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Application of Petri Net Theory for Modelling and Validation of Menthol Biosynthesis

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ABSTRACT

An essential step in network modelling is to validate the network model. Petri net theory provides algorithms and methods, which can be applied directly to metabolic network modelling and analysis in order to validate the model. This paper describes the thriving application of Petri net theory for model validation of biosynthesis of menthol using the well-established Petri net analysis technique of place and transition invariants. Because of the complexity of metabolic networks and their regulation, formal modelling is a useful method to improve the understanding of these systems. A petri net representation, its validation and simulation of biosynthesis of menthol from geranyl diphosphate (GPP) has been performed with the objective of understating new insights of the structure of this pathway affecting the synthesis of menthol. The model has been validated for its P-invariant and T-invariant. T-invariant analysis suggest absence of any loop in the net which restore the initial state suggesting all reactions to be only forward. The net is covered by positive P-invariants and bounded. The net is utilized to simulate the time (pt) with concentrations of GPP, (–)-limonene, (+)-pulegone, (–)-menthone and (–)-menthol. Dimethylallyl diphosphate and isopentenyl diphosphate were the main precursors for this biosynthesis. Biological data needed for simulation where obtained from extensive survey of literature. The results were shown graphically and the nature of graphs represent the variation of concentrations with time.

KEYWORDS

Petri Net, Simulation, Modelling, Menthol biosynthesis, P-invariant, T-invariant.

INTRODUCTION

Biological processes are made up of many chemical reactions or other events that are involved in the persistence and transformation of life forms. In order to understand the relationship between these reactions in detail, it is necessary to view reaction network as a whole [1]. Computational dynamic models of metabolic networks have been developed in order to provide an overview of the biosynthetic processes involved in menthol biosynthesis pathway [2,3]. Using such models, changes in the concentration of each metabolite can be simulated and used to analyse how such changes may contribute to the entire biosynthetic process. By using computational models, researchers get an in-depth view of the entire biosynthesis process.

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Plants of genus Mentha produce a variety of economical important essential oil, which finds application in the form of herbal tea, antimicrobial [4], antiviral [5], antifungal [6] and antioxidant agents [7,8]. They also show radiation protective activity [8]. Because of high commercial value of menthol, large number of research articles on various aspects such as production [9], modelling and reaction mechanism [10] have appeared recently. Menthol, a cyclic monoterpene alcohol found in the essential oils of *Mentha canadensis* and *Mentha piperita* L, menthol along with menthone and isomenthone provide the cooling minty taste and smell to plants [11]. About 32000 tonnes of menthol is consumed annually as a flavouring agent.

Petri nets treatment of few metabolic pathways [12,13], simulation of a Petri net-based Model of the terpenoid biosynthesis pathway [14] and applications of Petri net theory for modelling and validation of the sucrose breakdown pathway in the potato tuber by Koch *et al.* [15] have been reported.

To the best of our knowledge there is no report of Petri Net modelling, validation and simulation of menthol biosynthesis. We have therefore undertaken the problem of Petri net modelling with the objectives of validation and simulation of the Menthol pathway. In this pathway geranyl diphosphate is coming from terpenoid backbone biosynthesis. Geranyl-PP synthesized by isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are main precursors for whole pathway. The biosynthesis of universal precursors IPP and DMAPP occurs by two independent pathways include the methylerythritol phosphate (MEP) and mevalonate (MVA) pathway [16].

Various computational approaches can be considered for building and simulating a biological pathway model [17,18]. The ordinary differential equations (ODEs) [19-21] are most popular way to describe such a model. While ODEs are typically derived from Michaelis-Menten equation it involves quantitative analysis of models for simulation and analysis of time dependent properties of a network [22]. Petri net presentations of biological pathways are prominent tool in system biology. Typically, there are three categories of biological pathways: metabolic pathways [15,23,24], gene regulatory networks [18, 25,26] and signalling pathways [27,28]. Biosynthesis of menthol comes into metabolic pathways category.

