Strain optimization for aromatic amino acids using an *Escherichia coli* kinetic model

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Abstract: Phenylalanine, tyrosine and tryptophan are highly important aromatic amino acids, vital for food and pharmaceutics industry. They aren't only valuable by themselves, as they are also precursors for other high-added value compounds. However, their production by microorganisms is highly regulated, which hampers industrial production. Using kinetic modelling, we aim to provide a new tool to identify targets for metabolic engineering, in order to increase these amino acids production. To do this, we created a model, that encompasses the central carbon metabolism all the way to these amino acids production pathways, including their regulation by feedback inhibition. Optimizations were then performed to obtain sets of targets for metabolic engineering, which were then compared to the existing strategies found in literature. We obtained solutions similar to the strategies found in literature, but also new strategies not yet reported, which could imply new chassis for aromatic amino acids production.

Keywords: phenylalanine, tryptophan, tyrosine, kinetic model, optimization, metabolic engineering.

1. INTRODUCTION

The three aromatic amino acids (AAA): L-phenylalanine (L-Phe), L-tryptophan (L-Trp) and L- tyrosine (L-Tyr) are compounds of great interest, both as individual metabolites as well as precursors for other high-added value compounds, namely in the food and pharmaceutics industries (Báez-Viveros *et al.*, 2004; Lütke-Eversloh and Stephanopoulos, 2007; Zhao *et al.*, 2011; Liu *et al.*, 2018; Singh and Tiwari, 2018). These AAA are usually produced by biosynthesis with genetically modified microorganisms, using simple carbohydrates, such as D-Glucose, constituting a clean and renewable alternative to chemical synthesis(Singh and Tiwari, 2018). However, due to the complexities and intrinsic regulation of metabolic networks, the yield coefficient of these biosynthetic processes is relatively low, creating a new challenge: improving AAA yield (Polen *et al.*, 2005; Juminaga *et al.*, 2012; Tröndle *et al.*, 2018).

Systems biology is a field that aims to understand and simulate these metabolic intricacies, to the point where it allows prediction of biological processes, providing tools for metabolic engineering of biological systems, thus redesigning said systems with a set of desired properties (Chassagnole *et al.*, 2002; Jahan *et al.*, 2016; Kim, Rocha and Maia, 2018). A tool commonly used in this field is the modelling of biological systems using mathematical equations, describing biological processes and enabling quantitative predictions of the cells' states (Chassagnole *et al.*, 2002; Lima *et al.*, 2016; Kim, Rocha and Maia, 2018).

The potential of modeling is great, but the lack of information on biological systems can become a hindrance, particularly to kinetic modelling, which relies in enzyme kinetics for its quantitative simulations (Chassagnole *et al.*, 2002; Kadir *et al.*, 2010; Peskov, Mogilevskaya and Demin, 2012; Jahan *et al.*, 2016). While some metabolic pathways, such as the glycolysis or the tricarboxylic acid cycle (TCA), have been greatly studied over the years, there is still much to learn regarding kinetic information on the dynamics of reactions for less studied pathways.

While smaller than stoichiometric models in sheer size, kinetic, or dynamic, models can provide a level of detail that stoichiometric models can't measure up to (Link, Christodoulou and Sauer, 2014; van Rosmalen *et al.*, 2021). The kinetic information present in this kind of models allows for quantitative predictions of metabolites, as well as foresight into inhibitory roles by different compounds, for instance, in end-product inhibition. This allows for more accurate metabolic simulations and for better target predictions regarding metabolic engineering purposes.

In this paper, we strive to predict metabolic engineering targets for the optimization of AAA production using a kinetic model created with *in vitro* enzyme kinetics. Inhibition parameters were also allowed as targets to enable solutions with reaction knockout (KO), over/under expression (OU) and with an increase/decrease in inhibition feedback resistance (FbR).

