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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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 7484 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 ± 1.5%; hemicelluloses: 34.9 g L−1 ± 0.3%; acetyl groups: 4.1 g L−1 ± 0.6% and lignin: 21.6 g L−1 ± 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 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 (pre-hydrolysis) 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 H2SO4 (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) H2SO4, 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 2755g 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 CaCl2 2H2O-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
1200 (Agilent, Palo Alto, CA, USA), using an UV detector (at 276 nm) and a quaternary pump. Separation was achieved at 35 °C using a Zorbax SB-Aq reverse-phase column (Agilent, Palo Alto, CA, USA) with a guard column and a linear gradient run in 35 min from 100% to 52% of A at a flow rate of 1 ml/min consisting of two solvents: solvent A (2.5% formic acid in water, vol./vol.) and solvent B (100% methanol).

OP samples were analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detection (FID) system (Agilent Technologies, Germany). OP samples were directly injected into the chromatograph and compounds were separated in a Zebros ZB-WAX (I = 60 m × ID = 0.25 mm × df = 0.25 μm) from Phenomenex (Torrance, CA, USA). Injections were made in split mode (1:10). The injector temperature was 250 °C, the oven was programmed for 15 min at 60 °C, increasing at 3 °C/min to 200 °C and FID detector temperature 260 °C. The carrier gas was hydrogen (1 mL/min).

Total phenolic compounds were determined by the Folin–Ciocalteau method using caffeic acid as standard. The color value (U/L) was measured by spectrophotometrically by triplicate at 520 nm using UV-VIS Cintra 6 spectrophotometer (GBC Scientific Equipment Ltd, Braeside, Australia) and calculated as OD_{520} × dilute factor × 1000.

3. Results and discussion

3.1. Product inhibition

One of the main goals to be overcome during the biotechnological production of value-added products is the inhibition caused by end products, which significantly reduces cell growth and metabolic activities, thus limiting its production, and consequently decreasing both fermentation productivity and product yield [23]. For instance, Fabre et al. [24] reported a complete inhibition of the aromatic compound 2-phenylethanol during the fermentation by *Kluyveromyces marxianus* at concentrations of 2000 mg/L. In this work, the toxicity of the end product was evaluated adding different concentrations of 4VP before inoculation of *E. coli* cells expressing the PAD gene. Fig. 1 shows the kinetics of 4VP formation. It can be observed that initial concentrations higher than 500 mg 4VP/L are detrimental for the decarboxylation of p-CA to 4VP, repressing almost completely the production at concentrations higher than 1000 mg 4VP/L. Similarly, Jung et al. [25] evaluated the inhibitory effect of 4VP during the growth of recombinant *E. coli* harboring *Bacillus amyloliquefaciens* PAD, observed a clear inhibitory effect using concentrations higher than 1000 mg/L of 4-VP, meanwhile Lee et al. [16] observed inhibition of 4VG using *Bacillus pumilus* cells at concentrations higher than 1500 mg 4VG/L. Consequently,
it can be inferred that the accumulation of vinyl derivatives as a product renders a critical constraint in the decarboxylation of hydroxycinnamic acids in aqueous media.

3.2. p-CA extraction from corn cobs

Corn cobs were assayed as source of p-CA to bioproduce 4VP: raw corn cobs and the solid residue obtained after diluted acid hydrolysis pre-treatment, taking into account that p-CA can be extracted from corn cobs and pre-treated corn cobs by alkaline hydrolysis using low concentrations of NaOH (0.5 N) at room temperature [8,22]. Pre-treatments of lignocellulosic materials is a common step of biorefineries to separate major components cellulose, hemicelluloses and lignin [26]. In this manner, all fractions can be used to obtain different products. In alkaline hydrolysis it is possible solubilize oligosaccharides from the hemicellulose fraction [8]. The solubilization of hemicelluloses can cause an increase in the viscosity of hydrolyzate [27], thus biotransformation process may be hampered. Torre et al. [8] detected in corn cob alkaline hydrolyzate the solubilization of xylooligosaccharides, arabinooligosaccharides, glucooligosaccharides and acetic acid.

