Use of fungal enzymes to study the degradation of specific plant polyphenols

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Summary

A fraction of procyanidin was purified from cider apple. This procyanidin (PC) was used as the sole carbon source in order to evaluate the potency of different filamentous fungi to grow and degrade them. Fifteen filamentous fungi were screened in a mineral medium with or without glucose. Almost all the strains were observed to have PC degradation ability. *Aspergillus fumigatus* was the most efficient fungus that degraded >50% of PC after 72h of growth. This strain was selected for further studies. The PC cultured samples were analyzed by thiolysis-HPLC method and constitutive units (catechin units) of PC polymer were quantified and characterized. Kinetics of PC degradation revealed that the terminal units of PC decreased during fermentation and on the contrary, an increase in the average degree of polymerization (DP*n*) from 8 to 36 was also observed at the end of fermentation. These findings suggest that terminal units were possibly modified by the enzymatic action of the fungus. Normal-Phase HPLC analysis showed that PC oligomers were degraded during the early phase.

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1. Introduction

A wide variety of secondary compounds accumulate in the plants including alkaloids, terpenes and phenolics. The group of phenolic compounds in plants known as tannins is distinguished from other secondary phenolics in their chemical and biological activities. Multiplicity of phenolic groups (polyphenols) is a characteristic feature of these compounds and the special property – ability to precipitate proteins, sets tannins apart from all other phenolics. Among these tannins are a group of polyphenolic structures called condensed tannins or proanthocyanidins. Proanthocyanidins are present in coffee and by-products such as coffee pulp, by forming condensed tannins that contribute to their astringency and bitterness preventing their use as animal feed.

Procyanidins (PC) are a group of polyphenols existing most widely in plants, namely condensed tannins. PC are oligomers and polymers constituted of catechin units linked mainly through C4 → C8 or C4 → C6 bond (Figure 1).

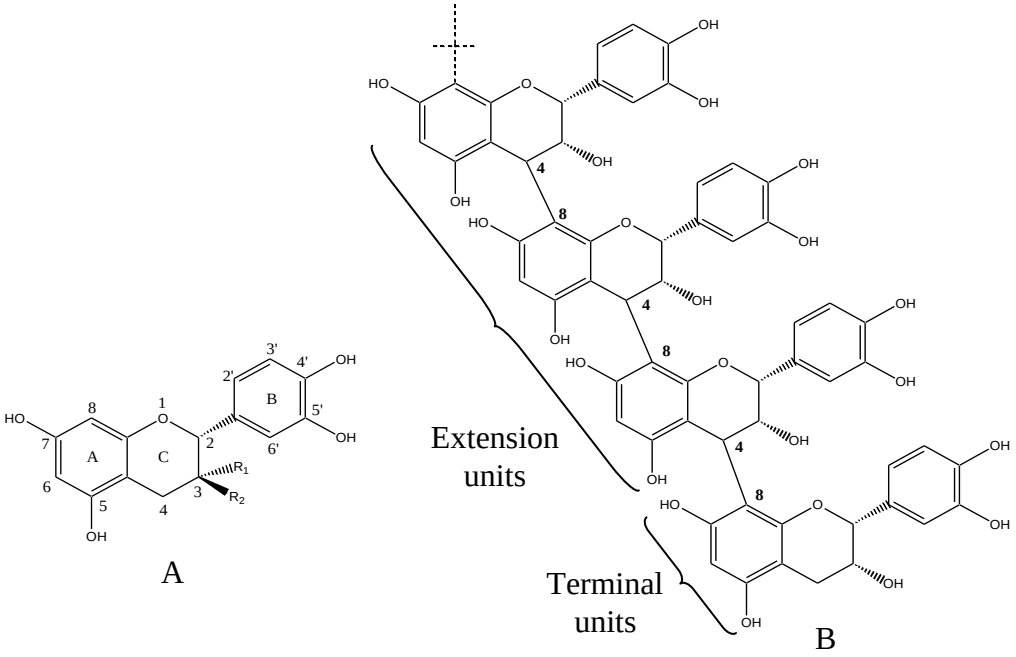

Figure 1. A) General monomeric structure of procyanidins. R_1 = H, R_2 = OH, (+)-catechin ; R_1 = OH, R_2 = H, (-)-epicatechin. B) polymers of procyanidins representing terminal (lower) and extension (upper) units

