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ORIGINAL PAPER

Ellagic acid production by *Aspergillus niger* in solid state fermentation of pomegranate residues

Armando Robledo · Antonio Aguilera-Carbó · Raúl Rodriguez · José Luis Martinez · Yolanda Garza · Cristobal N. Aguilar

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Abstract Two *Aspergillus niger* strains (GH1 and PSH) previously isolated from a semiarid region of Mexico were characterized for their effectiveness in converting pomegranate ellagitannins (ET) into ellagic acid (EA) in a solid state fermentation (SSF). Pomegranate seeds and husk were used as support for the SSF. Released EA was evaluated by liquid chromatography. Yields of 6.3 and 4.6 mg of EA per gram of dried pomegranate husk were obtained with *A. niger* GH1 and PSH, respectively. Total hydrolyzable polyphenols of pomegranate husk were degraded during the first 72 h of culture (71 and 61%, by GH1 and PSH strains, respectively). Tannin acyl hydrolase activity was not clearly associated with EA production. EA that accumulated in cultures of *A. niger* GH1 was remarkably pure after a simple extraction process. Pomegranate husk is a good support, and at the same time an excellent substrate in the production of high commercial interest metabolites like EA due the degradation of its ET content.

Keywords Pomegranate residues · Solid state fermentation · Ellagic acid

Introduction

Tannins are complex molecules characterized by the high content of polyphenols; according to their structures, polyphenols are divided into three groups: hydrolysable tannins, which have a sugar core linked by esterification to gallic acid (gallotannins) or to ellagic acid (EA) (ellagitannins); condensed tannins, formed from the monomer flavan-3-ol or flavan 3,4-diol; and complex tannins or catechin-gallates, which share the properties of hydrolysable and condensed tannins [6, 7]. Ellagitannins (ET) are esters of hexahydroxydiphenic acid (HHDP) linked to polyols, usually glucose or quinic acid [2]. When exposed to acids or strong bases, esters bonds are hydrolyzed and the HHDP acid spontaneously rearranged into the water-insoluble dilactone, EA [9]. ET are principally obtained from trees, such as oak (*Quercus* sp.) [11, 15] sweetgum (*Liquidambar styraciflua*), linden (*Tilia* sp.), aile (*Alnus* sp.), eucalyptus species [16], chestnut (*Castanea dentata*) [15, 16], pomegranate, guava, strawberry, raspberry, blackberry [15, 18], pistachio, mango, hazelnut, walnut, pepper, plum, apricot, peach, black raisin, red raisin, currant, tea, grape, wines, and aged brandies in oak casks [9].

Ellagic acid is an anti-mutagenic and anti-carcinogen starter [9]. Some studies suggested that EA decreased the abnormal cell growth in the human colon, prevented the development of cells infected with human papiloma virus (VPH), which is related to cervical cancer and promoted the apoptotic growth (natural death) of cancerous cells of the prostate [24]. The apoptotic process incited by this antioxidant (ellagitannin) also has beneficial effects on cancers of the breast, lungs, esophagus, and skin. Carcinogenesis inhibition by EA has been demonstrated in animals with tumors of the esophagus, tongue, lung, colon, liver, and skin [9]. Also, EA is a pharmacologically active compound and it has been found to control hemorrhages in animals and humans, as a result of its ability to activate the Hageman factor [9].

Production of EA has been poorly explored due to its high-production cost and the large amount of by-products

A. Robledo · A. Aguilera-Carbó · R. Rodriguez · J. L. Martinez · Y. Garza · C. N. Aguilar (✉)
Group of Food Bioprocesses, School of Chemistry, Universidad Autónoma de Coahuila, Unidad Saltillo, Blvd, Venustiano Carranza, PO BOX 252, 2500 Coahuila, Mexico
e-mail: cag13761@mail.uadec.mx

generated as a result of ellagitannins degradation, implying serious problems regarding the recovery and purification of EA [25, 26]. Recent studies on EA production are very scarce but they are very promising and they have reported the use of cranberry pomace fermented in a solid state culture using *Lentinus edodes* [28, 29]; authors attributed the catalysis to the enzyme β-glucosidase, Also, valonea tannins have been used to produce EA in axenic culture of *Aspergillus niger* SHL [14] or in co-culture of *Candida utilis* and *A. niger* [27]. However, low yields and long times are involved in the bioprocess.

