22.1 Introduction

The emergence of recombinant DNA technologies has provided the experimental means to not only explore cellular function but to alter it toward a specific goal, such as to solely synthesize and extract a protein or to catalyze the synthesis of an important chemical product. In that respect, we are currently experiencing the emergence of biotechnology as the technology of choice for the chemical and materials industries. In this context, we will discuss some current fine chemicals produced in a biotechnological process through recombinant microbial biosynthesis, the method typically followed by purification, and possibly additional chemical processes, to create high quality, low energy processes. Fine chemicals are defined as organic molecules whose production, typically in low amounts, has a designed goal in mind. As an alternative to chemical synthesis modes that usually require lengthy developmental times, and typically result in low yields due to their often-complicated chemical structure, many fine chemicals are readily suited for microbial biosynthesis since they occur as end points or intermediates of natural metabolic networks. For example, the cancer drug taxol, one of a few major isoprenoids we will discuss, was widely developed through chemical synthesis yet is now being explored for microbial biosynthesis to increase productivity and reduce cost and energy.

It is important to note that recombinant DNA technology, one of the enabling technologies for metabolic engineering, did not initiate the biotechnology era. Initially the development of chemical synthesis in microorganisms was highly reliant on experimentation via bioprospecting and random genetic alterations followed by assessment of the resulting physiology (trial-and-error methods). More recently a paradigm shift has occurred toward the more rational and systematic approach of microbial
bio-synthesis (Bailey, 1991; Stephanopoulos and Vallino, 1991) whereby taking advantage of the recent developments in molecular biology techniques, analytical methods and improved mathematical models. This approach to altering cellular physiology is commonly known as metabolic engineering. The field of metabolic engineering has been expanding since the early 1980s though the term was not actually coined until 1991 by Jay Bailey (Bailey, 1991). While this is not a chapter on metabolic engineering, these methods have been and will continue to be highly influential in the development of recombinant microorganism production platforms for fine chemicals.

One of the earliest accomplishments of recombinant DNA technology was the engineering of *Escherichia coli* for the synthesis of human insulin (Goeddel et al., 1979; Wetzel et al., 1981), whereby the cloning of A and B subunits of insulin separately into an *E. coli* expression plasmid resulted in the generation of the native human protein after the two subunits were chemically adjoined *in vitro*. In other early work, David Hopwood synthesized novel isochromanequinone antibiotics in *Streptomyces coelicolor* A3, initiating the era of fine chemical production from recombinant microorganisms (Hopwood et al., 1985). Recombinant DNA techniques that have been implemented include: (1) the addition or deletion of genes to increase the metabolic carbon flux to desired end products where overexpressions can involve single or multiple gene insertions or even the insertion of a whole genome (thereby forming a hybrid genome). Deletions are performed via direct selection or the probabilistic prediction of gene targets that, upon removal, result in carbon flow redistribution toward desired metabolic networks. For example, recently Causey et al. engineered efficient ethanol production in recombinant *E. coli* by developing deletion mutant strains with minimal energy requirements and lowered biosynthesis levels of acetate and other fermentative byproducts (Causey et al., 2004); (2) site specific or random point mutagenesis, directed evolution or other genetic altering mechanisms that are used to evolve targeted enzymes, metabolic pathways or entire genomes and (3) development of new approaches to alter the cellular physiology toward a specific goal by incorporating artificial circuit mimicry, cell-to-cell communication or synthetic regulatory processes. In this chapter, we will investigate a number of the fine chemicals being produced through the combination of these techniques with a particular emphasis on the metabolic engineering of two genetically tractable hosts, namely *E. coli* and *Saccharomyces cerevisiae*.

### 22.2 Flavonoids: A Natural Medicine

Flavonoids are plant secondary metabolites with over 8,000 configurations of the basic 15-carbon phenylpropanoid core that can be diversified through a variety of alkylation, oxidation, and glycosylation reactions. Flavonoids, being active estrogenic, antioxidant, antiviral, antibacterial, antiobesity, and anticancer molecules are of major interest for personal health applications (Forkmann and Martens, 2001). As such, they have generated extensive interest from pharmaceutical companies keen on their nutraceutical properties and as possible precursors for market pharmaceuticals. They are currently being used as dietary supplements and intensively investigated for use as treatments to many chronic human pathological conditions including cancer and diabetes (Allister et al., 2005; Caltagirone et al., 2000; Hou et al., 2004; McDougall and Stewart, 2005; Popiolkiewicz et al., 2005; Potter et al., 1998; Pouget et al., 2001; Zava and Duwe, 1997). For example anthocyanins (a class of colored glycosylated flavonoids) while of interest as possible replacement of banned or artificial dyes with real or perceived adverse effects, have come under increased attention as general antioxidants that may play a role in the reduction of various diseases (Hannum, 2004; Nakajima et al., 2001), such as obesity (Greenwald, 2004). As glycosylation reactions remain a challenging step for conventional organic chemistry in addition to the difficulties posed by acylations at specific positions, biochemical approaches for the large-scale production of these important molecules remain the only alternative to current methods reliant on plant extractions. This is especially true since the health-promoting effects so prevalent in these compounds have been the driving force toward the elucidation of their biosynthetic pathways with significant advances in the recent past.
22.2.1 Initiating the Biosynthesis of Flavonoids

