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BIO-CONVERSION STUDIES ON GALLIC ACID PRODUCTION FROM CHEBULIC MYROBALAN AND EMBLIC MYROBALAN BY *ASPERGILLUS NIGER* MTCC 281 AND *RHIZOPUS ORYZAE* MTCC 1987.

G.UMAKUMAR[1], MIR NAIMAN ALI[2]*, K. SAMUEL JOHN[3] AND HAJERA TABASSUM[4]

[1] Department of Microbiology, Mumtaz Degree & P.G College,Hyderabad, Andhra Pradesh (India).
[2]Assistant Professor, Department of Microbiology, Mumtaz Degree & P.GCollege, Hyderabad, Andhra Pradesh (India).
[3]Department of Microbiology, Mumtaz Degree & P.G College,Hyderabad, Andhra Pradesh (India).
[4]Researcher, College of Applied Medical Sciences King Saud UniversityRiyadh, (Saudi Arabia).

MIR NAIMAN ALI
Assistant Professor, Department of Microbiology, Mumtaz Degree & P.GCollege, Hyderabad, Andhra Pradesh (India).

*Corresponding author

ABSTRACT

In the present work, bio-conversion studies were performed on gallic acid, an important precursor for pharmaceutical industries engaged in manufacture of trimethoprim. Microbiological hydrolysis of two gallotanin rich substrates Chebulic myrobalan and Emblic myrobalan was studied by two fungal strains *Aspergillus niger* MTCC 281 and *Rhizopus oryzae* MTCC 1987. Comparative studies were performed using shake flask technique in different variations; high yield of gallic acid was produced by *Aspergillus niger* from Chebulic myrobalan.

KEY WORDS

Gallic acid, trimethoprim, myrobalan, bio-conversion, Aspergillus niger, Rhizopus oryzae

INTRODUCTION

Gallic acid is an important precursor for trimethoxybenzaldehyde which in turn is a precursor for the production of trimethoprim. Trimethoprim is a broad spectrum antibiotic, which is largely imported by pharmaceutical companies engaged in trimethoprim synthesis (1). Gallic acid has enormous applications in many fields, including dye-making, pharmaceutical, leather industry, food industry and chemical industries (2). It can be even used in the manufacture of ordinary writing inks and dyes, in photographic development and in the enzymatic synthesis of propylgallate. Besides,in tanning industry, it is used for homogenization of tannins for the production of pyrogallol and gallic acid esters. Gallic acid is used in staining fur, leather and hair (3, 4). Gallic acid is regarded as a non-toxic substance to man. Furthermore, considerable health promoting effects have been ascribed to gallic acid. Gallic acid is characterized as antimicrobial, antioxidative, anticarcinogenic hypotensive, serum lipid reducing, antiatherogenic, anticariogenic and heavy metal chelating agent (5). Gallic acid is also found to show cytotoxic activity against cancer cells, without harming normal cells. Gallic acid, being a sweetener and health promoter, makes it a compound with unique advantages. The current global requirement of gallic acid is around 8,000 tons/year and despite the immense commercial importance of gallic acid little work has been done on development of a process for gallic acid production at fermenter level (6). 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. At present gallic acid is produced industrially by acid hydrolysis of naturally occurring gallotannins (6). Due to high cost, low yield of desired product and production of large toxic effluent by acid hydrolysis, an enzyme based eco-friendly technology for gallic acid production is urgently required.

Chebulic myrobalan and Emblic myrobalan (Amla fruit) are rich source of tannins as they contain about 30-40% gallotannins. Chebulic myrobalan in particular has 18 amino acids, sugars, phosphoric acid, succinic acid. Also it contain more vitamin C and 14 other macro and micro-nutrients such as Se, K, Mn, Fe and Cu (10, 63·5, 32, 30 and 28·5%, respectively). Emblic myrobalan has higher concentrations of most minerals and amino acids such as glutamic acid, proline, aspartic acid, alanine, and lysine in 29.6%, 14.6%, 8.1%, 5.4% and 5.3% respectively. It also contains gallic acid 1.32%; tannins, sugars 36.10%; gum 13.75%; albumin 13.08%; crude cellulose 17.08%; mineral matter 4.12% and moisture 3.83%. Amla fruit ash contains chromium, 2.5 ppm; zinc, 4 ppm; and copper, 3 ppm.

