



Metabolic engineering of *Corynebacterium glutamicum* for L-tyrosine production from glucose and xylose

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ARTICLE INFO

Keywords:

Corynebacterium glutamicum
Rational design
L-Tyrosine overproduction
Sustainable process

ABSTRACT

Microbial production of aromatic compounds is an attractive and sustainable biotechnological approach. With this motivation, here metabolic engineering of *Corynebacterium glutamicum* for L-tyrosine (L-Tyr) overproduction was attempted by pushing the carbon flux more towards L-Tyr. Translational start codon exchanges of prephenate dehydratase (*pheA*), anthranilate synthase (*trpE*), and phenylalanine aminotransferase (*pat*) genes revealed that reduced expression of *pheA* was the major contributor to increased L-Tyr titer while codon exchange in *trpE* was effective to a lower extent. Overexpression of *aroE* and *qsuC*, encoding shikimate dehydrogenase and 3-dehydroquinate dehydratase, respectively, and of *dapC* (cg1253), which is predicted to encode prephenate aminotransferase, were futile to increase L-Tyr titer. Similarly, deletion of the *qsuABD* gene cluster had also not enhanced titer. As for increasing precursor supply, deletion of *ptsG* of glucose uptake and overexpression of inositol permease (*iolT2*) and glucokinase (*glcK*) were not effective, but with utilization of xylose, enabled by overexpression of xylose isomerase (*xyIA*) and xylulokinase (*xyIB*), titer improved. Highest L-Tyr titer using the construct was 3.1 g/L on glucose and 3.6 g/L on a 1:3 (w/v) mixture of glucose and xylose. This result displays the potential of the constructed strain to produce L-Tyr from lignocellulosic renewable carbon sources.

1. Introduction

Corynebacterium glutamicum lies in the heart of white biotechnology as a model for rational design of cells into microbial cell factories. Following its recognition as a natural L-glutamate producer (Kinoshita et al., 1957), a new era for *C. glutamicum* has started with the emergence of metabolic engineering. Different strains have then been rationally designed to synthesize a wide range of chemicals, fuels, materials and healthcare products (Becker et al., 2018; Wendisch, 2020; Kurpejović et al., 2021; Wolf et al., 2021). Construction of *in silico* models based on its genome sequence (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) and flux analysis have further helped to relieve the bottlenecks in strain development efforts (Stephanopoulos et al., 1998; Torres and Voit, 2002; Klaffl et al., 2013; Kulkarni, 2016).

L-Tyrosine (L-Tyr) is among the three aromatic amino acids of protein biosynthesis. It is also the precursor of numerous metabolites with defense, electron transport, structural support, neurotransmitter, UV protectant, analgesic, and antioxidant functions that find applications in

food, pharmaceutical, chemical and cosmetic industries (Schenck and Maeda, 2018). Due to its relatively low total market demand, its production processes have received limited attention. With the realization of the different classes of specialized compounds derived from L-Tyr, such as L-DOPA, melanin, naringenin, and resveratrol, its biotechnological synthesis has started to gain increasing interest (Chávez-Béjar et al., 2012).

Initial efforts for industrial L-Tyr production relied on extraction from protein hydrolysates using a chemical approach. Later, an enzymatic approach utilizing tyrosine phenol-lyase has been established for a more economical price (Lütke-Eversloh et al., 2007). Metabolic engineering has then shifted economically competitive L-Tyr synthesis to the use of rationally designed cells. Currently, industrial L-Tyr production is largely dependent on engineered *Escherichia coli* strains (Chávez-Béjar et al., 2012). Its fast growth rate and ease in genetic modifications have placed particular emphasis on *E. coli* as a producer of aromatic amino acids and aromatic compounds (Floras et al., 1996; Chávez-Béjar et al., 2012). Unfortunately, phage contamination and concerns on food safety

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restrict its applications (Tanji et al., 2008). In this respect, genetic stability, low protease activity, broad spectrum of carbon utilization on top of its safety for human use render *C. glutamicum* superior over *E. coli*. More importantly, *C. glutamicum* lacks endotoxins as present in lipopolysaccharides of *E. coli*, which is advantageous for the production of feed and food compounds and even more so for pharmaceuticals (Kogure and Inui, 2018).

Despite its leading role as a bacterial platform for the production of amino acids, strain development efforts for L-Tyr biosynthesis have not gone beyond classical strain breeding based on random mutations and screening/selection techniques (Hagino and Nakayama, 1973). This approach was used to isolate the *C. glutamicum* ATCC 21573 strain with a maximum L-Tyr titer of 12.3 g/L under optimized conditions (Hagino et al., 1974). Later, (Ikeda and Katsumata, 1992) were able to engineer a *C. glutamicum* strain for 26 g/L L-Tyr production. However, both the host of production (Hagino and Nakayama, 1975) and the donors of regulation-insensitive enzymes (Hagino and Nakayama, 1974) were analog resistant mutants derived from wild-type cells using random mutagenesis. Such undirected mutagenesis suffer from genome instability; indeed, the maximum titer we could obtain was ~ 0.2 g/L L-Tyr with *C. glutamicum* ATCC 21573. Therefore, engineering endeavors are required to get stable constructs for industrial applications (Darmon and Leach, 2014).

Current work undertakes the effort to achieve L-Tyr overproduction in *C. glutamicum* by (i) channeling the flux from aromatic amino acid biosynthesis towards L-Tyr by reducing the expression of genes involved in L-phenylalanine (L-Phe) and L-tryptophan (L-Trp) synthesis and increasing the expression of the aminotransferase gene of the arogenate (pretyrosine) pathway, (ii) increasing the flux through shikimate pathway by overexpression of the genes that encode shikimate dehydrogenase and 3-dehydroquinone dehydratase, (iii) inhibiting the flux

towards the shikimate pathway degradation product, protocatechuate, and (iv) increasing phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) pools. All target genes are depicted in Fig. 1.

2. Materials and methods

2.1. Prediction of the missing prephenate aminotransferase

The *N*-succinylidiaminopimelate aminotransferase of *Mycobacterium tuberculosis* has been reported to display prephenate aminotransferase activity (Graindorge et al., 2014). Therefore, taking the nucleotide sequence of this gene as the template (UniProt accession: O50434), the nucleotide sequences of all aminotransferase proteins inferred from the genome sequence of *C. glutamicum* (Marienhagen et al., 2005) have been aligned using the BlastN suite (Boratyn et al., 2013). The similarities between the aligned amino acid sequences of enzymes from *M. tuberculosis* and *C. glutamicum* have been displayed using ClustalΩ (Sievers et al., 2011).

