BIOSYNTHESIS OF $C_{10}H_{16}$ (LIMONENE) BY MEVALONATE PATHWAYS AND ITS PHARMACOLOGICAL APPLICATIONS

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ABSTRACT

Limonene is a transparent, colourless compound (chemical) has D and L isomer; which is found in oil of citrus fruits peels. It is a monoterpene (monocyclic) hydrocarbon and used in the production of several commodity chemicals, biodiesel products, medicinal compounds and pharmaceutical and it is also an important precursor of many flavouring. Limonene is having low toxicity and can be used for several purposes. Overproduction of Limonene can be achieved by genetic engineering of Mevalonate pathway and 2-C-methyl-D-erythritol-4-phosphate pathway (MEP and MVA) in microorganisms by introducing limonene synthase. Limonene has several applications in industrial products and also can be used in clinical or medical field. Moreover the metabolic engineering of the pathways is potential production platform for any valuable limonene derivative. Limonene is flammable, function as biofuel. Limonene is naturally occurring in plants but genetically engineered microbes could produce sufficient quantity of limonene to cover the need but yet requires more efforts in labs to engineer more effective and active microbes for overproduction of limonene by developing Geranyl Pyrophosphate (GPP) pool in microbes.

Keywords: MEP (Mevalonate pathway), MVA (2-C-methyl-D-erythritol-4-phosphate pathway), BUN (Blood Urea Nitrogen), IDP (Isopentenyl diphosphate) and DMADP (dimethyl allyl diphosphate), HMGR (hydroxymethylglutaryl_CoA reductase), GPPS (Guanosine pyrophosphohydrolase/synthetase).

INTRODUCTION

Limonene is a liquid hydrocarbon belongs to monoterpene family of terpenoids, colourless organic compound with D and L isomers. D-isomer smells like an orange (Fahlbusch *et al*., 2003) and L-isomer has fragrance similar to a resin of pine tree or wood turpentine (Fig. 1).

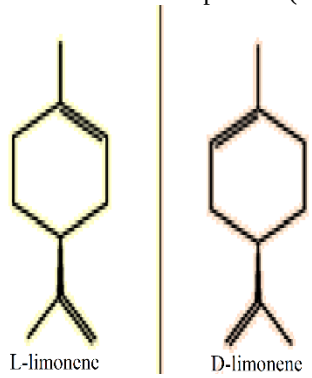


Fig 1. structures

Limonene takes its name from the peel of lemon. Biological sources produce only one enantiomer such as citrus fruits because limonene is a chiral organic molecule. Racemic mixture of limonene both D and L forms are called as dipentene (Simonsen, 1947). Using techniques such as centrifugal separation or steam distillation D-limonene can be obtained from citrus fruits.

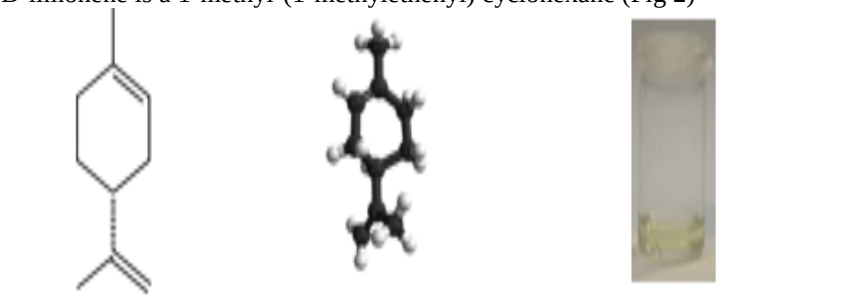
CHEMISTRY OF LIMONENE

1. D-LIMONENE INGESTION

D-limonene is considered safe while using as a flavouring agent. Fluctuation exists in dietary uptake of d – limonene which varies with the variation of food that is consumed. It is estimated as 0.27mg/kg of human body weight per 24 hours. For a 60 kg body it is 0.27 milligram X 60kg = 16.2 milligram which is daily requirement of a human body. In some areas the uptake is much higher that 20-40 milligram per day and 50-90 milligram per day, it is due to ingestion of citrus fruits and peel (Hakim *et al*., 2000).

