J Chem Technol Biotechnol 80:1154–1159 (2005)
DOI: 10.1002/jctb.1302

Production of ellagic acid from degradation of valonea tannins by *Aspergillus niger* and *Candida utilis*

Bi Shi,[1]* Qiang He,[1] Kai Yao,[2] Wen Huang[1] and Qing Li[2]

[1]The Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University, Chengdu 610065, People's Republic of China

[2]The Department of Food Engineering, Sichuan University, Chengdu 610065, People's Republic of China

Abstract: Two fungal strains, effective in converting valonea (*Quercus aegilops*) tannins into ellagic acid (EA), were isolated from soil contaminated by valonea tannins, and were tentatively identified as *Aspergillus niger* and *Candida utilis*. Properties of EA accumulation by the two isolates from valonea tannins' fermentation were studied. Both the strains preferred sucrose to glucose as the additional carbon and energy source. The most suitable concentrations of valonea tannins in fermentation media for EA accumulation by *A niger* and *C utilis* were 5.0 and 9.0 g dm^{-3} with EA yields of 12.1 and 8.9% (w/w), respectively. The optimal temperature for the two strains was 28 °C, while the preferred pH values of the fermentation media were 4.5–5.0 for *A niger* and 4.8–5.2 for *C utilis*. The tannin tolerances of *A niger* and *C utilis* were adapted to 20 and 25 g dm^{-3} by gradually increasing the concentrations of valonea tannins in the culture media. The adapted strain of *A niger* was able to completely degrade 20 g dm^{-3} valonea tannins with an EA yield of 14.3% in 9 days. Meanwhile, the adapted strain of *C utilis* decreased the valonea tannins' level from 25 to 9.1 g dm^{-3} with an EA yield of 11.48%. The degradation ability of *A niger* came from tannase whose activity in the medium was 63 U cm^{-3} at the ninth day of fermentation, and that of *C utilis* was due to both tannase and polyphenol oxidase (PPO) whose activities were 32 U cm^{-3} and 29 U cm^{-3} at the ninth day. The coculture of both the adapted strains could completely degrade 25 g dm^{-3} valonea tannins in only 7 days with a remarkably increased EA yield (21%). The activities of tannase and PPO of the coculture at the seventh day were 66 U cm^{-3} and 47 U cm^{-3} respectively, which proved the synergistic effect of the two enzymes on valonea tannins' degradation and EA accumulation.

Keywords: *Aspergillus niger*; *Candida utilis*; biodegradation; ellagic acid; valonea tannins; coculture

INTRODUCTION

Ellagic acid (EA, Fig 1) is a naturally occurring phenolic compound existing in many plants, such as grape, strawberry, raspberry, pecan, walnut, valonea, etc. During the past few decades, ellagic acid has been reported to have antimutagenic, anticarcinogenic and antioxidative activities, and shown good prospects in various applications.[1–3] Ellagic acid is usually present in plant sources as ellagitannins which primarily consist of glucose esterified with hexahydroxydiphenic acid, gallic acid and their derivatives. To obtain ellagic acid from plant sources, some studies on hydrolyzing ellagitannins with acid or base have been reported.[4,5] Due to the difference in plant sources, the variation of ellagitannin structures and the difficulty of purification, these chemical methods often led to low yield of ellagic acid and considerable impurities. In this study, the biodegradation of valonea tannins (Fig 1) was investigated. As typical ellagitannins, valonea tannins contain a large percentage of hexahydroxydiphenoyl groups (HHDP, Fig 1), having the potential to yield a high level of ellagic acid. In the presence of tannase, the ester bonds between HHDP groups and glucose in valonea tannins could be broken,[6] and then HHDP could be converted into EA through coupled oxidation which might be mediated by the action of polyphenol oxidase (PPO).[7] Although tannins usually inhibit the growth of microorganisms, certain strains, in their natural milieu, are resistant to tannins.[8] Several studies on tannin biodegradation have appeared. Some of them investigated the production of tannase,[9] but the rest were focused on tannin elimination in natural environments, such

* Correspondence to: Bi Shi, The Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University, Chengdu 610065, People's Republic of China
E-mail: Sibitannin@vip.163.com
Contract/grant sponsor: National Science Fund for Distinguished Young Scholars; contract/grant number: 20325619
Contract/grant sponsor: High Tech Research and Development (863) Programme; contract/grant number: 2003AA 647020
(*Received 10 March 2004; revised version received 8 November 2004; accepted 10 January 2005*)
Published online 22 July 2005

