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Mini Review

Metabolic engineering of functional phytochemicals

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Abstract

Phytochemicals belonging to the group's phenols, terpenes, betalains, organosulfides, indoles and protein inhibitors are important components in fruits, vegetables, legumes, whole grains and nuts that have health promoting benefits and a variety of applications in food and pharmaceutical industries. Initially only a few of these important phytochemicals are produced commercially by chemical synthesis. However, recent developments in the field of biotechnology have provided metabolic engineering strategies that use microorganisms as cell factories for high production of these products. This review will discuss the general biosynthetic pathways, metabolic engineering and optimization strategies of functional phytochemicals that have received a lot of attention from investigators.

Introduction

Phytochemicals are important components in fruits, vegetables, legumes, whole grains and nuts that have been shown to play a key role in health maintenance and onset of diseases. The vast arrays of phytochemicals are grouped into phenols, terpenes, betalains, organosulfides, indoles and protein inhibitors. However, among these major groups, the flavonoids and carotenes have been of special interest partly due to their health promoting benefits and applications in food and pharmaceutical industries (Pekkarinen *et al*., 1999; Valcic *et al*., 1999 ; Shahidi and Ho, 2000; Bradamante *et al*., 2004; Choi *et al*., 2008).

Initially only a few of these important phytochemicals were produced commercially by chemical synthesis. However, recent developments in the field of biotechnology have provided metabolic engineering strategies that use microorganisms as cell factories for high production of phytochemicals (Lee and Schmidt-Dannert, 2002; Deavours and Dixon, 2005; Yan *et al*., 2005; Leonard *et al*., 2007; Chemler and Koffas, 2008). These developments have contributed immensely to independence from agriculture and environmental conditions that may result to shortages (Krings and Berger, 1998). Moreover, the chemical synthesis of these compounds is associated with production of racemic mixtures and unfriendly environmental conditions coupled with high cost of precursors (Barghini *et al*., 2007). Most importantly also is the consumer demand for naturally healthy products (Walton *et al*., 2003).

Metabolic engineering leads to the establishment of new metabolic pathways and suppressing or removal of existing pathways to enhance the formation of a desired product by recombinant DNA techniques. This review will discuss the general biosynthetic pathways, metabolic engineering and optimization strategies of functional phytochemicals that have received a lot of attention from investigators.

Phenolics

Polyphenols are found in fruits, vegetables, cereals, legumes and beverages. They are generally characterised by the presence of hydroxyl groups with one or more phenol ring, which in addition to other structural elements are used as a basis for their classification. They comprise the flavonoids (quercetin, kaempferol, catechins), phenolic acids (ellagic acids), hydroxycinnamic acids (caffeic acid), tryrosol esters (tyrosol), stilbenoids (resveratrol) and punicalagins (pomegranates). The importance of these compounds lies in their ability to affect molecular targets in chronic diseases. They have been found to inhibit cyclo-oxygenases (Moroney *et al*., 1988), telomerase and lipoxygenase (Yamamoto *et al*., 1984), induce cell cycle regulation and platelet functions (Demrow *et al*., 1995). Recent studies have

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proven the anti-diabetic properties via the modulation of insulin secretion by pancreatic B cells (Chemler *et al*., 2007).

Flavonoids

Flavonoids are synthesized from phenylalanine or tyrosine in the phenylpropanoid pathway via the an ordered series of reactions catalysed by phenylalanine ammonia lyase (*PAL*), cinnamate – 4 – hydroxylase (*C4H*), 4 – coumarate:coenzyme A ligase (*4CL*), chalcone synthase (*CHS*) and chalcone isomerase (*CHI*) to produce flavonoids in plants (Limem *et al*., 2008) (Figure 1).

A lot of success has been achieved in the biosynthesis of flavonoids by inserting plant biosynthetic pathways in recombinant strains of E. coli. Sequel to the discovery of *4CL* in Streptomyces coelicolor capable of activating cinnamic acid to cinnamoyl-CoA, engineering techniques have been successful in the heterologous production of plant specific flavones. A plasmid constructed with ribosome binding sequence placed in front of each of the three genes (*PAL*, *4CL* and *CHS*) and transcribed by a single promoter placed in front of PAL produced high yield of flavanones (Hwang *et al*., 2003).

