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Improving process conditions of hydroxytyrosol synthesis by toluene-4-monooxygenase

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ABSTRACT

Toluene-4-monooxygenase from *Pseudomonas mendocina* KR1 was recently engineered for the synthesis of hydroxytyrosol, a potent antioxidant. Following a 190-fold improvement in the enzyme activity by protein engineering means, improving the process conditions of this biocatalytic route was under taken for developing a liter-scale bioprocess. The growth stage was improved by selection of a rich media and harvesting the cells at the end of the logarithmic stage. The biotransformation stage was optimized by evaluating substrate concentration, cell density, and different operational modes. It was found that although reusing the cells in successive batch modes is feasible, their activity is dramatically decreased after the first use. In comparison, the activity of the cells following subsequent substrate addition in a fed batch mode was only slightly decreased. Furthermore, a better yield was obtained by extending the duration of the biotransformation stage, rather than adding more substrate. An overall concentration of 133 mg/L HTyr, corresponding to a volumetric productivity of 54 mg/L/h and a yield of 48% was achieved by a batch mode using 2 mM substrate. This is an order of magnitude improvement compared with the enzyme productivity before the process optimization. The use of beads conjugated with phenylboronic acid residues for adsorbing the product from the biotransformation bulk was evaluated. Though the recovery yield and purity were shown to be oppositely dependent, an average recovery procedure led to 2-fold purification of HTyr resulting in 84% purity with 70% recovery yield.

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1. Introduction

There is an increasing interest in the use and application of biocatalytic processes at an industrial scale [1–4]. Hence, as the scale of the biocatalytic process increases, different considerations become more significant, and the process is required to overcome several limitations; mainly, low solubility of the substrate, toxicity of either substrate and/or products, low enzyme productivity, and metabolic diversity which leads to undesired by-products or further degradation of the desired molecules. For overcoming such weaknesses, efforts can be carried out from the point of view of either the biocatalyst (exploiting protein engineering to adjust the biocatalysts to a specific process) or the process (exploiting different biochemical tools for adjusting the process in a way that optimizes the biocatalyst activity) [1,4].

The biocatalytic process described in the present study is the whole cell biotransformation of 2-phenylethanol (PEA) to form the substituted catechol hydroxytyrosol (HTyr). HTyr, a commercially valuable antioxidant, is naturally present in olives and has been shown to be beneficial in preventing various diseases, such as diabetes, atherosclerosis and cancer [5–7]. Recently, we reported the designing of toluene 4-monooxygenase (T4MO) variants for the biosynthesis of HTyr by whole (resting) cell biotransformation [8–10]. T4MO, the biocatalyst chosen to perform this reaction, is an O_2-dependent multicomponent monooxygenase which requires NADH as a cofactor [11]. Hence, performing the reaction with a whole cell system enables NADH regeneration saving the tedious and costly process of protein isolation and purification. Moreover, the formation of undesired byproducts or further product degradation is minimized by the heterologous expression of the enzyme in *Escherichia coli* TG1 cells [11].

Earlier attempts to enhance the production of HTyr from PEA focused on the biocatalyst itself, by exploiting protein engineering to improve its catalytic activity [8–10]. These attempts lead to the discovery of a variant, TmoA I100A/E214G/D285Q, which exhibited an initial oxidation rate of 4.4 ± 0.3 nmol/min/mg protein, which is 190-fold faster than the rate obtained by wild-type [10]. However, the productivity achieved by the engineered biocatalysts is not yet ideal nor economically feasible for industrial applications. Hence, with the aim of enhancing HTyr production using recombinant *E. coli* cells, we changed the focus towards the process itself, to optimize the biocatalyst activity.

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An industrial process must address issues, such as reaction volume, substrate supply, product protection and recovery and biocatalyst reuse, in order to obtain the highest possible product concentration in the shortest possible time with the lowest costs [1,3,12]. In general, the decision regarding which method to apply is specific to a particular process or objective. For example, when the biocatalyst costs are high it is necessary to optimally exploit the biocatalyst, emphasizing the yield of product per catalyst quantity. Whereas, when bioproduction of an expensive substance is sought, the yield of product from the substrate should be the emphasized [4,13].

In addition to the production process, product recovery and purification must also be considered. The importance of product recovery comes to fruition not only in its contribution to the total process cost and yield but it also has an important role when the product is inhibitory, as in the case of catechol production in general and HTyr in particular. Exclusion of products via adsorption onto solid matrices has been employed for the recovery of a relatively large variety of substances, including toxic catechols [14,15]. With the intention of removing the HTyr from the biotransformation bulk, it should be noted that the main compounds which play a role during PEA biotransformation are the substrate (PEA), which is a substituted benzene, the intermediate tyrosol products (*m*- and *p*-tyrosol), which are substituted phenols, and the final product, HTyr, a substituted catechol. Hence, the different characteristics of the compounds and the presence of a reactive diol group in the desirable product can be employed in order to form a selective reversible complex with a solid adsorbent.