The presence of PC in plant is associated with defence against insects, herbivores and pathogenic microorganisms [1]. PC bind strongly to proteins and enzymes, playing a role in the inhibition of gastrointestinal microflora; this property has obvious nutritional consequences [2-3]. However, it has also been reported that different microorganisms are resistant to condensed tannins, as they could grow and degrade these compounds. In almost all the cases, monomeric PC have been tested [4] and the degradation of polymeric PC have been less studied. Among these, fungi have been found to degrade polymeric condensed tannins [5-6]. Indeed, the major disadvantage in the study of polymeric PC degradation is that there is no commercial PC substrate available for

fermentation studies and it is necessary to purify them. Many foods have been reported for their procyanidin content [7-8]. Moreover, their quantification is also difficult by classical HPLC methods. Thiolysis reaction in combination with reversed-phase HPLC analysis has allowed the general quantification of procyanidins in a large variety of cider and dessert apples [9-10]. The reaction allows the depolymerization of PC by the cleavage of interflavan linkages in acid conditions in the presence of a nucleophile as toluene-α-thiol, whose terminal units are liberated as monomeric units [(+)-catechin or (-)-epicatechin] whereas extension units are transformed into benzyl-thioether adducts [11-12]. The application of this method has showed a large variation in concentration and in the mean procyanidin chain lengths among apple cultivars. The estimation of size of the procyanidin molecule is known as average degree of polymerization (DP*n*). From the values obtained by terminal and extension units of reversed-phase HPLC analysis after thiolysis reaction, it is possible to calculate the DP*n*. In addition, normal-phase HPLC technique can be used to separate PC oligomers on the basis of their molecular weight [13].

The objective of the present study was to evaluate the ability of different filamentous fungi to degrade apple-procyanidin, in liquid culture. The strain that exhibited the best degradation profile was selected and further detailed studies. Techniques such as thiolysis-HPLC and Normal Phase-HPLC were used in order to separate and quantify the constitutive units resulting from the procyanidin degradation fractions.

2. Materials and Methods

2.1 Chemicals

(+)-Catechin, (-)-epicatechin, were obtained from Sigma Chemicals Co. France. Acetonitrile (HPLC grade) and acetic acid were purchased from Biosolve (France). Benzylmercaptan was obtained from Merck (Germany) and (-)-epicatechin-benzylthioether standard was provided by N. Marnet (URC-BFL, INRA, Le Rheu, France).

2.2 Microorganisms and inoculum preparation

Fifteen strains of filamentous fungi obtained from IRD (Institut de Recherche pour le Développement) collection were evaluated for their potency to degrade procyanidins. Strains were grown and maintained on Potato Dextrose Agar (Sigma, France) in 250 mL Erlenmeyer flasks. The flasks were stored at 4^{o}C and sub-cultured at regular intervals. Six-days-old fully sporulated flasks were used for inoculant preparation. Spores were harvested with the aid of a magnetic stir bar by the addition of 30 ml of sterilized distilled water containing 0.01% (v/v) of Tween 80 into the flasks under constant agitation. The suspension of spores obtained was used as inoculum.

2.3 Culture Medium

Modified synthetic medium ('basal medium') was used [14] for culturing, which contained (in $g \cdot l^{-1}$ of distilled water): glucose 2; $SO_4(NH_4)_2$ 1.0; KH_2PO_4 1.3; Na_2HPO_4 0.12; $MgSO_4$ 0.3; Urea 0.3 and 1 ml of oligo-element solution ($FeSO_4.7H_2O$ 5.0; $MnSO_4.H_20$ 1.6; $ZnSO_4.7H_2O$ 1.4; $CaCl_2$ 2.0). The pH of the oligo-element solution was adjusted to 6 using NaOH (3 M). Both the medium and solutions were sterilized at 121°C and 15 psi for 20 min. Procyanidin (from apple)-PC was dissolved in 10 ml of the basal medium and the solution was added aseptically to the culture medium by filtration (Millipore filter- 0.22 µm, PVDF) with a final concentration of 2 $g \cdot l^{-1}$.

