

Development of Petri Net-Based Dynamic Model for Improved Production of Farnesyl Pyrophosphate by Integrating Mevalonate and Methylerythritol Phosphate Pathways in Yeast

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Abstract In this case study, we designed a farnesyl pyrophosphate (FPP) biosynthetic network using hybrid functional Petri net with extension (HFPNe) which is derived from traditional Petri net theory and allows easy modeling with graphical approach of various types of entities in the networks together. Our main objective is to improve the production of FPP in yeast, which is further converted to amorphaadiene (AD), a precursor of artemisinin (antimalarial drug). Natively, mevalonate (MEV) pathway is present in yeast. Methylerythritol phosphate pathways (MEP) are present only in higher plant plastids and eubacteria, but not present in yeast. IPP and DAMP are common isomeric intermediate in these two pathways, which immediately yields FPP. By integrating these two pathways in yeast, we augmented the FPP synthesis approximately two folds higher (431.16 U/pt) than in MEV pathway alone (259.91 U/pt) by using HFPNe technique. Further enhanced FPP levels converted to AD by amorphaadiene synthase gene yielding 436.5 U/pt of AD which approximately two folds higher compared to the AD (258.5 U/pt) synthesized by MEV pathway exclusively. Simulation and validation processes performed using these models are reliable with identified biological information and data.

Keywords Amorpha diene · Artemisinin · Farnesyl pyrophosphate · Hybrid functional Petri net with extensions · Methylerythritol phosphate pathway · Mevalonate Pathway

Abbreviations

AD	Amorphaadiene
HFPNe	Hybrid functional Petri net with extension
MEV	Mevalonate
MEP	Methylerythritol 4-phosphate

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FPP	Farnesyl pyrophosphate
IPP	Isopentenyl pyrophosphate
DMAPP	Dimethylallyl pyrophosphate
GPP	Geranyl pyrophosphate

Introduction

Over the last few years, usage of terpenoids has increased exponentially in medicine and aromatics. They offer a viable commercial alternative to chemically synthesized products of similar use. Terpenoids are typically extracted from plants [1] and microorganisms. Terpenoids, being secondary metabolites, produced in very small quantities and scale up with existing plant and microorganism strains [2] are not cost effective. With commercial and medicinal uses of plant terpenoids on the rise, there is a need to increase the yield of terpenoid biosynthesis. Development of simulated models will diminish the troubleshooting and points the bottlenecks during the experimentation by understanding the relation between the complex biological pathway structures and dynamics of the system [3]. Various computational, mathematical, and Petri net models are developed in order to understand the relation between numerous pathways. This gives thorough observation of the troubleshoots, which solved easily or should find the alternatives [4–6]. In addition to this, flux analysis enables us to estimate and enumerate the flow of carbon and energy within a given system of bioprocesses. Flux analysis helps not only to build a better target model but also to eliminate unwanted side effects that might potentially be encountered in engineered organisms [7].

Biological pathways fall under three categories: gene regulatory networks, metabolic pathways, and signaling pathways [8] whose behavior widely described using methods like ordinary differential equations (ODEs), partial differential equations (PDEs) and non-ODE approaches [9, 10]. The present model deals with metabolic pathways and explains the improved production of farnesyl pyrophosphate (FPP), an intermediate for all major isoprenoids or terpenoids synthesis through two independent pathways (mevalonate (MEV) and non mevalonate (MEP)) in yeast. The MEV pathway is frequently found in the eukaryotic cytoplasm, while the MEP pathway is observed in the eubacteria such as *Escherichia coli* and *Streptomyces cerevisiae* [11, 12] as well as in plant plastids. The MEP pathway is not found in animals or fungi, but both pathways are operational in higher-level plants such as *Arabidopsis thaliana* and *Helianthus annuus* and *Artemisia annua* [13].

