Mimicking natural metabolisms: cell-free flow preparation of dopamine

Silvia Donzella[a], Agostina Colacicco [a], Luca Nespoli [a], Martina L. Contente*[a]

To my daughter Camilla, for her birth

[a] Silvia Donzella, Agostina Colacicco, Luca Nespoli, Dr. Martina Letizia Contente
Department of Food, Environmental and Nutritional Sciences (DeFENS)
University of Milan,
via Celoria, 2, 20133 Milan (Italy)
E-mail: martina.contente@unimi.it

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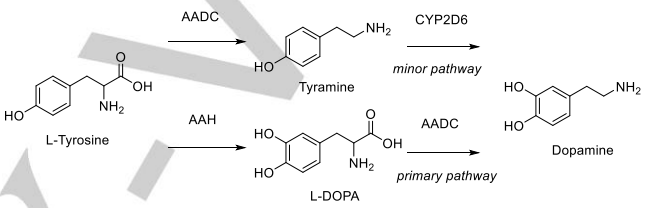
Abstract: The biosynthesis of dopamine (DA) from L-tyrosine as starting material is an excellent yet challenging strategy. Here we developed a versatile, multi-enzymatic platform for the biocatalytic preparation of DA in a continuous mode with excellent conversion (90%) and reaction time (45 min). The system exploits the immobilization of a decarboxylase from *Bacillus pumilis* (Fdc) and a tyrosinase from *Agaricus bisporus* (Tyr), which were combined to mimic the *in-vivo* synthesis of DA (both primary and secondary metabolisms) giving rise to an efficient strategy with a considerable reduction of process associated costs and environmental impact. To enhance the system automation an in-line purification *via* catch-and-release procedure was added.

Introduction

Catecholamines such as dopamine, epinephrine and norepinephrine are a major class of neurotransmitters modulating the activity of nervous system. In particular dopamine (DA) is involved in movement coordination and it is responsible for the reward-driven learning. Impairments in its synthesis and homeostasis causes several neuro-degenerative disorders such as Parkinson's disease, depression and anxiety.[1] DA precursor, levodopa (L-DOPA), is usually administered as a drug to increase DA production, especially in Parkinson's disease, where DA is not used due to its inability to cross the brain-blood-barrier. However, DA is employed in the treatment of low blood pressure and cardiac arrest.[2] It also demonstrated antioxidant and anti-inflammatory properties protecting the brain against free radical damages,[3] and preventing intestinal mucosal injuries by modulating eicosanoid production.[4]

In-vivo synthesis of DA is performed through 2 different pathways both starting from L-tyrosine as a substrate (Scheme 1): a minor one *via* tyramine and a primary metabolism *via* L-DOPA intermediate. While the first pathway consists in a 2-enzymatic step reaction with the involvement of an aromatic L-amino acid decarboxylase (AADC) followed by cytochrome 2D6 (CYP2D6) for the oxidation reaction, in the latter the generation of the catechol ring is performed through an aromatic amino acid hydroxylase (AAH) with the formation of L-DOPA which is subsequently decarboxylated *via* AADC.