Depending on their chemistry, flavonoid molecules can be classified in five major classes, namely flavones, flavonols, isoflavones, flavanols, and anthocyanins, that all are derived from the common flavanone precursors (Winkel-Shirley, 2001). Flavanones are synthesized from phenylalanine through a five-step enzymatic process (Figure 22-1). Phenylalanine is first converted into cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL) and then subsequently hydroxylated into \( p \)-coumaric acid by the enzyme cinnamate 4-hydroxylase (C4H). Next, \( p \)-coumaric acid is converted into a coumaroyl coenzyme A (CoA) ester by 4-coumaroyl:CoA ligase (4CL). Following the ligation reaction, chalcone synthase (CHS), the first committed step in flavonoid biosynthesis, catalyzes the sequential decarboxylative condensation of three acetate units from malonyl-CoA to 4-coumaroyl-CoA. This results in a linear phenylpropanoid tetraketide that forms 4,2',4',6'-tetrahydroxychalcone via intramolecular cyclization and aromatization (Austin and Noel, 2003). The formation of flavanones from chalcones then occurs through an isomerization performed by the enzyme chalcone isomerase (CHI). Various biosynthetic enzymes further down this upper pathway are accountable for catalyzing the conversion of flavanones into the plethora of flavonoid molecules with reactions involving hydroxylation, reduction, oxidation, glycosylation, methylation, and acylation (Figure 22.1). For more details on the pathway’s biochemistry, please see references Chemler et al. 2006, Leonard et al. 2005, Yan et al. 2005a. All of the flavonoid biosynthetic enzymes were originally thought to be plant derived, however reports have recently appeared demonstrating the presence of polyketide synthases in microorganisms that are also able to perform flavanone biosynthesis (Ueda et al., 1995).

22.2.2 Flavonoid Production in Plant Cell Cultures

Several attempts have been made to produce and extract flavonoids from plants with the purpose of utilizing them as nutraceuticals and natural colorants. Although colorant agents have been used in food technology for a very long time, this industry has not been comprehensively documented and tends to remain secretive. Size estimations of color agent markets in both volume and value are therefore difficult to ascertain (Marz, 1996), nonetheless the most recent data available estimates revenues for the overall European polyphenols market in 2008 at $144 million (Frost & Sullivan, 2003). Leading this market expansion is red fruit anthocyanins as well as green tea flavonoids followed by grape and olive polyphenols (NutraUSAingredients.com, 2004), with all compounds currently derived from plant extracts.

Additionally, bioreactor-based systems for mass production of flavonoids have been described for a few species (Kobayashi et al., 1993; Zhong et al., 1991), but to date economic feasibility has not been established, partly because of engineering challenges in large scale cultivation of plant cultures. One challenge is that plant cells tend to form aggregates that influence culture productivity (Hanagata et al., 1993) since cells within aggregates are not adequately exposed to the required lighting needed in flavonoid biosynthesis by plant tissue. For example, formation of PAL, a key enzyme in the biosynthetic pathway is promoted primarily by UV wavelengths, particularly those of the UV-B region (Wellmann, 1975). Other enzymes in the pathway, particularly those of the anthocyanin biosynthetic branch, appear to be regulated in part by UV and in part by the phytochrome, activating wavelengths of 700–800 nm (Meyer et al., 2002). In that respect, irradiance becomes a limiting factor to productivity not only when excluded from cells at the interior of an aggregate (Hall and Yeoman, 1986), but also when in a dense cell culture, at reduced cell dosing, or when the vessel wall composition selectively restricts certain wavelengths (Smith and Spomer, 1995).

Expression of flavonoid biosynthetic genes in transgenic plants has also been investigated for production of these ubiquitous secondary metabolites. In a recent study, CHI from *Saussurea medusa* was transformed into *Nicotiana tabacum* plants, a nonleguminous species, and resulted in an up to five fold total flavonoid production increase when compared to wild-type plants (Li et al., 2006). The flavonoid sub group isoflavonoids, predominantly synthesized in leguminous plants by isoflavone synthase (IFS) from flavanone, play key roles in plant physiology, acting as signal molecules in
FIGURE 22.1  (See color insert following page 13-20.) Metabolic network for biosynthesis of the variety of flavonoids is shown with relevant genes for each reaction highlighted. The structure of each class is shown with the R groups (i.e., H, OH) dependent on the substrate fed to the pathway.