In this case study, we designed a FPP biosynthetic network using hybrid functional Petri net with extension (HFPNe) which is derived from traditional Petri net theory [14] and allows easy modeling with graphical approach of various types of entities in the networks together. A Petri net is a graphical diagram consisting of circles and lines representing the current status of a rule-based state-dependent procedural system. For this reason, a Petri net is also called a place transition network. To support more complicated networks with varying degrees and kinds of control structures, HFPNe are used [15]. These networks support concepts essential to pathway design like quantitative (equation or value based) induction, inhibition, and repression. Petri net offers a versatile graphical language to design, integrate, and simulate multiple pathway networks [16]. Petri nets are suitable because of their intuitive graphical representation and their capabilities for mathematical analyses. Prospect to find out new option for amplifying the production of FPP was our major objective, which can be further utilized to synthesize variety of isoprenoids having medical as well as industrial importance.

Resources and Methods

Pathway Databases

The information and data desirable to build this model was obtained from biological databases: KEGG, BRENDA, ENZYME, IUBMB, MetaCyc, and PATHWAYDATABASE [17–21]. These databases include information on the substrates, products, enzyme consumption, and production rates involved in the MEV and MEP pathways. Along with the above data, stoichiometric and enzyme mechanisms are also taken into consideration in developing the dynamic model. This information was used to design the model layout and parameter assignments for each of the HFPNe elements.

Model Pattern Design

The HFPNe elements (place, transition, and arc (Fig. 1)) represent the metabolites and processes that comprise the MEV and MEP biosynthetic pathways. Continuous places correspond to the substrates, products, inhibitors, and enzymes in both the pathways. While continuous transitions correspond to biological processes such as synthesis, catalysis, and glycosylation, generic places represent the on/off switches and parameter modulators. The normal arc connects places (enzyme, substrate, and product) to transitions while inhibitory arcs connect inhibitors to their potential inhibited processes. All these entities were arranged according to the reaction stoichiometry and enzymatic mechanism in their order of incidence in the respective pathways. Each of the HFPNe elements possesses diverse features that characterize its function in the model. Parameter values are assigned to each of these attributes to control the behavior of the model during simulation. These values are generated based on the biological data obtained from databases.

Model Development

Developmental stages as well as the simulation and validation processes of the model were carried out using Cell Illustrator 5.0 www.cellillustrator.com (developed by Human genome center, Institute of medical science. The University of Tokyo, Japan).

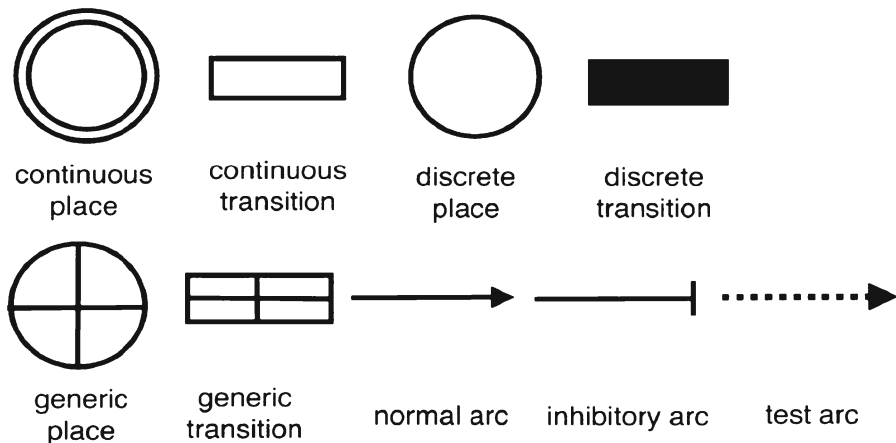


Fig. 1 Basic entities of HFPNe method

Simulation and Validation

Simulations were carried out with four different conditions:

1. Conventional production of FPP through the MEV pathway.
2. Conventional production of GPP2 through the MEP pathway.
3. Combined production of FPP through integrated MEV and MEP pathways.
4. Production of amorphaadiene through integrated MEV and MEP pathways.

Simulation results were calculated as concentration (unit) versus time (pt) graphs. Petri net time (pt) indicates virtual Petri net time that do not match to real time; concentration also is given in general concentration units (unit) that do not specifically correspond to standard concentration units such as mM and μM . The changes in metabolite concentrations (unit), over time predicted by each simulation were confirmed against known biological data to identify breaks and variations. Unmatched biological processes were re-examined and previous developmental steps (pattern design and parameter assignment) were repeated. The simulation process was then reexecuted and revalidated using cell illustrator. The entire process was carried out repetitively in order to rule out contradictions and obtain a best possible system.