Scheme 1. *In-vivo* synthesis of dopamine

Although several studies have been focused on the characterization of the single enzymes involved in the DA biosynthesis, little effort has been paid to the development of efficient enzymatic approaches for the preparation of DA from its amino acid precursor, leaving its industrial production limited to chemical synthesis.[5–7] Enzymatic cascade reactions reported so far consist in both batch and in continuous microfluidic methodologies. In the first case the transformation of L-tyrosine was performed through a tyrosine hydroxylase for L-DOPA preparation, followed by a decarboxylase for DA generation.[5] Although the successful reaction, it was performed on micro-scale biotransformation (100 µL) just for analytical purpose (LC-MS analysis). In the second example, the use of a tyrosinase as a catalyst to produce L-DOPA posed a challenge due to over-oxidation side-reactions which usually end up with the formation of melanin as a major product. Moreover, a high decrease of the enzymatic activity under continuous operations was observed (35% in 24 h, an additional 38% between 36 and 48 h).[7] The production of DA was subsequently achieved using a L-DOPA decarboxylase which demonstrated better performance and stability.[7] Similar molecules such as norpseudoepinephrine ((1*R*,2*R*)-NPE) and norepinephrine ((1*R*,2*S*)-NE) have been successfully produced at 10 mM scale *via* a one-pot two-step batch cascade starting from pyruvate. Employing a thiamine diphosphate (ThDP)-dependent acetohydroxyacid synthase I (AHAS-I) followed by (*S*)- or (*R*)-selective ω-transaminases (TAs), (1*R*,2*R*)-NPE and (1*R*,2*S*)-NE have been obtained with 85% and 80% m.c., respectively.[8]

Today a lot of chemical commodities are produced using biocatalytic approaches, either by fermentation or enzyme-mediated synthesis. These goods range from food and beverage, cleaning products, cosmetics and pharmaceuticals[9–12] leading to the development of smart and efficient strategies for process optimization and large scale production. In this context flow

biocatalysis, by combining the advantages of flow facilities (*i.e.,* better parameter control, higher mass and heat transfer, modularity), with the mild reaction conditions and the selectivity of biocatalysts (*i.e.,* water media, room temperature, chemo-, regio-, and stereoselectivity) represents an emerging technology which improves reaction productivity minimizing waste and energy consumption.[13–15] The integration of biocatalysts, both whole cells and cell-free enzymes, into continuous flow reactors usually involves immobilization techniques which not only enhance the catalyst stability but also enable its convenient isolation and reuse, thus improving the cost-efficiency of the process.[16–18] Moreover, by sequentially connecting bioreactors filled with enzymes from different sources, it is possible to create *ex-vivo* artificial metabolic pathways that do not exist in nature as well as increase the productivity of well-known biological routes for the preparation of valuable products.[19–24] When compared with a microbial machinery, optimized multi-step enzymatic reactions in flow avoid all unnecessary pathways and energy draining processes which are typical of a cell, dramatically impacting on the final overall yield.

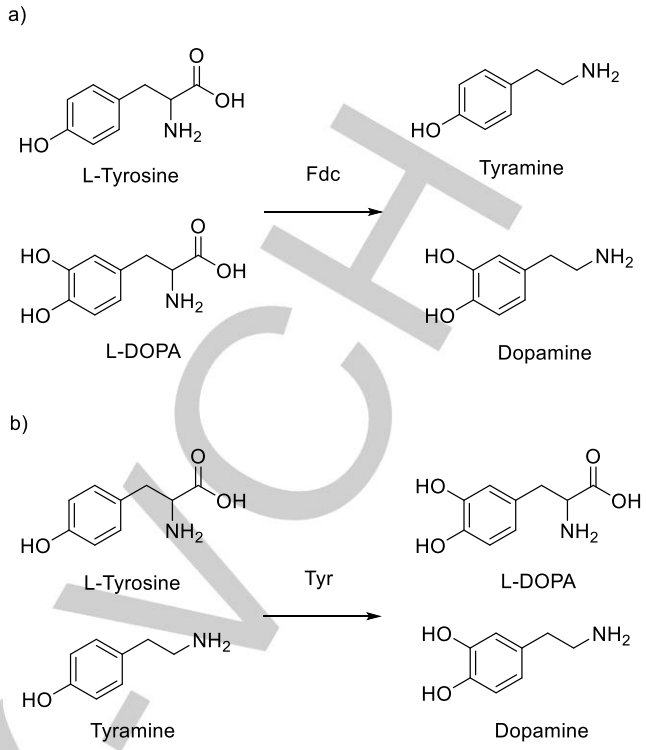
In this work flow biocatalysis has been utilized as key technique to mimic the two biological pathways for the preparation of DA. Employing different combinations of Fdc a decarboxylase from *Bacillus pumilis* and a tyrosinase from *Agaricus bisporus* (Tyr), both immobilized onto agarose beads, DA was obtained in high yields (85%) and excellent residence times (45 min).

