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Biosynthesis of Curcuminoids with Insights into the Phylogeny of *Curcuma* and Genetic Engineering of Curcuminoid Production

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ABSTRACT

Turmeric or the 'Golden spice of India', has benefits that are utilized in food as well as a cure for various diseases since time immemorial. Produced by the plant genus *Curcuma* of the family Zingiberaceae, turmeric has properties that have been employed by various pharmaceutical industries. Around 53 species of *Curcuma* are found in India, with *Curcuma longa* being the most abundantly cultivated. They are widely found in Maharashtra, West Bengal, Uttar Pradesh, Kerala, Karnataka, Tamil Nadu and the North Eastern states.

The content of curcuminoids varies from species to species. Tropical and sub-tropical countries have around 70 rhizomatous perennial species widely distributed. The entire plant is a source of starch, carbohydrates, proteins, vitamins, minerals, and fats, whereas the rhizome is highly lavish in curcuminoids. Species like *Curcuma longa*, and *Curcuma zedoaria*, have been reported to have the highest content of curcuminoids among the cultivars produced in the Indian subcontinent. *Curcuma longa* and *Curcuma zedoaria* have the highest commercial value worldwide. *Curcuma caesia* and *Curcuma angustifolia* have been highly studied to decipher the content of secondary metabolites in the Genus *Curcuma*.

Curcuma is of importance because of its ability to produce polyphenolic compounds or curcuminoids, that possess anti-inflammatory, antioxidant, anticancer, antimicrobial, anti-fibrotic, ypocholesterolemic, antirheumatic, antihepatotoxic, antivenomous, antidiabetic, nociceptive, and gastroprotective properties. Curcuminoids make up turmeric, namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin, derived from *Curcuma* sp. Due to such exclusive performance and their potential usage in curing various diseases like cancer, curcuminoids have been a center of attraction for decades now. With the function as a nontoxic iron chelator possessing free radical scavenging properties and limited bioavailability, the compulsive need for retaining curcuminoids as a drug has led to various research and breakthrough discoveries about the plant.

Studies on a genetic level and a detailed analysis of the chemical nature of curcuminoids have led to the plausible conclusion that the curcuminoid-producing factors follow a casket and can only work in a particular linked pattern, diminishing the potency for the production of a drug that separately utilizes the benefits of the three curcuminoids (curcumin, bisdemethoxycurcumin, demethoxycurcumin). One way put forward is the discovery of the novel polyketide found in *Oryza sativa*, having the potential to produce curcuminoids in a one-pot synthesis pattern when rightly used by bioengineering.

Various research laboratories have successfully produced curcuminoids, in vitro, with the aid of bioengineered microbes. This review provides a detailed study of the biosynthesis of curcuminoids in

Curcuma sp. with the help of type III polyketides and condensation of carboxylic acid co-esters (CoA), along with the simultaneous analysis of the consumption of the pathway in a few bioengineering projects including *Oryza sativa*, paving way for a future with copious production of curcuminoids.

Keywords: *Curcuma longa*, curcuminoids, biosynthesis, Type III Polyketide syntheses, CURS, DCS, CUS

1. INTRODUCTION

Curcuminoids are polyphenolic compounds or more specifically, lipophilic diarylheptanoids with the chemical formula C21H20O6 and a molecular weight of 368.4 grams per mole [1]. These compounds are derived from the plant *Curcuma longa* and other *Curcuma* species, used as a spice, colouring agent, and herbal drug in Asian countries like India and China [2]. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin are commercially sold as turmeric [3].

Studies have shown, that rhizomes containing other secondary metabolites and volatile oils bear antiseptic and aromatic properties. Phenols, flavonoids, and tannins are highly present in *C. longa. C. angustifolia* contains alkaloids in the largest amount [4]. Sesquiterpenoids and monoterpenoids make up curcuma oil [5]. All *Curcuma* species are abundant in alkaloids, flavonoids, terpenoids, phenols, tannins, and saponins. The following table (Table 1.0) lists the potential uses of the secondary metabolites found in *Curcuma* sp. [6]

SECONDARY METABOLITES	USES
PHENOLIC ACID	Potential antioxidant
FLAVONOIDS	Potential antioxidant
TANNINS	Potential antioxidant, analgesic, anti-inflammatory activity, astringent
TERPENOIDS	Analgesic, anti-inflammatory
SAPONINS	Potential anti-carcinogenic
ALKALOIDS	Inhibits DNA topoisomerase and shows anti-microbial activity.

Table 1.0:- Potential Uses Of Secondary Metabolites Extracted From *Curcuma* sp.
Source: Dutta. (2015)

In brief, 253 compounds have been detected or extracted from the entire plant of *C. longa* alone. 109 sesquiterpenes, 68 monoterpenes, 22 diarylheptanoids and diarylpentanoids, 8 phenylpropenes, 4 sterols, 2 alkaloids, and 14 other compounds [7]. The pharmacological properties of a few *Curcuma* species are listed in Table 1.1.

SPECIES	BIOLOGICAL ACTIVITIES	USAGE
Curcuma aeruginosa	Antimicrobial, cytotoxicity; antinociceptive, antipyretic; uterine relaxant; antioxidant; inhibition of nitric oxide production; and anti-inflammatory.	Treatment of amebic dysentery, stomach ache, ulcer, indigestion, sprains, bruises, and cosmetics.
Curcuma amada	Antifungal and insecticidal; CNS depressant and analgesic; antiulcer; anti-tubercular; antioxidant, antimicrobial, cytotoxic and antiplatelet; anti-inflammatory; anticancer; and larvicidal.	Used as an alexiteric, antipyretic, aphrodisiac, laxative, mood disorders, biliousness, bronchitis, stomachic, carminative, healing, and sprain.
Curcuma angustifolia	Antimicrobial, antiulcerogenic; antioxidant; antifungal and antibacterial.	Used as a tonic, for dysentery, gastrointestinal disorders, body pain, inflamed mucous membranes, and stop bleeding of cattle injured by leech.
Curcuma caesia	Smooth muscle relaxant; anxiolytic, CNS depressant and neuropharmacological; antimicrobial; antiulcerogenic; antitumor and antioxidant; anticancer; antihepatotoxicity and nephrotoxicity.	Treatment of tonsillitis, sprains, bruises, asthma, piles, leukoderma, epilepsy, jaundice, dysentery, diarrhea, cough, cough, constipation, and well urination.
Curcuma longa	Anticarcinogenic and chemopreventive; anti-Alzheimer's disease; antiallergic; antioxidant; α-amylase inhibitor; neurotoxin-inhibitory; anti-inflammatory; antiobesity; antidiabetic; antiproliferative; hypoglycemic; hypolipidemic; antiarthritic; Immunomodulating; gastroprotective, antiulcerogenic; larvicidal; angiogenic; anti vasoconstrictive; antibacterial; neuroprotective;	Treatment against biliary diseases, hepatic disorders, anorexia, coryza, dyspepsia, rheumatism, sinusitis, antiseptic, gastroprotection, tonic, stimulant, and blood purifier.

	scolicidal; antifungal and anti-aflatoxigenic	
Curcuma zedoaria	Antimicrobial; antiallergic; Antihypertensive; antinociceptive and analgesic; hemagglutinating, antimutagenic and antioxidant; antiulcerogenic; antiproliferative; antifungal; larvicidal and pupicidal; cytotoxicity; anti-inflammatory and antinociceptive; and antiplatelet aggregation.	Used as rubefacient, carminative, expectorant, demulcent, diuretic, and stimulant.

Table 1.1:- Pharmacological Properties Of Certain *Curcuma* sp.
Source: Kaliyadasa & Samarasinghe. (2017)

Curcuma is a sterile genus and asexually reproduces from the rhizome. It is thought to have risen by vegetative propagation and selection of a hybrid between *Curcuma aromatica* (wild turmeric) and some other closely related species [8]. Though used in Asia for thousands of years, a reported discovery of a 'yellow colouring matter' from the rhizomes of *C. longa* led to the revelation of curcuminoids by Vogel and Pelletier [9]. The health benefits of the golden spice have been utilized for ages but not very efficiently because it fails to be converted into a drug. Although the total number is controversial, about 90-100 species of *Curcuma* are economically accepted and put to ethnobotanical uses. *C. amada* (mango ginger), *C. angustifolia* (wild arrowroot), *C. zedoaria* (zedoary), and *C. aromatica* (Cochin turmeric) are species holding great economic value [8]. The cultivars produced in Andhra Pradesh and Odisha yield the highest amount of curcumin, and turmeric from Assam is regarded for its superior quality and has the largest commercial market [3].

Curcumin, bisdemethoxycurcumin and demethoxycurcumin, are the major curcuminoids present in *C. longa*. The biosynthetic pathway involves enzymes and aromatic compounds that result in these three major curcuminoids. Type III Polyketide Synthases (PKSs) are the enzymes employed for the synthesis [2]. Curcumin synthase (CURS: CURS1, CURS2 and CURS3) and Diketide CoA synthase (DCS) were the type III PKS found to be involved in the synthesis of curcumin, bisdemethoxycurcumin and demethoxycurcumin. Availability of substrate, expression level of enzymes, and substrate specificity influence the composition of curcuminoids [2].

Many naturally occurring small molecules used in chemotherapy, antibiotics like macrolides and tetracycline are produced by PKSs [10]. Engineering type III PKS into *E. coli* has led to the generation of novel polyketides that have been used in producing plant-specific, unnatural curcuminoids by co-expression and reconstruction of bioengineered microbes, with respective genes of interest [11].

The genome of *Curcuma* is polyploid (2n = 63-105) [13, 14, 15] with around 56,036 genes identified, 74% of which are repeat elements and 94% of the genes with MSA (Multiple signs of Adaptive evolution). Obligatory genes involved in the biosynthetic pathway are the Curcumin synthase 1 (CURS1), Curcumin synthase 2 (CURS2), Curcumin synthase 3 (CURS3) genes, and the Diketide CoA (DCS) gene. These genes are exclusive to the *Curcuma* genus [12]. Growth stages and genotypes affect gene expression, significantly impacting curcuminoid balance in turmeric rhizomes. All three genes confer a more effective

bioprotectant activity than any one alone due to synergistic effects [13]. Enormous interspecific hybridization and polyploidization, intraspecific variations are large, hindering the construction of a compatible and congruent phylogenetic tree [14]. After decades of research, a legitimate biosynthetic pathway is available to us today. However, there is still a major link missing between the simultaneously occurring conversions (pathways) of CoA esters into secondary metabolites (curcumin, demethoxycurcumin, and bisdemethoxycurcumin.

2. THE CURCUMINOIDS OF *Curcuma longa*

Curcuminoids are the major secondary metabolites that impart the pharmacological characteristics of turmeric. These are compounds possessing anti-bacterial properties and are biologically active. Curcumin (CUR), demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) along with other metabolites of curcumin compose turmeric. In 1953, Srinivasan determined the existence of other curcuminoids through chromatography, after Vogel and Pelletier discovered curcumin [15]. The chemical names and formulas are given in Figure 1.

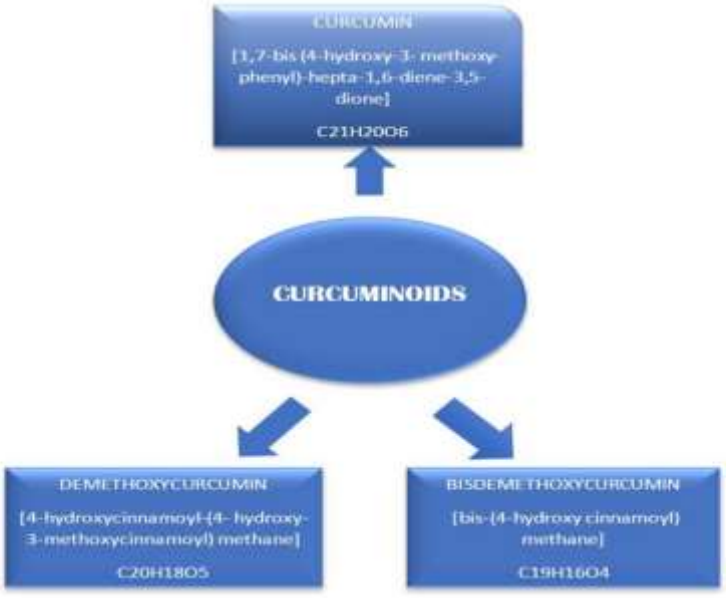

Fig. 1:- Derivatives of Curcumin

Two benzene methoxy rings joined by unsaturated chains are shared between the metabolites. Keto-enol tautomerism, α, β unsaturated β-diketo linker, and an aromatic methoxy phenolic group are responsible for the important functions of CUR, DCM, and BDCM. The metabolites exist in a keto-enol, trans-trans form [17, 18].

Linker gives flexibility to the structure and the aromatic group imparts hydrophobicity [17]. The hydrophobic nature of the central ring prohibits its solubility in water (acidic and neutral pH) but makes it highly soluble in acetone, methanol, and ethanol.

