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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Tyrosinase-based production of L-DOPA by *Corynebacterium glutamicum*

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Abstract

An increase in the number of elderly people suffering from the symptoms of Parkinson's disease is leading to an expansion in the market size of 3,4-dihydroxyphenyl-L-alanine (L-DOPA), which is the most commonly used drug for the treatment of this disease. Need for better quality products through economically feasible and sustainable processes makes biotechnological approaches attractive. The current study is focused on heterologous expression of *Ralstonia solanacearum* tyrosinase in *Corynebacterium glutamicum* cells to produce L-DOPA during growth on glucose or glucose/xylose mixtures. Whole-cells pre-grown on glucose were further exploited for biotransformation of L-tyrosine to L-DOPA. To prevent L-DOPA oxidation, not only the most commonly used agent, ascorbic acid, but also for the first time, thymol was evaluated. The highest L-DOPA titer was 0.26 ± 0.02 g/L at the end of growth on a mixture of 1% xylose and 3% glucose in the presence of 200 µM thymol as the oxidation inhibitor. The ability to co-utilize glucose and xylose to reach this titer could make these cells ideal for L-DOPA production using hydrolyzed lignocellulosic biomass. When the pre-grown cells were further used for biotransformation, the highest L-DOPA yield was 0.61 ± 0.02 g/gDCW with 4 mM ascorbic acid. Since L-tyrosine biotransformation is primarily dependent on tyrosinase activity, yield in this route could be improved by optimizing reaction conditions. As the industrial workhorse for amino acid production, these *C. glutamicum* cells will clearly benefit from strain development efforts and bioprocess optimization towards sustainable and economically feasible L-DOPA production.

Key points

- *Fermentative L-DOPA production was achieved in C. glutamicum.*
- *Tyrosinase produced by C. glutamicum cells successfully transformed L-Tyr.*
- *Thymol proved to be a significant oxidation inhibitor for L-DOPA production.*

Keywords *Corynebacterium glutamicum* · L-DOPA · Tyrosinase · Ascorbic acid · Thymol

Introduction

Rational design of microbial cells for the biosynthesis of compounds of pharmaceutical significance including drugs, drug-like molecules, or even drug precursors/intermediates is receiving increasing attention in biotechnology (Krämer et al. 2003; Camacho-Zaragoza et al. 2016; Li et al. 2018). Biotechnological synthesis routes offer the advantages of avoiding organic solvents, heavy metal catalysts, and strong acid/base solutions that are commonly required in synthetic chemistry-based approaches (Jeandet et al. 2013). Furthermore, by nature, when using a microbial cell, synthesis can usually be achieved under mild temperatures and atmospheric pressure. To this end, several platform strains have also been developed for the production of a wide range of molecules (Wendisch et al. 2006; Huang et al. 2014; Rodriguez et al. 2015; Kallscheuer et al. 2016; Matsumoto et al. 2017).

3,4-Dihydroxyphenyl-L-alanine (L-DOPA) is an amino acid that is used primarily for the treatment of Parkinson's disease. As the number of elderly people suffering from the symptoms of Parkinson's disease grows, the market size for L-DOPA is expanding (Koyanagi et al. 2005). Its current commercial production is mostly based on asymmetric

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hydrogenation, which suffers from poor conversion rates and low enantioselectivity (Knowles 2004). The complicated reaction scheme and harsh reaction conditions make chemical L-DOPA synthesis rather unattractive.

The alternative biotechnological approach relies on the activity of tyrosine phenol-lyase, tyrosinase, or 4-hydroxyphenylacetate 3-monooxygenase (Min et al. 2015). All these three enzymes have their discrete drawbacks; thus, each should be considered along with its advantages for the system in which it is used. Tyrosine phenol-lyase (EC 4.1.99.2) catalyzes the production of L-DOPA from pyruvate, ammonia, and catechol (Koyanagi et al. 2005). The requirement for the toxic precursor catechol would be the major disadvantage of this enzyme. On the other hand, tyrosinase and *p*-hydroxyphenylacetate 3-hydroxylase both use L-tyrosine as the precursor. Tyrosinase (EC 1.14.18.1) is a copper-containing oxidoreductase with two distinct activities: the cresolase activity that catalyzes the *ortho*-hydroxylation of L-tyrosine to L-DOPA and catecholase activity that catalyzes the oxidation of L-DOPA to L-dopaquinone (Min et al. 2015). L-Dopaquinone is further converted to melanin through a non-enzymatic route (Fig. 1). Unfortunately, catecholase activity reduces L-DOPA titer. Furthermore, some enzymes may also require unique adaptor proteins which makes it challenging to express active tyrosinases in different hosts (Garcia-Borron and Solano 2002; Min et al. 2010). *p*-Hydroxyphenylacetate 3-hydroxylase (EC 1.14.14.9) is a $FADH_2$-dependent monooxygenase, which normally catalyzes the regioselective hydroxylation of *p*-hydroxyphenylacetate at the C-3 position to yield 3,4-dihydroxyphenylacetate (Lee and Xun 1998). As a consequence of its broad substrate spectrum, it has also been found to hydroxylate L-tyrosine to L-DOPA. However, its need for NADH as a cofactor necessitates continuous reducing power for its regeneration (Wei et al. 2016).

