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Production of biovanillin from wheat bran

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Abstract

The fine specialty chemicals production from agro-industrial by-products such as cereal bran is a topic of high interest in wheat-producing countries. In this work, the possibility of obtaining vanillin from bioconversion of ferulic acid derived from enzymatic hydrolysis of wheat bran was explored. Freely available ferulic acid was first obtained through wheat bran hydrolysis by using commercially available, technical-grade enzyme preparations. Then, resting cells of an engineered *E. coli* strain, JM109(pBB1), containing the genes responsible for conversion of ferulic acid into vanillin, were used to transform ferulic acid available in the hydrolyzed mixture into vanillin. A vanillin molar yield of 50% was obtained, but most of the vanillin produced was reduced to vanillyl alcohol. The purification of the hydrolyzate with a ionic exchange resin to remove most of the carbohydrates allowed to obtain both higher bioconversion yields (up to 70%) and lower vanillin reduction. This is the first work in which vanillin is produced from ferulic acid obtained through wheat bran hydrolysis. The results obtained demonstrate the potential application of the proposed strategy in vanillin biosynthesis from agro-industrial by-products.

Keywords: Biovanillin; Wheat bran; Agro-industrial by-products; Ferulic acid; Bioconversion

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most important and widely used flavour in the food industry and it is the main component of vanilla. Natural vanillin is obtained from *Vanilla planifolia*, which, however, can supply only less than 1% of the annual market demand [1]. The majority of vanillin currently on the market is chemically produced from guaiacol or lignin [2,3]. Biotechnological production of vanillin via microbial bioconversion of substrates such as eugenol or ferulic acid is a feasible alternative way of obtaining vanillin and it has gained interest in the last years as vanillin thus produced can be labelled as "natural" by the European and US legislation [1,4–7].

In the last years, several studies have been focused on the production of specialty chemicals such as flavour compounds from wastes or surplus deriving from the agro-industry as a possible way of both disposing and valorising them [8]. Some raw materials, such as cereal bran, can be of interest in vanillin production as they are a potential source of ferulic acid. Cereal bran represents one of the most widely produced agricultural processing by-products [9]. The cell walls of higher plants, such as wheat, maize and rice, contain high amounts of ferulic acid which acts as a cross-linking agent among carbohydrates present in the wall [10,11]. Ferulic acid is linked to arabinoxylans through ester links [9]; therefore, for its use in vanillin production, it is necessary to break the ester bonds, which can be done either chemically or through the action of specific enzymes [12]. The latter option is a particularly interesting choice in order to produce "natural" vanillin [13–15]. A number of studies have therefore focused on the development of enzymatic methods to recover ferulic acid form cereal bran. Feruloyl esterases, capable of hydrolyzing the ester bond between ferulic acid and polysaccharides, have been isolated mainly from fungal strains, belonging to the genera *Fusarium* [15–17], *Neurospora* [18], *Aspergillus* [9,19,20], *Penicillium* [21], *Talaromyces* [22], *Humicola* [14] and *Sporotrichum* [23] and *Neosartorya* [24]. Several of the isolated enzymes, used in combination with other

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extracellular enzyme activities such as cellulases and hemicellulases, which degrade the polysaccharide fraction of the plant tissue, thus helping the access of feruloyl esterases to their substrate, allowed a nearly complete recovery of ferulic acid from wheat bran [14,25] and from corn bran [24]. In the work of Shin et al. [24], ferulic acid obtained from corn bran was solvent extracted and converted into vanillin with a yield of 43%.

Wheat cultivation is widely diffused in Italy: 7.8 million tonnes have been produced in 2005, from which approximately 1.6 million tonnes of bran have been obtained [26]. Wheat bran is currently used to produce animal feeds. However, there is great interest towards innovative strategies for valorizing it through its transformation into added value biomolecules. In the present work, freely available ferulic acid was first obtained from wheat bran purchased from a wheat mill in Italy using commercially available and technical-grade enzyme preparations. Then, a microbial biocatalyst consisting of an engineered *E. coli* strain, JM109(pBB1), containing the genes responsible for conversion of ferulic acid to vanillin in *Pseudomonas fluorescens* BF13 [27] was used to convert ferulic acid available in the hydrolyzed mixture into vanillin.

2. Materials and methods

2.1. Chemicals

Wheat bran employed in this work was purchased from Molino Grassi S.p.A. (Fraore, Parma, Italy); it has a humidity content of 10%. "Food-grade" ferulic acid (purity grade of 97%) was obtained from Wuhan Yuancheng Co. Ltd (China). HPLC solvents were from Carlo Erba, Milan, Italy. Reagents for culture media were from Biolife Italia, Milan, Italy, whereas salts for culture media and buffers were from Sigma–Aldrich, Milan, Italy.

2.2. Microorganisms, buffers and media

Escherichia coli JM109(pBB1) was used in this study: this strain contains a pJB3Tc19 plasmid derivative carrying a catabolic cassette for the conversion of ferulic acid into vanillin [27]. Luria Broth (LB) was prepared according to Sambrook et al. [28]. Saline buffer (pH 7) had the following composition (g/L): Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NH_4Cl, 1; NaCl, 0.5. Bioconversion buffer is saline buffer additioned with 1% (v/v) LB.