2. METHODS

2.1 Designing an Escherichia coli (E. coli) kinetic model

Unlike genome scale metabolic models, which cover thousands of metabolic reactions and encompass entire pathways, kinetic models can only cover tens of reactions

symbolizing one or a few pathways (Stalidzans *et al.*, 2018). While this simplifies the creation of a metabolic pathway structure, each individual reaction is more complex due to the kinetic information added to it. At the same time, kinetic models are hindered by the lack of kinetic information on the reaction dynamics (Chassagnole *et al.*, 2002).

The central carbon metabolism (CCM) of *E. coli* has been extensively studied and several dynamic models have been made to depict this pathway (Chassagnole *et al.*, 2002; Kadir *et al.*, 2010; Peskov, Mogilevskaya and Demin, 2012; Jahan *et al.*, 2016). However, none of them have the reactions needed for AAA production. Therefore, a new model was needed that included all previous studied pathways, while also including the shikimate pathway and AAA formation reactions, as well as any other reactions considered necessary.

This new model has its structure and kinetics based on existing models found in the literature: glycolysis and PPP was based on Chassagnole *et al.*, 2002, TCA was based on Peskov, Mogilevskaya and Demin, 2012, acetate formation was based on Kadir *et al.*, 2010, ammonia (NH4) intake was based on Bruggeman, Boogerd and Westerhoff, 2005, and the electron transport chain (ETC) and ATP synthase was based on Peercy *et al.*, 2006, Taylor, Korla and Mitra, 2013, and Ederer *et al.*, 2014. Co-metabolites (ATP, NAD, NADP, etc.) maintenance and equilibrium were based on Chassagnole *et al.*, 2002 and Peskov, Mogilevskaya and Demin, 2012. The model can be found in https://github.com/andrepacofonseca/model.git.

With this work's objective being optimization of AAA production, there was the need to extend this model into L-Phe, L-Trp, and L-Tyr pathways. To do this, a thorough analysis into the AAA was made, collecting information about the shikimate pathway, a common pathway leading to these three amino acids, and about each AAA individual pathway. This information was collected through the literature and with the help of BRENDA (Chang *et al.*, 2021). Stoichiometry of reactions and pathway structure was also conferred from an *E. coli* stoichiometric model, iJO1366 (Orth *et al.*, 2011). In case no kinetic information was found on BRENDA or on the literature, empirical kinetics or parameters were used, depending on how much information was lacking. The AAA biosynthesis is extremely regulated, with several enzymes of their pathways being feedback inhibited by their end-product (L-Phe, L-Trp and L-Tyr), in fact the very first reaction of the shikimate pathway, which converts erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) into the first metabolite of the shikimate pathway, 2-dehydro-3-deoxy-D-arabino-heptonate 7-phosphate (2DDA7P), is carried out by 3 isozymes, each regulated by a different AAA. In this work, care was taken to properly represent this inhibition, which was deemed as vital for later simulation and optimization work. The basic structure of the model is shown in Figure 1.

2.2 Simulation of a kinetic model

For both the simulation and optimization of the kinetic model, a python package called optimModels was used. This package allows for kinetic model simulation and *in silico* strain design optimization by finding combinations of knockout (KO) and