Results and Discussion

Free enzyme batch reactions. To mimic *in-vivo* preparation of DA, single step reactions with free enzymes were firstly investigated. Fdc, a decarboxylase from *Bacillus pumilis* so far reported just for the preparation of vinyl guaiacol from ferulic acid as intermediate for vanillin synthesis, [25–27] and the commercially available tyrosinase from *Agaricus bisporus,* successfully used for the flow preparation of hydroxytyrosol from tyrosol,[21] were selected as catalysts. In particular, Fdc was here employed to decarboxylate L-tyrosine with the formation of tyramine and L-DOPA for DA preparation (Scheme 2a). In both cases complete conversion was observed after 6 h of reaction at 10 mM scale. To the best of our knowledge this is the first time this enzyme has been applied for amino acid decarboxylation, thus expanding Fdc substrate scope.

The formation of the catechol ring starting from L-tyrosine or tyramine (Scheme 2b) was here performed using Tyr, which demonstrated to be able to accept both the substrates (50%, 46% m.c. respectively, 24 h). Notably, the *in-vivo* oxidation was carried out through two different enzymes in the primary and secondary metabolism: CYP2D6 and AAH respectively (Scheme 1), whereas here was performed by the same biocatalyst. As previously reported, the addition of ascorbic acid (1.5 eq) was necessary to avoid any further oxidation side-reaction, especially the formation of reactive ortho-quinones which can covalently bond to the enzyme or autoxidize producing brown pigments.[21,28]

Immobilization of Fdc and Tyr. A key point for successful enzymatic systems, especially for industrial applications, is the robustness and longevity of the catalyst, which can be dramatically enhanced by immobilization.[16,17]



Scheme 2. Batch biotransformations with a) Fdc or b) Tyr. Reaction conditions: 10 mM or 20 mM substrate, phosphate buffer 100 mM, pH 7.0, 1 mg/mL free enzyme or 50 mg (enzyme loading: 1 mg/g_{matrix}), T = 28 °C. For Tyr reactions 1.5 eq. of ascorbic acid was added.

In order to achieve durable catalysts to be used specifically at high flow rate for scalable productivity, a strategy involving covalent bonds between the enzymes and the matrix has been selected. Among the available supports, hydrophilic agarose beads previously functionalized with aldehyde groups (*i.e.,* glyoxyl-agarose) have been selected, thus guarantying excellent immobilization yield (>99% for both the proteins) and very good retained activity for Fdc and Tyr (50% and 48%, respectively) when immobilized with an enzymatic loading of 1 mg_{enzyme}/g_{matrix}).[18,29] Batch reactions to test the catalytic performance of agarose immobilized enzymes (Imm-Fdc and Imm-Tyr) were carried out in phosphate buffer pH 7.0. The substrate concentration was fixed at 20 mM, observing maximum conversions between 75 and 100% for oxidation (24 h) and decarboxylation (5 h) reactions respectively (Table 1). It is of note that the immobilized system afforded much higher activity and substrate tolerance than the free enzyme: in fact, the enzyme concentration (0.05 mg/mL) required for this level of reactivity was 20-times lower than for free Fdc and Tyr (1 mg/mL) even if a 2-fold higher substrate concentration was used.