Tannase (Tannin acyl hydrolyses-EC 3.1.1.20) is the enzyme responsible for the bioconversion of hydrolysable tannins (gallotannins) to gallic acid. It is an extracellular, inducible enzyme that hydrolyzes ester and depside bonds only in hydrolysable tannins, releasing gallic acid and glucose (7). Many microbes produce tannase enzyme, especially in fungi, *Aspergillus* and *Rizopus* species are potent producers of tannases (2, 8). Keeping in view the importance of gallic acid and development of an ecofriendly process, the present study was undertaken. Myrobalan is a potential raw material for the production of gallic acid, thus for the present study two myrobalan substrates - Chebulic myrobalan and Emblic myrobalan (Amla fruit) were taken and microbial bio-conversion of gallotannins from Chebulic

myrobalan and emblic myrobalan to gallic acid, by *Aspergillus niger* MTCC 281 and *Rhizopus oryzae* MTCC 1987 was carried out. Comparative studies were performed with two substrates and two fungal strains. If the utilization of these two substrates for gallic acid production and yield of gallic acid can be optimized, it will be of great economic importance for India as India has to import large quantities of gallic acid in order to fulfill its requirement.

MATERIALS AND METHODS

Substrates:

Emblic myrobalan and Chebulic myrobalan were purchased and collected from a local market of Hyderabad. These substrates have high content of tannins about 30-40% .These fruits were dried, milled to get the particle size below 5 mm and used for the present work.

Microorganisms:

In the present work, two fungal strains: *Aspergillus niger* MTCC 281 and *Rhizopus oryzae* MTCC 1987 were obtained from MTCC (Microbial type culture collection centre and Gene bank) Chandigarh, India. Fungal cultures were revived and maintained on Czapek-dox agar slants and plates.

***Inoculum Preparation*:**

For the preparation of inoculum the fungal cultures were plated on Czapekdox agar plates. The plates were incubated at 30^0C for 72 hrs until the mycelium sporulates black conidia. Inoculum was produced in 250 ml Erlenmeyer flasks containing 100 ml Czapekdox broth by transferring 2 discs from the agar plates. The flasks were incubated for another 72 hrs at 30^0C till the mycelial mat develops. This mycelial mat was used as inoculum for Gallic acid Production (8).

***Tannin Estimation*:**

Total tannin content of raw material was estimated, following the procedure given by Haggerman et al (9) with bovine serum albumin (BSA) as protein standard.

Bio-conversion setup for Gallic acid Production:

Milled pieces of Emblic myrobalan and Chebulic myrobalan (5gms each) were added in 100 ml of fermentation medium in 250 ml Erlenmeyer flasks. All flasks were autoclaved at 10 lbs for 20 minutes, cooled and inoculated with inoculum mats and incubated at 30°C in an orbital shaking incubator (90 rpm) for 72 hours. Gallic acid was estimated for every 12 hours. Microbial bio-conversion was set up with three different variations as shown below (Table-1):

Table-1

Set up of Bio-conversion experiments

ORGANISM	VARIATION-1	VARIATION-2	VARIATION-3
Aspergillus niger	EM + DW	EM + CZP	EM + SB + DW
	CM + DW	CM + CZP	CM + SB + DW
	EM + CM+ DW	EM + CM + CZP	EM + CM + SB + DW
Rhizopus oryzae	EM + DW	EM + CZP	EM + SB + DW
	CM + DW	CM + CZP	CM + SB + DW
	EM + CM+ DW	EM + CM + CZP	EM + CM + SB + DW

EM- Emblic Myrobalan; CM- Chebulic Myrobalan; DW- Distilled water; CZP- Czapek dox broth ; SB- Sugarcane bagasse

Estimation of Gallic acid:

Gallic acid in the culture broth was estimated by the method of Bajpai and Patil 1996 (10). Fermented broth was filtered through Whatman No. 1 paper and the filtrate was diluted 500 fold in 0.2 M acetate buffer, pH 5.0. The absorbance was recorded at two selective wavelengths of 254.6 and 293.8nm. The concentration of gallic acid was measured using specific extinction efficient, by the following equation:

Concentration of Gallic acid *(µg*mL-1)

= 21.77 (A254.6) -17.1 7 (A293.8).