Some other metabolites of curcumin that reside in turmeric and exhibit medicinal properties are curcumin sulphate, curcumin glucuronide, dihydroxy curcumin, tetrahydroxy curcumin (THC), octa hydro curcumin (OHC), and hexahydroxy curcumin (HHC) [15]. In most cultivars, the concentration of curcumin is 77-99%, demethoxycurcumin is 17-25% and bisdemethoxycurcumin is 3-5% [16, 17]. BDMC is the most stable among them all.

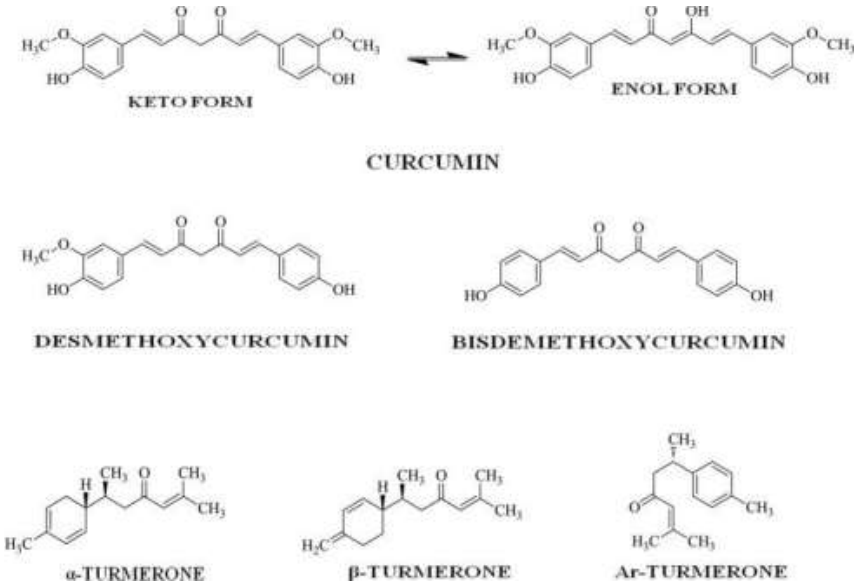

Fig. 2:- Chemical structures of important constituents present in turmeric

Source: Amalraj et al. (2017)

Anti-oxidant, anti-inflammatory, and anti-cancer effects are regulated by THC to a great extent. Platelet aggregation epistasis and anti-cancer effects are shown by HHC while OHC exhibits anti-inflammatory plus antioxidant activities [16, 17].

With the purpose of treatment and prevention of diseases, curcumin vitally is thought to be effective in the pathogenesis of molecular targets of various diseases. Regulation of cytokines, enzymes, kinases, growth factors and transcription factors, metastasis, apoptotic molecules, and receptors, employed in all phases of disease development can be manipulated by curcumin [16, 17, 18]. Related molecular targets of curcumin in various diseases are listed in Table 2.0.

DISEASES	MOLECULAR TARGETS OF CURCUMIN
Cancer	Cyclin D1, c-myc, Bcl-2, Bcl-Xl, cFLIP, XIAP, Ciap1 caspase-8, 3, 9, p53p21, DR4, DR5, JNK, Akt, AMPk, NF-κB
Obesity	MAPk, Wnt/β- catenin, NF-κB, TNF-α, IL-6
Diabetes Mellitus	Antioksidant, PPAR-γ, GLUT2, GLUT3, GLUT4, AMP-Kinase, HO-1
Cardiovascular Diseases	JAK2/STAT3, TNF-α, HO-1, NF-κB, il-6
Neurodegenerative Disease	Antioksidant, NF-κB, Kv1.3

	channels
Inflammatory Bowel Disease	NF-κB, COX-2, 5-LOX, iNOS
Allergy Asthma	Histamine release, Th2 response, cytokines
Psoriasis	IL-17A, IL-17F, IL-22, IL-1B, IL-6, TNF-α, Keratinocyte proliferation

Table 2.0:- Molecular Targets Of Curcumin During Pathogenesis Of Various Diseases.

Source: Kocaadam & Şanlier (2017)

Santosh. K Sundar et al (2007) analysed the function of curcuminoids in the suppression of tumor necrosis factor (TNF)-induced nuclear factor-κB (NF-κB) activation, revealing a pattern of the relative potency of curcuminoids (most to least potent) indicated below.

CUR> DMC> BDMC

This suggests that the presence of the methoxy group on the phenyl ring is essential. CUR, DMC, and BDMC exhibit activity in inhibiting cell growth and exerting anti-inflammatory effects by suppressing NF-κB and cyclin D1, along with inhibiting anti-apoptotic gene products, leading to anti-proliferative effects. They induce cytochrome C release, activate anti-angiogenic effects by downregulating the Vascular Endothelial Growth Factor (VEGF), and also trigger the activation of caspases and p53 [16].

The diseases or activities along with the type of curcuminoid acting on them and their effects are listed in Table 2.1

DISEASE/ACTIVITY	CURCUMINOIDS EMPLOYED	EFFECT OF TREATMENT
Oxidative Stress And Inflammation	CUR	Curcuminoid therapy changes the circulating concentration of the protein. Short-term supplementation with a curcumin-piperine combination significantly improves the oxidative and inflammatory status in patients with MetS.
Neuroprotective Activity	CUR	Inhibition of 6-OHDA induced neurotoxicity in SH-SY5Y cells.
Alzheimer's Disease	CUR, DMC, BDMC	Curcuminoids and their components showed dose-dependent inhibition in the frontal cortex and hippocampus with ex vivo AChE assay. Effective in memory enhancement.
Antitumor	CUR, DMC, BDMC,	DCM is a better inhibitor than CUR and

Activity	Cyclocurcumin	BDMC due to the presence of phenolic hydroxyl groups, methoxyl groups, and the diketone moiety. CUR affects the WT1 binding of the protein promoter.
Antioxidant Activity	CUR, DMC, BDMC	Good antioxidant capacity
Anticancer Activity	CUR, DMC, BDMC	CUR, DMC, and BDMC significantly decreased urokinase plasminogen activator. Three forms of curcuminoids significantly inhibited collagenase, MMPs.
Cardio-Protective Activity	CUR	Some amelioration in cardiac function, infarct size, and serum biochemical markers were noted.
Radioprotective Activity	CUR	Less cell necrosis
Sexually Transmitted Diseases	CUR	Box-Behnken Design has maximum residence time, good efficacy in terms of contraception, and the highest user compliance.
Antifungal Activity	CUR, DMC	The antifungal effect of CUR was stronger than that of DMC due to the existence of the methoxy group.
Anti-Inflammatory Activity	CUR, BDMC	Inhibition of calmodulin-dependent protein kinase II or extracellular signal-regulated kinase 1/2 leads to a reduction in inhibition by BDMC of LPS-induced inducible nitric oxide synthase expression and nitric oxide production.
Arsenic Toxicity	THC	A significant protective effect on mitochondria is a crucial element involved in both triggering and mediating the hepatoprotective response in hepatic cells.

Table 2.1:- Effects Of Treatment On Various Diseases Of Different Curcuminoids

Source: Amalraj et al. (2017)

3. THE TYPE III POLYKETIDE SYNTHASES

The type III Polyketide Synthases (PKS) are keto synthase homodimers that catalyse the conversion of a

starter substrate into an extender substrate through iterative decarboxylative Claisen condensation [18]. They catalyze reactions resulting in polyketide synthesis like flavonoids, stilbenes, and curcuminoids in plants. More than 20 PKSs are used commercially as drugs (like erythromycin, lovastatin, and tetracycline). PKS I, PKS II, and PKS III are so far discovered and classified. New members of the PKS family are being mined from natural as well as heterologous expressions, and are being engineered into industrial organisms viz. *Saccharomyces cerevisiae*, *Escherichia coli*, and *Streptomyces coelicolor* [19]. The superfamily of homodimeric enzymes such as thiolases, to which the Type Polyketide Synthases belong, possesses a highly homologous structure with a conserved catalytic triad, Cys- His- Asn as well as a CoA binding region [20]. The Type III PKSs do not use an ACP domain and are mainly involved in acyl thioester condensation into functional secondary metabolites [18]. The core β-keto synthase (KS) catalyzes the synthesis of polyketide backbones belonging to the superfamily of thiolase enzymes, that are involved in the catalysis of Claisen condensation reactions including acetoacetyl-CoA thiolase, ketoacyl thiolase, HMG- CoA synthase, β- ketoacyl ACP synthase I, II, III, and β- ketoacyl CoA synthase [19]. A five layered α-β-α-β-α core structural fold, location of the extensive interface of dimerization, active site placement, and usage of some catalytic cysteine (Cys) residue for attachment of substrates, are found to be conserved throughout the superfamily [19].

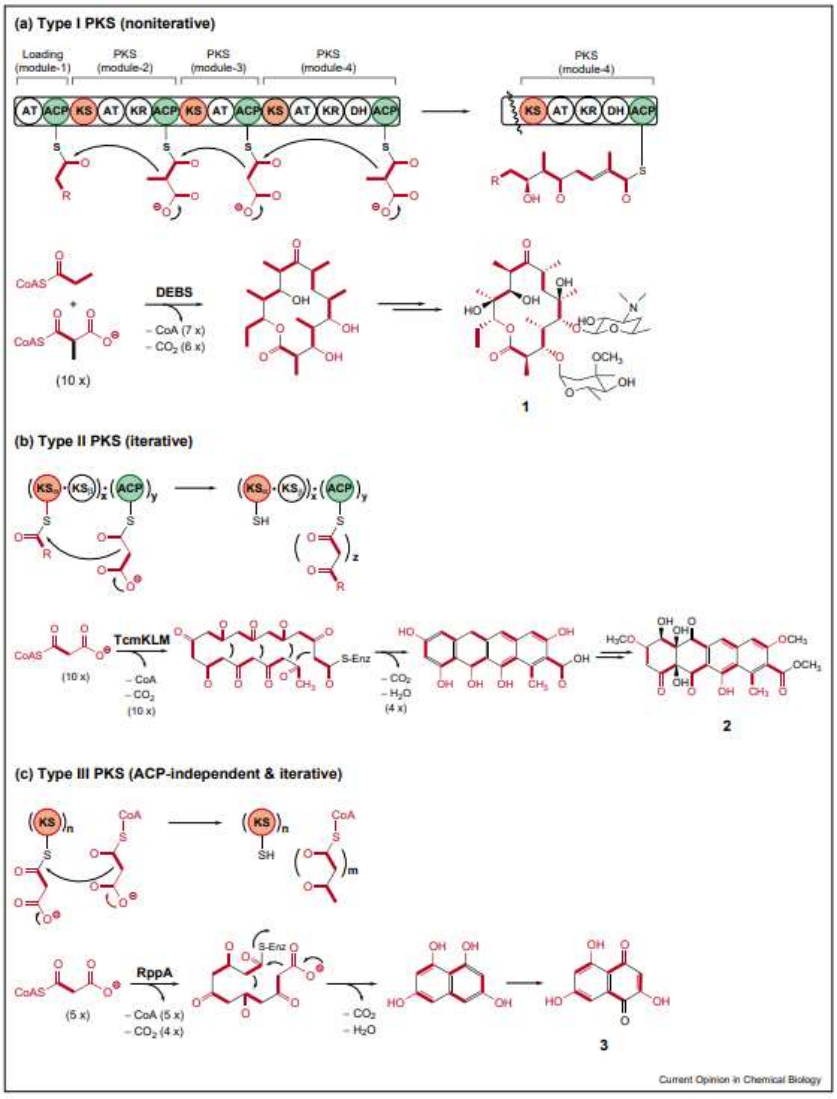

Fig 3:- Structure And Mechanism of Polyketide Synthases (PKSs). Source: Ben Shen (2003)

The main role of all PKSs is to catalyse the incorporation of acetate units in an expanding polyketide chain and to possess AT, ACP, and a β-keto domain [16]. Type I, type II, and type III PKSs are prevalent in living organisms like fungi, bacteria, plants, and a few animals [18, 19, 20, 21, 22]. Gene duplication or loss of function of fatty acid synthesis genes (FAS) has made Type I and Type II PKSs more evolved/advanced than Type III PKSs [18]. The kinetics of the association and dissociation of CoA thioesters may be altered on account of the increase or decrease of CoA interactions thus deciding the fate of Type III PKS reaction intermediate [18]. In 1970, Chalcone Synthase (CHS), involved in flavonoid synthesis was the first type III PKS ever discovered. CHS uses 4-coumaroyl CoA as starter substrates [11, 19]. To generate a poly-β-keto chain, the type III PKSs condense a starter unit with a series of extender units. An ACP-independent mechanism is utilized in the procedure. Acyl CoA thioesters are harnessed by a single enzyme that also catalyzes the transfer of the acyl group between CoA and an active site. As an acyl carrier, CoA is mandatory in both primary and secondary metabolism of polyketide biosynthesis [21].