2.2. Genetic techniques and strain construction

Molecular techniques were performed using standard cloning protocols as described (Sambrook et al., 1989). The constructed plasmids were transformed using the CaCl₂ method into *E. coli* DH5α for screening (Seidman et al., 2001). Then confirmed plasmids were transformed to *C. glutamicum* by electroporation (Eggeling and Bott, 2005).

Chromosomal alterations and gene deletion in *C. glutamicum* were done with two-step homologous recombination (Eggeling and Bott, 2005) via suicide vector pK19mobsacB (Schäfer et al., 1994). The purified PCR products of genes of interest were simultaneously ligated and cloned into single-digested pK19mobsacB by Gibson Assembly. The

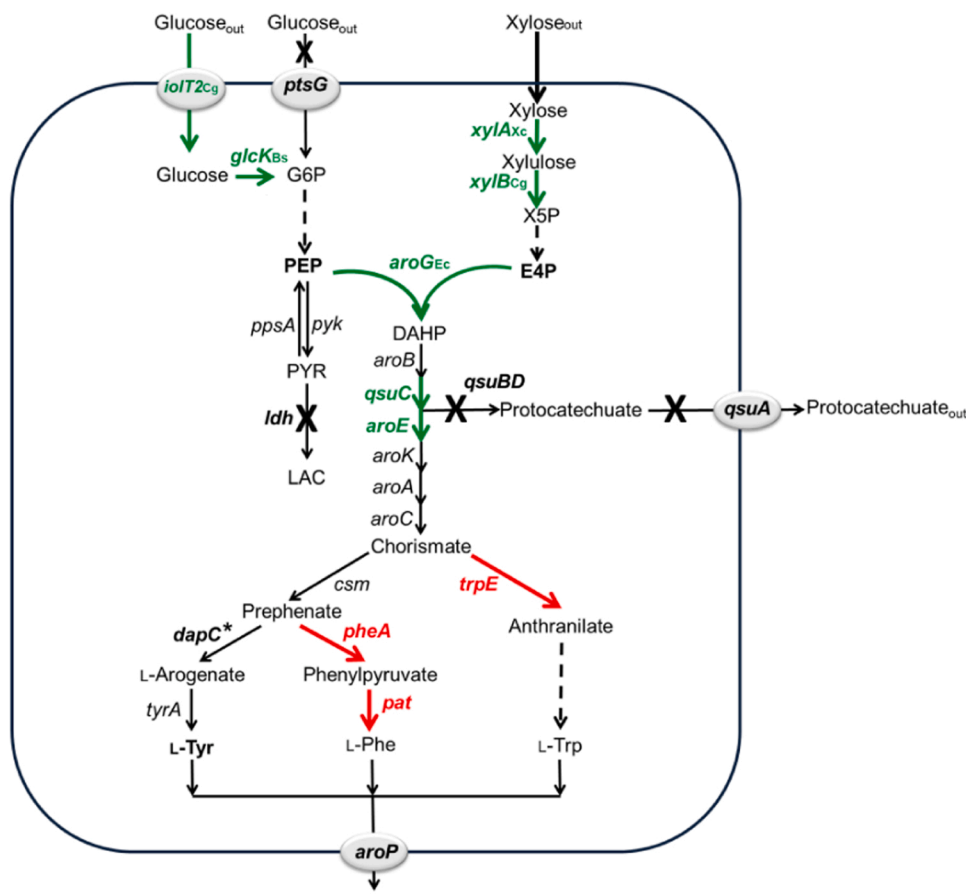


Fig. 1. Schematic representation of L-Tyr production by *C. glutamicum*. Green gene names and arrows represent chromosomal and plasmid overexpression, red gene names and arrows represent start codon replacement for reduced expression, X symbol on a pathway represents gene deletion, the gene with the asterisk has a predicted prephenate aminotransferase activity, and dashed arrows represent multiple reactions. *ptsG*: glucose-specific enzyme II component of PTS, *iolT2c_G*: inositol transporter from *C. glutamicum*, *glcKbs*: glucose kinase from *Bacillus subtilis*, G6P: glucose-6-phosphate, PEP: phosphoenolpyruvate, *ppsA*: PEP synthase, *pyk*: pyruvate kinase, *PYR*: pyruvate, *ldh*: lactate dehydrogenase, LAC: lactate, *xylAxc*: xylose isomerase from *Xanthomonas campestris*, *xylBcg*: xylulokinase from *C. glutamicum*, X5P: xylulose-5-phosphate, E4P: erythrose 4-phosphate, *aroGec*: 3-deoxy-7-phosphoheptulonate synthase from *Escherichia coli*, DAHP: 3-deoxy-7-phosphoheptulonate, *aroB*: 3-dehydroquinone synthase, *qsuC*: 3-dehydroquinone dehydratase, *qsuB*: 3-dehydroshikimate dehydratase, *qsuA*: shikimate dehydrogenase, *aroE*: shikimate dehydrogenase, *qsuA*: putative shikimate importer, *aroK*: shikimate kinase, *aroA*: 5-enolpyruvylshikimate-3-phosphate synthase, *aroC*: chorismate synthase, *trpE*: anthranilate synthase, L-Trp: L-tryptophan, *cg0975*: chorismate mutase, *tyrA*: arogenate dehydrogenase, L-Tyr: L-tyrosine, *pheA*: prephenate dehydratase, *pat*: phenylalanine aminotransferase, L-Phe: L-phenylalanine, *aroP*: aromatic amino acid transporter.

transfer of constructed plasmids was performed via trans-conjugation using *E. coli* S17 as donor strain (Walter et al., 2020).

The plasmids and strains used in this work are summarized in Tables 1 and 2. The oligonucleotides used in this work are summarized in Table S1.

2.3. Bacterial growth

A single colony was inoculated in 5 mL BHI broth and incubated at 30 °C, 200 rpm for 8 h. 2 mL of the over-day pre-culture was inoculated into 50 mL CGXII medium (Eggeling and Bott, 2005) with 4% glucose in a 500 mL baffled flask and incubated overnight at 30 °C and 150 rpm.