Table 1 shows the concentrations of p-CA and FA: the main hydroxycinnamic acids released after alkaline hydrolysis of corn cobs and diluted acid pre-treated corn cobs. Other phenolic compounds are solubilized in alkaline hydrolysis such as syringic acid, p-hydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, gallic acid, vanillic acid [28], but were not released in significant concentrations in this work. Using raw corn cobs directly as substrate, p-CA and FA were produced in a ratio of 1.8. Conversely, using the solid residue obtained after pre-hydrolysis, the concentration of p-CA increased from 1482.9 ± 32.1 mg/L up to 2570.7 ± 54.2 mg/L, meanwhile the FA decreased from 845.4 ± 25.3 mg/L to 416.8 ± 32.4 mg/L, thus implementing the p-CA/FA ratio to 6.2. The concentration of p-CA in alkaline hydrolyzate from pre-treated corn cobs was higher than that obtained from non pre-treated corn cob alkaline hydrolyzate. These results suggest that during the acid hydrolysis pre-treatment, p-CA was scarcely solubilized, at the time that the physical structure was modified, thus improving the p-CA extraction during the alkaline treatment. According to Xu et al. [29] p-CA is present more extensively esterified to lignin in the cell wall, and hemicelluloses are a physical barrier which could hinder the release of p-CA from lignin of cell wall by alkaline hydrolysis. Dilute acid pre-treatment was shown to be able to selectively remove hemicelluloses from different lignocellulosic materials [22,30], and although a small amount of lignin can be solubilized during the dilute acid hydrolysis, the majority is resistant to the attack by dilute acids. [29] However, Salgado et al. [22] observed an important reduction of lignin fraction (approximately 55% of the initial lignin) during the pre-hydrolysis. This percentage increased up to 86.5% after both sequential treatments, still remaining a small amount of lignin (4.5%) in the solid after sequential dilute acid and alkaline hydrolysis. However, the direct alkaline hydrolysis of the raw solid, only allowed releasing 17.6% of the initial lignin. Despite the lignin reduction observed during the dilute acid hydrolysis, there were no significant losses of p-CA (37.5 mg/L) in this step. Accordingly, p-CA was not released from brewer's spent grain during acid pre-treatment [11]. Additionally, a process of detoxification of hydrolyzates, consisting in acidification and further neutralization was assayed to formulate culture media ready to be used during the production of 4VP by two-phase extractive fermentation. Detoxification treatment removed color substances and reduced total phenolic compounds. Polyphenols responsible of black color can be removed by acid precipitation [31]. Additionally, the acidification reduced 24.8% and 31.8% the amount of p-CA and FA, respectively, in alkaline hydrolyzates obtained from raw corn cobs. However, these reductions were more drastic in alkaline hydrolyzates from pretreated corn cobs, with percentages of p-CA and FA reduction of 40.7% and 51.4%, respectively.

3.3. Production of 4VP by two-phase extractive fermentation

The application of in situ recovery techniques and integrated fermentation-separation processes, often synonymously called extractive fermentation, is a technique to improve some biotechnological processes [23]. Liquid–liquid two-phase systems can be used to overcome microbial limitations in a simple submerged fermentation, such as low substrate solubility, substrate/product inhibition and product further degradation [32]. In this study, fermentation medium (AP) was mixed with an organic solvent (OP), thus recovering 4VP during fermentation. Consequently, the concentration of toxic compounds in the medium was considerably reduced, avoiding the problems of product inhibition observed, at the same time that product recovery from fermented media was facilitated. In addition, it is avoided the problem derived from a poor solubility of 4VP in AP which could limit its production.