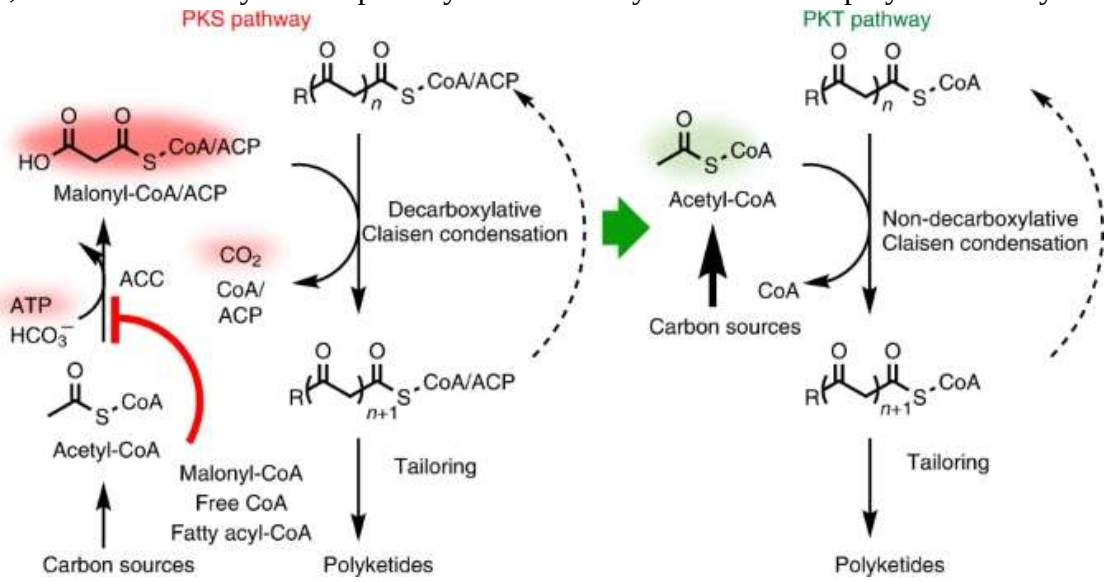

Fig. 4:- Polyketide Synthase Pathway

Source: Zaigo et al (2020)

In fatty acid biosynthesis, malonyl CoA formation is the first step, thus malonyl CoA is a readily available primary metabolite and is a common extender unit employed by all types of PKSs [21]. At the beginning of the enzyme reaction, loading of the starter substrate, elongation of the polyketide chain through decarboxylative condensation with the extender substrate, and termination of the resultant intermediate via cyclization take place within a single active site [20]. Diverse biosynthetic pathways in plants are achieved due to minor differences in the structure and capacity of the active site cavity [19, 22]. This cavity determines the preference of started and extender substrates thus deciding the fate of the entire reaction.

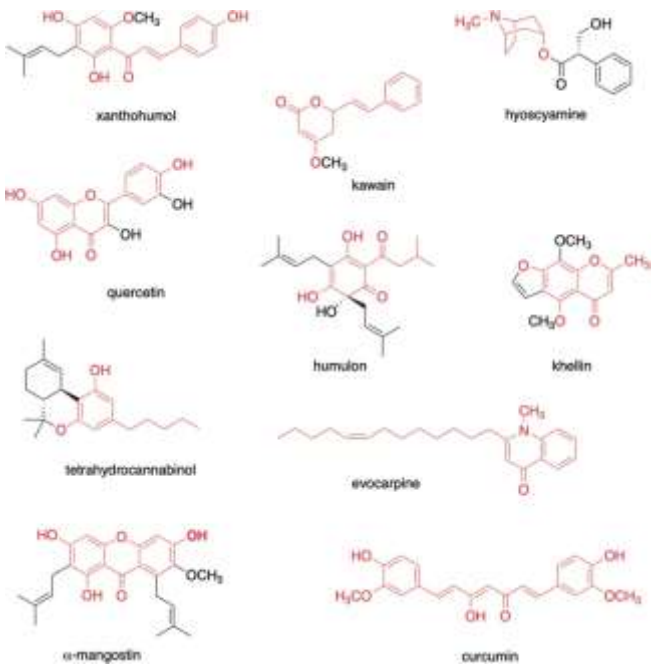

Fig. 5:- Typical Plant Secondary Metabolites Produced By Plant Type III PKSs
Source: Abe I (2020)

Curcuminoids synthesizing type III PKSs are Curcumin synthase and its isozymes (CURS 1, 2, 3), and Diketide synthase (DCS). 47-63% of the sequence identity of CURS and DCS is similar to other plant PKSs and 63% is identical to each other. Another type of Curcuminoid Synthase, CUS (curcuminoid synthase) is extracted from *Oryza sativa* (Poaceae) and has been inculcated in vitro production of curcumin [21, 22]. In *Oryza sativa*, the catalytic condensation of three substrates accomplishing a one-pot synthesis of curcuminoids is brought about by a single enzyme. The catalytic repertoires of the type III polyketide synthase family have been significantly expanded, providing a broader platform for the manipulation of enzyme reactions for the production of medicinally important, natural, and unnatural molecules [20].

A characteristic active site structure of curcumin synthase, CURS, with a rare hydrophobic pocket in the CoA binding tunnel, formed by the alteration of the active site Ser338 with Gln338 and the varied orientation of the gatekeeper Phe265 was revealed by X-ray crystallization. Curcuminoid synthase, CUS of *Oryza sativa* involved in the one-pot creation of the curcuminoid scaffold from malonyl CoA and p-coumaroyl CoA, controls the order of substrate loading at the active site, initiated with the loading of bulky p-coumaroyl CoA, the small malonyl CoA, and lastly the bulky p-coumaroyl CoA again. The X-ray crystal structure of CUS disclosed the unparalleled architecture of the active site. The two bulky coumarates and the malonate substrate can be consecutively loaded to the huge downward expanding active site with ease. Fixed by the hydrogen bonding Glu202-Tyr207-H2O-Asn142-Ser351 residue, which is an H2O molecule present at the proximity of Cys 174 in the catalytic center [20]. The amino acid sequence of *O. sativa* CUS shares 40–51% identity with other plant type III PKSs; 49% identical to *Medicago sativa* CHS, 51% identical to *C. longa* DCS, and 45% identical to *C. longa* CURS. A comparison of the sequences revealed that the Cys-His-Asn catalytic triad and most of the characteristic active-site residues of the CHSs are well conserved in CUS. However, Thr132, Thr197, Gly256, and Phe265, lining the active-site cavity of *M. sativa* CHS, are characteristically substituted with Asn, Tyr, Met, and Gly, respectively, which may account for the catalytic activity of CUS [24]. In addition to *O. sativa* CUS, a novel PKS3 from the Chinese club moss *Huperzia serrata* (Lycopodiaceae) also catalyzes

the one-pot condensation of feruloyl-CoA or p-coumaroyl-CoA or cinnamoyl-CoA and malonyl-CoA to yield a series of curcuminoids [25].

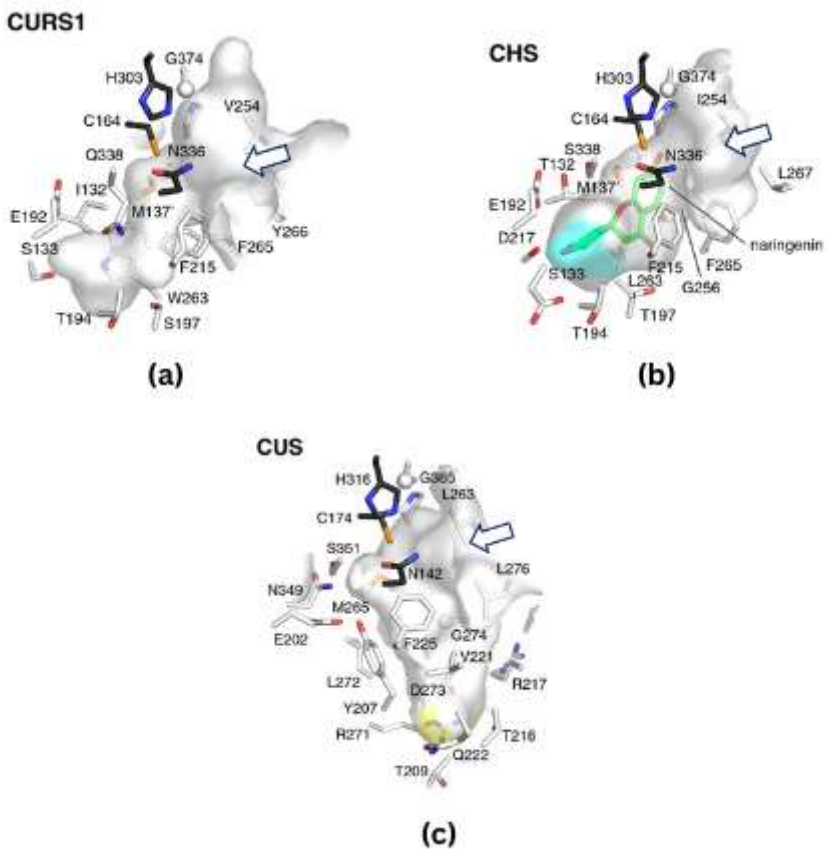

Fig. 6:- Active Site Cavities Observed In The Crystal Structures Of (a) *Medicago Sativa* CHS, (b) *C. Longa* CURS 1 and (c) CUS From *Oryza Sativa.* Source: Abe I (2020)

A close relationship can be drawn between the land plant CHS and non-CHS type III PKSs. Many enzymes involved in the biosynthesis of secondary metabolites like acridone synthases, pyrone synthases, bibenzyl synthases, p-coumaroyl triacetic acid synthase fall in the non-CHS category which evolved through repeated gene duplication, functional diversification, and repeat mutations from their ancestral plant enzymes.

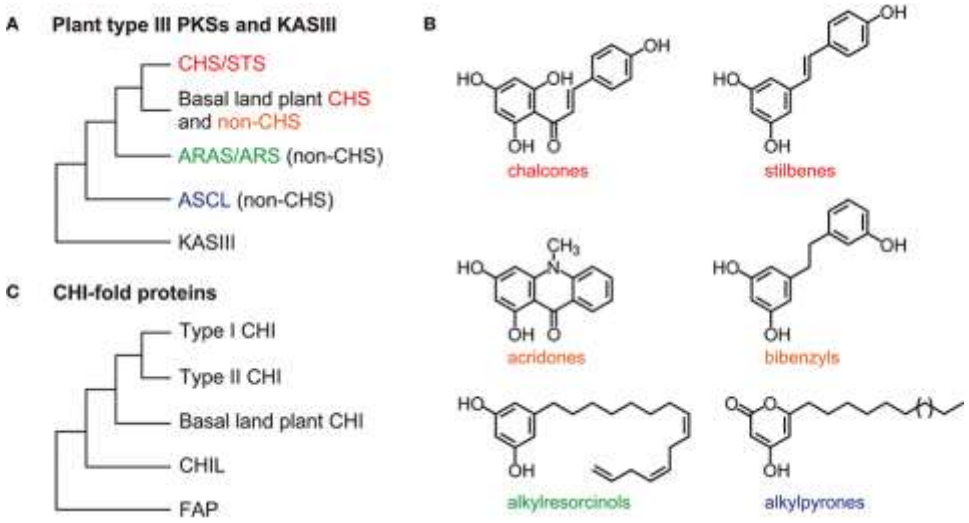

Fig. 7:- General overview of the type III PKS and CHI-fold protein phylogenies. (A) Relationships among the CHS/STS proteins, the basal land plant CHS, and non-CHS proteins of the plant type III PKSs. The non-CHS proteins include the ARAS/ARS proteins and the ASCL families. The overall three-dimensional protein structure is conserved in the type III PKSs and an *E. coli* KASIII enzyme (the αβαβα-fold). (B) Examples of type III PKS products. (C) The CHI-fold proteins in the CHI, CHIL, and FAP families share a common folded protein structure (the open-faced β-sandwich fold). ARAS, alkylresorcylic acid synthase; ARS, alkylresorcinol synthase; ASCL, anther-specific chalcone synthase-like enzyme; CHIL, CHI-like protein; CHI, chalcone isomerase; CHS, chalcone synthase; FAP, fatty-acid-binding protein; KASIII, 3-ketoacyl-ACP synthase isoform III enzyme; PKSs, polyketide synthases; STS, stilbene synthase. Source: Yonekura-Sakakibara et al (2019).

4. BIOSYNTHESIS OF CURCUMINOIDS IN *Curcuma longa* AND *Oryza sativa*

4.1. Biosynthesis of Curcuminoids in *Curcuma longa*

The rhizome of *C. longa* is the richest source of curcuminoids in the entire plant. Curcuminoids are small molecular weight, diarylheptanoid polyphenolic compounds found in *Curcuma* species. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin make up turmeric that is commercially sold in the form of a yellow dye, colouring agent, cosmetic beneficiary, and spices for different cuisines.

