutes conversion period while 4% wheat bran was required to get 70 mg gallic acid in the time period of 30 minutes.

Introduction

Gallic acid, a phenolic compound, chemically known as 3,4,5, trihydroxybenzoic acid is an important raw material, having specific use in the pharmaceutical industry. The global demand for gallic acid is more than eight thousand tons per year. Conventionally gallic acid is produced by acid hydrolysis of tannic acid but it has cost, yield and low purity disadvantages. Alternatively, gallic acid can be produced by the microbial hydrolysis of tannic acid by tannase (tannin-acylhydrolase EC 3.1.1.20), an inducible enzyme, that is secreted by microorganisms. The present day trend is the utilization of waste material for production of byproducts which boosts up high economic returns in many industries. Same strategy is used in designing the present project, with two step bioconversion of tannin rich substance to get Gallic acid, a very important raw material of pharmaceutical. Gallic acid is natural

secondary metabolite present in the tissues of certain plants. Gallnuts, tea leaves, oak barks, blue berries are few examples to cite with. It is normally found in ester forms. Gallic acid is mainly used in the manufacturing of trimethoprim, an antibacterial agent (Kar and Banerjee, 2000). Gallic acid possesses antioxidant and anticancerous activity also. It inhibits histamine release and pro inflammatory cytokine production in mast cell (Sang-Hyun *et al.,* 2005). Microbial production of tannase enzyme on agricultural waste substrate and further its use in the bioconversion of tannins into gallic acid is designed with the biotechnological, environmental as well as cost effective approach. The tannin content in the substrate is degraded by the enzyme tannase into gallic acid and glucose (Paranthaman *et al.,* 2010). Microbial production of tannase, especially from fungi, is well documented (Aguilar *et al.,* 2001), (Aissam, 2005), (Batra and Saxena, 2005), (Mondal *et al.,* 2001), (Treviño-Cueto *et al.,* 2005). *Aspergillus flavus, Penicillium chrysogenum, Trichoderma viride* are commonly reported fungi. However, the reports on tannic acid hydrolysis are limited. Mainly *Aspergilli* have been used for hydrolysis of tannic acid to yield gallic acid (Lekha and Lonsane, 1994; Pourrat *et al.,* 1985, 1987; Seth and Chand, 2000; Vermeire and Vandamme, 1990; Shete, 2011). Among bacteria *Klebsiella pneumoniae* and *Corynebacterium* sp. have been reported to produce gallic acid from crude extract of tara gallotannin (Deschamps and Lebeault, 1984). Banerjee and Mukherjee (2005) had used *Rhizopus oryzea* and *Aspergillus foetidus*, in co-culture method. Few reports are available on the use of powdered fruits of *Terminalia chebula* and pods of *Caesalpinia digyna* as substrate for solid state fermentation for tannase and gallic acid production. Paddy soil was used by Paranthaman *et al.* (2010) while, Bajpai and Patil (2008), has reported use of tannic acid concentrate extracted from *Quercus infectoria* gall nuts in the submerged fermentation.

The main hindrance in the development of a successful bioconversion process is the sensitivity of the microorganisms to tannic acid and the oxidation of the unused tannic acid. This limits the use of high tannic acid concentration during bioconversion process resulting in low productivity. Therefore, we have developed a strategy to overcome the above mentioned problems using screened and acclimatized fungal strains isolated from the soil, and achieve bioconversion of natural tannin in the tea leaves to get gallic acid. Parameter optimization was carried out so as to design a ready to transfer technology.

Materials and Methods

Primary screening for selecting tannase producer

Primary screening for tannase producing microorganisms was processed by enrichment technique. For this 1 gm of sample namely, field soil, used tea leaves powder and wheat flour remains of flour grinder mill collected from areas near and around the city was added in 5o ml nutrient broth for getting bacteria as well as in 50 ml potato dextrose broth for getting fungi. The broths were incubated at 30°C for 48 hours. After incubation the flasks were observed for the growth of organisms. A loopful content from these flasks were streak inoculated on the nutrient agar and potato dextrose agar supplemented with 0.5% tannic acid. The plates were incubated at 30°C for 72 hours and then observed for the development of microbial growth with the zone of tannic acid clearance around the

colony. Similar screening was carried out using other natural sources namely pomegranate peels, tamarind seeds and covers, coffee beans, paddy straws, cotton seed compress, amla leaves and acacia leaves.