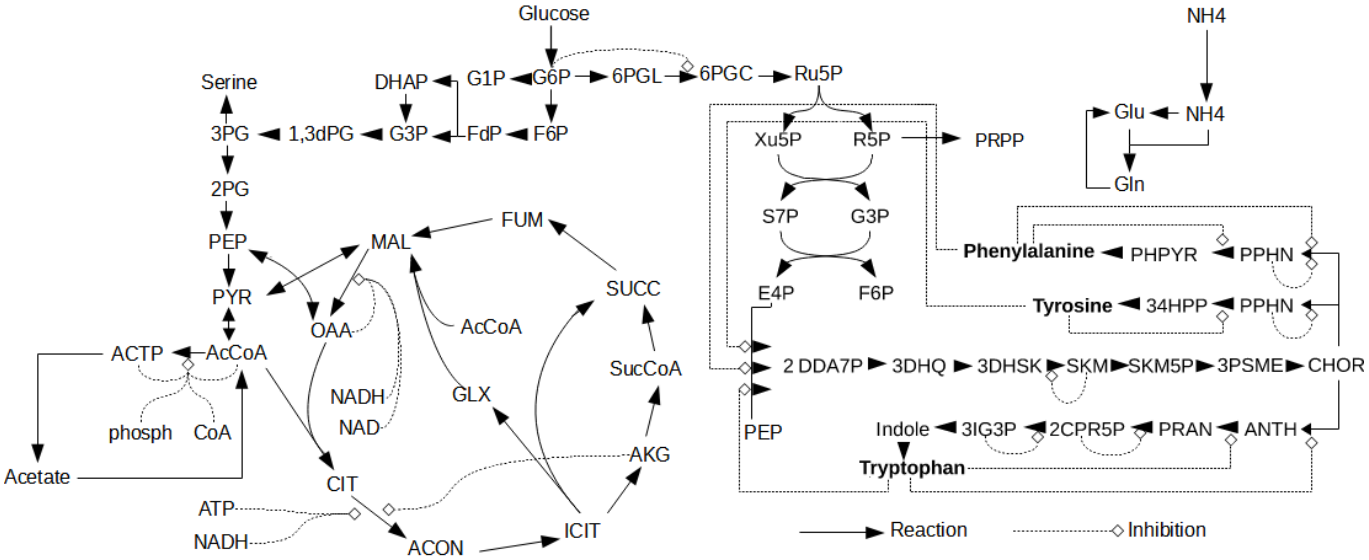

Figure 1. Basic structure of the kinetic model, including glycolysis, PPP, TCA, acetate formation, NH4 intake, shikimate pathway and AAA formation. ETC and ATP synthase are not shown in this image. Glucose, acetate and NH4 are external metabolites. Black arrows: reactions; dotted lines and white diamonds: inhibition. G6P: glucose 6-phosphate; G1P: glucose 1-phosphate; F6P: fructose 6-phosphate; FdP: D-fructose 1,6-biphosphate; G3P: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate; 1,3dPG: 3-phospho-D-glyceroyl phosphate; 3PG: 3-phospho-D-glycerate; 2PG: D-glycerate 2-phosphate; PYR: pyruvate; CoA: Coenzyme A; AcCoA: acetyl CoA; OAA: oxaloacetate; CIT: citrate; ACON: cis-aconitate; ICIT: isocitrate; AKG: 2-oxoglutarate; SucCoA: succinyl CoA; SUCC: succinate; FUM: fumarate; MAL: L-malate; GLX: glyoxylate; ACTP: acetyl phosphate; phosph: phosphate; 6PGL: 6-phospho-D-glucono-1,5-lactone; 6PGC: 6-phospho-D-gluconate; Ru5P: D-ribulose 5-phosphate; Xu5p: D-xylulose 5-phosphate; R5P: D-ribose-5-phosphate; S7P: sedoheptulose-7-phosphate; 3DHQ: 3-dehydroquinate; 3DHSK: 3-dehydroshikimate; SKM: shikimate; SKM5P: shikimate 5-phosphate; 3PSME:5-O-(1-carboxyvinyl)-3-phosphoshikimate; CHOR: chorismite; PPHN: prephenate; PHPYR: phenylpyruvate; 34HPP: 3-(4-hydroxyphenyl)pyruvate; ANTH: anthranilate; PRAN: N-(5-phospho-D-ribosyl)anthranilate; PRPP: 5-phospho-alpha-D-ribose 1-diphosphate; 2CPR5P: 1-(2-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate; 3IG3P: C'-(3-indolyl)-glycerol 3-phosphate; Glu: glutamate; Gln: Glutamine.

over/under expression of genes or reactions. The package can be found in https://github.com/saragcorreia/optimModels.

The kinetic model is prepared for simulation of a glucose pulse with a dilution rate of 0.1 h^{-1}, a glucose initial concentration of 2 mM and a glucose concentration in the feed of 110.96 mM. The sole carbon source for this model is glucose, the intake of which occurs by means of a phosphotransferase system (PTS), as described by Chassagnole *et al.*, 2002. All simulations had a total run time of 72000 s.