Results and Discussion

Outlines of the Model

All of the metabolites involved in the pathways are represented using HFPNe elements and are interconnected to each other based on their reaction stoichiometry and enzymatic mechanisms. The network consists of 84 continuous transitions representing various reactions and production/degradation processes; 61 places representing 56 metabolites; three on/off switches; and two parameter modulators (Fig. 2). Eighteen main enzymatic reactions are involved in this pathway network. MEV pathway network consists of ten main enzymatic reactions whereas MEP pathway network contains eight main enzymatic reactions.

Conventional Production of FPP Through the MEV Pathway

The MEV pathway consists of ten main enzymatic reactions (Fig. 3). In the mevalonate pathway, three molecules of acetyl-coenzyme-A (CoA) couple to yield 3-hydroxy-3-methylglutarylCoA (HMG-CoA), which is subsequently reduced by the enzyme HMG-CoA reductase to yield mevalonic acid (MVA). In the next two steps, mevalonate kinase and mevalonate 5-phosphate kinase catalyze MVA to form mevalonate 5-diphosphate, which is subsequently decarboxylated to yield isopentenyl pyrophosphate (IPP) [23, 24]. The mevalonate pathway provides IPP for the synthesis of some sesquiterpenes, sterols, and triterpenes and is localized in the cytosol. The behavior of each reaction in the pathway is described using HFPNe element [9, 22]. All simulations carried out in this study are based on known information retrieved from literature and database like KEGG, BRENDA and MetaCyc. Usually, in yeast, FPP produced through the MEV pathway can be assumed as test or control for our

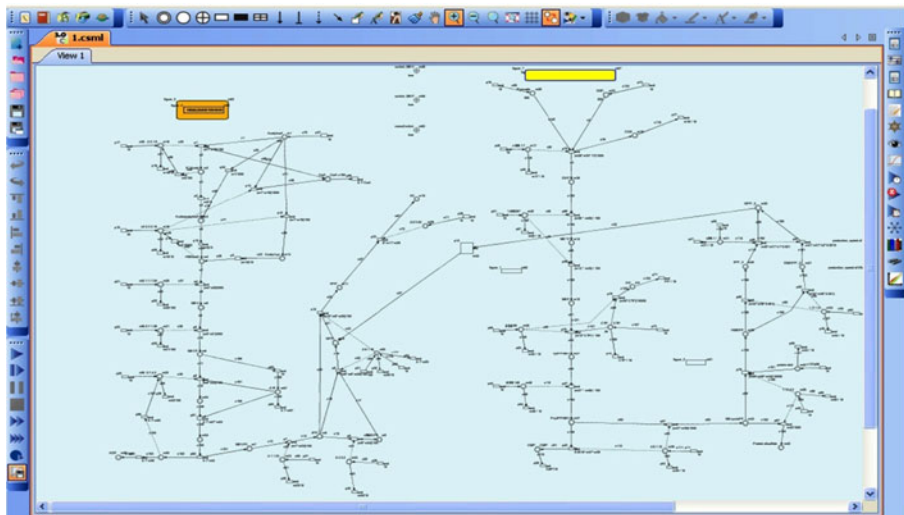


Fig. 2 Outline of the integrated MEV and MEP pathway. The MEV pathway is on the *left* and the MEP pathway on *right* and both are connected through GPP₂ flux (MEP) directed towards GPP (MEV). All metabolites are symbolized using continuous places. Each *transition entity* represents a biological process

experiment. Most important step of this pathway is synthesis of mevalonate catalyzed by HMGR. This situation yields 259.91U (Fig. 4) of FPP per 100 pt (sec). At the end of the simulation, the universal precursors IPP and DAMP were accumulated in low concentrations, 3.21U and 0.023U, respectively.

