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KEY WORDS: Ellagic Acid; Solid State Fermentation; *Aspergillus niger*; *Rhizopus oryzae*; Pomegranate Husk

BIOCONVERSION OF ELLAGITANNINS TO ELLAGIC ACID FROM POMEGRANATE PEELS BY SOLID-STATE FERMENTATION USING *ASPERGILLUS NIGER* AND *RHIZOPUS ORYZAE*

R. Sundaralingam — Assistant Professor Department Of Microbiology, Madras Christian College (Autonomous),Tambaram.

S. Premina — Assistant Professor Department Of Microbiology, Madras Christian College (Autonomous),Tambaram.

Dr. S. Niren Andrew* — Assistant Professor Department Of Microbiology, Madras Christian College (Autonomous),Tambaram. * Corresponding Author

ABSTRACT

Ellagic acid an effective polyphenol antioxidant found in many fruits and vegetables. The antioxidant properties of ellagic acid have incited preliminary research into the impending health benefits of ellagic acid consumption. In the present research, an attempt is made to extract ellagic acid from pomegranate peels using solid state cultures of *Aspergillus niger*, *Rhizopus oryzae* and mixed cultures of both. The fungus has the potential to convert ellagitannins in pomegranate to ellagic acid as an intermediary metabolite. Test organisms were isolated from soil, identified and screened for production of tannase enzyme. Solid state fermentation of pomegranate husk (10% w/v) using *A. niger*, *R. oryzae* and mixed cultures was performed for the production of ellagic acid. Samples were collected daily from the fermentation broth and physico-chemical analyses like changes in the total tannin content and tannase enzyme assay were performed. After fermentation the production of ellagic acid in the medium was determined using HPLC. The amount of ellagic acid produced was 9.1mg (for *A.niger*), 79.1mg (for *R. oryzae*), 69.6mg (for mixed culture of both *A. niger* and *R.oryzae*).

INTRODUCTION

In recent years researchers' have started to focus on phytochemical compounds produced by plants for their medical property. Among them Ellagic acid is a phenolic compound which exhibit anti-mutagenic and anti-carcin ogenic activity. Ellagic acid was studied in the year 1960s mainly for its effect on blood clotting. With the publication of several works studies in the mid of 1990s, Ellagic acid began to be promoted as a means of preventing and treating cancer.

Ellagic acid is a stable polyphenol antioxidant found in many fruits and vegetables that includes rasberries, strawberries, cranberries, walnuts, pecans, pomegranate and other plant foods. The anti-proliferative and antioxidant properties of Ellagic acid have incited preliminary research into the potential health benefits of Ellagic acid consumption (Krishna Veni *et al.,* 2012). Ellagic acid can be readily absorbed through the gastrointestinal system in mammals. It manages oxidation stress related disease by its direct involvement in quenching free radical from biological system. Ellagic acid can be readily absorbed through the gastrointestinal system in mammals. It manages oxidation stress related disease by its direct involvement in quenching free radical from biological system. In plants, Ellagic acid is present in the form of ellagitannins.

Pomegranate (*Punica granatum* L.) fruits are widely consumed fresh and can be found in commercial products as juice, jam, and wine. Pomegranate fruit husk/peel is a rich source of hydrolyzable tannin belonging to ellagitannins. These ellagitannins are extracted in significant quantitates from the husk in the commercial pomegranate juice industries. Pomegranate fruit peel, a by-product of the pomegranate juice industry, is therefore an inexpensive and abundant source of ellagitannins (Pharkphoom *et al.,* 2010).

Ellagic acid – rich pomegranate fruit peel extract (EARPPE) has antioxidant, anti-inflammatory, anti-mutagen and anti-cancer properties. Preliminary studies have proved that the ellagic showed the anti-cancer activity on cancer cells of the breast, esophagus, skin, colon, prostate and pancreas. EARPPE has also antiviral and antibacterial activities. Extraction of pomegranate peel and recovery of the extract rich in ellagic acid is the most important steps in pomeg ranate extract which possess effective medicinal and phar macological properties. Most of the physical and chemical extraction process of ellagic acid are time consuming, involves lengthy operation technique, usage of bulk amount of solvents and ultimately thermal decomposition of the target molecules at continuous high temperature (Samir Noori and Mohammed Jawad, 2019).

Pomegranate peel is a highly nutritive by-product of pomegranate fruits. It has medicinal properties such as antibacterial, anti-oxidants, anti-cancer activity, anti-atherosclerotic and wound healing properties (Rajan *et al.,* 2011).

Chemical extraction of ellagic acid uses acid-methanol mixture as solvents and concentrated hydrochloric acid or sulphuric acid as mediators to hydrolyze the rich ellagitannin plant materials (Lei *et al.,* 2001; Wilson and Hagerman, 1990), resulting in production of ellagic acid that are highly contaminant, expensive, aggressive and have low yields (Aguiera-carbo *et al.,* 2009).