2.4 Culture conditions

For the screening studies, liquid culturing was carried out in Petri dishes containing 10 ml of medium inoculated with $2x10^{10}$ spores per gram carbon source. For the kinetic profiling of *Aspergillus fumigatus*, 15 ml of culture medium taken in a 100 mL Erlenmeyer flask was inoculated with the same concentration of spores per gram carbon source. The flasks were incubated on a rotary shaker at 120 rpm at 30°C. A medium without inoculum was used as a positive control to determine whether the added polyphenols underwent oxidation during the experiment. All the experiments were carried out in duplicate.

2.5 Biomass determination

Fungal biomass was measured by gravimetry. Culture medium was filtered using a Millipore membrane filter of 0.45 µm (PVDF) diameter. The mycelia retained were washed with deionised water and dried in an oven at 60°C for 48h. Biomass concentration obtained was reported in grams of dry weight per litre of culture medium ($g \cdot l^{-1}$).

2.6 Extraction and purification of procyanidins (from apple)

PC was extracted from apple cider (cultivar *Marie ménard*). Freeze-dried apple powder was sequentially treated with hexane (once), methanol-acetic acid (3 times) and aqueous-acetone (3 times) as previously reported by [15]. Then, the fraction aqueous-acetone (HA) was pooled and excess solvent was eliminated by rotoevaporation. The purification of polymerized PC was carried out on an HPLC system (a binary pump Dynamax SD 300 Raining, an UV-Visible Dynamax YV1 detector and the EZ-Chrom-Waters software) at room temperature and at a flow rate of 30 $ml \cdot min^{-1}$. The HA freeze-dried extracts (4 g) were dissolved in 40 ml of acid aqueous solution (2.5%, CH_3COOH) and filtered through a membrane of 1.2 µm (Millipore, PVDF). The filtered solution was injected on a LiChrospher 100 RP-18 column (20 × 5 cm, 12µm) (Merck, Germany). The column was first washed with the aqueous acid solution for 50 min. After 10 minutes, procyanidins were recovered in a flask, thanks to a gradient of acetonitrile (50%). The eluant was monitored at 280 nm. Excess acetonitrile and acetic acid were eliminated by rotoevaporation at 30°C, and the concentrated product was lyophilized.

2.7 Thiolysis-HPLC analyses

The thiolysis reaction was carried out according to the procedure described by Guyot *et al.* [16]. Forty milligram of freeze-dried apple powder PC was dissolved in 10 ml of methanol (anhydrous) while, 2 ml of the fungal culture broth were lyophilized and after, solubilized in 2 ml of methanol (anhydrous). The thiolysis reaction of samples was carried out in a glass vial of 250 µl as: 50 µl of samples were introduced into the vial containing 50 µl of acid methanol solution (0.4 N, HCl), and 100 µl of benzyl-thio-ether solution (5% in anhydrous methanol). Vials were then closed, mixed and incubated at 40°C for 30 min. The reaction was stopped by introducing the vials into ice. After, the vials were stored at 4°C until analysis. The apparatus used was a Waters HPLC system (U.S.A.) with a 717 plus autosampler equipped with a cooling chamber for samples, a 600^{E} multisolvent pump and a 996 photodiode array detector and the Millenium 2010 manager system. Mixture of compounds (10µl) was separated at 30°C on an end-capped Purospher RP-18 column of 250 × 4 mm, 5µm (Merck, Germany) at a flow rate of 1 $ml \cdot min^{-1}$, by a gradient elution system (eluant A: aqueous acetic acid, 2.5%, v/v and eluant B: acetonitrile. Initial, 3% B; 0-5 min, 9% B linear, 5-15 min, 16% B linear and 15-45 min, 50% B linear, followed by washing and reconditioning of the column) and monitored at 280 nm.

2.8 Analyses of total procyanidins by Butanol-HCl

For the estimation of total PC from fungal liquid cultures as reported by [17], it was mixed with reactive Butanol-HCl. Each sample (250 µl) was taken into a glass tube of 10 ml and 3.5 ml of Butanol-HCl added. The tubes were closed and incubated at 95°C in a water bath for 1 h. Then, the tubes were cooled at room temperature for 15 min. PC content was estimated at 550 nm and quantified by comparing to a PC standard curve.