To investigate the effects of performed mutations, cells were first grown in 96-well U-bottom plates. 5–10 µL of the overnight preculture was used to inoculate the 220 µL of CGXII medium with 4% glucose in wells of a 96-well U-bottom plate. When necessary, growth media was supplemented with 0–2 mM L-Phe, L-Trp, or L-Tyr. The plates were incubated at 30 °C in continuous fast shaking rate mode in BIOTEK-ELx808 instrument. Growth was monitored by continuously measuring absorbance at OD₆₀₀ of the cells in the 96-well U-bottom plates.

For measuring growth of the WDD strain, 50 mL CGXII medium with 1% glucose and 1 mM L-Tyr was inoculated with 1–2 mL of overnight preculture to obtain a starting OD₆₀₀ of 0.5.

L-Tyr production was achieved in 500 mL baffled flasks containing 50 mL CGXII medium with 4% glucose using a 1–2 mL inoculum from an overnight preculture. The starting OD₆₀₀ was adjusted to 1. Cells were incubated at 30 °C and 150 rpm up to 48 h. Cell-free supernatants were used for L-Tyr analysis.

Table 1

Plasmids used in this work.

Plasmid	Description	Source
pK19mobsacB	Km ^R ; <i>E. coli</i> /C. glutamicum shuttle vector for construction of insertion and deletion mutants in <i>C. glutamicum</i> (pK19 oriV _{Ec} sacB lacZ)	(Schäfer et al., 1994)
pK19-trpE-TTG	pK19mobsacB with a construct for replacement of <i>trpE</i> start codon, ATG to TTG	This work
pK19-pheA-TTG	pK19mobsacB with a construct for replacement of <i>pheA</i> start codon, ATG to TTG	This work
pK19-pat-TTG	pK19mobsacB with a construct for replacement of <i>pat</i> start codon, ATG to TTG	This work
pK19-Δ <i>qsu</i> ABCD::P _{nif} - <i>qsu</i> C	pK19mobsacB with a construct for <i>qsu</i> ABCD replacement (cg0501-cg0504) by <i>qsu</i> C (cg0503) with an artificial RBS under control of <i>C. glutamicum</i> promoter P _{nif}	(Walter et al., 2020)
pK19-Δ <i>iolR</i> ::P _{nif} - <i>aroE</i>	pK19mobsacB with a construct for <i>iolR</i> deletion (cg0196) by <i>aroE</i> (cg1835) integration with an artificial RBS under control of <i>C. glutamicum</i> promoter P _{nif}	(Walter et al., 2020)
pSH1-Δ <i>ptsG</i> ::P _{nif} - <i>iolT</i> Best	Km ^R ; <i>E. coli</i> /C. glutamicum shuttle plasmid overexpressing <i>iolT2</i> gene from <i>C. glutamicum</i> and <i>glcK</i> gene from <i>Bacillus subtilis</i> subsp. <i>Subtilis</i> str. 168, constitutive expression	(Pérez-García et al., 2016)
pK19-Δ <i>dapC</i>	pK19mobsacB with a construct for <i>dapC</i> (cg1253) deletion	This work
pSH1- <i>xylA</i> _{Xc} - <i>xylB</i> _{Cg}	Km ^R ; <i>E. coli</i> /C. glutamicum shuttle plasmid overexpressing <i>xylA</i> gene from <i>Xanthomonas campestris</i> and <i>xylB</i> gene from <i>C. glutamicum</i> , constitutive expression	(Pérez-García et al., 2021)
pEKEx2	Km ^R ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression; <i>P_{tac}</i> , <i>lacIq</i> pBL1 oriVCG pUC18 oriV _{Ec}	(Eikmanns et al., 1991)
pEKEx2- <i>dapC</i> _{Cg}	pEKEx2 harboring <i>dapC</i> (cg1253) from <i>C. glutamicum</i>	This work

Table 2

Strains used in this work.

Strains	Description	Reference
<i>C. glutamicum</i> ATCC 13032	<i>C. glutamicum</i> wild-type strain	(Kinoshita et al., 1957)
WDD	Δ <i>dapC</i> mutant of <i>C. glutamicum</i> 13032	This work
EV	WDD harboring pEKEx2	This work
Dap	WDD harboring pEKEx2- <i>dapC</i> _{Cg}	This work
ARO02	<i>C. glutamicum</i> C1 * Δ <i>ldhA</i> Δ <i>vdh</i> ::P _{ivc} - <i>aroG</i> _{Ec} ^{Δ146N}	(Walter et al., 2020)
AROM1	<i>trpE</i> -TTG mutant of ARO02	This work
AROM2	<i>pheA</i> -TTG mutant of ARO02	This work
AROM3	<i>pheA</i> -TTG mutant of AROM1	This work
AROM3EV	AROM3 harboring pEKEx2	This work
AROM3Dap	AROM3 harboring pEKEx2- <i>dapC</i> _{Cg}	This work
AROM3X	AROM3 harboring pSH1- <i>xylA</i> _{Xc} - <i>xylB</i> _{Cg}	This work
AROM4	<i>pat</i> -TTG mutant of ARO02	This work
AROM21	Δ <i>qsu</i> ABCD::P _{nif} - <i>qsu</i> C mutant of AROM2	This work
AROM22	Δ <i>iolR</i> ::P _{nif} - <i>aroE</i> mutant of AROM21	This work
AROM23	Δ <i>ptsG</i> ::P _{nif} - <i>iolT</i> Best mutant of AROM2	This work
<i>E. coli</i> S17-1	<i>recA pro hsdR</i> RP4-2-Tc::Mu-Km::Tn7	
<i>E. coli</i> DH5α	<i>F-thi-1 endA1 hsdR17(r, m) supE44 lacU169 (Φ80lacZ1M15) recA1 gyrA96</i>	(Hanahan, 1983)

2.4. L-Tyr detection and quantification

Cells were screened for L-Tyr production using Arnow's method (Arnow, 1937). 1 mL of cell-free supernatants were mixed with 1 mL of mercury sulfate reagent, boiled for 10 min, cooled, and then mixed with 1 mL of sodium nitrate reagent. Final volume of the reaction was adjusted to 5 mL with distilled water. The presence of L-Tyr was confirmed by measuring absorbance at 530 nm.

