Improved Synthesis of Flavonoids by Simulation of Nargenine Chalcone Biosynthetic Reaction

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MS and PWR designed the study, performed the statistical analysis and wrote the protocol and first draft of the manuscript. Authors RNK and SA performed computational work and managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Metabolic Control Analysis provides a quantitative description of concentration dynamics with the change in system parameters. A metabolic Control Analysis aids determination of the threshold value of metabolites involved in a reaction and also helps to understand the role of various parameters in a reaction. In this work, a metabolic model of a Naringenine chalcone biosynthetic reaction is defined and a time series simulation was carried out based on the law of Mass action. Initial concentration of p-Coumaroyl-CoA and Malonyl-CoA were taken $5.0 \times 10^{-2}$ mM $2.2 \times 10^{-3}$ mM.
respectively. This concentration was then simulated over time for 10 seconds to find the steady state. Final concentration of Naringenine chalcone, COA, and CoA becomes 8.593946e-004 mM after 5.00 second of simulation at reaction constant 6.587753e-005 mM^2*ml/s. Steady state solution shows that Initial concentration of Naringenine chalcone was 2.199777e-003 mM which is eventually converted into 2.785128e+013 seconds half-life concentration of product at 7.898e-017 mM/s rate and 0.0000000e+000 mM^2*ml/s rate constant. Phenylpropanoid pathway was analysed to predict all the enzymes that can maximise and minimise the concentration of Malonyl-CoA and P-Coumaroyl-CoA which leads to flavonoid biosynthesis. In the Phenylpropanoid pathway four enzymes Phenylalanine/tyrosine ammonia lyase, trans-cinnamate 4-monoxygenase, Phenylalanine ammonia lyase, maximise the flavonoid biosynthesis. This analysis shows that other enzymes minimise the concentrations of Malonyl-CoA and P-coumaroyl-CoA, these are Cinnamoyl-CoA reductase, shikimate O hydroxyl transferase HCT), Oxidoreductase. Furthermore, Protein domain analysis of chalcone synthase mutants ( 1jwX Medicago sativa and 4yjy from Oryza sativa) was done to predict structural features to understand reaction mechanism and structure-based engineering to maximise flavonoid biosynthesis. Natural sequence variation CHS G256A, G256V, G256L, and G256F mutants of residue 256 reduce the size of the active site cavity but quick diversification of product specificity occurs. The threshold concentration of Malonyl-CoA and P-coumaroyl-CoA were predicted, maximisation of this concentration leads to enhanced flavonoid biosynthesis. Inhibition of few enzymes may also maximise the flavonoid biosynthesis if appropriate inhibitors are used and a constant supply of Malonyl-CoA and P-Coumaroyl-CoA is maintained using activator molecules. Chalcone synthase Mutants diversify product specificity that occurs without loss of catalytic activity and any conformational changes.

**Keywords:** Naringenine chalcone; phenylpropanoid pathway; flavonoid biosynthesis; metabolic control analysis.

### ABBREVIATIONS

CHS : Chalcone Synthase  
PKS : Polyketide Synthase  
AA : Amino acid  
MCA : Metabolic Control Analysis

### 1. INTRODUCTION

Biosynthesis of secondary metabolites can be affected by different reasons. Analysis of reaction kinetics is essential to understand the dynamic changes that occur in a complex biosynthetic pathway. Polyketides are synthesised from acyl CoA precursor by Polyketide synthase. Wide variety of these secondary metabolites are found in bacteria, fungi, and plants [1]. Three distinct classes of Polyketide synthases are found [2] Large Modular and multifunctional enzymes Type I PKSs, Monofunctional and dissociable complexes enzymes type II PKSs are usually found in bacteria [3]. Small Homodimeric enzymes Plant PKSs, type III group [4] as well as in bacteria and fungi [5]. Type III PKSs catalyse decarboxylative condensations of malonyl units with a CoA-linked starter molecule in several iterations [3]. A typical type III PKS reaction is accomplished in 3 steps (i) loading of a starter molecule (ii) the extension of the polyketide chain (iii) cyclisation of the linear intermediate [6]. Two novel type III PKS involved in the biosynthesis of long chain pyrones, the study of steady state kinetic parameter shows that PKS18 protein has several folds higher catalytic efficiency for long chain acyl Co-A as compared with small chain precursors. PKS synthesises polyketide by initialising a starter molecule and followed by repetitive decarboxylation and condensation of Co-enzyme. A analogue of simple carboxylic acid PKS uses a single active site for multiple condensation reaction [7]. Flavonoid biosynthetic pathway derives their substrate from the shikimate pathway [8]. Chalcone synthase is the key enzyme that synthesises Naringenine chalcone using P-coumaroyl-CoA and Malonyl-CoA. This Naringenine chalcone acts as a substrate in various biosynthetic pathways and is converted into isoflavonoids, flavones, and flavonols. In Synthetic biology biological systems are artificially designed and manipulated in organisms by remodeling their metabolic pathways to synthesise product with desired activity [9]. This scientific approach began to emerge in the 1980s, and in 1991, Bailey formulated the expression “metabolic engineering” to describe the techniques and systems to enhance metabolite synthesis in organisms [10]. Manipulating the endogenous pathway or importing components of a heterogenous pathway are the two different
approaches to synthesise the desired product or new enzyme which may be non-native to the host [11]. This "combinatorial biosynthesis" method has been practiced using the enzymes of plant metabolic pathways of different organisms in host organisms for over 30 years [12]. Few microbial hosts are widely used for metabolic engineering such as Escherichia coli, Saccharomyces cerevisiae and Pseudomonas species that produce large quantities of natural products, in large cultures fermentation using optimisation approaches and genetic engineering methods [13].

Metabolic pathways interact and create complex metabolic networks to modulate the range of biosynthetic products that are analysed and synthesised in metabolic engineering. This needs integrative information systems, which represent genes, enzymes, and metabolic pathways on the same interface [14]. Dynamic models facilitate interactive modeling and simulation of complex metabolic processes. Metabolic engineering can be applied in the biomedicine and biotechnology field especially in the detection of specific genetic defects [15].

In this work, a metabolic model of a CHS catalysed Naringenine Chalcone Biosynthetic reaction is defined, and a time series simulation is carried out. The initial concentrations of p-Coumaroyl-CoA and Malonyl-CoA in M. tuberculosis were taken from previous existing literature i.e. 5.0*10^-2 mMol and 2.2*10^-3 mMol respectively [16]. This concentration was then simulated over time for 10 seconds to find the steady-state.