Table 1. Comparison between batch reactions with free and immobilized enzymes

Entry	Enzyme[a]	Substrate	Substrate (mM)	m.c . (%)[b]	Time (h)
1	Free-Fdc	L-Tyrosine	10	>99	6
2	Imm-Fdc	L-Tyrosine	20	>99	5

3	Free-Fdc	L-DOPA	10	>99	6
4	Imm-Fdc	L-DOPA	20	>99	5
5	Free-Tyr	L-Tyrosine	10	50	24
6	Imm-Tyr	L-Tyrosine	20	75	24
7	Free-Tyr	Tyramine	10	46	24
8	Imm-Tyr	Tyramine	20	70	24

Reactions conditions: 10 or 20 mM substrate, 28 °C, phosphate buffer (100 mM, pH 7.0). For oxidation reaction 1.5 eq of ascorbic acid have been added. [a] Catalyst concentration: 1 mg/mL for free enzymes, 50 mg/mL for immobilized ones (enzyme loading: 1 mg/g_{matrix}). [b] m.c.: molar conversion. For analytical details see Supplementary Information.

Previously attempts to immobilize Fdc mainly rely on the use anion-exchange carriers (*i.e.,* Sepabeads EC-EA, Sepabeads EC-Q1A, Diaion HPA25L, and Diaion WA21J) thus creating an ionic bonding between the catalyst and matrix. Due to the lability of this type of bond, especially at high flow rates and pressurized systems, a covalent bond-based immobilization was preferred. Moreover high amount of immobilized catalyst was necessary for the ferulic acid-to-4-vinyl guaiacol transformation (5 mg_{enzyme}/50 mg_{resin},1 mL reaction) at 10 mM scale.[30] Examples of tyrosinase immobilization on copolymer matrices,[31] carbon nanotube,[32] gold nanoparticles,[33] alumina,[33] chitosan[34], alginate, polyacrylamide, and gelatine gels[35] have been reported in many industrial applications such as phenol removal from waste water[36] and preparation of bioactive compounds.[37] However, some of these methods are rather complicated and do not give good enzyme stability or retention of the desired activity as observed for the covalent glyoxyl-agarose immobilization here proposed. Possibly due to the characteristic multi-point attachment of the covalent enzyme-to-matrix connection and the consequent protein structural stabilization,[38] 40-times more active biocatalysts were obtained. This peculiar behavior has been previously observed by us working with an acyl-transferase from *Mycobacterium smegmatis* which was immobilized employing the same strategy here exploited and aldehyde-agarose as support.[29]

Single step reaction optimization. Before the connection of different bioreactors for DA obtainment, the single reactions were optimized under flow conditions. Different residence times were tested for both decarboxylation and oxidation biotransformations (Table 2), employing L-tyrosine as substrate. The best enzymatic performance was obtained after 15 min of residence time employing imm-Fdc (Entry 2, Table 2), while 30 min were necessary for imm-Tyr (Entry 5, Table 2). For this latter step an inlet of air forming an air/liquid biphasic system guarantying the oxygen supply was required, while the addition of 1.5 eq ascorbic acid avoided the formation of over-oxidized by-products, resulting in clean reactions. Notably, the same bioreactors have been used for all the experiments without any loss of activity. The enhanced catalytic performance obtained under flow mode (*i.e.*, reduction of the reaction times, improved yields) can be explained considering the efficient mixing and mass transfer in the biphasic flow stream, better control of the reaction parameters (*i.e.,* temperature, residence time) thus circumventing some critical issues typical of batch biotransformations.

Table 2. Optimization of the reaction parameters

Entry	Enzyme	Rt (min)[a]	m.c. (%)[b]
1	Fdc	7	75
2	Fdc	15	>99
3	Tyr	7	40
4	Tyr	15	75
5	Tyr	30	88

Reactions conditions: 20 mM substrate, 28 °C, phosphate buffer (100 mM, pH 7.0). For oxidation reaction 1.5 eq of ascorbic acid have been added. Immobilized enzyme loading: 1 mg/g_{matrix}. [a] rt: residence time. [b] m.c.: molar conversion. For analytical details see Supplementary Information.