Conventional Production of GPP₂ Through the MEP Pathway

MEP pathway usually present in higher plant plastids and eubacteria are involved in the production of mono- and diterpenes. MEP pathway starts from transketolase type condensation of pyruvate and glyceraldehyde-3-phosphate to form Deoxyulose5-phosphate (DXP), followed by the rearrangement and reduction of DXP to MEP. MEP transforms to the cytidine 5-diphosphate derivative and sequential phosphorylation at C₃ and cyclization leads to 2-C-methylerythritol-2,4-cyclodiphosphate. Loss of CMP forms MEcycPP which is further converted to 1-hydroxy-2methyl-2-(*E*)-Butenyl 4-diphosphate. IPP and DMAPP are produced as final products. DXP synthase is the first enzyme and rate limiting enzyme in this pathway which leads to the production of DXP. The constructed MEP Perti net model produced 229.0U of the GPP₂ per 100 pt (Fig. 5). At the end of the simulation, precursors accumulated. Unconverted or accumulated IPP₂ is more (99.0U/100 pt) than DAMP₂ concentrations (30U/100 pt)..

Combined Production of FPP Through Integrated MEV and MEP Pathways

MEV and MEP pathways are integrated by routing the GPP₂ flux towards GPP which finally leads to the improved production of FPP. The fifth reaction of MEV pathway acts in two-substrate/two-product sequential enzymatic mechanism. Studies have