The biosynthetic pathway for the production of curcuminoids in *C. longa* involves type III polyketide synthase enzymes, carboxylic acid coenzyme A esters as the starter substrate, and malonyl CoA as the extender substrate. A polyketide chain is the result of the cyclization. The concentration of p-Coumaroyl CoA and feruloyl CoA in vivo regulates the composition of curcuminoids [2].

The amalgamation of curcuminoids is derived from a phenylpropanoid pathway, two phenylpropanoid units form curcuminoids. These are chemically derived from phenylalanine and are connected by a central carbon unit acquired from malonyl CoA [25]. In vitro, reaction products have a lesser amount of curcuminoids than the rhizome of turmeric. Leaves of turmeric also contain dihydroxy curcuminoids synthesized via double bond reduction of the curcuminoids [25].

Two type III PKSs are employed in the synthesis process, namely Curcuminoid synthase (CURS) and Diketide synthase (DCS). Until the discovery of CURS 2 and CURS 3, CURS 1 was believed to be at work along with DCS in yielding curcuminoid scaffold in *C. longa*. Both the enzymes have separate substrate affinity [2]. Expression levels of all three polyketide synthases are essential to determine the composition of the curcuminoid mixture along with the availability of substrates (p-coumaroyl CoA and feruloyl CoA). Feruloyldiketide CoA is formed from feruloyl CoA, catalyzed by DCS. CURS catalyzes the formation of curcuminoids from feruloyl CoA and Feruloyldiketide CoA. The integrated action of CURS and DCS catalyzes the biosynthetic pathway of curcuminoid synthesis [2, 21, 25].

Phenylalanine in the system is first converted to p- coumaroyl CoA which acts as a starter substrate for DCS. DCS along with malonyl CoA transforms p-coumaroyl CoA into p-coumaroyldiketide CoA. It is further acted upon by CURS, where CURS 1, CURS 2, CURS 3 converts it to demethoxycurcumin and CURS 3 singly converts it to bisdemethoxycurcumin and curcumin. Another pathway acts simultaneously where DCS prefers feruloyl CoA as a starter substrate and works more efficiently. CURS3 acts on Feruloyldiketide CoA to form demethoxycurcumin and CURS1, CURS2, and CURS3 together form curcumin [2, 27, 25, 26]. The incorporation of the methyl ethers at the 3´ position of Curcumin (before and after curcumin scaffold formation) is yet to be figured out. By condensing the Diketide CoA produced from starter substrates, acted upon by DCS, and CURS curcuminoids are produced [25].

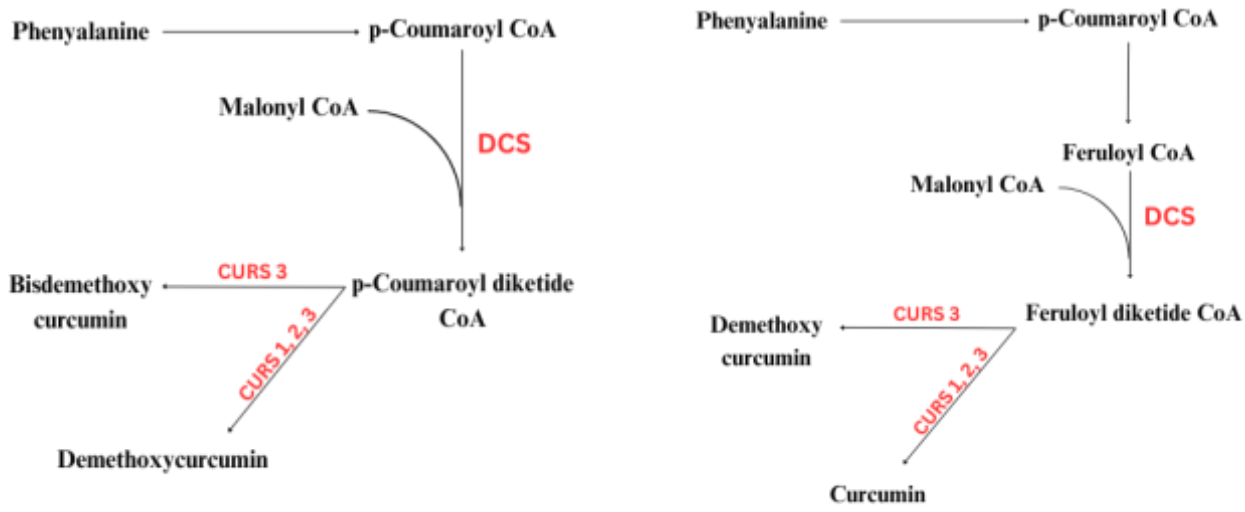

Fig. 8:- Biosynthetic Pathway Of Curcuminoids in *Curcuma longa*

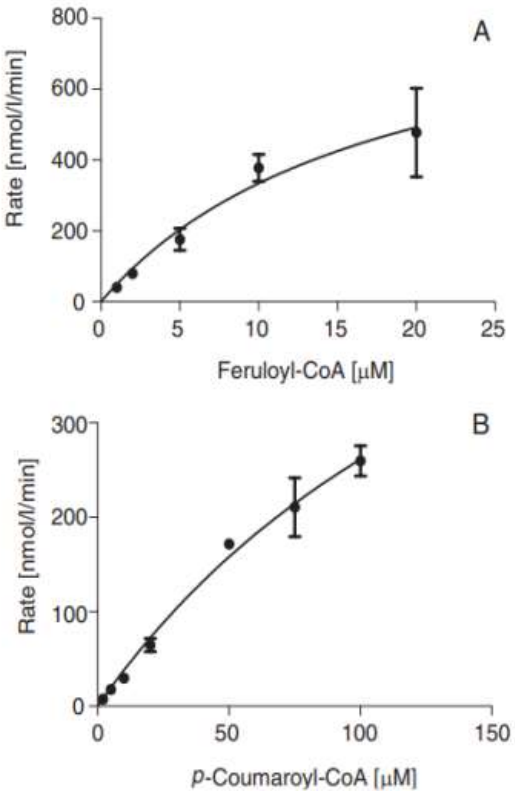

Fig. 9:- Kinetic analysis of CURS. The rate-feruloyl-CoA concentration profile (A) for cinnamoylferuloylmethane synthesis from feruloyl-CoA and cinnamoyldiketide-NAC are shown. Similarly, the rate-p-coumaroyl-CoA concentration profile (B) for cinnamoyl-p-coumaroyl methane synthesis from p-coumaroyl-CoA and cinnamoyldiketide-NAC are shown. Source: Katsuyama et al. (2009)

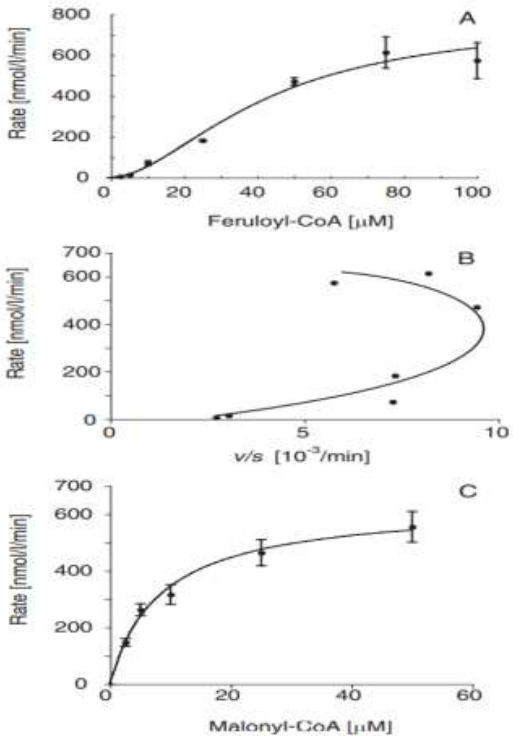

Fig. 10:- Kinetic analysis of DCS. The rate-feruloyl-CoA concentration profile (A), Eadie-Hofstee plot (B), and the rate malonyl-CoA concentration profile (C) are shown. The data, obtained from three independent experiments, suggest that DCS is an allosteric enzyme. Source: Katsuyama et al. (2009)

Perhaps CURS1 and CURS2 use feruloyl CoA for starters and catalyze the condensation with p-coumaroyldiketide CoA, employing feruloyldiketide CoA as an extender substrate for the formation of curcumin and demethoxycurcumin. Whereas CURS 3 is involved in the formation of all three curcuminoids by catalysis of the condensation of feruloyl CoA/p-coumaroyl CoA with p-coumaroyldiketide CoA/feruloyldiketide CoA as an extender substrate.

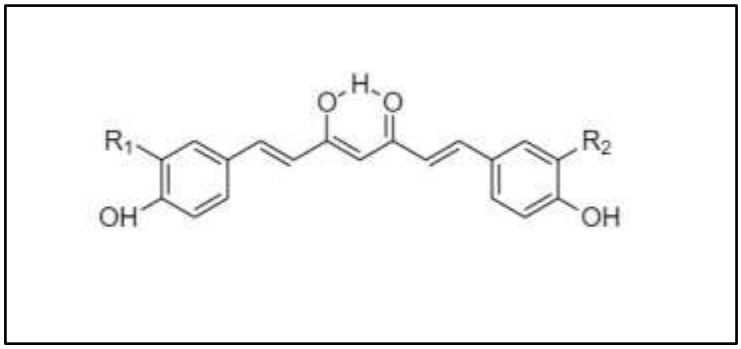

Fig.11:- Structure of Curcuminoids

R1 = R2 = OCH_3 (curcumin)

R1 = OCH_3; R2 = H (demethoxycurcumin)

R1 = R2 = H (bisdemethoxycurcumin)

4.2. Biosynthesis of Curcuminoids in *Oryza sativa*

Oryza sativa belonging to the family Poaceae is phylogenetically different from Zingiberaceae but they possess genes that code for curcuminoid synthesis [18]. CUS or curcuminoid synthase is a type III PKS regulating the formation of bisdemethoxycurcumin, dicinnamoyl methane, and curcumin [19, 29]. CUS has been a topic of discussion because of its ability to perform a 'one-pot' synthesis of the C6-C7-C6 diarylheptanoid scaffold of bisdemethoxycurcumin. The presence of a unique downward expanding active site architecture with a long tunnel, sufficient to accommodate two coumaroyl units (C6-C3) and one malonyl unit at once, and the characteristic putative nucleophilic water molecule suggests the employment of catalytic machinery by CUS for the one-pot production of bisdemethoxycurcumin [24].

Diketide CoA is initially synthesized by CUS and the β-keto acids derived from diketide CoA are incorporated as second extender units to form curcuminoids. Aromatic esters (4-coumaroyl CoA, cinnamoyl CoA, or feruloyl CoA) along with malonyl CoA are employed by CUS. Both aromatic CoA and malonyl CoA act as starters [28].

The nucleophilic water molecule in the structure of CUS is utilized to terminate the initial polyketide chain elongation at the diketide stage. The cleavage of the thioester bond in the enzyme-bound intermediate generates 4-coumaroyl diketide acid. Asn142 and Tyr207 orient the α-β unsaturated carboxyl of the diketide acid, post formation of 4-coumaroyl diketide acid, and the acid carboxyl forms hydrogen bonds with Ser351 and converts to carboxylate anion by proton abstraction via thiolate anion of the catalytic Cys174. The second 4-coumaroyl CoA acts as a starter and is subsequently loaded at the catalytic center Cys174 of the active site which is reactivated with His316, accommodating the diketide intermediate into the downward expanding tunnel of the active site. The final tail-tail coupling with the coumaroyl diketide acid occurs due to the reorientation of the enzyme-bound coumaroyl monoketide. Tyr207, Leu263, and Pro386 provided by the non-polar environment assist the decarboxylative condensation reaction to produce and release bisdemethoxycurcumin [20, 24].

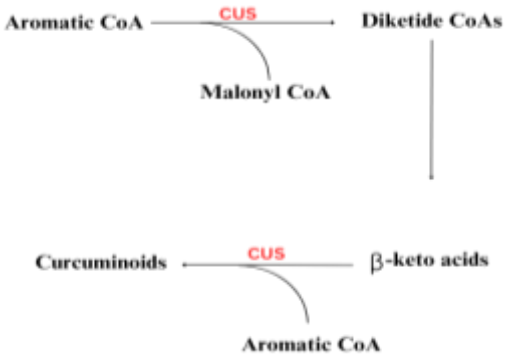

Fig. 12:- Biosynthetic Pathway Of Bisdemethoxycurcumin in *Oryza sativa*

Hence CUS catalyzes the synthesis of bisdemethoxycurcumin from 2(p-coumaroyl CoA) and 1(malonyl CoA), dicinnamoyl methane from cinnamoyl CoA, and curcumin from feruloyl CoA. Condensation of 2(malonyl CoA) to p-coumaroyl CoA, produces triketide pyrones as by-products.

