CHEMICAL ENGINEERING TRANSACTIONS, Volume 14, 2008
Edited by G. Marino, E. Bardone, A. Viglia
Copyright © 2008, AIDIC Servizi S.r.l., ISBN 978-88-95608-02-0

Bioconversion of ferulic acid obtained from wheat bran into vanillin

Luigi Sciubba[1], Diana Di Gioia[1], Leonardo Setti[2], Maurizio Ruzzi[3], Fabio Fava[1]
[1] DICASM, Engineering faculty, University of Bologna, Italy
[2] Department of Industrial Chemistry and Materials, University of Bologna, Italy
[3] Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, Viterbo, Italia

In this work the possibility of producing vanillin through microbial bioconversion from ferulic acid obtained from the enzymatic hydrolysis of wheat bran has been explored. The biocatalyst employed was *Escherichia coli* JM109/pBB1, a recombinant strain containing the genes from *Pseudomonas fluorescens* BF13 for the transformation of ferulic acid into vanillin. The substrate of the bioconversion, performed with resting cells of *E.coli* JM109/pBB1 grown on LB rich medium, was the crude hydrolizate obtained from wheat bran or a buffer containing ferulic acid recovered from the crude matrix with different methods, as adsorption on ion exchange resins, liquid-liquid extraction and solid phase extraction; these operations allowed the selective recovery of ferulic acid from carbohydrates present in the hydrolizates, thus obtaining an aqueous phase rich in reducing sugars. The removal of reducing sugars allowed to obtain higher bioconversion yields. The results of this work showed that vanillin can be obtained at interesting yields from ferulic acid extracted from wheat bran.

1.Introduction

Vanillin (4-hydroxy-3-methoxy-benzaldheyde) is one of the most common flavouring compounds in the food industry and its production reaches about 12000 tons per year. It is usually obtained from *Vanilla* pods, through a long and expensive process (the price of natural vanilla is about 2000-4000 $/kg), or through chemical synthesis, leading to a cheaper product (15$/kg) but of lower quality [Walton *et al.*, 2003]. Biotechnological production of vanillin from selected substrates (such as ferulic acid) could be an interesting alternative, as it is cheap and permits the labelling of the product as natural according to the US and European legislation [EC directive 88/388, 1988]. Ferulic acid is a phenolic compound present in large amount in the cell wall polysaccharides, such as wheat bran, which is an agricultural by-product obtained in huge quantity in Italy (1.6milion tons) and currently used only for animal feeds. Ferulic acid is linked to the cell wall through ester bonds and can be released breaking these bonds through enzymatic hydrolysis [Di Gioia *et al.*, 2007]; then it can be converted into vanillin using as the biocatalyst a recombinant strain, *Escherichia coli* JM109/pBB1, containing the genes from *Pseudomonas fluorescens* BF13 for the transformation of ferulic acid into vanillin [Barghini *et al.*, 2007]. In this way a waste matrix such as wheat bran could be valorized, being a source of ferulic acid, to be transformed into a fine chemical product as vanillin.

2.Materials and methods

2.1 Micorganisms, buffers and media

Escherichia coli JM109/pBB1 was used in this study; this strains contains a plasmide derivative carrying a catabolic cassette for the conversion of ferulic acid into vanillin [Barghini *et al.*, 2007]. Rich medium LB was prepared according to Sambrook et al. Saline buffer (pH7) had the following composition(g/L): Na_2HPO_4 6,0; KH_2PO_4 3,0; NH_4Cl 1,0; NaCl 0,5.

2.2 Wheat bran hydrolizates production

Wheat bran was suspended in distilled water in the ratio 1:7 (7 liters of water per kg of wheat bran), then the suspension was subjected to thermal treatment (121°C for 20 min in an autoclave). Then the enzyme preparation, Fungamyl Super AX® (1%w/w) + Celluclast BG®(1%w/w), was added and this mixture was incubated at 30°C for 20h.

The hydrolizate was filtered on a paper, centrifuged at 6000rpm for 10 minutes, sterilized through a 0,20 µm porosity filter and stored at -20°C. The hydrolizate had a concentration of 200mg/L of ferulic acid, 50g/L of total sugars (30g/L of reducing sugars), pH=5,8 [Di Gioia *et al*., 2007].

