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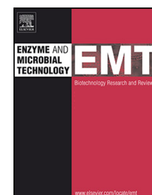

Bioproduction of 4-vinylphenol from corn cob alkaline hydrolyzate in two-phase extractive fermentation using free or immobilized recombinant *E. coli* expressing pad gene

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ABSTRACT

In situ extractive fermentation was used to produce 4-vinyl derivatives from hydroxycinnamic acids extracted from corn cobs by recombinant *Escherichia coli* cells expressing *Lactobacillus plantarum* phenolic acid descarboxylase (PAD) gene. This microorganism mainly produced 4-vinylphenol (4VP) from *p*-coumaric acid (*p*-CA). In the first study , we observed that the concentrations of 4VP are higher than 1 g/L which had a negative impact on decarboxylation of *p*-CA to 4VP by recombinant *E. coli* cells. Because of this, and in order to improve the downstream process, a two-phase aqueous-organic solvent system was developed. The results of the extractive fermentation indicated that it was possible to use hydrolyzates as aqueous phase to bioproduce 4VP, and recover simultaneously the product in the organic phase containing hexane. The detoxification of pre-treated corn cob alkaline hydrolyzate improved 4VP production up to 1003.5 mg/L after 24 h fermentation (Q_P = 41.813 mg/L h). Additionally, preliminary experiments using cells immobilized in calcium alginate showed to be a good system for the biotransform of *p*-CA to 4VP in extractive fermentation, although the process hindered partially the recovery of 4VP in the organic phase.

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1. Introduction

4-Vinylphenol (4VP), also known as 4-hydroxystyrene or hydroxystyrene monomer (CAS 2628-17-3), is a volatile compound which can be produced from *p*-coumaric acid (*p*-CA). Due to its inherent reactivity, it can only be marketed in maximum alcoholic solution (propylene glycol) concentrations not exceeding 10% (w/w) of the vinyl monomer [1]. 4VP finds application as a polymer precursor or as a flavoring substance in perfumery, food and beverages industries being approved as FEMA GRAS (Flavor and Extract Manufacturer's Association; General Regarded as Safe) [2]. In addition, 4VP can be used as antifungal agent [3]. The chemical synthesis can be carried out by Knoevenagel condensation of benzaldehydes with malonic acid and classical acidic [4]. Chemical synthesis can need expensive reagents and harsh conditions and low yields are obtained [2]. The biotechnological production is possible by enzymatic decarboxylation of *p*-CA [5,6].

Lignocellulosic materials are a source of *p*-CA which is known to be esterified essentiality only to the lignin [7]. Significant amounts of *p*-CA can be released from corn cobs. These agro-industrial wastes are generated during shelling of corn, being estimated that for every 100 kg of corn grain approximately 18 kg corn cobs are produced [8]. *p*-CA is a potential precursor in the biocatalytic production of value-added aromatic natural products [9]. Lignocellulosic materials are treated to fractionate their principal components cellulose, hemicelluloses, lignin or extracts. Dilute acid pre-treatment is commonly used during fractionation of lignocellulosic materials for the selective extraction of hemicelluloses [10]. Alkaline hydrolysis is a process that easily dissolves lignin, allowing for the complete utilization of the lignocellulosics with a low environmental impact [11].

Two-phase biotransformation system (TPBS) or extractive fermentation is a technique that allows recovering the product along of fermentation. This system has been widely used in biotechnology

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for the extraction and purification bioproducts [12,13]. It is an ideal system for the retrieval of hydrophilic products as vinyl derivatives, since the coexistence of two phases (aqueous and organic) permits the ready separation of the lipophilic products vinyl derivatives from the more hydrophilic substrate hydroxycinnamic acids [14]. This system has been used for the biotechnological production of 4VP, 4 vinyl guaiacol, lauryl lactone or 2-phenylethanol [1,14–16]. In problems with inhibition by product, extractive fermentation can be a good solution for simultaneous cell cultivation and downstream processing of product.

Combined of TPBS and immobilization of microorganisms allow to reuse cells and also prevent direct contact of cell with organic phase, therefore avoiding phase toxicity [17]. There are several immobilization methods including calcium alginate. This immobilization system not need hard conditions, has a low cost method and it is common its use in foods [18].

This work researched the product inhibition of 4VP in recombinant *Escherichia coli* cells overproducing the phenolic acid decarboxylase (PAD) enzyme from *Lactobacillus plantarum* CECT 748^T and the use of a TPBS process in alkaline hydrolyzate of corn cobs to avoid this problem.

2. Materials and methods

2.1. Chemicals and raw material

The phenolic acids, ferulic acid (FA) (128708) and *p*-CA (C9008) as well as the corresponding 4-vinylphenol derivatives, 4-vinyl guaiacol (4VG) (W267511) and 4VP (W373923), respectively, were purchased to Sigma-Aldrich (Madrid, Spain) as standards for compound identification by HPLC. Tryptone (403682), NaCl (131659), yeast extract (403687), and methanol HPLC grade (221091) were supplied by Panreac (Barcelona, Spain), meanwhile ampicillin (A9518) was supplied by Sigma-Aldrich, S. A. (Madrid, Spain).