2. CHEMICAL STRUCTURE AND CHARACTERISTICS OF D-LIMONENE

D-limonene is a 1-methyl-(1-methylethenyl) cyclohexane (Fig 2)



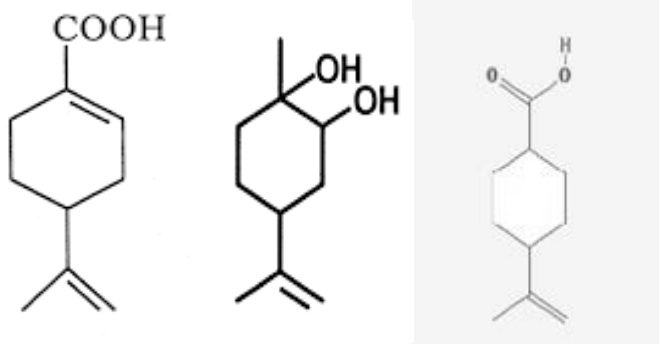
General structure *Ball and stick Model* *Liquid form of limonene*

Fig 2. Structures of D-limonene is a 1-methyl-(1-methylethenyl) cyclohexane having a molecular formula $C_{10}H_{16.}$

The density of limonene is 0.88411 gram per cm^3, with a molar mass 136.24 gram per mole. The melting point is ranging from -73.35 to -74.35 ^{0}C (-100.03 ^{0}F to -101.83^{0}F) and boiling point of limonene is 175 ^{0}C to 176 ^{0}C (347 ^{0}F to 349 ^{0}F). It is, frequently soluble in alcohol, Benzene, Chloroform, ether etc and insoluble in inorganic solvent water. It rotates the plane polarized light to right between the degrees 87^0 - 102^0 (Table 1).

METABOLISM OF D-LIMONENE

D-limonene has half-life in human body is about 12 – 24 hours (Crowell *et al*., 1992). The metabolites of D-limonene is excreted out of the body through urine (Igimi *et al*., 1974) (Kodama *et al*., 1976). If dosing of D-limonene is continued for two days there would be no accumulation of metabolites within the body (Vigushin *et al*., 1998). In human body the D-limonene metabolites are Perillic acid, dihydroperillic acid, limonene-8, 9-diol, limonene-1, 2-diol and other include such as perillic acid isomer (Chow *et al*., 2002) (Vigushin *et al*., 1998) (Crowell *et al*., 1994) (Poon *et al*., 1996). (Fig 3)



Perillic Acid Limonene-1, 2-diol dihydroperillic acid

Fig 3. D-limonene metabolites are Perillic acid, dihydroperillic acid, limonene-8, 9-diol, limonene-1, 2-diol

Taking 850.49 grams to 1133.98 grams of limonene which contains 445-595 milligrams of (+) Limonene, the concentration peak of Perillic acid can be observe in human body which declines with passage of time. Maximum

perillic acid concentration was observed in human study of taking lemonade. The concentration was 2.08 to 13.98 micro mole which was not detected after 24 hours of lemonade intake.

Table 1. Properties of limonene.

Properties	**(+) - limonene or (R) – limonene or (D) – limonene**	**(-) – limonene or (S) – limonene or (L) – limonene**	**Dipeptene**
Chemical Abstracts Service Registry Number or CASRN	5989-27-5	5989-54-8	138-86-3
Compound Name	(R)-1-methyl-4-(1methylethenyl) cyclohexene	(S)-1-methyl-4-(1methylethenyl) cyclohexene	1-methyl-4-(1methylethenyl) cyclohexene
Chemical formula of Compound	$C_{10}H_{16}$	$C_{10}H_{16}$	$C_{10}H_{16}$
Molecular weight of Chemical Compound	136.23 g/mol	136.23 g/mol	136.23 g/mol
Melting point of Compound in (°C)	-74.35	-74.35	-95.9
Boiling point of Compound in (°C)	175.5–176.0	175.5–176.0	175.5–176.0
Density	0.8411 g/cm3 at 20 °C	0.8422 g/cm3 at 20 °C	0.8402 g/cm3 at 20 °C
Vapour pressure	190 $N.m^{-2}$ or $Kg.m^{-1}.s^{-2}$ at 20°C	190 $N.m^{-2}$ or $Kg.m^{-1}.s^{-2}$ at 20°C	190 $N.m^{-2}$ or $Kg.m^{-1}.s^{-2}$ at 20°C
Water solubility	13.8[a] mg/litre at 25°C	-	-
Henry's law constant	34.8[b] kPa m3/mol at 25°C	-	-
Log Kow	4.23[c]	-	4.83[d]

a- United States Environmental Protection Agency (US EPA), Duluth, Minnesota, 1991. Assessment Tool for the Evaluation of Risk (ASTER) database, Environmental Research Laboratory, Massaldi and King, 1973

b- United States Environmental Protection Agency (US EPA), and Syracuse Research Corporation (SRC), calculated value by Environmental Fate database, Office of the Toxic Substances, New York, NY, 1995).

c- Calculation by United States Environmental Protection Agency, 1990a and 1994.

d- Calculation by United States Environmental Protection Agency, 1994. Log Octanol–Water Partition Coefficient Program [LOGKOW], Syracuse Research Corporation (SRC), New York, NY.

EXCRETION

D-limonene is mostly excreted through urine in form of its metabolites. The excretory metabolites in urine are glucuronides which is the two isomers of monohydroxylated limonene, limonene-8, 9-diol, Perillic acid and dihydroperillic acid (Vigushin *et al*., 1998) (Poon *et al*., 1996). In urine 7-11% in form of Perillic acid and its metabolites and 25-30% limonene-8, 9-diol its glucuronide are excreted (Kodama *et al*., 1976).