Currently, the only commercialized biotechnological process is an in vitro enzymatic conversion that uses the tyrosine phenol-lyase synthesized by *Erwinia herbicola* (Iizumi et al. 1991). Recent studies show that a biotechnological process involving microbial fermentation to achieve in vivo L-DOPA synthesis is a favorable approach to improve the conversion rate and economize the process (Min et al. 2015; Wei et al. 2016; Fordjour et al. 2019). Thus, the focus here will be the construction of a microbial cell to produce L-DOPA with a tyrosinase enzyme to circumvent toxic catechol utilization and to enable production from lignocellulosic biomass. The constructed cells will be suitable for an economically feasible and sustainable process.

Tyrosinase enzymes, which are widely distributed in both microbial and plant sources, usually require unique cofactors and/or adaptor proteins. Cofactors, especially, *o*-diphenol derivatives, such as L-DOPA and (+)-catechin may be essential for initial activity (Zaidi et al. 2014). For this reason, tyrosinase-based microbial L-DOPA synthesis has been primarily limited to natural tyrosinase producers (Ali et al. 2005, 2007; Krishnaveni et al. 2009; Surwase and Jadhav 2011; Surwase et al. 2012; Patil et al. 2013). The catecholase activity of the enzyme is a critical parameter in optimizing L-DOPA biosynthesis, since it lowers titers by oxidizing L-DOPA. The tyrosinase from the Gram-negative plant pathogen *Ralstonia solanacearum* has been shown to possess an exceptionally high ratio of cresolase/catecholase (oxidation of L-DOPA to L-dopaquinone) activities. Furthermore, its activity does not depend on the presence of a cofactor, e.g., L-DOPA, to eliminate the characteristic lag period of tyrosinases. These properties, which are rare among other tyrosinases, increase its potential to be used in L-DOPA synthesis

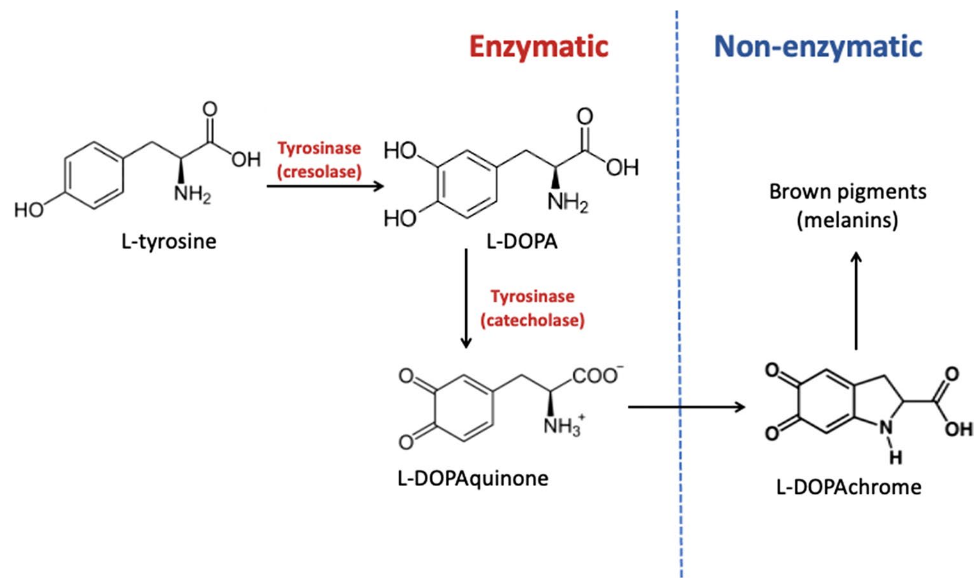

Fig. 1 Conversion of L-Tyr to L-DOPA and its downstream metabolites

(Hernández-Romero et al. 2006). Indeed, Nakagawa et al. (2011) reported L-DOPA production by *Escherichia coli* with this tyrosinase.