2.3. Enzyme preparations employed in the study

Cytolase® M102 is a pectinase obtained from DSM (Heerlen, The Netherlands); Pentopan 500BG®, commercialized as a preparation with a xylanase activity, Fungamyl Super AX®, a preparation containing α-amylase and pentosanase activity, Celluclast BG®, a cellulose, and Termamyl® 120 L, an α-amilase, were purchased from Sigma–Aldrich (Milan, Italy). Feruloyl and caffeoyl esterase activities were evaluated with the spectrophotometric method described by Ralet et al. [29] by using the corresponding methyl esters as substrates. Activity was expressed as μmoles of ferulic acid or caffeic acid released × mg^{-1} of enzymatic extract × min^{-1}. Pectinase, amylase and xylanase activities were assayed as described in Bailey et al. [30] by using 1% (w/v) pectine, α-amilose and xylane as substrates, respectively. Activity was expressed as μmoles of reducing sugars released × mg^{-1} of enzymatic extract × min^{-1}. β-Glucosidase activity was assayed spectrophotometrically with *o*-nitrophenyl-β-*D*-galactoside as the substrate according to Phillips [31]. Activity was expressed as μmoles of *o*-nitrophenol released × mg^{-1} of enzymatic extract × min^{-1}. Laccase activity was determined by measuring the change in absorbance at 502 nm due to the oxidative coupling of guaiacol and 3-methyl-2-benzothiazoline hydrazone as described elsewhere [32].

2.4. Wheat bran hydrolyzates production

Forty grams of wheat bran were dispensed in a 0.5 L Duran bottle and 0.28 L of water added. The bottle was subjected to a thermal treatment (80 °C for 180 min or 90 °C for 180 min in an oven or 121 °C for 20 min in autoclave). Then the enzyme preparation was added individually or in combination to the pre-treated bran and then the reaction mixture was incubated at 30 °C for 20 h. Samples were taken at the 5th hour and 20th hour of incubation and ferulic acid released was evaluated via high performance liquid chromatography-diode array detector (HPLC-DAD) as described later. The wheat bran hydrolyzate was centrifuged (6000 × *g* for 10 min), filter sterilized by a Cellulose Acetate filter (0.20 μm porosity, Albet, Barcelona, Spain) and stored at −20 °C in aliquots. pH of enzymatic hydrolyzates thus obtained was in the range 5.8–6.3. Chemical hydrolysis of wheat bran was obtained by treating wheat bran suspension with 2N NaOH as described by Courtin and Delcour [33].

2.5. Bioconversion experiments performed with resting cells of E. coli strain JM109(pBB1)

In order to prepare resting-cell experiments, a 1-L flask containing 0.1 L of LB plus ampicillin (50 μg/mL) was inoculated (2% v/v) with an overnight *E. coli* JM109(pBB1) culture. The flask was incubated at 37 °C and 150 rpm until the culture reached an A_{600} of 1.5, usually after 3.5 h of incubation. Cells were harvested by centrifugation (Beckman J2-HS centrifuge, Beckman Coulter, USA), washed with saline buffer, pH 7 and resuspended either in the bioconversion buffer supplemented with ferulic acid or directly in the enzymatic hydrolyzate, at the appropriate dilution in order to have a cell concentration of 4 mg (wet weight)/mL. Twenty milliliters of cell suspension were dispensed in 100 mL sterile flasks, which were then incubated at 30 °C and 150 rpm. One-milliliter samples were taken out at the beginning of the experiment and at pre-established times (generally after 1 h, 2 h, 3 h, 4 h and 20 h of incubation), acidified with 25 μL of 2N thrichloroacetic acid, centrifuged at 12,000 × *g* for 10 min and analyzed via HPLC-DAD. The extract was employed at three different concentration: (i) undiluted, with pH corrected to 7 at the beginning of the experiment, (ii) diluted in bioconversion buffer to have the extract at 75% (v/v) of the total volume and (iii) diluted in bioconversion buffer to have the extract at 50% (v/v) of the total volume. pH was checked in the diluted samples at the beginning of each experiment and when necessary adjusted to 7. Each experiment was performed in duplicate. Cell-free samples were prepared as control and processed in the same way. Ferulic acid molar conversion percentage was calculated by dividing its concentration at the end of the experiment by its initial concentration. Vanillin molar yield was calculated by dividing the concentration of vanillin produced by the initial concentration of ferulic acid.

