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Production profiles of phenolics from fungal tannic acid biodegradation in submerged and solid-state fermentation

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ABSTRACT

Potent antioxidant phenolics are derived from tannin biodegradation. Understanding of biodegradation pathways through the identification of the intermediates molecules of great value like tannins is important to pursuit the production of bioactive monomers. Biodegradation of tannins remains poorly understood due to their chemical complexity and reactivity. Tannic acid biodegradation by *Aspergillus niger* GH1 in submerged fermentation (SF) and solid state fermentation (SSF) was evaluated by liquid chromatography coupled to mass spectrometry (LC–MS). Both cultures were kinetically monitored for the biodegradation profiles during 72 h. Differences in tannic acid composition were evidenced and the consumption of substrate and identification of biodegradation intermediates were achieved. The mechanism of tannic acid degradation by *A. niger* GH1 is by degradation of high molecular weight gallotannins and highly polymerized tannins to small molecules like gallic acid, digalloyl glucose and trigalloyl glucose. Important differences on time of substrate uptake and product release were revealed.

1. Introduction

Tannins are naturally occurring polyphenols mainly as secondary plant metabolites [1–3]. They form precipitable complexes with proteins. Within classification of tannins there are hydrolysable and no hydrolysble tannins [4]. Gallotannins are a type of hydrolysable tannins. Khanbabaee and Van Ree [3] describe them as all those tannins in which galloyl units are bound diverse polyols units.

Biotechnological degradation is one of the most efficient way to degrade high molecular weight tannins into small molecules that may have important biological activities and high added-value. Microbial ability to assimilate tannins differs between yeast, bacteria and fungi. Yeast are able to degrade efficiently gallotannins but lose largely this ability for high molecular weight compounds. Bacteria have the ability to degrade gallotannins and ellagitannins. However, fungi can degrade efficiently these two (gallotannins and ellagitannins) different types of tannins [2].

Well-known enzymes involved in the degradation of gallotannins are tannases, which were probably the most studied in the biodegradation of tannins, its action is on the esters links of gallotannins and it may be obtained from microbial, plant or animal sources but, the microbial source is the most important [5]. It has been reported that tannase acts on ester bonds in hydrolysable and complex tannins, particularly on gallotannins releasing glucose and gallic acid [6,7].

Very few studies on tannic acid biodegradation have been reported, Goel et al. [8] reported that the degradation products from tannic acid by *Enterococcus faecalis* are gallic acid, pyrogallol and resorcinol. Rodriguez et al. [18] also stated the biodegradation of tannic acid to gallic acid and pyrogallol by *Lactobacillus plantarum*. Pepi et al. [9] reported glucose and gallic acid from hydrolysis of tannic acid from bacteria *Serratia* spp. and *Pantoea* sp. However, these identified compounds are the final products of microbial hydrolysis. These products have been reported to possess important biological activities as antioxidant, anti-microbial, antiviral, anti-inflammatory, anti-thrombotic, cardioprotective effects even inhibition of cancer cells [10,11]

Biodegradation of tannic acid or similar tannins remains poorly understood due to chemical complexity and reactivity of this kind of molecules, but it is known that the biodegradation of hydrolysable tannins provides an important molecules such as gallic acid and ellagic acid which present antiviral, antimicrobial, anticancer and antioxidant properties [12–15]. Therefore the understanding of the biodegradation pathway through the identification of the intermediates molecules of great value is important

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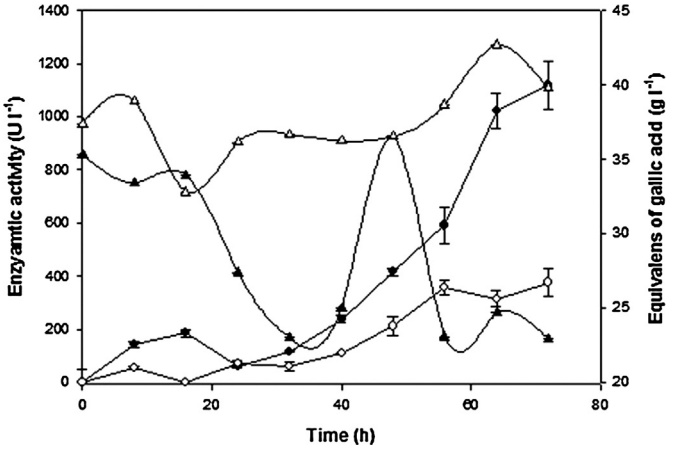

Fig. 1. Tannase enzymatic activity and quantification of total polyphenols in equivalents of gallic acid in solid state fermentation (SSF) and submerged fermentation (SF) during fermentation period. Enzymatic activity acid in SSF (–●– EA SSF), enzymatic activity in SF (–○– EA SF), gallic acid in SSF (–▲– GA SSF), gallic acid in SF (–△– GA SF).

to persuit the production of bioactive monomers. In this study the kinetic of fungal biodegradation of tannic acid and the intermediates molecules released path under SF and SSF were traced and identified by LC–MS.