Metabolic control analysis provides a focused approach to identify and characterise metabolic reactions in cell behavior, which may be used as targets for developing processes. Phenylpropanoid pathway was analysed to predict all the enzymes that can maximise and minimise the concentration of Malonyl-CoA and P-Coumaroyl-Co A which leads to flavonoid biosynthesis. Potential enzymes and weak enzymes were analysed in phenylpropanoid pathways. Protein domain analysis of chalcone synthase was done to predict structural features product binding site, active site, Malonyl-CoA binding site, and dimer interface to understand reaction mechanism and structure based engineering to maximise flavonoid biosynthesis.

Fig. 1. Reaction mechanism of Naringenine Chalcone Biosynthesis
1.1 Naringenine Chalcone Biosynthetic Reaction

The synthesis of many chalcones and releases of Co-A enzyme for the elongation process, the reaction is catalysed by Chalcone Synthase. Binding of substrate and intermediate in polyketide elongation can be deduced from the position of CoA Thioester and product analogue in the active site of CHS. p-Coumaroyl CoA binds to CHS at Cys164, His303 attack the thioester linkage which transfers Coumaroyl moiety to Cys 164 and Asn 336 makes H bond with Carbonyl Oxygen of thioester which stabilizes the formation of reaction intermediates. This allows dissociation of CoA from the enzyme and binding of malonyl CoA. Methylene carbon of Malonyl-CoA comes in close proximity to the carbonyl carbon of coumaroyl thioester which is covalently attached. A carbanion is formed as a result of decarboxylation. Thiolate anion of Cys164 is released and the coumaroyl group is transferred to the moiety of the Co-A thioester. This elongated diketide Co-A is trapped at an oxanion hole by Cys164 which releases Co-A and permits two additional round of elongation to synthesise tetraketide reaction intermediates [17].

2. MATERIALS AND METHODS

2.1 KEGG

Database Phenylpropanoid pathway and annotation of Naringenine Chalcone biosynthetic reaction is retrieved from the KEGG database.

2.2 Gepasi

Gepasi is a software system for the simulation of chemical and biochemical kinetics. Kinetic modeling is about describing the system evolution in time. Using kinetic modeling, the state of the system can be predicted when it stops evolving in time. Such states are called steady states. Gepasi provides multiple kinetic types that are predefined, few are given here(i) the common Henry-Michaelis-Menten (ii) inhibition or activation mechanism (iii) Hill Kinetics and it is equivalent for reversible reaction(iv) several allosteric and multireactant mechanisms.

Table 1. Annotation of reaction produces CoA and Naringenine chalcone obtained from Kegg database

| Entry         | Reaction                                                      | Annotation                                      |
|---------------|----------------------------------------------------------------|------------------------------------------------|
| Name          | Malonyl-CoA:4-Coumaroyl-CoA malonyltransferase (cyclizing)     |                                                |
| Definition     | p-Coumaroyl-CoA + 3 Malonyl-CoA => Naringenine chalcone + 4 CoA | S1a-S2a:*C1b-C1b                               |
| Equation       | C00223 + 3 C00083 <==> C06561 + 4 C00010 + 3 C00011            | C5a-C5a:C8y+*S2a:C2b+05a-C2b+05a                |
| Reaction class | RC00004 C0010_C00083                                           |                                                |
|                | C0010_C00223                                                  |                                                |
|                | RC02726 C00222 C06561                                         |                                                |
| Enzyme         | 2.3.1.74 Naringenine-chalcone synthase;chalcone synthase;flavanone synthase;6'-deoxychalcone synthase; chalcone synthetase;DOCS; EC 2.3.1.170 (6' deoxychalcone) | Substrate: CHSmalonyl-CoA [CPD:C00083]; 4-coumaroyl-CoA [CPD:C00223] |
| Pathway        | m00941                                                        | Flavonoid biosynthesis                         |
|                | m01100                                                        | Metabolic pathways                             |
|                | m01110                                                        | Biosynthesis of secondary metabolites          |
| Module         | M00137                                                        | Flavanone biosynthesis, phenylalanine => Naringenine |
| Orthology      | K00660                                                        | chalcone synthase [EC:2.3.1.74]                |
2.3 Chalcone Synthase

Structures Chalcone Synthase 2 from *Oryza sativa* (4jyy) and *Medicago sativa* (1jwx) is retrieved from (PDB database). Chalcone Synthase annotation is obtained from the literature [18–20].

2.4 Pymol

Pymol visualisation tool (http://www.pymol.org) is an open-source software, created by [21] to be used for visualisation of structural features, specific active site, Malonyl-CoA binding site, product binding site and dimer interface in chalcone synthase 2 structure of *Oryza sativa* and *Medicago sativa*.

2.5 Input

Data about Naringenine chalcone biosynthetic reaction was collected from the existing literature. The reaction to be simulated was entered on the add reaction page on Gepasi using standard notation.

2.6 Definition of Reaction Kinetics

Substrates were displayed in the input window involved in Naringenine chalcone biosynthesis. Mass action (reversible) kinetics was used among the list of all the predefined reactions in the model and list of possible kinetic type. This facilitates Pharmacokinetic simulation using Compartment and metabolite properties. Multiple compartments can be defined with their volume; volume variation is included in calculations to facilitate chemical and pharmacokinetic simulation. Metabolites were defined by property set. Initial concentration may be allowed to vary or fixed, program calculate arbitrary functions for parameters, given by the user [22].

2.7 Initialisation

The concentrations of metabolites (using initial concentrations) were scanned from a minimum concentration (1mMol) to maximum (2mMol) using regular grid and 32 iterations.

Concentrations of the substrates and products were initialised. The concentration of the metabolites involved in the reaction was entered as $5 \times 10^{-2}$, $2.2 \times 10^{-3}$ mMol for p-coumaroyl-CoA and malonyl-CoA respectively, and 0mMol for Naringenine chalcone, CO2, and CoA [16], then simulation was carried out using the Evolutionary Programming method.

2.8 Scanning of Concentration during Simulation

After Simulation, the concentration was scanned between a minimum (1mM) and maximum values (2 mM) to observe the changes. The simulation can also be observed as a dynamic graph in running simulation (time course window).

2.9 Optimisation

The concentrations were optimised over the generations. The optimisation was achieved by minimising the function which was applied to minimise from initial concentration to infinity value over hundred (100) and five hundred (500) number of generations and populations respectively.

2.10 Fitting

Regression is the process of adjusting the values of some parameters of a model such that the model can reproduce the behaviour of the real system. Usually, this means that several parameters are adjusted in simulation to match a set of (with minimum deviation) experimentally measured variables.

The fitting results show real behaviour. Optimisation is the search for a maximum or minimum value of a function. It is used in pharmacology to minimise the concentration of toxic substances over many generations. The Steady State concentration of metabolites was minimised using the evolutionary programming method for 100 generations of 500 people population. Then, the model was optimised using the evolutionary method for 100 generations using 500 populations to minimise the concentration of reactants: p-coumaroyl-CoA and Malonyl-CoA (Fig. 2).