PAL - phenylalanine lyase
C4H - cinnamate 4-hydroxylase
4CL - 4-coumaryl-CoA lyase
CHS - chalcone synthase
CHI - chalcone isomerase
DFR - dihydroxyflavanone reductase
ANS - anthocyanidin synthase
IFS - isoflavone synthase
3GT - 3-O-glucosyltransferase
FSI - flavone synthase
FHT - flavanone 3b-hydroxylase
FLS - flavonol synthase
plant–bacteria interactions, specifically plant nodulation (for reviews, see Cornwell et al., 2004 and Dixon, 2004). Because leguminous plants are not widely consumed, engineering of isoflavonoid biosynthesis in more commonly utilized crop plants have been investigated to increase the availability of these health-promoting metabolites in human diets. Tobacco again was used as a case study where, soybean IFS1 was cloned into a binary vector consisting of the cauliflower mosaic virus 35S promoter. The microorganism Agrobacterium tumefaciens was transformed with the IFS carrying plasmid for the subsequent transfection of tobacco plants (Yu et al., 2000). Even though the transgenic tobacco plants successfully synthesized isoflavonoids, the amounts were low, due to competing pathways leading to the biosynthesis of another flavonoid class, the anthocyanins. This diversion of metabolic flux, as well as issues inherent with plant cell suspension cultures (Hellwig et al., 2004), are some of the reasons why until now no such system has been established for the commercial, large-scale production of flavonoids despite a more than 10 year effort in this field.

22.2.3 Pathway Expression in Recombinant Hosts

The important pharmacological properties of flavonoids and the limited availability of purified forms from plants have inspired the production of these secondary metabolites in recombinant hosts. E. coli and S. cerevisiae have been engineered to harbor flavonoid biosynthetic pathways and synthesize flavanones (Hwang et al., 2003; Kaneko et al., 2003; Miyahisa et al., 2005b; Yan et al., 2005b), flavones (Leonard et al., 2005), flavonols (Leonard et al., 2006; Miyahisa et al., 2005a), and anthocyanins (Yan et al., 2005a). In all cases, the biosynthetic genes were episomally inserted into E. coli or S. cerevisiae using coreplicable plasmids.

22.2.3.1 Recombinant E. coli for Flavonoid Biosynthesis

Among the numerous natural pigments found in plants, anthocyanins are the largest water soluble group and can be found in most flower petals, leaves, and fruit skins. Anthocyanins are derived from the flavanones naringenin and eriodictyol through four consecutive synthesis steps (Figure 22.1). The enzyme flavanone 3β-hydroxylase (FHT) first converts flavanones to dihydroflavonols which are then reduced by dihydroflavanone 4-reductase (DFR) to form the leucoanthocyanidins. Further processing by the enzymes anthocyanidin synthase (ANS) and UDP-glucose:flavonoid 3-O-glucosyltransferase (3-GT) forms the first stable anthocyanins, such as B-ring monohydroxylated pelargonidin 3-O-glucoside (with an orange/red color) and the dihydroxylated cyanidin 3-O-glucoside (with a red color). It is important to note that anthocyanin a-glycons (known as anthocyanidins) are unstable metabolites therefore the requirement for a sugar side-chain is essential to form a stable soluble product.

In the study by Yan et al., the four step metabolic pathway was constructed in E. coli using biosynthetic genes derived from heterologous origins. After using PCR to place the genes under trc promoters and bacterial ribosomal binding sites, the enzymes were cloned into the low-copy number vector pK184 for transformation. The constructed recombinant pathway enabled the conversion of naringenin and eriodictyol to produce the corresponding glycosolated anthocyanins, albeit at low production levels (Yan et al., 2005a). This was the first demonstration of production of plant-specific anthocyanins by a microorganism, opening the way for optimization and further synthesis of other natural and nonnatural anthocyanins via enzyme and pathway engineering (Yan et al., 2008, Leonard et al., 2008). It was noted that the production of the side product flavonols existed due in part to an alternate reaction of ANS which was assumed to be the cause of the low production levels (Yan et al., 2005a).