The effect of the variation of some parameters such as pH of the bioconversion buffer, strain growth temperature, bioconversion temperature and dissolved oxygen concentration on the bioconversion rate and extent was also studied. For this purpose, an extract prepared with 1% (w/w) Cytolase M102® and 0.1% (w/w) Termamyl® 120 L with a ferulic acid concentration of 0.9 mM was used and it was diluted in the bioconversion buffer at 75% (v/v). In order to evaluate the effect of pH on the process, the bioconversion buffer was prepared at different pHs (6.0, 7.0, 7.5 and 8.0) by changing the molar ratio of the two phosphate salts. Two growth temperatures (30 and 37 °C) and two bioconversion temperatures (30 and 37 °C) were also studied. Finally, the bioconversion was performed in a 3 L stirred tank reactor (Solaris Biotechnology s.r.l., Porto Mantovano, Italy) filled with 2 L medium (enzymatic hydrolyzate diluted in the bioconversion buffer at 75% (v/v)) at pH 7 and 30 °C at two different dissolved oxygen concentration: 3.75 mg/L and 7.50 mg/L, corresponding to 50% and 100% of saturation.

2.6. Wheat bran hydrolyzate purification and its use in bioconversion experiments

Ferulic acid was selectively recovered from the crude enzymatic hydrolyzate (obtained with 1% (w/w) Cytolase M102® and 0.1% (w/w) Termamyl® 120 L), after separating the polysaccharide suspension filtering it on a paper, by

employing an Amberlite IRA 95 resin. The resin was washed with distilled water and then with 1N HCl. Then, the resin was washed with distilled water several times until pH of eluted water was 4.5. The activated resin was then added to the enzymatic hydrolyzate which was mixed at 50 rpm for 3–4 h. The hydrolyzate was then removed and the resin was treated with ethanol additioned with 4% (v/v) HCl for 1 h at room temperature to mobilize sorbed ferulic acid. The alcoholic ferulic acid rich extract thus obtained was neutralized by using 2N NaOH and then concentrated with a rotavapor. Both carbohydrates and ferulic acid concentration were evaluated in the extract prior to its use in bioconversion experiments performed as previously described. The purified extract was added to *E. coli* JM109(pBB1) bioconversion medium to reach a final ferulic acid concentration of 0.25 mM, 0.50 mM and 1 mM.

2.7. Bioconversion experiments performed with growing cells of E. coli strain JM109(pBB1)

They were performed in shaken flasks by using an enzymatic hydrolyzate (obtained with 1% (w/w) Cytolase M102® and 0.1% (w/w) Termamyl® 120 L) as the only source of carbon and energy. Three different conditions were assayed: (i) the enzymatic hydrolyzate was used as it was obtained (i.e. with no dilution and no pH correction), (ii) diluted at 50% (v/v) in the bioconversion buffer (pH 7) and (iii) undiluted but with pH corrected to 7 (with 2N NaOH at the beginning of the experiment). *E. coli* JM109(pBB1) strain was grown overnight at 37 °C and 150 rpm in LB plus ampicillin (50 μg/mL). This culture was used to inoculate (2% v/v) a fresh LB culture which, when its A_{600} reached the value of 1.5 (about after 4.5 h of incubation), was used to inoculate (2% v/v) 500 mL flasks containing 50 mL of enzymatic hydrolyzate under the three conditions described above. The flasks were incubated at 37 °C and 150 rpm for 48 h and samples were periodically removed to evaluate the concentration of viable cells and of ferulic acid, vanillin and related metabolites via HPLC-DAD. Un-inoculated controls were prepared and treated in the same way.

2.8. Analytical methods

Total carbohydrates and reducing carbohydrate concentration were evaluated according to Daniels et al. [34]. Viable cell concentration was evaluated on LB agar plates. Qualitative and quantitative analyses of ferulic acid, vanillin and vanillyl alcohol in the hydrolyzates and in the cultures was done via a HPLC-DAD system (Beckman Coulter, USA) equipped with a Beckman Ultrasphere 4.6 mm × 250 mm ODS column (particle diameter = 5 μm). Column temperature was 35 °C; injection volume was 20 μL. The eluents employed were: (A) water additioned with 1% (v/v) acetic acid and (B) methanol additioned with 1% (v/v) acetic acid. The solvent gradient method employed was: initial solvent composition, 90% (A) and 10% (B); solvent composition changed to 55% (A) and 45% (B) in 7 min; solvent composition changed to 62% (A) and 38% (B) in 30 min; isocratic elution for 10 min; back to initial solvent composition in 10 min. pH and dissolved oxygen concentration were measured by using selective probes (81–04 model and 97–08 model, respectively, ATI-Orion, Boston, MA).

3. Results and discussion

3.1. Screening of the enzymatic activities present in commercial enzyme preparations

Five enzyme products, commercialized with different trade names and peculiar enzyme activities, i.e. Cytolase® M102, Pentopan 500BG®, Fungamyl Super AX®, Celluclast BG® and Termamyl® 120 L, were assayed for their pectinase, xylanase, amylase, glucosidase, cellulase, feruloyl esterase, caffeoyl esterase and laccase activities. The results, presented in Table 1, showed that all products possessed additional activities with respect to that or those for which they are commercialized. In particular, Cytolase® M102 showed, in addition to the pectinase activity, a remarkable xilanase activity and feruloyl and caffeoyl esterase activities. Celluclast BG®, which is commercialized as a cellulase, presented relevant pectinase and xylanase activities, but no esterase activities. Fungamyl Super AX® and Pentopan 500BG® showed very high feruloyl and caffeoyl esterase activities. Termamyl® 120 L, an α-amilase, evidenced a strong amylase activity, but no other evident enzyme activities. Laccase activity was not evidenced in any of the enzyme preparations, which is a desirable feature in order to employ them to release ferulic acid from plant cell wall, due to the capability of laccase to oxidase/polymerize low molecular weight phenolic compounds such as ferulic acid [35,36].