Petri nets: Petri nets are a special class of networks, introduced in 1962 by Carl Adam Petri [29] that provide a convenient language and graphical representation for many areas of science and engineering. A Petri net (PN) [30-33] is a bipartite graph (Fig. 1) consisting of places, transitions and arcs. Places are represented by circles; transitions are presented by rectangles and arcs are represented by directed arrows. Arcs connect places to transition and transition to places. Places contain tokens, arcs may contain waits, tokens stand for discrete elements such as number of moles or number of molecules. Waits over arcs present stoichiometry of the reaction. Each transition (event) is associated with a finite number of input places (pre-conditions) and output places (post-conditions). A transition is enabled when the number of tokens in its input places is greater than or equal to the weights on the arcs connecting the places to the transition. A transition with no input places, called a source transition, is always enabled. An enabled transition can fire, depositing tokens in its output places, again

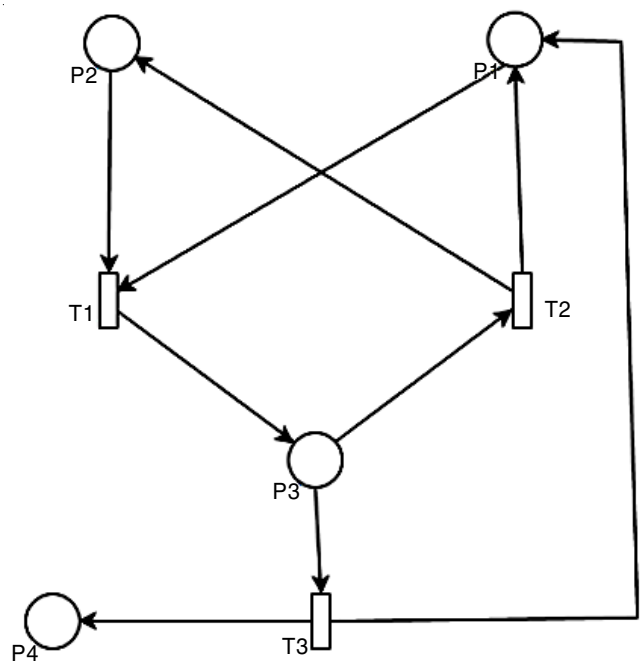

Fig. 1. A Bipartite Graph showing places transitions and arcs

their number determined by the arc weights. A transition with no output places, called a sink transition, can fire when enabled consuming the tokens from its input places.

Obviously, for a coupled chemical reaction, a Petri net will consist of a set of places, a set of transitions, a set of arcs, a set of weights and marking. We can therefore, define Petri net (PN) as a tuple of the above sets.

Definition 1: A Petri net is a four-tuple:

$$N = \{P, T, E, W, M0\}$$

where:

P = {Pl, P2, P3. ... Pm} is a finite set of places

T = {t1, t2, t3. ... tn} is a finite set of transitions

$E \subseteq (P \times T) \cup (T \times P)$ is a set of arcs

$W: E \rightarrow \{1, 2, 3. ...\}$ is a weight function

Petri nets were originally designed to represent discrete, concurrent processes of technical systems. Since its development in 1962, there have been many extensions to the concepts which are graphically shown in Fig. 2.

In stochastic Petri nets (SPN) a probability term decides the firing of a transition [34]. In continuous Petri nets (CPN)

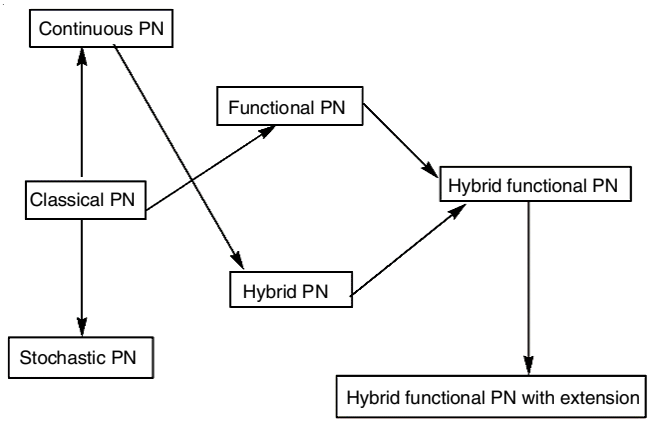

Fig. 2. Extensions to Petri nets

places are represented by double circles, transitions are represented by hollow rectangles and tokens are real numbers [35]. Transitions possess a firing quantity which can be any quantity between 0 and m where m is the token in the input place. A simple example of continuous Petri net is pumping of a liquid between two vessels pictorially is shown in Fig. 3.