Out of all isolates 13 fungal colonies and 2 bacterial colonies with marked clear zone were selected for further work. The cultures were maintained on respective media supplemented with 0.5% tannic acid.

Initially, the tannin hydrolysis ability of selected isolates was confirmed by spot inoculating the isolates on tannin agar medium having composition yeast extract 1%, sucrose 1%, sodium chloride 0.5%, tannin 0.5% and agar-agar 2.5%. Further screening was done on tannin agar medium containing 0.5% tannin as the sole carbon source.

Acclimatization of the selected strains for increasing concentration of tannins

Selected fungal isolates were acclimatized to increasing concentrations of tannic acid from 0.5% to 20% in yeast extract supplemented medium. After each step the ability of organism to hydrolyze tannic acid was confirmed by allowing their growth in minimal medium containing K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $CaCl_2.7H_2O$, $MnCl_2.6H_2O$, $NaMoO_4.2H_2O$ and $FeSO_4.7H_2O$ supplemented respective amount of tannic acid.

Selection of natural tannins

After selecting proper fungal strains with ability to hydrolyze tannic acid at high concentration of 20%, various natural sources of tannin were collected. The sources were decided according to available references. Pomegranate peels, tamarind seeds and covers, coffee beans, paddy straws, cotton seed compress, amla leaves and acacia leaves were collected from Nanded city and around, while the major ingredient used i.e. used tea leaves were collected from different hotels in the city. All materials were dried and grinded. They were sieved before use.

Tannase production

In the initial stage tannase production was studied in production medium containing 0.5% tannic acid. The medium was formulated with the addition of phosphate, magnesium, potassium and chloride according to the need of fungal growth. Inoculum was developed in potato dextrose broth incubated at 30°C for 72 hours. The production medium was inoculated with 5% inoculum and allowed to ferment at 30°C for 72 hours. Tannase produced was assayed spectrophotometrically.

In the next step tannase production was carried out using 1% natural source in the production medium. Natural tannin sources used were tea leaves, coffee beans, wheat bran, pomegranate peels, amla leaves, cotton seed compress and tamarind seed powder.

Enzyme assay

Tannin acyl hydrolase (tannase) assay was performed spectrophotometrically as per Lubuchi et al method. 1ml crude enzyme was added to 1ml of 1% tannic acid substrate and the reaction was allowed to take place at 30^oC for 15 minutes. The reaction was terminated by adding 5ml ethanol. Then absorbance was recorded at 300 nm. Enzyme activity as units/ml was calculated using the formula - Units/ml of enzyme = $(A_o - A_s) \times 20.3 \times 1 \times 1.04 / 0.71 \times 0.25 \times 15$ min. where,

A_o is the initial absorbance and A_s is the absorbance of the reaction mixture. One unit of the enzyme activity was defined as the amount of enzyme which hydrolyses 1mM of ester bond in tannic acid per minute. Depending upon the amount of the substrate hydrolyzed, the amount of the enzyme present in the reaction mixture was calculated.

Optimization of parameters for tannase production on tea leaves

Tannase production was optimized w.r.t. pH and temperature of the medium and duration of incubation, to get maximum tannase activity by spectrophotometric method.

Bioconversion of natural tannins in to gallic acid

6ml of crude tannase extract was allowed to react with equal volume of tea extract which is natural tannin rich substrate, for 30minutes. The resultant mixture was then assayed for the presence of gallic acid. Similarly the experiments were performed using wheat flour as the substrate.

Effect of substrate concentration on gallic acid production

Bioconversion process was carried out using extracts of tea leaves as well as wheat flour bran as the substrate with varying concentrations as 0.5%, 1%, 2%, 3% and 4%. Solid state waste treatment process [SSWT] was performed using same substrate moistened with the sterile distilled water. Gallic acid produced was allowed to precipitate from the mixture. The supernatant was decanted and the precipitate was dissolved in warm acidified water. The mixture was then filtered through whatman no. 1 filter paper and dried at 40^0C for overnight period. The faint yellow crystals obtained were then weighed carefully.