2.3 Bioconversion experiments performed with resting cells of *Escherichia coli* JM109/pBB

A 1L-flask containing 100mL of rich medium LB+ampicillin (50µg/mL) was inoculated with 2%v/v with an over-night *E.coli* JM109/pBB1 culture, for the growth phase. The flask was incubated at 37°C and 150rpm until the culture reached an A_{600} of 1,5, usually after 3,5h of incubation. Then the cells were harvested by centrifugation (6000rpm, 10 minutes), washed with saline buffer pH7, resuspended in the bioconversion buffer (the crude hydrolizate or a buffer containing purified ferulic acid) at the concentration of 4mg wet biomass/mL, in a 100mL flask containing 20 mL of this solution and incubated at 30°C. Each hour 1-mL samples were taken out, acidified with 25ml of TCA 2M centrifuged at 12000rpm for 10 minutes and analyzed through HPLC-DAD reverse phase system.

2.4 Wheat bran hydrolizate purification

2.4.a Adsorption on ion exchange resins Amberlite IRA® 95

Ferulic acid was recovered by adding ion exchange resin Amberlite IRA® 95 (6%w/v, 50rpm, 4h) to the crude hydrolizate, then the hydrolizate was removed and the resin was washed with ethanol additioned with 4%HCl for 1h at room temperature. The alcoholic ferulic acid rich extract thus obtained was neutralized by using NaOH 2N and concentrated through evaporation in rotavapor; the solution was diluted in saline buffer in order to obtain a concentration of ferulic acid of 100mg/L. This operation allowed a ferulic acid recovery of 80% and a removal of 90% of reducing sugars.

2.4 b Liquid-liquid extraction with ethyl acetate

5mL of ethyl acetate were added to an equal volume of crude hydrolizate (pH=5,8), shaked and centrifuged for 5 minutes at 3000rpm, then the organic phase was taken out and put in a 50mL bottle; then 5mL of ethyl acetate were again added to the aqueous phase, centrifuged, then the two phase were collected and HPLC analyzed.

The organic phase was evaporated through rotavapor, the concentrated ferulic acid was resuspended in saline buffer in order to obtain a concentration of 100mg/L. The

recovery of ferulic acid was about 80% , while the reducing sugars removal was near to 95%.

2.4c Solid phase extraction with ISOLUTE ENV$^{+®}$columns

The columns (20mL volume, 1g of packing) were first washed with 9mL of methanol 9mL of distilled water, then the crude hydrolizate was introduced. The columns were washed with 20mL of distilled water and the adsorbed ferulic acid was eluted twice with 12mL of absolute ethanol. The organic phase was evaporated through rotavapor, the concentrated ferulic acid was resuspended in saline buffer in order to obtain a concentration of 100mg/L. Ferulic acid recovery was about 95% and the reducing sugars removal was 95%. The aqueous phase (30g/L of reducing sugars) was sterilized through filtration, in case diluted in distilled water or additioned with yeast extract, and dispensed into sterile flasks.

3.Results

The first experiment was performed with resting cells of *E.coli* JM109/pBB1 grown on LB rich medium using the crude hydrolizate as the bioconversion matrix; it was employed undiluted (200mg/L of ferulic acid, 30g/L of reducing sugars), diluted 3:1 (150mg/L of ferulic acid, 22g/L of reducing sugars)and diluted 1:1 in saline buffer (100mg/L of ferulic acid, 15g/L of reducing sugars). Ferulic acid was quickly transformed into vanillin (Fig.1), but the product was converted, since the second hour of bioconversion, into vanillyl alcohol. Therefore the molar yields obtained in this set of experiments reached a maximum of 50% in the 3:1 diluted buffer and of 37% utilizing the 1:1 diluted matrix.