2.11 Evolutionary Programming

The fitting (regression) of Naringenine Chalcone biosynthetic reaction model was performed to reproduce the behavior of a real system for 100 generations and 500 populations using evolutionary programming method. Evolutionary Programming was used for fitting (regression) to simulate the behaviour of a real system using a hundred (100) generations and five hundred
(500) populations. Fitting, or regression, is the process of adjusting the values of some parameters of a model such that the model can reproduce the behaviour of the real system. Usually, this means that several parameters are adjusted in simulation to match a set of experimentally measured variables with minimum deviations. The fitting or regression results can be obtained from the menu bar (Figs. 2 and 3).

3. RESULTS AND DISCUSSION

3.1 Simulation of Narinegenin Chalcone Biosynthetic Reaction

3.1.1 Results of integration

Initial concentrations of p-Coumaroyl-CoA and Malonyl-CoA were 1.000000e+000 mM and 2.000000e+000 mM respectively after (5.00e+000 s) then changed into 8.422792e-001 mM and 1.842279e+000 mM respectively at -1.065877e-008 mM*ml/s J(R1). Product concentrations (Naringenine chalcone and CoA) were very low 1.157721e+000 mM at initial stage. At -1.915410e-008 mM*ml/s J(R1), final concentration of both reactants became 1.620410e+000 mM and final concentration of both products became 1.379590e+000 mM. At equilibrium constant J(R1) 1.257098e-008 mM*ml/s, final concentration of both reactants became 1.152292e+000 mM and concentration of Naringenine Chalcone became 1.847708e+000 mM, CoA concentration was significantly raised up to 8.477076e-001 mM. At equilibrium constant 0.000000e+000 mM*ml/s J(R1), final concentration of both products became 2.000000e+000 mM and 1.000000e+000 mM respectively. Significant predictions were obtained at 1.257098e-008 mM*ml/s equilibrium constant J(R1) among all these predictions (Table 2).

Steady state analysis shows same prediction at this state. At J(R1) -4.201448e-010 mM*ml/s, concentration of p-Coumaroyl CoA is 8.422792e-001 mM, which is higher than 1.842279e+000 mM of Malonyl-CoA at 4.201e-010 mM/s reaction rate constant. Reaction rate was -4.201e-010 mM/s for product formation and steady state concentration was 1.157721e+000 mM with half life of 2.755528e+009 seconds. Eventually at 0.000000e+000 mM*ml/s equilibrium constant J(R1), concentration of reactants become 1.620410e+000 mM and concentration of all the products become 1.379590e+000 mM at 0.000e+000 mM/s reaction rate with half life of 1.797693e+030 s (reactants and products), these results shows sustainability in product formation. Again steady state concentration was changed at same reaction rate and half life, concentration of p-Coumaroyl-CoA and Malonyl-CoA was 1.152292e+000 mM, concentration of Naringenine chalcone and CoA were 1.847708e+000 mM and 8.477076e-001 mM at this state, subsequent concentration of Coumararoyl CoA and Naringenine chalcone became 2.000000e+000 mM and 1.000000e+000 mM for Malonyl-CoA and CoA at same parameters. These results show that product concentration of CoA is significantly increased and this may favour the formation of diverse flavonoids. Although Naringenine chalcone concentration is not reached up to desired level.

In last two state product concentration of Naringenine chalcone were 1.847708e+000 mM and 2.000000e+000 mM. Coenzyme A concentration was 8.477076e-001 mM and 1.000000e+000 mM (Table 3). At 6.587753-005 mM/s J(R1), initial concentration of reactant p-Coumaroyl Co A and Malonyl Co-A were 5.0000-002 mM & 2.20000-003 mM. The concentration of Co A is significantly increased in the last two states which are in favour of diverse kinds of flavonoid formation. After optimisation it was increased up to 4.914061e-002 mM and 1.340605e-003 mM. Initial concentration (0.000000e+000 mM) of products for Naringenine chalcone, CO2 and Co A, increased up to 8.593946e-004 mM after optimisation at 6.587753e-005 mM*ml/s (Table 4). Here, Naringenine Chalcone concentration is significantly increased, this indicate improved prediction in favour of Naringenine Chalcone Biosynthesis.

Steady state Analysis: Initial concentration of p-Coumaroyl CoA and Malonyl Co-A 4.78022e-002 mM. 2.199777e-003 mM converted into 2.199777e-003 mM product concentration at 7.898298e-017 mM. Steady state solution shows that Naringenine chalcone, CO2 and Co A was 2.199777e-003 mM which is eventually converted into 2.785129e+013 s half life concentration of product at 7.898e-017 mM/s rate and J(R1) 0.000000e+000 mM*ml/s rate constant. Concentration of Naringenine Chalcone become 2.199777e-003 mM, when p-Coumaroyl-CoA and 3 Malonyl-CoA concentration become 2.226928e-007 mM and 4.780022e-002 mM respectively at the rate of 7.898e-017 mM.
Concentration dynamics follows the law of Mass Action as revealed by above results and depicted by time course graph. This shows the fall in concentrations of substrates (Coumaroyl-CoA and Malonyl-CoA) with time and the rise in the concentration of the product. The concentration of Naringenine Chalcone attains a constant value after time 9.2 seconds. Reactants concentrations decrease as the product concentrations increased. When product concentrations increased it starts to be converted into substrate concentrations. The rate is proportional to the product of the concentrations of the two reactants because the collision frequency increases with concentration for a bimolecular reaction.

The Law of Mass Action expresses an equilibrium system, According to the Law of Mass Action expression of Naringenine chalcone Biosynthetic Equation

\[
[Naringenine \text{ chalcone} + [4\text{CoA} + [3\text{CO}_2 / [p-C\text{oumaroyl} - \text{CoA} + [3\text{Malonyl} - \text{CoA} = J][R1}
\]

\[
[Naringenine \text{ chalcone}(8.593946e - 004 \text{ mM})][\text{CoA}(8.593946e - 004 \text{ mM})][4 \text{Co}_2(8.593946e - 004 \text{ mM})]3 / [p-C\text{oumaroyl} - \text{CoA}(4.914061e - 002 \text{ mM})][3\text{Malonyl} - \text{CoA}(1.340605e - 003 \text{ mM})]3 = J[R1(6.587753e - 005 mM * ml/s)
\]

\[
[Naringenine \text{ chalcone}(2.199777e - 003 \text{ mM})][4\text{CoA} (2.199777e - 003 \text{ mM})][4 \text{Co}_2(2.199777e - 003 \text{ mM})][p-C\text{oumaroyl} - \text{CoA}(4.780022e-002 \text{ mM})][\text{Malonyl} - \text{CoA}(1.842279e+000 \text{ mM})]3 = J[R1(-7.898298e-017 mM*ml/s)]
\]