2.3 Optimization for AAA

Evolutionary algorithms (EA) have been shown to be possible approaches for target prediction in metabolic engineering (Evangelista *et al.*, 2009), giving a mix of solutions containing KO or OU of the maximum enzyme reaction rate (Vmax) for any set of reactions. This method works by multiplying the parameters with a factor, which can go from 0 to any positive number higher than 1. Should the factor value (f) be 0<f <1 then it is considered an under expression of the reaction. In the case that f>1, then it is an over expression. If it is 0, then it is considered a reaction KO. If f is 1 then there is no change to the parameter. In this work we also used an EA optimization approach. However, we included inhibition parameters (Ki) alongside OU of reactions Vmax as possible values for the set of solutions, to provide better and more complete metabolic engineering strategies. Due to mathematical constraints on the reaction kinetics, Ki cannot have a value of zero, so KO was not a possibility, as can be exemplified in (1), where Km is the Michaelis-Menten constant. However, under expression of Vmax can be considered as KO, should it be biologically viable.

$$\frac{Vmax * A * B}{(Km1 + A) * \left(1 + \frac{Inhib}{Ki}\right) * \left(Km2 * \left(1 + \frac{Inhib}{Ki}\right) + B\right)} \quad (1)$$

To check for the most important targets, sets of solutions were divided in size with up to three or five alterations (the word alterations will from now on be used whenever a mix of knockouts, overexpressions, underexpressions and/or mutations are referred to). All Vmax and Ki belonging to the reactions present in Figure 1 were selected as possible parameters for OU, including drain reactions for the three AAA, AcCoA, Serine, PRPP, 3DHQ, 3DHSK, Glu and Gln. Some parameters were not selected as possible targets, since they may not be biologically viable to alter, such as the ETC, ATP synthase and co-metabolites pathways (ATP, NAD, NADP, etc.). Each optimization run had 200 generations, with a population of 100 for each generation. The objective function for the optimization was the concentration of a single AAA (L-Phe, L-Trp or L-Tyr), depending on the AAA that was being optimized. The results were later filtered based on the highest fitness found, with only solutions having >90% of the highest fitness value being selected for further analysis. Since the dynamic model doesn't incorporate genes, the optimization results were given in parameter changes for specific reactions. Therefore, all solutions were translated into genomic alterations in section 3 of this paper, for a better comprehension of the reader.

3. RESULTS

3.1 Wild type (WT) simulations

OptimModels allows the simulation of the kinetic model in an unaltered state (referred to as the WT) and with altered parameters (representing strain mutations). It's by comparing the changes between these two simulations that we assess the impact brought by specific mutated strains. Some of the metabolic concentrations of a WT simulation are shown in Table 1.

3.2 Optimization

The optimization process resulted in several possible strategies for creating *E. coli* producing strains, for all three AAA.

Table 1 Steady state concentration of AAA and other metabolites in a WT simulation.

Metabolite	Concentration (mM)	Metabolite	Concentration (mM)
L-Phe	0.85560	PYR	0.30351
L-Trp	0.01198	2DDA7P	0.02283
L-Tyr	0.44248	CIT	0.10101
G6P	3.44617	AKG	1.29453
E4P	0.10386	Acetate	0.00038
PEP	2.59049	Gln	0.07388
ATP	4.14085	Glu	16.87863

The best optimization solution found for L-Phe includes feedback resistance of pheA and aroH, over expression of tyrB and aroB, and under expression of a reaction unique to the model that represents the use of L-Phe by the cell: Phe_drain. As can be seen in Table 2, $aroH^{fbr}$, ↑aroB and ↓Phe_drain are always present among the top results obtained for L-Phe, implying their importance in L-Phe regulation. Other common targets include ↑tyrB and ↑pheA. Since L-Phe is important to the cell's metabolism, optimizations without Phe_drain were made, to ensure viable metabolic engineering strategies, without altering L-Phe's natural intake by the cell. In these new results, apart from the already mentioned alterations, ↑aroC and ↑aroH were also common in the solutions.