2.9 Normal Phase-HPLC analyses

The HPLC apparatus used was an HP quaternary gradient pump 1100 series (Agilent Technologies), autosampler and data were processed by Chemstation® 2D software. A binary gradient was applied (eluent A (%): dichloromethane (10), methanol (86), formic acid (2), water (2); eluent B (%): dicloromethane (82), methanol (14), formic acid (2), water (2)): initial, 100% B; 0-30 min, 80% B; 30-35 min, 75% B; 35-40 min, 0%B; 40-42 min, 100% B. The eluents were filtered using a 0.22 µm (Millipore, PTFE) filter. Two millilitres of fungal culture from the liquid fermentation, containing polymeric PC were lyophilized and resuspended in 2 ml of eluent B. Twenty microliters were injected into a Silica column (250 × 4.6 mm ; 5 µm, Luna, Phenomenex) with a flow rate of 1 $ml \cdot min^{-1}$ at 30°C. The analysis of procyanidin was carried out at 280 nm.

3. Results and Discussion

3.1 Procyanidin purification

The cider apple variety (*Marie ménard*) used for PC polymer extraction was observed to have an average degree of polymerization (DP*n*) of 8. This fraction was used as carbon source for fungal growth. The distribution of constitutive units and DP*n* of purified PC fraction from cider apple is presented in Table 1. The (-)-epicatechin was the major structure of flavan 3-ol found as terminal and extension units but a small percentage of (+)-catechin as terminal units was also observed.

Table 1. Proportion of constitutive catechin units and average degree of polymerization (DPn) from cider apple purified fraction. ECA_e = extension (-)-epicatechin; ECA_t = terminal (-)-epicatechin, and CAT_t = terminal (-)-catechin units.

	Procyanidin fraction from cider apple procyanidin
ECA_e (%)	87.8
ECA_t (%)	10.4
CAT_t (%)	1.7
$ECA_t + CAT_t$ (%)	12.1
DP*n*	~ 8.2

3.2 Procyanidin degradation by cultures of filamentous fungi

In order to evaluate the PC consumption and biomass production, fifteen strains of filamentous fungi were cultivated in a basal medium containing PC (2 $g \cdot l^{-1}$) as the sole carbon source. After 72h of incubation almost all fungal strains were able to grow in liquid cultures. It was also observed that these strains degraded condensed tannins with different capacities (from 3.1 to 71.9 %) but low levels of biomass production were observed (from 0.01 to 0.22 mg of dry cell weight ml^{-1}) (Table 2). PC seemed to be used by filamentous fungi as a carbon source to produce the biomass. Previous studies described that tannins (hydrolysable and condensed) can be used as carbon and energy source for fungal growth but higher biomass production was observed with cultures grown on hydrolysable tannins. In support to this, Bhat *et al.* [18] has reported that the strain *A. niger* exhibited better and faster growth on hydrolysable tannin-medium and had a growth inhibition on condensed tannin-medium.

Table 2. Biomass production and procyanidin (PC) degradation at 72 h by fifteen filamentous fungi strains cultivated in a mineral medium with procyanidins.

Stains of filamentous fungi	**Biomass**		**PC degradation**	
	[a]mg of dry cell weight ml^{-1}	[b]mg of dry cell weight ml^{-1}	[a]%	[b]%
Trichoderma harzianum	0.12 ± 0.08	0.10 ± 0.02	45.2 ± 1.91	13.4 ± 0.10
Rhizopus sp.	0.09 ± 0.02	0.14 ± 0.08	3.1 ± 1.10	12.9 ± 0.81
Penicillium sp.	0.11 ± 0.03	0.27 ± 0.06	52.7 ± 3.08	55.1 ± 2.60
Rhizopus sp.	0.22 ± 0.03	0.36 ± 0.09	54.4 ± 4.03	15.0 ± 4.47
Geotrichum sp.	0.17 ± 0.05	0.43 ± 0.34	59.2 ± 1.90	51.1 ± 1.67
Rhizopus sp.	0.06 ± 0.03	0.49 ± 0.03	15.5 ± 5.03	30.8 ± 0.83
Aspergillus niger	0.22 ± 0.04	0.60 ± 0.03	31.1 ± 4.10	49.0 ± 3.69
Fusarium sp.	0.11 ± 0.01	0.63 ± 0.05	53.9 ± 0.48	54.1 ± 3.00
Penicillium sp.	0.17 ± 0.09	0.63 ± 0.01	45.2 ± 4.02	47.3 ± 2.44
Aspergillus fumigatus	0.16 ± 0.08	0.65 ± 0.04	71.9 ± 2.25	57.2 ± 3.88
Aspergillus sp.	0.14 ± 0.11	0.69 ± 0.01	58.5 ± 1.52	47.2 ± 1.05
Aspergillus awamori	0.21 ± 0.04	0.71 ± 0.08	33.9 ± 4.13	40.5 ± 6.52
Penicillium sp.	0.15 ± 0.01	0.71 ± 0.06	28.2 ± 0.98	54.7 ± 4.52
Rhizopus stolonifer	0.15 ± 0.03	0.74 ± 0.06	49.6 ± 1.19	53.2 ± 4.52
Rhizopus microsporus	0.01 ± 0.03	0.77 ± 0.06	28.9 ± 0.09	44.6 ± 1.60