Artificial metabolisms for DA preparation. Mimicking of cellular enzymatic cascades can be achieved through the assembly of multi-step enzymatic reactions in a continuous flow bioreactor, which also significantly increases the reaction efficiency and sustainability of the process.[11,23] Challenges involved in setting up multi-enzymatic processes in flow mode include the number of reaction steps, cost of the biocatalysts, residence times, solvent compatibility of the different steps, need for intermediate work-up.[13–15] Here, both the minor metabolism (Scheme 3, A) and the primary pathway (Scheme 3, B) were successfully developed *ex-vivo* by combining Fdc and Tyr enzymes in different ways. In the first case L-tyrosine (20 mM, 113 µL/min as flow rate) was decarboxylated by imm-Fdc (1 mg/$g_{matrix,}$ 1.7 mL reactor volume) obtaining tyramine as intermediate with full conversion. Subsequently air and ascorbic acid (113 µL/min as total flow rate) were added to the flow stream in order to produce DA (90% m.c., 45 min rt) employing imm-Tyr (1 mg/$g_{matrix,}$ 6.8 mL reactor volume). In the second cascade L-tyrosine was firstly oxidized by imm-Tyr (113 µL/min as total flow rate, 3.4 mL reactor volume) producing L-DOPA (88% m.c.), which was then decarboxylated through imm-Fdc (113 µL/min as flow rate, 1.7 mL reactor volume) to obtain DA (>99% m.c., 45 min rt). In both cases an in-line purification step was performed employing a catch-and-release procedure, thus enhancing the system automation while reducing the manual handling operations. This strategy involves supported boronic acid able to trap DA (Scheme 3, A) and L-DOPA (Scheme 3, B) forming a cyclic borate with their catechol group, leaving the unreacted substrates (tyramine or L-tyrosine) and the excess of ascorbic acid in the exiting flow stream. DA and L-DOPA were then released using 1 N HCl (Step 2, Scheme 3). Before the biotransformation of L-DOPA into DA (Scheme 3, B) NaOH was added to the solution to restore pH 7.0. After the collection of 50 mL (7 hours of continuous operation) 130 and 123 mg of pure DA were collected from the cascade A and B respectively (Table 3).