Fig. 2. Modelling and simulation of Naringenine biosynthetic reaction, Time course graph after 7.2 seconds
Table 2. Illustration of kinetic parameters and reaction components with initial and final concentration of reactants and products of reversible reaction $R_1$ (Mass action (reversible)) at $k_1=1.0000e+000$; $k_2 = 1.0000e+000$, Compartment $V$(compartment) = $1.0000e+000$. The concentration of metabolites is scanned from a minimum concentration (1mMol) to maximum (2mMol)

| Kinetic parameters results of integration after (5.00e+000 s) | Reaction Components | Initial | Final       |
|--------------------------------------------------------------|---------------------|---------|-------------|
| $J( R_1) = -1.065877e-008$ mM*ml/s                         | p-Coumaroyl-CoA     | 1.000000e+000 mM | 8.422792e-001 mM |
|                                                             | 3Malonyl-CoA        | 2.000000e+000 mM | 1.842279e+000 mM |
|                                                             | Naringenine chalcone| 1.000000e+000 mM | 1.157721e+000 mM |
|                                                             | 3CO2                | 1.000000e+000 mM | 1.157721e+000 mM |
|                                                             | 4CoA                | 1.000000e+000 mM | 1.157721e+000 mM |
| $J( R_1) = -1.915410e-008$ mM*ml/s                         | p-Coumaroyl-CoA     | 2.000000e+000 mM | 1.620410e+000 mM |
|                                                             | 3Malonyl-CoA        | 2.000000e+000 mM | 1.620410e+000 mM |
|                                                             | Naringenine chalcone| 1.000000e+000 mM | 1.379590e+000 mM |
|                                                             | 3CO2                | 1.000000e+000 mM | 1.379590e+000 mM |
|                                                             | 4CoA                | 1.000000e+000 mM | 1.379590e+000 mM |
| $J( R_1) = 1.257098e-008$ mM*ml/s                          | p-Coumaroyl-CoA     | 1.000000e+000 mM | 1.152292e+000 mM |
|                                                             | 3Malonyl-CoA        | 1.000000e+000 mM | 1.152292e+000 mM |
|                                                             | Naringenine chalcone| 2.000000e+000 mM | 1.847707e+000 mM |
|                                                             | 3CO2                | 1.000000e+000 mM | 8.477076e-001 mM |
|                                                             | 4CoA                | 1.000000e+000 mM | 8.477076e-001 mM |
| $J( R_1) = 0.000000e+000$ mM*ml/s                          | p-Coumaroyl-CoA     | 2.000000e+000 mM | 2.000000e+000 mM |
|                                                             | 3Malonyl – CoA      | 1.000000e+000 mM | 1.000000e+000 mM |
|                                                             | Naringenine chalcone| 2.000000e+000 mM | 2.000000e+000 mM |
|                                                             | 3CO2                | 1.000000e+000 mM | 1.000000e+000 mM |
|                                                             | 4CoA                | 1.000000e+000 mM | 1.000000e+000 mM |
Table 3. Steady-state solution for the reaction shows substrate concentration between 1 to 2 nmol, half-life, and rate of reaction to attain chemical equilibrium

| Steady-State Solution (Results of integration) After 5.00e+000 s | Reaction Components | Concentration | Tt (half-life) | Rate |
|---------------------------------------------------------------|--------------------|---------------|---------------|------|
| J(R1) = -4.201448e-010 mM*ml/s                               | p-Coumaroyl-CoA    | 8.422792e-001 mM | 2.004735e+009 s | 4.201e-010 mM/s |
|                                                               | 3Malonyl-CoA       | 1.842279e+000 mM | 4.384867e+009 s | 4.201e-010 mM/s |
|                                                               | Naringenine chalcone | 1.157721e+000 mM | 2.755528e+009 s | -4.201e-010 mM/s |
|                                                               | 3CO2               | 1.157721e+000 mM | 2.755528e+009 s | -4.201e-010 mM/s |
|                                                               | 4CoA               | 1.157721e+000 mM | 2.755528e+009 s | -4.201e-010 mM/s |
| J(R1) = 0.000000e+000 mM*ml/s                                | p-Coumaroyl-CoA    | 1.620410e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 3Malonyl-CoA       | 1.620410e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | Naringenine chalcone | 1.379590e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 3CO2               | 1.379590e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 4CoA               | 1.379590e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
| J(R1) = 0.000000e+000 mM*ml/s                                | p-Coumaroyl-CoA    | 1.152292e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 3Malonyl-CoA       | 1.152292e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | Naringenine chalcone | 1.847708e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 3CO2               | 8.477076e-001 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 4CoA               | 8.477076e-001 mM | 1.797693e+308 s | 0.000e+000 mM/s |
| J(R1) = 0.000000e+000 mM*ml/s                                | p-Coumaroyl-CoA    | 2.000000e+000 mM, | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 3Malonyl-CoA       | 1.000000e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | Naringenine chalcone | 2.000000e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 3CO2               | 1.000000e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
|                                                               | 4CoA               | 1.000000e+000 mM | 1.797693e+308 s | 0.000e+000 mM/s |
Table 4. R1 (Mass action (reversible)), $k_1 = 1.0000e+000$, $k_2 = 1.0000e+000$, $V$ (compartment) = $1.0000e+000$ Optimisation of CHS by minimizing the function from initial concentration to infinity value over 100 generations for 500 populations

| Kinetic Parameters | Results of Integration | Reaction Components | Initial | Final |
|--------------------|------------------------|---------------------|---------|-------|
| R1 (Mass action (reversible) (after $1.00e+001$ s)) $J(R1) = 6.587753e-005$ mM*ml/s | p-Coumaroyl-CoA | 5.000000e-002 mM | 4.914061e-002 mM |
|                    | 3Malonyl-CoA           | 2.200000e-003 mM   | 1.340605e-003 mM |
|                    | Naringenine chalcone   | 0.000000e+000 mM   | 8.593946e-004 mM |
|                    | 3CO2                   | 0.000000e+000 mM   | 8.593946e-004 mM |
|                    | 4CoA                   | 0.000000e+000 mM   | 8.593946e-004 mM |
| (after $5.00e+000$ s) $J(R1) = 6.587753e-005$ mM*ml/s | p-Coumaroyl-CoA | 5.000000e-002 mM | 4.914061e-002 mM |
|                    | 3Malonyl-CoA           | 2.200000e-003 mM   | 1.340605e-003 mM |
|                    | Naringenine chalcone   | 0.000000e+000 mM   | 8.593946e-004 mM |
|                    | 3CO2                   | 0.000000e+000 mM   | 8.593946e-004 mM |
|                    | 4CoA                   | 0.000000e+000 mM   | 8.593946e-004 mM |