One of the biggest challenges however for engineering the flavonoid (and many other phytochemical’s) biosynthetic pathway in E. coli is the functional expression of plant cytochromic P450 monooxygenases. This is a class of enzymes catalyzing regiospecific and stereospecific oxidation of nonactivated carbohydrates at moderate temperatures. As such, they are heavily involved in the functionalization of various natural products in general and flavonoids in particular. Two important requirements of P450s are their attachment to the eukaryotic cell’s endoplasmic reticulum (ER) membrane and a P450
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reductase for transporting electrons from the NADPH donor to the heme core of the P450 complex. These requirements are the reasons why functional expression of these enzymes in *E. coli* is a challenging task: as a prokaryote, *E. coli* lacks ER and at the same time it does not have a P450-redox partner protein. Efforts to engineer a “soluble” version of the P450 monooxygenases has generally resulted in enzymes with low solubility and the formation of inclusion bodies, especially in cases of high expression levels, something that results in extensive cell lysis.

Such was the case for the generation of an active flavonoid 3’5’-hydroxylase derived from *Catharanthus roseus* in *E. coli* for the biosynthesis of hydroxylated flavonols, such as quercetin and myricetin (Leonard et al., 2006). Specifically, the nucleotides of the fifth codon were replaced to ATG along with the removal of four N-terminal codons. Moreover, the second codon of the shortened F3’5’H which encodes for leucine was changed into alanine. In order to compensate for the lack of a P450 reductase in *E. coli*, a shortened P450-reductase also derived from *C. roseus* was fused with the modified F3’5’H through a short linker sequence having no preference for the formation of secondary structures reducing interference with the folding of the two proteins. When the engineered chimera was expressed in *E. coli* together with a grafted flavanone biosynthetic pathway, small amounts of quercetin could be recovered from the culture media, but the tri-hydroxylated myricetin could only be produced when the recombinant strain was supplemented with the monohydroxylated flavanone naringenin. Since cell lysis was evident in the culture, it was obvious that several other parameters, including the use of weaker promoters and lower copy number plasmids are also required for biocatalysis optimization. Overall, key issues remain unaddressed for optimal flavonoid biosynthesis in *E. coli*, including further optimization of the functional expression P450 hydroxylases and increases to the intracellular pool of malonyl-CoA.

### 22.2.3.2 Flavonoid Biosynthesis in Recombinant Yeast

As a eukaryote, *S. cerevisiae* offers the advantage of supporting the functional expression of ER membrane-bound P450 monooxygenases. This feature, together with the expectation of better expression of the plant-derived flavonoid biosynthetic enzymes makes yeast an attractive alternative to *E. coli* as a production platform for flavonoid molecules. The first two enzymes of the phenylpropanoid pathway, namely PAL and 4CL were first positively expressed by Ro and Douglas in *S. cerevisiae* together with a P450 reductase (Ro and Douglas, 2004); this work allowed the investigation of the formation of “metabolons” or complexes between these two enzymes.

In another study, the synthesis of the flavonoid naringenin from cinnamic acid was afforded by a recombinant *S. cerevisiae* strain expressing 4CL, CHS, CHI, and the P450 cinnamate 4-hydroxylase (C4H) that performs the hydroxylation of cinnamic acid into p-coumaric acid (Yan et al., 2005b). An extension of this work involved the biosynthesis of flavones in yeast. Derived from flavonanes, the flavones such as apigenin and luteolin are plant flavonoids with potent medicinal properties (Caltagirone et al., 2000). They are synthesized by two distinct enzymes; the soluble flavone synthase I (FSI) that is found only among plants that belong to the *Apiceae* family (such as parsley), and the membrane-bound flavone synthase II (FSII) together with the yeast P450 reductase. Metabolically engineered *S. cerevisiae* overexpressing FSI together with a flavonone biosynthetic pathway resulted in 50% higher production than the FSII expressing recombinant strain (Leonard et al., 2006) with further optimization involving the use of alternative carbon sources, such as acetate.

### 22.3 Isoprenoids

The diverse group of molecules known as isoprenoids includes carotenoids, terpenes, sterols, polyprenyl alcohols, ubiquinone, and even prenylated proteins. Many isoprenoids currently have significant biotechnological value for their roles as natural food colorants (carotenoids), antioxidants, natural aromas, and flavors (terpenes), and for their antiparasitic and anticancer properties (Haynes and Krishna, 2004; Lee and Schmidt-Dannert, 2002). Their name originates from the five-carbon molecule isoprene, a molecule
ubiquitous in all plant species from which most isoprenoids are derived (Barkovich and Liao, 2001). Isoprene is used to synthesize the common precursor molecule isopentenyl diphosphate (IDP) by two major metabolic pathways: the mevalonic acid pathway or the deoxyxylulose 5-phosphate (DXP) pathway.