Boric acid gel is a commercial resin, capable of forming a complex with cis-diol groups of various compounds, under neutral or weakly alkaline conditions (resulting with the formation of reversible boronic esters) and releasing them in an acidic solution [16–18]. Lorand and Edwards were the first to show that such ester formation is more favorable in solutions of high pH where the boronate ion exists in high concentrations [19]. Following this observation, boronic acid derivatives ($RB(OH)_2$) on various solid supports have been investigated and applied for separation of various compounds, as saccharides, nucleotides and catechols, including L-DOPA, catechol estrogens, and catechol amines [16–18,20]. Consequently, beads conjugated with boronic acid residues can be employed for the recovery of HTyr. Such a specific complex which efficiently binds catechols but not phenols can enable simple purification of HTyr. Furthermore, linkage of HTyr to the beads through its reactive group provides protection by preventing its spontaneous oxidation. Therefore, utilizing beads conjugated with boronic acid can attain protection, recovery and purification of HTyr in one step.

In this work, we focus on several aspects of the HTyr biosynthesis process, aiming for improving its conditions and enhancing the final productivity. Accordingly, the biotransformation was scaled up to 1 L and several aspects were evaluated and optimized. Furthermore, the recovery and purification of the product, HTyr, by the use of boric acid gel was examined.

2. Materials and methods

2.1. Materials

2-Phenylethanol (PEA), *m*-tyrosol, *p*-tyrosol and boric acid gel beads (particle size 0.1–0.4 mm) were purchased from Sigma–Aldrich Chemical Co. (Sigma–Aldrich, Rehovot, Israel). Hydroxytyrosol was obtained from Cayman Chemical Co. (MI, USA). All standards were prepared as stock solutions in ethanol. All materials used were of the highest purity available and were used without further purification.

2.2. Bacterial strains and growth conditions

Escherichia coli TG1 (*supEhsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^+$ *lacI*^{q}*lacZ*ΔM15]) with the plasmid constructs was routinely cultivated at 37 °C in Terrific Broth (TB) medium supplemented with 100 μg/mL kanamycin to maintain the plasmid. To express the toluene monooxygenase genes stably and constitutively from a single promoter, the expression vector pBS(Kan)T4MO (henceforth T4MO) was constructed as described earlier [21]. All experiments were conducted by diluting overnight cells to an optical density (OD) at 600 nm of 0.1 and growing to an OD of 5.5. The exponentially grown cells were centrifuged at 8000 × *g* for 10 min at 25 °C in a Sigma-4K15 centrifuge (Sigma, Osterode, Germany) and re-suspended in potassium phosphate buffer (PB, 100 mM, pH 7.0).

2.3. Whole-cell enzymatic biotransformations

Whole-cell activity assays were performed in a similar manner to previous studies [8,10] except for using Erlenmeyer flasks containing 20 mL cells. The substrate, 2-phenylethanol (PEA) was added to the cells from a 400 mM stock solution in ethanol. All flasks were shaken at 30 °C, 250 rpm (Orbital shaker incubator, MRC, Holon, Israel) and samples were taken periodically and the reaction was stopped by removal of the cells via filtration. The progress of enzymatic hydroxylation of PEA was measured by reverse-phase HPLC.

A 1 L scale bioconversion of PEA was examined using two different strategies: batch bioconversion with successive reuse of cells, and fed-batch bioconversion in a bioreactor with periodic addition of substrate. All experiments were carried out at 30 °C in a 3 L BioFlo3000 reactor (New Brunswick Scientific, Edison, NJ, USA) which was stirred at 400 rpm. One liter of concentrated cell suspension (OD_{600} of 16) in potassium phosphate buffer (PB) was contacted with either 2 or 1.2 mM PEA (from a 400 mM stock solution in ethanol) and the bioconversion progress was monitored using the HPLC. For the purpose of reusing the cells, the cell suspension was harvested, resuspended in fresh buffer and returned to the bioreactor with the same operation conditions. The fed-batch bioconversions were carried out with subsequent additions of 1.2 mM PEA, added following 90% consumption of the initial PEA concentration (after 2 h of reaction).

2.4. HTyr recovery and purification using boric acid gel

The formation of a complex between dihydroxyboryl groups and vicinal diol groups such as in HTyr has been used as a basis for the separation of HTyr from the biotransformation broth. For that purpose, boric acid gel beads, i.e. cross-linked polymer beads conjugated with phenylboronic acid, were utilized. Prior to use, the beads were washed twice with the eluting solvent (0.025 N HCl) and the initial solvent (PB buffer, pH 8), as recommended by the supplier, and fine particles were decanted each time. Then, the beads were allowed to swell in PB buffer, pH 8 for about 4 h. After excluding the buffer, the biotransformation broth was applied to the activated beads. In a typical experiment, each milliliter of broth was contacted with 30 mg of beads. Following successive washing of the beads with PB buffer pH 8, to remove unbound substances, HTyr was eluted by the addition of 0.03 mL 0.025 N HCl (in either distilled water or ethanol) for each milligram of beads. Regeneration of the beads was performed by washing with sequential doses of ddH_2O, 1 N HCl, ddH_2O and 0.1 N NaOH.

The separation of the beads from each solution was performed by either filtration using two-compartment test tubes separated by a 10-μm-cutoff membrane (Whatman polypropylene mesh

VectaSpin Micro or VectaSpin 3™) or, for large quantities of beads, by centrifugation using a 50 mL polypropylene centrifuge tube.