Table 5. Steady-state solution after optimisation

| Steady-state solution (Results of integration) | Reaction Components | Concentration | $T_t$ (half-life) | rate |
|-----------------------------------------------|---------------------|---------------|------------------|------|
| After $1.00e+001$ s (chemical equilibrium) $J(R1) = -7.898298e-017$ mM*ml/s | p-Coumaroyl-CoA | 4.780022e-002 mM | 6.051965e+014 s | 7.898e-017 mM/s |
|                                                | 3Malonyl-CoA       | 2.226928e-007 mM | 2.819504e+009 s | 7.898e-017 mM/s |
|                                                | Naringenine chalcone | 2.199777e-003 mM | 2.785128e+013 s | -7.898e-017 mM/s |
|                                                | 3CO2               | 2.199777e-003 mM | 2.785128e+013 s | -7.898e-017 mM/s |
|                                                | 4CoA               | 2.199777e-003 mM | 2.785128e+013 s | -7.898e-017 mM/s |
| After $5.00e+000$ s $J(R1) = 0.000000e+000$ mM*ml/s | p-Coumaroyl-CoA | 4.780022e-002 mM | 6.051965e+014 s | 7.898e-017 mM/s |
|                                                | 3Malonyl-CoA       | 2.226928e-007 mM | 2.819504e+009 s | 7.898e-017 mM/s |
|                                                | Naringenine chalcone | 2.199777e-003 mM | 2.785128e+013 s | -7.898e-017 mM/s |
|                                                | 3CO2               | 2.199777e-003 mM | 2.785128e+013 s | -7.898e-017 mM/s |
|                                                | 4CoA               | 2.199777e-003 mM | 2.785128e+013 s | -7.898e-017 mM/s |
Optimisation is the search for a maximum or minimum value of a function. Optimisation is used in pharmacology to minimise the concentration of some toxic substances over many generations. Optimisation of the reaction was done by minimising the function from initial concentration to infinity value over 100 generations for 500 populations. The Steady-State concentrations of metabolites were minimised using the evolutionary programming method for 100 generations of 500 people population. Here in the above result, the minimum concentrations obtained of Coumaroyl-CoA and Malonyl-CoA were 4.914061e-002 mM and 1.340605e-003 mM respectively after 100 generation which are sufficient to initiate Naringenine Synthesis.

Finally, the model has been fitted again through the evolutionary method for 100 generations and 500 populations to represent the real behavior and to minimise the goodness of fit between the model results are similar to the optimisation results representing 0 goodness of fit value.

### 3.2 Structural and Conformational Changes in Chs 2 during Naringenine Chalcone Biosynthetic Reaction

Polyketide synthase Superfamily enzymes are found in plants, which include CHS stilbene synthase (STS), acridone synthase, pyrone synthase, bibenzyl synthase, and p-coumaroyl triacetic acid synthase [23]. Flavonoids are synthesised via the phenylpropanoid and polyketide pathway, one molecule of CoA-ester of cinnamic acid or derivatives such as coumaric or ferulic acid, and three molecules of malonyl-CoA undergo condensation in the presence of chalcone synthase, to produce Naringenine chalcone. Enzyme chalcone flavanone isomerise (CHI) converts chalcone into flavanone. These intermediates are further converted into leads to a different class of flavonoids in diverse reactions and pathways [24].

![Fig. 3. Modelling and simulation of Naringenine Chalcone biosynthetic reaction, Time course graph after 10 seconds attaining a constant value](image-url)
3.3 Phenylpropanoid Pathway Analysis

Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine. Phenylalanine is undergone subsequent deamination, hydroxylation methylation to produce coumaric acid and other acids with a phenylpropane (C6-C3) unit. Further Reduction of carboxyl groups of these acids produces a variety of aldehydes and alcohols. The alcohols are called monolignols, the starting compounds for the biosynthesis of lignin [25–27]. 4 coumarate Co A ligase, Phenylalanine/tyrosine ammonia lyase, trans-cinnamate4-monoxygenase, Phenyl alanine ammonia lyase work in Monolignol biosynthesis Flavanone biosynthesis module. Phenylalanine/tyrosine ammonia lyase only works in Flavanone biosynthesis module. These enzymes are necessary for the synthesis of flavonoid precursors (Malonyl-CoA and P-coumaroyl-CoA) (Table 6). p-coumarate 3-hydroxylase and cytochrome P450 family 98 subfamily enzymes divert the precursor molecules into multiple pathways, Phenylpropanoid biosynthesis (Aec00940); Biosynthesis of secondary metabolites(ec01110). These two enzymes indirectly lead to the Biosynthesis of other secondary metabolites. Biosynthesis of flavonoids gets affected (Table 7, Fig. 4). Previous studies have reported use of increased intracellular malonyl-CoA concentration to gain high yield of flavonoids using coordinated overexpression of four acetyl-CoA carboxylase (ACC) subunits and biotin ligase from Photorhabdus luminescens [28,29]. Malonate assimilation pathway from Rhizobium trifolii ensures constant supply of malonyl-CoA [30,31]. Malonyl-CoA is derived from the carboxylation of acetyl-CoA in E. coli, S. cerevisiae, and C. glutamicum, increasing ACC activity is required to use acetyl-CoA pool [31,32]. Modulation of Central Carbon metabolism is a focus of research to increase availability of acetyl-CoA as precursor molecule for malonyl-CoA synthesis and all malonyl-CoA-derived products. In E. coli, deletion of acetate and ethanol producing pathways that consume acetyl-CoA, leads to a 15-fold increased availability of malonyl-CoA. This further enhances the synthesis of chlorogluicinol (1280 mg/L) [33]. Another Pathway is glycolytic flux based on acetyl-CoA, partial elimination of anaerobic pyruvate carboxylation facilitate to the malonyl-CoA-dependent synthesis of noreugenin (53 mg/L). This is a pentaketide from the medicinal plant Aloe arborescens, in C. glutamicum [34].

Over expression of genes encoding the phosphoglycerate kinase and pyruvate dehydrogenase glycolytic pathway by increasing flux enable the accumulation of Naringenin (474 mg/L) in E. coli [28].

The ACC-independent formation of malonyl-CoA in Rhizobium trifolii is derived from malonate assimilation pathway that increased polyphenolic polyketide pinocembrin biosynthesis up to 15-fold (final titer 480 mg/L) using E. coli [30,35]. In S. cerevisiae, overexpression of AAE13 (Arabidopsis thaliana), encoding the malonyl-CoA synthetase, significantly increased microbial synthesis of resveratrol and fatty acid [36].