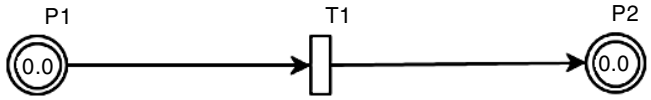

Fig. 3. CPN model of pumping liquid. T1 is continuous transition, P1, P2 continuous places containing real amount

Hybrid Petri net (HPN) consists of continuous part as well as discrete part. A simple example of HPN is pumping of a liquid from one tank to the anther operated by switches. The tanks containing liquid are continuous parts and switches used for starting or stopping the pump are discrete parts. The system is shown in Fig. 4.

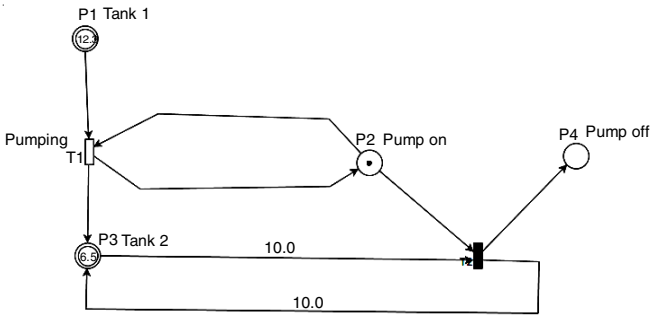

Fig. 4. HPN model of pumping liquid

Places P1, P3 and transition T1 are continuous parts P1 contain 12.3 units of liquid and P2 contain 6.5 units of liquid and firing capacity of T1 is 0.1. P2, P4 and transition T2 are discrete parts. P2 and P4 are switches and T2 is a discrete transition. In the current state the switch P2 contain a token and in 35 units of time pumps 3.5 units of liquid from p1 decreasing its content to 8.8 units and deposit 3.5 units to P3 making its total content to 10 units. This enables T2 which consumes a token room P2 deposits to P4 and disables T1.

Hybrid functional Petri net (HFPN) is an extension to HPN, it contains two additional arcs namely test arcs and inhibitory arcs. These arcs can be only input arcs. A test input arc is directed from a place of any kind to a transition of any kind. It does not consume the content of the source place. Inhibitory arcs prevent the transition from firing if the content of the input place is greater than or equal to weight of the inhibitory arc. The continuous transition in HFPN is similar to that used in HPN and some function can be assigned to it for firing rate. Besides these, it is also possible to assign any function (values of places) to arcs, such possibilities are helpful to model composition of tetramer in terms of monomers as shown in Fig. 5. V1, V2 and V3 can be functions of values of places P1 and P2. As four monomers are needed for one tetramer v2 can be written as 4*v3 [26].

Hybrid Functional Petri nets with extension [HFPNe] [36,37] takes into account the spatial information such as position and speed of a place. Besides using real and integer

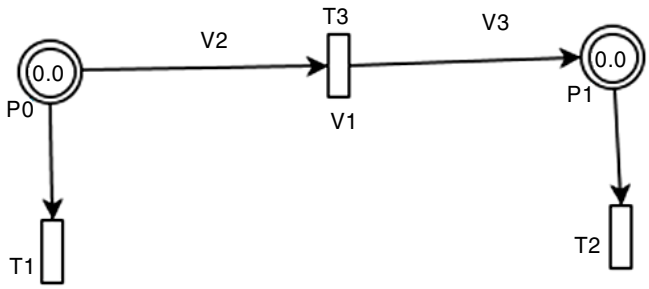

Fig. 5. A HFPN model for composition of tetramers out of monomer

values, it is also possible to use strings (DNA sequences) and objects with variables and methods. HFPNe also use generic places (mRNA sequence, phosphorylation state of a protein) and generic transitions (complex reactions by updating the state of connected entities). The entities used in HFPNe are shown in Fig. 6.