[a] without glucose, [b] with glucose

Addition of glucose (0.2 $g \cdot l^{-1}$) significantly increased biomass production in all fungal cultures (from 0.10 to 0.77 mg of dry cell weight ml^{-1}) at 72h. On the other hand, PC degradation profile by the cultures in the presence of glucose was also determined. Glucose did not show any enhancement of procyanidin degradation in all fungal cultures since the percentage of PC degradation on *Tricoderma harzianum*, *Rhizophus* sp.,

Geotrichum sp., *Aspergillus fumigatus* and *Aspergillus* sp. cultures were lower than that obtained with glucose-medium (Table 2), although these differences were really marked in only three strains. Other studies on sorghum condensed tannin degradation by *Penicillium* sp. in an aqueous mineral medium with or without glucose (0.1 $g \cdot l^{-1}$) showed a significant diminution of tannins in both media. The percentage of condensed tannin degradation was higher in glucose-containing medium [5]. Among the strains screened, *A. fumigatus* showed the best PC degradation profile in the both culture media and hence this strain was selected for further studies.

3.3 A. fumigatus growth and procyanidin degradation

A variety of enzymes produced by *Aspergilli* are involved in the degradation of organic substances I particular plant cell wall polysaccharides [19]. The nutritional properties of *A. fumigatus* and their occurrence in the soil and their presence on natural by-products substrates [20] suggest that this fungus may play an important role in aerobic degradation of organic matter in nature. Degradation of hydrolysable tannins by different *Aspergillus Sp.* has been reported by Batra and Saxena [21] and among them the strain *A. fumigatus* was reported to be an efficient tannase-producer. However, information regarding condensed tannin degradation by fungi in general is limited as one of the major problems in this study of microbial degradation of PC is the lack of pure substrates.

The growth of *A. fumigatus* at two concentrations of PC extracted from cider apple was tested (2 or 4 $g \cdot l^{-1}$). Kinetic profiling was carried out in order to obtain a final carbon source concentration of 4 $g \cdot l^{-1}$ at initial time, in a medium where glucose was present or absent (0 or 50 % of total carbon source). A time course of procyanidin degradation, biomass content and PC degradation were determined (Figure 2).