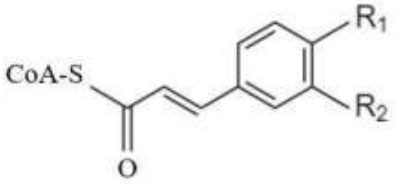

Fig. 13:- Structure Of Curcuminoids Synthesized By Cus In *Oryza Sativa*.

R1 = OH; R2 = H (4-coumaroyl CoA)
R1 = R2 = H (cinnamoyl CoA)
R1 = OH; R2 = OCH3

5. GENETIC BASIS OF CURCUMINOID SYNTHESIS AND INSIGHTS INTO THE PHYLOGENY OF GENUS *Curcuma.*

The attempt to analyze the genetic basis of the biosynthetic pathway of curcuminoids in *C. longa* is partially successful to this date. Extensive studies and research have been conducted to comprehend the genetics involved by transcriptome analysis of leaves and rhizomes of various cultivars over the past decade to generate helpful insights for a potent phylogenetic tree. The original stem of the vascular plant lineage was the rhizome, although very little is known about the genetic basis of rhizome identity, development, and growth in general [35]. *Curcuma* has a short flowering season and the floral morphology has high similarities among the species with varying inflorescence positions which were used in earlier intragenic classification of *Curcuma* [30].

In an attempt to generate extensive transcriptome data of *C. aromatica* and *C. longa* (lowest to highest curcuminoid content) using deep sequencing, Sheeja et al (2015) showed that the transcriptome of C. longa has a 46.24% average GC content, indicating the species to be marginally AT-rich. Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl CoA/quinate hydroxycinnamoyl transferase (HCT), coumarate 3- hydroxylase (C3H), caffeoyl CoA 3- O-methyltransferase (COMT), diketide synthase (DCS), curcumin synthase 1 (CURS1), curcumin synthase 2 (CURS2), curcumin synthase 3 (CURS3), and curcuminoid synthase (CUS) genes were involved in the biosynthesis [31].

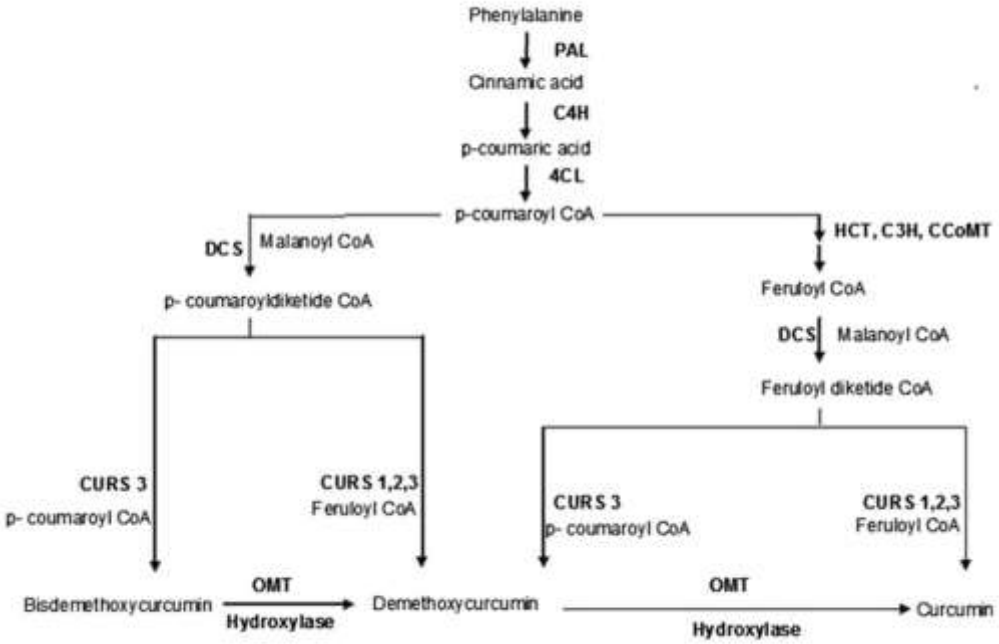

Fig. 14:- Biosynthetic pathway of curcuminoids in turmeric. Source: Sheeja et al (2015)

Note: PAL phenylalanine ammonia lyase, C4H cinnamate 4- hydroxylase, 4CL 4-coumarate-CoA ligase, HCT hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase, C3H p-coumarate 3-hydroxylase, CCOMT caffeoyl CoA O-methyltransferase, DCS diketide CoA synthase, CURS curcuminoid synthase, OMT (O-methyl transferase).

Statistical analysis of ginger and *Curcuma* showed that transport and metabolism genes, protein biosynthesis genes, and genes related to protein modification are more highly expressed in rhizomes than in leaves [29]. The curcuminoid content of cultivars growing in varied climatic regions differs indicating a strict influence of genes and agro-climatic conditions. Differential association of CURS and DCS gene expression was found to be associated with curcuminoid content. As suggested earlier, the relative expression of DCS, CURS1, and CURS2 is higher in rhizomes than in any other plant organ. Exclusively, CURS3 is equally expressed in both rhizomes and leaves. Growth stage and genotype significantly affect the gene expression resulting in an impression on the curcuminoid balance in turmeric rhizome. From the active vegetative state to the rhizome development stage, the gene expression increases and gradually decreases from maturity to senescence [13, 31].

DCS and CURS3 have a similar pattern of expression are distinct from CURS1 and CURS2. DCS and CURS3 expression had a consequential effect on the total curcuminoid content. The expression patterns of the four basic genes involved in curcuminoid biosynthesis exhibit a positive and negative correlation with the production. DCS is positively associated, while CURS3 is negatively correlated to all three curcuminoid manufacturing. CURS1 has a negative association with curcumin while CURS2 is positively associated with demethoxycurcumin. The transcription of the DCS gene majorly affects the content of curcumin in different cultivars [13].

Other factors like hybridization, introgressive agroclimatic conditions, microenvironment of the root zone and shoot just above the soil, and genotype-environment interaction also have an impact on the curcuminoid content [13, 14, 29, 30]. Tissue-specific and temporal variations influence the expression of diketide CoA synthase and curcumin synthase gene.

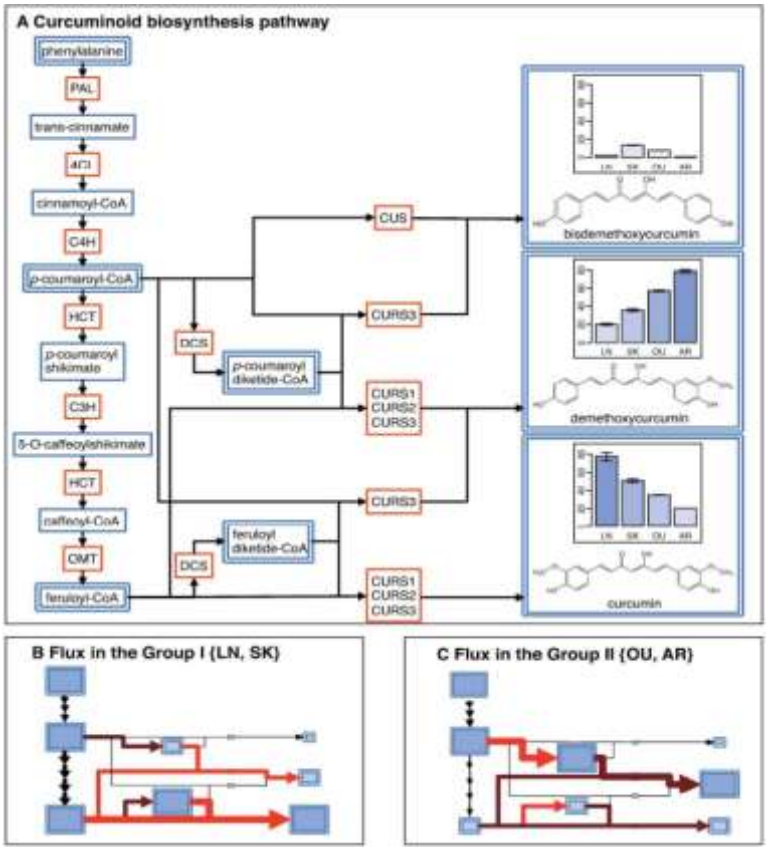

Fig. 15i:- Curcuminoid biosynthesis pathway. (A) The pathway from phenylalanine to curcuminoids. The relative contents depicted in the bar graphs for curcumin, demethoxycurcumin,

and bisdemethoxycurcumin, respectively, are 77.4%, 20.2%, and 2.4% for CL; 50.9%, 35.8%, and 13.3% for SK; 35.1%, 57.2%, and 7.7% for OU; and 20.4%, 78.8%, and 0.8% for AR. The error bars show the standard deviation of three replicate measurements. (B) and (C) Schematic illustrations of flux distributions in different specimens. According to their curcuminoid content, we compared these four specimens in two groups: Group I comprised LN and SK (in which curcumin was the largest component); Group II comprised OU and AR (in which demethoxycurcumin was the largest component). The boxes correspond to the double-lined boxes in (A). Bright and dark red arrows represent relative differences in expression levels for DCS vs CURS1 and CURS2. The sizes of the dashed and solid blue boxes show the expected and observed concentrations of the metabolites. Source: Li et al (2015)

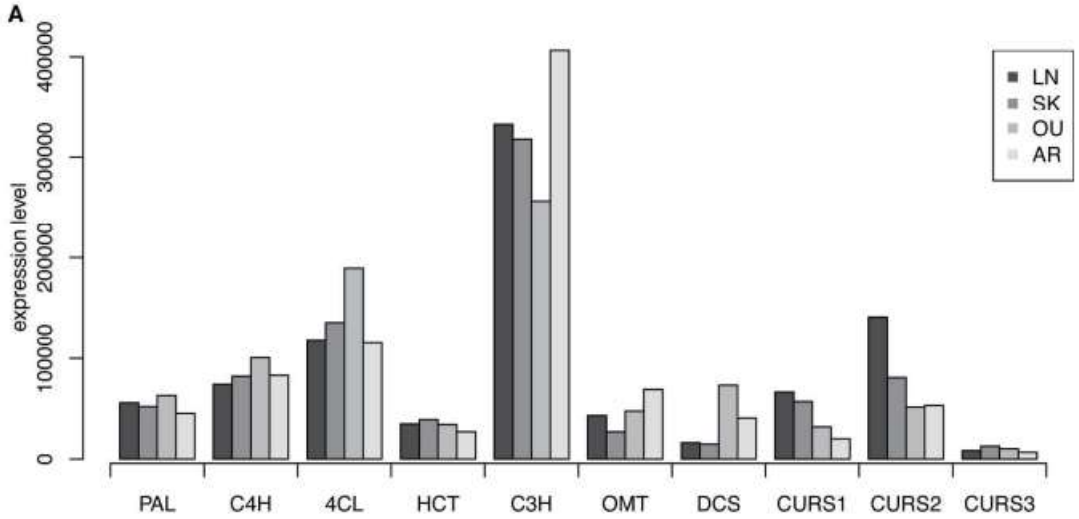

Fig. 15ii:- Gene expression profiles of the enzymes in the curcuminoid biosynthesis pathway. (A) Expression level of each enzyme. The order of enzymes corresponds to the position in the curcuminoid synthesis pathway. (B-D) Significance of expression difference. We compared expression levels of enzyme pairs along with the curcuminoids synthesis pathway. Each bar represents the p-value of the U test whether the expression levels of adjacent enzyme pairs such as (PAL, C4H), (C4H, 4CL), X were equal between specimen sets such as {LN, SK} vs. {AR, OU}, etc. The dashed lines represent thresholds p=0.05. Source: Li et al (2015)

Species identification or phylogenetic reconstruction based on the morphological characters of *Curcuma* are difficult due to enormous interspecific hybridization and polyploidization leading to the great intraspecific disparity. Intrinsic taxonomical and biological problems of the genus have made systematic studies incomplete and confusing. High variance in somatic chromosomes (2n = 20-105) has rendered formulated phylogenetic trees for *Curcuma* to be incompatible and conflicting [30].

The matK (Maturase K) plastidial gene showed potential for composing and evaluating an efficient phylogenetic tree of the genus *Curcuma*. Mat K sequence shows considerable variation between different *Curcuma* sp. to elucidate the evolutionary relations and placement of taxa. Since the matK region shows less divergence, has a sufficient genetic distance between species, exhibits better performance and variability, and evolves much faster in angiosperms, it has been widely accepted as a potential barcode [14]. Kumar R et al (2017) [14, 30] formulated a phylogenetic tree based on the efficiency of matK and rbcL plastid marker genes for species discrimination among the genus *Curcuma*, illustrated in the

following figure (Fig. 16i & Fig. 16ii) [14, 30].