### 22.3.1 Microbial Biosynthesis of Carotenoids

Carotenoids, a subfamily of isoprenoids, are synthesized from the general terpenoid pathway and involve geranylgeranyl diphosphate (GGDP) synthase (encoded by \textit{crtE}) and phytoene synthase (\textit{crtB}) for the production of the carotenoid phytoene (Figure 22.2). Subsequent desaturation by phytoene desaturase (\textit{crtI}), and further enzymatic modifications generate a repertoire of carotenoid molecules responsible for the yellow, orange, and red pigments naturally synthesized in bacteria, algae, and fungi. Carotenoids typically have a 40-carbon (C₄₀) backbone formed from the condensation of four isoprene units, however the existence of 30, 45, and 50-carbon backbones has also been shown (Chemler et al., 2006; Tao et al., 2005b; Tobias and Arnold, 2006). In efforts to enable biochemical production of these fine chemicals, the enzymes responsible for carotenoid synthesis have been successfully expressed in noncarotenogenic microbes such as \textit{E. coli} and yeast with the resulting recombinant strains currently under further optimization.

Typically synthesis of carotenoids begins with head-to-head condensations of IDP to form the twenty-carbon GGDP molecule by \textit{ispA} and \textit{crtE}. This is then followed by the unification of two GGDP molecules, again via a head-to-head condensation, by \textit{crtB}. The further desaturation by phytoene desaturase results in the synthesis of the first colored carotenoid lycopene, a compound of high nutritional importance present in high natural concentrations within tomatoes. Lycopene is made up of 11 \textit{trans} double bonds.

![FIGURE 22.2](image_url) The biosynthetic pathway for production of carotenoids where the molecular structure of each molecule is shown to illustrate the lengthening of the carbon chain through addition of IDP or GGDP. Enzymes included are id\textit{i}, isopentyl diphosphate isomerase; \textit{ispA}, farnesyl diphosphate synthase; \textit{crtE}, geranylgeranyl diphosphate synthase; \textit{crtB}, phytoene synthase; \textit{crtI}, phytoene desaturase; \textit{crtY}, lycopene cyclase.
in the carbon chain that result in a highly active molecule. This accessibility of double bonds allows for its easy modification by lycopene cyclase (crtY) to form the cyclic molecule, β,β-carotene. Lycopene, and β,β-carotene can be processed by a number of enzymes to yield the multitude of linear and cyclic compounds making up the carotenoid family. The C₅₀ compounds are formed through the extension of the C₄₀ backbone by prenyl-transferase like enzymes while C₃₀ compounds are made from condensations of two C₁₅ farnesyl diphosphate (FDP) molecules. In yeast, ergosterol (provitamin D2) is the principal isoprenoid molecule as it is an essential part of the yeast membrane and is derived from FDP. Redirection of the flux away from ergosterol and into GGPP and subsequent carotenoids was achieved through the insertion of a plasmid containing Erwinia uredovora crtE, crtB, and crtI genes under the control of various S. cerevisiae promoters. The engineered recombinant yeast strain produced lycopene up to 113 μg/g dry weight where a similarly engineered strain harboring a plasmid containing the additional Erwinia uredovora crtY gene resulted in the production of β-carotene (103 μg/g dry weight) (Yamano et al., 1994).

The availability of genetic elements, such as transposons and transformation techniques that permit a more high-throughput genetic manipulation of E. coli have allowed the application of stochastic methods for the generation of lycopene overproducing strains (Alper et al., 2005a). More specifically, the genes encoding for 1-deoxy-D-xylulose 5-phosphate (dxs), FDP synthase (ispA), and isopentenyl diphosphate (IPP) isomerase (idi) were first grafted into the E. coli chromosome while episomally expressing the crtEB1 operon. Of these two inserted gene sets, the first enabled the conversion of the glycolytic metabolites pyruvate and glucose 3-phosphate into the required precursor isopentenyl diphosphate which is then converted to lycopene by the enzymes encoded within the crtEB1 operon. Introduction of random gene deletions by transposon-based mutagenesis (Alexeyev and Shokolenko, 1995) resulted in the identification of three genes whose deletion improved lycopene biosynthesis (Alper et al., 2005b; Alper et al., 2005c). The genes identified as positive deletions encode for rssB, a gene controlling macromolecule degradation, and two hypothetical proteins yjjP, yjjD. To further improve lycopene production, the authors combined the single mutations found from the transposon library screening with a set of gene deletions predicted to result in lycopene production increases based on the results of flux balance analysis and minimization of metabolic adjustment simulations (Segre et al., 2002; Varma et al., 1993). After identification, combinatorial deletions were performed that resulted in strains producing lycopene over 10 mg/g dry cell weight.