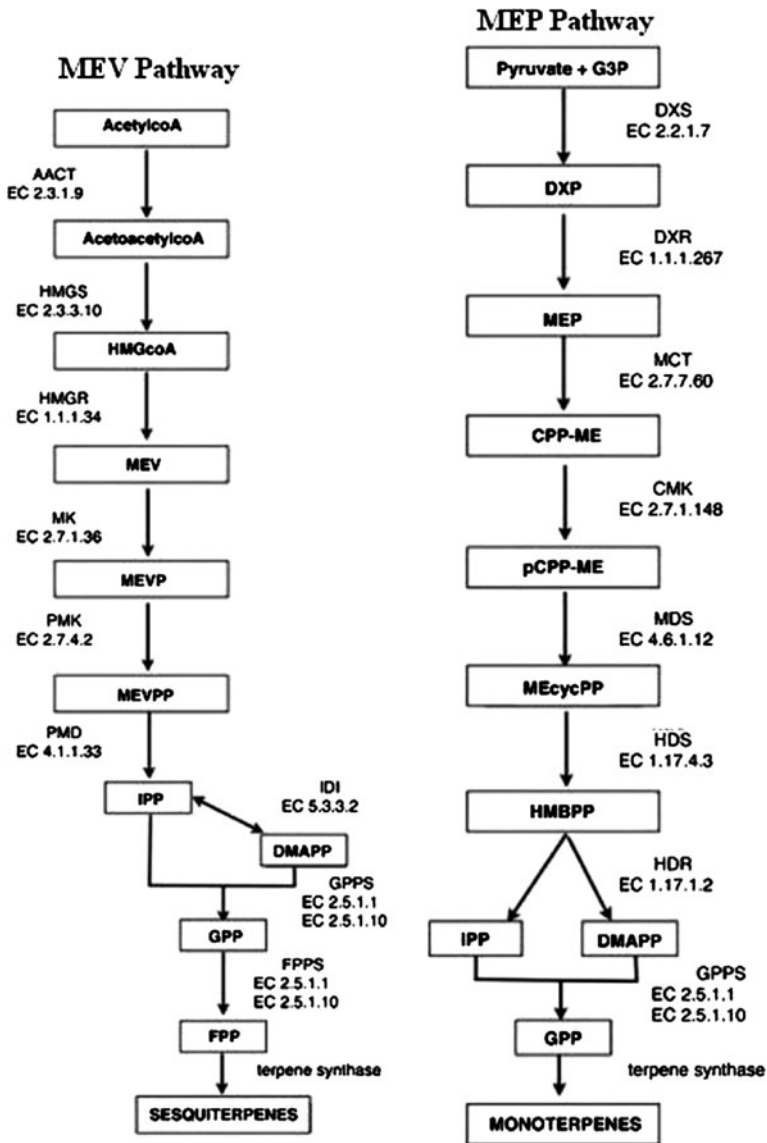


Fig. 3 Sequential enzymatic steps involved in synthesis of sesqui- and monoterpene independently by MEV and MEP pathways. *HMG-CoA* Hydroxymethyl glutaryl-CoA, *MEV* Mevalonate, *MEVP* Mevalonate phosphate, *MEVPP* Mevalonate diphosphate, *G3P* Glyceraldehyde-3-phosphate, *DXP* Deoxyxylulose-5-phosphate, *MEP* Methylerythritol 4-phosphate, *CPP-ME* 4(cytidine 5'diphospho)2-C-methyl-D-erythritol, *pCPP-ME* 4(cytidine 5'diphospho)2-C-methyl-D-erythritol 2-phosphate, *MEcycPP* 2-c-methylerythritol-2,4-cyclo-diphosphate, *HMBPP* 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate, *IPP* Isopentenyl pyrophosphate, *DMAPP* Dimethylallyl pyrophosphate, *GPP* Geranyl pyrophosphate, *FPP* Farnesyl Pyrophosphate, *AACT* Aceto acetyl transferase, *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *MK* Mevalonate kinase, *PMK* Phosphomevalonate kinase, *PMD* MEVPP decarboxylase, *IDI* Isopentenyl diphosphate isomerase, *GPPS* GPP synthase, *FPPS* FPP synthase, *DXS* DXP synthase, *DXR* deoxyxylulose phosphate reductoisomerase, *MCT* MEP cytidyltransferase, *CMK* CPP-ME kinase, *MDS* MEcycPP synthase, *HDS* HMBPP synthase, *HDR* HMBPP reductase

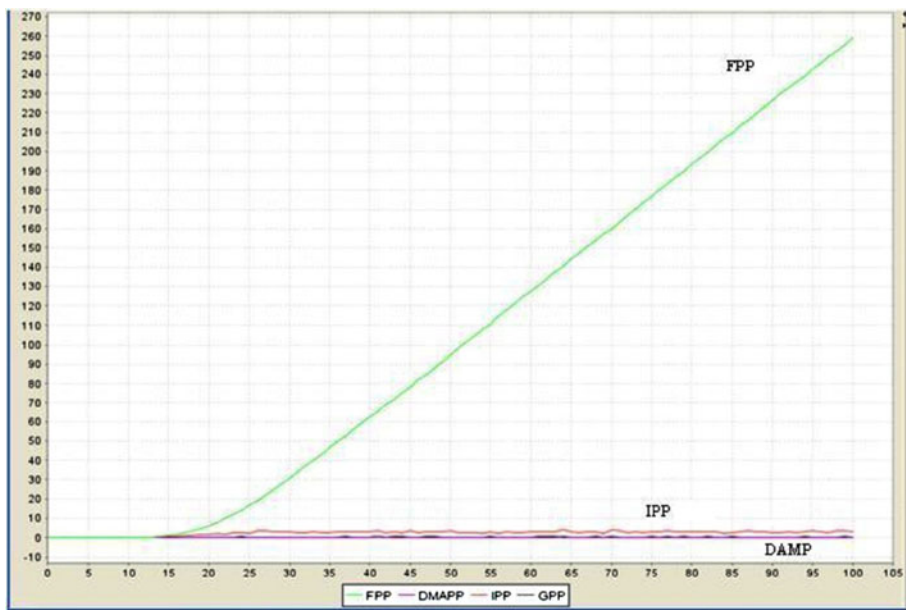


Fig. 4 Concentration changes of main precursors in MEV pathway over time (pt). Universal precursors *DAMP* and *IPP* concentrations are completely converted to *GPP* and further to *FPP* (concentration in Unit (U) on *Y*-axis whereas time (pt) is on *X*-axis)