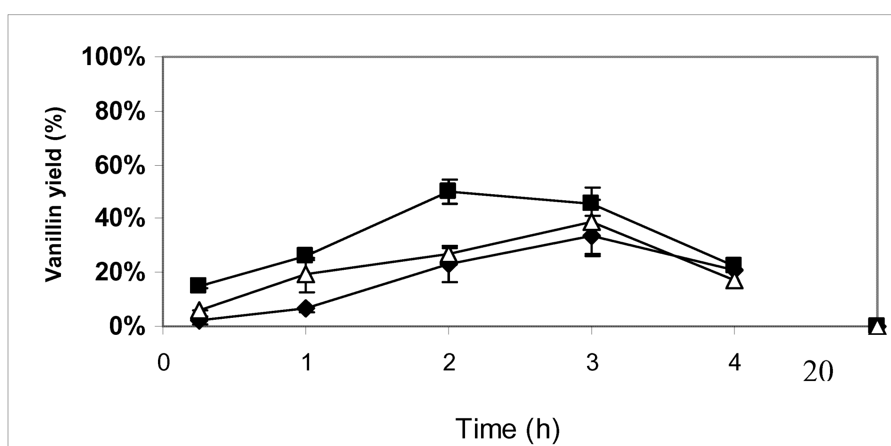

Fig.1: Vanillin molar yields obtained using the undiluted hydrolizate(◆), the hydrolizate diluted 3:1(■), and diluted 1:1 (△)

The following experiment was performed by adding 100mg/L of food grade ferulic acid both to the undiluted hydrolizate (300mg/L of ferulic acid, 30g/L of reducing sugars)

and to the 1:1 diluted hydrolizate (200mg/L of ferulic acid, 15g/L of reducing sugars) in order to explore the effects of higher concentration of substrate, with the same carbohydrate concentration, on the bioconversion process. Also in these experiments, the yields were not high and reached a maximum of 50% in the diluted hydrolizate (data not shown), suggesting that the low bioconversion yield could be due to the high reducing sugars concentration rather than the high ferulic acid initial concentration.

Therefore in the next experiments differents methods for the separation of ferulic acid from the carbohydrates of the crude hydrolizate were employed, i.e. a)adsorption on ion exchange resins, b)liquid-liquid extraction with ethyl-acetate (LLE), c)solid phase extraction (SPE).

In the first experiment of this set, the use of ion exchange resins Amberlite IRA® 95 allowed a recovery of ferulic acid of 80% and a removal of reducing sugars of 90%, obtaining a purified hydrolizate, which was diluted in order to obtain 50,100 and 200mg/L of ferulic acid, corresponding to 0,25 0,5 and 1,0 g/L of reducing sugars respectively. The bioconversion made on this matrix with resting cells of *E.coli* JM109/pBB1 showed a rapid conversion of ferulic acid into vanillin obtaining a molar yield of about 70% (Fig.2), suggesting that the elimination of sugars had a positive effect on the bioconversion process.

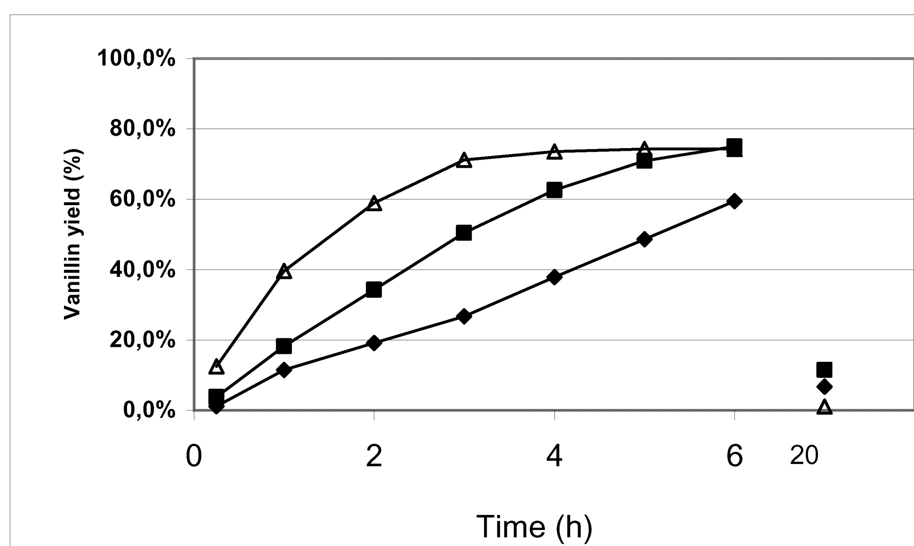

Fig.2: Vanillin molar yields obtained from 50mg/L(△), 100mg/L(■), and 200mg/L (◆) of ferulic acid recovered employing ion exchange resins Amberlite IRA® 95.