RESULTS

Gallic acid production was studied with respect to two gallotannin rich substrates- Emblic myrobalan and Chebulic myrobalan and two fungal organisms *Aspergillus niger* MTCC 281 and *Rhizopus oryzae* MTCC 1987. The fermentation process was monitored for 72 hrs with estimation of gallic acid every 12 hrs. The main focus of the work was to develop an economical and environmentally safe process for gallic acid production and hence comparative studies were performed with three different variations using two fungal strains. Shaking fermentation method was adopted. All experiments were performed in triplicates and mean values were considered. Following results were obtained:

1. Variation -I Substrates with Distilled water:

In Variation-I substrates were used with distilled water without any additional nutrients. With organism *Aspergillus niger* following results were obtained: for Emblic myrobalan gallic acid production steadily increased from 12 hrs to 36 hrs and then slowly declined till 72 hrs with a maximum production of 5.298g/100g at 36 hrs. For Chebulic myrobalan more amount of gallic acid was produced (6.86g/100g) and a similar trend was observed with a maximum production at 36 hrs. When both substrates were used in combination (Emblic+Chebulic myrobalan) gallic acid production increased from 12 hrs till 36 hrs and followed a similar pattern with a maximum yield of 5.328g/100g at 36 hrs. The results are shown in Table-2 and figure-1.

Table-2

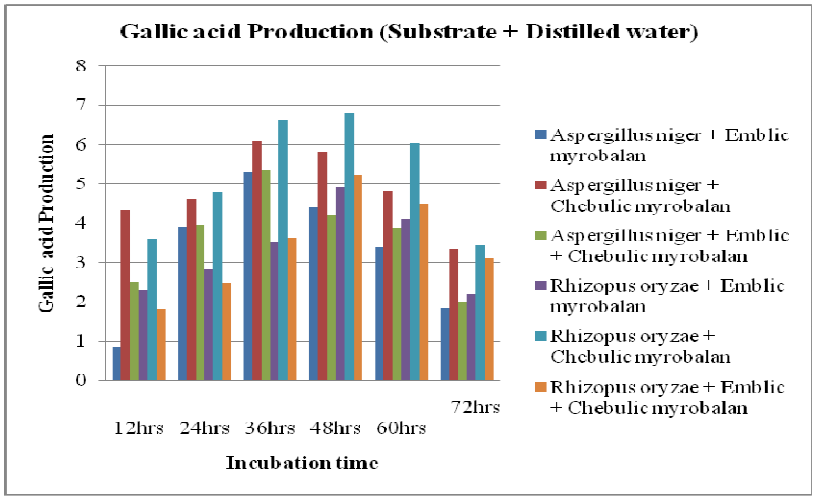
Gallic acid production (gm/100gm) with Substrate + Distilled Water.

Organism	Substrate	12hrs	24hrs	36hrs	48hrs	60hrs	72hrs
Aspergillus niger	**EM + DW**	0.858	3.906	5.298	4.378	3.394	1.84
	CM + DW	4.326	4.598	6.086	5.794	4.802	3.346
	EM + CM+ DW	2.486	3.934	5.328	4.184	3.876	2.005
Rhizopus oryzae	**EM + DW**	2.288	2.822	3.5	4.906	4.106	2.208
	CM + DW	3.588	4.788	6.602	6.78	6.014	3.448
	EM + CM+ DW	1.81	2.479	3.624	5.215	4.502	3.08

EM- Emblic Myrobalan; CM- Chebulic Myrobalan; DW- Distilled water;

With *Rhizopus oryzae* comparatively less amount of gallic acid was produced but the production phase continued upto 48 hrs. For Emblic myrobalan gallic acid production increased from 12 hrs upto 48 hrs and then declined, with a maximum production of 4.906g/100g at 48 hrs. For Chebulic myrobalan highest amount of gallic acid was produced 6.78g/100g at 48 hrs. For Emblic + Chebulic myrobalan combination 5.215g/100g of gallic acid was produced at 48 hrs.