HUMAN STUDY REGARDING D-LIMONENE

In a study five adult men were given one dose of 20 grams (+)-limonene. The subjects under study complained about tenesmus but in blood tests no effect was seen everything in blood was normal such as total protein, cholesterol, bilirubin, alkaline phosphatase regarding liver. There was not either any problem regarding Blood Urea Nitrogen (BUN) of kidney or amylase of pancreas function functions (Igimi *et al*., 1992).

D-limonene is safe for human even the concentration is increased up to 100 milligrams per kilogram of body weight except slightly fatigue for few hours after post ingestion of D-limonene (Crowell *et al*., 1994). In another study female rats were given 600 milligrams per kilogram of body weight of D-limonene and found low survival while in male rats nephropathy was noticed (National Toxicology Program 2007). D-limonene induced nephropathy in rats under study after sub-acute or chronic exposure. Nephropathy in rats is due to accumulation of $\alpha_{2\mu}$ - globulin in hyaline droplets, this kind of accumulation does not occur in human (Whysner and Williams, 1996).

OVERPRODUCTION OF D-LIMONENE

Green plants and several other microorganisms prepare essential secondary metabolites; these can be used by human in form of medicines, cosmetics, cuisines etc. There are about 60,000 secondary metabolites of terpenoids which can be used for several purposes by human (Cheng *et al*., 2007) (Xie *et al*., 2012). Terpenes are driven from five carbon units (C5) units called as Isopentenyl diphosphate and dimethyl allyl diphosphate (IDP & DMADP). These compounds are synthesized by Mevalonate pathway and 2-C-methyl-D-erythritol-4-phosphate pathway (MVA & MEP) (Smit and Mushegian, 2000) (Degenhardt *et al*., 2009) (Nagegowda, 2010) (Lombard and Moreira, 2011). (Fig 4)

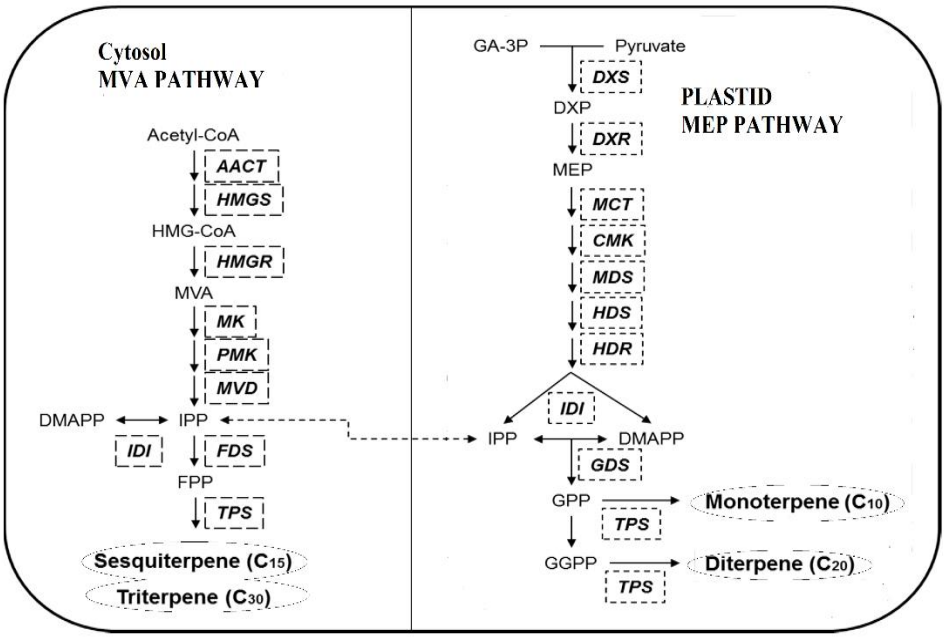


Fig 4. by Mevalonate pathway and 2-C-methyl-D-erythritol-4-phosphate pathway (MVA & MEP*)*