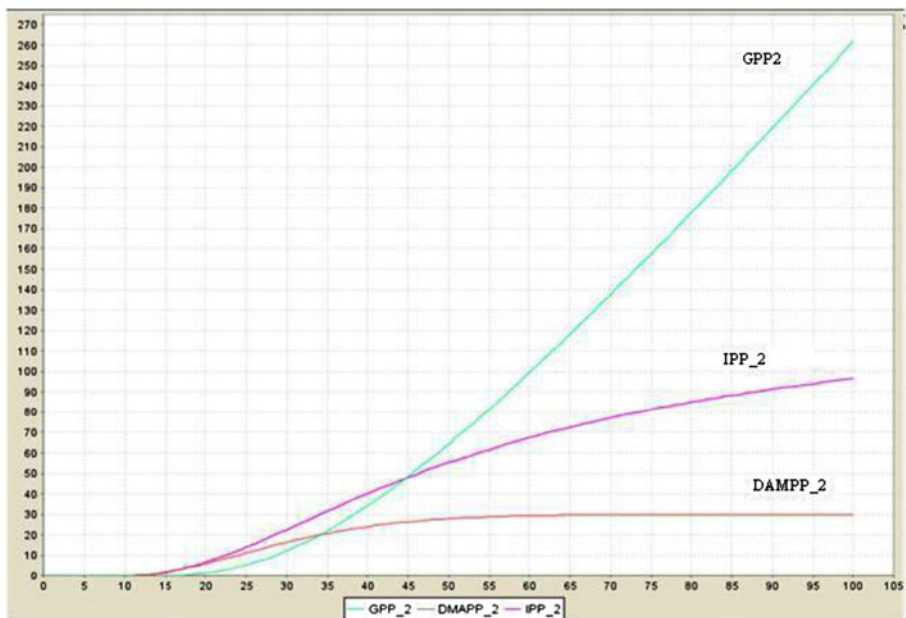


Fig. 5 Concentration changes of metabolites in MEP pathway over time. Concentration in Unit (U) on *Y*-axis whereas time (pt) is on *X*-axis

shown that high concentration of ATP halts the production of MEVPP and thus blocks the entire pathway from continuing to synthesize other intermediates.



ATP acts as a competitive inhibitor; this obstructs the downstream reactions in the pathway. However, the increase in the concentration of MEVP shows that the inhibitory effects of ATP could be reversed. Higher concentrations of MEVP inhibit the synthesis of ATP, lowering the concentration of ATP to a manageable level allows the synthesis of MEVPP well as downstream reactions. This model serves as a tool for better understanding of the reactions involved in both pathways and how they affect each other. Diversion of GPP₂ flux (MEP pathway) towards MEV pathway increased FPP production approximately two fold, 259.91 u/100 pt (sec) to 431.168 u/ 100 pt which will increase the sesquiterpene production and concentration of GPP and GPP₂, 0 and 4.11 U/pt, respectively (Fig. 6).

Production of Amorphadiene Through Integrated MEV and MEP Pathways

FPP is a common precursor for sesquiterpenoids like artemisinin which is well-known for its antimalarial, anti cancer, and anti viral activities. FPP is converted to amorphadiene (AD) (Fig. 7) by amorphadiene synthase (EC 4.3.2.24), which is an immediate precursor of artemisinin. Kinetic data of the enzyme obtained from the biological databases and this additional reaction was added to this model yielding 436.5 U/pt of AD which is approximately two folds greater than that of 258.5 U/pt AD given exclusively by MEV pathway (Figs. 8 and 9). Improved production of AD will have great impact on artemisinin productivity, which is not affordable to most of the malarial patients. No kinetic data is available for cytochrome P450 mono oxygenase (*CYP71A1*) which converts AD to artemisinin in order to design the model for enhanced production of artemisinin.