2.5. Analytical methods

The analytical method was the same as we described previously [9]. HPLC analysis was performed with an Agilent 1100-series instrument (Agilent Technologies, CA, USA) using an Eclipse XDB-C18 column (5 μm, 4.6 mm × 150 mm, Agilent Technologies, CA, USA) equipped with a photodiode array detector. The isocratic elution was performed with 85% acidic H_2O (0.1% formic acid) and 15% acetonitrile as the mobile phase at a flow rate of 1 mL/min. Compounds were identified by comparison of retention times and UV–visible spectra to those of the appropriate standards.

3. Results

3.1. Reaction optimization for synthesis of HTyr by whole-cell biotransformation

In order to gain further insight regarding the implementation of HTyr biosynthesis and for enhancing its yield, the PEA biotransformation was scaled up to 1 L and optimized. The biocatalyst used for all experiments was the best HTyr producing variant found to date, T4MO TmoA I100A/E214G/D285Q [10].

As the PEA biotransformation by T4MO is performed using resting cells, uncoupling the growth (catalyst production) and the biotransformation (reactant conversion) stages enabled the optimization of each step independently, which is a clear advantage over performing the bioconversion with growing cells, as the conditions for optimal cell growth and expression of heterologous proteins can be very different from those of the biotransformation. Notably, separating the biotransformation from cell growth enables modification of the medium thereby increasing the cell concentration in the biotransformation stage. The growth stage was improved by using a rich media (TB) which enabled higher biomass production than Luria Bertani (LB) medium (biomass concentration of 1.65 *vs*. 0.39 g dry cell/L was obtained with TB and LB mediums, respectively). Additionally, harvesting the cells at the end of the logarithmic stage yielded higher enzymatic activity (represented by substrate consumption) in comparison with cells from mid log phase or the stationary phase (Fig. 1). Further focus was placed on the biotransformation step.

In the earlier studies, *E. coli* cells expressing T4MO variants were shown to produce HTyr from PEA at an initial substrate concentration of 0.25 mM [8–10]. These experiments were performed using a set of glass vials, which were sequentially removed from incubation at each time period. To achieve gram quantity production of HTyr, the concentration of PEA as well as the scale of the biotransformation had to be increased. Nevertheless, HTyr, a substituted catechol, and the intermediate products, *m*- and *p*-tyrosol, could adversely affect biocatalyst performance due to toxicity. Thus, aiming toward the optimization of HTyr production, we initially evaluated the conversion kinetics using different ratios of substrate/cell concentrations. As a preliminary step before scaling up the biotransformation to 1 L, the volume of the reaction was increased to shake flasks containing 20 mL cell suspension. The highest HTyr concentration and conversion yield (product/substrate) were obtained with a biomass concentration of OD_{600} = 20 and an initial PEA concentration of 2 mM. Higher and lower biomass concentrations and/or initial substrate concentrations resulted in decreased conversion yields and product concentrations (Fig. 2). Noteworthy, is that increasing the biotransformation volume from 2 to 20 mL had no effect on the reaction kinetics (data not shown). It should also be noted

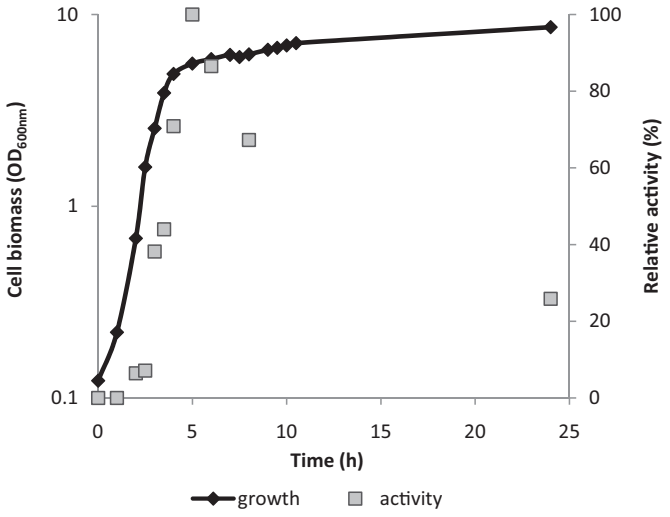

Fig. 1. Profiles of cell growth and relative PEA oxidation rate by T4MO I100A/E214G/D285Q in TB medium. Cells were grown in TB medium and aliquots were removed to evaluate the conversion of PEA to tyrosol and HTyr. Each activity point on the graph represents the decrease in PEA by the enzyme with the highest oxidation rate depicted as 100% (corresponding to 4.9 nmol/min/mg protein with 0.25 mM initial PEA concentration). PEA conversion was determined using HPLC analysis.

that the results demonstrated decomposition of HTyr with time due to spontaneous oxidation. All kinetic profiles revealed a preferred accumulation of *m*-tyrosol over the *para* isomer. Evaluating the initial oxidation rate of *m*- and *p*-tyrosol by T4MO I100A/E214G/D285Q variant demonstrated an improved oxidation rate of *p*-tyrosol, which was 3-fold faster than the *m*-tyrosol oxidation rate and yielded higher amounts of HTyr (data not shown). Consequently, it can be concluded that the formation of HTyr by the T4MO I100A/E214G/D285Q variant is mainly by oxidation of the *p*-tyrosol isomer, resulting with lower concentrations of *p*-tyrosol *vs.* *m*-tyrosol during the PEA biotransformations.