Monoterpene synthases are group of enzymes that belong to major group of terpene synthases which are responsible for the production of monoterpenes (C_{10}) [22, 23]. (E, E)-β- farnesene synthase is a multi-substrate enzyme which can be extracted from herb Mentha x piperita. Guanosine Diphosphate (GDP) is used by (E, E)-β-farnesene synthase as substrate and then can produce several different cyclic monoterpenes. Guanosine Diphosphate (GDP) is a ten carbon moiety ($C_{10}H_{15}N_5O_{11}P_2$) which acts as precursor for monoterpenes (C_{10}). After chemical procedure the enzyme produces monoterpenes such as limonene (48%) and terpinolene (15%) and also generates acylic monoterpene myrcene (15%) (Crock *et al*., 1997). 8-selinene and γ-humulene synthase in power to produce monoterpenes. When Guanosine Diphosphate (GDP) substrate is available (Table 2), germacrene C synthase from solanum lycopersicum can also produce limonene (Colby *et al*., 1998). A plasmid (pBbE1a_GPPS_LS) is designed containing a gene whose product is geranyl pyrophosphate synthase (GPPS) from Abis grandis which converts the IPP/DMAPP to Geranyl Pyrophosphate (GPP), this plasmid also carrying a gene which produces limonene synthase from Mentha spicata, which converts Geranyl Pyrophosphate (GPP) to limonene (Redding Johanson *et al*., 2011). This process does not produce to detectable amount. Two plasmids were pBbA5c_MevT_MBI and pTrc_GPPS_LS and then the products of these plasmids for limonene were examined through GC/MS, which produced limonene at rate of 2 milliliter per liter.

Phosphomevalonate kinase (PMK) and mevalonate kinase (MK) are two mevalonate pathway proteins as in saccharomyces cerevisiae, their genes would be boosted with stronger promoters and product level is enhanced (Redding Johanson *et al*., 2011). It is also seen that the function of hydroxymethylglutaryl_CoA reductase (HMGR) is not to mark and accumulates in cell under study (Pitera *et al*., 2011). Replacement of both enzymes HMG_CoA synthase and HMG_CoA reductase by other balanced enzymes from staphylococcus aureus increase production of amorpha-4, 11 diene (Tsuruta *et al*., 2009). By combing increasing of Phosphomevalonate kinase (PMK) and mevalonate kinase (MK) levels and replacing the HMG_CoA synthase and HMG_CoA reductase (HMGS and HMGR) from saccharomyces cerevisiae with those from staphylococcus aureus (MTSA operon) leads to an increase in production of limonene from 2 milligrams per liter to 70 milligrams per liter. By combing these improvements with truncation of Guanosine pyrophosphohydrolase/synthetase (GPPS) and attains increase in production to 167-

fold from 2 milligrams per liter to 335 milligrams per liter of limonene. Reduction in number to decrease metabolic burden on cell by preparing assembled plasmid and transformed into Escherichia coli reduce the production of limonene to 187 milligrams per liter. When two strains as 2p_SA_f_tr and 1pC are prepared and induced using IPTG levels (500µM or 25,100) shows maximum limonene production at 100µM IPTG for strain 2p_SA_f_tr and for strain 1pC occurs at 25µM IPTG. The maximum production for both strains is almost same that is about 435 milligrams per liter (Alonso-Gutierrez *et al*., 2013).

Table No. 2 Overview of Enzymes that produce limonene.

Enzyme	**Species**	**Substrate**	**Terpenoid**	**Refrence**
β-Bisabolene synthase	Santalum austrocaledonicum	Guanosine Diphosphate (GDP)	Limonene, terpineol	(Jones *et al*., 2011)
(E)-β-farnesene synthase	Mentha x piperita	Guanosine Diphosphate (GDP)	Limonene, α – terpineol	(Crock *et al*., 1997)
(E,E)-α-farnesene synthase	Cucumis sativus	Guanosine Diphosphate (GDP)	Limonene, terpinolene, myrcene	(Mercke *et al*., 2004)
α-Bisabolene synthase	Abies grandis	Guanosine Diphosphate (GDP)	camphene, sabinene, limonene, β-pinene, β-myrcene, α-Pinene	(Bohlmann and Croteau, 1999)
Germacrene C synthase	Solanum lycopersicum	Guanosine Diphosphate (GDP)	Limonene	(Colby *et al*., 1998)
δ-Selinene synthase	Abies grandis	Guanosine Diphosphate (GDP)	Limonene	(Steele *et al*., 1998)
γ-Humulene synthase	Abies grandis	Guanosine Diphosphate (GDP)	Limonene, terpinolene, myrcene, (E)-β-ocimene, camphene, α-pinene, β-pinene, sabinene, α-thujene, α-terpinen	(Steele *et al*., 1998)
Terpene synthase	Arabidopsis thaliana	Guanosine Diphosphate (GDP)	α-Pinene, sabinene, β-pinene, β-myrcene, limonene, (E)-β-ocimene	(Chen *et al*., 2003)

Both microbes Saccharomyces cerevisiae and Escherichia coli are suitable for production of limonene because they use either MEP or mevalonate pathway which produces isoprenoid precursors such as Geranyl Pyrophosphate (GPP). These microbes can be engineered for monoterpene production. Geranyl Pyrophosphate (GPP) is intermediate product in MEP or mevalonate pathway which is converted to large chain isoprenoids (Burke and Croteau, 2002) Expressing plant limonene synthase in suitable microbes higher limonene production can be achieved (Carter *et al.*, 2003) but low availability of GPP the amount of limonene is not so high. Escherichia coli results in high production of limonene, yeast produce also limonene but the amount is less than Escherichia coli (Table 3).