For L-Tyr optimization, the best solution includes feedback resistance for aroH, increasing feedback inhibition of pheA (+inhib), over expression of tyrB and aroB and under expression of Tyr_drain (reaction which represents use of Tyr by the cell, similar to Phe_drain. There is also one for L-Trp, called Trp_drain). In Table 2 it can be seen that ↑aroB, ↑tyrB,

pheA^{+inhib} and ↓Tyr_drain are indispensable for better L-Tyr production. Other common targets include ↑aroG and ↓pheA.

Similar to L-Phe, more optimizations were made, but without Tyr_drain. In these results, apart from the previously mentioned alterations, ↑aroC and ↑aroL were also common targets. These results seem similar to L-Phe, which is to be expected since they share a common pathway, all the way to the reaction PPHN.

For L-Trp optimization, the best solution includes feedback resistance of trpD, overexpression of trpE, trpD and trpC, and under expression Trp_drain. In Table2 it is shown that ↑trpD^{fbr}, ↑trpE and ↓Trp_drain are indispensable for a higher L-Trp concentration. Other common targets include increasing pheA inhibition by L-Phe or aroG^{fbr}.

Like for the previous AAA, more optimizations were made without Trp_drain. Apart from the previous alterations, ↓ppc and ↑sucA were also common. When comparing to L-Phe and L-Tyr, SHK pathway reactions seem of less importance for L-Trp optimization. As seen in Table 2, the mutated strains obtained by optimization, even with a smaller number of alterations, show higher [AAA] than the WT strain, although expectedly lower than solutions with more alterations.

3.3 Optimization results vs experimentally tested strategies

There are several examples in the literature of successful strategies for AAA increased production. These use some of the over/under expressions or feedback resistant mutants that are present in our own optimizations' solutions. However, our goal was not only to find new targets for metabolic engineering, but also to discover new and different sets of targets.

In the literature, changes to pheA expression are the most used target for L-Phe producing strains, whether by over expression, feedback resistance mutation or a combination of both (Sugimoto *et al.*, 1987; Backman *et al.*, 1990; Tatarko and Romeo, 2001; Gottlieb, Albermann and Sprenger, 2014; Ding *et al.*, 2016; Liu *et al.*, 2018). Our best optimization solutions also present pheA^{fbr} or pheA↑ as an ideal target. The first reaction of the shikimate pathway, determined by the genes aroG, aroF and aroH is also overexpressed or mutated to reduce feedback inhibition. Either aroF or aroG are found in literature as possible targets (Báez-Viveros *et al.*, 2004; Yakandawala *et al.*, 2008; Gottlieb, Albermann and Sprenger, 2014; Liu *et al.*, 2018). In this our solutions differ from the literature, presenting aroH as the best target out of the three isozymes. In the literature we found no strategies where reactions that use L-Phe as reactant are knocked out or under expressed. This could mean the inviability of our strategies that possess these reactions, represented as Phe_drain. Further studies into the L-Phe pathway would be needed to properly assess this hypothesis, since Phe_drain represents a broad range of reactions. When it comes to L-Tyr producing strains, the ones found in literature have tyrA and aroG as their main targets for over expression or feedback resistance mutations

Table 2 Optimization solutions for each AAA and corresponding AAA concentration. ↑ Stands for over expression, ↓ for under expression, ^{fbr} for feedback resistance and $^{+inhib}$ for increasing inhibition.