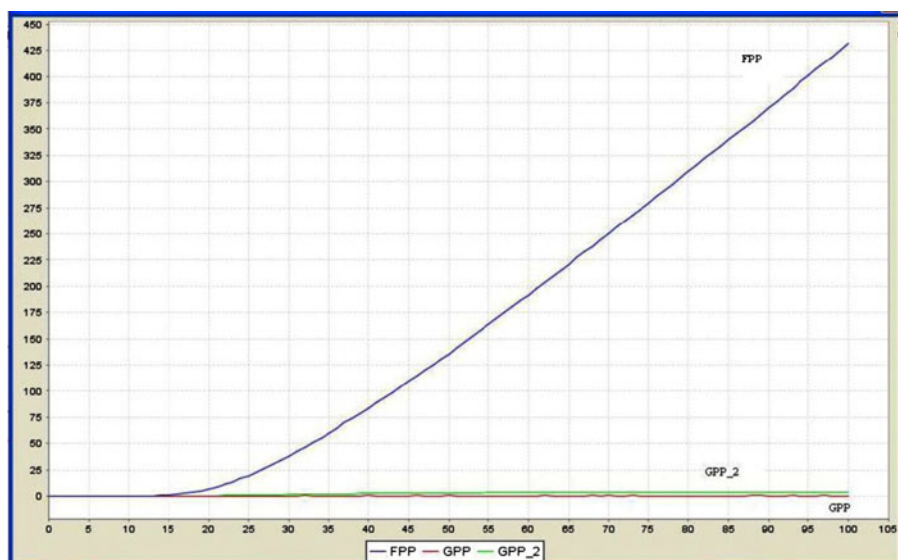


Fig. 6 Concentraions of FPP, GPP and GPP2 after the integration of MEV and MEP pathways. After the integration of two pathways, FPP concentrarion was increased, whereas GPP, GPP₂ converted to FPP

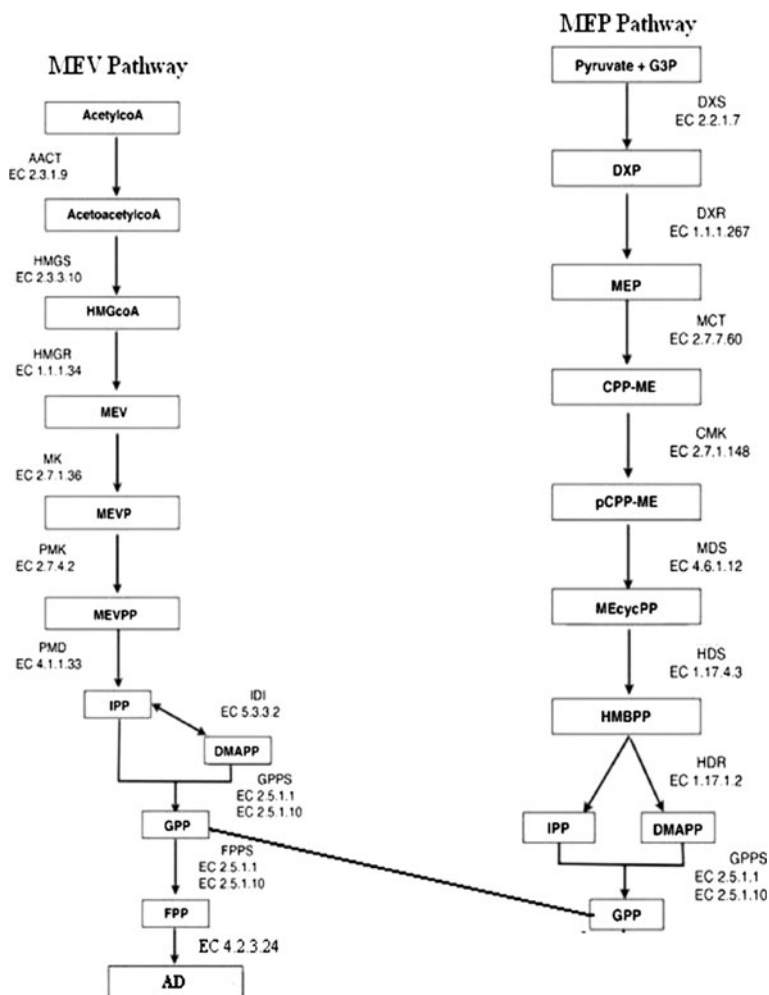


Fig. 7 Integrated *MEV* and *MEP* pathways for synthesis of *AD*. *GPP* flux from *MEP* pathway is directed towards *MEV* pathway which will successfully increase the *FPP* synthesis further it was converted to *AD*, an immediate precursor of artemisinin (Abbreviations are same as in Fig. 3)

Conclusions

HFPNe technique enables complicated modeling tasks to be viewed and solved in a graphical manner. The model serves as a tool to better understand the reactions involved in combinatorial *FPP* synthesis and how they interact to each other. This gives needful information for finding the alternatives for production of isoprenoids. Apart from this, most of the acetyl-CoA in the *MEV* pathway is either transported from the cytosol into the mitochondrion for oxidation by TCA cycle or utilized in fatty acid and ergosterol synthesis. Channeling more acetyl-CoA into the mevalonate pathway by limiting acetyl-CoA transport to the mitochondrion or inhibiting ethanol and fatty acid synthesis, it can be possible to further increase the *FPP* production which is having tremendous significance in chemical industry and medicine.