Yeast, however is more suitable for production of limonene because it can tolerate pH extremes, osmotic pressure and other culture infections (Gruchattka *et al*., 2013). Streptomyces species and Cyanobacteria also produce monoterpenes (Giglio *et al*., 2011). Cyanobacteria use CO_2 and light for production of limonene (Davies *et al*., 2014) carbon from CO_2 could contribute appreciable platform for production. Overexpress of MEP or mevalonate pathway through terpene synthase could produce sesquiterpenes, amorphadiene etc. along with sesquiterpenene synthases in yeast strain (George *et al*., 2015; Martin *et al*., 2003). By adding of mevalonate pathway in Escherichia coli can produce limonene (Alonso-Gutierrez *et al*., 2013; Dunlop *et al*., 20011; Willrodt *et al*., 2014). Mevalonate pathway side effect is acetate formation in Escherichia coli which is correlated with limonene production (Willrodt *et al*., 2014).

Table 3. Microbes engineered to produce limonene.

Microbe	**Engineered design**	**Limonene synthase**	**Maximum limonene production per liter of culture**	**References**
Escherichia coli BLR (DE3) codon+ (recA deficient derivative of BL21 (DE3))	Abies grandis GPPS (Oregon fir, Grand fir, Great silver fir, Giant fir, Lowland fir, White fir,)	Mentha spicata (Garden mint, Spearmint, Mackerel mint)	5 milligrams per liter	Carter *et al*., 2003
Escherichia coli (DH1 ΔacrAB)	HMG-CoA synthase and HMG-CoA reductase from Staphylococcus aureus MVK, PMK, and PMD Codon optimized from Saccharomyces cerevisiae One plasmid containing the mevalonate pathway genes and one plasmid with Geranyl Pyrophosphate Synthase and Limonene Synthase IDI and AACT from Escherichia coli GPPS from Abies grandis (garden mint)	Mentha spicata	57 milligrams per liter	Dunlop *et al*., 20011
Escherichia coli (DH1)	HMG-CoA synthase and HMG-CoA reductase from Staphylococcus aureus MVK, PMK, and PMD codon optimized from Saccharomyces cerevisiae AACT and IDI from Escherichia coli Geranyl PyroPhosphate Synthase from Abies grandis (garden mint)	Mentha spicata	430 milligrams per liter	Alonso-Gutierrez *et al*., 2013.
Escherichia coli BL21(DE3)	From Saccharomyces cerevisiae HMG-CoA synthase, HMG-CoA, MVK, PMK, and PMD Escherichia coli's AACT and IDI Geranyl pyrophosphate synthase from Streptomyces sp. strain KO-3988 or Geranyl pyrophosphate synthase from Abies grandis (spearmint)	Mentha spicata	1.35 grams per liter	Willrodt *et al*., 2014
Synechocystis species (PCC 6803)	IDI, DXS, and CrtE of Synechocystis	Schizonepeta tenuifolia	56 microgram per liter per culture per day	Kiyota *et al*., 2014
Synechococcus spechies (PCC 7002)	ΔglgC and Wild-type background were compared	Mentha spicata	4 milligrams per liter	Davies *et al*., 2014
Saccharomyces cerevisiae (AE9)	Yeast Farnesyl pyrophosphate synthase (ERG20 K197G) mutated to partly produce Geranyl Pyrophosphate	Perilla frutescens	490 micrograms per liter of L-limonene, 120 micrograms per liter D-limonene	Jongedijk *et al*., 2015
Saccharomyces cerevisiae (EPY210C)	HMG-CoA reductase from Saccharomyces cerevisiae upc2–1 transcription factor	Mentha spicata, Citrus limon	1.48 milligrams per liter	Behrendorff *et al*., 2013

Enzymes from MEP can be added to Cyanobacteria, synechocystis sp. PCC 6803 can increase limonene titers (Kiyota *et al.*, 2014). Limonene can be overproduced by expression of a shortened version of 3-hydroxyl-3-methylglutaryl _CoA reductase (HMGR) (Alonso-Gutierrez *et al*., 2013; Dunlop *et al*., 20011; Willrodt *et al*., 2014; Behrendorff *et al*., 2013). Availability of Geranyl Pyrophosphate can be achieved by expression of shortened versions of microbial Geranyl-Geranyl Pyrophosphate or Farnesyl Pyrophosphate synthases. These shortened or truncation leads to enzymes which produce Geranyl Pyrophosphate (GPP) (Narita *et al*., 1999) which is substrate of limonene. Several disadvantages are reported in this method such as negative impact on enzyme kinetics (Reiling *et al*., 2004) and production by products due to solvolyses of Geranyl Pyrophosphate (GPP) (Fischer *et al*., 2011) (Jongedijk *et al*., 2015). Geranyl Pyrophosphate synthase can be added for Geranyl Pyrophosphate (GPP) production