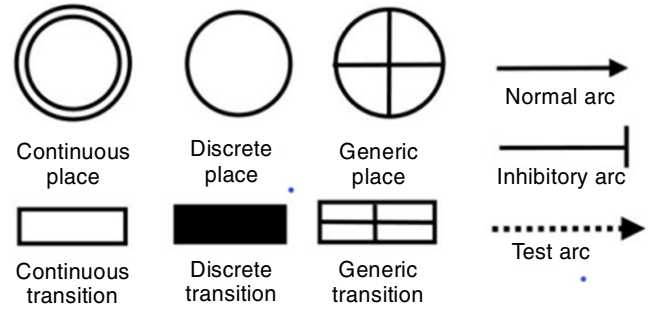

Fig. 6. Basic entities in the HFPNe architecture

RESULTS AND DISCUSSION

Biosynthesis of menthol: Details of biosynthesis of menthol is available in literature [41], therefore, only a short description of pathway relevant to the present manuscript is given here. T(i) represent transition numbers in the Petri net model given in Fig. 7. The first step (T0) involves the condensation of isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by specific prenyltransferase geranyl diphosphate synthetase (GPPS), to the universal monoterpene precursor geranyl diphosphate (GPP) which cyclizes *via* (–)-limonene synthase (LS) to (–)-limonene (T1). An oxygen atom at an allylic position in limonene is then introduced (T2) by an enzyme (–)-limonene-3-hydroxylase (L3OH) to produce (–)-*trans*-isopiperitenol which is subsequently oxidized (T3) to (–)-isopiperitenone by (–)-*trans*-isopiperitenol dehydrogenase (iPD). In the next step (T4), (–)-isopiperitenone is converted to (+)-*cis*-isopulegone by (–)-isopiperitenone reductase enzyme (iPR) using NADPH and then, (+)-*cis*-isopulegone is converts (T5) to (+)-pulegone with (+)-*cis*-isopulegone isomerase enzyme (iPI). (+)-Pulegone is the first pathway intermediate to be found in appreciable amount. It is further converted to (+)-menthofuran [T6], (–)-menthone [T7] and (+)-isomenthone [T8] (as an isomer of menthone) by (+)-menthofuran synthase (MFS) and (+)-pulegone reductase (PR), respectively. (–)-Menthone is then transformed to (–)-menthol by enzyme (–)-(3R)-menthol reductase (MDEH). There is evidence that (+)-(3S)-neomenthol reductase (NR)converts (+)-isomenthone