Corn cobs employed in this work were donated by farmers in the area of Ourense (Galicia, Spain), dried at room temperature and milled to a particle size suitable for acid hydrolysis (<5 mm). Corn cobs were characterized by quantitative acid hydrolysis [19]. The composition expressed in percentage (oven-dry basis) was: cellulose: $31.5\,g\,L^{-1} \pm 1.5$; hemicelluloses: $34.9\,g\,L^{-1} \pm 0.3$; acetyl groups: $4.1\,g\,L^{-1} \pm 0.6$ and lignin: $21.6\,g\,L^{-1} \pm 1.5$ [18]. Zhang et al. [20] carried out a complete characterization of corn cobs (% dry mass): extract total, 19.63 (15.31 water extracts and 4.32 ethanol extracts); cellulose, 22.27; hemicelluloses, 28.30 (18.99 xylose, 4.32 galactose, 6.24 arabinose, 2.46 mannose); lignin total, 28.6 (8.65 acid soluble and 19.95 acid non soluble); ash, 6.58.

2.2. Bacterial and growth conditions of inocula

Microorganism used in fermentations was recombinant *E. coli* cells expressing the PAD gene from *L. plantarum* [21]. Strain was stored in cryovials at −80 °C (20% glycerol as cryoprotector) to be inoculated into fermentation media. Growth media for inocula were prepared in Erlenmeyer flasks of 250 mL with 25 mL of sterile Luria–Bertani (LB) medium containing 10 g tryptone/L, 10 g NaCl/L, 5 g yeast extract/L and 100 mg ampicillin/L, at 37 °C and 150 rpm in orbital shakers for growth inocula. pH was adjusted to 7 and sterilized in autoclave (121 °C, 15 min). 4% of inocula were added in each experiment [22].

2.3. Product inhibition assays

Fermentations to evaluate product inhibition were performed in Erlenmeyer flasks of 250 mL containing 150 mL of sterile LB medium (sterilized in autoclave at 121 °C during 15 min) at 37 °C and 115 rpm in orbital shakers (Optic Ivymen System, *Comecta* S.A., distributed by Scharlab, Madrid, Spain). Agitation and volume of work for 4VP production were previously optimized by Salgado et al. [22]. The inhibition of 4VP was determined by including 4VP at various concentrations (500, 1000, 1500, 2000 mg 4VP/L) and 1500 mg *p*-CA/L before inoculation. One additional experiment was performed without 4VP addition as a control. Substrate and 4VP solutions were sterilized by ultrafiltration using 0.22 μm membranes (Nalgene). Samples of 1 mL were recovered along of fermentation in sterile conditions. Samples were centrifuged and analyzed by HPLC. All assays were performed in duplicate.

2.4. Preparation of media with alkaline hydrolyzates

Materials were fractionated by two different procedures: alkaline hydrolysis or sequential stages of acid hydrolysis (prehydrolysis) and alkaline hydrolysis.

Pre-hydrolysis of corn cobs was carried out with diluted sulfuric acid (3%) during 15 min in ISO glass bottles with caps inside autoclave at 130 °C with a liquid/solid ratio of 8 g/g following the procedure described by Bustos et al. [19].

Raw corncob or solids from the acid treatment were hydrolyzed with solutions of NaOH (0.5 N) at room temperature in Erlenmeyer flasks at 150 rpm, using a liquid/solid ratio of 0.084 g solid/g NaOH solution [8]. After 6 h, solids were separated by vacuum filtration, liquors were neutralized with H_2SO_4 (72%, w/v) to pH 7, filtrated by vacuum again and stored at 4 °C for analysis by HPLC.

Detoxification of hydrolyzates media was performed by acidification, reducing the pH of both alkaline hydrolyzates to pH 3 with 98% (w/w) H_2SO_4, followed by centrifugation to remove the precipitate. Finally, supernatants were neutralized to pH 7 with NaOH (5 N) and filtered through Whatmann filter paper No. 1.

2.5. Two-phase biotransformation system

Fermentations were carried out in Erlenmeyer flasks of 250 mL with ground glass stoppers with a final volume of 150 mL. Media composition was 75 mL of aqueous phase (hydrolyzate sterilized by filtration) and 75 mL of organic phase (hexane). Table 1 shows the hydrolyzates employed in media 1 to 4. Firstly, aqueous phase was inoculated with 4% of overnight grown inocula, and then the organic phase was added. A sample of aqueous phase (AP) and organic phase (OP) was taken at each time along the fermentation. Fermentations were carried out in orbital shakers at 110 rpm at 37 °C. Experiments were performed in triplicate.

2.6. Cell immobilization

The Ca-alginate method [18] was used to immobilize recombinant *E. coli* cells expressing the *L. plantarum* PAD gene. Microorganism was grown in the same medium that inoculum. After overnight growth, biomass was recovered by centrifugation (Hettich Zentrifugen, Germany) at 2755*g* for 15 min at 4 °C and then cells were suspended in 4 mL of water. A cell suspension containing 1.5 g/L was added into 46 mL of sterilized solution of sodium alginate at 4% (w/v). This suspension was pumped with a peristaltic pump (Master flex, Cole Palmer instrument Co.) and dripped into $CaCl_2{\cdot}2H_2O$-water 2% (w/v) to form cell beads with the average diameter of 0.5 mm. After washed with sterilized water, the beads were cultured in fermentation media.

2.7. Analytical methods

AP samples, containing the phenolic compounds, were filtered through 0.2 μm-pore membranes (Sartorius, Goettingen, Germany) in order to analyze the compounds by High Performance Liquid Chromatograph (HPLC), in a chromatograph Agilent model

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