3.2. Wheat bran hydrolysis by employing different enzyme preparations

The above mentioned enzymes were used, separately or as mixtures of two or three of them, to release ferulic acid from wheat bran suspensions, prepared through the thermal pretreatment as described in the method section. The results of the hydrolysis test, in term of ferulic acid released, by single enzymes are presented in Fig. 1A. No ferulic acid was released from wheat bran suspensions in the absence of any enzymes. Free ferulic acid was generated by the three enzymes which had previously shown to possess feruloyl esterase activity. In particular, Fungamyl Super AX® evidenced a strong activity after 5 h incubation, whereas the activity of Pentopan 500BG® and Cytolase® M102 was greater at longer incubation times. The maximum amount of ferulic acid released by using single enzyme preparations was 0.95 ± 0.09 g of ferulic acid/kg of

Table 1
Enzyme activities (expressed as μmol of target substrate released × mg^{-1} of enzymatic extract × min^{-1}) present in the technical grade enzymes used in this work

Enzyme	Enzymes activities						
	Pectinase	Xylanase	Amilase	Glucosidase	Feruloyl-esterase	Caffeoyl-esterase	Laccase
Cytolase® M102	10709.5	1584.8	1031.5	5.8	237.1	127.44	0
Celluclast BG®	6899.2	2239.0	943.7	3.5	0	0	0
Fungamyl Super AX®	10515.7	1808.3	33001.4	5.3	8064.5	15615,4	0
Pentopan 500BG®	15635.5	2174.6	1031.0	6.7	20340.5	27692.3	0
Termamyl® 120 L	1711.0	140.4	45429.7	0	0	0	0

The activities for which each enzyme is commercialized are underlined.

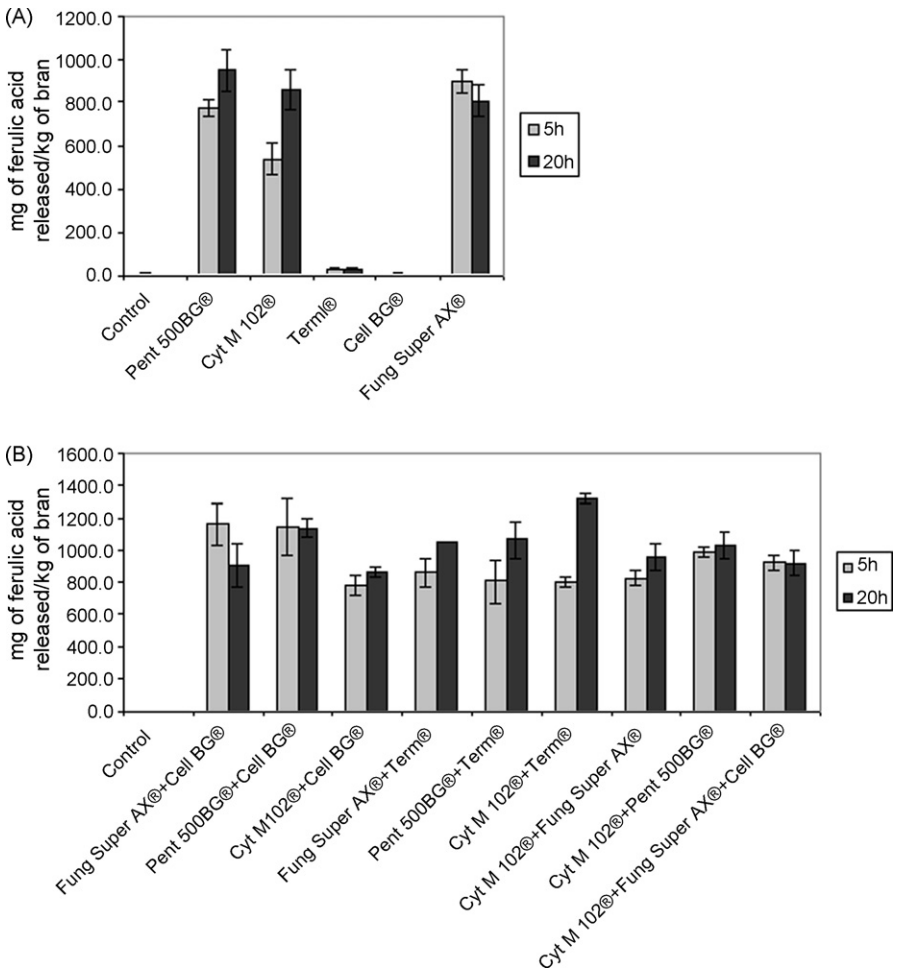

Fig. 1. Amount of ferulic acid released from pretreated wheat bran by employing single enzymes (A) or a mixture of them (B).