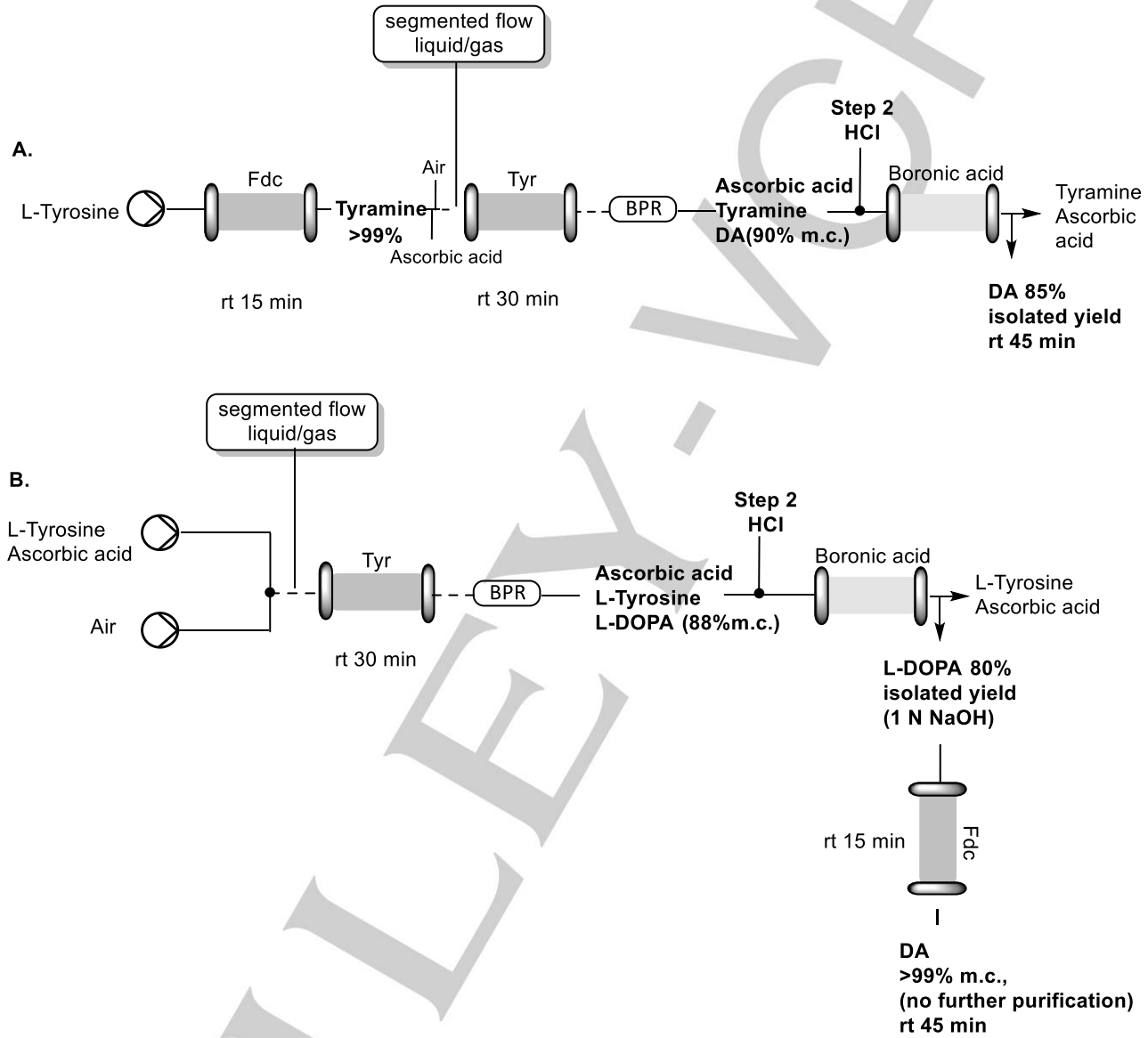
Table 3. Continuous preparation of dopamine (20 mM) in flow reactor.

Entry	Cascade[a]	DA isolated yield (mg)[b]	Space time yield (mg d^{-1})[c]

1	A	130	446
2	B	123	422

[a] Referring to Scheme 3
[b] Considering 7 h (50 mL)
[c] Considering 24 h

mushroom tyrosinase (Tyr) and a *Bacillus pumilis* decarboxylase (Fdc). Starting from the commercially available and cheap L-tyrosine, DA was successfully prepared in 45 min of residence time. After the collection of 50 mL (7 h of continuous operation) 130 and 123 mg of pure DA have been collected respectively from cascade A and B. This methodology, avoiding tedious intermediate work-up and purification steps, allowed for a



Scheme 3. Flow synthesis of DA mimicking the *in-vivo* minor metabolism (A) and the primary pathway (B). Reaction conditions: 20 mM substrate, 1.5 eq ascorbic acid. The solutions were prepared in 100 mM phosphate buffer pH 7.0. Immobilized enzyme loading: 1 mg/g_{matrix} BPR: 40 psi. rt: residence time.

Conclusion

Within the scope of Green Chemistry, a minimization of waste deriving from chemical reactions needs to be taken into account, as well as cost-efficient processes compatible with the time scale industry. To fit these purposes, a flow preparation of DA, mimicking the *in-vivo* metabolisms (minor and major pathways) was developed using different combinations of a commercial considerable reduction of process associated costs and environmental impact. The immobilization technique here employed exploits a covalent bond between the catalysts and glyoxyl-agarose (enzyme loading 1 mg/$g_{agarose}$, immobilization yield >99%, retained activity for Fdc 50% and for Tyr 48%) to ensure the reuse of the enzymes and enhance their stability in the operational conditions. The design of programmed routes for the *ex-vivo* synthesis of valuable molecules merging the versatility of flow reactors with the specificity of biocatalysts, goes with no doubts beyond academic development, contributing favorably to advances in modern chemistry as well as the promotion of flow biocatalysis as a robust and reliable technique to address industrial needs.