L-Tyr detection and quantification was achieved by high-performance liquid chromatography (HPLC) using an Agilent 1100 system equipped with a 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-Tyr (0.02–1 mg/mL) was prepared in HPLC-grade water. Cell-free supernatants were used for analysis. The retention time for L-Tyr was 3.4 min.

The L-Tyr titers were measured after 48 h of incubation, which were approximately 50% higher than the values obtained after 24 h.

2.5. Quantification of reducing sugars

Glucose and xylose in the growth media was measured using 3,5-dinitrosalicylic acid (DNS) method (Harisha, 2007).

3. Results

3.1. Metabolic engineering of the branch point metabolites chorismate and prephenate to reduce formation of L-Trp and L-Phe

Chorismate is the common precursor of the three aromatic amino acids L-Trp, L-Phe and L-Tyr, with anthranilate synthase, TrpE, and chorismate mutase, Csm, competing for its conversion towards L-Trp (via TrpE) and prephenate, the common precursor of L-Phe and L-Tyr (Fig. 1). Prephenate, in turn, is also a branch point metabolite that is converted by prephenate dehydratase, PheA, and phenylalanine aminotransferase, Pat, to yield L-Phe. By contrast, the enzyme (and its gene) converting L-prephenate to L-arogenate remains elusive, while arogenate dehydrogenase, TyrA, is known to synthesize L-Tyr from L-arogenate. For over-production of L-Tyr, conversion of chorismate to L-Tyr has to be enhanced and/or formation of L-Phe and L-Trp, as side-products, has to be avoided. While it is known that deletion of *trpE* is favourable for production of L-Phe and N-methyl-L-phenylalanine (Kerbs et al., 2021),

this approach results in L-Trp auxotrophy. Similarly, deletion of *csm* resulted in auxotrophies for L-Phe and L-Tyr in strains overproducing L-Trp and indole (Ferrer et al., 2022). Here, we aimed at branch point engineering without generating auxotrophies for L-Trp and L-Phe.

Therefore, the translational start codons of the genes *trpE*, *pheA*, and *pat* have been changed from the canonical ATG to the less preferred TTG in the chromosome. This is expected to reduce expression of each gene by about 7–8 fold (Schneider et al., 2012). Since *C. glutamicum* has not yet been rationally designed for L-Tyr overproduction, while an L-Trp overproducer has been reengineered for L-Tyr production (Ikeda and Katsumata, 1992), we chose strain ARO02 as the base strain because it has been rationally engineered from the wild-type. Due to expression of a feedback resistant form of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase from *E. coli*, ARO02 produced little shikimate and anthranilate (Walter et al., 2020). The descriptions of the constructed strains are given in Table 1.

Microbial growth obtained in 96-well microtiter plates are presented in Fig. 2. Strains AROM1 and AROM4 with the start codon exchanges in *trpE* and *pat*, respectively, displayed no substantial reduction in growth, and additional L-Trp or L-Phe has not further improved their growth (Figs. 2A, 2D). On the other hand, the start codon exchange of *pheA* in AROM2 and AROM3 strains severely hampered growth without L-Phe addition, as seen in Figs. 2B and 2C. Supplementation of L-Phe (2 mM)

completely restored the growth of both strains.

Based on the fact that excess L-Phe may act as a feedback inhibitor for L-Phe and L-Tyr synthesis, different L-Phe concentrations have been tested to fine-tune its concentration for supplementing growth of the AROM2 strain in 500 mL baffled flasks. The obtained results indicated that as L-Phe concentration was increased from 0.1 to 0.5 mM, growth rate gradually increased and with 0.5 mM L-Phe, growth was completely restored and the wild-type growth rate was reached. A further increase in L-Phe has made no additional change on the growth rate. Moreover, L-Phe concentrations above 0.5 mM decreased L-Tyr titer by 50% (Fig. S1). To this end, 0.5 mM L-Phe was used in further experiments with AROM2 and all other strains that contained a start codon exchange in the *pheA* gene.

3.2. Metabolic engineering of the branch point metabolites chorismate and prephenate to enhance conversion of chorismate to L-Tyr

As the *C. glutamicum* aminotransferase yielding arogenate from prephenate was unknown (Marienhagen et al., 2005) thus unavailable for metabolic engineering, we aimed at identifying it. Notably, prephenate aminotransferase functionality was identified as a side activity of *N*-succinyldiaminopimelate aminotransferase (encoded by gene Rv1178) from *M. tuberculosis* (Graindorge et al., 2014). Comparing this