Previous studies carried out by our group have suggested that pomegranate residues can be used as an attractive alternative substrate for EA biotechnological production [2, 3]. This study was carried out to evaluate the potential use of pomegranate residues as supports in solid state fermentation (SSF) for EA production as result of the hydrolysis of ellagitannins.

Materials and methods

Microorganisms

Aspergillus niger GH1 and PSH strains from DIA/UAdeC (Depto. Investigacion en Alimentos/Universidad Autonoma de Coahuila) collection (Fungal strains are crio-conserved at −50°C) were used in this study. Both strains have been previously reported as high-level tannin tolerant and tannase producers [10].

Raw material

The pomegranate fruits used in this study were acquired from an orchard located in the region of Sabinas, Coahuila, México. The peels and seeds were separated from fruits and both samples were dehydrated to 60°C for 48 h. Samples were pulverized to a 30 mesh particle size in an industrial homogenizer (5 l; model LP12 Series 600-182, JR Maquinaria para mercado S.A. de C.V., México).

Physicochemical characterization

Total dry matter, moisture, ash, crude fats, protein, crude fiber, and pH were determined by following the official procedures of AOAC [5]. Total sugars were determined using the colorimetric method reported by Dubois et al. [12]. Briefly the method consisted in the spectrophotometric evaluation at 480 nm of the reaction of sugars present in samples with the phenol-sulfuric acid reagent. Reducing sugars content was evaluated using the colorimetric method reported by Millet [20]. The determination consisted in the evaluation of reduction reaction of dinitrosalicylic acid reagent in presence of sugars. EA was evaluated by the HPLC described at details in the next section [19]. Total hydrolyzable phenols were determined using the method reported by Makkar et al. [17] using gallic acid as standard molecule.

Tests of support in solid state fermentation

Water absorption index (WAI), critical humidity point (CHP), and water activity (a_W), were evaluated to know the potential use as a support in SSF of both residues (seeds and husk). The WAI was determined according to the methodology described by Anderson et al. [4]. About 1.25 g of residue were added to 15 ml of distilled water and the suspension was mixed for 10 min and placed into a 50 ml measured centrifugation tube. Centrifuge was operated at 18,000*g* for 10 min. The supernatant was decanted and the gel weight was reported. WAI was expressed as g gel/g dry support. The CHP was estimated using a thermo-balance by placing 1 g of sample impregnated with water at saturation (WAI result) at a temperature of 120°C by 60 min [5]. The a_W of the samples was evaluated using the AquaLab device (series 3B V 3.4 of Decagon Devices Inc., USA)

Invasion capacity on the pomegranate residues

The fungal growth was evaluated in Petri dishes, where the pomegranate residues were moisturized (70%) with the Czapek-Dox medium [g/l: $NaNO_3$ (7.65); KH_2PO_4 (3.04); $MgSO_4$ (1.52); KCl (1.52)], using pomegranate residue as sole energy and carbon source. Spores of *A. niger* GH1 and PSH (2×10^7 spores) were inoculated at the center of plates. Radial growth was monitored kinetically. All these assays were in triplicate and the growth rate was expressed in mm/h.

Solid state fermentation

Typical batches of column reactors containing 3 g of sample and 7 ml of Czapek Dox mineral medium inoculated with 2×10^7 spores per gram of support were incubated at 30°C. Samples were monitored kinetically during 144 h and the consumption of total sugars [12] and ellagitannins content [17] were evaluated, fungal biomass was spectrophotometrically evaluated by the glucosamine content [8, 23] and reported as mg of biomass per gram of dried solid support. Tannin acyl hydrolase was assayed by the HPLC method reported by Aguilar et al. [1]. Enzyme solution (0.05 ml) was incubated with 0.80 ml of 1.0% (w/v) tannic acid, in 0.2 M acetate buffer (pH 5.0) at 30°C for 30 min and then the reaction was stopped by addition of 0.20 ml tricloroacetic acid (TCA). Reaction mixture was filtered

(nylon membrane, 0.45 μm) and injected into HPLC equipment (Varian, ProStar System) with PDA detector at 254 nm. Separation was carried out with an Optisil ODS column (5 μm; 250 × 4.6 mm) at a temperature of 30°C. A tannase unit was defined as amount of enzyme able to release a μmol of gallic acid per min under assay conditions; and EA accumulation was determined by HPLC [19]. It was necessary to make some modifications described in the next section. Samples were analyzed in triplicates.