The robustness and ease in handling have made *E. coli* a commonly preferred platform strain. However, phage contamination is a major issue with *E. coli*, which is difficult and costly to overcome on industrial scale production (Wegrzyn et al. 2006). In this regard, *Corynebacterium glutamicum*, the industrial workhorse for amino acid production (Wendisch 2020), may also serve as a suitable alternative host for the biosynthesis of the amino acid L-DOPA. The major advantages of production with *C. glutamicum* can be summarized as: (i) it grows well in cost-effective minimal media, (ii) it grows fast and reaches high cell densities, (iii) it is genetically stable, (iv) it is free of endotoxins, and on top of all, (v) it has been used for safe production of generally recognized as safe (GRAS) status food ingredients (Lee et al. 2016; Nutrition C for FS and A. 2019; Li et al. 2020). A number of aromatic compounds have been produced by engineered *C. glutamicum* (Kallscheuer et al. 2019; Wendisch and Lee 2020).

The primary goal of the present work was to achieve L-DOPA production in *C. glutamicum* using the *R. solanacearum* tyrosinase (Hernández-Romero et al. 2006). For this production, not only the most preferred carbon source, glucose, but also the ability of this bacterium to co-utilize mixed carbon sources (Wendisch et al. 2000), specifically glucose and xylose, a major component of lignocelluloses, were exploited. Although *C. glutamicum* cells cannot naturally utilize xylose, upon heterologous expression of *xylA* and *xylB* genes, xylose is converted to D-xylulose-5-phosphate and fed to the pentose phosphate pathway (Meiswinkel et al. 2013). The whole-cells grown on glucose were further used for whole-cell biotransformation of L-tyrosine to L-DOPA. The second goal of this study was to inhibit the catecholase activity of the employed tyrosinase to prevent rapid oxidation of L-DOPA to dopachrome, which is a major drawback that leads to reduced L-DOPA titers. For this purpose, the potential of ascorbic acid (Ros et al. 1993) and thymol (Zolghadri et al. 2019) to prevent oxidation and increase final titer was compared and evaluated.

Materials and methods

Bacterial strains, plasmids, and reagents

C. glutamicum ATCC 13,032 was obtained from American Type Culture Collection. The shuttle vector pEKEx2 was a kind gift from B.J. Eikmanns. All strains and plasmids used in this work are summarized in Table 1. The codon optimized tyrosinase gene was synthesized by GenScript Biotech (NJ, USA) and its sequence has been deposited in GenBank under the accession number Seq1 MZ711563. Phusion High-Fidelity DNA Polymerase was purchased from Thermo Scientific (MA, USA), and restriction enzymes *Eco*RI and *Pst*I were from Promega (WI, USA). DNA ligase was supplied by Fermentas. Oligonucleotide primers were purchased from MedSanTek (Istanbul, Turkey). Ascorbic acid was purchased from Balmumcu Kimya (Istanbul, Turkey). Brain heart infusion (BHI) broth was from Biolife (Milan, Italy) and all other chemicals, including L-DOPA and L-tyrosine, were from Sigma-Aldrich (USA).

Construction of L-DOPA producer *C. glutamicum* cells

The codon optimized tyrosinase gene from *R. solanacearum* on pUC57 was amplified using the primer pairs tyrF (**5′-**AATTCTGCAGAAGGAGAATCAT**ATG**GTCGTTCGTCGC-**3′**) and tyrR (**5′-**CAGTGAATTCCTAAATAACCGCAACTTCAATAGATTCAGG-**3′**). The ribosome binding site is underlined and the start codon is given in bold on the forward primer. The PCR product was digested with *Eco*RI and *Pst*I and cloned into plasmid pEKEx2 with the ribosome

Table 1 Strains and plasmids used in the study

Name of plasmid or cell	Description	Reference
pUC57-tyr_{Rs}	Amp^r; *E. coli* cloning vector with *R. solanacearum* tyrosinase gene	GenScript
pEKEx2	Kan^r; *C. glutamicum-E. coli* shuttle vector for regulated gene expression; *Ptac*, $lacI^q$ pBL1 $oriV_{Cg}$ pUC18 $oriV_{Ec}$	(Eikmanns et al. 1991)
pEKEx2-tyr_{Rs}	pEKEx2 harboring *tyrosinase* from *R. solanacearum*	This study
pEKEx3	Spec^R; *C. glutamicum/E. coli* shuttle vector (*Ptac*, $lacI^q$; pBL1, $OriV_{Cg}$, $OriV_{Ec}$)	(Stansen et al. 2005)
pEKEx3-$xylA_{Xc}$-$xylB_{Cg}$	pEKEx3 harboring $xylA_{Xc}$ from *Xanthomonas campestris* and $xylB_{Cg}$ from *C. glutamicum*	(Meiswinkel et al. 2013)
E. coli TOP 10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen, USA
C. glutamicum ATCC 13,032	Biotin auxotroph, wild-type strain	(Kinoshita et al. 1957)
CgEKV-I	Wild-type cells harboring pEKEx2-tyr_{Rs}	This study
CgEKV-II	*CgEKV-I* cells harboring pEKEx3-$xylA_{Xc}$-$xylB_{Cg}$	This study