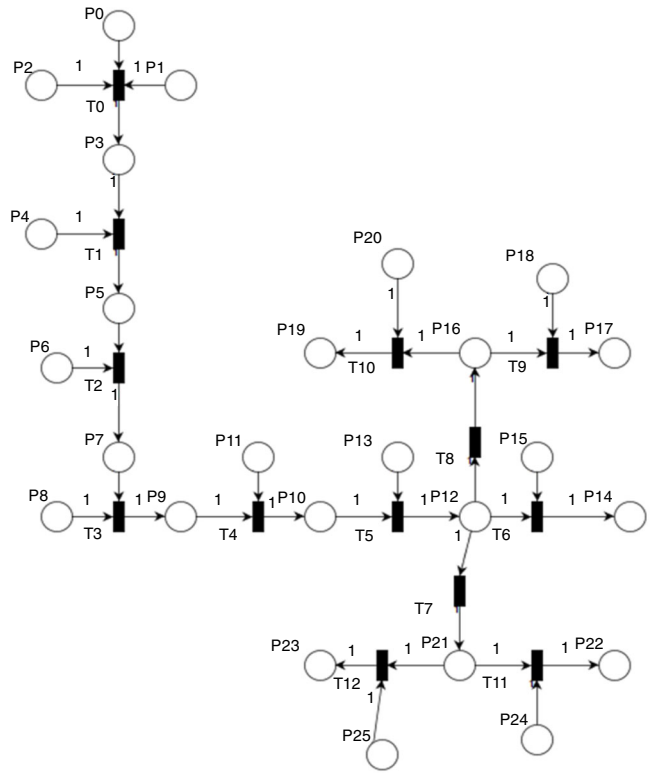

Fig. 7. Petri net representation of biosynthesis of menthol

to (+)-isomenthol and (–)-menthone to (+)-neomenthol. Similarly, enzyme (–)-(3R)-menthol reductase (MDEH) coverts (–)-menthone to (–)-menthol and (+)-isomenthone to (+)-neoisomenthol.

Petri net representation: Pictorial representation of the biosynthesis of menthol is taken from reference [38]. A simple Petri net representation of these reactions is shown in Fig. 7. The net consists of 15 places (P0, P1, P3, P5, P7, P9, P10, P12, P14, P15, P16. P17, P21, P22, P23) for substrates and products and 11 places (P2, P4, P6, P8, P11, P13, P15, P18, P20, P24 and P25) for different enzymes. It also consists of 13 normal transitions (T0-T12). This representation has advantages over simple representation by chemical equations. There is no notation for reactions in the chemical equations. In general, chemical reactions are models using ordinary differential equations which are sometime difficult to solve. On the other hand, transitions represent reactions in Petri net notations.

P-invariant and T-invariant: Although, there are few reports on the P-invariant and T-invariant [38-40] of few metabolic problems in literature there seems to be no report on validation of Petri net model of biosynthesis of menthol presented in this manuscript. Validation of the net has been made employing the technique P-invariant and T-invariant, respectively. In Fig. 8a, the net starts with a place and ends with a place no token goes out of the net and obviously preserves the token in Fig. 8b, the token entering at the source transition is given to sink and the state does not change of P-invariant and T-invariant. In Petri net theory, a P-invariant is a set of places over which the number of tokens remain constant and is independents of transitions.

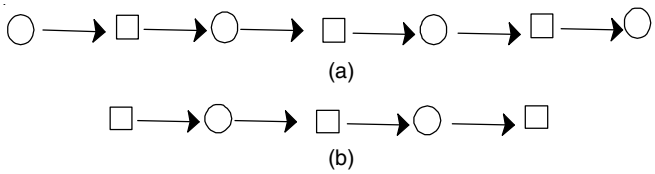

Fig. 8. (a) P-invariant and (b) T-invariant

Fig. 8a and b represents a place vector is called a P-invariant if it is a non-trivial non-negative integer solution of the linear equation xC=0. Here x is the vector of places and C is the P x T incident matrix. None zero entries in the set is called support of the invariant x. A net is covered by P-invariants if every place belongs to at least one of the P-invariant. A list of the sets of P-Invariants is shown in Table-1. The net is covered by positive P-Invariants and is bounded. There are a total of 12 P-invariants [15].

A transition vector is called T-invariant if it is non-trivial non-negative integer solution of the linear equation C; Y = 0. The set of none zero entries in the set is called support of the invariant y. A net is covered by T-invariants if every transition belongs to at least one of the T-invariant. T-invariant analysis suggest absence of any loop in the net which restore the initial state suggesting all reactions to be only forward. Construction of net and computations for P-Invariants and T-invariants for biosynthesis of menthol were made with the help of computer program called PIPEv4.3.0 (Platform Independent Petri Net Editor) [41]. All P-invariants start with a place and at a place suggesting the number of tokens to be conserved. P-invariants 5, 6, 8, 9 and 10 are special invariants involving only end products.