EA determination by HPLC

A gram of fermented pomace was mixed with an aliquot of 10 ml of 95% ethanol and homogenized for 1 min using an immersion blender (RIVAL immersion blender, model IB901 MX). Material homogenized was shaken (Bransonic, Model 2510R-MTH, Branson Corp, CT, USA) for 30 min in a 50 ml conical tube. Later, 1.5 ml aliquot was placed into a 2 ml conical tube and centrifuged at 36,000*g* for 20 min. The supernatant was decanted and the precipitate was resuspended in ethanol. The sample was shaken again for 30 min and the solution was transferred into clean test tubes and filled up to 5 ml with ethanol and shaken for 2 h. The sample with suspended material was filtered through 0.45 μm nylon membrane and injected into HPLC. Ethanolic extractions were obtained from the fermented material, to recover the EA produced as a result of ET hydrolysis. EA recovered was determined by HPLC (Varian Pro Star) using the Matějíček method [19] with a modification and a photodiode array detector (PDA Pro Star 330) at 254 nm. Separation was carried out with an Optisil ODS column (5 μm; 250 × 4.6 mm) at a temperature of 25°C. A linear gradient profile of mobile phase, consisting of acetonitrile (solvent A) and 0.3% acetic acid in water (v/v) (solvent B), 7–20% A (0–7 min), 20–30% B (7–12 min), 30% B (12–18 min), 30–60% B (18–20 min), 60–100% B (20–23 min), 100% B (23–30 min) y 7% B (30–31), and 7 min for baseline stabilization was applied at a flow rate of 0.6 ml/min. The sample injection was of 10 μl. The analytical method was validated according to the guidelines of the international conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) and Wang et al. [31] recommendations. To validate the method, standard solutions of different concentrations (0, 5, 10, 20, 30, 40, and 50 μg/ml) were prepared by dissolving EA in methanol and dilutions of this solution were made for the linear calibration curve, which was analyzed by linear regression analysis.

Calibration curve was generated using six concentrations (in tetraplicates) of EA standard solution, inter-day (different days) and intra-day (same day). The accuracy of the method was determined by recovery of known amounts of EA standard in hexaplicates [4].

EA characterization

Ellagic acid recovered as previously mentioned was characterized by HPLC (Varian) and FTIR (Perkin-Elmer, Foster City, USA) against an EA standard (Sigma Reagent, St.Louis, MO, USA), similarities of retention times and main substituent groups in both EAs were compared.

Results and discussion

Physicochemical characterization and EA content of pomegranate husk and seeds are presented in Table 1. Higher levels of total and reducing sugars, crude fiber and polyphenols were found in pomegranate husk. Total protein and crude fat were higher in pomegranate seeds. Chemical profiling demonstrated that both materials are good prospects for SSF. However, in this case, the total hydrolyzable polyphenols (THP) content of pomegranate husk is critical because it is the source of EA. Levels of THP were similar to the values reported in literature [26], while the free EA content in the pomegranate husk powder was 3.34 times higher than the level reported by Seeram et al. [26].

Results of the evaluation for the potential use as a support in solid cultures using pomegranate are given in Table 1. The WAI, is the quantity of water that can be absorbed by the support, where pomegranate husk recorded a higher WAI than the seeds, which can be attributed to its high crude fiber content and low-fat content. Materials with high WAI levels are preferred, since their moisture content can be modified during solid state culturing. The CHP, represents the quantity of water linked to the support, which cannot be employed by the microorganism and it is also strongly related to the a_W. Moo-Young et al. [22] recommended a 40% CHP limit for *A. niger* strains in solid state cultures, due to the need for modification of the moisture content in relation to the absorbed media. The a_W, is the quantity of water available for the metabolic functions of microorganism on the support. The values reported in the literature are between 0.88 and 0.99 of a_W with an optimum of 0.96 for the *A. niger* strain. The a_W-values for the pomegranate seed (0.796), was below the minimal requirement for the microorganism growth, while the husk (0.986), presented an optimum value.