Solutions (L-Phe)	**[L-Phe] (mM)**	**Solutions (L-Tyr)**	**[L-Tyr] (mM)**	**Solutions (L-Trp)**	**[L-Trp]**
pheA^{fbr}, aroH^{fbr}, ↑tyrB, ↑aroB, ↓Phe_drain	10.03	↑tyrB, ↑aroB, pheA^{+inhib}, aroH^{fbr}, ↓Tyr_drain	11.94	↑trpD^{fbr}, ↑trpE, ↑trpC, ↓Trp_drain	0.39
pheA^{fbr}, ↑aroH, ↑aroB, ↑tyrB, ↓Phe_drain	9.80	↑tyrB, ↑aroB, pheA^{+inhib}, ↑aroG, ↓Tyr_drain	11.94	↑trpD^{fbr}, ↑trpE, aroG^{fbr}, ↓Trp_drain	0.31
↑pheA, aroH^{fbr}, ↓tyrA, ↑aroB, ↓Phe_drain	8.37	↑tyrB, ↑aroB, ↓pheA^{+inhib}, ↓Tyr_drain	11.6	↑trpD^{fbr}, ↑trpE, pheA^{+inhib}, ↓Trp_drain	0.31
↑tyrB, aroH^{fbr}, ↑aroC, ↑aroB, ↑pheA	6.84	↑tyrB, ↑aroB, ↑aroC, aroH^{fbr}, ↑aroL	10.78	↑trpD^{fbr}, ↑trpE, ↑trpC, ↓ppc	0.25
↑tyrB, ↑aroH, ↑aroC, ↑aroB, ↑pheA	6.81	↑tyrB, ↑aroB, ↑aroC, ↓trpE, ↑aroL	3.93	↑trpD^{fbr}, ↑trpE, ↑trpC, ↑sucA	0.23
pheA^{fbr}, aroH^{fbr}, ↓Phe_drain	5.54	↑aroB, aroH^{fbr}, ↓Tyr_drain	6.05	trpD^{fbr}, ↑trpE, ↓Trp_drain	0.18
↑pheA, aroH^{fbr}, ↑aroB	2.28	↑aroB, ↑aroC, aroH^{fbr}	2.78	trpD^{fbr}, ↑trpE, trpC	0.09

(Lütke-Eversloh and Stephanopoulos, 2007, 2008; Chávez-Béjar *et al.*, 2008; Juminaga *et al.*, 2012; Kim *et al.*, 2015), with Santos, Xiao and Stephanopoulos, 2012 also knocking out pheA. The solutions obtained in this paper show an under expression of pheA, which does a similar, less definitive, role to pheA KO. The fbr mutations on tyrA and aroG are also present in the top optimization results (data not shown) although not as common as the solutions shown in Table 2. Another common over expression found in both literature and in this paper is for aroL (Lütke-Eversloh and Stephanopoulos, 2007; Juminaga *et al.*, 2012). Again, reactions that use L-Tyr as reactant are not found in these strategies, which could imply the inviability of altering these reactions. At the same time, it could also mean that there is no need to alter these reactions, with other alternatives being more worthwhile.

As for L-Trp, most strategies found in the literature aim to increase flux in the L-Trp specific pathway, with overexpression of trpDE (Zhao *et al.*, 2011; Liu, Duan and Wu, 2016) or even most of the pathway (Chen *et al.*, 2018; Tröndle *et al.*, 2018). These also include mutation to cause fbr in trpE, but not in trpD. In this paper's optimization results $trpD^{fbr}$ is a vital mutation for higher L-Trp production. Although $trpE^{fbr}$ is also a common occurrence in this paper's solutions, it provides a lower L-Trp concentration (data not shown). Unlike for L-Phe and L-Tyr, there is data in literature for the KO of tnaA (Zhao *et al.*, 2011; Tröndle *et al.*, 2018), a reaction that uses L-Trp as a reactant to produce indole and pyruvate. This means that this paper's strain solutions with Trp_drain (which represents tnaA and others) are viable. In fact, tnaA is a sort of regulator for L-Trp, preventing the cell from accumulating it.

The strategies found in the literature are composed of more than five alterations, while the solutions found in this paper only have up to five, making it difficult to properly compare entire strategies instead of individual targets. Further optimizations with higher number of possible parameter changes are thus needed. However, the initial results show high potential in using kinetic models for strain optimization. These results also show that a diverse strategy, including a mix of OE, KO and fbr mutations is highly desirable.

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