A major breakthrough in the biosynthesis of flavonoids followed the work of (Leonard *et al*., 2006) which devised a strategy for the functional expression of plant P450 flavonoid hydroxylase in *Escherichia coli*. Hydroxylated flavonoids were produced from p-coumaric acid precursor by expressing *4CL* together with *CHS*, *CHI*, flavanone 3ß- hydroxylase (*FHT*) and flavonol synthase (*FLS*). Production levels were later elevated with the use of suitable medium rather than high copy number of plasmids (Chemler *et al*., 2007) and by increasing precursor metabolites (maloyl-CoA) that resulted in the amplification of acetate assimilation pathways coupled with overexpression of acetyl-CoA carboxylase (*ACC*) (Leonard *et al*., 2007). Other researchers employed the use of compatible vectors, medium pH adjustments, mimicking enzyme complex that exist in plants (Yan *et al*., 2008).

Recently, Leonard *et al*. (2008) successfully produced strains that were capable of high flavonoid production. The study engineered an alternative carbon assimilation pathway coupled with the inhibition of competitive reaction pathways that resulted in an increased availability of precursors for flavanone and anthocyanin synthesis.

Phenolic acids

Vanillin

Among the phenolic acids, vanillin has attracted

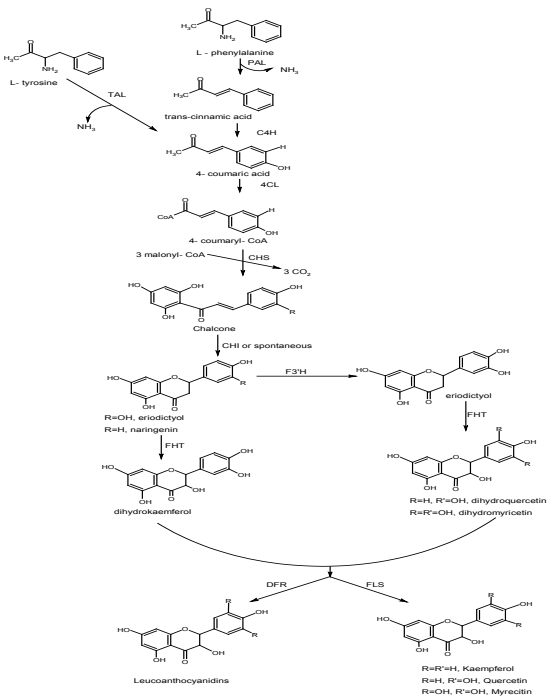

Figure 1. Biosynthetic routes of plant specific flavonoids: Enzyme names are abbreviated as follows: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), flavanone 3-hydroxylase (FHT), flavonols synthase (FLS), flavonoid 30-hydroxylase (F30H) or flavonoid 30,50-hydroxylase (F3050H), Phe ammonia-lyase (PAL), tyrosine ammonia lyase (TAL) (adapted from Limem *et al*.(2008)

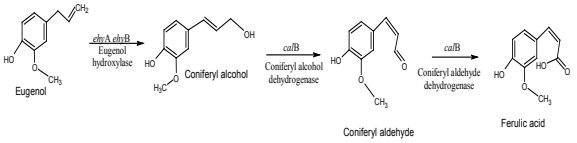

Figure 2. Metabolic pathway for the production of vanillin in Pseudomonas strains (adapted from Walton *et al*. (2000)

a lot of interest because of its use as flavour in food and cosmetic industries. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is found in vanilla orchids. The unravelling of genes responsible for bioconversion of ferulic acid to vanillin in *Pseudomonas* sp strain HR199 paved the way for the biosynthesis of vanillin. These genes fcs (feruloyl co-enzyme A) and ech (enoyl-coA hydratase) were cloned and expressed in *E. coli* to confirm their function. Results of the study indicated that the recombinant strains were able to transform ferulic acid to vanillin (Figure 2) (Overhage *et al*., 1999). Although this study successfully demonstrated the biotransformation of ferulic acid to vanillin in an economically feasible process, it relies solely on the use of expensive substrate ferulic acid.