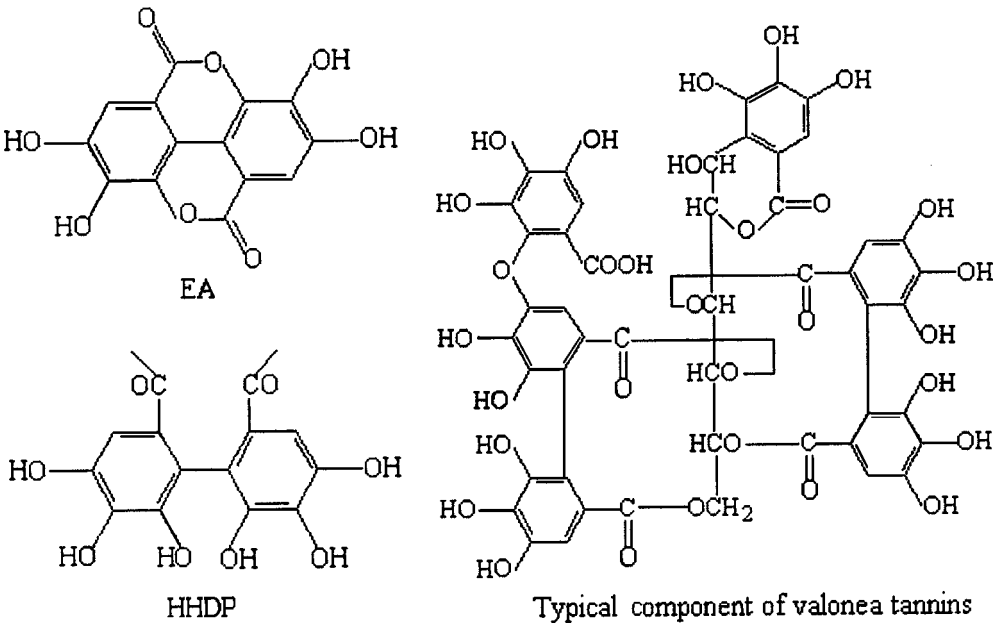

Figure 1. Structures of ellagic acid (EA), hexahydroxydiphenoyl group (HHDP) and valonea tannins.

as in tannery effluent[10] and waste water.[11] The present investigation reports the formation of tannin-degradation-products.

Two strains, one each of *A niger* and *C utilis*, were isolated and adapted, and their ability to convert valonea tannins into EA were studied. The growth of the two strains, the degradation of valonea tannins and the enzymes produced in fermentation media of monocultures of each strain and of coculture of both the strains were estimated and compared.

EXPERIMENTAL

Chemicals and culture media

Samples of valonea tannins, extracted from the fruit shell of *Quercus aegilops*, were supplied by Chengdu Tannin Extract Factory, Chengdu, China: the tannin content was 68.3% (w/w) measured by the method of Makkar *et al*.[12] Ellagic acid was purchased from Sigma Chemical Co, St. Louis, MO, USA, and the other reagents used were analytical grade. The enriched liquid medium was composed ($g\,dm^{-3}$) of $(NH_4)_2SO_4$ 5.0, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 1.0, KH_2PO_4 1.0, $CaCl_2.2H_2O$ 0.02, $FeCl_3.2H_2O$ 0.05, proteose peptone 5.0, yeast extract 2.0 and glucose 30; pH 5.0. The selective liquid medium contained ($g\,dm^{-3}$) $NaNO_3$ 2.0, K_2HPO_4 1.0, KH_2PO_4 1.0, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4$ 0.01 and valonea tannins $5.0\,g\,dm^{-3}$, pH 5.0. The selective agar medium, used for purification of the strains and preparation of inoculum for studies on the fermentation of valonea tannins, had the same composition as that of the selective liquid medium, except that 3% (w/v) agar[13] was added. The accumulation of EA was carried out in liquid fermentation medium, composed of the selective liquid medium, valonea tannins, and $30\,g\,dm^{-3}$ sucrose or glucose.