For this reason, in the last few years, several attempts have been made to develop a bioprocess to produce ellagic acid through fermentation technology.

Bioconversion is a process of conversion of organic materials, such as plant or animal waste, into usable products or energy sources by biological processes or agents, such as certain microorganisms. Ellagitannins are characterized as hydrol ysable conjugates containing one or more hexahyd roxy diphenoyl (HHDP) groups esterified to a sugar, mainly glucose. HHDP groups are released from the main structure leading to spontaneous conversion into ellagic acid by hydrolysis. Ellagic Acid is obtained from the ellagitannins when hexahydroxydiphenic acid (HHDP) group is released by chemical or enzymatic hydrolysis. Ellagitannins can be hydrolysed by various enzymes such as Tannase (tannin acyl hydrolase), Beta glucosidase, Polyphenol oxidase, Xylanase and Cellulase to form Ellagic acid (Aguilera-Carbo *et al.,* 2007).

In view of increasing medicinal properties of ellagic acid, this present investigation is undertaken to produce ellagic acid from pomegranate residues using *Aspergillus niger* and *Rhizopus oryzae*.

These fungi being filamentous in nature have good ability to grow on solid substrate. Also there are several reports that these fungi are resistant to tannins and grow on them as a carbon source. The tannase enzyme produced by the resistant fungi is known to degrade tannin. Deschamps *et al.,* (1983) found that *Aspergillus* sp. as one of the most efficient producer of tannase enzyme. Hadi *et al.,* in 1993 found the synthesis of an extracellular enzyme tannin acyl hydrolase by a newly isolated *R. oryzae*, showing its degradability of tannic acid.

MATERIALS AND METHODS

COLLECTION OF SUBSTRATE - POMEGRANATE PEEL POWDER

Red Pomegranates were collected from local markets, immediately after collection cleaning was done with running water. The peels were separated manually from the fruit, sun-dried and powdered, and then kept at room temperature for further study (Fig 1 and Fig 2).

ISOLATION AND IDENTIFICATION OF TEST ORG A NISMS

Soil samples were collected from various areas of Madras Christian College campus; bird dung and bread were also used for the current study. From these samples, test organisms were isolated by serious dilution and spread plating technique in Sabouraud Dextrose Agar (SDA). The test organisms were identified by Colony morphology and Microscopic examinations by LPCB mount.

SCREENING AND IDENTIFICATION OF TANNASE PRODUCING FUNGI

The isolated fungal strains were sub-cultured on Czapek-dox medium containing 1% (W/V) tannic acid and incubated at 25-27° C for 4 days. The selection is based on the zone of lysis and tannase activity. The identified strains were sub-cultured in Czapek-dox medium plates and incubated at 25-27° C for 72-96 hours. The pure cultures were sub-cultured in Czapek-dox medium containing 1% tannic acid containing tubes.

PREPARATION OF SUBSTRATE FOR SOLID-STATE FERMENTATION (SSF)

50 ml of Czapek-dox liquid medium was prepared Ehrlen-Meyer flasks. 10 g of substrate (processed pomegranate peel powder) was weighed and added to each 50 ml of Czapek-dox liquid medium. pH of the medium was adjusted to 4.5 – 5.0 using HCl or NaOH. Prepared medium was autoclaved at 121° C for 15 minutes.

PREPARATION OF SPORE SUSPENSION

Sub-cultured mycelial growth on Czapek-dox medium containing 1% tannic acid were collected using Tween 80 (0.1%) along with 10 ml of sterile distilled water. The spore suspension was agitated in vortex for 5 minutes and used for inoculum preparation.

INDUCED INOCULUM PREPARATION

100 ml of 1% tannic acid in Czapek-dox liquid medium was prepared in a conical flask and autoclaved. 10 ml of prepared spore suspension was added to Czapek-dox liquid medium and then incubated at room temperature.

SOLID STATE FERMENTATION OF POMEGRANATE SUBSTRATE WITH THE TEST ORGANISMS

Czapek-dox liquid medium were prepared in 4 different Ehrlen-Meyer flasks for 3 different cultures (*A. niger*, *R. oryzae* and mixed culture of both) and one as control. 10 g of substrate (processed pomegranate peel powder) was weighed and added to each 50 ml of Czapek-dox liquid medium and autoclaved. After cooling, 10 ml of the prepared spore suspension of different organisms were added to corresponding medium and incubated at room temperature. Samples were collected daily, filtered, centrifuged and the chemical changes were monitored using standard methods.

QUANTITATIVE ANALYSIS

Quantitative analysis such as determination of reducing sugar (DNS method), soluble protein (Lowry method), extracellular tannase and hydrolysable tannin (Folin-Ciocalteau method) were done on daily basis.