Further studies to establish eugenol transformation to ferulic acid were exploited by (Overhage *et al*., 2003) in a two-step biotransformation strategy. Recombinant *E. coli* strains (carrying *vaoA*, *calA* and

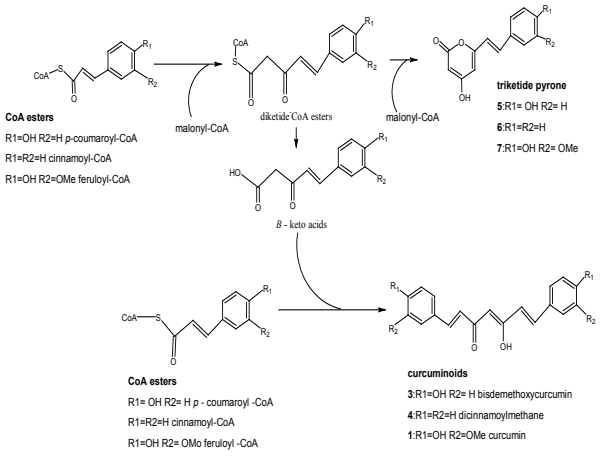

Figure 3. Biosynthesis routes of curcuminoids by recombinant *E. coli* (adapted from Katsuyama *et al*. (2008))

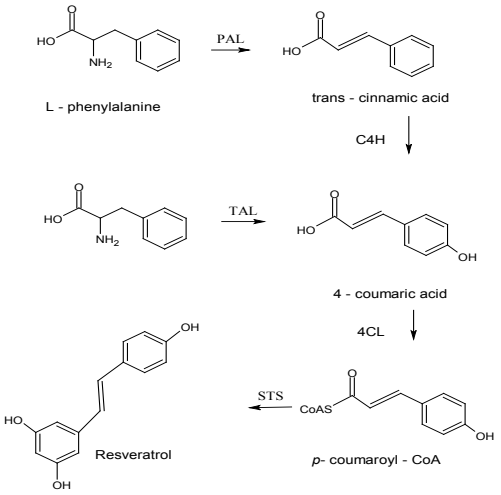

Figure 4. Biosynthesis pathway of resveratrol (adapted from Zhang *et al*. (2006))

calB encoding vanillyl alcohol oxidase, coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase respectively) were used to produce ferulic acid from eugenol and subsequently, *E. coli* (carrying *fcs* and *ech* encoding feruloyl coenzymeA and enoyl coA hydratase respectively) was used to convert the ferulic acid to vanillin.

Genes encoding feruloyl-CoA synthetase and feruloyl hydratase in Pseudomonas fluorescence have recently been expressed in *E. coli*. Interestingly, production yield of vanillin was increased using low copy plasmid of cells from actively growing cultures, bioconversion at 30^{o}C and reuse of biomass (Barghini *et al*., 2007).

Curcumin

Curcuminoids produced specifically by plants of the order *Zingiberales*, have been widely used in Asia as food additives because of their aromatic, stimulant and colouring properties and as traditional medicines for a variety of illness (Katsuyama *et al*., 2008).

The efficient production of curcuminoid in *E. coli* has been attributed to the use of curcuminoid synthase (*CUS*) – a type III polyketide synthases (*PKSs*) and exogenous supplementation with corresponding precursors. Katsuyama *et al*., (2008) efficiently produced curcuminoids in recombinant *E. coli* constructed with an artificial biosynthetic pathway comprising *PAL* (from Rhodotorula rubra), *4CL* (from *Lithospernum erythrorhizon*) and *CUS* (from *Oryza sativ*a) in tyrosine and/or phenylalanine medium (Figure 3). Improved yields were achieved upon removal of *PAL* in a different system that increased the p-coumaroyl-CoA concentration but supplementing with phenylpropanoid acids (p-coumaric acid, cinnamic acid and ferulic acid).

Stilbenoids

Resveratrol (3,5,4-transhydroxystilbene) is present in grapes. Studies have demonstrated the health benefits of this compound to be a good anti-carcinogenic (Signorelli and Ghidoni, 2005). Resveratrol biosynthesis begins with the deamination of phenylalanine by phenylalanine ammonia lyase (*PAL*) to produce cinnamic acid, which is then hydroxylated by cinnamate-4-hydroxylase (*C4H*) to form 4-coumaric acid. This product is attached to CoA by 4-coumarate-CoA ligase (*4CL*). Next, stilbene synthase (*STS*) condenses 4-coumaroyl-CoA with three molecules of malonyl-CoA to form resveratrol. In heterologous expression systems, tyrosine ammonia lyase (*TAL*) can replace *PAL* and *C4H* by producing 4-coumaric acid from tyrosine (Figure 4) (Kyndt *et al*., 2002).