It has been reported that *A. niger* strains possess tannin-protein complex degrading activity at a pH range of 3.5–6.5 with an optimum value of 5.0 at 30°C [2]. pH-values for seed and husk pomegranate residues presented permissible pH-values for enzyme synthesis. Both strains grew on the pomegranate husk with higher growth rates (0.40 mm/h-GH1 and 0.41 mm/h-PSH) than the growth rates observed on the seeds (0.23 mm/h-GH1 and 0.24 mm/h-PSH). With

Table 1 Physicochemical characterization of the pomegranate seed and husk powder samples and results of their characterization as support for SSF process

Dry base analysis		Quantity per 100 g of sample	
		Seed (g)	Husk (g)
Dry matter		74.33 ± 0.09	94.45 ± 1.25
Moisture		25.66 ± 0.09	5.5 ± 1.25
Ash		3.62 ± 0.13	3.59 ± 0.08
Crude fat		10.33 ± 0.17	3.57 ± 0.38
Total protein		10.42 ± 2.61	1.26 ± 0.17
Crude fiber		12.12 ± 2.10	17.75 ± 1.61
Total sugars		10.13 ± 0.33	16.08 ± 0.61
Reducing sugars		4.67 ± 0.02	4.34 ± 0.01
Total hydrolysable phenols		0.002 ± 0.00015	6.11 ± 1.83
Ellagic acid		0.00048 ± 0.00002	0.26 ± 0.08
Characteristic			
WAI (g gel/ g dry sample)		1.62 ± 0.020	4.84 ± 0.006
CHP (%)		64.55 ± 2.170	22.08 ± 0.630
PH		5.37 ± 0.354	5.70 ± 0.420
a_W	Without media	0.156 ± 0.015	0.189 ± 0.018
	With media	0.796 ± 0.003	0.986 ± 0.001
Growth rate of A. *niger* (mm/h)	PSH with media	0.243 ± 0.021	0.413 ± 0.011
	GH1 with media	0.231 ± 0.046	0.405 ± 0.000

WAI water absorption index, *CHP* critic humidity point, a_W water activity

these results it can be concluded that the pomegranate husk is a better support for the SSF.

Fungal invasion was associated with biodegradation of the ET present in the pomegranate husk, for the EA production. Simple stereoscopy (Laica-40X) of the fermented pomegranate residues revealed the growth of fungi in the support. Environmental scanning electron microscopy (Phillips X-L30 ESEM) demonstrated that fungi grew invading and penetrating the support (Fig. 1). A short time of lag phase was exhibited by the fungi and the biomass production was maximum at 96 h of fermentation (Fig. 2c). *A. niger* PSH grew faster than GH1 strain; however, the maximal biomasses were not significantly different. Tannin acyl hydrolase activity (Fig. 2d) was clearly associated with biomass production. The presence of tannin acyl hydrolase implies that a great amount of hydrolysable tannins are gallotannins. Maximal enzyme activities values were obtained at 96 h of culture and these were not associated with EA accumulation. Similar patterns were previously reported by Aguilar et al. [1].

After 144 h of fermentation, *A. niger* GH1 and PSH consumed 83 and 88% of total sugars, respectively, in pomegranate husk (Fig. 2a). Earlier, Fadavi et al. [13] reported the high content of glucose and fructose in pomegranate husk. ET content evaluated as THP decreased 71 and 61% after 72 h of fermentation by GH1 and PSH, respectively (Fig. 2a). ET from pomegranate husk are mainly monomeric ET, its isomers and EA glycosides (hexosides, pentosides, ramnosides, etc.) [26].