Carotenoid biosynthesis in E. coli has also been achieved and optimized using directed protein evolution to develop novel compounds with unique properties. Notably, the generation of novel acyclic carotenoids was achieved by gene shuffling crtI genes derived from Erwinia herbicola and Erwinia uredovora. After an opening round of shuffling with the crtI genes, the resulting library was introduced into E. coli harboring wild-type crtE and crtB for subsequent selection of clones that confer carotenoid colorations. One clone with yellow coloration (I25) and one with pink (I14) were isolated. Further sequence analysis of the mutated crtE gene isolated from I14 revealed two amino acid mutations and a replacement of the 39 N-terminus amino acids of crtE from E. uredovora with that of E. herbicola. Sequence analysis of crtE isolated from I25 showed two amino acid changes from the original sequence. In order to extend the breeding of novel cyclic carotenoids, crtY from E. uredovora and E. herbicola were introduced separately into the carotenoid pathway containing crtE from clone I14. When the wild-type crtE was introduced in the recombinant E. coli expressing the carotenoid pathway, a bright yellow–orange coloration was produced. However, replacing the wild-type crtE with the desaturase from I14 resulted in bright yellow coloration. A library of crtY was also created by shuffling the crtY genes from the two origins, and introduced into the E. coli carrying the I14 desaturase pathway. Out of 4,500 clones screened, 25 colonies with different colorations were selected. Sequencing of the cyclase isolated from a bright red clone revealed the generation of two amino acid changes within the E. uredovora cyclase, without a recombination event occurring. One reaction product extracted from this colony was identified to be torulene, a compound not native to the recombinant metabolic pathway introduced. A similar approach has recently been presented where carotenoid production alterations from E. coli by random chromosomal mutations were used to produce novel carotenoids (Tao et al., 2005a).
22.3.2 Coenzyme Q_{10}: The Ubiquitous Quinone

Coenzyme Q_{10} (CoQ_{10}) is widely regarded as one of the most important lipophilic antioxidants that can prevent the generation of free radicals as well as oxidative modifications of proteins, lipids, and DNA. Many human pathological conditions are associated with reduced levels of CoQ_{10}, including cardiac disorders, neurodegenerative diseases, and cancer, all of which are usually treated with dietary CoQ_{10} supplements. There are two major components of this molecule, the quinine ring and the long isoprene tail, having uniquely defined functions. The quinine component allows CoQ_{10} to transfer electrons while the isoprenoid side-chain holds it within the mitochondrial or cytoplasmic membrane. To satisfy demand for this important fine chemical, CoQ_{10} has been synthesized by conventional chemical synthesis (Negishi et al., 2002), semichemical synthesis (Lipshutz et al., 2002) and more recently from native and recombinant microbial strains (Park et al., 2005; Yoshida et al., 1998).

While the metabolic pathways for the synthesis of CoQ_{10} in eukaryotes and prokaryotes differ, both require common assembly steps, namely assembly of the quinonoid ring and generation of the decaprenyl diphosphate tail. Synthesis of the quinoid nucleus is accomplished by the shikimate pathway via chorismate and p-hydroxybenzoate in bacteria or, in higher eukaryotes, by tyrosine that is supplied through dietary means due to the absence of the shikimate pathway. Yet uniquely, *S. cerevisiae* can synthesize CoQ_{10} from either chorismate or tyrosine. The isoprene tail, most commonly decaprenyl diphosphate (DPDP), is synthesized through consecutive condensations of IDP to form FDP followed by subsequent condensations of FDP by DPDP synthase. In the final steps, the head and tail groups are unified by phenylation using 4-hydroxybenzoate polyprenyltransferase (HBPT), a membrane bound protein known to have wide substrate specificity (Melzer and Heide, 1994). Additional modifications, such as decarboxylations, hydroxylations, and methylations follow to finally synthesize CoQ_{10}. A more detailed review of the complete synthesis can be found in Choi et al. (2005). Table 22.1 lists the major enzymes for the biochemical synthesis and their corresponding genes in yeast and *E. coli*.

An early study of bacterial production identified three efficient CoQ_{10} producing strains from the species *A. tumefaciens*, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans*, and the discovery of two mutated over producer strains; one mutant *A. tumefaciens* and a mutant *R. sphaeroides* (Yoshida et al., 1998). Before additional mutations were introduced, an original set of 34 strains across the three species were grown in flask fermentation cultures. Interestingly, a number of the parental strains achieved high production levels, but for many of the cultures, extractions proved difficult owing to the high viscosity of the fermentation media and were thus excluded from further study. Following selection, two parent strains belonging to the *A. tumefaciens* species were subjected to random mutagenesis by chemical treatment by N-methyl-N’-nitro-N-nitrosoguanidine (NTG). After 90 hr fermentations, CoQ_{10} production in mutant strains ranged from 60 mg/L up to 1 10 mg/L as compared to the wild-type *A. tumefaciens* production of at most 50 mg/L. Higher concentrations in mutant strains were attributed to the introduced genetic alterations, although mutation sites were never identified (Yoshida et al., 1998).