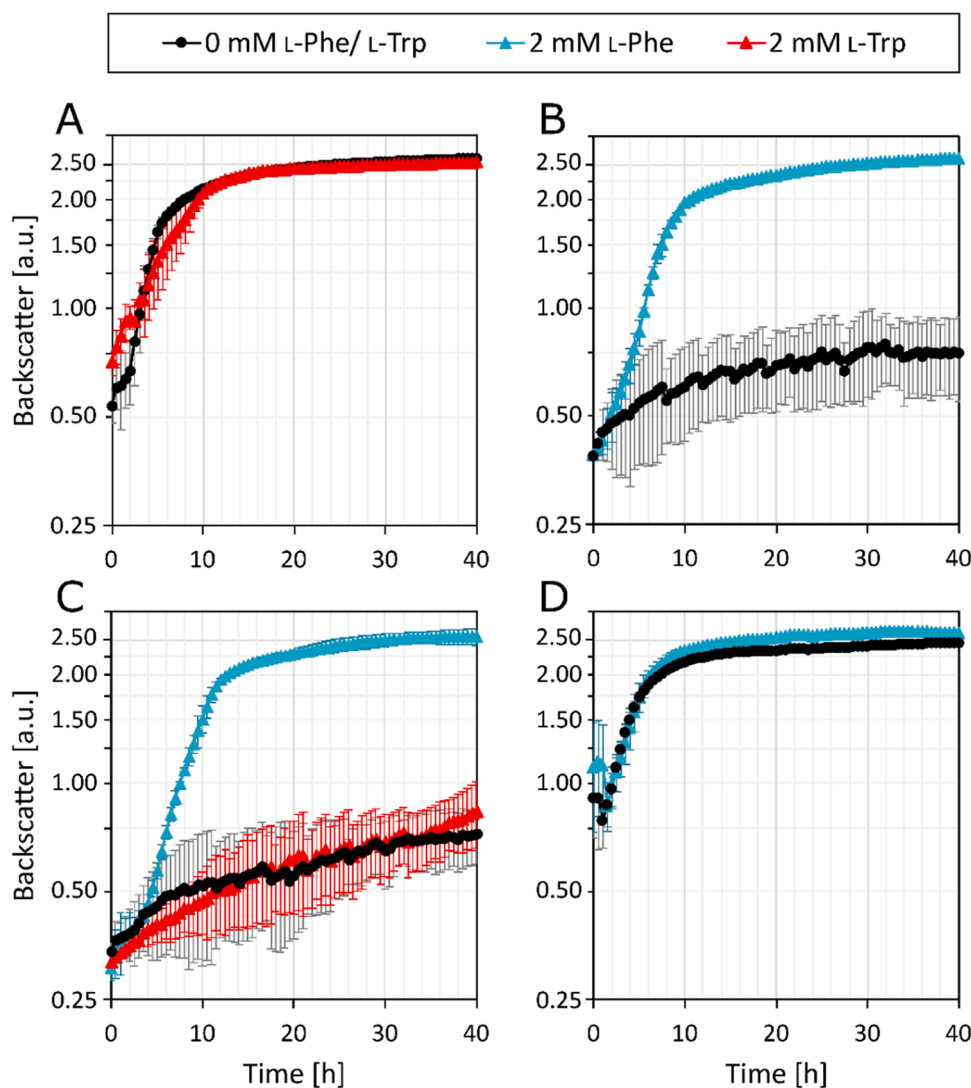


Fig. 2. Growth of constructed mutants in 96-well plates. A) AROM1, B) AROM2, C) AROM3, D) AROM4. Each curve represents means and standard deviations of three biological replicates. Backscatter signal is given in arbitrary units (a.u.).

amino acid sequence to the aminotransferases encoded by *C. glutamicum* indicated that *N*-succinyldiaminopimelate aminotransferase DapC (cg1253) was the most similar protein (Fig. 3). DapC is required for *L*-lysine (*L*-Lys) biosynthesis via the succinylase variant of the pathway, but its absence can be compensated for by the dehydrogenase pathway (Cremer et al., 1990; Sonntag et al., 1995). In addition to its role in the succinylase variant of *L*-Lys biosynthesis, we speculated that DapC could constitute the missing aminotransferase in *L*-Tyr synthesis and consequently could be involved in *L*-Tyr overproduction. Indeed, the query coverage obtained from BlastP alignment (Altschul et al., 1990) for DapC and prephenate aminotransferase of *Arabidopsis* was > 75% and the coverages obtained for DapC and the aminotransferases with *L*-Lys as the major substrate were > 80%, while the percentages of the sequences aligned were much lower with the other aminotransferases listed in (Koper et al., 2022).

In a loss-of-function analysis, *dapC* was deleted from the *C. glutamicum* wild type chromosome. In preliminary growth experiments in 96-well plates, the *dapC* deletion mutant (WDD strain) grew slower than *C. glutamicum* wild type in the absence of *L*-Tyr, while growth was comparable in the presence of 0.5 mM or higher *L*-Tyr concentrations (Fig. S2). Thus, deletion of *dapC* resulted in a bradytrophism for *L*-Tyr. As expected from the presence of the dehydrogenase variant of *L*-Lys biosynthesis, deletion of *dapC* did not result in *L*-Lys auxotrophy (Fig. S3).

To exclude potential polar or other secondary effects of the *dapC* deletion, a genetic complementation test was performed. WDD strain was transformed with either empty vector or the vector carrying *dapC* gene to yield strains EV and Dap, respectively. Growth experiments were performed without *L*-Lys supplementation, while either 0 or 1 mM *L*-Tyr was added. The absence of *dapC* slowed growth unless external *L*-Tyr was added, resulting in a significantly reduced growth rate of EV (Fig. 4). By contrast, upon plasmid-borne expression of *dapC* in the *dapC* deletion mutant (strain Dap), the growth rate was comparable to that of the wild type in the absence as well as in the presence of externally added *L*-Tyr (Fig. 4). Thus, the observed *L*-Tyr bradytrophism is due to the absence of DapC, indicating that DapC plays a role in *L*-Tyr biosynthesis.

3.3. *L*-Tyr production by strains with engineered branch point metabolites chorismate and prephenate

First, all constructed strains with start codon replacements in *trpE*, *pheA*, and *pat* were examined for their potential to overproduce *L*-Tyr and the results obtained are given in Table 3. *L*-Tyr titer of AROM4 was quite similar to the titer obtained from the parent strain, ARO02, which was around 60 mg/L after 48 h incubation. Hence, there was no apparent effect of the mutation in *pat* for *L*-Tyr production. Changing the start codon of *trpE* has doubled *L*-Tyr titer in AROM1, as compared to the parent strain. On the other hand, the AROM2 strain with the start codon replacement in *pheA*, had an *L*-Tyr titer of almost 44-fold higher, as compared to the parent strain, ARO02. The titer was 3.1 g/L in the AROM3 strain with replacements in both *trpE* and *pheA*. This obtained value was only about 10% higher as compared to AROM2 but 50-fold higher as compared to ARO02 strain. There was no significant change in *L*-Tyr titer with the replacement in the *pat* gene in AROM4.

Next, *dapC* was overexpressed in AROM3 to test if DapC activity may limit *L*-Tyr production by the strain with the highest *L*-Tyr titer. Both strains, AROM3EV with the empty vector and AROM3Dap overexpressing *dapC*, produced around 3 g/L of *L*-Tyr (Table 3), indicating that *dapC* overexpression has no effect on *L*-Tyr overproduction. Thus, *L*-Tyr production by the currently best strain AROM3 did not benefit from *dapC* overexpression under the current conditions.