Isolation, cultivation and adaptation of strains

As a source of inoculum, soil samples containing valonea tannins were obtained from the Chengdu Tannin Extract Factory, China. Both enrichment and selection cultures were aerobically carried out at $2\,rad\,s^{-1}$ and 28 °C for 48 h. After incubation, $2\,cm^3$ of the selection culture liquid was inoculated onto selective agar plates and was incubated at 28 °C for 48 h. After two successive subcultures on selective agar plates, two colony-isolates that had fastest growth rate were obtained. Biochemical tests, carried out by The Microorganism Laboratory of Sichuan University (China), tentatively identified the isolates as *Aspergillus niger* and *Candida utilis*, according to the method of Kathleen and Arthur.[14] Under the growth conditions of pH 5.0, 28 °C and $2\,rad\,s^{-1}$, the two strains were successively adapted to higher tannin concentrations in the selective liquid medium with valonea tannin contents of 5.0, 10.0, 15.0, 20.0 and $25\,g\,dm^{-3}$. The course of valonea tannins' fermentation by *A niger* and *C utilis* was initiated by placing four $4\,mm^2$ agar disks taken from pure culture of each strain into $500\,cm^3$ flasks containing $100\,cm^3$ fermentation fluid medium. Coculture of *A niger* and *C utilis* was initiated by simultaneous inoculation of two $4\,mm^2$ agar disks of each strain. Monocultures of *A niger* and of *C utilis* and the coculture were repeated three times.

Determination of cell growth, tannin and ellagic acid contents

The fermentation fluid samples ($2\,cm^3$) were centrifuged at $0.5\,rad\,s^{-1}$ for 10 min, and the absorbance of the supernatant at 660 nm was measured as a growth parameter of strains.[15] Meanwhile, the samples were centrifuged at $70\,rad\,s^{-1}$ for 10 min, and the sediment containing microbial cells, EA and tannins was used for determination of residual tannins by the method of

Makkar *et al*.[12] For EA measurement, this sediment was added to ethanol (5 cm^3) and vibrated at 45 °C for 2 h for complete dissolution of EA. After filtration, four volumes of distilled water were added into the ethanol solution and the crystals of EA were obtained after standing at 4 °C for 24 h. The crystals were washed with distilled water and methanol successively, and then redissolved in ethanol. The content of EA in the ethanol solution was determined by a UV spectrophotometer at 255 nm on the basis of standard EA solutions. The EA yield was calculated as weight percentage of the accumulated EA to the original valonea tannins. All samples were analyzed in triplicate.

Enzyme assays

Cell-free enzyme extracts were prepared by ultrasonication as described earlier.[16] All samples and the controls were analyzed in triplicate.

Tannase

Tannase was assayed by the method of Sharma *et al*[17] with some modifications. The principle of the method is to determine the gallic acid generated from methyl gallate. The substrate solution (0.01 mol dm^{-3} methyl gallate prepared in 0.05 mol dm^{-3} citrate buffer, pH 5.0), enzyme preparation and buffer (0.05 mol dm^{-3} citrate buffer, pH 5.0) were pre-incubated at 30 °C for 5 min before assay. One cm^3 substrate solution was added into blank, test and control tubes, and then 1 cm^3 buffer was pipetted into the blank tube and 1 cm^3 enzyme preparation was pipetted into a test tube. After the three tubes had been incubated at 30 °C for 5 min, 1.2 cm^3 of methanolic Rhodanine (0.667%, w/v) was added to all the tubes which were then kept at 30 °C for 5 min. Hereafter, 0.8 cm^3 of 0.5 mol dm^{-3} potassium hydroxide was added to each tube and the tubes were incubated at 30 °C for 2.5 min. Then 1 cm^3 of the enzyme preparation was added into the control tube. Finally, each tube was diluted with 4.0 cm^3 distilled water, incubated at 30 °C for 10 min, and their absorbances at 520 nm (characteristic absorbance of gallic acid) were recorded against water. The enzyme activity was calculated from the difference of absorbances:

$$\Delta A_{520} = (A_{\text{test}} - A_{\text{blank}}) - (A_{\text{control}} - A_{\text{blank}})$$

One unit of enzyme activity was defined as μmol of gallic acid formed min^{-1}.