For further optimization of the process, the reuse of cells was evaluated as well as fed-batch operation (step-wise addition of substrate to the same cell suspension). The biotransformation was

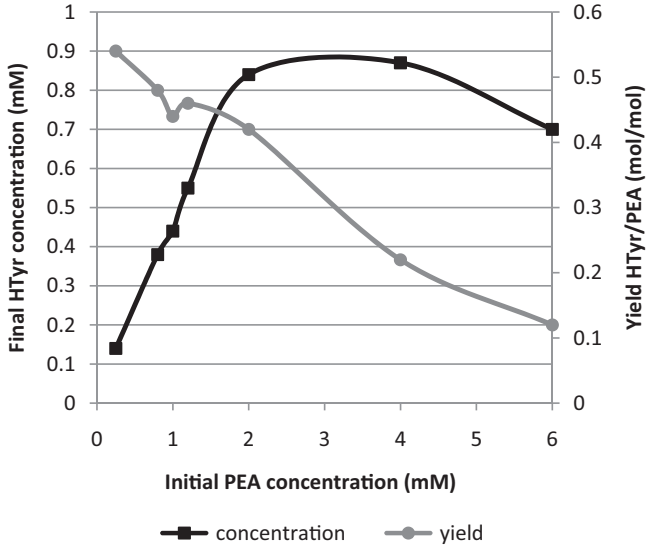

Fig. 2. Final HTyr concentration and yield as a function of initial PEA concentration. All biotransformations were monitored for 3 h while calculations were made at a time point in which maximum HTyr concentration was observed. Biotransformations were performed using 20 mL of biomass at an OD_{600} of 20 suspended in PB (pH 7). Shaking was carried out at 250 rpm at 30 °C.

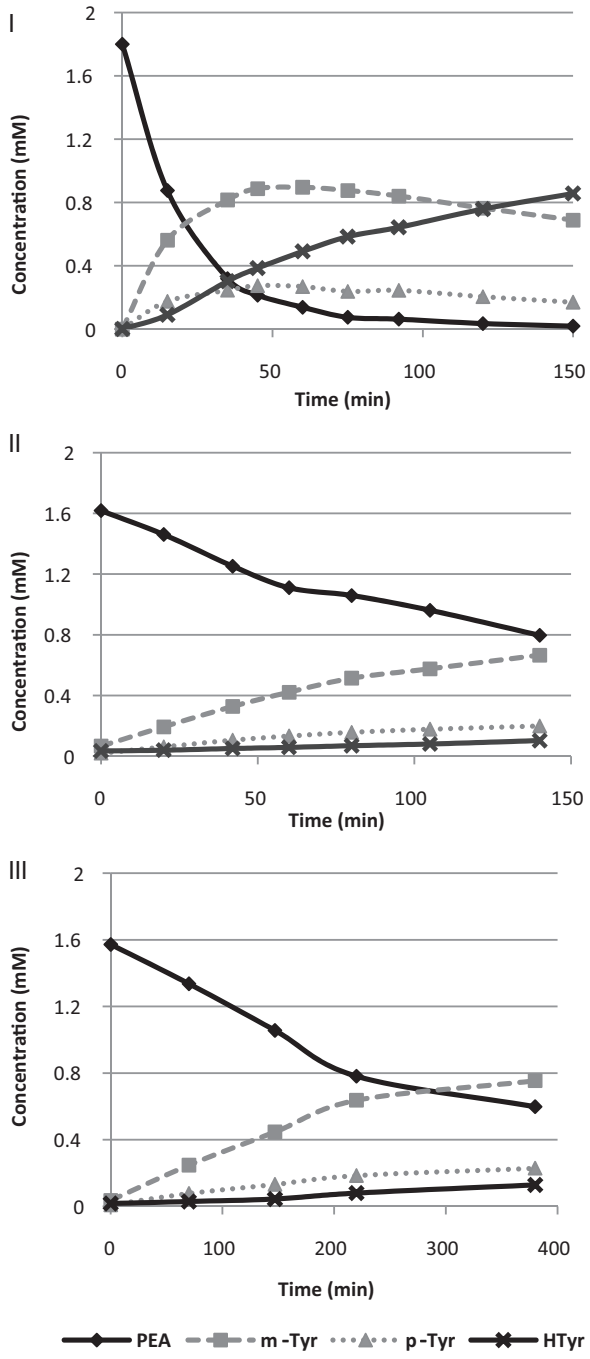

Fig. 3. Time course of three successive batch biotransformation reactions (I, II, III) with reused cells. The batch bioconversion was carried out at 30 °C in a 3 L bioreactor with 1 L of cell suspension and after each cycle, the cells (OD_{600} of 16) were harvested and re-suspended in fresh buffer containing 1.6 mM PEA. Analytes were measured with HPLC.

performed, in both cases, in a 3 L stirred bioreactor using T4MO I100A/E214G/D285Q cells, grown in 3L TB medium to the end of the logarithmic phase and resuspended in PB pH 7 to a final volume of 1 L (resulting with an OD_{600} of 16). The progress of the bioconversion was monitored by HPLC analysis and the time frame was set to be 2.5 and 2 h, for the sequential cycles and fed-batch operation, respectively, in order to reduce oxidative degradation of HTyr.