3.4 Domain Analysis to Predict Structural Features of Mutant Chalcone Synthase in Medicago sativa and Oryza sativa

These enzymes PKS (monomer size of 42–45 kDa) catalyse a series of decarboxylation, condensation, and cyclisation reactions [24]. Ferrer et al., analysed the three-dimensional structure of alfalfa CHS2, is an enzyme from Medicago sativa. Homodimer structure has two structural domain in each monomer of alpha CHS2 structure, each alfalfa CHS2 monomer composed of two structural domains [37]. The upper catalytic domain has four amino acids (Cys164, Phe215, His303, and Asn336) at the active site. The lower domain of CHS has a large pocket for the tetraketide formation which participate in chalcone formation i.e., Naringenine and Resveratrol) [24].

Prediction of the domain and structural features CHS like cd00831 domain (from 19 a.a. to 387 a.a.) was predicted in chalcone synthase 2 structure (4YJY) Oryza sativa with 476.331 bit score and from 16 a.a to 384 a.a. was predicted in Chalcone synthase 2 structure (1JWX) of Medicago sativa with 479.028 bit score. This domain belongs to cl09938 superfamily Chalcone and stilbene synthases; plant-specific polyketide synthases (PKS) and related enzymes, also called type III PKSs. Dimeric iterative PKS generates an array of diverse products after loading starter molecule at the active site using coenzyme A esters, followed by carboxylation condensation reaction which extends the polyketide chain.

Domain analysis reveals active site C167, H306, N339, and Malonyl-CoA binding site K58, R61, M62, F218, F268, G309, P310, A311 in chalcone
synthase of *Oryza sativa*. The product binding site and dimer interface were also predicted. (Table 7 and Fig. 5). Amino acid F218 is located at the surface of the Malonyl-CoA binding site, Amino acid G259 is located on the product binding site. Any change at this position can make a significant contribution to product specificity if implemented similarly as in the case of Mutant chalcone synthase 2 of *Medicago sativa*.

Domain analysis reveals active site C164,H303,N336 and Malonyl-CoA binding site K55,R58,M59,S215,F265,G306,P307,A308 in chalcone synthase of *Medicago sativa*. The product binding site and dimer interface were also predicted. (Table 8 and Fig. 6). Amino acid F218 is located on the surface of the Malonyl-CoA binding site. Amino acid G256 is located at the product binding site.

CHS active site consists of Gly256 residue on the surface that is in direct contact with the polyketide chain derived from Malonyl-CoA. Cavity and polyketide chain-length determination may be connected using position 256 in type III PKS. Tetraketide lactones are produced in more amounts using p-Coumaroyl-CoA as a starter molecule with G256A and G256V mutants. Further restrictions in cavity volume similarly in the G256L and G256F mutants, yield increasing levels of the styrylpyrone bis-noryangonin from a triketide intermediate. Natural sequence variations CHS G256A, G256V, G256L, and G256F mutants of residue 256 reduce the size of the active site cavity but quick diversification of product specificity occurs without the associated loss of substantial catalytic activity and any conformational changes due to evolution of side chain in CHS-like enzymes at position 256 [17].

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**Fig. 4. Phenyl Propanoid Pathway Potential nodes (green box) and weak nodes*red colour)* responsible for the biosynthesis of flavonoid as a precursor are as follows: 4 coumarate Co A ligase(6.2.1.1), Phenylalanine/tyrosine ammonia lyase (4.3.1.25), trans-cinnamate 4-monooxygenase (1.14.1491), Phenylalanine ammonia lyase (4.3.1.24). Involvement of Weak nodes (red outline) show less flavonoid biosynthesis, these are as follows: Cinnamoyl Co A reductase (1.2.1.44), shikimate O hydroxyl transferase(23.1.133 HCT), Oxidoreductase(1.14.13)
Table 6. Enzymes of phenylpropanoid pathways that lead to flavonoid biosynthesis. Multiple pathways are M00039 Monolignol biosynthesis, phenylalanine/tyrosine; M00137 Flavanone biosynthesis, phenylalanine =>Naringenine, ec00360 Phenylalanine metabolism; ec00940 Phenylpropanoid biosynthesis; ec01100 Metabolic pathways; ec01110 Biosynthesis of secondary metabolites

| SN | Name of Enzyme                          | EC number | Reaction                                                                 | Module/Pathway               |
|----|----------------------------------------|-----------|--------------------------------------------------------------------------|------------------------------|
| 1  | 4 coumarate Co A ligase                 | 6.2.1.12  | ATP + trans-Cinnamate + CoA <=> AMP + Diphosphate + Cinnamoyl-CoA         | M00137                      |
|    |                                        |           |                                                                          | M00039                      |
| 2  | Phenylalanine/tyrosine ammonia lyase    | 4.3.1.25  | (1) L-phenylalanine = trans-cinnamate + NH3 [RN:R00697]; (2) L-tyrosine = trans-p-hydroxycinnamate + NH3 [RN:R00737] | M00039; ec00360 ; ec00940 ; ec01100 ; ec01110 |
| 3  | trans-cinnamate4-monoxygenase           | 1.14.14.91| trans-Cinnamate + [Reduced NADPH---hemoprotein reductase] + Oxygen <=> 4-Coumarate + [Oxidized NADPH---hemoprotein reductase] + H2O | M00039 M00137               |
| 4  | Phenyl alanine ammonia lyase            | 4.3.1.24  | L-phenylalanine = trans-cinnamate + NH3 [RN:R00697]                       | M00039M00137                |
Table 7. Enzymes of phenylpropanoid pathways that suppress the flavonoid biosynthesis are given. rn00945 Stilbenoid, diarylheptanoid, and gingerol biosynthesis, ko00940 Phenylpropanoid biosynthesis, ko01110 Biosynthesis of secondary metabolites degradation of aromatic compound rn00945 Stilbenoid, diarylheptanoid, and gingerol biosynthesis degradation of an aromatic compound, rn01100 Metabolic pathways, rn01220; Degradation of aromatic compounds; ec00940 Phenylpropanoid biosynthesis; ec01110 Biosynthesis of secondary metabolites