QUANTIFICATION OF ELLAGIC ACID BY HPLC

Samples were filtered using Whatman filter paper (0.2 µm) followed by membrane filtration under suction pump and analyzed by HPLC system using Shimadzu LC 10AT VP. Separation was carried out using Phenomenex C- 18, 250 x 4.60 mm column. The mobile phase consisted of acetonitrile: water (50:50) and the flow rate was 0.5 ml / min. 20 µl of sample were injected and ellagic acid was detected at 280 nm. The separation was carried out simultaneously for standard and controls.

RESULTS AND DISCUSSION

Ellagic acid is a naturally occurring dietary antioxidant, antim utagen and anticancerous with strong anti-inflammatory activities. In the present study, an attempt was made to produce this antioxidant using the aqueous polyphenolic extract of pomegranate residues as the carbon source during the solid-state fermentation by *Aspergillus niger*, *Rhizopus oryzae* and mixed culture of both *Aspergillus niger* and *Rhizopus oryzae*. These fungi demonstrated its capacity to degrade hydrolysable tannins, ellagitannins to ellagic acid by producing the enzyme tannase.

Isolated *Aspergillus niger* and *Rhizopus oryzae* was identified through microscopic and macroscopic observation.

Solid state fermentation of pomegranate husk using *A. niger*, *R. oryzae* and mixed cultures was performed for the production of ellagic acid (Fig 3). Samples were collected daily from the fermentation broth and quantitative analysis like reducing sugar concentration, determination of soluble protein content, tannase enzyme assay and concentration of hydrolysable tannins were performed as per standard methods (Fig 4).

DETERMINATION OF SUGAR CONCENTRATION

For all the three types of inoculum (*A.niger*, *R.oryzae* and Mixed culture of both), the concentration of reducing sugar increased with the incubation time till 12th day and after that there was a decrease in the concentration. Initially the concentration of reducing sugar was 1.14 mg and the maximum concentration achieved on 12th day was 3.08 mg, 2.82 mg and 3.03 mg for *A.niger*, *R.oryzae* and Mixed culture respectively.

DETERMINATION OF SOLUBLE PROTEIN

For *A. niger*, the concentration of protein increased with rise in incubation time till 12th day and after that there is a decrease in sugar concentration, whereas for *R.oryzae* and mixed culture the concentration of protein increased with rise in incubation time till 11th day and after that there is a decrease in protein concentration. Initially the concentration of soluble protein was 0.37 mg and the maximum concentration achieved was 1.03 mg, 1.04 mg and 1.08 mg for *A.niger*, *R.oryzae* and Mixed culture respectively.

TANNASE ENZYME ASSAY

Tannase enzyme activity was increased till 9th day for *A. niger* (8.37 units/ml), 10th day for *R. oryzae* (6.25 units/ml) and 11th day for Mixed culture (8.48 units/ml). After that the enzyme activity got decreased. The results were depicted in Chart 1.

Tannase is an easily inducible enzyme and hence tannin substrate in the medium induces the production of tannase which in turn convert hydrolysable tannin to ellagic acid.

Pomegranate peels, rich in ellagitannins was used as a substrate in the medium induces the production of tannase which in turn convert the hydrolysable tannin to ellagic acid. Pomegranate peels rich in ellagitannins are used as a substrate in the present study. Aguilar *et al.,* 2008 utilized pomegranate peel (*Punica granatum*) and creosote bush (*Larrea tridentate*) in this study for the production of nutraceuticals.Ventura *et al.,* 2007 utilized tar bush (*Fluorensia cernua*) for the production of ellagic acid.

ESTIMATION OF HYDROLYSABLE TANNIN

Initial tannin content was found to be 1.46 mg/g of substrate which got reduced during the fermentation. After fermentation tannin content reduced to 0.24 mg/g for *A. niger*, 0.29 mg/g for *R. oryzae* and 0.41 mg/g for mixed cultures; same was shown in Chart 2.

These fungi reduce the high tannin content in pomegranate peels demonstrating the ability of the fungal strain to degrade hydrolysable tannins. No reduction of tannin was observed in the control. Similar results were given by Ventura *et al.,* 2007 stating that the concentration of tannin got reduced at the end of fermentation.

QUANTIFICATION OF ELLAGIC ACID BY HPLC

After fermentation the production of ellagic acid in the medium was determined using HPLC. Based upon the retention time and peak value, the amount of ellagic acid produced by *A. niger* and *R. oryzae* is about 0.91 mg/gm and 7.9 mg/gm respectively; and with mixed culture the amount of ellagic acid was produced is 6.9 mg/gm. HPLC analysis of Standard ellagic acid and ellagic acid produced from test organisms were depicted in Fig 5.