The data obtained from the three successive cycles of reused cells revealed that the cells' activity is dramatically decreased after the first use (Fig. 3). While the first bioconversion yielded 0.86 mmol of HTyr after 150 min, the second and third

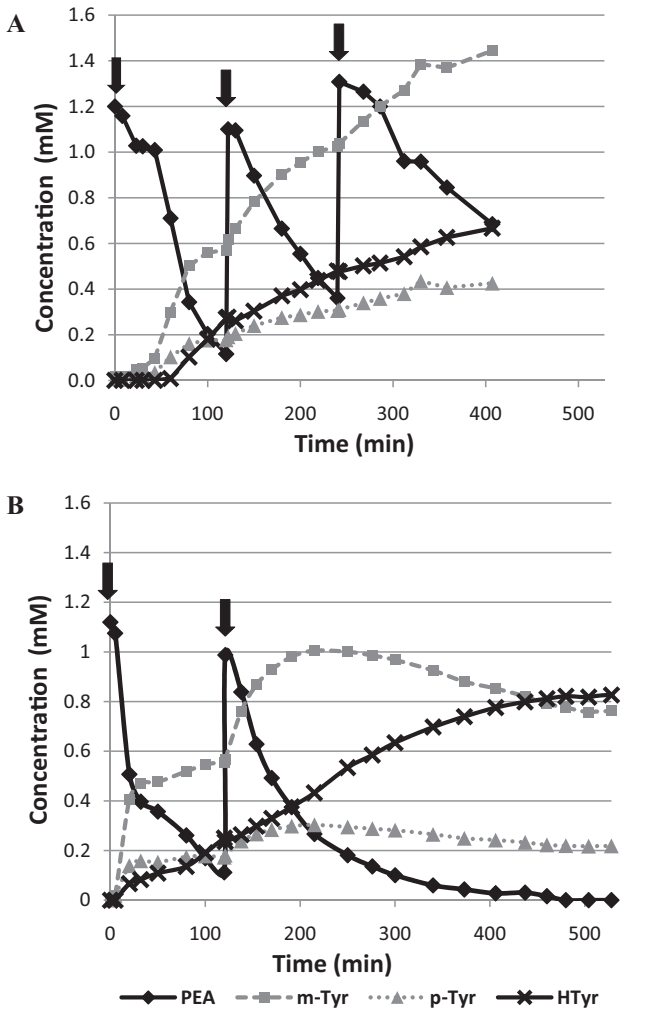

Fig. 4. Time course of the fed-batch biotransformation with three (A) and two (B) substrate additions. The bioconversion was carried out at 30 °C in a 3 L bioreactor using 1 L cell suspension at a biomass concentration of 16 OD_{600}. The black arrows indicate the point of substrate addition (1.2 mmol PEA).

bioconversions yielded with only 0.1 mmol HTyr after 140 and 380 min, respectively (in the first 150 min the third bioconversion yielded only 0.04 mmol HTyr).

The results obtained from the fed-batch experiment with three steps of substrate additions are illustrated in Fig. 4A. From the data it can be concluded that the cells remained active after 407 min of reaction, although the rate of the PEA hydroxylation declined with time. Approximately 160 nmol/h/mg protein was obtained at the first step compared with 112 and 64 at the 2nd and 3rd steps, respectively. Likewise, the first bioconversion step yielded 22.5% HTyr (from 1.2 mM PEA), while following the second step the total yield decreased to 20% (out of 2.4 mM PEA) and after the third step to 18.6% HTyr (out of 3.6 mM PEA). Summing the amount of all analytes at each point during the bioconversion, showed a decrease of 7, 9 and 10% in the total amount at the first, second and third bioconversion steps, respectively. Hence, it could be approximated that no more than 10% of the HTyr formed during the fed-batch bioconversion was decomposed over its course.

Repeating the fed-batch experiment without the third substrate addition (Fig. 4B) reveals that a better impact is achieved by extending the duration of the biotransformation stage, rather than adding more substrate (as in Fig. 4A). In particular, while the amount of HTyr formed after 120 min (following single substrate addition) as well as after 240 min (following two substrate

Table 1
Summary of the biotransformation performance using different operation modes.

Operation mode	Details	Volume (L)	Total PEA amount (mmol)	Overall HTyr concentration (mM)	Time (min)	Space time yield (mmol/L/h)	Yield HTyr/PEA (mol/mol)
Batch	First use of cells	1	2	0.86	150	0.344	0.476
	Second use of cells	1	2	0.10	140	0.043	0.059
	Third use of cells	1	2	0.13	380	0.021	0.075
	Total	3	6	0.36	670	0.032	0.182
Fed batch-A	First addition	1	1.2	0.27	120	0.135	0.225
	Second addition	1	2.4	0.48	240	0.120	0.200
	Third addition	1	3.6	0.67	407	0.099	0.186
Fed batch-B	First addition	1	1.2	0.25	120	0.125	0.208
	Second addition	1	2.4	0.83	528	0.093	0.346

additions) was similar between both operations (0.27 and 0.48 mM for the first operation *vs*. 0.25 and 0.53 mM for the second operation, respectively), the amount of HTyr accumulated after 407 min was different (0.67 mM *vs.*0.78 mM, respectively). Allowing the biotransformation to continue, even after the substrate was completely consumed, promoted the conversion of *m*- and *p*-tyrosol to HTyr.