Recombinant DNA technology has also been applied in the biosynthesis of CoQ_{10} through multiple gene insertions. Park and colleagues performed batch and fed-batch fermentations of *E. coli* BL21 strains episomally expressing DPDP synthase (*ddsA*) from *Gluconobacter suboxydans* (Park et al., 2005). The *ddsA* gene was introduced to the cells by two different coreplicable plasmids: (1) a high-copy number plasmid (pUC19) resulting in strain pYCDdsA, and (2) a low-copy number plasmid (pACYC184) to yield the pACDdsA strain. The low-copy pACDdsA strain consistently outperformed the high-copy pYCDdsA strain to achieve CoQ_{10} production levels of 0.97 mg/L with 103 g dry cell weight/L in batch cultures and a final concentration of approximately 25.5 mg/L under fed-batch conditions. While titers are still low relative to chemical synthesis, these studies illustrate the limited effort needed to achieve competitive titers through microbial production of this complex isoprenoid.
22.3.3 Terpenoids

Terpenoids represent a diverse class of natural, high-value chemicals containing more than 30,000 different structures. Commercial applications for terpenoids include flavor and fragrance additives, essential oil constituents and an expanding role in pharmaceuticals where commercial generation of terpenoids is generally achieved through chemical synthesis or plant extraction. Conventional chemical synthesis uses a series of isoprene condensations and a cyclization to form terpenoids although production quantities of critical terpenoids are generally obtained by extraction from a variety of plant tissues (Watts et al., 2005). Both conventional chemical synthesis and extraction are highly expensive and low-yielding processes, therefore an opportunity exists for the engineering of terpenoid biosynthesis in recombinant organisms. This is especially beneficial since microbial production means efficient enzymatic cyclization reactions can occur by a variety of terpene cyclases, thus offering more variation in terpenoid conformation (Kim et al., 2006; Picaud et al., 2006). Similarly to classes of flavonoids, many terpenoids require the use of membrane-bound cytochrome P450 monooxygenases, a challenging hurdle to overcome in prokaryotes. As with most critical fine chemicals being investigated for their pharmaceutical potential, two critical terpenoids, artemisinin and taxol, are especially important since the current demand exceeds production capabilities (Hezari et al., 1995).

22.3.3.1 Artemisinin

Artemisinin is a sesquiterpene found in sweet wormwood (Artemisia annua) that is derived from amorphalide, a cyclization product of FDP. Malaria-causing Plasmodium strains have begun to develop resistance to traditional antimalarial compounds, such as chloroquine, cycloguanile, and sulfadoxin. As such, the potential of artemisinin is becoming increasingly important as an alternative treatment to this deadly disease (Liu et al., 2006; Rathod et al., 1997). Current extraction methods for artemisinin are inefficient and result in inadequate production levels that can not accommodate the growing global demand for inexpensive anti-malarial drugs. This is especially true among countries of the developing world where malaria infections are the most frequent.
Considering these factors, recombinant strains are beginning to show potential as microbial factories capable of producing artemisinin. In one of the first attempts, an engineered \textit{E. coli} strain was developed capable of synthesizing the precursor amorphadiene (Martin et al., 2003). In this study, mevalonate kinase, phosphomevalonate kinase, and mevalonate pyrophosphate decarboxylase from \textit{S. cerevisiae}, together with IDP isomerase and farnesyl pyrophosphate synthase from \textit{E. coli} were first sewn together under the control of the \textit{lac} promoter to form a synthetic operon cloned onto a coreplicable plasmid. The synthetic operon was transformed and coexpressed with a codon modified amorphadiene synthase (Martin et al., 2003) resulting in a recombinant strain able to produce up to 24 mg/L of amorphadiene. Further analysis revealed this to be an underestimate on production levels in that losses from stripping to the air during fermentation were not considered significant, but is now known to be prevalent for isoprenoid products. To rectify this issue, a two-phase partitioning bioreactor (TPPB) strategy was implemented that resulted in the separation of the hydrophobic product, amorphadiene, from the fermentation broth. By doing so, the previously engineering strain had improved product titers of up to 20-fold, generating approximately 500 g/L of amorphadiene (Newman et al., 2006).