Polyphenol oxidase

The substrate solution (0.1 mol dm^{-3} pyrocatechol prepared by 0.05 mol dm^{-3} citrate buffer, pH 5.0), enzyme preparation and buffer (0.05 mol dm^{-3} citrate buffer, pH 5.0) were pre-incubated at 30 °C for 5 min before assay. Buffer (3.4 cm^3), substrate solution (1 cm^3) and enzyme preparation (1 cm^3) were successively added into the test tube and then kept at 30 °C for 10 min.[18] The test tube was then immediately kept in boiling water bath for 2 min to stop the enzyme reaction. The absorbance at 420 nm (characteristic absorbance of oxidized pyrocatechol) was recorded against water. The control tube containing 1 cm^3 enzyme solution was kept in a boiling water bath for 2 min and then 3.4 cm^3 buffer and 1 cm^3 substrate solution were added. The tube was kept at 30 °C for 10 min and the absorbance at 420 nm was recorded against water. The enzyme activity was calculated from the difference of absorbances:

$$\Delta A_{420} = A_{\text{test}} - A_{\text{control}}$$

One unit of enzyme activity was defined as an increase in absorbance by 0.001 min^{-1}.

RESULTS AND DISCUSSION

The EA-accumulating ability of the isolated strains

By enrichment culture of soil samples containing valonea tannins and subsequent selection culture with valonea tannins as sole carbon and energy source, two strains tolerant to valonea tannins were isolated and were tentatively identified as *Aspergillus niger* and *Candida utilis*. The effects of culture conditions, such as additional carbon source, valonea tannins' concentration in medium, cultivation temperature and initial medium pH, on the production of EA by the two strains were studied. It was shown that both glucose and sucrose promoted the yield of EA (Table 1), suggesting that the additional carbon sources favored the growth of strains or the production of enzymes involved in EA liberation. Previous studies[6] on tannin biodegradation by *A niger* reported that glucose was a better carbon and energy source for the growth of the strain, but our results showed that sucrose was more suitable for EA production by *A niger* and *C utilis*. Considering the fact that the degradation of tannins is based on catalytic reactions of enzymes produced during the growth of strains, therefore, it could be deduced that the enzymes needed for EA accumulation are not completely the same as those for tannins' elimination. Indeed, the pathways of tannin degradation are versatile, but the formation of EA, as a specific pathway of degradation, might need specific enzymes. Based on these observations, all further experiments in liquid fermentation medium were carried out by

Table 1. Effect of additional carbon and energy source on ellagic acid accumulation by *A niger* and *C utilis*[a]

	EA yields (%, w/w) with following additives:		
Strain	Control	Glucose	Sucrose
A niger	4.6	10.0	12.1
C utilis	2.8	8.1	8.9

[a] Cultivation was carried out in liquid fermentation media (pH 5.0, valonea tannins 5.0 g dm^{-3} for *A niger*, and 9.0 g dm^{-3} for *C utilis*, sucrose or glucose 30 g dm^{-3}) at 28 °C and 2 rad s^{-1} for 7 days.

using sucrose as the additional carbon and energy source.

Most fungi resistant to tannins are able to grow under a high concentration of tannic acid. The minimum inhibitory concentration (MIC) usually reaches 20–30 g dm^{-3} and a few strains show good growth even when the concentration of tannic acid is as high as 100 g dm^{-3}.[6] The tolerance of the isolated *A niger* and *C utilis* strains to valonea tannins was relatively lower. The most suitable concentrations of valonea tannins in liquid fermentation medium for EA production by *A niger* and *C utilis* were 5.0 and 9.0 g dm^{-3} respectively (Fig 2(A)). *A niger* preferred a lower concentration (5.0 g dm^{-3}) of valonea tannins, but showed a higher EA yield (11.5%), whereas *C utilis* had a preference for a higher concentration of valonea tannins (9.0 g dm^{-3}) with an EA yield of 8.7%. For both strains, with the increase in the tannins content, the EA yield first increased and then decreased, indicating that an optimum concentration of valonea tannins was required to induce the production of tannin-degrading enzymes, and that at higher concentrations the growth of both strains was inhibited, resulting in a decrease in EA yields.