(Willrodt *et al.*, 2014) but not all Geranyl Pyrophosphate synthase are suitable for metabolic engineering. The most suitable enzymes for this purpose are homodimeric Geranyl Pyrophosphate synthase from Arabidopsis (Bouvier *et al*., 2000) and Abies (Carter *et al*., 2003). Heterodimeric Geranyl Pyrophosphate synthase can also be used for this purpose such as from menthe x piperita (Chang *et al*., 2010). The most effective Geranyl Pyrophosphate synthase ever used for limonene production is that from Abies grandis (Alonso-Gutierrez *et al*., 2013; Carter *et al*., 2003; Willrodt *et al*., 2014) that produce Geranyl Pyrophosphate (GPP) as substrate for limonene (Carter *et al*., 2003).

Table 4. The Enhancement of Terpenoids Synthesis in Different Studies.

Chemical Product	Product Increased	Overexpressed Gene	Reference
Isoprene	0.4-fold	1-deox-xylulos-5-phosphate synthase 1-deoxy-d-xylulose-5-phosphate reductoisomerase	Xue and Ahring, 2011
Phytoene	2.1-fold	Isopentenyl-diphosphate delta isomerase	Kajiwara *et al*., 1997
Isoprene	2.3-fold	1-deoxy-xylulos-5-phosphate synthase 1-deoxy-d-xylulose-5-phosphate reductoisomerase	Zhao *et al*., 2011
β-Carotene	2.7-fold	Isopentenyl-diphosphate delta isomerase	Kajiwara *et al*., 1997
Abietadiene	2.8-fold	1-deoxy-xylulos-5-phosphate synthase 1-deoxy-d-xylulose-5-phosphate reductoisomerase Isopentenyl-diphosphate delta isomerase	Morrone *et al*., 2010
Squalene	2.9-fold	1-deoxy-xylulos-5-phosphate synthase Isopentenyl-diphosphate delta isomerase	Ghimire *et al*., 2009
Limonene	3.4-fold	1-deoxy-xylulos-5-phosphate synthase Isopentenyl-diphosphate delta isomerase	Fu-Liang *et al*., 2014.
Carotenoids	3.5-fold	1-deoxy-xylulos-5-phosphate synthase 1-deoxy-d-xylulose-5-phosphate reductoisomerase Isopentenyl-diphosphate delta isomerase	Albrecht *et al*., 1999
Isoprene	4.0-fold	1-deoxy-xylulos-5-phosphate synthase (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate synthase (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate reductase Isopentenyl-diphosphate delta isomerase 4-diphosphocytidyl-2-c-methyl-d-erythritol kinase 1-deoxy-d-xylulose-5-phosphate reductoisomerase 2-c-methyl-d-erythrtol-4-phosphate cytidyltransferase 2-c-methyl-d-erythritol 2, 4-cyclodiphosphate synthase	Zurbriggen *et al*., 2012
Lycopene	4.5-fold	Isopentenyl-diphosphate delta isomerase	Kajiwara *et al*., 1997
Isoprene	4.8-fold	1-deoxy-xylulos-5-phosphate synthase 1-deoxy-d-xylulose-5-phosphate reductoisomerase Isopentenyl-diphosphate delta isomerase	Lv *et al*., 2013
Taxadiene	15.6-fold	1-deoxy-xylulos-5-phosphate synthase Isopentenyl-diphosphate delta isomerase (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate reductase 2-c-methyl-d-erythritol 2, 4-cyclodiphosphate synthase	Ajikumar *et al*., 2010
Levopimaradiene	611.3-fold	1-deoxy-xylulos-5-phosphate synthase Isopentenyl-diphosphate delta isomerase (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate reductase 2-c-methyl-d-erythritol 2, 4-cyclodiphosphate synthase	Leonarda *et al*., 2010

Expression of Geranyl Pyrophosphate synthase in microbes appears to have strong effects on the production of limonene (Alonso-Gutierrez *et al*., 2013). Two rate limiting enzymes are overexpressed on vector Pet21a+ in bacteria Escherichia coli, these are 1-deoxy-xylulose-5-phosphate synthase and Isopentenyl diphosphate isomerase

(DXS & IDI) of MEP which results in production of limonene to 17.4 milligrams per liter at 48 hours. The synthesis of limonene is increased to 35.8 milligram per liter by two phase culture system composed of normal-hexadecane (having composition-1/50, V_{org}/V_{aq}) composed to the initial quantity which is 4.87 milligram per lite. This initial titer is produced by p15T7-Is-gpps strain of E. coli with engineered having genes that encodes enzymes limonene synthase (LS) and a geranyl diphosphate synthase (GPPS) for limonene synthesis. The gene of geranyl diphosphate synthase (GPPS) is obtained from Abis grandis and gene of limonene synthase (LS) from menthe spicata (Fu-Liang *et al*., 2014). The rate limiting enzymes can be over expressed to enhance the production of different terpenoids to higher folds (Table 4).