2. Materials and methods

2.1. Culture medium and microorganisms

Aspegillus niger GH1 (DIA-UAdeC collection, preserved at −20 °C in protect-cryoblocks) was inoculated on dextrose potato agar (PDA) (Bioxon de México S.A. de C.V.) previously sterilized. Culture was incubated by three days at 30 °C. Fresh spores were scraped with a sterilized solution of Tween 80 (0.1% v/v) and counted in a Neubauer chamber. The culture medium was formulated as follows ($g\,l^{-1}$): KH_2PO_4 (4.38), $(NH_4)_2SO_4$ (8.76), $MgSO_4{\cdot}7H_2O$ (0.88), $CaCl_2{\cdot}7H_2O$ (0.088), $MnCl_2{\cdot}6H_2O$ (0.018), $NaMoO_4{\cdot}2H_2O$ (0.0088) and $FeSO_4{\cdot}7H_2O$ (0.12). The medium was sterilized and cooled; then tannic acid (40) was added. Medium was filtered through 0.45 μm membrane. Culture medium was inoculated with a spores suspension at concentration of 1×10^6 spores ml^{-1} of medium.

2.2. Solid state fermentation (SSF)

Polyurethane foam (PUF) was employed as support for fermentation. PUF was ground, washed and dried. PUF was deposited into Erlenmeyer flasks and culture medium was poured. Culture conditions were: temperature 30 °C, incubation time 72 h, sampling every 8 h. The extracts of culture were obtained by compression of fermented material then filtered through 0.45 μm membrane. Extracts were stored at −20 °C, lyophilized and reduced into homogeneous powder.

2.3. Submerged fermentation (SF)

Steril Erlenmeye flasks (250 ml) contained 30 ml of culture media inoculated as described above, were cultivate at 30 °C during 72 h at 250 rpm. Samples were taken every 8 h, filtered through 0.45 μm membrane. Extracts were stored at −20 °C, lyophilized and reduced into homogeneous powder.

2.4. Analysis of extracts

Tannase activity was assayed by the method reported by Sharma et al. [16], defining one tannase unit as the amount of enzyme needed to release one micromole of gallic acid per minute under assay conditions. Quantification of total polyphenols was evaluated with the method reported by Ventura et al. [17].

2.5. Extraction of polyphenols

Solid phase extraction (Waters-SepPak C_{18}-5 g Voc. 20 cc) cartridges was employed to realize a purification of extracts of fermentation. Each sample (obtained as homogeneous powder) was weighted (80 mg) and dissolved into 5 ml acidified water (1% acetic acid). Column was conditioned with methanol (20 ml) and then equilibrated with 40 ml of acidified water (1% acetic acid). Sample was loaded and slowly eluted on the cartridge. Then, acidified water (40 ml) was eluted to remove the non-retained material. The polyphenol fraction was recovered by eluting 40 ml of methanol/acidified water (1:1). Finally the column was washed with pure methanol. The aqueous methanol fraction was evaporated until complete removal of methanol. Then samples were frozen at −25 °C and lyophilized.

2.6. LC–MS

Analyses were carried out using an HPLC-DAD–MS system with a SCMA1000 vacuum membrane degasser (ThermoQuest, San Jose,

Table 1
LC–UV–MS analysis of components of tannic acid. Number of peak found, retention time (RT), name of identified compound, wavelength maximum (λ), molecular weight of each compound, $[M-H]^{-1}$ and de main MS/MS product ions.

Peak	RT	Compound	Λ_{max}	Molecular weight	$[M-H]^-$	Main MS/MS products ions
1	4.88	Gallic acid	271	170	169	125
2	8.78	Digalloylglucose	275	484	483	331, 271
3	11.3	Digalloylglucose	275	484	483	331, 271
4	12	Unknown	274	322	321	
5	13.82	Trigalloylglucose	271	636	635	465, 482
6	15.55	Trigalloylglucose	271	636	635	465, 482
7	17.03	Trigalloylglucose	278	636	635	465, 483
8	19.73	Tetragalloylglucose	275	788	787	635
9	21.03	Tetragalloylglucose	278	788	787	617/635
10	23.3	Pentagalloyglucose	275	940	939	
11	23.8	Pentagalloylglucose	275	940	939	
12	24.22	Pentagalloylglucose	275	940	939	787
13	25.45	Hexagalloylglucose	276	1092	1091	
14	26.25	Hexagalloylglucose	276	1092	1091	989, 1081
15	27.92	Heptagalloylglucose	275	1244	1243	519
16	29.18	Octagalloylglucose	274	1396	1395	1235, 1336, 1089