bran. Wheat bran hydrolysis was then performed with different enzyme mixtures (Fig. 1B). A marked activity was observed in the presence of all the enzyme product combinations, in particular in those containing enzymes having esterase activity, i.e. Cytolase® M102, Pentopan 500BG® and Fungamyl Super AX®, with enzymes presenting cellulase or amylase activity, such as Celluclast BG® or Termamyl® 120 L (Fig. 1B). Two enzyme mixtures showed a large release of ferulic acid after only 5 h of incubations, i.e. Fungamyl Super AX® plus Celluclast BG® (used at the concentration of 1 g/100 g of bran each), which released 1.17 ± 0.13 g of ferulic acid/kg of bran, and Pentopan 500BG® plus Celluclast BG® (used at 1 g/100 g of bran each), which released 1.14 ± 0.06 g of ferulic acid/kg (Fig. 1B). The larger ferulic acid release was obtained with the mixture composed of Cytolase® M102 plus Termamyl® 120 L (the former used at 1 g/100 g of bran and the latter at 0.1 g/100 g of bran), which released 1.32 ± 0.04 g of ferulic acid/kg of bran after 20 h of incubation (Fig. 1B). This amount corresponded to about 30% of chemically bounded ferulic acid, as determined by its comparison with the amount ferulic acid (4620 mg/kg of bran) released by alkaline hydrolysis of an identical suspension of the same wheat bran. These results pointed out that the combination of different enzymes activities is needed to release ferulic acid from cell wall polysaccharides as already evidenced in several studies present in the literature [9,14,24,25]. However, the release of ferulic acid from wheat bran performed in the present study was less efficient with respect to that obtained with enzymes purified from several fungal strains [14,16,17,19], thus indicating a limit in the use of commercial technical grade enzyme preparations to hydrolyze ferulic acid. The low efficiency in ferulic acid release may be ascribed to the not efficient feruloyl esterase activity of the enzyme preparations employed towards wheat bran molecular structure; on the other hand, it is well known in the literature that purified feruloyl esterases isolated from microorganisms can display very different activity towards ferulic acid esters present in wheat bran [16,17].

In order to increase the yield of ferulic acid hydrolysis, an attempt was made to optimize the thermal treatment preceding the hydrolysis step. Three different temperatures (80 °C, 90 °C and 121 °C) were assayed. The experiments, performed with all the three above mentioned enzyme mixtures, evidenced that the highest release of ferulic acid was obtained with a treatment of 121 °C for 20 min (data not shown), which corresponded to the experimental conditions which had been applied to all the extracts previously prepared. These treatment conditions have therefore been applied to the preparation of all the extracts used for the successive experiments. The release of ferulic acid from wheat bran was accompanied by a concomitant marked release

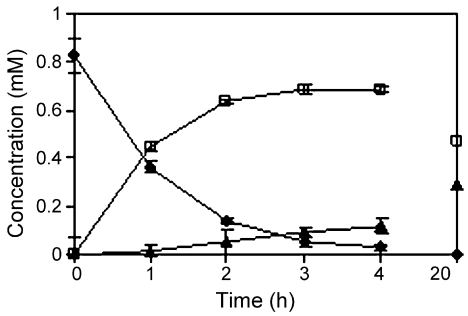

Fig. 2. Biotransformation of "food-grade" ferulic acid (●) and production of the related metabolites (□: vanillin, ▲: vanillyl alcohol) by resting cells of *E. coli* strain JM109(pBB1). The results are the average (±S.D.) of duplicate experiments.

of carbohydrates; as an example, the occurrence of total carbohydrates and reducing carbohydrates in 5 enzymatic extracts prepared independently with Cytolase® M102 plus Termamyl® 120 L was of 47.40 ± 0.75 g/L and 29.40 ± 0.73 g/L, respectively.

3.3. Bioconversion of "food-grade" ferulic acid by resting cells of E. coli strain JM109(pBB1)

The ability of JM109(pBB1) strain to convert ferulic acid into vanillin was initially evaluated by using LB grown cells suspended in bioconversion buffer amended with "food-grade" ferulic acid at an initial concentration of 1.00 mM, which corresponded to that generally occurring in the enzymatic hydrolyzates. The results are presented in Fig. 2, which shows a rapid consumption of ferulic acid which is converted into vanillin with a molar yield of 83.1% at the 3rd hour of incubation. Vanillyl alcohol is produced at low concentrations since the 2nd hour of incubation, reaching the amount of 0.12 ± 0.02 mM at the 4th hour, when all the initially added ferulic acid had been consumed. By prolonging the incubation time, vanillin tended to be reduced to the corresponding alcohol (Fig. 2). These bioconversion results are similar to that obtained in a previous work [27] by using "analytical grade" ferulic acid and, therefore, confirm the efficient biocatalytic potential of the employed strain.