Figure-1
Gallic acid produced in Variation-I



2. Variation -II Substrates with Czapek dox broth:

High yield of gallic acid production was observed in Variation-II. The results obtained for *Aspergillus niger* showed more amount of gallic acid production (Table-3 and figure-2). For Emblic myrobalan similar pattern of increase in production of gallic acid was observed with time as observed for Variation-I. The production increased from 12 hrs to 36 hrs and then slowly declined upto 72 hrs. Maximum amount of gallic acid was produced at 36 hrs with a yield of 4.624g/100g. The amount of gallic acid produced for Chebulic myrobalan (11.205g/100g) at 36 hrs was highest in Variation-II when compared to other two variations. After 36 hrs the production declined. Similarly for Emblic + Chebulic myrobalan combination the gallic acid production increased from 12 hrs to 36 hrs and again declined till 72 hrs. At 36 hrs 8.433g/100g of gallic acid was produced.

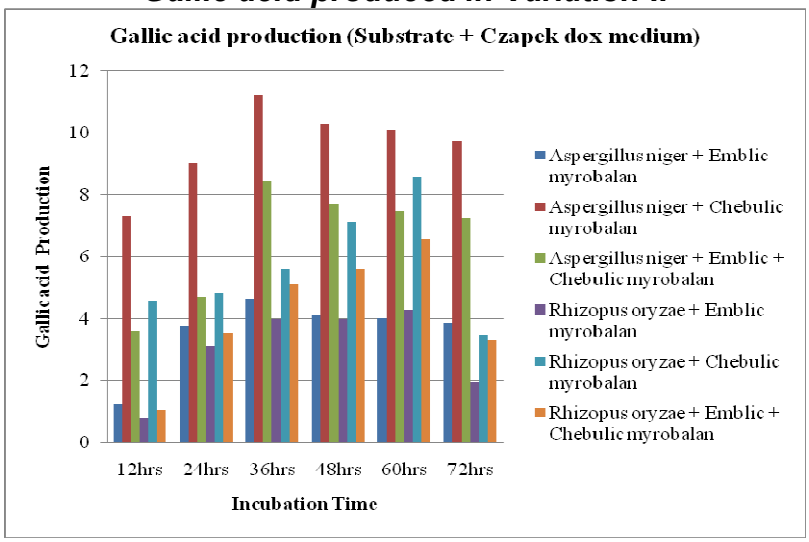
Table -3:
Gallic acid production (gm/100gm) With Substrate + Czapek dox medium.

Organism	Substrate	12hrs	24hrs	36hrs	48hrs	60hrs	72hrs
Aspergillus niger	EM + CZP	1.216	3.728	4.624	4.09	4.01	3.86
	CM + CZP	7.282	8.988	11.205	10.296	10.088	9.724
	EM + CM+ CZP	3.57	4.69	8.433	7.708	7.456	7.219
Rhizopus oryzae	EM + CZP	0.768	3.128	3.972	3.982	4.244	1.908
	CM + CZP	4.53	4.82	5.592	7.126	8.568	3.448
	EM + CM+ CZP	1.036	3.51	5.074	5.594	6.548	3.28

EM- Emblic Myrobalan; CM- Chebulic Myrobalan; CZP- Czapek dox broth ;

With *Rhizopus oryzae* gallic acid production started from 12 hrs and interestingly continued upto 60 hrs and then declined till 72 hrs. For Emblic myrobalan maximum amount of gallic acid was produced at 60 hrs with a yield of 4.244g/100g. For Chebulic myrobalan 8.568g/100g of gallic was produced at 60 hrs and then declined. For Emblic + Chebulic myrobalan combination similar pattern of gallic production was recorded with a maximum production of 6.548g/100g at 60 hrs. The production then declined till 72 hrs.