Determination of effective duration for bioconversion of tannic acid into gallic acid

The tannase extract was allowed to react with the tannin rich material viz. tea leaves and wheat flour bran for 15 min, 30min, 60min and 90 minutes. The gallic acid crystals were obtained as above and weighed carefully.

Results and Discussion

Isolation of tannase producing microorganisms

13 fungal colonies and 2 bacterial colonies were obtained with marked clear zone of tannic acid hydrolysis. During acclimatization 10 fungal cultures had shown capacity to tolerate tannic acid concentration as high as 20%. They were labeled as F1, F2, F3, F4, F5, F6, F7, F8, F9 and F10. Bacterial cultures however could not be acclimatized above 3% tannic acid concentration in the medium (Table 1).

Determination of tannin hydrolysis capacity of the isolates

Hydrolysis capacity (HC) of the selected isolates is expressed as ratio of diameter of zone of hydrolysis in mm to the diameter of the colony in mm (Table 2, Figure 1) when grown on tannin agar with 0.5% tannic acid for 24hrs, 48hrs and 72 hrs. Cultures F4 and F5 showed highest capacity of tannin hydrolysis with HC 2.09 at 72 hours of incubation.

Tannase production studies

During the tannase production study F4, F5, F9 and F10 proved more efficient than others. pH 6 and temperature 30°C were recorded as the optimum parameters for tannase production by these fungi. A

maximum yield (13.33 gm/l) of gallic acid was obtained with F5 using optimized conditions. This fungal isolate was further used for bioconversion process.

Table.1 Characters of selected fungal cultures on potato dextrose agar plate incubated at 30°C. for 72 hours

Sr. No.	Isolate Code	Spore colour	Mycelium
1	F1	Black	Septate, branched
2	F2	Black	Septate, branched
3	F3	Green	Septate, branched
4	F4	Black	Septate, branched
5	F5	Black	Septate, branched
6	F6	Black	Septate, branched
7	F7	Light Green	Septate, branched
8	F8	Yellowish Green	Septate, branched
9	F9	Dark green	Septate, branched
10	F10	Black	Septate, branched

Table.2 Tannic acid hydrolysis capacity of selected fungal culture

Sr. No.	Isolate Code	HC at 24hrs	HC at 48hrs	HC at 72hrs
1	F1	1.33	1.36	1.43
2	F2	1.16	1.16	1.28
3	F3	1.5	1.15	1.08
4	F4	1.44	1.44	2.09
5	F5	1.20	2.04	2.09
6	F6	1.33	1.39	1.61
7	F7	1.28	1.18	1.13
8	F8	1.44	1.14	1.26
9	F9	1.23	1.26	2.03
10	F10	1.07	1.19	2.06

Table.3 Effect of different concentrations of substrate and reaction time on gallic acid production by *A. niger* isolate

Sr. No.	Reaction time (min)	0.5% [S] Wheat Bran (gallic acid in mg)	0.5% [S] Tea leaves (gallic acid in mg)	2%[S] Wheat Bran (gallic acid in mg)	2%[S] Tea leaves (gallic acid in mg)	4%[S] Wheat Bran (gallic acid in mg)	4%[S] Tea leaves (gallic acid in mg)
1	15	34	60	55	60	70	70
2	30	40	60	60	70	64	80
3	60	68	70	72	70	70	80
4	90	60	60	67	64	65	69