3.4. Metabolic engineering to increase precursor supply for *L*-Tyr production

Further work aimed to increase flux through the shikimate pathway and prevent flux towards the degradation product of this pathway, protocatechuate. To this end, first the gene cluster responsible for the expression of the genes involved in the synthesis of this product was targeted. The *L*-Tyr titer obtained with the AROM21 strain, constructed by the deletion of *qsuABD* and overexpression of *qsuC* under the *P_{trf}* promoter, was comparable to that of AROM2 (Table 3). Additionally, the AROM22 strain was constructed by integration of *aroE* under the *P_{trf}* promoter as the *iolR* gene was deleted. Interestingly, there was no change in *L*-Tyr titer, as compared to AROM2 with the overexpression of *aroE*, either.



Fig. 3. Amino acid sequence alignment of *M. tuberculosis* Rv1178 with *C. glutamicum* DapC (Cg1253).

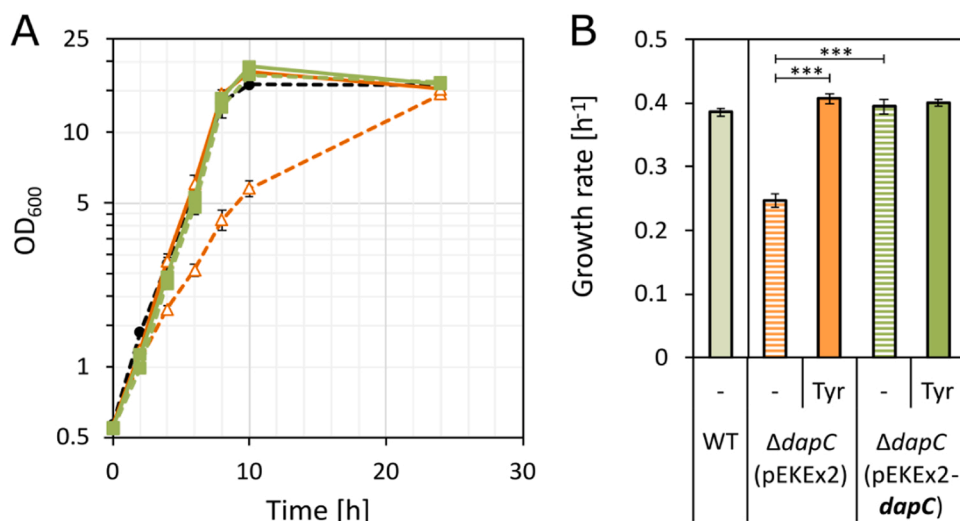


Fig. 4. Growth of A) *C. glutamicum* WT: black circles, EV: empty triangles with dashed lines, EV with 1 mM L-Tyr: empty triangles with straight line and B) comparison of growth rates in the presence of *dapC* and L-Tyr. EV: $\Delta dapC$ (pEKEEx2), Dap: $\Delta dapC$ (pEKEEx2-*dapC*). The strains were grown in CGXII medium with 10 g/L glucose in 500-mL baffled flasks. Means and standard deviations of triplicate cultures are depicted, and significance was calculated by unpaired two-sided Student's t-test with ***: $p < 0.001$.

Table 3

Final OD₆₀₀ values and L-Tyr titers of ARO02 and its mutants. Production was achieved in 500-mL baffled flasks for 48 h. Each data is average of three independent experiments.

Strain	OD ₆₀₀	L-Tyr (g/L)	mg L-Tyr/ g Glucose
ARO02	55 ± 1	0.06 ± 0.01	1.5
AROM1	42 ± 3	0.14 ± 0.01	3.5
AROM2	25 ± 7	2.70 ± 0.08	67.5
AROM3	24 ± 1	3.1 ± 0.10	77.5
AROM4	52 ± 3	0.07 ± 0.01	1.8
AROM21	22 ± 3	2.50 ± 0.08	62.5
AROM22	20 ± 1	2.50 ± 0.01	62.5
AROM3Dap	18 ± 1	3.01 ± 0.07	75.3

Next, to increase phosphoenolpyruvate availability for the initial reaction of the shikimate pathway by rerouting glucose uptake was attempted. To this end, the *ptsG* gene was inactivated by integration of both *iolT2* from *C. glutamicum* and *glcK* from *Bacillus subtilis* to block the phosphotransferase system (PTS) (Pérez-García et al., 2016) and enable glucose uptake through the inositol permease. Since inositol induces inositol utilization genes (Lindner et al., 2011), first inositol concentration required to activate glucose utilization via the inositol permease in AROM23 was investigated. The presence of inositol enhanced growth but even with 2 mM inositol, growth rate was very low, as well as the final cell concentration (Fig. S4). Then L-Tyr production of AROM23 grown in 500-mL baffled flasks was evaluated. L-Tyr titer was only 2.1 ± 0.1 g/L in the absence of inositol and 1.2 ± 0.2 g/L with 2 mM inositol, thus, both lower than that of the parent strain.

In conclusion, when using glucose as the sole carbon and energy source, the highest titer was obtained with the AROM3 strain.

3.5. L-Tyr production on glucose/xylose mixtures

In contrast to glucose, xylose does not depend on PEP for internalization, which is one of the two precursors of the shikimate pathway. Thus, further work was pursued to investigate L-Tyr titer with cells grown on glucose/xylose mixtures. To this end, in order to enable xylose utilization, the strain with the highest titer on glucose, AROM3, was transformed with pSH1-*xyIA_{Xc}xyIB_{Cg}* plasmid and the resulting strain was designated as AROM3X.

The final OD₆₀₀ and L-Tyr titers obtained with AROM3X are summarized in Table 4.

The titers obtained with AROM3X and AROM3 with 40 g/L glucose as the carbon and energy source were comparable (Table 3; Table 4).

Table 4

Final OD₆₀₀ and L-Tyr titers of AROM3X. Production was achieved in 500-mL baffled flasks for 48 h. Each data is average of three independent experiments.

Carbon source	OD ₆₀₀	L-Tyr (g/L)	mg L-Tyr / g carbon source
Glu (40 g/L)	25	3.2	80.0
Glu (30 g/L) + Xyl (10 g/L)	24 ± 1	3.2 ± 0.07	80.0
Glu (10 g/L) + Xyl (30 g/L)	24 ± 3	3.6 ± 0.10	90.0
Xyl (40 g/L)	21 ± 2	1.7 ± 0.10	42.5

This was not unexpected and indicated that plasmid pSH1-*xyIA_{Xc}xyIB_{Cg}* was not a metabolic burden. As expected from its entry via the pentose phosphate pathway, xylose-based L-Tyr production was lower (approximately half of that obtained with glucose; Table 4). This drop was not seen when mixtures of glucose and xylose were used. Notably, the highest titer of 3.6 ± 0.1 g/L was obtained with the glucose:xylose ratio of 1:3.