binding site using standard cloning protocols (Sambrook et al. 1989). The constructed plasmid was transformed using the $CaCl_2$ method into *E. coli* T10 for screening (Seidman et al. 2001). The gene was verified by DNA sequencing. The pEKEx3-*xylA*Xc-*xylB*Cg plasmid with the xylose utilization genes has been constructed in a previous work (Meiswinkel et al. 2013). The selected plasmids were transformed into *C. glutamicum* cells by electroporation (Li et al. 2010).

L-DOPA production during microbial growth

C. glutamicum cells were routinely grown aerobically at 30 °C in BHI for multiplication and maintenance. Standard CGXII defined medium that contains 4% glucose as the sole carbon and energy source for production was used as the basic production medium (Keilhauer et al. 1993). When needed, growth medium was supplemented with 25 µg/mL kanamycin and/or 100 µg/mL spectinomycin. CGXII medium was supplemented with copper sulfate pentahydrate (0–2 mM) to activate the tyrosinase and with tyrosine (1 mg/mL) to achieve L-DOPA synthesis. Then, 100 mM stock thymol solution prepared in dimethyl sulfoxide (DMSO) or 500 mM stock ascorbic acid solution prepared in water was added at different final concentrations to prevent L-DOPA oxidation. The maximum DMSO concentration (1%) was below the amount that inhibited cell growth.

C. glutamicum CgEKV-I and CgEKV-II cells were used for L-DOPA synthesis during growth on glucose and mixed sugars, respectively. Cells were grown for 8 h in test tubes with 5 mL BHI medium on a rotary shaker at 200 rpm as the first preculture. Two milliliters from this preculture was used to inoculate 50 mL of standard CGXII medium in 500-mL baffled Erlenmeyer flasks. These cells were cultivated overnight in a rotary shaker at 30 °C and 150 rpm. Using this overnight culture, the main production culture of 50 mL CGXII medium was inoculated to an optical density of 4.0 at 600 nm. Heterologous gene expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside 1 h after inoculation. Bacterial growth during L-DOPA synthesis was monitored by measuring absorbance at 600 nm.

To investigate the utilization of xylose, keeping the total carbon source concentration at 4%, glucose concentration was gradually decreased as xylose concentration was increased.

L-DOPA production via biotransformation

C. glutamicum CgEKV-I cells that have synthesized L-DOPA during growth were further used for a biotransformation reactions. Cell pellets were removed from fermentation broth by centrifugation for 10 min at 10,000 rpm and 4 °C. After washing twice with distilled water, cells were resuspended in 10 mL sterile distilled water. To a 15-mL falcon tube containing 10 mL of water with 1 mg/mL L-tyrosine, 50 µL of cell resuspension (final 0.66 mgDCW/mL) was added and the tubes were incubated at 30 °C. The broth was tested for L-DOPA production after 48 h. To preserve tyrosinase activity, couple of drops of chloroform was added to each sample. The same thymol and ascorbic acid stock solutions prepared for fermentative synthesis were used to prevent L-DOPA oxidation during biotransformation.

L-DOPA detection with Arnow's test

Arnow's test for L-DOPA detection is based on the fact that L-DOPA gives a yellow color when mixed with nitrous acid; this color changes to dark orange when found in excess of sodium hydroxide (Arnow 1937). This test was used only for preliminary detection of L-DOPA. Melanin synthesis interfered with the test; therefore, Arnow's test was not suitable for quantification.

The cell-free supernatants from the fermentation broth or from the biotransformation reactions were used for the test. To detect L-DOPA, 1 mL of supernatant was mixed with 1 mL 0.5 M HCl, 1 mL nitrite-molybdate reagent, 1 mL 1 M NaOH, and 1 mL dH_2O. Then, absorbance at 530 nm was measured. L-DOPA in the supernatant was estimated using a calibration curve prepared with standard L-DOPA.