TABLE-1
LIST OF P-INVARIANTS CALCULATED FROM Fig. 7

P-invariant	Places																								
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	1			1			1	1		1	1		1		1		1	1		1		1	1			
2	1			1		1		1		1	1		1		1		1	1		1		1	1	1		
3											1	1	1		1		1	1		1			1	1	1	
4													1	1	1		1	1		1		1	1	1		
5															1	1										
6																		1	1							
7			1	1		1		1		1	1		1		1		1	1		1		1	1	1		
8																				1		1				
9																							1		1	
10																								1		1
11							1	1		1	1		1		1		1	1		1			1	1	1	
12							1	1	1		1		2		1		1			1			1	1	1	

Simulation: Transitions are associated with reaction velocities which can be used for computing amount of a substrate at different Petri net times. It is possible that an enzyme can catalyses two different substrates to produce two different products with different velocities. It is possible in Petri nets to differentiate these two cases. Examples are catalysis by NR and MDEH on (–)-menthone and (+)-isomenthone.

To produce different products with different rates. Similarly, PR catalyzes (+)-pulegone to produce (–)-menthone and (+)-isomenthone. These processes can be better understood by Petri net modelling.

To represent reaction velocity in the main pathway, we adopt one of the most known and used method of Michaelis-Menten kinetics. Let us consider a petri net for an enzymatic reaction with two places S and P for substrate and product respectively and continuous transition T whose firing speed is given by:

$$\frac{-d[S]}{dt} = \frac{d[P]}{dt} = \frac{V_{max}[S]}{(K_m + [S])}$$

here [S] and [P] represent the contents of continuous places S and P, respectively and V_{max} is a constant number representing the maximum reaction rate and K_m is the Michaelis Menten constant.

The transition will fire when [S] > 0. Necessary data needed for these computations were collected from literature and are listed in Tables 2 and 3.

Variation in concentration of IPP and DMAPP in the MEV pathway of terpenoid synthesis is shown in Fig. 9. Initial concentrations are taken from literature [14]. Increase in concentration of IPP is much faster as compared to that of DMAPP. The sharp decrease in concentrations of both precursors can be attributed to the fast production of GPP. Concentration (μM) changes in metabolites in a enzyme catalyzed reaction with time (pt) is depicted in Fig. 10. When the concentration of GPP increases over time, IPP &DMAPP and GPPS production decelerates at 30pt (time) and remains constant. However, a vast increase in GPP concentration is observed. Similar trend is seen in the case of GPP, (–)-limonene and (-)-limonene synthase. In general, it is clear from Figs. 10-14 that concentration of product increases, concentration of substrate decreases and amount of catalyst remains constant. The simulation and validation processes performed using the

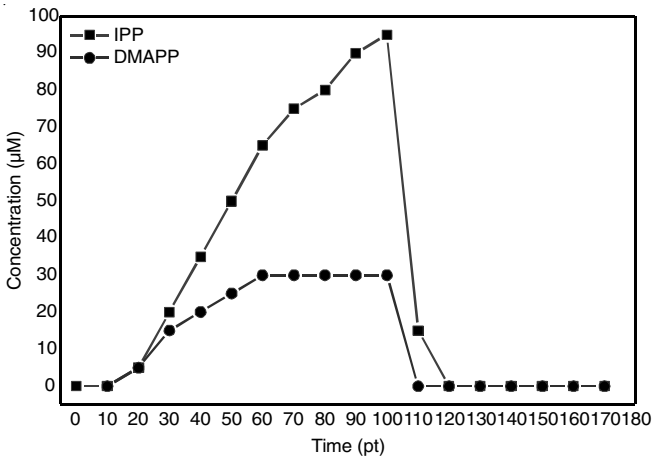

Fig. 9. Variation in concentration of IPP & DMAPP in the MEV pathway of terpenoid synthesis are taken from literature