3.4. Bioconversion of ferulic acid in crude enzymatic extracts by resting cells of E.coli strain JM109(pBB1)

The enzymatic extract employed in these experiments, prepared as described in Section 2 by employing 1% (w/w) Cytolase M102® and 0.1% (w/w) Termamyl® 120 L, had a pH of 6.3 and a ferulic acid concentration of 0.97 mM. LB grown JM109(pBB1) cells were suspended in the extract (i) undiluted, (ii) diluted in bioconversion buffer to have the extract at 75% (v/v) of the total volume and (iii) diluted in bioconversion buffer to have it at 50% (v/v) of the total volume. The same experiments were also performed with cell-free reaction media showing no transformation of ferulic acid occurring in the extracts. As shown in Fig. 3, vanillin was significantly produced under all conditions, but its concentration started to decrease since the 3rd hour of incubation with the concomitant accumulation of vanillyl alcohol. After 20 h of incubation, neither vanillin nor vanillyl alcohol were present, whereas a residual concentration of ferulic acid was evidenced (Fig. 3). The vanillin molar yields of the bioconversion experiments are presented in Fig. 3D. The highest yield (50%) was obtained by using the extract at 75% (v/v) in bioconversion buffer after 2 h of incubation, then it decreases as vanillyl alcohol started to be produced from vanillin. This yield corresponded to a vanillin concentration of 0.39 mM (i.e. about 60 mg/L); almost the same amount of vanillin (0.36 mM) was produced with the undiluted extract after 3 h of incubation, but, at this point, greater concentrations of residual ferulic acid and of vanillyl alcohol

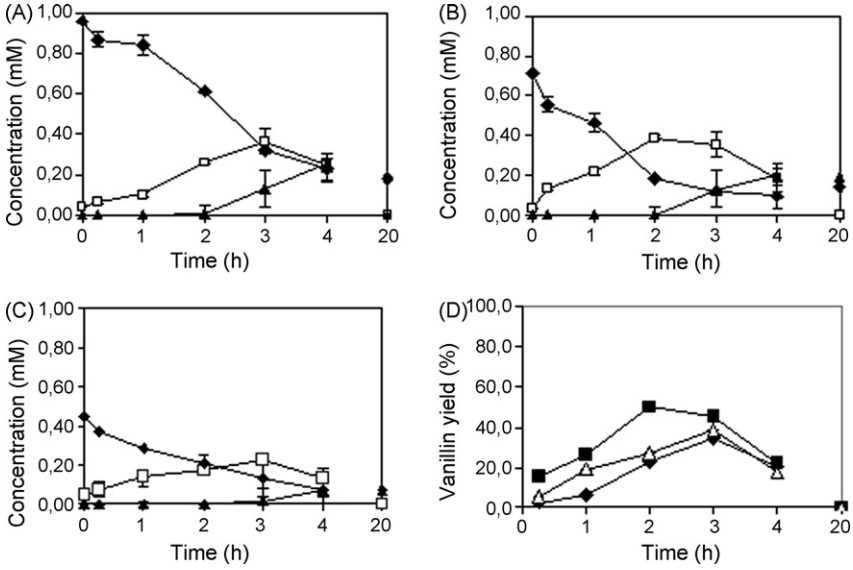

Fig. 3. Biotransformation of ferulic acid (●) and production of the related metabolites (□: vanillin, ▲: vanillyl alcohol) by resting cells of *E. coli* strain JM109(pBB1) in the presence of crude extract obtained with 1% (w/w) Cytolase M102® and 0.1% (w/w) Termamyl® 120 L undiluted (A), diluted at 75% (v/v) in bioconversion buffer pH 7 (B) and diluted at 50% (v/v) in bioconversion buffer pH 7 (C). (D) Vanillin yield of the experiments performed with the undiluted extract (♦), extract diluted at 75% (v/v) (■) and at 50% (v/v) (△). The results are the average (±S.D.) of duplicate experiments.

were present. Therefore, JM109(pBB1) displayed much lower bioconversion yields and selectivity when applied to wheat bran extract with respect to "food-grade" ferulic acid and the adverse effects increased with the concentration of extract in the reaction medium. The transformation of toxic aromatic aldehydes such as vanillin into the correspondent reduction products is a defence strategy widely diffused in recombinant *E. coli* ferulic acid catabolyzing strains [37–39]. This side reaction, catalyzed by broad substrate specificity dehydrogenases, was very probably responsible for the vanillin transformation into vanillyl alcohol observed during the second part of the experiment (Fig. 3A–C). This feature was more evident in the assays containing the enzymatic extracts as the source of ferulic acid with respect to those performed with traditional bioconversion medium supplemented with "food-grade" ferulic acid (Figs. 2 and 3), probably for the high carbohydrate concentration present in the hydrolyzates. Reducing carbohydrate concentration analyses performed both at the beginning and at the end of the bioconversion experiments (i.e. after 20 h of incubation) showed a marked consumption of carbohydrates, whose concentration was reduced by 37–38% (of the initial amount) in the undiluted sample and in that diluted at 75% (v/v) and by 57% in the sample diluted at 50% (v/v). These findings indicated that the experiments were performed under partial growing-cell conditions and this may have stimulated the production of oxidative enzymes. In addition, other phenolic compounds present in the extract may also be used as the carbon source by the *E. coli* strain thus activating oxidase activities responsible for vanillin oxidation.