During the course of fermentation, a slight decrease in the pH of the medium could be observed. Each strain had a suitable pH range, within which the EA yield was nearly constant. The maximum EA accumulation (11.5%) of *A niger* was attained when the initial medium pH was 4.5–5.0, while that of *C utilis* (8.7%) was attained at pH 4.8–5.2 (Fig 2(B)). For *A niger* and *C utilis* strains, the optimal culture temperature for EA accumulation was 28 °C, where the maximum EA yields were 12.1% and 8.9% respectively. In fact, no significant EA accumulation could be observed below 20 °C (Fig 2(C)).

To enhance the EA-producing capacity of *A niger* and *C utilis*, adaptations of both strains to higher tannin levels were carried out. As a result, the tannin tolerances of *A niger* and *C utilis* increased to 20 and 25 g dm^{-3} respectively, and very little growth inhibition was observed.

The monocultures of *A niger* and *C utilis*

Based on the results of single parametric experiments described above, the monocultures of adapted *A niger* and *C utilis* were investigated in detail and the growth of the strains, the residual contents of valonea tannins and the enzymes produced in the media are shown in Fig 3.

There was a 2-day lag for growth of *A niger*, but only 1 day for *C utilis*, which implied that *C utilis* could adapt more readily to a tannin environment having comparatively a higher content of valonea tannins (25 g dm^{-3}). After the period of lag, *A niger* had a faster growth within a shorter time than did *C utilis* and the absorbance of fermentation fluid of *A niger* at 660 nm peaked on the sixth day while that of *C utilis* was maximum on the eighth day. After an incubation of 9 days, the residual valonea tannins in both media remained essentially unchanged. The *A niger* strain had completely degraded 20 g dm^{-3} valonea tannins, and produced 63 U cm^{-3} of tannase in the fermentation medium in which no polyphenol oxidase activity was detected. The strain of *C utilis* produced 32 U cm^{-3} of tannase and 29 U cm^{-3} of polyphenol oxidase and degraded valonea tannins to a residual concentration of 9.1 g dm^{-3} from an initial level of 25 g dm^{-3}. It is well known that tannase is an ubiquitous enzyme existing in the plant kingdom and has strong ability to degrade tannins.[9,11] The higher degradation degree of valonea tannins by *A niger* indicated a prominent role of tannase in hydrolysis of galloyl and hexahydroxydiphenoyl esters attached to glucose in ellagitannin molecules. Consequently, the lower content of tannase produced by *C utilis* might be the reason for higher residual valonea tannins in the medium. The *C utilis* strain degraded 15.9 g dm^{-3} valonea tannins, equal to 80% of the tannins degraded

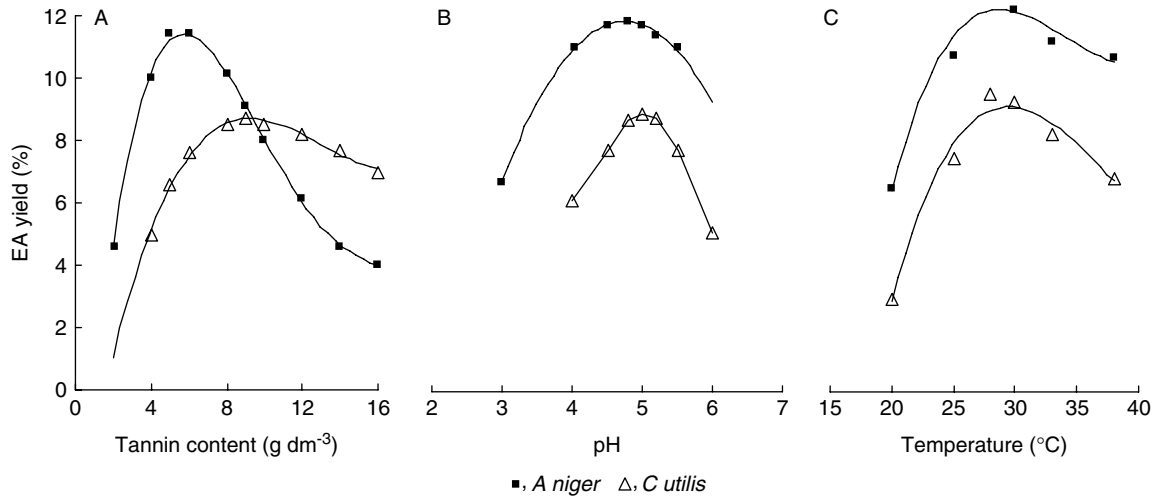

Figure 2. Effects of valonea tannin concentration (A), initial media pH (B) and culture temperature (C) on EA accumulation by *A niger* and *C utilis*. The strains were cultivated at 2 rad s^{-1} for 7 days in liquid fermentation medium in the conditions: (A) 30 °C, pH 5.0; (B) 30 °C, valonea tannins 5.0 and 9.0 g dm^{-3} for *A niger* and *C utilis*, respectively; (C) pH 5.0, valonea tannins 5.0 and 9.0 g dm^{-3} for *A niger* and *C utilis*, respectively.