| S.N. | Enzyme Name                                      | EC number   | Catalysed Reaction                                                                 | Module/Pathway                  |
|------|-------------------------------------------------|-------------|-----------------------------------------------------------------------------------|---------------------------------|
| 1    | cinnamoyl-CoA reductase;                        | 1.2.1.44    | cinnamaldehyde + CoA + NADP+ = cinnamoyl-CoA + NADPH + H+ (RN:R02506)              | M00039                          |
| 2    | p-coumarate 3-hydroxylase                       | 1.14.13.    | p-Coumaroyl-CoA <=> Caffeoyl-CoA (R07436)                                         | m00940; m00945; m00941; m01100; ko01110; m01220 |
| 3    | enzyme not yet characterized                    | 1.14.13.-   | 4-Hydroxycinnamyl aldehyde <=> Caffeic aldehyde (R06579)                           | ko00940; ko01110               |
| 4    | p-coumarate 3-hydroxylase                       | 1.14.13.    | 4-Coumarate <=> Caffeate (R07826)                                                 | ko00940; m01100; ko01110 m01220 |
| 5    | shikimate O-hydroxycinnamoyltransferase         | E2.3.1.133  | 4-coumaroyl-CoA + shikimate = CoA + 4-coumaroylshikimate (RN:R02416)              | M00039                          |
| 6    | cytochrome P450 family 98 subfamily A polypeptide 9 | 1.14.13.-   | N1,N5,N10-Tricoumaroyl spermidine <=> N1,N5,N10-Tricaffeoyl spermidine             | ko00940; ko01110               |
| 7    | trans-4-coumaroyl-CoA 2-hydroxylase;            | 1.14.11.62  | p-Coumaroyl-CoA + 2-Oxoglutarate + Oxygen <=> 2,4-Dihydroxycinnamoyl-CoA + Succinate + CO2 R12306 | ec00940;ec01110 |
| 8    | cinnamoyl-CoA reductase;                        | 1.2.1.44    | cinnamaldehyde + CoA + NADP+ = cinnamoyl-CoA + NADPH + H+ (RN:R02506)              | M00039                          |
Table 8. Results on Domain analysis, structural features with a range of amino acids are illustrated here

| Query               | Specific active site | Product binding site | Malonyl-CoA binding site | Dimer interface                                      |
|---------------------|----------------------|-----------------------|--------------------------|------------------------------------------------------|
| 4YJY Oryza sativa   | C167,H306, N339      | E195,I196,T197,G219,I257,D258,G259,T267,F268,S341,P378 | K58,R61,M62,F218,F268,G309,P310,A311 | L94,D95,Q98,D139,M140,Y145,K149,P155,N156,V157,S158,R159,L160,M161,Q165,G166,R175,V176,D179,E182,N183,N184,A245,Q247,T248,I249,G259,H260,L261,R262,T381 |
| 1JWX Medicago sativa| C164,H303, N336      | E192,V193,T194,G216,I254,D255,G256,T264,F265,S338,P375 | K55,R58,M59,S215,F265,G306,P307,A308 | L91,D92,Q95,D136,M137,Y142,K146,P152,Y153,V154,K155,R156,Y157,M158,Q162,G163,R172,L173,D176,E179,N180,N181,T242,Q244,T245,I246,G256,H257,L258,R259,T378 |
Fig. 5. Structural features of Chalcone synthase (4yjy) from *Oryza sativa*, are depicted.

Fig. 6. Structural features of F215S mutant Chalcone synthase (1jwx) from *Medicago sativa*, are depicted.
Type III polyketide assembly can be modified by altering the preference for starter molecules. Wild-type CHS does not accept N-methylanthraniloyl-CoA as a substrate. Phe-215 and Phe-265, which are situated at the opening of the active site, mutants were created and screened against aliphatic and aromatic CoA-linked starter molecules. Substitution of Phe-215 by serine yields a CHS mutant that preferentially accepts this CoA-thioester substrate to generate a novel alkaloid, namely N-methylanthraniloyl triacetic acid lactone. Fig. 6 [37].

4. CONCLUSION

Metabolic control analysis revealed minimised concentrations of coumaroyl-CoA and malonyl-CA, that are 4.914061e-002 mM and 1.340605e-003 mM respectively. These concentrations can be obtained after 100 generations. This reveals that product concentration would be significantly decreased or there may be no synthesis below this threshold concentration. Final predicted concentration of Naringenine chalcone, 3CO2,4CoA is 8.593946e-004 mM after 5.00 second of simulation at reaction constant (J (R1) 6.587753e-005 mM*ml/s. This is associated with the production of diverse kinds of flavonoids. Analysis of the Phenylpropanoid pathway shows that the concentration of potential enzymes can be increased to achieve the above results. These enzymes are 4 coumarate Co A ligase, Phenylalanine/tirosine ammonia lyase, trans-cinnamate 4-monoxygenase, Phenylalanine ammonia lyase. Other enzymes minimise the concentration of Malonyl-CoA and Coumaroyl CoA, these enzymes are Cinnamoyl Co A reductase, shikimate O hydroxyl transferase (HCT), Oxidoreductase. Inhibition of these enzymes may maximise the flavonoid biosynthesis if appropriate inhibitors are used and a constant supply of Malonyl-CoA and P-Coumaroyl-CoA is maintained using activator molecules. Protein domain analysis of mutant chalcone synthases(source PDB) was done to predict structural features product binding site, active site, Malonyl-CoA binding site, and dimer interface to understand reaction mechanism which modulates flavonoid biosynthesis. Phe-215 and Phe-265, which are situated at the opening of the active site, mutated by substitution of Phe-215 by serine yields a CHS mutant that preferentially accepts this CoA-thioester substrate to generate a novel alkaloid. In CHS 2 structure of Medicago, sativa serine is introduced at 215. The same changes can be done in the CHS structure of Oryza sativa to obtain specific flavonoids using genetic engineering methods.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Wang J, Zhang R, Chen X, Sun X, Yan Y, Shen X, et al. Biosynthesis of aromatic polyketides in microorganisms using type II polyketide synthases. Microb Cell Fact [Internet]. 2020 May 24 [cited 2020 Nov 3];19. Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7247197/
2. Risdian C, Mozef T, Wink J. Biosynthesis of Polyketides in Streptomyces. Microorganisms [Internet]. 2019 May 6 [cited 2020 Nov 3];7(5). Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6560455/
3. Lussier F-X, Colatriano D, Wiltshire Z, Page JE, Martin VJJ. Engineering Microbes for Plant Polyketide Biosynthesis. Comput Struct Biotechnol J [Internet]. 2013 Feb 22 [cited 2020 Nov 3];3. Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3962132/
4. Pandith SA, Ramazan S, Khan MI, Reshi ZA, Shah MA. Chalcone synthases (CHSs): The symbolic type III polyketide synthases. Planta. 2019;251(1):15.
5. Yu D, Xu F, Zeng J, Zhan J. Type III polyketide synthases in natural product biosynthesis. IUBMB Life. 2012;64(4):285–95.
6. Lim YP, Go MK, Yew WS. Exploiting the Biosynthetic Potential of Type III Polyketide Synthases. Molecules [Internet]. 2016[cited 2020 Nov 3];21(6). Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6274091/
7. Taura F, Iijima M, Yamanaka E, Takahashi H, Kenmoku H, Saeki H, et al. A Novel Class of Plant Type III Polyketide Synthase
Involved in Orsellinic Acid Biosynthesis from Rhododendron dauricum. Front Plant Sci [Internet]. 2016 Sep 27 [cited 2020 Nov 3];7.
Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5037138/

8. Tzin V, Galli G. The Biosynthetic Pathways for Shikimate and Aromatic Amino Acids in Arabidopsis thaliana. Arabidopsis Book [Internet]. 2010 May 17 [cited 2020 Nov 3];8.
Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3244902/

9. Liang J, Luo Y, Zhao H. Synthetic biology: Putting synthesis into biology. Wiley Interdiscip Rev Syst Biol Med. 2011;3(1):7–20.