MVA pathway in yeast *Yarrowia lipolytica* is engineered for increase limonene biosynthesis. A maximum amount of limonene is produced which is 23.56 milligram per liter. This amount is 226-fold increase than the initial yield (Cao *et al*., 2016).

APPLICATION OF LIMONENE

1. CLINICAL USE

Limonene acts as a solvent for cholesterol and used to dissolve the gall stones containing cholesterol. D-limonene also have strong effect on cancer activity, working as chemopreventive. It can also be used against heartburns. It is effect also against peristalsis and also can be used against several kinds of microbes in form of nanoemulsified. It is also having activity against free radicals.

a. GALLSTONES

In an experiment the gallstones are dissolved just in two hours. In Vivo, the gallstones were dissolved after the infusion of d-limonene which was excreted through bile duct. 20 milliliter through of d-limonene after every 48 hours patients with gallstone surgery dissolved overlooked gallstones. In some patients the dissolution of gallstones occurred just within six days (Igimi *et al*., 1976). This experiment was carried out on 200 patients with supplement of 20 – 30mL of D-limonene (97% solution) dissolved fully or partially gallstones. (Table 5).

Table 5. Number of patients with completely of partially dissolution of gallstones.

Size of gallstone	No. of Patients	Dissolution of gallstones	Percentage
0.5 to 1.5 cm (average 1 cm)	96	Completely	48%
0.5 to 1.5 cm (average 1 cm)	29	Partially	14.5%
0.5 to 1.5 cm (average 1 cm)	16	Completely along with hexamethaphophate a chelating agent dissolves bilirubin calcium stones (HMP)	8%

Treatment of patients with gallstones took time from few weeks to four months (Igimi *et al*., 1991).

b. HEARTBURNS

19 patients in an experiment suffering from chronic heartburn - Gastroesophageal reflux disorder (GERD) were given D-limonene capsule containing 1,000 mg per day. After a period of two weeks (14 days) total 89% of patients achieved complete relief from GERD (Wilkins, 2002).

c. PERISTALSIS

In a study D-limonene increases the resting tone of rat vas deferens and guinea pig ileum tissues. Some scientists believe that D-limonene is supporting healthy peristalsis (Lis-Balchin *et al*., 1996)

d. CANCER PREVENTIVE

D-limonene is chemopreventive against several types of cancer which is proved by several experiments. Chemically induced mammary cancer in rodents is reduced by D-limonene. The subjects are given either pure D – limonene or peel oil (Elegbede *et al*., 1984; Elson *et al*., 1988; Maltzman *et al*., 1989; Wattenberg, 1983). The inhibition of cancer occurs in initiation or promotion phases (Crowell, 1999; Uedo *et al*., 1999; Yano *et al*., 1999). D-limonene is also active in prevention of other cancers such as liver cancer, adenoma cancer pulmonary cancer, and forestomach tumors (Dietrich and Swenberg, 1991; Wattenberg *et al*., 1989; Wattenberg and Coccia, 1991). D-limonene performs its anti-carcinogenic activity during phase I and II by enhancing the activity of cytochrome p450 protein which metabolizes and deforms the carcinogens to less toxic forms and stops the activity of cancer causing agents on DNA. D-limonene accelerates the gastrointestinal UDP-glucuronosyl transferase (UGT) performance in rodents such as rats. It also stops tumor cell growth or proliferation and inhibits protein isoprenylation, which is anti – cancer activity of D – limonene (Crowell, 1999). It inhibits the gastric cancer by decreasing DNA synthesis and

increasing the programme cell death. It also decreases the activity of ornithine decarboxylase (Uedo *et al*., 1999; Yano *et al*., 1999). D – limonene inhibits the expression of oncogenes (Giri *et al*., 1999; Kaji *et al*., 2001). D-limonene helps in immune response by enhancing phagocytosis, microbiicidal activity and production of nitric oxide (Del Toro-Arreola *et al*., 2005). A female with breast cancer in a pharmacokinetics study showed effective and strong response to D-limonene at a rate of dose 8g/m^2/day (Fig. 5).