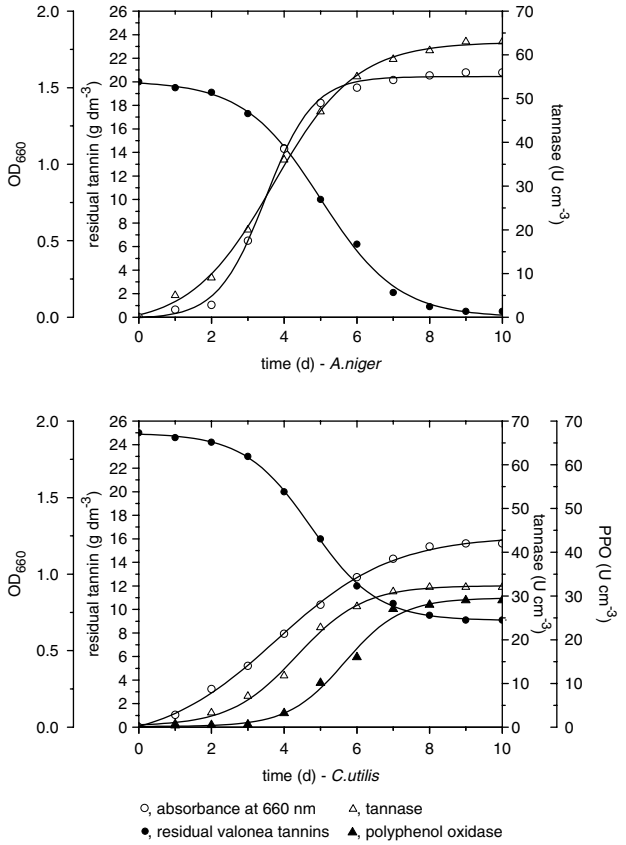

Figure 3. Degradation of valonea tannins and production of enzymes by *A niger* and *C utilis*. The cultivations were carried out at pH 5.0, 28 °C and 2 rad s^{-1}. The original valonea tannin contents were 20 g dm^{-3} for *A niger* and 25 g dm^{-3} for *C utilis*. Assays were done at indicated time for absorbance at 660 nm, residual valonea tannins, tannase and polyphenol oxidase.

by *A niger*, at 50% activity level of tannase when compared with *A niger*. This implies that PPO might be also contributing to degradation of valonea tannins and the accumulation of EA, which is in agreement with the reports[7,18] that PPO plays an important role in oxidative breaking and coupling of tannins.

The coculture of *A niger* and *C utilis*

The maximum EA yields from valonea tannins' fermentation by monocultures of *A niger* and *C utilis* were 14.3% and 11.8% respectively (Fig 4). The purpose of employing a coculture of *A niger* and *C utilis* was to convert valonea tannins into ellagic acid more effectively. This approach is based on the fact that, in many cases, the biodegradation of natural products can be significantly enhanced by using a coculture of microbes.[15,19] *A niger* and *C utilis* displayed different characteristics in valonea tannins' degradation. The former microbial strain had a lower tolerance to valonea tannins (20 g dm^{-3}), produced higher activity of tannase with faster growth and did not have PPO activity in the medium. The latter strain had a higher tolerance to valonea tannins (25 g dm^{-3}) with a shorter lag of growth, yielded lower activity of tannase, but produced PPO in the fermentation medium. Therefore, the synergistic effect of coculture

