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Selection of Co-Substrate and Aeration Conditions for Vanillin Production by *Escherichia coli* JM109/pBB1

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Summary

Yeast extract, Luria-Bertani medium and tryptone were tested as co-substrates for vanillin production from ferulic acid by resting cells of *Escherichia coli* JM109/pBB1. Yeast extract proved to be the best component for sustaining such a bioconversion, which is not self-sustained from the bioenergetic point of view. Tests were also performed under variable aeration conditions by simultaneously varying the ratio of medium to vessel volume and the agitation speed. The results of these tests suggest that, under excess aeration, a non-specific oxidase activity was very likely responsible for the oxidation of a significant portion of vanillin to vanillic acid, thus reducing the vanillin yield.

Key words: Escherichia coli, vanillin, ferulic acid, resting cells, vanillic acid

Introduction

Vanillin is a flavour compound used in food applications, fragrances and pharmaceutical preparations, which is nowadays mainly produced by chemical synthesis (*1*). Most of the synthetic vanillin is used as an intermediate in the production of herbicides, antifoaming agents or drugs (*2*) and household products. The increased demand for natural products in food industry (*3*) as well as the high cost of natural vanillin extracted from vanilla pods (*4,5*) have recently stimulated the search for alternative production of this compound naturally (*6*).

The microbial transformation of ferulic acid, a phenolic compound from lignin degradation (*7*), is recognised as the most interesting alternative to produce natural vanillin (*8*). Free ferulic acid can be released from agricultural residues by a combination of physical and enzymatic treatments (*9*), and then transformed into vanillin, vanillic acid and protocatechuic acid using fungi, bacteria or genetically engineered microorganisms (*10*). It is only recently that reasonable vanillin concentrations have been obtained from ferulic acid using the gram-positive bacteria *Amycolatopsis* sp. (*11*) or *Streptomyces setonii* (*12*). However, the optimisation of the process, needed to exploit it at industrial level, appears to be a very difficult task with these microorganisms; therefore, the construction of new recombinant strains able to specifically convert ferulic acid to vanillin is very attractive.

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Consistently with the results of Overhage *et al.* (*13*), some of the authors have recently confirmed that co-expression of feruloyl-CoA synthetase and feruloyl-CoA hydratase/aldolase genes from *Pseudomonas fluorescens* BF13 conferred to different *Escherichia coli* strains the ability to produce vanillin from ferulate-rich substrates (*14*). All recombinant strains behaved as interesting vanillin producers at 30 °C and starting mass concentrations as dry matter of biomass (X_o) and ferulic acid (S_o) of 5.6 g/L and 300–400 mg/L, respectively, with *E. coli* JM109/pBB1 exhibiting the highest conversion yield (0.96 mol/mol).

Tests performed at different temperatures and X_o = 5.6 g/L and S_o = 190 mg/L evidenced that this recombinant strain achieved maximum values of vanillin volumetric (91 mg/L·h) and specific productivities (18 mg/g·h) at 30 °C after only 1 h of bioconversion, while longer time (t = 4 h) was needed to get the highest conversion yield (0.86 mol/mol). Additional tests performed at variable starting substrate concentration ($20 \le S_o \le 2000$ mg/L) and X_o = 5.0 g/L and 30 °C showed that the ferulic acid-to-vanillin yield was higher than 0.80 mol/mol only within the first 4 h of bioconversion at $S_o \le 400$ mg/L, whereas a decrease in vanillin yield took place at higher S_o values, thus pointing out a substrate inhibition effect (*15*). This substrate inhibition of vanillin production proved to be stronger than the effects observed in common fermentation systems *(16,17)*, most likely due to the impossibility of resting cells to face the growing maintenance requirements.

As it is well known, the use of a co-substrate is an essential requisite to sustain the metabolism of resting cells utilizing metabolic routes, like those responsible for ferulic acid to vanillin bioconversion, which are ineffective or scarcely effective from the bioenergetic point of view. The required co-substrate has to be chosen among those carbon sources which are, as is well known, unable to exert enzyme catabolic repression and this selection should be made by simultaneously optimising the aeration conditions that directly influence the ATP yield.