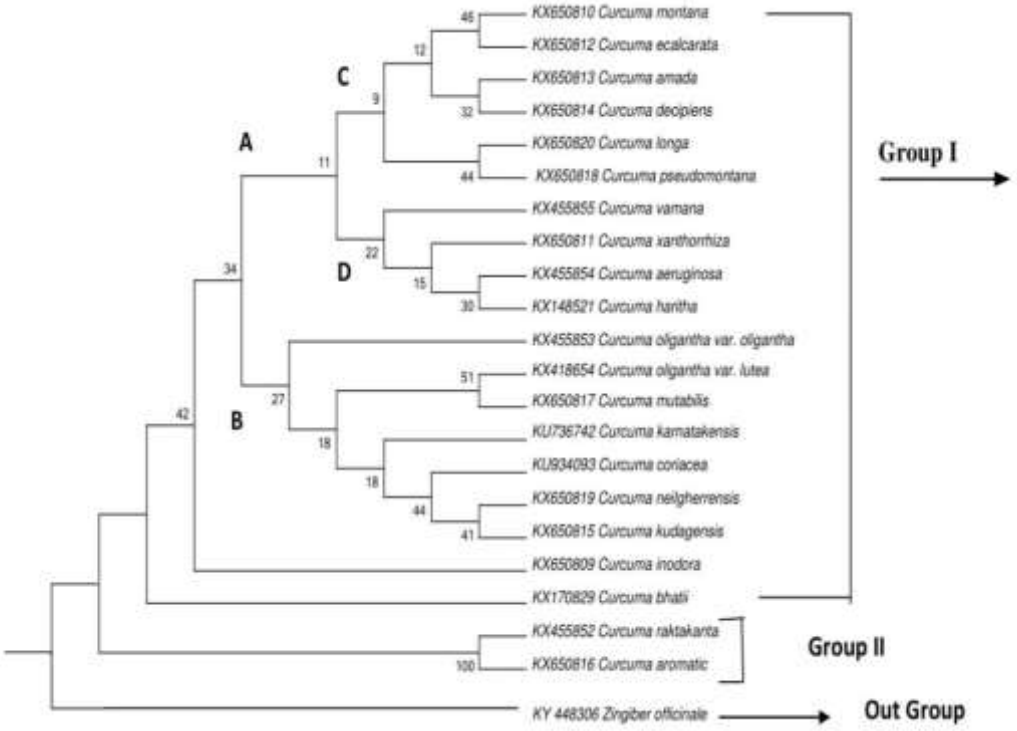

Fig. 16i:- Phylogenetic tree of matK showing Evolutionary relationship between 20 curcuma species and on variety. Bootstrap values from 1000 replications were employed for the MP method. Source: Santhoshkumar R. and Yusuf A.(2018)

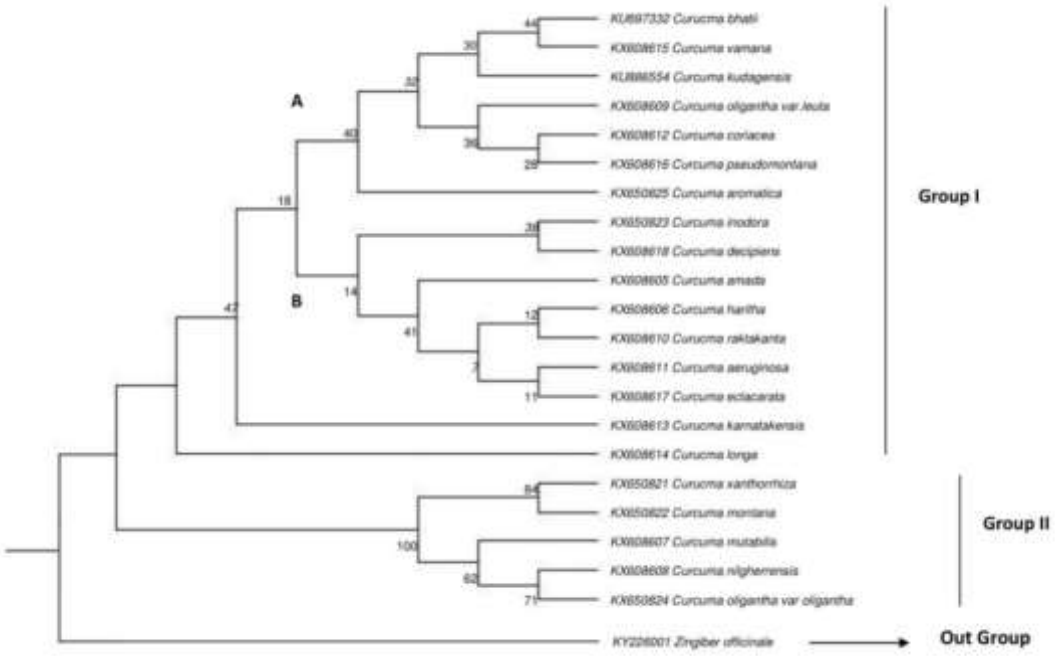

Fig. 16ii:- Phylogenetic tree of rbcL showing Evolutionary relationship between 20 curcuma species and on variety. Bootstrap values from 1000 replications were employed for the MP method. Source: Santhoshkumar R. and Yusuf A.(2018)

Santhoshkumar and Yusuf (2018) infer that Group 1, comprising of *Curcuma raktakanta* and *Curcuma*

aromatica, demonstrated no variation, both represented by a 100% bootstrap value. Group II exhibited two branches and two monoclades. Branch 1 bifurcates into clades A and B, with *Curcuma kudagensis* and *Curcuma neilgherrensis* falling under clade A, displaying a 41% similarity, while *Curcuma mutabilis* and *Curcuma oligantha var. lutea* are categorised under clade B, demonstrating a 51% similarity. *Curcuma karnatakensis*, *Curcuma oligantha var. oligantha*, and *Curcuma coriacea* form a monoclade within clade B. Branch II branched into clades C and D; clade C comprises one subclade and two monoclades, with *Curcuma haritha* and *Curcuma aeruginosa* categorized under the same clade, and *Curcuma vamana* and *Curcuma zanthorrhiza* form a separate monoclade. Clade D further branches into three subclades, D1, D2, and D3, with *Curcuma longa* and *Curcuma pseudomontana* placed within the same clade. Similarly, *Curcuma amada* and *Curcuma decipiens* are grouped, while *Curcuma montana* and *Curcuma ecalcarata* share a subclade within D1, exhibiting a 46% similarity. *Curcuma inodora* and *Curcuma bhatii* forms a monoclade.

6. BIOENGINEERING MICROBES FOR HETEROLOGOUS PRODUCTION OF CURCUMINOIDS

Curcuminoids found in turmeric have their benefits and ethnobotanical usage. Since all three curcuminoids, make up a single unit of secondary metabolites naturally, availing the advantages of discrete curcuminoids has been difficult. With the advancement in genetic engineering, various heterologous organisms have been used to carry out heterologous production of curcuminoids. The expression levels of genes and enzymes are fine-tuned to upscale the fabrication of curcumin, demethoxycurcumin, bisdemethoxycurcumin, and other curcumin derivatives.

In many instances, it is seen that to generate an optimal biosynthetic metabolic flux, the enzyme concentrations are to be regulated at a particular appropriate ratio rather than their maximal expression [32]. It began with mutating *Escherichia coli* strains for in vivo production of curcuminoids with the help of CUS from *Oryza sativa* [11]. Six genes were identified to be a part of the artificial biosynthesis pathway namely *optal, sam5, com, 4cL2nt, dcs,* and *curs.* A T7 promoter, an RBS upstream, and a T7 terminator downstream of the gene were parts of each gene [33].

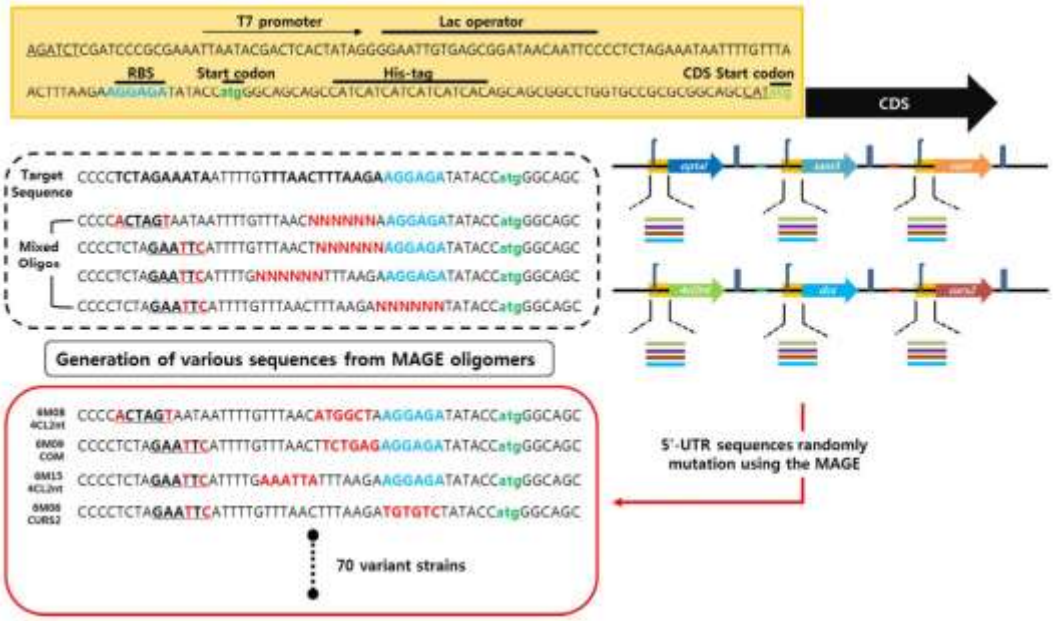

Fig. 17:- Diversification of the 5′-UTR sequence for each curcumin biosynthetic gene from the

MAGE oligomers. Source: Kang et al (2018)

Note: The yellow box highlights the synthesized 90-mer oligonucleotide sequence derived from the pET28 expression vector, which is consistently found at the beginning of the coding sequences for all six genes. The dashed box indicates the partial sequences within the 90-mer oligonucleotides that include six degenerate bases (shown in red) and the SpeI or EcoRI restriction sites (underlined). The red line box marks the altered sequences of the corresponding genes in the variants. For detailed 90-mer oligonucleotide sequences and the modified sequences of the variants, refer to the supplementary data.

Co-expressing CUS with phenylalanine ammonia lyase (PAL) from *Rhodotorula rubra* and 4-coumarate CoA ligase (4CL) from *Lithospermum erythrorhizon* reconstructed into *E. coli* for the production of plant-specific curcuminoids. These engineered strains were used to manufacture a series of unnatural curcuminoids by providing several carboxylate precursors [11]. Incubation of the *E. coli* strains carrying out artificial biosynthesis with the aid of CUS and enzymes of the phenylpropanoid pathway, supplemented with an exogenous supply of tyrosine and phenylalanine (precursors of phenylpropanoid pathway) resulted in the in vivo production of bisdemethoxycurcumin, dicinnamoyl methane, and cinnamoyl p-coumaroyl methane [11].

Combinatorial biosynthesis and the 'one-pot' synthesis characteristic of the CUS enzyme were engineered in *E. coli* and manipulated to increase production. Designing a new set of gene clusters by combining enzyme-encoding genes from different species for the synthesis of bioactive compounds in heterologous hosts was the major objective of such projects. Since the other genes required for the phenylpropanoid pathway are already available in the plant kingdom, the introduction of only one or two genes facilitates heterologous production in plants. Large-scale downstream purification and microbial fermentation can be easily attained since microbes do not have a competitive pathway against the transgenic metabolism [34].

CUS is preferential over CURS and DCS because of its unique 'one-pot' synthesis pattern of working. CUS alone is capable of catalyzing both the steps involving CURS and DCS, disobeying the head-to-tail polyketide assembly model. Owing to its unique architecture of the downward expanding active site and its ability to accommodate two coumaroyl molecules and one malonyl molecule simultaneously, makes CUS easier to use in artificial biosynthesis. DCS and CURS have been utilized in vitro production but rarely in vivo. CUS is best suited for the in vivo synthesis of polyketides [12, 34, 33].

The artificial biosynthesis in *E. coli* begins with PAL (phenylalanine ammonia-lyase) obtained from the yeast *Rhodotorula rubra*. L-phenylalanine is converted to cinnamic acid by PAL which was seen to have tyrosine ammonia lyase (TAL))-like activity. Tyrosine acts as a precursor for conversion to coumaric acid. 4-coumarate CoA ligase (4CL) from *Lithospermum erythrorhizon* (Le4CL1) converts carboxylic acids to CoA esters and then to curcuminoids by CUS. Since malonyl CoA is naturally produced in microorganisms, and is a vital substrate in the biosynthetic pathway, acetyl CoA carboxylase (ACC) from *Corynebacterium glutamicum* is overexpressed in the strains to increase the pool of intra-cellular malonyl CoA, as the naturally produced ones are used up for synthesis of phospholipids and fatty acids, leaving only a scarce amount to be employed for secondary metabolite production. The recombinant *E. coli* strains are cultivated in a suitable medium (M9) supplied with antibiotics and tyrosine or phenylalanine [11, 32, 33, 34] resulting in the formation of unnatural and asymmetrical curcuminoids.