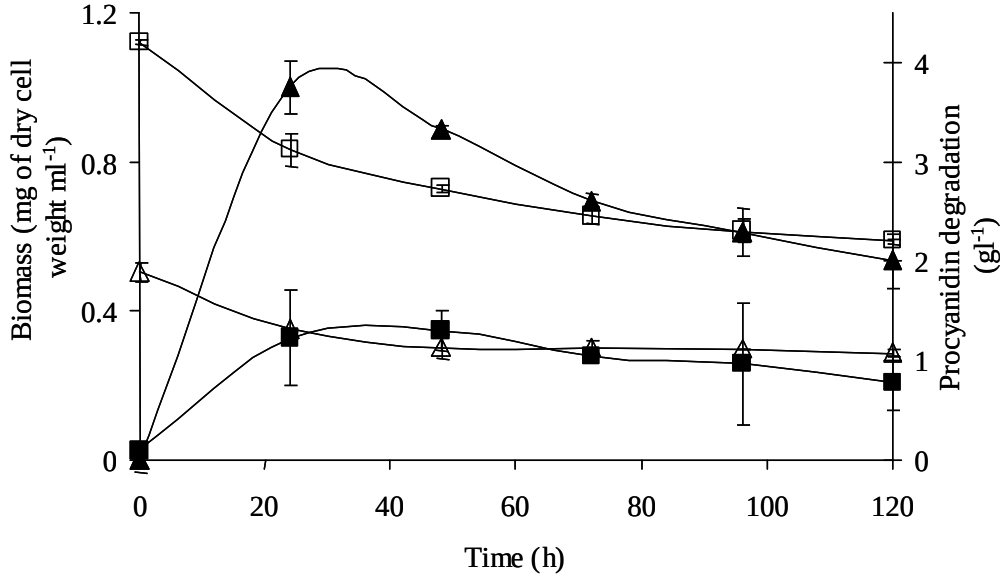

Figure 2. Kinetics of procyanidin degradation containing 4 $g \cdot l^{-1}$ of PC (□) or 2 $g \cdot l^{-1}$ glucose + 2 $g \cdot l^{-1}$ PC (Δ). Biomass production in a medium containing 4 gl^{-1} of PC (■) or 2 $g \cdot l^{-1}$ glucose+ 2$g \cdot l^{-1}$ PC (▲).

When glucose was present, maximal biomass (1.01 mg of dry cell weight ml^{-1}) was reached at 24 h of culturing and it was almost conserved until 36 h. After that, a decrease of growth was observed. Total PC seemed to be consumed in 60 h of culturing with a PC degradation rate of 0.015 $g \cdot l^{-1} h^{-1}$. On the other hand, a higher PC degradation rate (0.032 $g \cdot l^{-1} h^{-1}$) was observed in the medium when PC was used as the only source of carbon but a strong decrease in biomass was also observed (0.31 mg of dry cell weight ml^{-1}). It has already been observed that fungal hyphae complexes strongly with oligomeric and monomeric condensed tannins [22] and it is well know that tannins, particularly condensed tannins, form large complexes mainly with proteins and other macromolecules [23]. Thus, the decrease in the biomass could be due to complex formation of fungal biomass with polymeric procyanidins. However, PC degradation was observed from initial time of fermentation. Thus, in order to understand the role of *A. fumigatus* in PC degradation, a study was conducted to evaluate the DP*n* from kinetic culture when biomass was produced in glucose containing medium. The thiolysis-HPLC reaction is a technique strongly used to characterize and quantify constitutive units from a polymer of PC [16, 24]. Moreover, it is possible to know the DP*n*, thanks to the relation between these units (terminal and extension). A consecutive increase in the DP*n* was observed, from 8.2 at initial to 36.3 at the end of fermentation (Figure 3).

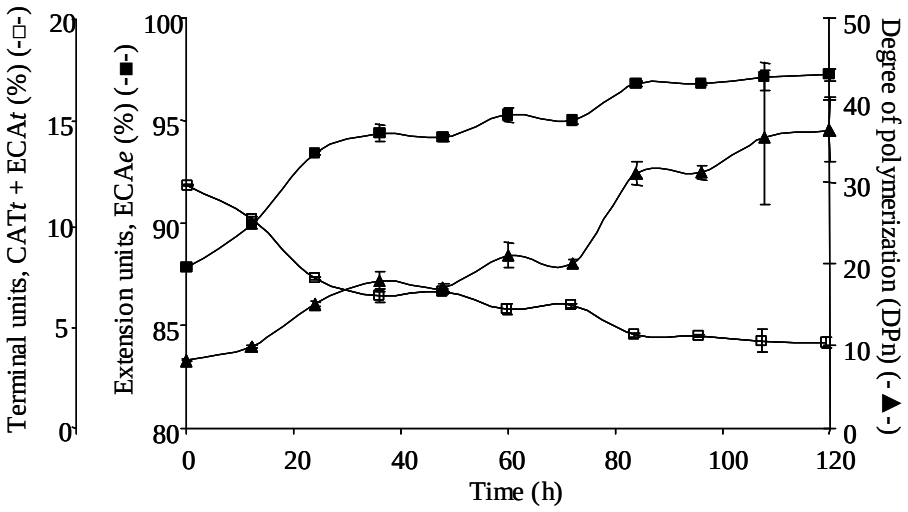

Figure 3. Kinetics of total terminal units (CAT_t + ECA_t) (□), extension units (■) and measure of DPn (▲) resulting from *A. fumigatus* fermentation of apple procyanidin extract.

These results are in agreement with the decrease of the total terminal units, [(+)-catechin and (-)-epicatechin] from 12.1 to 2.7% . This result is correlated with an increase in extension units (from 87.8 to 97.2%) resulting therefore in an increase in the DP*n* (Figure 3).