The metabolic engineering approach employed in the production of resveratrol accompanied the exogenous supplementation of recombinant strains with precursors. Studies by (Watts *et al*., 2006) have grafted and expressed the genes responsible for the biosynthesis of stilbenes from two different plants viz *Arabidopsis thaliana* (4-coumaroyl CoA ligase *4CL*) and *Arachis hypogaea* (stilbene synthase *STS*) in *E. coli*. The recombinant strains were grown in the presence of increasing concentrations of phenylpropionic acid precursor (4-coumaric acid) to produce stilbene resveratrol and piceatannol. A similar strategy has been carried out in *Saccharomyces cerevisiae* paving the way for exploiting yeast in the production of resveratrol (Beekwilder *et al*., 2006).

A different metabolic engineering approach employed in the production of resveratrol was demonstrated using un-natural fusion proteins that offer strategies for improving pathway yields by co-localization of enzymes. A construct encoding a translational fusion protein of *4CL* and *STS* (*4CL*:*STS*) was generated and transformed into *S.*

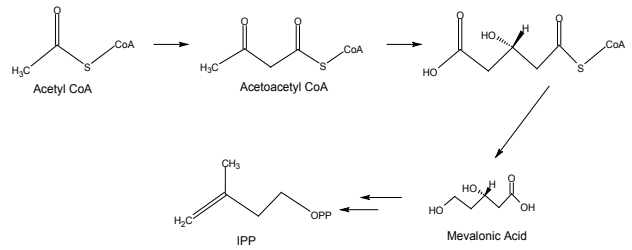

Figure 5. Pathways to IPP. MVA pathway (Barkovich and Liao, 2001)

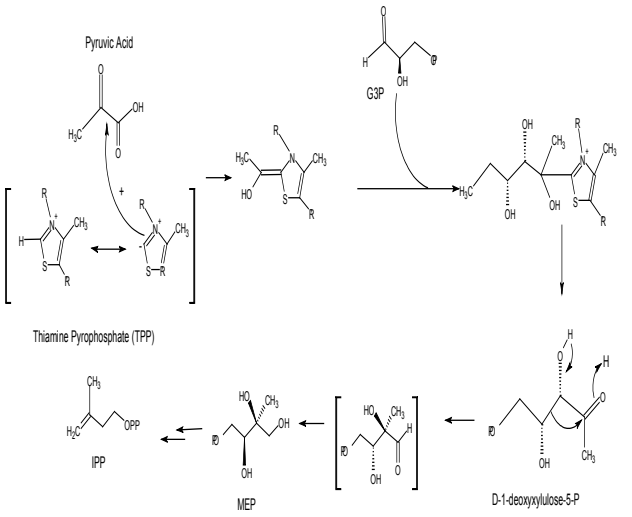

Figure 6. Pathways to IPP. (A) G3P pathway (adapted from Barkovich and Liao, 2001)

cerevisiae. Expression of *4CL*:*STS* fusion protein in the presence of 4-coumaric acid produced increasing yields of resveratrol for up to 20 hours (Zhang *et al*., 2006).

Terpenes (Isoprenoids)

Among the terpenes, attention has been focused more on the carotenoids (β-Carotene, Lycopene etc) which are natural pigments found mostly in vegetables and fruits like in carrots, pumpkins, maize, tangerine, orange, grapefruit watermelon etc). Investigators have reported a number of functions of β-Carotene which are independent of their antioxidant role (Toma *et al*., 1995; Santos *et al*., 1996; Hughes *et al*., 1997). They have also been shown to function as chain breaking antioxidants protecting cells and other body components from free radical attack (Rock *et al*., 1996). Apart from their potential health benefits, they are used industrially as animal feed additives and colourants in food and cosmetics (Das *et al*., 2007).

Carotenoids are tetraterpenes derived from 5-carbon units of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Two biosynthetic pathways viz G3P (glyceraldehydes 3- phosphate/pyruvate) and MVA (mevalonic acid) have been identified for terpenes, both of which lead to the synthesis of IPP (Figures 5 and 6). This is followed by isomerization of IPP to dimethylallyl

Table 1. Characteristics of the respective Crt enzymes derived from *Erwinia* species and the marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes* sp. strain PC-1