Experimental Section

Chemicals

Reagents and solvents were obtained from commercial suppliers and were used without any further purification. Flow biotransformations were performed using a R-series modular flow chemistry system (RS-200) purchased from Vapourtec equipped with HPLC pumps, R-series software, fraction collector and Omnifit glass columns (6.6 mm internal diameter (i.d.) × 100 mm length or 10 mm i.d. × 150 mm length). Pressure was controlled by using backpressure regulators. TLC analyses were performed on commercial silica gel 60 F254 aluminium plates; L-tyrosine, mushroom tyrosinase from *Agaricus bisporus* (8503 U/mg), ascorbic acid, polymer-supported boronic acid and reference products were purchased from Merck (Merck KGaA, Darmstadt, Germania). Plain agarose (6BCL) was purchased from Agarose Bead Technologies (ABT), Madrid, Spain.

Cloning, overexpression, purification and activity assay of Fdc

Fdc protein expression, purification, and free enzyme activity measurements (10 U/mg) were performed following previously reported protocols.[26,39] One unit (U) of activity is defined as the amount of enzyme which catalyzes the consumption of 1 µmol of ferulic acid per minute (See Supplementary Information). Protein concentration was determined by Bradford assay by measuring the absorbance at 595 nm of a solution containing the correct amount of protein sample opportunely diluted in water with 1 mL of Bradford reagent. A standard curve was built with BSA protein.

Activity assay of Tyr

Activity measurements were performed spectrophotometrically at 475 nm by determining the formation of red-colored dopachrome at 25 °C in a half-microcuvette (total volume 1 mL) for 3 min. One unit (U) of activity is defined as the amount of enzyme which catalyzes the consumption of 1 µmol of L-DOPA per minute under reference conditions, namely 2 µL Tyr (5 U/mL), 333 µL L-DOPA (2 mg/mL), in 665 µL of 100 mM phosphate buffer pH 7.0. The specific activity resulted 82 U/mg.

Aldehyde agarose immobilization

Aldehyde agarose immobilization was performed as previously described.[18] Briefly, 1 g of epoxy-agarose was incubated with 10 mL of 100 mM H_2SO_4 overnight under orbital shaking. Then, the support was filtered and washed 5 times with 10 volumes of H_2O. The resulting glyceryl-support was oxidized with 10 mL of 30 mM $NaIO_4$ for 2 h under orbital shaking. Finally, the glyoxyl-support was washed 5 times with H_2O and stored at 4 °C until use.

For the enzyme immobilization, 10 mL of enzyme at the desired concentration in 100 mM sodium bicarbonate buffer at pH 10.0 were mixed with 1 g of glyoxyl-support and incubated under orbital shaking for 3 hours. Then, the suspension was filtered and the immobilization yield (%) was calculated by measuring the remaining activity in the flow-through. The immobilized enzyme was incubated with 20 mM piperidine-borane to reduce the imine bonds between the enzyme and the aldehyde groups on the support. The immobilized enzyme was washed with the appropriate buffer for its storage.

Batch reactions

Batch reactions using free Fdc and Tyr or imm-Fdc and imm-Tyr were performed in 10 mL screw cap tubes; 1 mL reaction mixture in 100 mM phosphate buffer pH 7.0, containing 10 or 20 mM substrate, 1 mg/mL enzyme for free enzymes or 50 mg/mL for imm-enzymes (1 mg/g_{matrix}) and 1.5 eq. of ascorbic acid for oxidation reactions were left under gently shaking at 28 °C. 50 µL aliquots were quenched with hydrochloric acid (HCl) 0.2% every hour and analyzed *via* HPLC (See Supplementary Information).

HPLC analysis

See Supplementary Information.