Figure.1 Tannic acid (HC) Hydrolysis capacity by selected fungal cultures

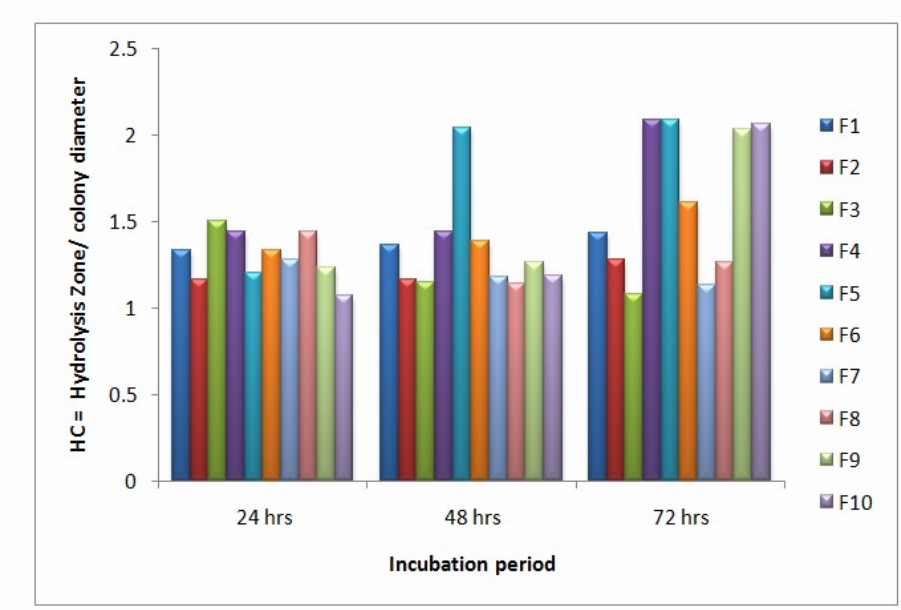
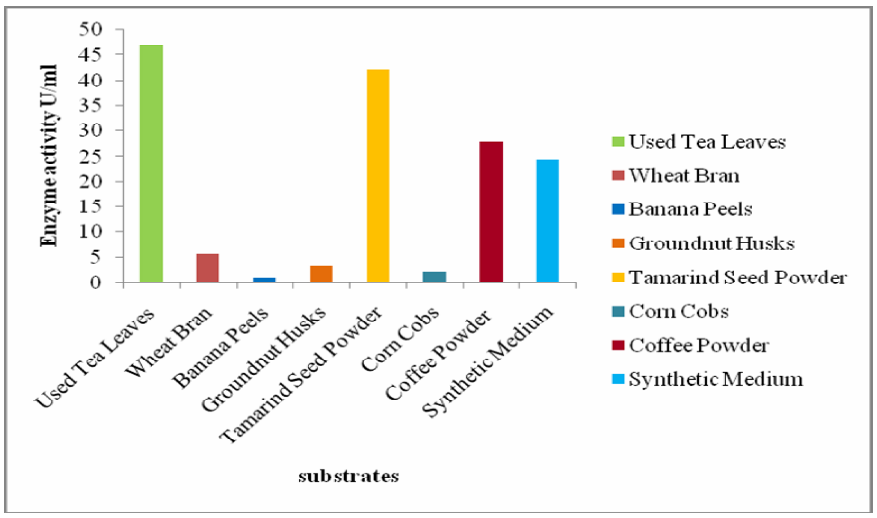


Figure.2 Tannase production on different natural tannin rich substrates



Identification of F5

The isolate F5 was identified as *Aspergillus niger* on the basis of cultural and morphological characters

Bioconversion of tannins from tealeaves and wheat bran

Bioconversion studies showed that maximum i.e. 70mg gallic acid was obtained using tealeaves extract at the concentration of 2% within duration of 30 minutes. The amount did not increase with further increase in incubation period. However same amount of gallic acid was obtained within 15 minutes of conversion period when 4% substrate was used. With 4% substrate concentration 80mg gallic acid was obtained within 30 minutes period. This suggests that a period of 15-30 minutes is enough for tannin conversion using tannase

extract obtained by *Aspergillus niger* fermentation. The same enzyme extract showed effective conversion of tannin at 2% concentration within 30 minutes for wheat substrate (Table 3).

Aspergillus niger isolated during the project period has good ability to produce tannin hydrolyzing enzyme. Thus the isolate can be used as a source of the enzyme for tannin bioconversion process. Two step bioconversion method is easy to handle and can give good yield of the gallic acid. The enzymatic conversion also helped in easy detection and partial purification of gallic acid that can be obtained as faint yellow crystals. The project directs for the microbial production of gallic acid, a value added pharmaceutical intermediate. Second major contribution is the utility of major kitchen waste viz. tea leaves. Its extract contains available amount of tannic acid for microorganisms. Thus it can be a very useful and economical raw material for microbial industries. After using it for extract preparation, remaining part can be further used as fertilizer for the bushes and plants even in the kitchen garden. Further studies are needed to go for the large scale production of gallic acid by two step bioconversion process.

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