High-performance liquid chromatography for L-DOPA

High-performance liquid chromatography (HPLC) analysis was carried out using an Agilent 1100 system with C18 Zorbax column (250 × 4.6 mm, 5 µm) and a UV detector at 280 nm. 0.1 N acetic acid–methanol (10:1) mobile phase (Yang et al. 2001) with a flow rate adjusted to 1.2 mL/min for 10 min at 30 °C was used (Surwase et al. 2012). Injection volume was 20 µL. Standard L-DOPA (0.01–1.5 mg/mL) and L-tyrosine (0.02–1.5 mg/mL) were prepared in HPLC-grade water and cell-free supernatants were used for analysis. The retention time obtained was ~ 2.7 min for the standard L-DOPA and ~ 3.5 min for the standard L-tyrosine.

Results

L-DOPA production by fermentation from simple sugars

R. solanacearum tyrosinase gene (Hernández-Romero et al. 2006) has been successfully introduced into *C. glutamicum* to produce L-DOPA from L-tyrosine. Tyrosinases are copper-containing enzymes; thus, L-DOPA synthesis depends on copper ions. To this end, initial effort has focused on optimizing the concentration of copper ions for production. First

CgEKV-I cells grown in the presence of different concentrations of copper ions were examined for growth (Fig. 2a) and then growing cells were screened for L-DOPA production using Arnow's test. When glucose was the sole carbon and energy source, there was no detectable L-DOPA synthesis in the absence of the copper ions. Then, the copper ion concentration was gradually increased to find the most favorable amount for production. Maximum L-DOPA titer was detected with 0.4 mM copper ions. The titer with 0.2 mM copper ions was approximately 60% lower than that obtained with 0.4 mM. The presence of 0.8 mM significantly retarded growth, while higher copper ion concentrations totally inhibited growth and abrogated L-DOPA synthesis (Figure S1). With this finding, 0.4 mM copper sulfate was selected as the optimal concentration for further experiments (Figure S2).

When tyrosinase was expressed by *C. glutamicum* cells, the color of the culture broth gradually got darker (Figure S3). This is a strong indication of formation of L-DOPA oxidation products (Figure S4). Different approaches have been pursued to tackle this issue. Here, the effect of two oxidation inhibitors, ascorbic acid and thymol, was evaluated to prevent L-DOPA degradation in order to increase the final titer. While the presence of low concentrations of ascorbic acid (0.1, 1, 2, and 4 mM) showed no effect on L-DOPA titers, the presence of high concentrations (50 mM) completely inhibited bacterial growth (Fig. 2b). Titer obtained with low ascorbic acid concentrations was 0.027 ± 0.009 g/L, quite similar to titer obtained without any L-DOPA oxidation inhibitors. Therefore, no further optimization with ascorbic acid was carried out. To this end, only different concentrations of thymol were tested to reduce L-DOPA oxidation. After determination of the allowable thymol concentrations (Fig. 2c), production was carried out with CgEKV-I and CgEKV-II cells during growth on glucose, xylose, and their mixtures w/o thymol (Table 2). L-DOPA titers at the end of growth on 4% glucose in the two cells were comparable at ~ 0.03 g/L without thymol addition. When CgEKV-II cells were grown on a mixture of 3% glucose and 1% xylose, titer was only slightly lower. Increasing the xylose concentration to 2% abolished L-DOPA synthesis.

In general, thymol had a favorable effect in preventing L-DOPA oxidation which consequently increased titer, but its concentration should be carefully adjusted. During growth of CgEKV-I cells on glucose, the effect of 100 µM thymol was negligible. As thymol concentration increased to 200 and 400 µM, L-DOPA titer increased 2- and 3.7-fold, respectively, when compared to the condition with no thymol. No L-DOPA accumulated with 600 µM thymol and 1 mM thymol completely inhibited growth (Fig. 2c).

Table 2 L-DOPA production during microbial growth. All cultures were supplemented with 0.4 mM $CuSO_4 \cdot 5H_2O$

Strain	C-sourse	Thymol (µM)	Titer ($g \cdot L^{-1}$)
CgEKV-I	4% G	0	0.036 ± 0.002
	4% G	100	0.032 ± 0.019
	4% G	200	0.074 ± 0.030
	4% G	400	0.133 ± 0.035
CgEKV-II	4% G	0	0.035 ± 0.004
	3% G + 1% X	0	0.028 ± 0.005
	3% G + 1% X	100	0.047 ± 0.002
	3% G + 1% X	200	0.255 ± 0.020
	3% G + 1% X	400	0.076 ± 0.021
	2% G + 2% X	0	< 0.004
	1% G + 3% X	0	< 0.004