Hexane has been proposed in 4VP biotransformation in biphasic reactions [1]. However, the use of organic solvents in biocatalytic processes can reduce the enzymatic activity. Thus, in order to evaluate whether a solvent is suitable for bioprocesses, it is taking into consideration the partition coefficient between octanol and water (log P). It was considered that solvents with a log P higher than 2 are suitable for biocatalytic systems [17]. The log P of hexane is 3.29 [1] so that can be used as OP in two-phase extractive fermentations to produce 4VP by recombinant E. coli. Furthermore, Jung et al. [25] studied remaining PAD activity after incubation with different organic solvents and they observed that the effect of hexane in PAD activity was lower than the other solvents effect. Consequently, in this study, the in situ extractive fermentation was performed to evaluate the continuously recuperation of vinyl derivatives produced in AP by recombinant E. coli using hexane as OP. Fermentations were carried out with crude or detoxified hydrolyzates from direct alkaline hydrolysis of corn cobs (p-CA-AH) or sequential acidic and alkaline hydrolyses (p-CA-AAH). Table 2 shows the results obtained in all fermentations at the maximal concentration of 4VP (time 24 h) as a sum of AP and OP, and at the end of fermentation (time 87 h) with both phases separately in order to appreciate the transport from 4VP from the AP to the OP.

The production of 4VP was affected by the process of detoxification. Thereby, fermentations performed using detoxified hydrolyzates achieved higher productions, yields and global volumetric productivity of 4VP. Overlimiting is a common process employed for detoxification of acid diluted hydrolyzates [33], which allows precipitating toxic compounds at high pH values [34]. This detoxification process consists in increasing the pH of the hydrolyzate to extreme values, occurring substance precipitation. The pH of hydrolyzates after alkaline hydrolysis reached up to 12, being further reduced to pH 3 by acidification using H2SO4, and final neutralization to pH 7 with NaOH, producing the precipitation of compounds.

The 4VP concentration was split into two portions, AP and OP. Figure 2a–d display the production of 4VP and 4VG during fermentation (bottom of the figure) and the migration of these compounds into the OP (top of the figure). As it was expected, it was observed a higher concentration of 4VP in AP at the beginning of the fermentation. However, the concentration of 4VP was continuously decreasing in AP, increasing simultaneously in OP, during the course with time of fermentation. Except with detoxified pre-treated corn cob alkaline hydrolyzates (AHH), 4VP was fully recovered at the end of fermentation.

The maximum concentration of 4VP was obtained in fermentation using detoxified AHH, considering the sum of both phases at 24 h (1003.5 mg/L) which also represents the highest global
Finally, AVG was also produced although in smaller amounts, and the highest product concentration (101.1 mg/L) was achieved after 24 h of fermentation. Additionally, in all fermentations, the volumetric productivity of 4-vinylphenol was lower than that of AVG. Although Salgado et al. [22] reported that PDA had a preference for p-CA metabolism instead of FA degradation in media, this was not observed in the present study. Furthermore, it was observed that the FA was not released from the FA-CA mixture in the absence of AVG production. This finding is consistent with previous reports by Salgado et al. [22] and Abalo et al. [20].

**Table 2**

Fermentative parameters of extractive fermentations.

| Exp. | Culture | Det. | Imm. | t<sub>max</sub> (h) | [4VP]<sub>max</sub> (mg/L) | [4VG]<sub>max</sub> (mg/L) | Y<sub>4VP</sub>-CA | Q<sub>4VP</sub> | Q<sub>4VG</sub> | t<sub>f</sub> (h) | [4VP]<sub>f</sub> (mg/L) | [4VG]<sub>f</sub> (mg/L) | FA<sub>cons</sub> (mg/L) |
|------|---------|------|------|---------------------|---------------------------|---------------------------|-----------------|----------------|----------------|----------------|----------------|----------------|
| F1   | AH      | −    | −    | 24                  | 685.71                    | 87.79                     | 0.79            | 1.21           | 28.56         | 1.01           | 87             | 0.0            | 21.73 ± 3.22 |
| F2   | AHH     | −    | −    | 24                  | 290.78                    | 101.06                    | 0.70            | 1.32           | 12.11         | 1.16           | 87             | 0.0            | 44.21 ± 5.51 |
| F3   | AH      | +    | −    | 24                  | 641.42                    | 89.32                     | 0.64            | 0.66           | 26.72         | 1.06           | 87             | 0.0            | 15.58 ± 0.71 |
| F4   | AHH     | +    | −    | 24                  | 1003.51                   | 76.01                     | 0.86            | 1.34           | 41.81         | 0.87           | 87             | 0.0            | 18.79 ± 0.78 |
| F5   | AH      | +    | +    | 21                  | 1013.38                   | 74.78                     | 1.07            | 0.32           | 48.25         | 1.90           | 38             | 0.0            | 16.11 ± 0.09 |
| F6   | AHH     | +    | +    | 24                  | 852.81                    | 74.04                     | 0.79            | 0.49           | 37.21         | 1.96           | 38             | 0.0            | 15.06 ± 0.51 |