3.6. L-Tyr yield on substrate

L-Tyr yield on substrate was 77.5 mg L-Tyr / g glucose in AROM3, while it improved to 90.0 mg L-Tyr / g carbon source in AROM3X. These numbers are comparable with the yields reported by Lütke-Eversloh and Stephanopoulos (Lütke-Eversloh and Stephanopoulos, 2007, 2008). Using minimal media with glucose as the sole carbon source, yield was 37 mg L-Tyr / g glucose with feedback inhibition resistant derivatives of DAHPS and bifunctional chorismate mutase/prephenate dehydrogenase, while it improved to 102 mg L-Tyr / g glucose when limitation of precursor substrates was further considered (Lütke-Eversloh and Stephanopoulos, 2007). With releasing bottlenecks in the aromatic amino acid biosynthesis route, it was possible to get a yield of ~ 150 mg L-Tyr / g glucose (evaluated from the presented plots) (Lütke-Eversloh and Stephanopoulos, 2008). The L-Tyr yield on glucose remained between 60 and 100 mg L-Tyr per g glucose in those studies using complex medium for production (Chávez-Béjar et al., 2008; Na et al., 2013).

4. Discussion

In microbial cells, shikimate pathway is the route for aromatic amino acid synthesis, which starts with the condensation of the metabolic precursors PEP and E4P. Then, diversion to L-Trp, L-Phe, and L-Tyr is achieved at the chorismate branch point. Therefore, studies targeting aromatic amino acid synthesis commonly focus on shikimate pathway and the branched terminal aromatic amino acid pathways. These

pathways constituted the major routes of alteration for aromatic amino acid overproduction also in *C. glutamicum* (Averesch and Krömer, 2018).

Interestingly available hosts and enzymes used for L-Tyr overproduction in *C. glutamicum* do not go beyond those derived from auxotrophic and antimetabolite resistant strains obtained using classical mutagenesis (Hagino and Nakayama, 1973, 1974, 1975; Hagino et al., 1974; Ikeda and Katsumata, 1992). Rational design of *C. glutamicum* for aromatic amino acid overproduction focus to a large extent on either L-Phe or L-Trp, due to their higher market sizes. For L-Phe overproduction, commonly the feedback resistant form of prephenate dehydratase, encoded by *pheA*, has been used (Zhang et al., 2013, 2015), while L-Trp overproduction has necessitated silencing of chorismate mutase in addition to the overexpression of *trpD* and feedback resistant form of *trpE* (Veldmann et al., 2019). Since available data indicated that the activities of prephenate dehydratase and anthranilate synthase component 1 should be important to channel flux from chorismate towards L-Phe and L-Trp, respectively, their activities were genetically reduced via start codon exchanges in *pheA* and *trpE* in ARO02. This was, in turn, expected to increase flux towards L-Tyr. Indeed, the changes in the expression of the two feedback regulated enzymes were found to be important for channeling carbon flow towards L-Tyr overproduction. Clearly the impact of *pheA* was higher than that of *trpE*. On the other hand, the start codon replacement in *pat*, the second enzyme specific for L-Phe synthesis, was found to be futile for L-Tyr overproduction.

As pointed out in a recent review (Koper et al., 2022), there are still uncharacterized aminotransferases, possibly with multiple substrates, even in model organisms. Indeed, *C. glutamicum* is one of those. In order to further increase carbon flow from prephenate to L-Tyr, an enzyme catalyzing the amination of prephenate to aroenate was searched. Available data suggest that among the two alternative routes for the post-chorismate branch of the pathway leading to L-Tyr, *C. glutamicum* uses the aroenate route (Fazel and Jensen, 1979). In this route, an aminotransferase is required for the amination of prephenate to aroenate, which is then converted to L-Tyr by the aroenate dehydrogenase encoded by *tyrA*. Evidence shows that the *N*-succinyldiaminopimelate aminotransferases in actinobacteria, *Streptomyces avermitilis* and *M. tuberculosis*, display prephenate aminotransferase activity (Graindorge et al., 2014). Furthermore, the fact that the 3-deoxy-7-phosphoheptulonate synthase and chorismate mutase of *M. tuberculosis* and *C. glutamicum* share significant structural and mechanistic similarities (Burschowsky et al., 2018) also suggested a link between the aminotransferases of these two microorganisms. The deletion of the predicted aminotransferase transformed the wild-type *C. glutamicum* strain into an L-Tyr bradytroph. This result supported the role of *dapC* in L-Tyr biosynthesis but has also shown that there should be at least one other aminotransferase involved for this amination. However, overexpression of *dapC* had no influence on L-Tyr titer, presumably due to the fact that aminotransferases commonly operate near equilibrium (Taylor et al., 1998).

The shikimate pathway is exclusively used for the synthesis of aromatic compounds. Thus, it would be reasonable to assume that increasing flux through this pathway could improve L-Tyr production. Different enzymes of this pathway have been targeted by various studies for achieving higher rates of flux through the shikimate pathway for shikimic acid, aromatic molecules or related compounds (Zhang et al., 2015; Kogure et al., 2016; Purwanto et al., 2018). Here, shikimate dehydrogenase, encoded by *aroE*, and dehydroquinase dehydratase encoded by *qsuC* (*aroD*) have been integrated into the genome under the strong *P_{trf}* promoter to increase flux through the shikimate pathway and the *qsuABD* genes encoding enzymes involved in the quinate/shikimate degradation pathway have been deleted to block protocatechuate synthesis. Unlike in shikimate (Kogure et al., 2016) and 4-hydroxybenzoate overproduction (Purwanto et al., 2018), neither of the considered modifications had a significant effect on L-Tyr product titer when compared to AROM2. One of the reasons for this could be that AroE requires NADPH as a cofactor and its availability was not sufficient.