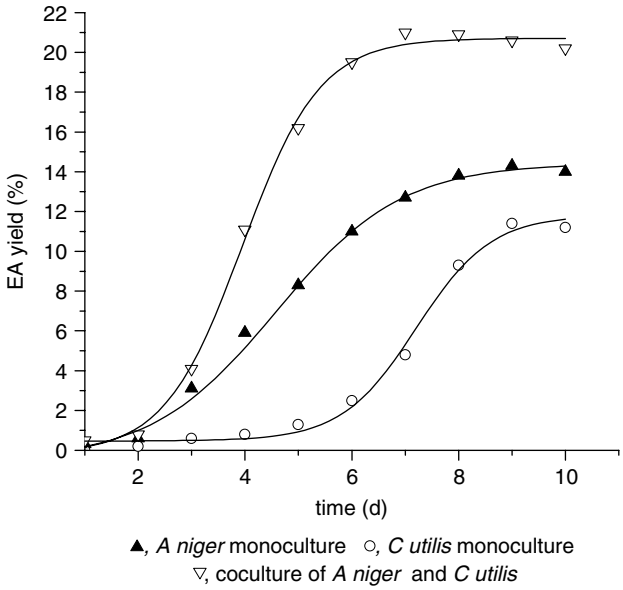

Figure 4. Time courses of EA accumulation by *A niger* monoculture, *C utilis* monoculture and the coculture of *A niger* and *C utilis*.

of *A niger* and *C utilis* on increasing EA yield was desirable. The coculture was carried out by using the same inoculum size as those for *A niger* and *C utilis* monocultures. The time course of EA formation by monocultures of *A niger* and *C utilis* and of the coculture of the two strains indicated that the profile of the coculture was similar to that of *A niger* monoculture, but the coculture provided higher EA yield (Fig 4).

In comparison with both the monocultures (Fig 3), the general growth of strains of the coculture was faster and the maximum amount of biomass was reached within a shorter time (4 days), as shown in Fig 5. As a result, a substantial degradation of valonea tannins was achieved in 7 days. The activity of tannase yielded by the coculture was 66 U cm^{-3}, nearly equal to that of *A niger* monoculture, but a higher PPO

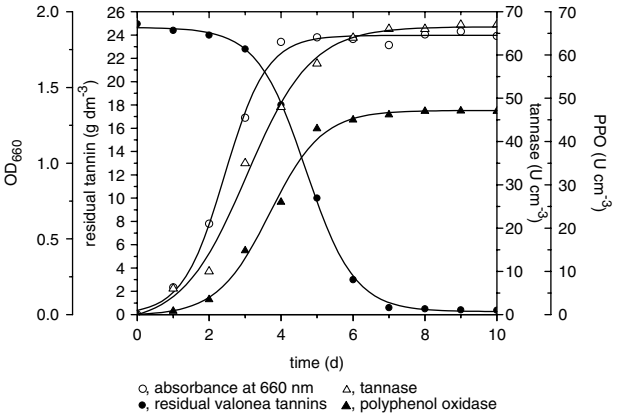

Figure 5. Degradation of valonea tannins and production of tannase and PPO by coculture of *A niger* and *C utilis*. The cultures were grown at pH 5.0, 28 °C and 2 rad s^{-1}. The original valonea tannins content was 25 g dm^{-3}. The cultures were periodically monitored for absorbance at 660 nm, residual valonea tannins, tannase and polyphenol oxidase.

activity (47 U cm^{-3}) was observed in the coculture medium. So, it could be deduced that the coculture was not the simple summation of the monocultures, but was the result of a synergistic effect of the two strains. From the first to the fifth day, the tannase and PPO activities of the coculture increased quickly, accompanied by a fast increase in EA yield (Fig 4). The highest EA yield (21%) of the coculture was attained in 7 days, while those of both the monocultures were attained in 9 days (Fig 4). Compared with EA yields of *A niger* and *C utilis* monocultures, the EA yield of the coculture was increased by 1.47- and 1.78-fold respectively. This remarkable increase in EA yield of the coculture was possibly due to the increased PPO activity in the medium since the activity of tannase had no substantial change compared with that of *A niger* monoculture (Fig 3), indicating that PPO might cause the conversion of the intermediate HHDP groups to EA through coupled oxidation.[18]

Although numerous studies have been conducted on biodegradation of tannins and on the degradation mechanism of some simple tannins, such as gallotannin,[20] 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 stage, and further studies on ellagic acid accumulation by biodegradation of valonea tannins should be carried out to exploit the potential of various strains for commercial applications.