G glucose, *X* xylose

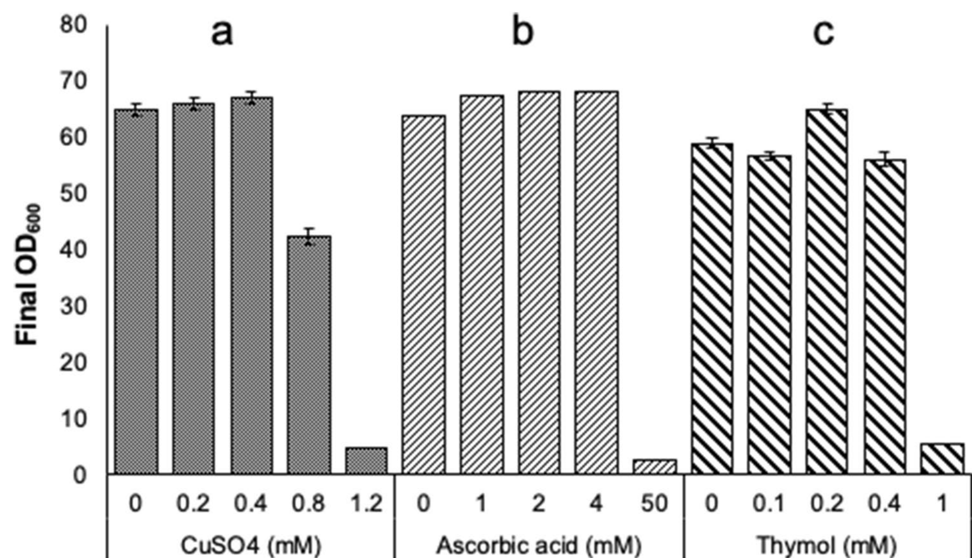

Fig. 2 The effect of copper ions (**a**), ascorbic acid (**b**), and thymol (**c**) on the final OD600 of CgEKV-I

When CgEKV-II cells were growing on a mixture of glucose and xylose, the effect of thymol was rather different. Already with 100 µM thymol, the titer increased 1.7-fold. With 200 µM thymol, the titer was 255.0 ± 20 mg/L, almost ninefold higher than that obtained without thymol. This was the highest titer obtained with *C. glutamicum* cells. Even though growth was not affected, titer dropped significantly with 400 µM thymol.

Whole-cell biotransformation of L-tyrosine to L-DOPA using CgEKV-I cells

In the second part of the study, whole-cell biotransformation of L-tyrosine to L-DOPA was studied using CgEKV-I cells that have heterologously expressed tyrosinase. Pre-grown cells were incubated with L-tyrosine in distilled water without any buffering agent and L-DOPA formation was measured in the presence and absence of the cofactor copper and the oxidation inhibitors ascorbic acid or thymol (Table 3).

In the absence of copper ions and any of the oxidation inhibitors, L-DOPA titer was as high as 0.39 ± 0.05 g/L, though there was a significant amount of oxidation products, as suggested by the final dark color of the reaction mixture. Intracellular copper seemed to be sufficient for the catalysis. The presence of additional copper ions in the reaction mixture somehow reduced L-DOPA titer to approximately half of that obtained without copper ions, possibly by increasing the activity of the tyrosinase to convert L-DOPA to subsequent compounds. The presence of neither ascorbic acid nor thymol has improved final titer. However, with either of these oxidation inhibitors, L-DOPA oxidation was reduced; the color of the resulting reaction mixtures was light brown.

Table 3 Summary of whole-cell biotransformation to L-DOPA using CgEKV-I cells

$CuSO_4$ (mM)	Thymol (µM)	Ascorbic acid (mM)	Titer ($g \cdot L^{-1}$)	$g \cdot gDCW^{-1}$*
0	0	0	0.39 ± 0.05	0.58 ± 0.07
0.4	0	0	0.18 ± 0.06	0.27 ± 0.09
	100	0	0.20 ± 0.05	0.30 ± 0.07
	200	0	0.19 ± 0.03	0.29 ± 0.05
	400	0	0.22 ± 0.03	0.34 ± 0.03
0	400	0	0.31 ± 0.05	0.48 ± 0.07
0.4	0	1	0.20 ± 0.02	0.30 ± 0.03
	0	2	0.22 ± 0.02	0.33 ± 0.04
	0	4	0.22 ± 0.02	0.33 ± 0.05
0	0	4	0.40 ± 0.01	0.61 ± 0.02

*Per liter tyrosine solution containing 0.66 $mgDCW \cdot ml^{-1}$ *C. glutamicum* CgEKV-I. 26.4 gDCW was obtained from 1 l of cultivation using CGXII defined medium with glucose as carbon source