With the point mutation (Ser361Phe) in 6-phosphogluconate dehydrogenase gene (*gnd*; cg1643), this bottleneck can be relieved (Ohnishi et al., 2005). Shikimate titer in a strain with this mutation was 40% higher (Sato et al., 2020).

E. coli has been intensively engineered using analogous approaches to enhance L-Tyr production. Although *E. coli* and *C. glutamicum* have slightly different routes and regulation patterns for L-Tyr synthesis, it might be possible to briefly compare them. First of all, the feedback inhibition resistant derivatives of DAHPS and the bifunctional chorismate mutase/prephenate dehydrogenase were primarily employed in *E. coli* for L-Tyr overproduction (Lütke-Eversloh and Stephanopoulos, 2007, 2008; Kim et al., 2015; Xu et al., 2020). A similar approach has been pursued here using a feedback-resistant DAHPS. However, *E. coli* possesses bifunctional enzymes common to L-Tyr and L-Phe, consequently it is not trivial to target one amino acid at a time (Ikeda, 2006). On the other hand, *C. glutamicum* possesses discrete enzymes; thus, it was possible to finely tune the prephenate node here by start codon changes in prephenate dehydratase and phenylalanine aminotransferase. As an additional attempt around the prephenate node, the unidentified aminotransferase was predicted. Downstream of the shikimate pathway, the contribution of anthranilate synthase to L-Tyr production was shown here for the first time. Regarding the bottlenecks of aromatic amino acid biosynthesis route, various overlapping strategies have been pursued on the two hosts, but they displayed different outcomes as exemplified by shikimate dehydrogenase (Lütke-Eversloh and Stephanopoulos, 2008).

Apart from changes in the shikimate and downstream pathways, an alternative approach pursued for aromatic amino acid overproduction has been the modification of cells for increased PEP and E4P pools (Averesch and Krömer, 2018). In addition to its major uptake route via PTS, which consumes PEP, *C. glutamicum* has been shown to use the inositol permease for glucose uptake, which does not consume PEP (Lindner et al., 2011). Thus, rerouting glucose to the permease should increase PEP availability. However, efficient glucose uptake by the permease necessitates overexpression of a glucokinase gene in addition to either *iolT1* or *iolT2* (Lindner et al., 2011). Therefore, in this study, the glucokinase from *B. subtilis* and *iolT2* from *C. glutamicum* (Pérez-García et al., 2016) were together integrated into *ptsG* locus to inactivate the PTS. Contrary to expectations, L-Tyr titer obtained with the constructed AROM23 strain was 1.3-fold lower than that obtained with its parent strain AROM2, despite the effort to increase PEP availability. However, retaining the PTS system for overproduction of shikimate was reported to be favorable (Sato et al., 2020), which is compatible with the results of this study. Another possibility to increase the PEP pool for L-Tyr production in AROM3X, could be to inactivate the PEP carboxylase gene, *ppc*, to inhibit the conversion of PEP to oxaloacetate (Walter et al., 2020). The overexpression of *aroB* in AROM3X can also be considered to increase carbon flux towards shikimate for L-Tyr production since (Sato et al., 2020) reported a 1.5-fold increase in shikimate production with this modification. In order to increase E4P availability for the committed step of the shikimate pathway, AROM3 with the highest L-Tyr titer on glucose, was considered for utilization of glucose, xylose, and their mixtures. Intracellular xylose is converted to xylulose 5-phosphate upon the synthesis of xylose isomerase and xylulokinase and subsequently is directed to pentose phosphate pathway. In this route, xylose was expected not to only contribute to E4P pool but also to PEP pool, since xylose doesn't rely on PEP for internalization. When possible limitation of precursor substrates in *E. coli* was considered, phosphoenolpyruvate synthetase and transketolase were overexpressed to improve titer (Lütke-Eversloh and Stephanopoulos, 2007). However, in *C. glutamicum*, overexpression of transketolase alone only barely enhanced aromatic amino acids yield (Ikeda et al., 1999), therefore it was not considered further in this work. Alternatively, there was an endeavour to overcome this limitation by impeding glucose uptake through PTS and by enabling xylose utilization.

Here, it was demonstrated that xylose could be metabolized by

AROM3X and when a glucose:xylose mixture of 1:3 was used, a statistically significant improvement of 12.5% ($p = 0.01$) in L-Tyr titer was observed as compared to AROM3. Apart from overcoming the limitations in precursor substrates, this finding also provides the basis for sustainable L-Tyr overproduction from lignocellulosic feedstocks with high xylose content. Many lignocellulosic feedstocks contain high concentrations of xylose and glucose. Therefore, renewable carbon sources with high xylose contents (Narisetty et al., 2022) could serve as suitable sources for AROM3X strain for the bio-friendly production of L-Tyr and value-added chemicals derived from L-Tyr such as tyramine, L-DOPA, and flavonoids (Wu et al., 2018).

5. Conclusion

In this study, a *C. glutamicum* strain for L-Tyr production has been engineered. Results have demonstrated that coupling of the feedback resistant form of DAHP synthase with reduced expression of prephenate dehydratase for lower activity may be adequate for L-Tyr overproduction. Considering that *C. glutamicum* is the model organism for amino acid production, titer would definitely benefit from further under controlled conditions with optimized feeding strategy. Furthermore, L-Tyr production with glucose/xylose mixtures suggested that the constructed strain can be used as a platform for the production of L-Tyr and L-Tyr-derived molecules not only from the most abundant carbon source glucose but also from renewable carbon sources with high glucose and xylose contents.

Funding

This work was supported in part by The Scientific and Technological Research Council of Turkey (TUBITAK) grant 120N728 and by the Bundesministerium für Bildung und Forschung (BMBF) grant 01DL21001.

CRedit authorship contribution statement

EK, VFW, and BSA designed the study. EK, AB, GMB, and NJ carried out the experimental work and analyzed the data. All authors contributed to the discussion of the results and commented on the manuscript. All authors approved the article for publication.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Berna Sariyar Akbulut reports financial support was provided by Scientific and Technological Research Council of Turkey.

Data availability

Data will be made available on request.

Acknowledgements

EK thanks Federation of European Microbiological Societies (FEMS) for the research grant (Grant Number FEMS-GO-2019–509).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jbiotec.2022.12.005](https://doi.org/10.1016/j.jbiotec.2022.12.005).

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