For these reasons, the effects of co-substrate and aeration on vanillin production from ferulic acid have been jointly investigated in this work using *E. coli* JM109/pBB1 under conditions of negligible substrate inhibition ($S_o \cong$ 200 mg/L).

Materials and Methods

Microorganism growth and bioconversion conditions

Cells of *E. coli* JM109/pBB1, a recombinant strain producing key enzymes of ferulate catabolic pathway from *P. fluorescens* BF13 (feruloyl-CoA synthetase and feruloyl-CoA hydratase/aldolase), were used as a biocatalyst in this work. Recombinant plasmid pBB1 was generated by cloning a 5000-bp fragment including the first three genes of the ferulate upper pathway and the entire promoter region from ferulate-degrading *P. fluorescens* strain BF13 into the low-copy vector pJB3Tc19 (*18*). Donor fragment contained an engineered mutation in vanillin dehydrogenase (*vdh*) gene, which prevented oxidation of vanillin to vanillate in bioconversion experiments with ferulic acid as substrate. The DNA sequence of the entire ferulate operon from *P. fluorescens* BF13 is available from the EMBL nucleotide sequence database under accession no. AJ536325.

The cells were transferred into 250-mL Erlenmeyer flask (EF) containing 25 mL of the medium of Luria-Bertani (LB) (*19*) and ampicillin (100 mg/L), or tetracycline (15 mg/L), and then cultivated overnight on a rotary shaker at 37 °C (200 rpm). Fresh LB medium (30 mL) plus antibiotic was seeded with 0.6 mL of the above pre-culture in 100-mL EFs. Cells were collected by centrifugation (6000 rpm, 10 min) during the late log phase, washed with M9 saline phosphate buffer (Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NH_4Cl 1 g/L and NaCl 0.5 g/L), and inoculated into 50 mL of the bioconversion medium (M9 buffer amended with ferulic acid 190 mg/L and yeast extract (YE) 50 mg/L). Bioconversion experiments were performed in 250-mL EFs at 30 °C and a starting biomass concentration (X_o) of 5.1 g/L. Further tests were performed under the same conditions using LB medium (1 % volume fraction) or tryptone (100 mg/L) as co-substrates instead of YE. The aeration conditions were varied by decreasing the ratio of the medium volume (V_m) to that of the flasks (V_f = 250 mL) from 0.5 to 0.2 and/or raising the agitation speed from 100 to 200 rpm. A further bioconversion test with starting addition of N_2 was used as anaerobic reference.

Each set of batch runs was carried out in triplicate at almost constant starting biomass concentration (X_o = 3.0–3.5 and 5.1 g/L for tests at variable co-substrate and aeration conditions, respectively). The standard deviations of vanillic acid and vanillin concentrations varied in the ranges of 3.2–6.5 % and 1.5–6.1 %, respectively; therefore, no additional statistical analysis of data was considered to be essential. No formation of vanillyl alcohol was observed under the experimental conditions used for these experiments; a limited reduction of vanillin to the corresponding alcohol occurred only when cells were reused for several cycles (data not shown).

Analytical determinations

Biomass concentration was determined by optical density measurements at 600 nm. Vanillic acid, vanillin and ferulic acid were analysed by a HPLC 1100 HP provided with a Vydac 201TP54 C18 reverse-phase column and UV/Vis detector (254 nm, 35 °C). The mobile phase (0.5 mL/min) was prepared by mixing 40 % methanol and 60 % of a 6.8 g/L of KH_2PO_4 water solution and adjusting the pH to 2.5 with H_3PO_4.

Results and Discussion

Selection of co-substrate

Since vanillin is produced using metabolic routes other than glycolysis that do not include any ATP formation (*8*), the resting cells need to catabolize some carbonaceous co-substrates to energetically sustain their long-term viability in view of possible continuous application. The significance of complex media components was demonstrated by Rabenhorst (*20*), who observed a strong influence of the co-substrate (yeast extract and casein hydrolysate) and aeration on directing the euge-

nol metabolism of a new *Pseudomonas* isolate to certain different aroma components (vanillic acid, ferulic acid and coniferyl alcohol) instead of a crude mixture.