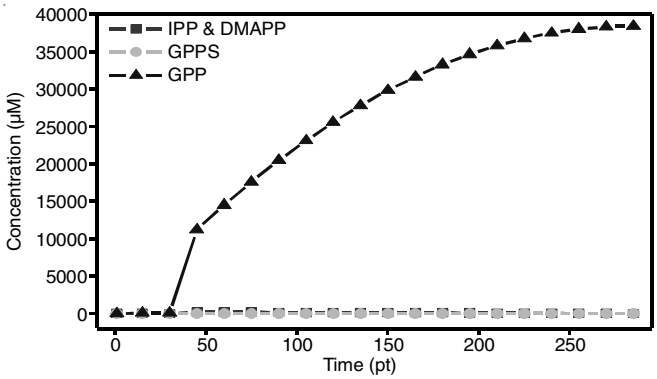

Fig. 10. Variations of the concentrations of IPP, DMAPP, GPPS and GPP with time

TABLE-2
K_m AND K_{cat} CONCENTRATIONS ABSTRACTING REACTION VELOCITY (V) OF IMPORTANT REACTANTS OF MENTHOL BIOSYNTHESIS

Names of metabolites	K_m (μm)	K_{cat} (S^{-1})	Reaction velocity ($V_{max} = K_{cat}/K_m$); (M^{-1}, s^{-1})	References to K_m & K_{cat} values
GPP	2.2	2.1	0.95	14
(-)-Limonene	0.2	0.04	0.2	48
(-)-*trans*-Isopiperitenone	72	0.002	0.000027	46
(-)-Isopiperitone	1	1.3	1.3	46
(+)-Pulegone	2.3	1.8	0.782	46
(-)-Menthone	3.0	0.6	200000	47

TABLE-3
PLACES HAVING NON-ZERO INITIAL VALUES

Metabolite name	Variable	Initial value*	Comment
IPP	P_0	95	Concentration of IPP
DMAPP	P_1	30	Concentration of DMAPP
GPP	P_2	28.5	Concentration of GPP
(-)-Limonene	P_3	3.2	Concentration of (-)-Limonene
(+)-Pulegone	P_{11}	0.16	Concentration of pulegone
(-)-Menthone	P_{23}	4.29	Concentration of (-)-Menthone
(-)-Menthol	P_{25}	0.06	Concentration of (-)-Menthol

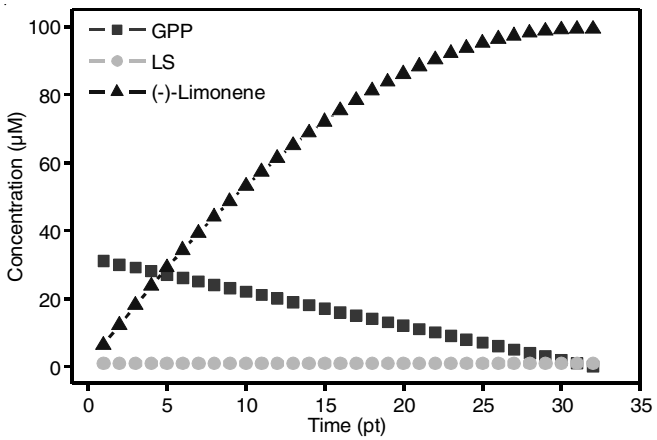

Fig. 11. Concentration (µM) changes in metabolites affected by the enzyme catalyzed reaction time (pt), Synthesis of (-)-Limonene

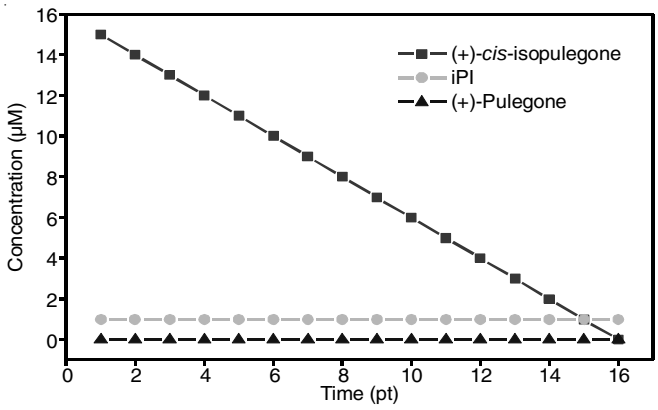

Fig. 12. Variation in the concentration of (+)-Pulegone and (+)-*cis*-isopulegone