10. Pearsall SM, Rowley CN, Berry A. Advances in Pathway Engineering for Natural Product Biosynthesis. ChemCatChem. 2015;7(19):3078–93.

11. Farré G, Blancquaert D, Capell T, Van Der Straeten D, Christou P, Zhu C. Engineering Complex Metabolic Pathways in Plants. Annual Review of Plant Biology. 2014;65(1):187–223.

12. Winter JM, Tang Y. Synthetic Biological Approaches to Natural Product Biosynthesis. Curr Opin Biotechnol. 2012;23(5):736–43.

13. Wilkinson B, Micklefield J. Mining and engineering natural-product biosynthetic pathways. Nature Chemical Biology. 2007;3(7):379–86.

14. Lechner A, Brunk E, Keasling JD. The Need for Integrated Approaches in Metabolic Engineering. Cold Spring Harb Perspect Biol [Internet]. 2016 Nov [cited 2020 Nov 3];8(11).
Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5088530/

15. Kim OD, Rocha M, Maia P. A Review of Dynamic Modeling Approaches and Their Application in Computational Strain Optimization for Metabolic Engineering. Front Microbiol [Internet]. 2018; [cited 2020 Nov 3];9.
Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6079213/

16. Fukuma K, Neuls ED, Ryberg JM, Suh D-Y, Sankawa U. Mutational Analysis of Conserved Outer Sphere Arginine Residues of Chalcone Synthase. J Biochem. 2007;142(6):731–9.

17. Jez JM, Bowman ME, Noel JP. Structure-Guided Programming of Polyketide Chain-Length Determination in Chalcone Synthase. Biochemistry. 2001;40(49):14829–38.

18. Go MK, Wongsantichon J, Cheung VWN, Chow JY, Robinson RC, Yew WS. Synthetic Polyketide Enzymology: Platform for Biosynthesis of Antimicrobial Polyketides. ACS Catal. 2015;5(7):4033–42.

19. Heller W, Hahlbrock K. Highly purified “flavanone synthase” from parsley catalyses the formation of naringenin chalcone. Archives of Biochemistry and Biophysics. 1980;200(2):617–9.

20. Yahyaa M, Ali S, Davidovich-Rikanati R, Ibda M, Shachtier A, Eyal Y, et al. Characterization of three chalcone synthase-like genes from apple (Malus x domestica Borkh.). Phytochemistry. 2017;140:125–33.

21. DELANO WL. The PyMOL Molecular Graphics System. http://www.pymol.org [Internet]; 2002. [cited 2020 Nov 11]
Available:https://ci.nii.ac.jp/naid/10020095229/

22. Cascante M, Boros LG, Comin-Anduix B, de Atauri P, Centelles JJ, Lee PW-N. Metabolic control analysis in drug discovery and disease. Nature Biotechnology. 2002;20(3):243–9.

23. Raharjo TJ, Chang W-T, Choi YH, Peltenburg-Looman AMG, Verpoorte R. Olivetol as product of a polyketide synthase in Cannabis sativa L. Plant Science. 2004;166(2):381–5.

24. Dao TTH, Linthorst HJM, Verpoorte R. Chalcone synthase and its functions in plant resistance. Phytochem Rev. 2011;10(3):397–412.

25. Nair RB, Bastress KL, Ruegger MO, Denault JW, Chapple C. The Arabidopsis thaliana REDUCED EPIDERMAL FLUORESCENCE1 Gene Encodes an Aldehyde Dehydrogenase Involved in Ferulic Acid and Sinapic Acid Biosynthesis. Plant Cell. 2004;16(2):544–54.

26. Baucher M, Halpin C, Petit-Conil M, Boerjan W. Lignin: Genetic engineering and impact on pulping. Crit Rev Biochem Mol Biol. 2003;38(4):305–50.

27. Rogers LA, Dubos C, Cullis IF, Surman C, Poole M, Willment J, et al. Light, the circadian clock, and sugar perception in the control of lignin biosynthesis. J Exp Bot. 2005;56(416):1651–63.

28. Xu P, Ranganathan S, Fowler ZL, Maranas CD, Koffas MAG. Genome-scale metabolic
network modeling results in minimal interventions that cooperatively force carbon flux towards malonyl-CoA. Metab Eng. 2011;13(5):578–87.

29. Leonard E, Lim K-H, Saw P-N, Koffas MAG. Engineering central metabolic pathways for high-level flavonoid production in Escherichia coli. Appl Environ Microbiol. 2007;73(12):3877–86.

30. Leonard E, Yan Y, Fowler ZL, Li Z, Lim C-G, Lim K-H, et al. Strain Improvement of Recombinant Escherichia coli for Efficient Production of Plant Flavonoids. Mol Pharmaceutics. 2008;5(2):257–65.

31. Wu J, Yu O, Du G, Zhou J, Chen J. Fine-Tuning of the Fatty Acid Pathway by Synthetic Antisense RNA for Enhanced (2S)-Naringenin Production from L-Tyrosine in Escherichia coli. Appl Environ Microbiol. 2014;80(23):7283–92.

32. Cronan JE, Waldrop GL. Multi-subunit acetyl-CoA carboxylases. Progress in lipid research. 2002;41(5):407–35.

33. W Z, Sb R-P, Z S, H Z. Improving cellular malonyl-CoA level in Escherichia coli via metabolic engineering. Metab Eng. 2009;11(3):192–8.

34. Milke L, Kallscheuer N, Kappelmann J, Marienhagen J. Tailoring Corynebacterium glutamicum towards increased malonyl-CoA availability for efficient synthesis of the plant pentaketide noreugenin. Microbial Cell Factories. 2019;18(1):71.

35. An JH, Kim YS. A gene cluster encoding malonyl-CoA decarboxylase (MatA), malonyl-CoA synthetase (MatB) and a putative dicarboxylate carrier protein (MatC) in Rhizobium trifolii--cloning, sequencing, and expression of the enzymes in Escherichia coli. Eur J Biochem. 1998;257(2):395–402.

36. Wang Y, Chen H, Yu O. A plant malonyl-CoA synthetase enhances lipid content and polyketide yield in yeast cells. Appl Microbiol Biotechnol. 2014;98(12):5435–47.

37. Ferrer J-L, Jez JM, Bowman ME, Dixon RA, Noel JP. Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nature Structural Biology. 1999;6(8):775–84.