E. coli JM109/pBB1 was then tested in batch bioconversion experiments to select the best co-substrate for the transformation of ferulic acid to vanillin. To identify medium components that were able to sustain vanillin production, three bioconversion buffers were prepared, using either YE or tryptone as a supplement, or adding directly LB medium, which contains both ingredients. The amount of single ingredients was the same as in the LB medium supplement. The results of these tests, which were performed at a starting biomass concentration of 5.1 g/L, are illustrated in Fig. 1. It can be seen that YE was a better stimulating agent, providing, after only 3 h of bioconversion, maximum values of vanillin concentration (84 mg/L) and molar yield (0.56 mol/mol) about 27 % higher than those obtained with tryptone. On the other hand, the use of LB medium did not result in any appreciable increase in the conversion yield with respect to YE (only 3.6 %); therefore, the successive bioconversions were performed using the latter co-substrate, which is significantly cheaper.

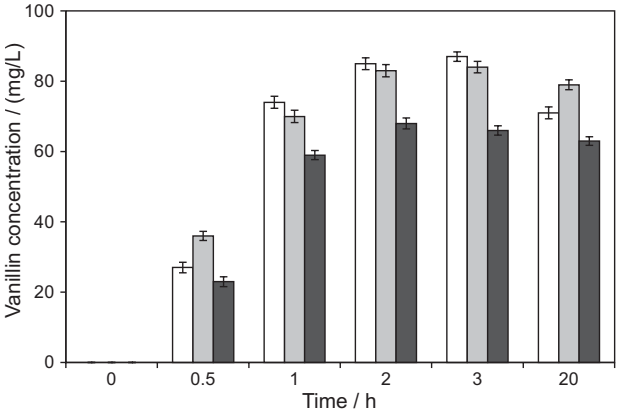

Fig. 1. Time behaviour of vanillin concentration during ferulic acid bioconversions by *E. coli* JM109/pBB1 performed in EFs. Bioconversion media were constituted of M9 saline phosphate buffer supplemented with (□) LB medium, (▒) YE or (■) tryptone. S_o = 190 mg/L; X_o = 5.1 g/L; T = 30 °C; Agitation speed = 150 rpm

Effect of aeration on ferulic acid-to-vanillin bioconversion

To study the aeration effect on the production of vanillin in EFs, we followed a simple protocol, which was similar to that for 2,3-butanediol production described by Jansen *et al*. (*21*), and widely reviewed for xylitol production by Parajó *et al*. (*22,23*), *i.e*. simultaneously increasing the agitation rate from 100 to 200 rpm and decreasing the ratio of medium to vessel volumes (V_m/V_f) from 0.5 to 0.2. A bioconversion with starting addition of N_2 was used as anaerobic reference test.

As shown in Fig. 2A, vanillin concentration increased progressively either with time or with aeration level up to a threshold value of 160–162 mg/L and then decreased to 146 mg/L under excess aerobic conditions (V_m/V_f = 0.2 at 200 rpm). In particular, the consumed ferulic acid was almost completely bioconverted to vanillin (0.98 mol/mol) under optimal conditions (V_m/V_f = 0.5; 100 rpm), whereas this yield was shown to decrease under excess aeration.

An opposite behaviour was observed for vanillic acid (Fig. 2B), which was produced at low concentrations (up to 9.2 mg/L) under intermediate aerobiosis. Predictably, the high aeration level ensured by V_m/V_f = 0.2 at 200 rpm caused the final concentration of this compound to rise up to 18 mg/L, most likely due to the stimulation of a non-specific oxidase activity. In addition, the formation of this product was clearly favoured by an increased bioconversion time.

The results in Fig. 2, taken together, point out that the best biotransformation results were obtained under oxygen limited conditions (V_m/V_f = 0.5, 100 rpm) and anaerobic conditions (the supply of N_2).

In spite of the very low S_o values investigated in this study, these preliminary tests make up an attempt of selecting a new recombinant strain, whose resting cells could be used, after optimisation, in an immobilized-cell system for continuous vanillin production.