Figure-2
Gallic acid produced in Variation-II



3. *Variation -III Substrates with Sugarcane bagasse*:

In Variation-III sugarcane bagasse was used along with distilled water in order to stimulate good growth and metabolism of the organism. Interestingly the results obtained are quite encouragive. With *Aspergillus niger* similar pattern of increased gallic acid production rate was observed till 36 hrs and then declined till 72 hrs. For Emblic myrobalan 6.298g/100g of gallic acid was produced at 36 hrs which is maximum production. For Chebulic myrobalan 10.486g/100g of gallic acid was produced at 36 hrs and for Emblic + Chebulic myrobalan combination 7.328g/100g at 36 hrs (Table - 4 and figure-3).

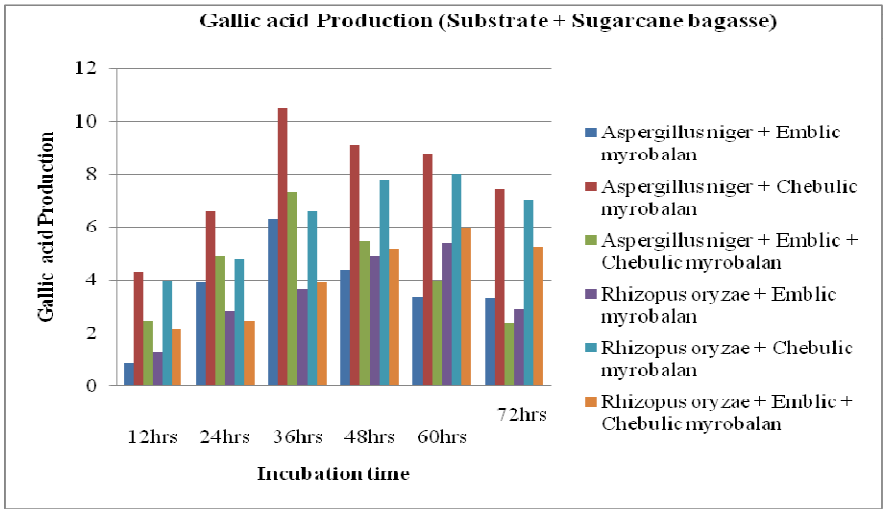
With *Rhizopus oryzae* gallic acid production phase increased upto 60 hrs and then declined. For Emblic myrobalan maximum amount of 5.406g/100g of gallic acid was produced at 70 hrs, followed by 8.012g/100g for Chebulic myrobalan and 6.002g/100g for Emblic + Chebulic myrobalan combination. The results obtained for Variation-III clearly highlight increased production of gallic acid in presence of sugarcane bagasse.

Table-4
Gallic acid production (gm/100gm) with Substrate + Sugarcane baggasse.

Organism	Substrate	12hrs	24hrs	36hrs	48hrs	60hrs	72hrs
Aspergillus niger	EM + SB	0.858	3.906	6.298	4.378	3.394	3.34
	CM + SB	4.326	6.598	10.486	9.094	8.802	7.446
	EM + CM+ SB	2.486	4.934	7.328	5.484	3.976	2.405
Rhizopusoryzae	EM + SB	1.288	2.822	3.667	4.906	5.406	2.908
	CM + SB	3.988	4.788	6.602	7.782	8.012	7.048
	EM + CM+ SB	2.178	2.479	3.924	5.215	6.002	5.28

EM- Emblic Myrobalan; CM- Chebulic Myrobalan; SB- Sugarcane bagasse

Figure-3
Gallic acid produced in Variation-III



DISCUSSION

Gallic acid production was studied by most of the workers using different substrates as it is an important precursor for pharmaceutical industries engaged in production of trimethoprim. N. Lokeswari et al., (1) used seed powder of *Terminalia chebula,* B. Kar et al., (3) used powder of teri pod (*Caesalpinia digyna*) by a solid state fermentation process, N.Y.Sariozlu et al., (4) used Gall nuts and Sumac leaves, N Lokeswari et al., (6) used plant extract of *Anacardium occidentale*, H. Pourrat et al., (11) also used Sumac leaves for gallic acid production, B. Bajpai et al., (12) used dry powder of tannic acid by aqueous extraction of Chinese gall nuts. All these workers extracted tannins from the substrate and then used for gallic acid production, on contrary in our study an attempt was made to use raw substrates in different combinations for bio-conversion studies in order to develop an economical

process. The results obtained in our study are in accordance with the previous studies mentioned above.