Interestingly, when the cells were incubated with L-tyrosine and one of the oxidation inhibitors, in the absence of copper ions, L-DOPA titers were 0.31 ± 0.05 mg/L with 400 µM thymol (~30% L-tyrosine conversion) and 0.40 ± 0.01 mg/L with 4 mM ascorbic acid (~40% L-tyrosine conversion). The latter was the highest titer under the reaction conditions tested, which corresponded to 0.61 ± 0.02 mg/gDCW. The titer obtained with ascorbic acid was very close to the value obtained in its absence but the major point in these conversions was that the color of the resulting reaction mixtures was absolutely transparent; i.e., oxidation inhibitors prevented the formation of dopachrome and its subsequent non-enzymatic conversion to melanin.

In short, when CgEKV-I cells that have synthesized L-DOPA during growth were directly used as the tyrosinase source, not only significant biotransformation was achieved in water, but the simplicity of the reaction mixture should facilitate downstream applications (Figure S5, for HPLC analysis of the reaction mixtures from biotransformation and of the supernatants obtained at the end of growth).

Discussion

Microbial production of valuable compounds including Parkinson's disease drug L-DOPA has been a subject of interest in the last decade. Current L-DOPA production processes involve toxic substrates such as catechol (Lee et al. 1996; Koyanagi et al. 2005) and use industrially unfavorable microorganisms (Ali et al. 2005; Agarwal et al. 2016), which are easily contaminated or prone to phage invasion. The requirement for complex and high-cost nutrients in current production media further challenges the economic feasibility and scale-up of the processes (Wei et al. 2016; Fordjour et al. 2019).

Here, two *C. glutamicum* strains capable of heterologous expression of *R. solanacearum* tyrosinase are used; while the host of expression was the wild-type cells in the first one, the second host was additionally overexpressing xylose utilization genes, $xylA_{Xc}$ and $xylB_{Cg}$. Setting off from the fact that lignocellulosic wastes contain significant amounts of xylose, overexpression of these additional genes should enable the use of lignocellulosic biomass as a carbon and energy source for sustainable L-DOPA production. *C. glutamicum*'s ability to tolerate inhibitory byproducts in lignocellulosic hydrolysates, such as phenolic compounds and furfurals, constitutes an advantage to make this strain an ideal candidate to be used for this production using hydrolyzed lignocellulosic biomass (Gopinath et al. 2011). To our knowledge, this is the first study which investigates *C. glutamicum* for L-DOPA production.

Generally, L-DOPA synthesis with tyrosinases has been restricted to biotransformation of L-tyrosine by natural

tyrosinase producers that include fungi such as *Aspergillus oryzae* and *Yarrowia lipolytica* and bacteria such as *Brevundimonas* sp. SGJ and *Bacillus* sp. JPJ (Ikram-ul-Haq and Qadeer 2002; Ali et al. 2005 2007; Surwase and Jadhav 2011; Surwase et al. 2012). The major disadvantage of fungal strains for which the titer may reach 3 g/L with optimized strains and parameters (Ali et al. 2007) is the long periods required for fungal growth, up to 120 h, which may adversely affect productivity. This calls into question the suitability of the fungal systems for scale-up. The bacterial strains used for the same biotransformation give ~ 0.4 g/L and ~ 0.5 g/L of L-DOPA with *Brevundimonas* sp. SGJ (Surwase et al. 2012) and *Bacillus* sp. JPJ (Surwase and Jadhav 2011), respectively, in the absence of significant optimization. Only after substantial optimization and cell recycling, titer reaches ~ 3.8 g/L with *Brevundimonas* sp. SGJ. In all the mentioned studies, various concentrations of ascorbic acid have been used as a reducing reagent to prevent oxidation to dopachrome.

Among the microbial systems used for in vivo L-DOPA production, *Acremonium rutilum* is a fungal strain with a titer of 0.9 g/L L-DOPA (Krishnaveni et al. 2009). With 72–120-h cultivation periods, productivity is 7.4–12 mg/(L·h). The engineered strains with tyrosinase or only tyrosine hydroxylase activity were intended to produce plant alkaloids and L-DOPA was an intermediate in their production (Nakagawa et al. 2011; Matsumura et al. 2018). While the former was able to synthesize ~ 0.3 g/L L-DOPA with the *Streptomyces castaneoglobisporus* tyrosinase, the latter synthesized ~ 1 g/L L-DOPA with *Drosophila melanogaster* tyrosine hydroxylase. Operating in fed-batch mode for 90 h, L-DOPA productivity was 3–11 mg/(L·h) with these *E. coli* cells. Both *E. coli* cells were obtained following substantial genetic modifications and were cultivated in media that possessed components of rich growth media, such as yeast extract, tryptone, and beef extract, which also render the bioprocess unsuitable for scale-up.