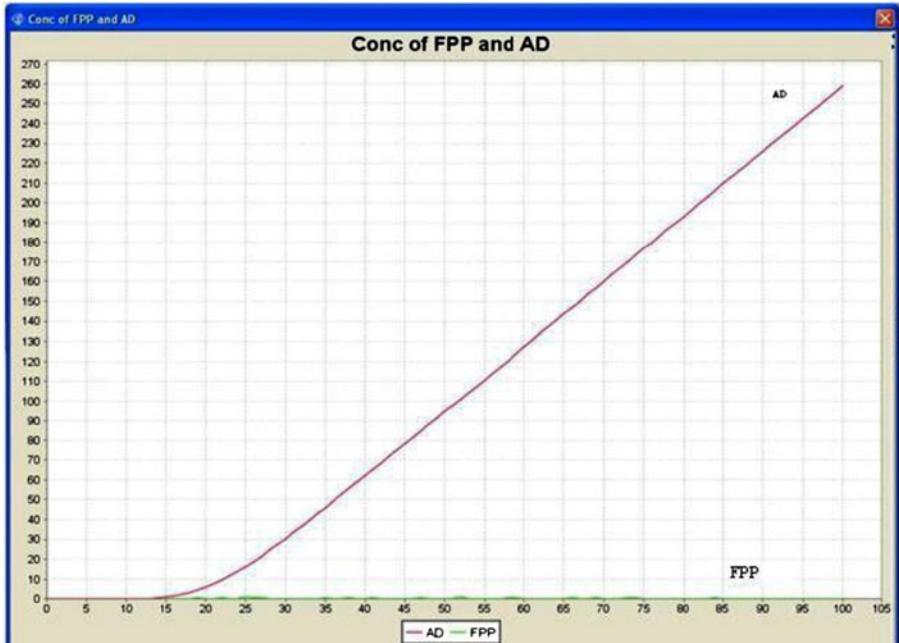


Fig. 8 Concentrations of *FPP* and *AD* in MEV pathway .Total *FPP* converted to *AD*, no accumulation *FPP* observed

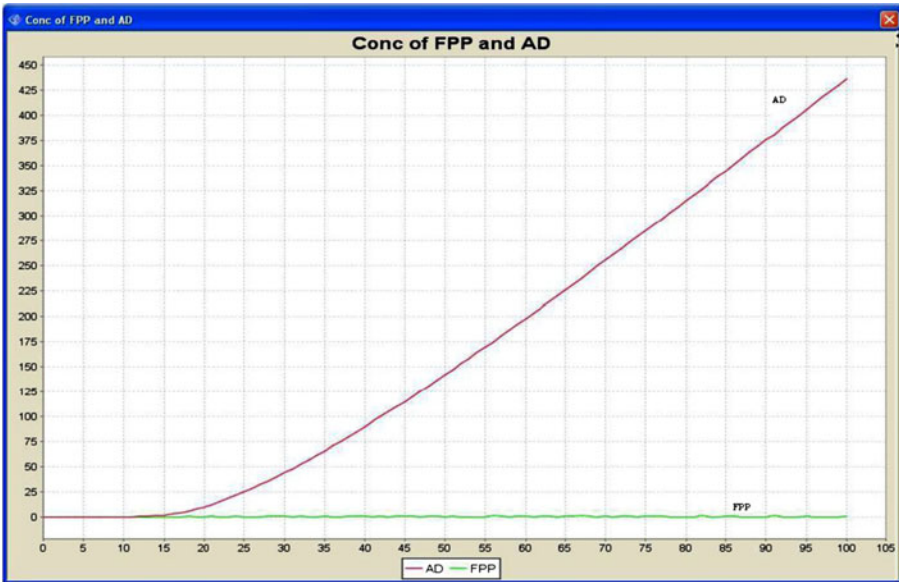


Fig. 9 Concentration of *FPP* and *AD* in integrated MEV and MEP pathways. Total available *FPP* is converted to *AD* and its concentration increased by two folds

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