When two different fungal strains were used, it was observed that *Aspergillus niger* performed better and produced higher amounts of gallic acid when compared with *Rhizopus oryzae* in all the three variations (Figure-1, 2 and 3). A difference in gallic acid production phase was observed in these two organisms: *A. niger* has given high yield but the production phase continued only up to 38 hrs whereas *R. oryzae* in comparison gave slightly less yield of gallic acid but the production phase continued up to 48 hrs. Another interesting difference in the performance of *R. oryzae* was observed with respect to production phase, when the substrates were used in combination with Czapek dox medium and sugarcane bagasse the ability to produce gallic acid was prolonged up to 60 hrs (Table- 3 and 4 and Figure-2 and 3), giving a clue that both these fungal organisms have the potential to exploit at industrial level for gallic acid production.

Upon comparison of different combinations of substrates used, it was observed that highest amount of gallic acid was produced from Chebulic myrobalan followed by the Chebulic + Emblic myrobalan combination and Emblic myrobalan. These results indicate that when myrobalan fruits are used as substrates, either chebulic myrobalan alone or a combination of chebulic and emblic myrobalan can be used for high yield of gallic acid.

The overall results indicated that when organisms were allowed to grow on raw substrates with distilled water, lesser amounts of gallic acid were produced and when additional nutrients were provided in the form of Czapek dox medium and sugarcane bagasse (Variation-II and III) higher amounts of gallic acid were produced and gallic acid production phase was prolonged for a higher duration especially in case of *R. oryzae* (Table-5 and Figure-4).

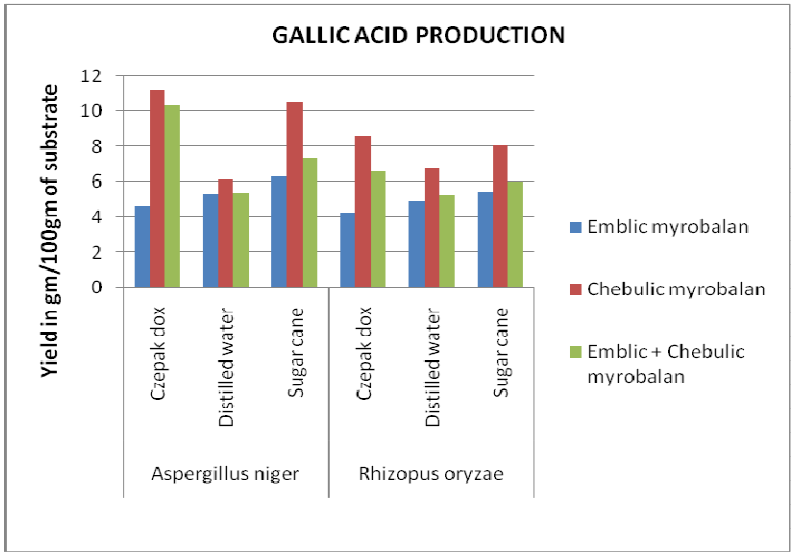
Table-5

Overall results of gallic acid production for three variations using Aspergillus niger and Rhizopus oryzae

Organism	Variation	**Emblic myrobalan**	**Chebulic myrobalan**	**Emblic + Chebulic myrobalan**
Aspergillus niger	**DW**	4.624	11.205	10.296
	CZP	5.298	6.086	5.328
	SB	6.298	10.486	7.328
Rhizopus oryzae	**DW**	4.244	8.568	6.548
	CZP	4.906	6.78	5.215
	SB	5.406	8.012	6.002

DW- Distilled Water; CZP- Czapek dox broth ; SB- Sugarcane bagasse.

Figure-4
Comparison of Gallic acid produced by Aspergillus niger and Rhizopus oryzae in three variations



CONCLUSION

After performing bio-conversion studies with *Aspergillus niger* and *Rhizopus oryzae* and based on the results obtained, we conclude that raw substrates like myrobalan fuits can be used as potential substrates (rather than extracted tannins) for gallic acid production, *Aspergillus niger* is better gallic acid producing strain, shaking fermentation method can be adopted and sugarcane bagasse can also be used for enhanced production of gallic acid.

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