Genotype	Source	Enzyme common names	Substrates	Products	Requirements
CrtE	*Erwinia*	GGPP synthase	FPP GGPP	GPP GGPP	
CrtB	*Erwinia, A. aurantiacum*	Phytoene synthase	GGPP	Phytoene	
CrtI	*Erwinia, A. aurantiacum*	Phytoene desaturase	Phytoene	Lycopene Lycopene Lycopene	FAD
			z-carotene		
			Neurosporene		
CrtY	*Erwinia, A. aurantiacum*	Lycopene cyclase	Lycopene	*β*-carotene	$NADH_2$
			□-carotene	*β*-carotene	
CrtX	*Erwinia*	Zeaxanthin glucosyl-	Zeaxanthin	Zeaxanthin-b-D-diglucosid	UDP-Glucose
		transferase			
CrtZ	*Erwinia, A. aurantiacum, Alcaligenes* PC-1	*β*-carotene hydroxylase	*β*-carotene	Zeaxanthin	O_2Fe^{2+}, 2-oxolase
			B-cryptoxanthin	Zeaxanthin	glutarate
			Canthaxanthin	Astaxanthin	
CrtW	*A. aurantiacum, Alcaligenes* PC-1	b-carotene ketolase	*β*-carotene Echinenone Zeaxanthin	Canthaxanthin Canthaxanthin	O_2Fe^{2+}, 2-oxolase
		(*β*-carotene oxygenase)		Adonixanthin, (astaxanthin)	glutarate

(adapted from Misawa and Shimada, 1998)

pyrophosphate (DMAPP) in the presence of isopentenyl pyrophosphate isomerase (*idi*). These two species condense in the presence of geranyl pyrophosphate (GPP) and geranyl pyrophosphate synthase. Longer chain lengths are formed by further iteration of this process (Barkovich and Liao, 2001).

The genes responsible for the biosynthesis of carotenoids have been characterised in *Erwinia* species (epiphytic bacteria) and *Agrobacterium aurantiacum* (marine bacterium) (Table 1). A lot of studies have been published on the metabolic engineering of carotenoids in non – carotenogenic bacteria (*E. coli* and *Zymomonas mobilis*) and yeast (*Candida utilis* and *S. cerevisiae*) by redirecting carbon flux for the biosynthesis of isoprenoid compounds in addition to insertion of *crt* genes and introduction of corresponding microbial *crt* genes under the control of yeast derived promoters and terminators respectively (Misawa and Shimada, 1998). Other investigators increased the supply of IPP and DMAPP precursors via the expression of key enzymes in MEP and MVA pathways (Wang and Keasling, 2002; Yuan *et al*., 2006). A comparative account of the strategies involved in production of lycopene in transformed *E. coli* revealed that induction of MVA levels via lycopene incorporation of exogenous MVA operons increases the production of lycopene than overexpression of deoxyylose 5-phosphate synthase (*DXS*) (Yoon *et al*., 2007).

Another metabolic engineering approach apart from the classical recombinant DNA technology of transforming *E. coli* cells with gene clusters encoding carotenoid biosynthetic enzymes is the ordered gene assembly in *Bacillus subtilis* (OGAB). A method for

the assembly of multiple genes with a designated order on *B. subtilis – E. coli* shuttling vectors allows one – step assembly of multiple DNA. Using this method, rearrangement of *crt* genes from *Pantoea ananatis* was done in newly designed operons. Transformed *E. coli* strains successfully produced zeaxanthin which is comparable to the classical recombinant DNA techniques (Nishizaki *et al*., 2007).

Optimization of carotenoids has been done using a variety of strategies. Studies by Kim *et al*. (2006) demonstrated the construction of inducible operons that was scaled up gradually to accommodate β-carotene production. Findings of the study showed the efficient production of β-carotene on low copy based vectors which were consistent with previously published data (Jones *et al*., 2000). Several interesting results of gene function, recurrence and divergent phenotypes obtained from combinatorial gene knockout targets for the improvement of recombinant strains demonstrated that many diverse genotypes can yield same overall phenotypes, pointing to the high degree of complexity in metabolic landscapes (Alper and Stephanopoulos, 2008).

Conclusions

The understanding of biosynthetic pathways leading to the production of phytochemicals in plants has been the major success to engineering their production in recombinant bacterial and yeast strains. Strategies developed through the insertion of plant genes of biosynthetic pathways have been successful in the production of desired phytochemicals from natural, cheap and environmentally friendly sources. Different optimization methods have also been successful in attaining production scale at industrial levels.

Although a lot of progress has been made in the metabolic engineering of phytochemicals, attention needs to be focused on other functional phytochemicals like indoles and protein inhibitors which also have wide applications in food and pharmaceutical industries. Other microorganisms with metabolic engineering potentials should also be explored.

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