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Short Communication

Biotransformation of ferulic acid to 4-vinylguaiacol by a wild and a diploid strain of *Aspergillus niger*

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ABSTRACT

Ferulic acid biotransformation has a number of interesting industrial uses. Ferulic acid biotransformation by the wild strain *Aspergillus niger* C28B25 and a diploid strain DAR2, obtained by parasexual recombination, was studied. The wild strain of *A. niger* C28B25 biotransforms ferulic acid to vanillic acid (VA); while the diploid strain DAR2 preferentially decarboxylates ferulic acid to 4-vinylguaiacol (4VG). The latter was identified by mass spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, and quantified by HPLC. The diploid strain *A. niger* DAR2 and the wild strain showed a ferulic acid conversion of 64% and 36%, respectively. Molar yields show that the formation of 4VG was preferred, being as much as 4.4 times higher than the formation of VA in diploid strain cultures. Differential regulation of enzymes involved in the biotransformation of ferulic acid may explain the accumulation of 4VG by diploid DAR2. This strain produced both 4VG and VA.

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1. Introduction

Ferulic acid (FA) is one of the most abundant phenolic compounds, it is well known for its antioxidant properties; it occurs in plant cellular walls either freely or linked covalently to biopolymers (Mathew and Abraham, 2006). The compound 4-vinylguaiacol (4VG) is a particularly valuable product in the food industry, for example, being used as flavouring in beers such as Belgian wheat and German Rauch (Mathew et al., 2007). It is also used as a starting material for the manufacture of chemical commodities such as vanillin, and in the ophthalmic field for preparing a solution containing flavouring agents (US Patent 20060292188). The aim of this work was to study the biotransformation of FA by the wild and the diploid strain of *Aspergillus niger* C28B25. The latter had been obtained previously by parasexual recombination (Montiel-González et al., 2002).

2. Methods

2.1. Microorganism

Two strains of *A. niger* were used: the wild strain C28B25 and diploid strain DAR2, The wild and diploid strains of *A. niger* were maintained in potato dextrose agar (PDA), and in Czapek Dox agar (Difco), respectively.

2.2. Inoculum preparation

Spore suspensions for both strains were obtained from cultures that had been incubated for 96 h at 30 °C in 250 ml Erlenmeyer flasks containing 50 ml of corresponding maintenance agar. The spores were counted using a Neubauer hematocytometer and the inoculum was adjusted to 10^{7} spores/ml.

2.3. Cultures for ferulic acid biotransformation

The composition of the culture medium was as follows (g/l): 3, sucrose; 3, yeast extract; 3, $NaNO_3$; 0.5, K_2HPO_4; 0.5, $MgSO_4$ and 0.5, KCl. FA was first diluted in methanol and filtered through a 0.20 μm Millipore membrane, and added to reach a final concentration of 800 mg/l. Fermentations were carried out in 35 ml glass bottles containing 10 ml of broth. Incubation was realized at 30 °C for 120 h at 100 rpm. Samples were taken in duplicate every 12 h, mycelium was removed by filtration and biomass quantified by gravimetry. Aliquots were analyzed for FA, products, sugar concentrations and pH.

The molar yield of product formation to FA consumed was expressed as:

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$$Y = \frac{P_f - P_0}{FA_0 - FA_f}$$

where P_0 and P_f are the initial and final molar concentration of the product respectively. FA_0 and FA_f are the initial and final concentration, respectively. FA conversion (%) was expressed as follows:

$$\text{FA conversion}(\%) = \frac{FA_0 - FA_f}{FA_0} * 100$$

2.4. Analytical techniques

2.4.1. Preparative chromatography (PC) and purification of compounds

Cultures were carried out in 500 ml Erlenmeyer flasks with 300 ml of medium for product purification and identification; cell-free samples were obtained at 120 h. Samples were extracted three times with chloroform, and then the solvent was removed under vacuum at 40 °C. Extracts were resuspended in 2 ml of methanol. PC of extracts was carried out on a silica glass plate using toluene, 1,4-dioxane, acetic acid (90:25:4) as the mobile phase (Hopper and Mahadevan, 1997) fractions were analyzed by HPLC and characterized by GC–MS and NMR.

2.4.2. Phenolic and sugar analysis by HPLC

Phenolic and sugar content were analyzed by HPLC as described by Barghini et al. (2007) and Tovar-Castro et al. (2008), respectively.

2.4.3. NMR

The NMR assays were recorded on a Brucker DMX 500 MHz spectrometer (Karmakar et al., 2000.). Chemical shifts values (ppm) and coupling constants (J) were reported in hertz (Hz): ^{1}H NMR: δ (ppm) 6.93 (d, 1H, J = 1.9 Hz, H_e), 6.91 (ddd, 1H, J_1 = 0.5, J_2 = 1.9, J_3 = 8.0 Hz, H_d), 6.86 (d, 1H, J = 8.0 Hz, H_e), 6.63 (dd, 1H J_1 = 10.8, J_2 = 17.6 Hz, H_c), 5.67 (br s, 1H, OH), 5.58 (dd, 1H J_1 = 0.9, J_2 = 17.5 Hz, H_a), 5.12 (dd, 1H, J_1 = 0.9, J_2=10.8 Hz, H_b), 3.90 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 146.57 (C-3-Ar), 145.63 (C-4-Ar), 136.62 (C-7), 130.27 (C-1), 120.05 (C-6), 114.34 (C-5), 111.44 (C-8), 107.99 (C-2), 55.56 (C-9).