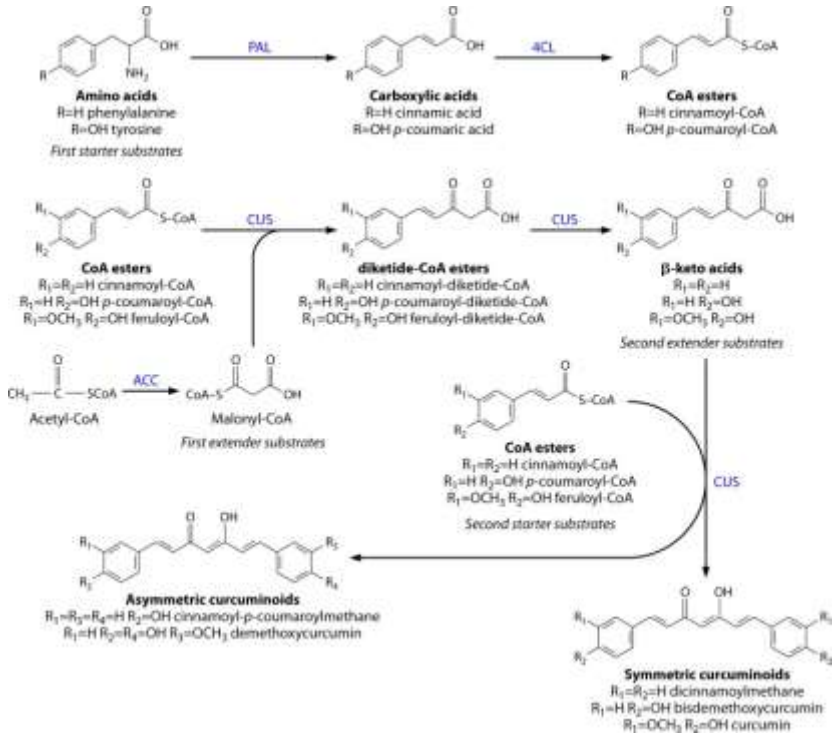

Fig. 18:- Biosynthesis of curcuminoids by recombinant *Escherichia coli*, using tyrosine and/or phenylalanine as starter substrates that are converted to carboxylic acids by PAL (phenylalanine ammonia-lyase). The carboxylic acids, which can also be added directly to the medium, are converted into the corresponding CoA esters by 4CL (4-coumarate-CoA ligase) (top), which is followed by several reactions catalyzed by curcuminoids synthase (CUS) (bottom). Malonyl-CoA is overproduced by ACC (acetyl-CoA carboxylase). Source: Rodrigues et al. (2015)

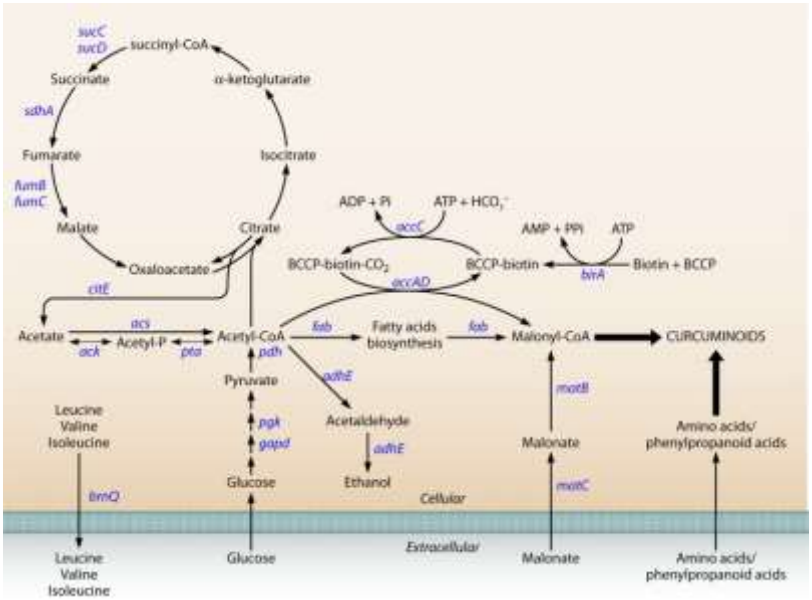

Fig. 19:- Metabolic pathways connecting malonyl-CoA with curcuminoid biosynthesis in *Escherichia coli.* Source: Rodrigues et al. (2015)

The initial idea to design an artificial biosynthetic pathway was inspired by bioengineering projects involving stilbenes and flavonoid production using Chalcone synthase (CHS) [11]. Appropriate usage of synthetic enzymes decides the fate or the rate of success of a biosynthetic pathway. *E. coli* is the most

commonly used heterologous host in many genetic engineering ventures. Eukaryotic microbes like *Saccharomyces cerevisiae* have been employed in the production of resveratrol, naringenin, and other polyketides. *S. cerevisiae* provides unique advantages over *E. coli*, being a eukaryote. Having a food-grade status makes it more viable to use in pharmaceuticals and human nutrition, intracellular compartments are similar to that of plants, hence unlike *E. coli, S. cerevisiae* does not lack post-translational machinery. Perhaps utilization of a eukaryotic host would be more efficient [34].

Pseudomonas putida, a saprophytic bacterium with strong solvent tolerance and robust metabolism makes it an attractive host for bioremediation and metabolic engineering. Exclusively for *Pseudomonas putida*, a pathway was constructed. Coumarate was exogenously added and the native feruloyl/coumaroyl CoA synthetase of *P. putida* (FCS) activated the coumarates. CUS was used to condense coumaroyl CoA with malonyl CoA to yield bisdemethoxycurcumin. CUS was introduced into *P. putida* via a plasmid vector and the CoA synthase fcs was expressed from its native chromosomal locus. Discovering the capability of *P. putida* for heterologous production of curcuminoids gave a new alternative for better production [39].

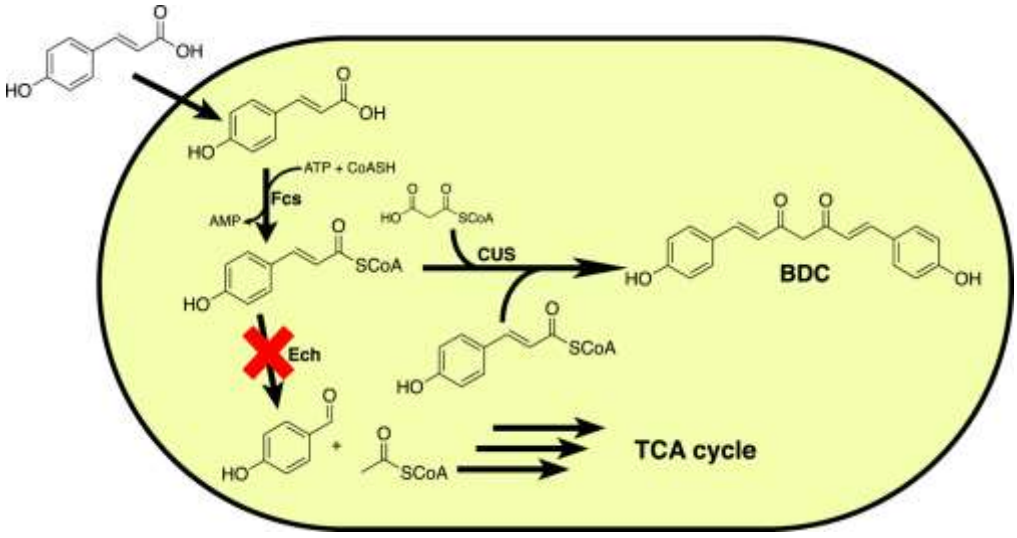

Fig. 20:- Diagram of the engineered *P. putida* strain used to produce bisdemethoxycurcumin.
Source: Incha et al. (2020)

Note: Fcs is the feruloyl/coumaroyl CoA synthetase that is naturally present in Pseudomonas putida KT2440. Ech is the native enoyl-CoA hydratase-lyase involved in the second step of coumarate catabolism, which was knocked out in our production host. CUS is the curcuminoid synthase originating from Oryza sativa. BDC refers to the final curcuminoid product, bisdemethoxycurcumin.

The efficiency of the artificial biosynthetic pathway depends on a lot of factors. There is room to improve the threshold production of curcuminoids via artificial pathways. Managing regulations of enzymes at the allosteric and transcriptional levels is mandatory. As for the Aromatic amino acid biosynthesis in *E. coli*, (Fig. 19) the most regulated step is catalyzed by 3-deoxy-D-arabinose-heptulosanate-7-phosphate (DAHP) synthase.

aroH, aroF, and aroG genes encode for three isozymes of DAHP synthase which are feedback regulated by their end products. The chorismate branch point is another regulatory step with enzymes chorismate mutase (tyrA) and prephenate dehydrogenatase (pheA), feedback inhibited by end products tyrosine and phenylalanine. In case of amino acid overproduction, the tyrosine repressor (TyrR) gene mediates transcriptional control. *aroH, aroF, and aroG* can be repressed by tyrA and tyrB. Elimination of tyrR is

essential for successful engineering. Modifying the sequence of repressed genes so that their products are not sensitive to feedback inhibition is another step taken to increase efficiency [34]. Inactivation of genes like *poxB, adhE,* and *fabF* combined with *acs* improves curcumin biosynthesis because of the increased flux of carbon to acetyl CoA and malonyl CoA, minimizing the flux to fatty acid biosynthesis. The gene *cur*A in *E. coli* is associated with curcumin reductase which can be knocked out to construct a viable strain to be employed in the process.

Medium copy number of plasmids in a strain facilitates high production of curcumin. The step catalyzed by CUS regulates the biosynthetic efficiency of the pathway. Increasing the hydrophobic compounds in the cell membrane of the microbes (membrane engineering leading to enhanced lycopene and β-carotene production) may enhance curcumin synthesis and storage [33].

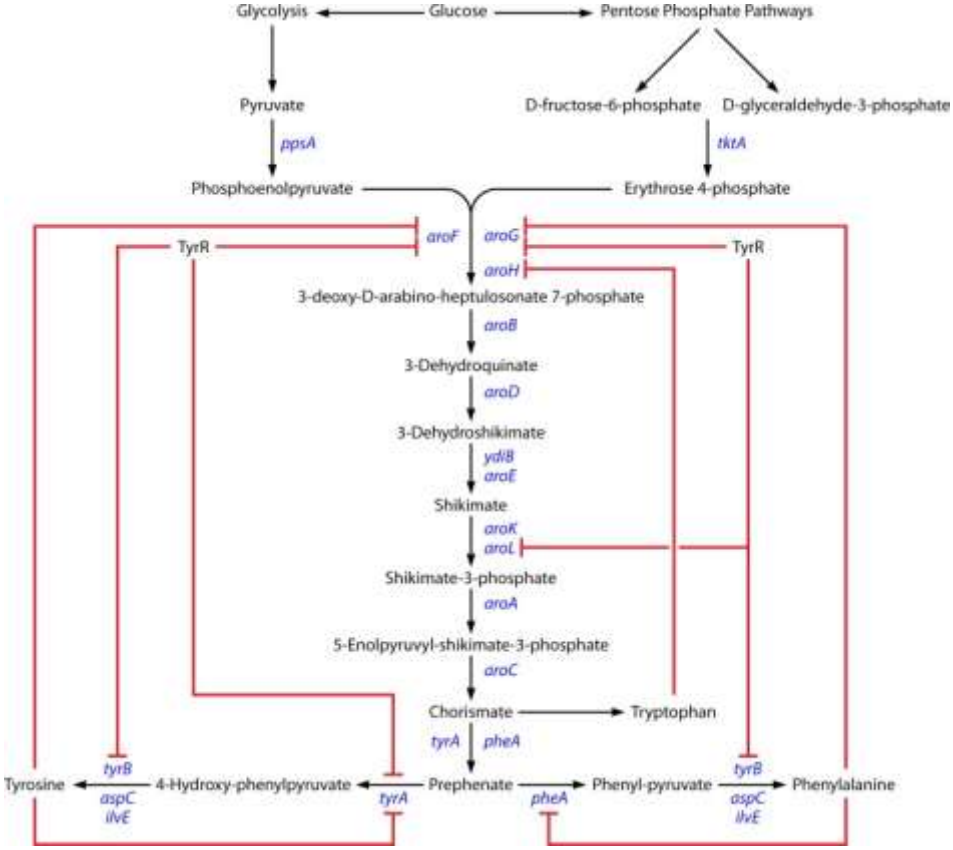

Fig. 21:- Biosynthesis of aromatic amino acids in *Escherichia coli*. Red lines show the regulation points.