AH, corn cobs alkaline hydrolyzates; AHH, pre-treated corn cob alkaline hydrolyzates; Det., detoxified (+); not detoxified (−); Imm., immobilized cells (+), free cells (−); t<sub>f</sub>, time of end fermentation; t<sub>max</sub>, time of maximum production; [4VP]<sub>max</sub>, 4-vinyl phenol maximum production; [4VG]<sub>max</sub>, 4-vinylguaiacol maximum production; p-CA<sub>cons</sub>, p-coumaric acid consumed at final of fermentation; FA<sub>cons</sub>, ferulic acid at final time of fermentation; Y<sub>4VP</sub>-CA, yield of 4-vinylphenol; Y<sub>4VG</sub>, yield of 4-vinylguaiacol at t<sub>max</sub>; Q<sub>4VP</sub>, volumetric productivity of 4-vinyl phenol at t<sub>max</sub>; Q<sub>4VG</sub>, volumetric productivity of 4-vinylguaiacol at t<sub>max</sub>.
3.4. Extractive fermentation with immobilized microorganism

In order to minimize the contact of free cells with OP, preliminary experiments were performed to evaluate the effect of immobilization of cells in alginate beads, therefore avoiding OP toxicity. However, the use of immobilized cells limits mass transfer compared with free cells [17]. In this study, it was observed that recombinant E. coli cells expressing the PAD gene immobilized in alginate beads supported 4VP by in situ extractive fermentation using hexane as OP, and AH and AHH as AP. Fermentative parameters are shown in Table 2. The production of 4VP in no pre-treated corn cob alkaline hydrolyzates (AH) increased using immobilized cells (up to 1013.4 mg/L) as the sum of two phases after 21 h fermentation rather than free cells (641.4 mg/L); however, using pre-treated corn cob alkaline hydrolyzates (AHH) this concentration decreased slightly, from 1003.5 mg/L using free cells, to 892.8 mg/L with immobilized biomass. However, this technology must be optimized, considering that 4VP was not fully recovered from AP, as it can be seen in Fig. 3a–b in both fermentations, remaining 497.3 and 437.6 mg/L of 4VP in AH and AHH, respectively. Thus, it was observed a negative effect of immobilized cells in the extraction process from AP to OP. In entrapment with calcium alginate some problems such as gel instability in the presence of organic solvents and mass transfer limitations it was observed [17].

4. Conclusions

This study has revealed that the biotransformation of p-CA to 4VP by recombinant E. coli cells expressing the PAD gene from L. plantarum was affected by the amount of 4VP in the culture medium. Media with an initial content exceeding 1000 mg 4VP/L had a strong inhibition effect on decarboxylation of p-CA to 4VP by PAD. For this reason and to facilitate the product recovery, an in situ extractive fermentation by two phase’s system was planned. Alkaline hydrolyzate from no pre-treated corn cobs (AH) and pre-treated corn cob alkaline hydrolyzates (AHH) showed to be a good source of p-CA. Crude or detoxified hydrolyzates were used as AP in extractive fermentation. Hexane was selected as OP, because it was able to recover effectively the 4VP from AP. The results of this studied demonstrated that detoxified hydrolyzates improved the production of 4VP, especially in AHH. It was also shown that 4VP could be easily recovered from fermented media. Finally, a preliminary study using cells immobilized in calcium alginate indicated that it was possible the biotransformation of p-CA to 4VP, improving the production of 4-vinylphenol using AH, however the recovery of 4VP from AP was hampered in immobilized cells. Effect of immobilized in recovery of 4VP is not clear, further detailed studies are required to confirm this assumption. These suggested the need to change OP during fermentation or to use an external solvent column. Further research might investigate this option, as well as the scale-up of the process and the number of cycles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2014.02.005.

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