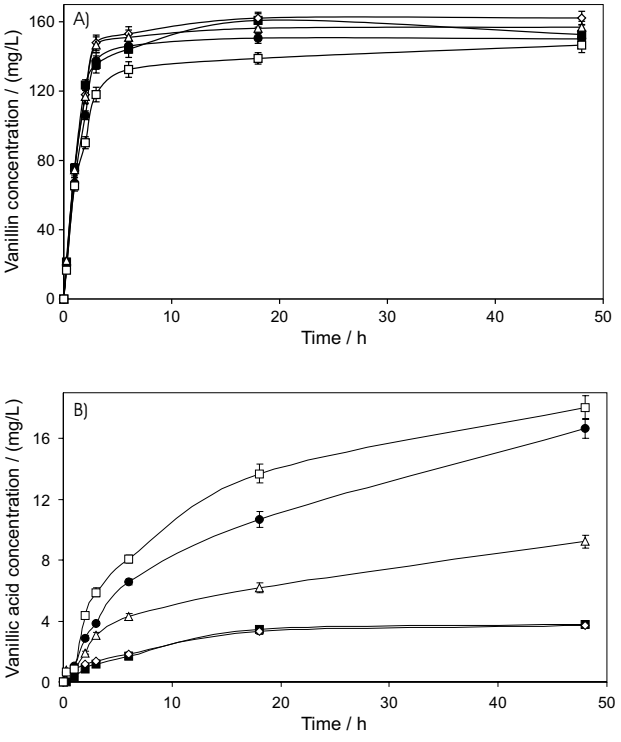

Fig. 2. Molar conversions of ferulic acid into (A) vanillin and (B) vanillic acid *vs.* time. Bioconversion conditions: (■) V_m/V_f = 0.5, 100 rpm (under N_2 supply); (◇) V_m/V_f = 0.5, 100 rpm; (△) V_m/V_f = 0.5, 200 rpm; (●) V_m/V_f = 0.2, 100 rpm; (□) V_m/V_f = 0.2, 200 rpm. S_o = 165 mg/L; X_o = 3.0–3.5 g/L; T = 30 °C

Conclusions

Yeast extract was shown to be the most advantageous co-substrate to sustain the batch bioconversion of ferulic acid to vanillin by resting cells of the transfor-

mant *E. coli* JM109/pBB1, providing, after 3 h of bioconversion, maximum vanillin concentration (84 mg/L) and molar yield (0.56 mol/mol) significantly higher than tryptone. Tests carried out in Erlenmeyer flasks under variable aeration conditions demonstrated that vanillin concentration was scarcely influenced (160 – 162 mg/L) up to a maximum threshold of the ratio of medium to vessel volumes of 0.5. Under these conditions, the bioconversion of ferulic acid to vanillin was almost complete at 100 rpm. An increased aeration level with respect to the optimum conditions caused the final concentration of vanillic acid to rise, probably due to a non-specific oxidase activity.

Further attempts in this field will deal with (*i*) a set of tests in a bioreactor to ensure rigorous control of the operating conditions, particularly concerning the aeration conditions, and process optimisation; (*ii*) the identification of the suspected oxidase activity; and (*iii*) continuous vanillin production by immobilized cells of this strain using optimal oxygenation level and residence time.

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Odabir kosupstrata i uvjeta aeracije za proizvodnju vanilina s pomoću *Escherichia coli* JM109/pBB1

Sažetak

Ekstrakt kvasca, podloga Luria-Bertani i tripton ispitani su kao kosupstrati za proizvodnju vanilina iz ferulične kiseline s pomoću stanica *E. coli* JM109/pBB1 koje ne rastu (»resting cells«). Utvrđeno je da je kvaščev ekstrat najbolji dodatak pri ovoj biokonverziji koja se s bioenergetskoga gledišta sama ne provodi. Provedena su ispitivanja pri različitim uvjetima aeracije, a istodobno su mijenjani omjer veličine podloge s obzirom na volumen Erlenmeyerove tikvice i brzina miješanja. Dobiveni rezultati pokazuju da uz pojačanu aeraciju dolazi do nespecifične oksidazne aktivnosti kojom se značajni dio vanilina provodi u vanilinsku kiselinu, snizujući time iskorištenje na vanilinu.