Single-step flow optimization

A solution of L-tyrosine (20 mM) or L-tyrosine (20 mM) and ascorbic acid (1.5 eq.) were prepared in phosphate buffer pH 7.0 and pumped through the reactors *via* HPLC pumps. In the case of oxidation reactions, the substrate solution and air were mixed in a T-tube in order to generate a liquid/gas segmented flow. The solution/biphasic flow was pumped through the column reactor filled with imm-Fdc or imm-Tyr (1 g, 1 mg/$g_{matrix,}$ 1.2 mL reactor volume). For oxidation reactions a BPR (40 psi) was applied to the system to keep a constant air/liquid segmented flow. The flow rate was varied and optimized depending on the desired residence time. A total flow rate of 80 µL/min or 40 µL/min was employed to obtain 15 or 30 min rt, respectively. The outlet flow was collected and analyzed through HPLC (See Supplementary Information).

Ex-vivo flow DA preparation

Continuous tandem flow biotransformations were performed using two Omnifit glass columns (6.6 mm i.d. × 100 mm length or 10 mm i.d. × 150 mm length) in line filled with 1 g of imm-Fdc (1.7 mL) and 2 or 4 g of imm-Tyr (3.4 or 6.8 mL), respectively. Solutions of L-tyrosine (20 mM) or L-tyrosine (20 mM) and ascorbic acid (1.5 eq.) were prepared in phosphate buffer 100 mM pH 7.0. In the case of oxidation reactions, the substrate solution and air were mixed in a T-tube to generate a liquid/gas segmented flow. The resulting flow streams were directed into the columns packed with the biocatalysts. The flow rate was varied and optimized. The flow streams were in-line purified following the catch-and-release procedure described below. The reactions were analyzed by HPLC using the conditions reported in Supplementary Information and the products collected for NMR analysis, see below.

Catch-and-release procedure

The in-line purification was performed as previously reported.[21] Briefly, the flow streams containing the unreacted substrates and the desired catechol compounds were flowed through a column packed with polymer-supported boronic acid (1.0 g, 1.4-2.2 mmol/g loading), 80 °C, BPR 1.0 bar, thus trapping DA or L-DOPA. The release of catechol products was performed by flowing 1 N HCl through the column at room temperature with a residence time of 30 min.

NMR spectra

NMR spectra were recorded on a Brucker Avance 600 MHz spectrometer employing the residual signal of the deuterated solvent as internal standard. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz (Hz). The solution containing tyramine or DA were basified (1 N NaOH) and extracted with ethyl acetate, while L-DOPA freeze-dried (BAXIT freeze dryer BXT-FD-12N) before NMR analysis (See Supplementary Information).

Acknowledgements ((optional))

Acknowledgements Text.

Keywords: Dopamine preparation • Flow biocatalysis • Metabolic pathways • decarboxylase from *Bacillus pumilis* • tyrosinase from *Agaricus bisporus.*

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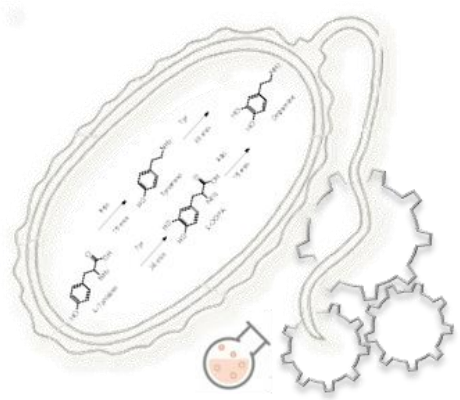

In this work dopamine *in-vivo* metabolism was reproduced employing a combination of immobilized enzymes integrated in a flow chemistry reactor. A decarboxylase from *Bacillus pumilis* and a tyrosinase from *Agaricus bisporus* have been selected for their cofactor-independency. The efficient *ex-vivo* cascade allowed for dopamine biosynthesis with excellent yields (85%) and rapid reaction times (45 min).

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