Summarizing the performance of the different operation modes of the scaled up biotransformations (Table 1) shows that the highest HTyr concentration was obtained for both the batch bioconversion (first use, before recycling the cells) and the two cycles of fed-batch bioconversion (0.86 and 0.83 mM, respectively). The highest space time yield and the overall yield of HTyr from PEA are obtained by the first batch bioconversion before recycling the cells. In addition, while the space time yields of both fed-batch bioconversions are identical, the overall yield of HTyr is much higher for the two cycles of fed batch bioconversion (0.83 mM). It should be noted that the yield can vary depending on the space time of the bioconversion, meaning that stopping the reaction at a shorter time period may result with higher space time yield and lower overall yield. Thus, calculating the yields of the second operation as if it would have stopped after 407 min, similar to the first operation, will give a yield of 0.323 mol/mol with space time yield of 0.115 mmol/L/h. These values are higher than those obtained for the first fed batch operation.

To conclude, from the results presented here, the most efficient mode for the biosynthesis of HTyr seems to be a batch or fed-batch bioconversion of 2 mM PEA without reusing the cells. In fact, in this research, we attained 133 mg/L HTyr, corresponding to a volumetric productivity of 54 mg/L/h and a yield of 48% (achieved by a batch bioconversion of 2 mM PEA). This is an order of magnitude improvement compared with the enzyme productivity before the process optimization (volumetric productivity of 6 mg/L/h and final HTyr concentration of 17 mg/L were obtained with the small scale biotransformation of 0.25 mM PEA).

3.2. Protection, recovery and purification of HTyr using boric acid gel

As the desirable product, HTyr, is a substituted catechol, it may undergo rapid self-oxidation or other cross-interactions with the resting cells or diverse environmental elements. Therefore, to obtain maximum yield and productivity, exclusion of the product from the biotransformation bulk should be considered. Consequently, it was hypothesized that HTyr, through its vicinal diolgroup, could bind to the boronic acid residues of the boric acid gel beads under weakly alkaline conditions (Fig. 5A) and be stabilized and removed from the solution. Then, under acidic conditions, HTyr could be eluted from the beads in its free, non-complexed form (Fig. 5B). The boronic acid residues, due to covalent linkage to the polymer, remain in the stationary phase. The formation of reversible boronic esters through the reactive diolgroup of HTyr can prevent its oxidation (while it is in bound form) and, moreover, this specific complex formation which efficiently binds catechols but not phenols can enable the separation of HTyr from other substance in the biotransformation bulk, like the tyrosol isomers. Therefore, this method could provide not just the recovery, but also the protection and purification of HTyr.

Hence, the use of boric acid gel beads for HTyr recovery and purification was assessed, using products from the biotransformation supernatant. Due to the fact that throughout the research the biotransformation was performed at pH 7, while the utilization of the beads may require higher pH, the effect of the loading buffer's pH on the HTyr linkage was evaluated. It was found that HTyr recovery by the beads is feasible at both pH 7 and pH 8, while better yields are obtained when the bead loading is performed at pH 8 (70% of the initial HTyr was removed from the solution and a recovery yield of 50% was obtained when PB pH 8 was used *vs.* 50% binding and 20% yield for PB pH 7). Consequently, performing the biotransformation itself at pH 8 was evaluated and the initial oxidation rate and regioselectivity obtained by both WT and mutant at pH 8 were found to be identical to that obtained at pH 7.

An additional important aspect for pH selection is the stability of HTyr in the medium. Therefore, the content of HTyr under various conditions was evaluated, showing that 100% of the HTyr kept at pH 7 is maintained even after 28 h at 30 °C or 144 h at 4 °C. On the other hand, the concentration of HTyr kept at pH 8 decreased to 94% in the first 2 h and to 60% after 18 h at 30 °C. This decline can be explained by the deprotonation of the diol group causing instability and oxidative degradation of HTyr at an alkaline environment. The decrease in HTyr concentration was accelerated in the presence of bacterial cells (either expressing the enzyme or not), probably due to various cross-interactions. Therefore, it was concluded that in order to protect the HTyr formed by the whole-cell catalysis, the biotransformation should be performed at pH 7 and the cells removed immediately when full conversion is attained. Since the use of boric acid beads for the recovery of HTyr requires basic conditions (pH 8) it is recommended to keep the solution at pH 7 and to adjust the pH prior to adsorption. Alternatively, *in situ* product removal (ISPR) may be used to remove and/or protect the HTyr from the biotransformation bulk as soon as it is formed.

In order to improve and facilitate the purification of HTyr, an elution step using ethanol at acidic pH was evaluated rather than a diluted HCl solution (0.025 N HCl in water), resulting with similar results to those obtained with the aqueous HCl solution. The use of an organic solvent can enable its evaporation thus enabling the concentration of HTyr. An additional advantage is the reduced oxidation of HTyr in the elution solvent, resulting with an improved storage environment.