The second recovery method for ferulic acid from the hydrolizates was liquid-liquid extraction with ethyl-acetate; in this way the 78% of the ferulic acid was recovered and the 95% of sugars was removed. Ferulic acid was resuspended in saline buffer in order to obtain 100mg/L of ferulic acid (reducing sugars concentration in this matrix was 0,5g/L). The molar yields of the bioconversion performed on this purified

solution were very high and reached a maximum of about 80% (Fig.3) after 4 hours of bioconversion.

The third recovery method, solid phase extraction on ISOLUTE ENV$^{+®}$ columns, allowed a recovery of 95% of ferulic acid and a removal of 95% of reducing sugars. The bioconversion performed on solution (100mg/L of ferulic acid, corresponding to 0,5g/L of reducing sugars) had a maximum molar yield of 75% (Fig.3).

Therefore the results of the latter experiments confirmed the positive effect of the removal of sugars on the bioconversion yields.

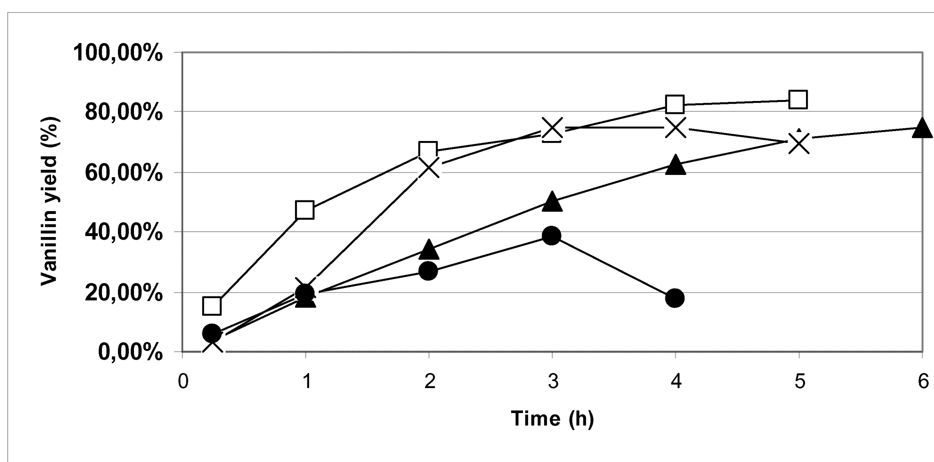

Fig.3: Vanillin molar yields reached using 100mg/L of ferulic acid obtained from the crude hydrolizate diluted 1:1(●), from adsorption on ion exchange resins(▲), liquid-liquid extraction with ethyl-acetate(□), solid phase extraction(X).

Conclusions

In this work the possibility of employing wheat bran as a source of ferulic acid for the bioconversion into vanillin has been explored.

The enzymatic hydrolysis of wheat bran allowed to obtain about 200mg/L of ferulic acid in a hydrolizate containing also 30 g/L of reducing sugars; however, the bioconversion yields obtained using the crude hydrolizate as the bioconversion matrix were not very high and reached a maximum of 50%, after 2hours of bioconversion diluting the hydrolizate in distilled water in the ratio 3:1.

Among the three different ferulic acid recovery methods employed in this study, i.e. adsorption on ion exchange resins Amberlite IRA® 95, liquid-liquid extraction with ethyl-acetate, solid phase extraction with ISOLUTE ENV$^{+®}$ columns, the most efficient was solid phase extraction, as ferulic acid recovery was 95%.

Maximum bioconversion yields were obtained, with resting cells of *Escherichia coli* JM109/pBB1, with ferulic acid recovered through liquid-liquid extraction with ethyl-acetate, and were about 80% starting from 100 mg/L of ferulic acid after 4 hours of bioconversion.

Therefore the results of this work showed that vanillin can be obtained at interesting yields from ferulic acid extracted from wheat bran, an agricultural by-product produced in large amounts in Italy.

References

[1] Walton N.J., Mayer M.J., Narbad A., 2003, Phytochemistry, 63:505-515
[2] EC directive 88/388 OJL 184 July, 15, 1988
[3] Di Gioia D. Sciubba L., Setti L., Luziatelli F., Ruzzi M., Zanichelli D., Fava F. 2007, Enzyme Microbial Technology, 41: 498-505
[4] Barghini P., Di Gioia D., Fava F., Ruzzi M., 2007, Microbial Cell Factories 6:13