Bioconversion was also performed by employing as the ferulic acid source a wheat bran hydrolyzate obtained with a different enzyme mixture, i.e.1% (w/w) Fungamyl Super AX® plus 1% (w/w) Celluclast BG®. The experiments were performed in all the conditions mentioned above, but no significant variation in vanillin molar yield were obtained (not shown) with respect to experiments performed with the extract prepared with Cytolase M102® and Termamyl® 120 L, thus indicating that there was not any significant influence on vanillin production yield by the enzyme system applied in the extract preparation.

3.5. Optimization of resting-cell bioconversion assays through improvement of bioconversion parameters and the use of purified extract

With the aim of increasing the vanillin yield of the bioconversion process, the effects of several bioconversion parameters, such as pH, temperature and dissolved oxygen concentration of the bioconversion medium and strain growth temperature, was studied. The vanillin yields of the bioconversion experiments performed at 30 °C and at pH 6.0, 7.0, 7.5 and 8.0 after 3 h of incubation were 35%, 51%, 37% and 29%, respectively, indicating that pH 7, which had already been used for the experiments previously performed, was the optimal one. The results of the experiments performed by lowering the strain growth temperature from 37 °C to 30 °C and by operating the bioconversion both at 30 °C and at 37 °C are presented in Fig. 4, which shows that only a slight increase in vanillin molar yield (about 5%) was obtained by lowering the growth temperature from 37 °C to 30 °C. No further increase of the bioconversion yield was obtained by changing dissolved oxygen concentration in the medium. In fact, the experiments performed in a stirred tank reactor at dissolved oxygen concentrations of 3.75 mg/L and 7.5 mg/L showed a vanillin bioconversion yield of 48% and 27%, respectively (data not shown). In addition, vanillyl alcohol formation did not change significantly. Therefore, the attempts to increase vanillin bioconversion yield by changing growth and bioconversion parameters did not show any significant improvements of vanillin production.

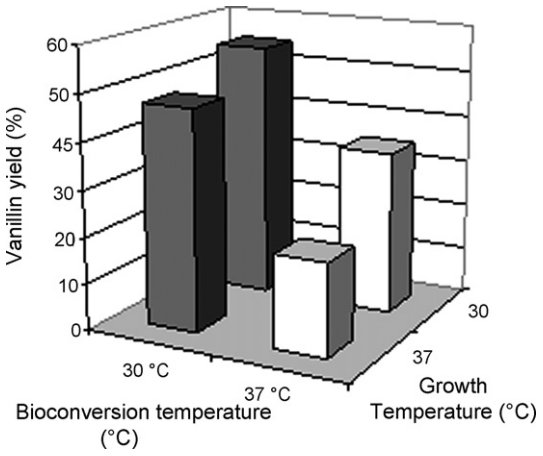

Fig. 4. Effect of the growth and bioconversion temperature on the vanillin production yield.

Experiments were then performed with enzyme extracts deprived of carbohydrates to evaluate the effect of these molecules in ferulic acid bioconversion. The treatment of the crude extract (obtained with 1% (w/w) Cytolase M102® and 0.1% (w/w) Termamyl® 120 L) with the Amberlite IRA95 resin resulted in an extract with a marked lowered content of reducing carbohydrates, which were 28% of those originally occurring in the extract. The alcoholic phase obtained from the resin washing had a ferulic acid concentration of 12.87 mM, which corresponded to a ferulic acid recovery of 80%. It was additioned to *E. coli* JM109(pBB1) bioconversion suspensions to have a final ferulic acid concentration of 0.25 mM, 0.50 mM and 1 mM. The results of the bioconversion experiments are presented in Fig. 5A–C and vanillin yields are reported in Fig. 5D. All ferulic acid added is transformed during the experiment and vanillin is produced with a yield up to 70% in 5 h starting from either 0.25 mM or 0.5 mM ferulic acid and 60% in 6 h from 1 mM ferulic acid. Thus, the purification of the hydrolyzate with a ionic exchange resin allowed to obtain both higher bioconversion yields and lower vanillin reduction to vanillyl alcohol than when crude hydrolyzates were applied. In addition, higher vanillin concentration in the medium, up to 0.6 mM, corresponding to 90 mg/L, was detected. These data support the hypothesis that carbohydrates had a negative impact on the ferulic acid bioconversion into vanillin. However, the maximum vanillin concentration achieved in these experiments is not competitive with the vanillin amount obtained in the presence of pure ferulic acid by the same strain (Fig. 2 and [27]) or by other strains described in the literature [1,39,40]. However, similar bioconver-