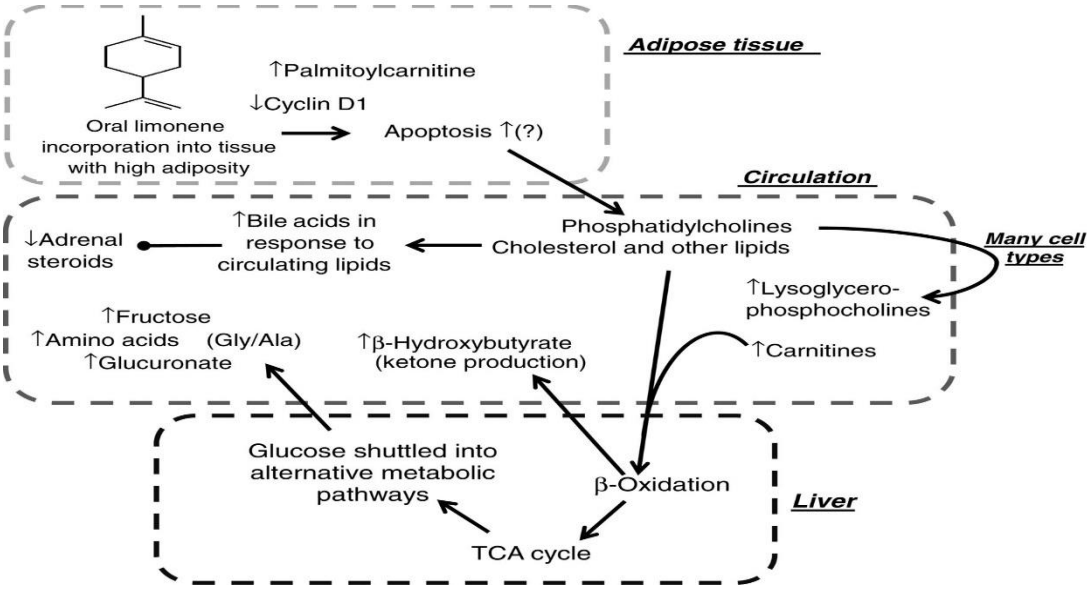


Fig 5. female with breast cancer in a pharmacokinetics study showed effective and strong response to D-limonene at a rate of dose 8g/m^2/day.

Three patients suffering from colorectal carcinoma decreased progression of the disease for more than period of six months with supplement of 0.5/m^2/day. Another patient with colorectal adenocarcinoma remained stable for 7.5 months with supplement of 2g/day (1g/m^2/day) (Vigushin *et al*., 1998). A study shows those people with cancer as epithelial cell carcinomas consume less citrus fruit peel. D-limonene also prevents skin cell carcinoma (Hakim *et al*., 2000).

Arrows indicate observed changes with limonene intercession. Limonene causes apoptosis or Programme Cell Death in different types of cells and is observed to deposit in adipose tissue (From top first box), this leads to the hypothesized changed metabolism in the liver (second box) and explain the known changes in circulation of metabolites (Third box). Gly & Ala.

e. AGAINST FREE RADICALS

In a study on Swiss mice with supplement of D – limonene epoxide 25 – 75 mg/kg body weight potentially stops the production of Nitrite ion (NO_2^-), hydroxyl radical (OH) and thiobarbituric acid reactive species (TBARS). In the same study it was also found that the D-limonene epoxide was effective against anxiety. Further it was determined that the activity of catalase and superoxide dismutase enzyme was increased to several folds against the free radicals (Cardoso de almeida *et al*., 2014)

f. ANTIMICROBIAL ACTIVITY

D-limonene is used to reduce the heat treatment of many microbes such as Listeria monocytogenes. When 0.5 mM of D-limonene is added to the heat medium the thermal resistance of L. monocytogenes is reduced to 5 times but when the same amount of Dlimonene is added to the heat medium in form of nanoemulsified the resistance to heat by L. monocytogenes is reduced to 100 times. This treatment to microbes can be used in food industry (Mate, *et al*., 2016). Bovine mastitis caused by Streptococcus uberis can be controlled by D – limonene. Minimum inhibitory concentration (MIC) of D-limonene for S. uberis is 3.3 to 52.5 milligrams per milliliter and Minimum Bactericidal Concentration (BAC) is 210 milligrams per milliliter (Montironi *et al*., 2016). D-limonene is alternative for most of antibacterial drug without side effects.

2. COSMETICS

Limonene is common in cosmetics industry. Having lemon like odor it is used in food manufacturing products to flavour cover the bitter taste of alkaloids. D-limonene is also used as fragrance in perfume industry including aftershave lotions, bath products etc.

3. BIOFUEL

Limonene is combustible, limonene is considered as a biofuel.

OTHER USES OF LIMONENE

Limonene exists being part of some glue and also occurs as a part in some wall paints. Limonene is an ingredient of almost all industrial air fresheners and air propellants. It is also used to remove adhesive postage stamps from envelope papers.

CONCLUSION

In conclusion, limonene has a wide range of applications in different field areas. This area is clinical, industrial, biomaterial and medical. Limonene is flammable and has function as biofuel. Limonene is naturally occurring in plants but genetically engineered microbes could produce sufficient quantity of limonene to cover the need but yet requires more efforts in labs to engineer more effective and active microbes for overproduction of limonene by developing Geranyl Pyrophosphate (GPP) pool in microbes. Limonene has antimicrobial activity this problem could be overcome by collecting limonene time to time from culture after biological synthesis.

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