Previous studies had shown that hydrolysable tannins could be depolymerized in order to produce the monomeric compound gallic acid, a product formed after depolymerization of tannic acid [25]. Our results suggest that degradation of PC polymers does not happen by a depolymerization, as it has been observed in hydrolysable tannins degradation, since no increase of monomeric catechin units was observed. In

contrary, a diminution of total terminal units was obtained. The results suggest that terminal units possibly are being modified by the presence of fungal enzymes and the new structures formed can not be identified from thiolytic reaction. A second explanation could be that the microorganism preferentially consumes the short chain oligomers, leaving aside large polymers. Sambandam and Mahadevan, [26] reported the degradation pathway of the monomeric condensed tannin by the fungus *Chaetomium cupreum*. The (+)-catechin was degraded to phloroglucinol carboxylic and protocatechuic acid by a catechin oxygenase. This pathway was also found in aerobic bacterial degradation [27] and recently, it was demonstrated that the filamentous fungus *Diaporthe* sp. oxidized C4 carbon of (+)-catechin and (-)-epicatechin to give 3,4-cis-dihydroxyflavan [28]. However, another explanation related to DP*n* was also considered. Thus, Normal-Phase HPLC analysis was conducted in order to separate procyanidin oligomers (Figure 4).

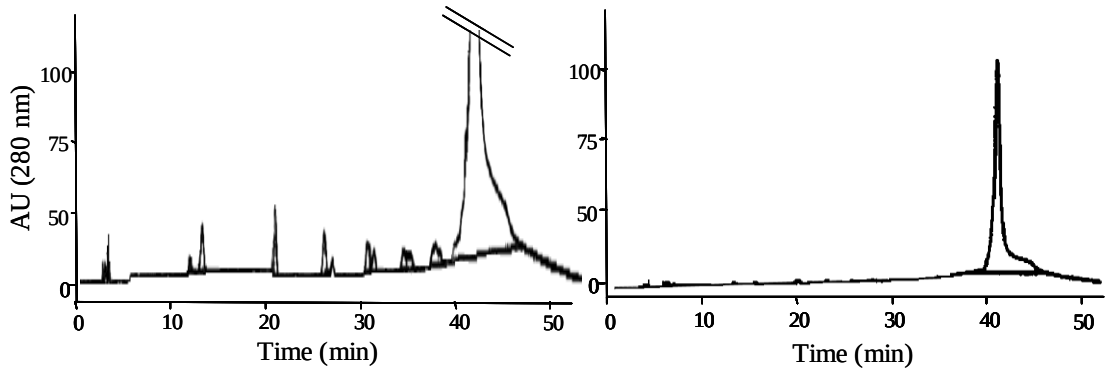

Figure 4. Profile of procyanidins by Normal-Phase HPLC. At the initial (A) and at the end (B) of the fermentation time.

Chromatogram profiles showed that lower PC oligomers diminished at the end of fermentation time and these phenolic compounds seemed to be degraded preferentially, leading to an accumulation of polymeric PC. The degradation of PC is possibly due to the presence of enzymes present during the initial fermentation time, degrading the lower weight PC which are more easily degradable than polymeric PC. Thus, decrease of PC's oligomers could be correlated to increase of DP*n*.

4. Conclusions

The present study revealed that almost all filamentous fungi screened, were able to grow and degrade procyanidins under liquid fermentation after 72h of culturing. It was demonstrated using the best strain, *A. fumigatus*, that DP*n* of procyanidin increased obtaining the highest value at the end of fermentation. These findings have had different explanations. However, thiolysis-HPLC reaction applied in fungal cultures containing PC resulted in a decrease of terminal units. It clearly points to the fact that the fungus produced an enzyme which could act on (-)-epicatechin or (+)-catechin units, but this reaction was not due to the depolymerization of PC as it has usually been observed with hydrolysable tannins. On the other hand, Normal-Phase HPLC analysis indicated that the oligomers of PC were degraded during the initial stages.

This is the first report wherein, the characterized procyanidin purified from cider apple was used as sole carbon source in order to evaluate the degradation potency of filamentous fungi. Moreover, thiolysis-HPLC method was used to quantify and characterize the DP*n* and constitutive units from fungal fermentation kinetics. Further studies are in progress on the capability of this fungal strain to degrade low molecular procyanidins (dimers) and evaluate/characterize the degradation products and also the enzymes involved in the procyanidin degradation pathway.

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