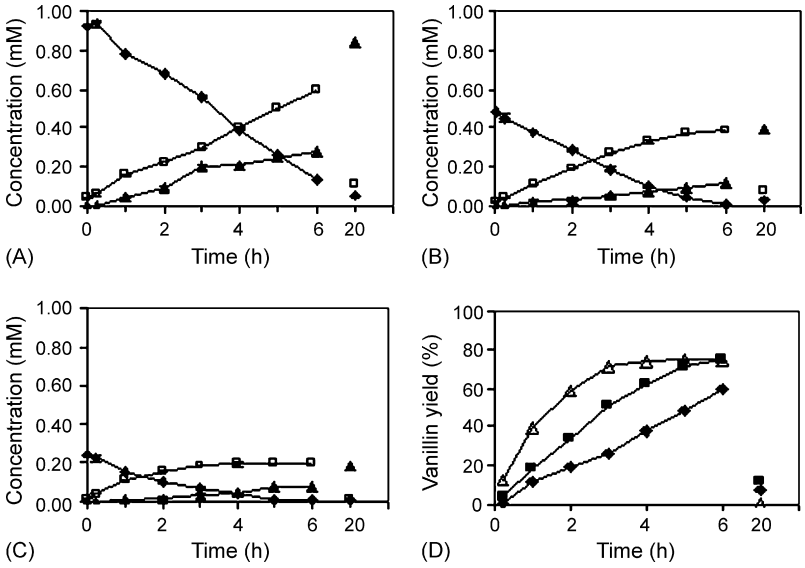

Fig. 5. Biotransformation of ferulic acid (●) desorbed from the Amberlite IRA95 resin at the concentration of 1 mM (A), 0.5 mM (B), 0.25 mM (C) and production of related metabolites (□: vanillin, ▲: vanillyl alcohol) by resting cells of *E. coli* strain JM109(pBB1). (D) Vanillin yield in the presence of 1 mM ferulic acid (⧫), 0.5 mM ferulic acid (■) and 0.25 mM ferulic acid (△). The results are the average (±S.D.) of duplicate experiments.

sion results were observed by Shin et al. [24] with a *Streptomyces* strain on ferulic acid (1 mM) extracted with an organic solvent from corn fiber hydrolyzates, showing a yield of 43% after 12 h.

3.6. Bioconversion of ferulic acid present in crude enzymatic extracts by growing cells of E. coli strain JM109(pBB1)

These experiments were aimed at evaluating the possibility of growing the strain on crude extracts, containing carbohydrates, and, at the same time, obtaining the bioconversion of ferulic acid into vanillin. The enzymatic extract obtained with Cytolase® M102 and Termamyl® 120 L already employed in the resting-cell experiments was used. *E. coli* strain JM109(pBB1) was found to grow on the extract under all the conditions assayed, i.e. by using the extract with (i) no dilution and no pH correction, (ii) no dilution but pH adjusted to 7.0 at the beginning of the experiment and (iii) a 50% (v/v) dilution in buffer at pH 7. The time course of the experiment performed under the last condition is reported in Fig. 6, which shows that cell concentration increased rapidly in the initial 20 h, after which a clear cell CFU/mL decrease was observed. This trend was also observed under all the other conditions assayed (data not shown). The percentage of conversion of ferulic acid was about 35% under the first and second conditions (data not shown), whereas it reached 93% in the presence of the diluted extract (Fig. 6). However, despite ferulic acid consumption, vanillin produced was very low under all the experimental conditions described as vanillin produced was promptly and extensively reduced to vanillyl alcohol. These findings support the evidence that strain growth on the extract had an adverse effect on vanillin production by inducing its reduction to the corresponding alcohol. Therefore, the use of wheat bran hydrolyzates as growth substrate for *E. coli* JM109(pBB1) did not seem to be a valuable approach

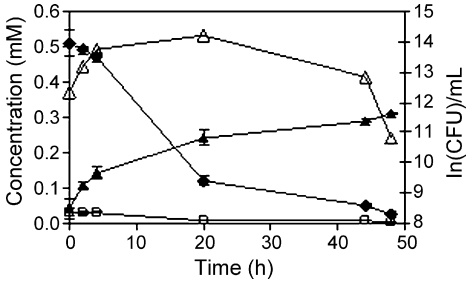

Fig. 6. Biotransformation of ferulic acid (●) present in the crude extract obtained with 1% (w/w) Cytolase M102® and 0.1% (w/w) Termamyl® 120 L diluted 1:1 in bioconversion buffer pH 7 by growing cells of *E. coli* JM109(pBB1). □: vanillin, ▲: vanillyl alcohol, △: cell growth. The results are the average (±S.D.) of duplicate experiments.

to obtain the bioconversion of ferulic acid into vanillin by our strain.

4. Conclusion

In this work, the possibility of obtaining vanillin from bioconversion of ferulic acid derived from enzymatic hydrolysis of wheat bran was explored. Technical mixtures of commercial enzyme preparations and the strain *E. coli* JM109(pBB1), containing the genes for the conversion of ferulic acid to vanillin deriving from a *Pseudomonas* strain [41], were used for such a purpose. The results obtained pointed out that vanillin can be obtained at interesting rates and yields from wheat bran, an agriculture by-product obtained in large amounts in Italy. To our knowledge, this is the first work in which it is demonstrated that vanillin is produced from ferulic acid obtained through the hydrolysis of such a matrix.

Currently, the possibility of employing other bacterial strains with a reduced capability of further metabolizing vanillin is

under study. This would allow to increase vanillin accumulation in the medium and, therefore, the productivity of the process.

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