From an economic perspective and the desire to reuse the beads, the performance of regenerated beads *vs.* new beads was evaluated. Consequently, it was found that the regenerated beads

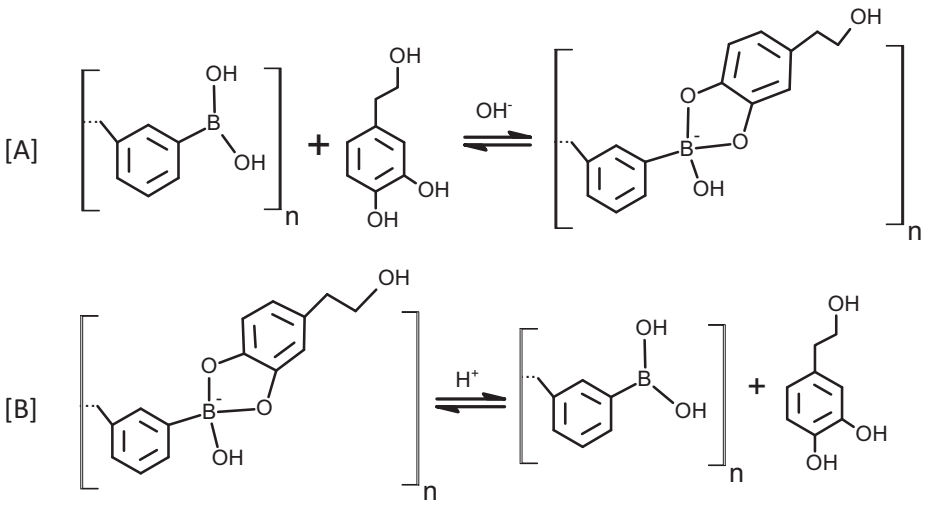

Fig. 5. Scheme for the adsorption (A) and elution (B) of HTyr from beads conjugated with boronic acid residues. At a neutral or weakly alkaline solution, the boronic acid residues are ionized and form a specific complex with the vicinal diol group of HTyr. This complex formation is reversible and is decomposed under acidic conditions, to give HTyr in its native form.

demonstrated the same recovery results as the new beads. Moreover, in both cases (regenerated and new beads) most of the PEA and tyrosol did not bind to the beads and were washed away in the flow-through and the subsequent washing steps.

It should be noted that the yield of recovered HTyr in the elution step and its purity are oppositely dependent on the washing steps. The more the beads are washed, the higher the likelihood for unbound or weakly bound substances, like *m*-tyrosol, to be removed from the beads, resulting with increased purity of the eluted HTyr. While at the same time, part of the bound HTyr is washed away as well, resulting with a lower recovery yield. Nonetheless, the effectiveness of the beads in purifying HTyr was assessed, using biotransformation supernatant containing in addition to 0.8 mM HTyr, also 0.02 mM PEA, 0.2 mM *p*-tyrosol and 0.7 mM *m*-tyrosol. It was shown, in a typical recovery procedure, that while most of the impurities were washed away in the flow-through and in two subsequent washing steps (100, 90 and 80% out of the initial PEA, *p*-tyrosol and *m*-tyrosol quantities, respectively), only 3% of HTyr was lost as well. Hence, following elution of the bound product, HTyr with an 84% purity was recovered. The recovery yield was 70% (mM HTyr in the elution recovered from mM in the biotransformation broth).

In order to further understand the recovery potential of the beads, the dependence of the recovery yield on the amount of beads used was evaluated. Increasing amounts of beads were added to aliquots of the biotransformation supernatant and the binding of HTyr to the beads was evaluated. A typical adsorption curve is obtained (Fig. 6) demonstrating that higher bead concentrations result in better binding of HTyr up to 100%.

4. Discussion

The study presented here aims to optimize a biocatalytic process for the synthesis of a potent antioxidant, HTyr, which holds tremendous potential for industry. The scale up of the PEA biotransformation to HTyr was studied in a 3 L bioreactor and different parameters were evaluated in order to gain further insight regarding factors that might hinder or favor the implementation of HTyr biosynthesis. In the course of this study, the PEA bioconversion was performed using resting (whole) cells, which enabled uncoupling of the growth and the biotransformation stages. Hence, the HTyr biosynthesis process can be divided into three partly separated stages – the growth of the cells, the biotransformation and the product recovery. Separating the stages simplifies the process and enables the optimization of each step independently. The growth stage was optimized by employing a rich buffered medium and selecting the best stage for cell harvest. Even though further optimization of the growth stage could be applied, particularly by employing large scale bioreactor growth, we have chosen to focus our efforts on scaling up the biotransformation stage.

The biotransformation stage was optimized by evaluating substrate concentration, cell density, and different operational modes (results are summarized in Table 1). Overall, the performance of the biotransformation was improved by up to an order of magnitude compared with the HTyr concentration and space time yield obtained before the process optimization (0.11 mM and 0.037 mmol/L/h, respectively), while the final yields of HTyr from PEA were similar (0.451 mol/mol obtained with the small scale biotransformation of 0.25 mM PEA). It was found that although reusing the cells is feasible (the cells remain active), their activity is dramatically decreased after the first use. In comparison, the activity of the cells following subsequent substrate addition, rather