Modification of EA determination by HPLC permitted a good resolution of EA and its quantification. The released EA in the fermented samples by SSF was higher using *A. niger* GH1 than PSH. EA production of 6.3 and 4.5 mg per gram of pomegranate husk was obtained with *A. niger* GH1 and PSH, respectively, after 120 h of culture (Fig. 2b). Differences in time of tannin acyl hydrolase production and EA accumulation can be explained by the fact that gallotannins are more biodegradable than ellagitannins as described recently by Mingshu et al. [21] and Aguilar et al. [3]. The EA production was higher than the values reported by other

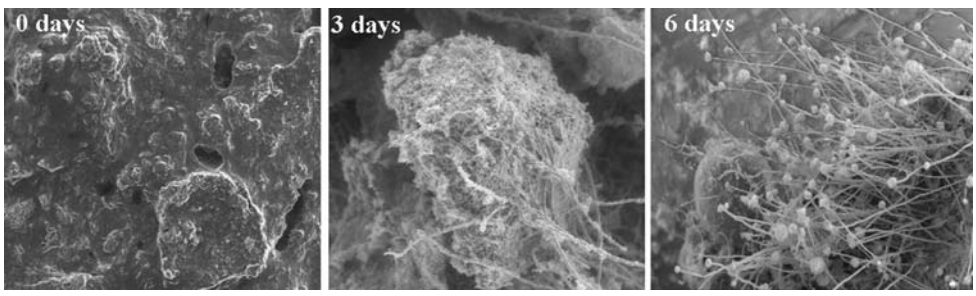

Fig. 1 Growth and invasion capacity of *A. niger* GH1 on pomegranate husk at 0, 3, and 6 days of SSF

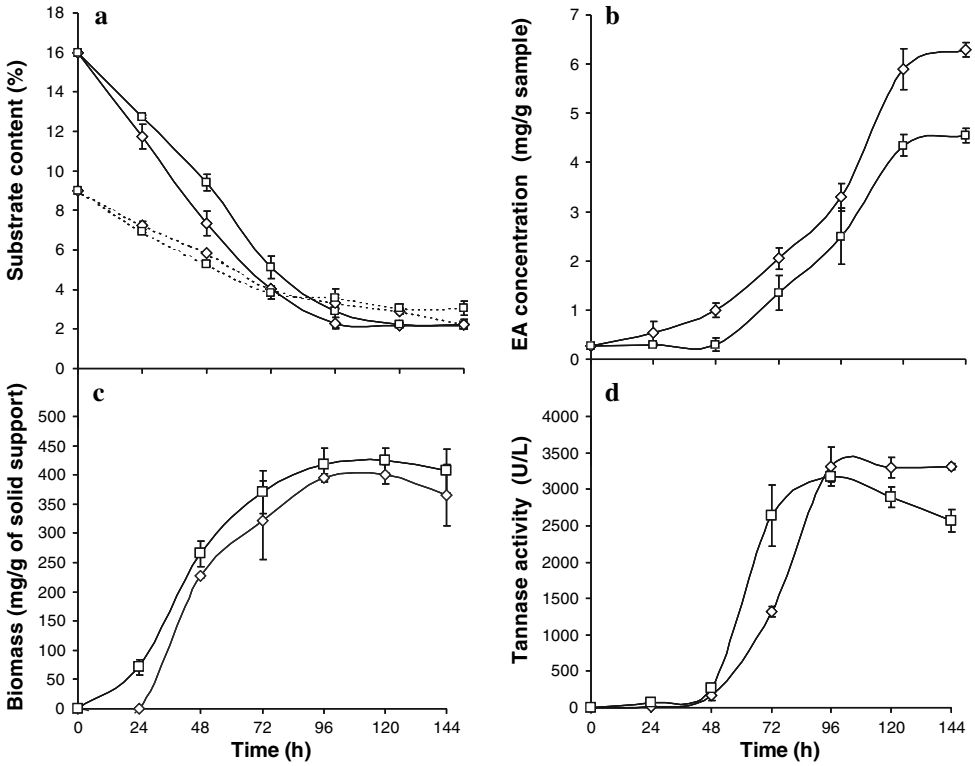

Fig. 2 Kinetics of fungal fermentation of pomegranate husk in SSF. **a** Total sugars consumption, *dotted lines* correspond to ET consumption (THP), **b** kinetics of ellagic acid production, **c** kinetics of fungal growth and, **d** tannase production by *A. niger* GH1 (*open diamond*) and PSH (*open square*)

authors [14, 27–29]; however, the optimization of the process to produce EA is necessary. In addition, EA accumulation was not associated with tannin acyl hydrolase activity and it partially supported the hypothesis proposed by Aguilar et al. [3] about the difference between the tannin acyl hydrolase (gallo-tannase) and the unknown "ellagi-tannase." For this reason it is very important to continue the investigations to elucidate the existence of these kind of new tannases.