Vattem and Shetty, 2002 reported the solid-state production of phenolic antioxidants from cranberry pomace using the fungus *Rhizopus oligosporus* with maximum ellagic acid production of 400 mg/100gm of pomace after 12 days of fungal fermentation.

In view of the increasing therapeutic application of ellagic acid from pomegranate which is a rich source of ellagitannins, the precursor of ellagic acid. The ellagitannins punicalagins is the substance responsible for the antimicrobial activity of pomegranate.

CONCLUSION

Pomegranate is an ancient fruit with an illustrious medical history and has been the subject of classical reviews for over 100 years (Lloyd, 1897; Li *et al.,* 2002). The peels of Pomegranate (*Punica granatum* L.) are a rich source of phenolic compounds like hydrolyzable ellagitannin that possess strong antioxidant activities. The objective of the present study was production of Ellagic acid from pomegranate peels by bioconversion using *Aspergillus niger* and *Rhizopus oryzae* through solid state fermentation. From the present study it can be concluded that SSF is an effective method to improve producing bioactives from pomegranate peels. According to Hölker and Lenz, (2005), solid-state fermentation is considered as the ideal fermentation process to produce fungal secondary metabolites in shorter fermentation times compared to submerged cultures; also reducing costs and operation time. Further it is reported that ellagic acid possess anti-microbial, antioxidant and anti-cancer activities, which should be studied in the future.

Fig 1: Dried Pomegranate Peels

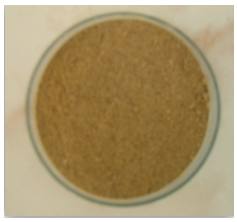
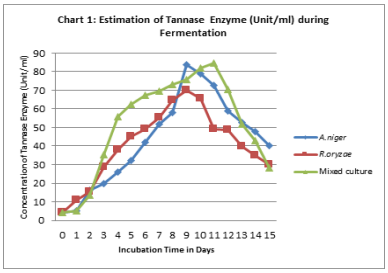


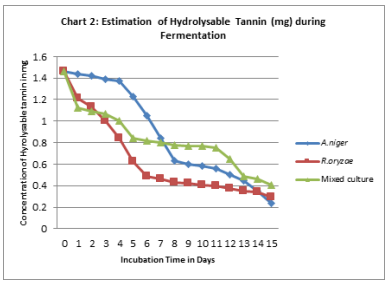
Fig 2: Coarsely powdered Pomegranate Peels

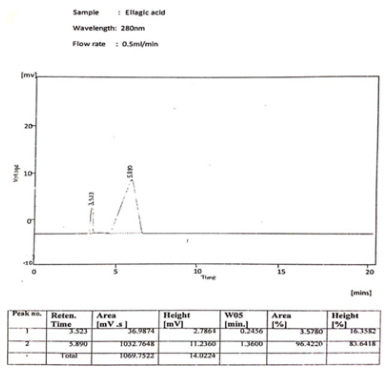
Fig 3: Solid State Fermentation using the test organisms

Fig 4: Filtration of Pomegranate peel extract

Quantitative Analysis

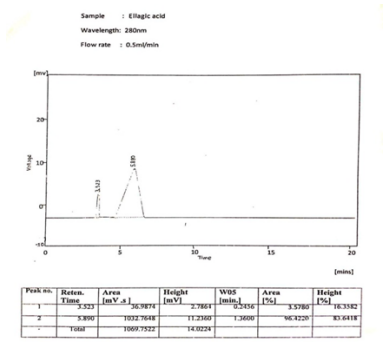






Peak no.	Reten. Time	Area [mV .s]	Height [mV]	W05 [min.]	Area [%]	Height [%]
1	3.523	36.9874	2.7864	0.2456	3.5780	16.3582
2	5.890	1032.7648	11.2360	1.3600	96.4220	83.6418
-	Total	1069.7522	14.0224			

Fig 5: HPLC Analysis of Control, Standard Ellagic Acid and Ellagic acid produced by Test Organisms



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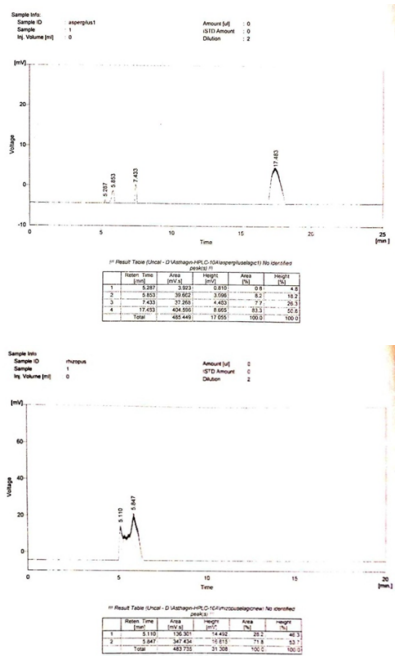

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