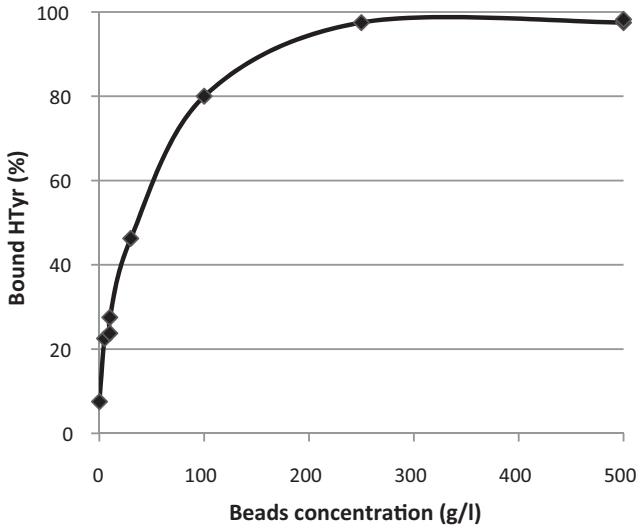

Fig. 6. Binding of HTyr as function of the boric acid gel beads concentration. The examination was carried out with aliquots of the biotransformation supernatant, containing a HTyr concentration of 0.8 mM in batch mode at pH 7.

than re-suspending the cells with fresh medium, was only slightly decreased. Therefore stepwise substrate addition was concluded to be preferable. Furthermore, comparing the results of the two fed-batch experiments revealed that a better yield is obtained by extending the duration of the biotransformation stage, rather than adding more substrate. Accordingly, for improving HTyr production, it is preferable to extend the time between substrate additions to allow the conversion of the intermediate products.

With the intention of excluding HTyr from the biotransformation bulk, while providing its protection and purification, the approach of solid-phase extraction using boric acid gel beads was employed. The results demonstrated the feasibility of the boric acid beads for HTyr recovery and the high dependence of the recovery yield on the beads concentration. Furthermore, it was seen that the percent of recovered HTyr from the beads in the elution step and its purity are oppositely dependent on the number of washing steps. In either case, a recovery yield or purity of 100% was achievable.

Use of boric acid beads was investigated by Chauhan et al. for the recovery of L-erythrulose [22]. Although the recovery yields were not addressed by the authors, calculations made out of the reported data give recovery yields of ~60%. Interestingly, the yields reported for the biotransformation with the beads (as ISPR concept) were the same and even lower than those obtained without the beads (for example, reaction yield of 87% was reported in a fed-batch reaction with ISPR, compared to 100% yield without ISPR) [22]. Nevertheless, employing the beads for the biotransformation facilitated the potential recovery of the product. Similar to our results, the stability of L-erythrulose was reported to be lower at alkaline pH (in particular at values greater than 8).

Despite the specific complex of boronate/vicinal-diol ester formation, secondary interactions can also be influential. Such secondary interactions include hydrophobic interactions which can cause nonspecific adsorption of analytes (due to the phenyl ring comprising the boronate ligand), ionic interactions (the negative charge of the active tetrahedral boronate can lead to ionic attraction or repulsion) and hydrogen bonding (through the hydroxyl groups) [23]. Moreover, the dependence of the complex formation on pH can also affect the efficiency of HTyr recovery and purification. In order to attain an efficient recovery, a stable complex between the vicinal diolgroup of the analyte (HTyr) and the boronate residues must be formed. Such complex formation was shown to be more favorable in solutions of high pH in which the boronate ion exists in higher concentrations [19]. Hence, raising the environment pH may improve the recovery and purification of HTyr thereby requiring changing the phosphate buffer to a more appropriate one. Alternatively, due to the fact that the boronate complex formation greatly depends on the ionization characteristic of the boronate ligand, substituting the aromatic ring of the phenylboronic derivatives (e.g. by introducing an electron-withdrawing group) can enable stable complex formation under more favorable pH conditions. This idea was previously applied by Dukler et al. for ISPR of a ketose product from aldose-containing medium [18].

An important issue that should be considered is whether the complete purification of HTyr is essential. In fact, as the enzyme fully consumes the substrate, the final biotransformation bulk comprises three main components: the final product (HTyr) and the intermediate products (*m*- and *p*-tyrosol). These phenolic compounds naturally present in olives [27–29] may also provide added health value. *p*-Tyrosol, also present in wine and *Rhodiola* species, has been reported to have various beneficial health properties, including antioxidant and anti-inflammatory activities [28,30–32]. In contrast with the numerous studies on *p*-tyrosol, there are only few reports regarding *m*-tyrosol, though it was shown to have bacteriostatic activity similar to that of HTyr and double that of *p*-tyrosol [33]. These beneficial properties may provide a motivation to use HTyr in combination with the phenolic derivatives, thereby reducing the purification efforts. *In vitro* and *in vivo* trails in which a mixture of HTyr with *p*- and *m*-tyrosol is evaluated in comparison to pure HTyr are needed.

5. Conclusions

To conclude, HTyr, an attractive antioxidant for cosmetic, pharmaceutical and food preparations, has to date, limited commercial availability accompanied by high prices (approximately US $50,000/kg (Cayman Chemical Co., USA)). Hence, an effective biocatalytic production of HTyr can have important industrial applications and high market potential. The current research addressed a novel and promising process for the biosynthesis of the commercially valuable HTyr from a low-cost starting material. The scale-up and optimization of the process resulted in complete substrate consumption and an overall yield of 48% HTyr. The productivity of the process was improved by an order of magnitude and the recovery and purification of HTyr from the medium was achieved using a simple solid phase extraction. In an industrial perspective, the results presented here are not yet ideal, though bearing in mind the high value of HTyr and the low cost of PEA, the market potential and cost performance aspects should also be considered. Hence, the results presented here offer potential for further development of this process at larger scale.

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