In the current study, the initial L-DOPA titer was ~ 0.04 g/L for fermentation with *C. glutamicum* cells. The main reason for this relatively low titer compared to the engineered *E. coli* was the oxidation of L-DOPA. This is a major challenge with tyrosinase enzymes. Different methods and diverse chemicals have been tested for their ability to inhibit tyrosinase activity for melanin formation (Da Silva et al. 2017; Zolghadri et al. 2019); however, many are toxic and economically unfeasible. Ascorbic acid stands out as a valuable agent due to its nontoxicity and availability (Kim and Uyama 2005). While it is known as the most commonly used agent to prevent oxidation (Ros et al. 1993), its effect has been reported to be temporary since it is chemically oxidized to a nonfunctional form, dehydroascorbic acid (Komthong et al. 2007). Here, ascorbic acid was not sufficient to prevent oxidation during growth. To this end, we sought different alternatives to increase titer in engineered *C. glutamicum* cells.

Due to their ability to prevent melanin overproduction in pharmaceutical and food applications, many studies have focused on finding efficient tyrosinase inhibitors of both natural and synthetic origin (Zolghadri et al. 2019). Among the reported inhibitors are thymol and its analogues (Ashraf et al. 2015; Da Silva et al. 2017). Thymol has been proven to display a different inhibitory mechanism; it acts as a redox inhibitor rather than neither a tyrosinase inhibitor nor a substrate (Satooka and Kubo 2011). Interestingly, thymol has not yet been evaluated as an oxidation inhibitor during L-DOPA synthesis.

In the presence of thymol, L-DOPA titer in *C. glutamicum* cells has improved to 0.13 ± 0.04 g/L with cells growing on glucose and to 0.26 ± 0.02 g/L with cells growing on a mixture of glucose and xylose. This titer obtained with a mixed carbon source was comparable to the value obtained with the same tyrosinase in *E. coli* cells and the shorter cultivation period also helped to keep the productivity at 12 mg/(L·h). Since L-DOPA is very sensitive to oxygen, the final L-DOPA titer will clearly benefit from further bioprocess optimization. Unlike *E. coli*, *C. glutamicum* cells gave this titer in a medium devoid of rich media components not suitable for scale-up which makes the bioprocess far from industrial feasibility. The ability to co-utilize the glucose and xylose mixture to reach this titer places this organism in the center of sustainable L-DOPA production using renewable carbon sources.

In the majority of processes developed for L-DOPA synthesis using tyrosinase, production is primarily based on whole-cell biotransformation using pre-grown cells. Based on this fact, the *C. glutamicum* CgEKV-I cells grown on glucose for L-DOPA synthesis were also exploited for L-tyrosine biotransformation. The effect of ascorbic acid was found to be more important during biotransformation. The titer of 0.40 ± 0.01 g/L with 4 mM ascorbic acid was comparable to the values reported for *Brevundimonas* sp. SGJ (Surwase et al. 2012) or *Bacillus* sp. JPJ (Surwase and Jadhav 2011). On the other hand, L-DOPA yield was 0.61 ± 0.02 mg/gDCW with *C. glutamicum* CgEKV-I while it was ~ 0.42 and ~ 0.50 g/gDCW with *Brevundimonas* sp. SGJ and *Bacillus* sp. JPJ, respectively. Only after reaction parameters of biotransformation with *Brevundimonas* sp. SGJ were optimized and cell recycling was adopted, titer increased by almost sevenfold. Considering that from a liter of cultivation as much as 26.4 ± 1.9 gDCW *C. glutamicum* is obtained while only ~ 0.5 gDCW *Brevundimonas* sp. SGJ and ~ 5.5 gDCW *Bacillus* sp. JPJ could be obtained, many batches of biotransformation can be achieved with *C. glutamicum* just from a single cultivation.

L-tyrosine biotransformation is primarily dependent on tyrosinase activity; thus, L-DOPA yield could be further

improved by optimizing reaction conditions. On the other hand, as the industrial workhorse of amino acid production, *C. glutamicum* cells will clearly benefit from strain development efforts and bioprocess optimization towards sustainable and economically feasible L-DOPA synthesis.

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Author contribution BSA and EK conceived and designed the study. EK carried out the experimental work. VFW reviewed the results and provided critical feedback for their interpretation. All authors contributed to the writing of the manuscript. All authors approved the article for publication.

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Declarations

Ethical approval This article does not contain studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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