The recovery protocol of EA described in Materials and Methods resulted in good purity of the EA released during the fungal fermentation. Figure 3 presents the spectrograms of EA. Hydroxyl and lactone substituents corresponded to 97% with respect to standard.

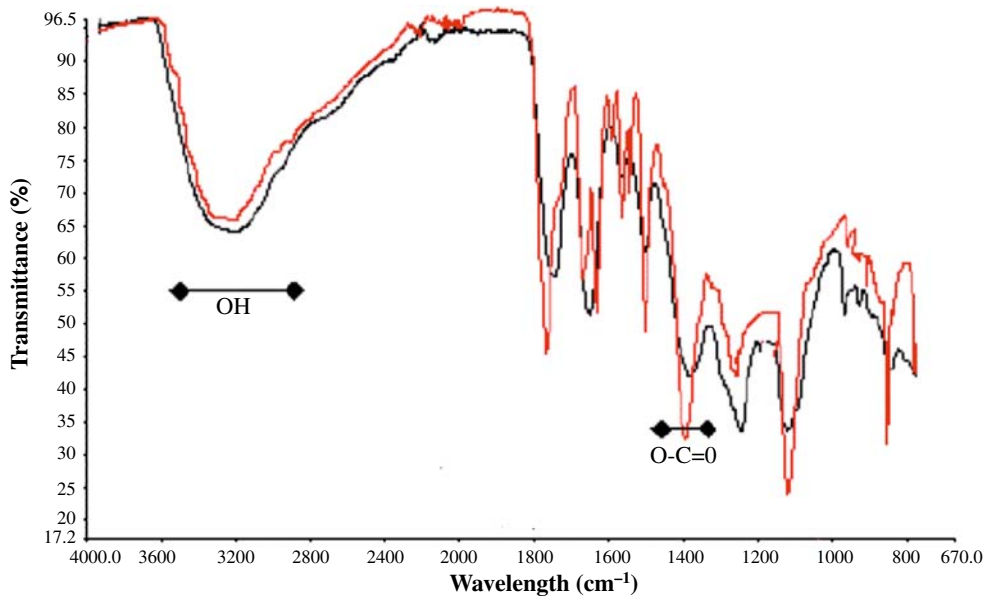

Fig. 3 FTIR-spectrogram of recovered and commercial EAs

Finally, although numerous studies have been conducted on biodegradation of tannins and on the degradation mechanism of some simple tannins, such as gallotannin [7], less is known about the pathways and the enzymes involved in breaking of complex tannins, especially about the accumulation mechanism of some intermediates by fungi and yeasts. This work is in an incipient but important stage, and further studies on EA accumulation by biodegradation of pomegranate tannins should be carried out to exploit the potential of *A. niger* GH1 for the production of important bioactive compounds like EA.

World production of pomegranate fruit was 1, 655, 500 tons in 2006: Iran was the leading producer with 650,000 tons and India was second with 600,000 tons. The rest of the countries produced the remaining 405,500 tons [30]. In 2006, Mexico produced 2,781 tons of pomegranate fruit for the juice industry [32], representing a generation of almost 1,500 tons of husk, which suggests the feasibility to yield 8 kg of EA per ton of pomegranate husk, and the possibility to consider this agro-industrial residue as sufficient to complete with alternative raw materials.

Conclusions

Pomegranate residues were physicochemically evaluated and characterized to be considered as a substrate for the production of high-value bioactive compounds via SSF. Pomegranate husk had an attractive level of THP to be bioconverted into EA during the SSF. *A. niger* GH1 degraded a major content of THP releasing a higher level of EA than *A. niger* PSH. Further studies on the optimization of EA production from pomegranate husk are needed.

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