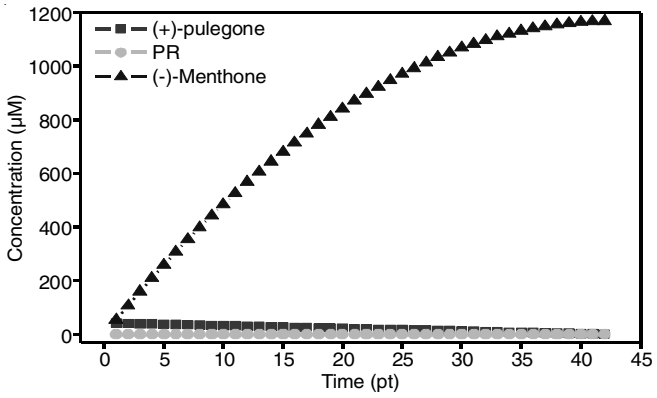

Fig. 13. Variation in the concentration of (-)-menthone and (+)-pulegone

model are consistent with known biological information and data. The intuitive approach introduced by the SPN technique enables intricate modelling tasks to be viewed and solved in a graphical perspective. The above petri net model drawn with the help of PIPEv4.3.0 [41] petri net software tool. The model serves as for better understanding the reactions involved in pathway. Perturbations performed on the model provide insight into the effects of manipulation of a single reaction on the whole network and thus should facilitate both industrial and biomedical menthol bioengineering.

The simulation and validation processes, as well as the developmental stages of the model performed in this study,

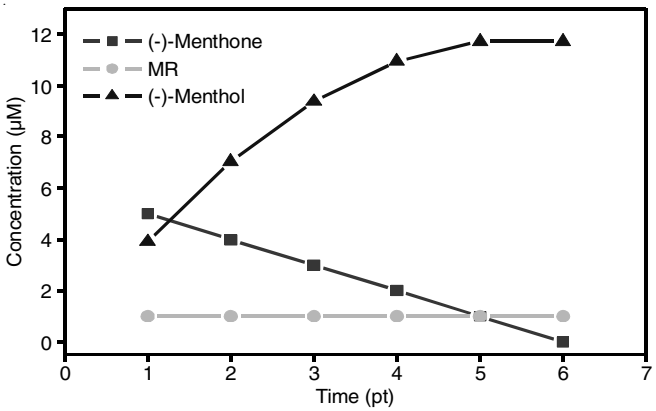

Fig. 14. Concentration (µM) changes in metabolites affected by the enzyme catalyzed reaction time (pt). When the concentration of menthol increases over time, menthone production decelerates about 1 pt; however, a vast increase in menthol concentration is observed

were carried out using a computer program written in FORTRAN 77. The model was validated against known data and information obtained from extensive literature and database searches carried out in the study. Simulation results are returned as concentrations (µM) *versus* Petri net time (pt) graphs. Here, we discussed the six simulated conditions.

Conclusion

In this paper, a Petri net modelling and simulation of the menthol biosynthesis pathway was discussed. Petri nets represent not only a unique formalism to depict biochemical networks, but also provide techniques, which can be applied for qualitative validation of the model before starting a quantitative analysis. A stochastic Petri net (SPN) was used since simple petri net is the best way of explaining the P and T invariants for modelling and studying biological systems. The SPN modelled and presented using PIPEv4.3.0 PN software tool [41]. The goal is to facilitate the behavioural studies between time (pt) and concentration (µM) of recognized metabolites. The simulations have shown the dependencies between several metabolites and enzymes of the modelled system. They allowed to have a general idea of the behaviour of the pathway, highlighting the irregularity resulting in the end of each execution scenario. This information are expected to identify reactions that could be experimentally manipulated to enhance the productivity of this medically and commercially important material menthol. In future, the existing net should be extended by other central biochemical processes (*e.g.* synthesis, catalysis, degradation, inhibition), rst of all to get deeper insights into the whole biosynthesis in the menthol plant and, as a side effect, to scrutinize Petri net methods for more complex networks.

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