2.4.4. GC–MS analysis

GC coupled to MS was performed and results were interpreted according to the corresponding decomposition patterns. GC–MS (m/z): 150 (M^+), 135 (M^+ $-CH_3$), 107 (M^+ $-CH_3$ and $CH_2{=}CH_2$) and 77 (Huang et al., 1993).

3. Results and discussion

Fig. 1 shows time course concentrations of FA, VA and 4VG for the wild and diploid strain of *A. niger* C28B25. Table 1 shows the conversion and yield values obtained for both strains after 5 days of cultivation.

Small changes in pH were observed: pH varied from 6.6 to 7.2, and 6.6 to 7 at the end of the cultures, for the wild and DAR2 strain, respectively. Sugar was totally consumed after 24 h, and higher values of biomass were reached by strain DAR2 (45 mg/ml) in comparison to the wild strain (35 mg/ml), suggesting a higher tolerance of DAR2 strain to FA toxicity (Tsioulpasa et al., 2002).

A FA conversion of 36% was observed (Table 1) and a maximal VA concentration of 137 mg/l was obtained, giving a yield of 57% for the wild strain. A similar concentration of VA was found by Ghosh et al. (2006), using *Paecilomyces variotii* MTCC 6581; after 16 days of cultivation, the productivity (mg/ld) was almost three times higher in the current study for the wild strain.

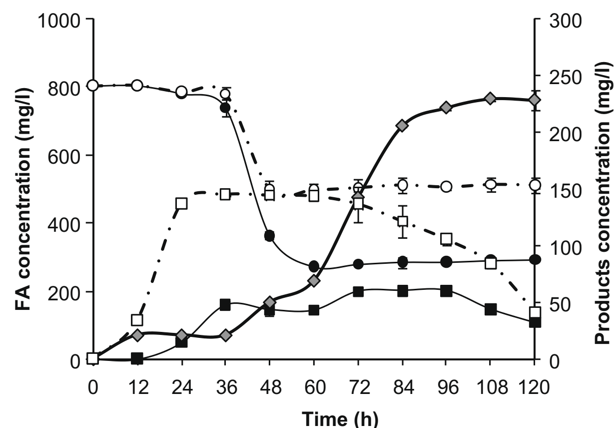

Fig. 1. Transformation of ferulic acid into vanillic acid and 4-vinylguaiacol by fungal strains *A. niger* C28B25 (wild type) and *A. niger* DAR2 (diploid). (○) ferulic acid *A. niger* C28B25, (●) ferulic acid *A. niger* DAR2, (□) vanillic acid *A. niger* C28B25, (■) vanillic acid *A. niger* DAR2, (♦) 4-vinylguaiacol *A. niger* DAR2.

Table 1
FA conversion and yield for the wild type strain and the DAR2 strain at 120 h of incubation.

Conversion	Wild type	DAR2
FA conversion (%)	36	64
Yield 4VG/FA (M/M)	ND	57
Yield VA/FA (M/M)	57	13

4VG: 4-Vinylguaicol; FA: ferulic acid and VA: vanillic acid.
ND: Not detected.

Low concentrations of methoxyhydroquinone for both strains were detected as well as other products that could not be identified and 15 mg/l of vanillyl alcohol was detected in DAR2 cultures. The main difference in the FA biotransformation was that the diploid strain DAR2 produced both 4VG and VA, while the wild strain produced mainly VA. Conversion of FA into products was 1.8 times higher in the diploid strain (64%) in comparison with the wild strain (36%). Molar yields show that the formation of 4VG was preferred, being as much as 4.4 times higher than the formation of VA in diploid strain cultures.

Biotransformation of FA by the diploid strain suggests a non-oxidative decarboxylation into 4VG, and this was reduced into vanillyl alcohol and methoxyhydroquinone. On the other hand, the wild strain biotransforms FA via a propenoic chain degradation to VA with subsequent oxidative decarboxylation into methoxyhydroquinone (Fig. 2). This suggests that the diploid strain biotransformed FA in the same way as *Paecilomyces variotii* or *Pestalotia palmarum* (Priefert et al., 2001). Although vanillin was not detected, this might be because vanillin is usually found at a low concentration and is rapidly metabolized (Karmakar et al., 2000).

Decarboxylation of FA could be part of a detoxification system to maintain the level of inhibitory compounds under a threshold concentration. A similar mechanism has been observed in the secondary metabolism, in particular in the phenylpropenoic acid pathway (Seshime et al., 2005).

Studies regarding the enzymes involved in decarboxylation of FA to 4VG have been performed mainly in bacteria and yeasts (Priefert et al., 2001). High concentrations of 4VG were obtained in *Bacillus coagulans* BK07 (908 mg/l) or *Debaryomyces hansenii* (1470 mg/l) cultures by Karmakar et al. (2000) and Mathew et al. (2007), respectively.