ACKNOWLEDGEMENTS

This project is financially supported by National Science Fund for Distinguished Young Scholars (20325619) and High Tech Research and Development (863) Programme (2003AA 647020).

REFERENCES

1 Meyer AS, Heinonen M and Frankel EN, Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry* **61**:71–75 (1998).

2 Bohn AA, Forsyth CS, Stoner GD, Reed DJ and Frank AA, Effect of cocaine, 95% oxygen and ellagic acid on the development and antioxidant status of cultured rat embryos. *Toxicology Letters* **95**:15–21 (1998).

3 Ogawa Y, Kanatsu K, Iino T, Kato S, Jeong YI, Shibata N, Takada K and Takeuchi K, Protection against dextran sulfate sodium-induced colitis by microspheres of ellagic acid in rats. *Life Sciences* **71**:827–839 (2002).

4 Daniel E-M, Ratnayake S, Kinstle T and Stoner G-D, The effects of pH and rat intestinal contents on the liberation of ellagic acid from purified and crude ellagitannins. *J Nat Prod* **54**:946–952 (1991).

5 Shi B, Di Y and He Y-J, Oxidizing degradation of valonea extract and utilization of the products—Part 1. Oxidizing degradation of valonea extract and characterization of the products. *J Soc Leath Technol Chem* **84**:258–262 (2000).

6 Ganga P-S, Nandy S-C and Santappa M, Effect of environmental factors on the production of fungal tannase. *Leather Sci* **24**:8–16 (1977).

7 Tremoleres M, Isolation and characterization of polyphenol oxidase from senescent of black poplar. *Phytochemistry II* **3**:301–305 (1985).

8 Scalbert A, Antimicrobial properties of tannins. *Phytochemistry* **30**:3875–3883 (1991).

9 Lekha PK and Lonsane BK, Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface and submerged fermentations. *Process Biochem* **29**:497–503 (1994).

10 Saxena RK, Sharmila P and Singh VP, Microbial degradation of health-risk compounds, in *Biotransformations, Microbial Degradation of Tannins*, ed by Singh VP. Elsevier Science Publishers BV, Amsterdam, pp 259–270 (1995).

11 Field JA and Lettinga G, Degradation of environmental pollutants by microorganisms and their metalloenzymes, in *Metal Ions in Biological Systems, Biodegradation of Tannins*, ed by Sigel H. Marcel Dekker Inc, New York, pp 61–97 (1992).

12 Makkar HPS, Blummel M, Borowy NK and Becker K, Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J Sci Food Agric* **61**:161–165 (1993).

13 Nelson KE, Pell AN, Schofield P and Zinder S, Isolation and characterization of an anaerobic ruminal bacterium capable of degrading hydrolyzable tannins. *Appl Environ Microbiol* **61**:3293–3298 (1995).

14 Kathleen P-T and Arthur T, Foundations, fungal classification and identification in microbiology, in *Eucaryotic cells and Microorganisms*, ed by Nancy M. McGraw-Hill Co, Singapore, pp 137–145 (1990).

15 Yutaka M, Characterization of a symbiotic coculture of *Clostridium thermohydrosulfuricum* YM 3 and *Clostridium thermocellum* YM 4. *Appl Environ Microbiol* **56**:37–42 (1990).

16 Hopper W and Mahadevan A, Utilization of catechin and its metabolites by *Bradyrhizobium japonicum*. *Appl Microbiol Biotechnol* **35**:411–415 (1991).

17 Sharma S, Bhat TK and Dawra RK, Isolation, purification and properties of tannase from *Aspergillus niger* van tieghem. *World J Microbiol Biotechnol* **15**:673–677 (1999).

18 Wang M and Zheng S-Z, Physiological and biochemical properties of the polyphenol oxidase in *Eichhornia crassipes*. *Journal of Fudan University (Natural Science)* **33**:157–163 (1994).

19 Ng TK, Bassat AB and Zeikus JG, Ethanol production by thermophilic bacteria: fermentation of cellulolytic substrates by coculture of *Clostridium thermacellum* and *Clostridium thermohydrosulfuricum*. *Appl Environ Microbiol* **41**:1337–1343 (1981).

20 Bhat TK, Singh B and Sharma OP, Microbial degradation of tannins—a current perspective. *Biodegradation* **9**:343–357 (1998).