Source: Rodrigues et al. (2015)

Incha et al. (2020) depicted that manipulating the efficiency of PAL enzymes by co-expressing with 4CL1 from *Arabidopsis thaliana* (At4CL1) and CUS from *Oryza sativa* in *E. coli* enhances the production of curcuminoids. PAL1, PAL3, PAL4 from *Trifolium pratense* were screened and PAL1 showed best results. ACC enzyme was not used in this combination of enzymes indicating that, in this case, the naturally produced malonyl CoA was enough to increase the production by around 3.4 times more than the conventional methods [34].

Wu et al. (2020), used the recent advancements in tools and methods of bioengineering leading to the production of curcuminoids found in turmeric in a single yield. A strain of *E. coli* COS6-T5M4DUΔmutS was created harboring all six genes (*optal*, *sam5*, *com*, *4cL2nt*, *dcs*, and *curs*) essential to reconstruct the artificial biosynthetic pathway of curcuminoids, successfully synthesized all three curcuminoids in vitro

[32].

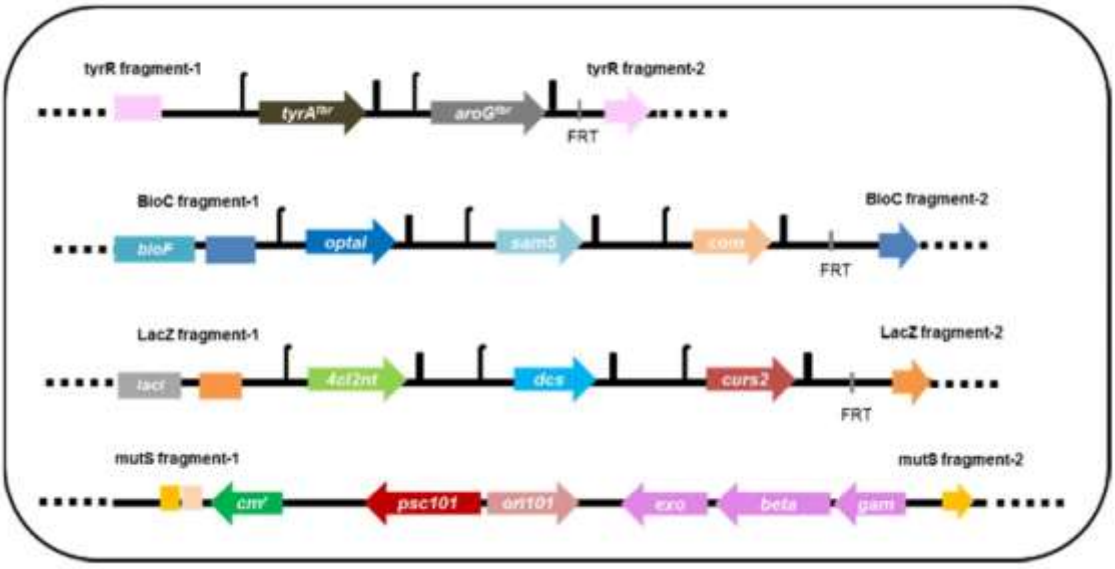

Fig. 22:- Strain (COS6-T5M4DUΔmutS) with the artificial curcumin biosynthetic pathway.
Source: Kang et al (2018)

Note: To optimize the expression levels of six genes for curcumin production, they were sequentially inserted into the L-tyrosine-producing E. coli strain ΔCOS1. This strain was genetically modified to include feedback-resistant versions of chorismate mutase (aroGfbr) and prephenate dehydrogenase (tyrAfbr) genes, and it has a knockout of the tyrosine repressor gene (ΔtyrR). The T5M module, containing the optal, sam5, and com genes for ferulic acid production, was inserted into the bioC gene, which encodes malonyl-acyl carrier protein (ACP) methyltransferase. The 4DU module, comprising the 4cl2nt, dcs, and curs2 genes for converting ferulic acid to curcumin, was inserted between the lacZ gene. Lastly, the portable λ recombination system was integrated at the mutS locus on the genome.

With an improving understanding and engineering of genes and strains involved in synthetic production, the efficiency and viability of the artificial synthesis of both natural and unnatural curcuminoids can be further studied and monitored to optimize yield and customise the engagement of curcuminoids in ethnobotanical usage.

7. CURCUMINOIDS AS DRUGS

The medicinal properties owned by turmeric are mainly imparted by curcuminoids. With 75% curcumin, 20% demethoxycurcumin, and 5% bisdemethoxycurcumin, turmeric has anti-inflammatory, antioxidant, anticancer, antimicrobial, anti-fibrotic, hypocholesterolemic, antirheumatic, anti-hepatotoxic, anti-venomous, anti-diabetic, nociceptive, gastroprotective properties (table 2.1). Yet these biomolecules are tough to be availed as drugs due to poor bioavailability.

Among the biomolecules of turmeric, curcumin is the most efficient and effective. Weak bioavailability and poor solubility are major drawbacks to curcumin's clinical translation and incorporation into pharmaceutical drugs. Susceptibility to degradation is pH dependent and lack of water solubility specifically makes it a class II drug. Bioavailability and gastrointestinal absorption are critical characteristics in the pharmacokinetics of any compound. Following oral administration curcumin is poorly absorbed. It degrades to Trans-6-(40-hydroxy-30-methoxy phenyl)-2, 4-hydroxy-5 hexanal, feruloyl methane, ferulic acid, and vanillin, under alkaline (pH>7) conditions. Under acidic conditions,

degradation is much slower.

Curcumin has quick hepatic and intestinal metabolism. 60-80% of the content is eliminated via feces, allowing the absorption of the small amounts within the intestine which is then subjected to metabolism in the liver and plasma. Extensive conversion to its water-soluble metabolites, sulphates, and glucuronides leads to excretion through urine. This renders a poor half-life to oral drugs of curcumin. Human Serum Albumin (HAS) transports hydrophobic and acidic drugs. The half-life of drugs is increased when they bind to serum proteins. The high rate of metabolic detoxification and strong interactions of curcumin with HAS create a less viable half-life of the drugs. Through glucuronidation and sulphation, curcumin undergoes rapid hepatic first-pass metabolism. The phenolic hydroxyl and enolic hydroxyl groups form a hydrogen bond with phospholipid polar groups, causing curcumin to form a complex with phosphatidylcholine which structurally protects it from degradation and augmentation through facilitated diffusion across lipophilic cell membrane.

Enhancing the bioavailability of curcumin-based drugs has been a challenge to date. Various methods and techniques have been invented to make the pharmacokinetic properties of curcuminoids more suitable. Taking the aid of techniques like polymerization with polylactic co-glycolic acid (PLGA) polymer, commercial formulations like nanoparticle formulations, and micellization, can help broaden the bioavailability and half-life. Compared to unformulated curcumin, including it with Solid Lipid Particles (SLP) pharmaceutical form keeps curcumin from rapid degradation and excretion, eventually improving the bioavailability by ameliorating the half-life and plasma conditions. The compulsive need to box the benefits of secondary metabolites of turmeric into a drug has led to the discovery of different ways to systematically imbibe the biomolecules with the usage of Tween 80, polysorbate 80, ceramic particles, PEG, alginate, PLGA, omega-3 fatty acids, chitosan, and other substances.

Micronizing the curcumin molecules to create a smaller diameter of drug particles has been reported to possess 9 times more bioavailability than unformulated curcumin. This enhances the augmentation to dissolution rate by increasing the surface area to drug ratio and directly affecting the bioavailability. Micellization techniques have sought ways to improve the solubility of curcumin. Incorporating curcumin in a Nano ionic surfactant like Tween 80 causes the formation of liquid micelles that ultimately improve absorption and dissolution. The most widely accepted commercial formulation for curcumin is the Nanoparticle dispersion mechanism where a colloidal Nanoparticle dispersion of curcumin has been produced, increasing the solubility and oral bioavailability up to 16 folds than unformulated curcumin [36, 37, 38, 39].

The successful formation of a drug containing the beneficial properties of turmeric, increasing the target efficiency and bioavailability of curcuminoids would be a ground-breaking advancement in scientific research, contributing to cures for various diseases and reducing fatality.

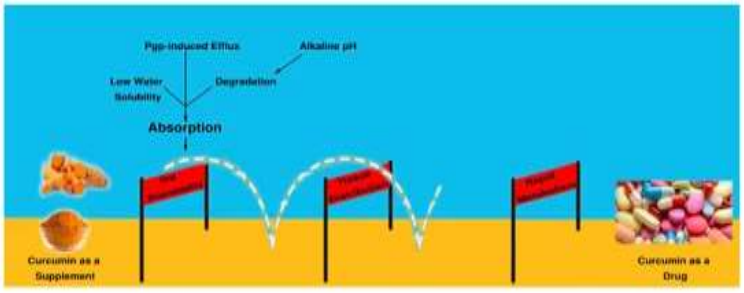

Fig. 23:- Obstacles against the marketing of curcumin as a drug. Challenges in curcumin oral bioavailability, distribution, and metabolism, the main pharmacokinetic parameters, emerged as

major obstacles limiting the therapeutic efficacy and marketing of curcumin. Source: Hassanzadeh et al. (2020)

8. CONCLUSION AND FUTURE PROSPECTS

The usage of natural ingredients containing beneficial biomolecules and metabolites has been a centuries-old tradition in South Asian countries. Without much knowledge about the chemistry behind the pharmacologically beneficial plants, ancient times witnessed the usage of *Curcuma longa* as a cure for various diseases.

Turmeric obtained from *Curcuma longa* of the family Zingiberaceae has proven to be highly advantageous because of the presence of special secondary metabolites called curcuminoids. The biosynthesis of curcuminoids is a complex, much-branched process employing enzymes of the type III polyketide family (CURSs, and DCS) leading to the formation of curcumin, demethoxycurcumin and bisdemethoxycurcumin. The main focus of the pathway is catalysis, by three different types of Type III polyketide converting aromatic CoA to curcuminoids in two simultaneously occurring pathways.

The content of curcuminoids in various species of *Curcuma* is widely affected by the genes and environment interaction. The expression patterns of both *dcs* and *curs* genes are regulatory and diverse. In total, six genes are found to be contributing to the biosynthesis of secondary metabolites of turmeric, namely *optal, sam5, com, 4cL2nt, dcs,* and *curs*. These genes have been recruited into various bioengineering projects. Both DCS and CURSs have varied regulation patterns on the content of curcuminoids produced in a plant. Up and down-regulations of DCS, CURS1, CURS2, and CURS3 genes affect the yield and intensity of curcuminoids in different cultivars.

Although the three curcuminoids exhibit medicinal properties, the conversion into drugs to feed a bigger commercial market is still under construction owing to poor bioavailability and pH-dependent solubility of these compounds. Attempts to produce curcuminoids in vitro and in vivo have been successful in the past decade after the discovery of a novel type of polyketide synthase CUS (curcumin synthase) from *Oryza sativa*. The one-pot synthesis property and deflection from the head-to-tail polyketide assembly model have made CUS the hero of engineering curcuminoid processes. The major biomolecule having the most health benefits is curcumin which is produced over 75% naturally. CUS facilitates the formation of bisdemethoxycurcumin from 2(p-coumaroyl CoA) and 1(malonyl CoA), dicinnamoyl methane from cinnamoyl CoA, and curcumin from feruloyl CoA where bisdemethoxycurcumin is mostly yielded. Employing the enzyme CUS in bioengineering projects and genetically modifying the genes to benefit the needs of mankind would prove to be a more beneficial procedure as it would promisingly increase yield and possibilities for the in vitro synthesis of mainly curcumin.

Tools and inventions to make pharmacological interventions and bioengineering procedures go hand in hand and would be a breakthrough in meeting the compulsive need of curcumin in the commercial and pharmaceutical market. Using more efficient eukaryotic microorganisms like *S. cerevisiae* in the modification of the yield of curcumin in laboratories and utilizing drug-making technologies, as mentioned earlier, to interweave in vitro production for the creation of a more efficient drug with increased bioavailability and half-life would be helpful. The link between the two pathways simultaneously producing different products using the same starter substrate and extender substrate is yet to be deciphered. The timing of the introduction of the functional groups, before and after the curcuminoid skeletal formation, remains a mystery.

The therapeutic effects of curcumin are indefatigable but because of its character to dissolve wholly in fat

and partly in water, the formation of a drug is challenging. The body absorbs curcumin partially because of a water-based digestive system when consumed raw. The amount that deliquesces is completely metabolized. The affinity of curcumin to be transformed into a drug is to be increased to ease the consumption, readiness, and effectiveness, as an agent of pharmacology. Regular consumption of Turmeric shows a big difference in the epidemiology of various diseases.

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