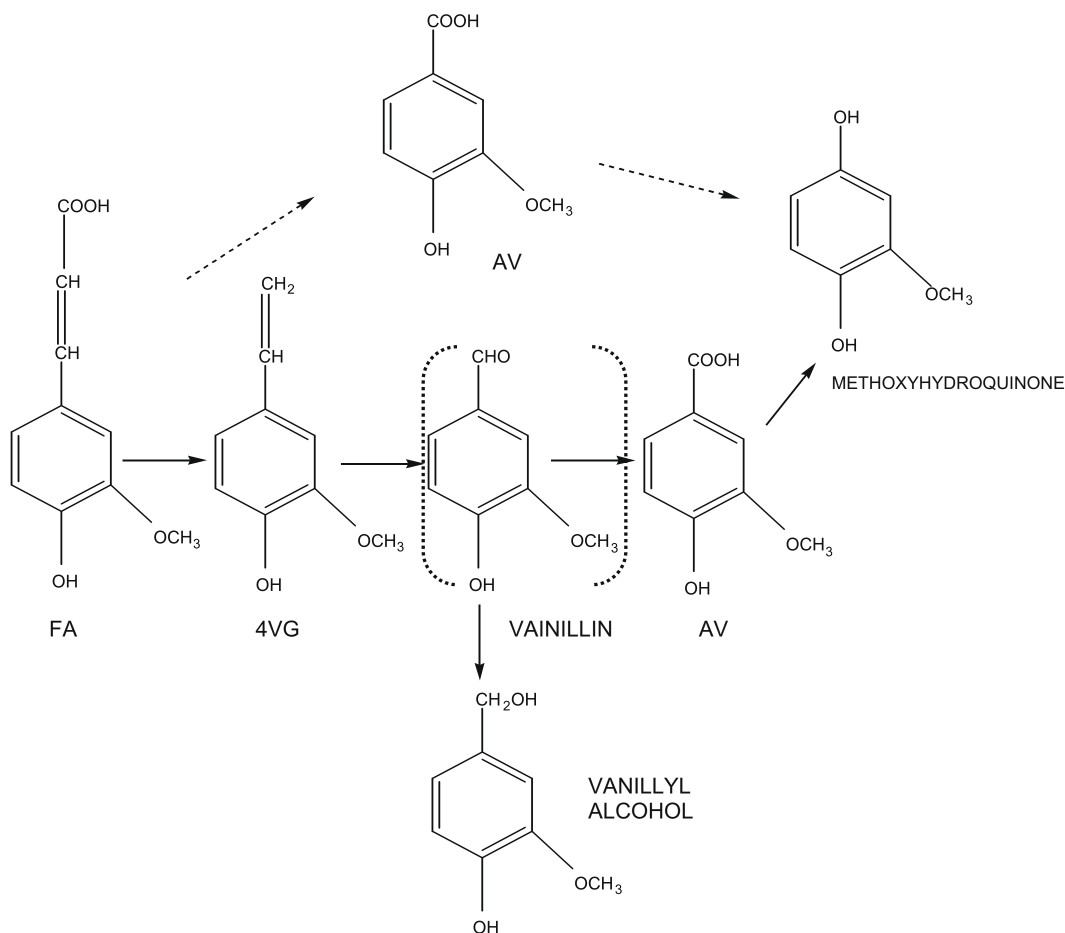

Fig. 2. FA biotransformation by strains *A. niger* C28B25 (dashed arrows) and DAR2. Dashed brackets indicate postulated product.

A. niger have been reported on FA biotransformation (Ward et al., 2006), nevertheless studies conducted on fungus regarding the enzymes involved in decarboxylation of FA to 4VG, are scatter. Deregulation of enzyme production has been reported for the diploid strain DAR2, used in this work, which was able to produce higher amounts of invertase and more rapidly in comparison to the wild type strain *A. niger* C28B25 (Montiel-González et al., 2002). In fact, the diploid strain showed variations in the proteins that regulate the expression of various hydrolytic enzymes (Loera and Córdova, 2003; Montiel-González et al., 2002). Accordingly, the differential regulation of enzymes involved in the biotransformation of FA may explain the accumulation of 4VG by the diploid strain, which could be advantageous for the formation of 4VG in a potential straightforward process, It is worth mentioning that the diploid strain is classified as a GRAS and thus it can be used to obtain 4VG for human consumption.

4. Conclusions

The wild strain of *A. niger* C28B25 biotransforms FA via a propenoic chain degradation to VA, with a lower FA conversion. The biotransformation of FA to 4VG by the diploid strain DAR2, preferentially followed the non-oxidative decarboxylation pathway, which could be advantageous for the formation of 4VG in a straightforward process. 4VG was confirmed by preparative chromatography techniques and identified by ^{1}H NMR, ^{13}C NMR and GC–MS. Differential regulation of the enzymes involved in the biotransformation of FA may explain the accumulation of 4VG by the diploid strain.

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