More recently, efforts have focused on engineering \textit{S. cerevisiae} to improve the production yields of amorphadiene by increasing precursor availability and to produce artemisinic acid, a later stage precursor to artemisinin. In a first step, the carbon flux leading to competing biosynthetic pathways was reduced in an effort to increase FDP production within the host. In the end, up regulation occurred for several genes responsible for FDP synthesis and at the same time the gene responsible for FDP conversion to sterols, squalene synthase, was down regulated (Ro et al., 2006). The resulting recombinant yeast produced 153 mg/L of amorphadiene after introduction of amorphadiene synthase from \textit{A. annua} (Ro et al., 2006). As with flavonols, the synthesis of artemisinic acid from amorphadiene requires functional expression of membrane bound cytochrome P450 monooxygenases. After isolation of genes encoding the oxidizing of amorphadiene in \textit{A. annua}, the cloning and expression of genes responsible for hydroxylation (CYP71AV1) and oxidation, a cytochrome P450 oxidoreductase (CPR) as a partner protein, led to the biosynthesis of artemisinic acid in recombinant yeast. As a result of the engineered mevalonate pathway and introduction of CYP71AV1 and CPR, high titers of up to 100 mg/L of artemisinic acid were found (Ro et al., 2006).

### 22.3.3.2 Taxol

In the early 1970s extensive characterization of extractions from the bark of a number of Northwestern United States trees, including one from the Pacific yew (\textit{Taxus brevifolia}) were undertaken in an effort to identify new natural products with potential therapeutic properties. With advances in chemical characterization, a highly influential terpenoid, named taxol, was eventually identified. Today this important chemical is increasingly used in cancer chemotherapy (Foa et al., 1994). Yet extraction from \textit{T. brevifolia} is highly inefficient (yielding only 1 mg of taxadiene from 750 kg of dry Pacific yew bark) and as a further result, the inefficient extraction has led to the depletion of natural resources thus driving the cost of taxol higher. The elucidation of the biosynthetic pathway to taxadiene, a precursor of taxol, opened the door to available approaches for production through chemical synthesis, but these processes remain cumbersome and sometimes require as many as 25 steps (Kingston, 1991; Shuler, 1994). In recent years, taxadiene production in microorganisms has been explored in an effort to lower costs and provide a simple mode of extraction.

Recombinant microorganisms provide an environmentally friendly and competitive approach for the possible large-scale production of taxadiene via its phosphate precursor, IDP, through a 3-step reaction pathway. First IDP is isomerized to form dimethlallyl diphosphate (DMADP) by IDP isomerase. In the second step, GGDP is formed from the condensation of three molecules of IDP with one molecule of DMADP by the enzyme GGDP synthase. These two steps, shown in Figure 22.3, are universal steps for the biosynthesis of various isoprenoids, such as carotenoids. Finally, taxadiene synthase catalyzes the cyclization of GGDP to form taxadiene (Lin et al., 1996). In a first attempt to achieve substantial biosynthesis of taxadiene in \textit{E. coli}, IDP isomerase, GGDP synthase, and a truncated taxadiene synthase were
overexpressed, together with the deoxyxylulose-5-phosphate (DXP) synthase from *E. coli*, to increase the availability of IDP (Huang et al., 2001). Truncation of taxadiene synthase was used to improve the solubility of the enzyme in the host cell. Heterologous genes were cloned separately into multiple coreplicable expression plasmids where the expression level of each gene was regulated by the strong T7 phage promoter. The recombinant *E. coli* strain achieved production levels of up to 1.3 mg taxadiene/L in batch fermentations, a significant improvement over plant extraction.

In a first attempt toward the complete synthesis of taxol, the tractable host *S. cerevisiae* was employed as it provides the ability (unlike the prokaryote *E. coli*) to functionally express P450 enzymes that are widely utilized in the taxol biosynthetic pathway (DeJong et al., 2006). In addition to the incorporation of GGDP synthase (GGDPS) and taxadiene synthase (TS), the three steps following taxadiene formation were also introduced to the recombinant yeast to product taxadiene-5α,10β-diol monoacetate. The three additional enzymes are taxoid 10β-hydroxylase (THY10b), taxadienol 5α-O-acetyl transferase (TAT) and the cytochrome P450 taxadiene 5α-hydroxylase (THY5a). Fermentations up to three days using the recombinant strains yielded 1.0 mg/L of the taxadiene intermediate, but only trace amounts (<25 μg/L) of the diol product. This indicated the ample cooperativity of GGDPS and TS but also highlights their poor expression with the rest of the biosynthetic pathway. To resolve the problem of poor functional expression in the rest of the taxol biosynthetic pathway, coordinated overexpression of the P450 oxygenases and P450 reductases has been suggested as a way to increase total pathway activity, thereby increasing the diol (end product) production. (DeJong et al., 2006; Jennewein et al., 2005). PCR differential display has identified a number of the genes actively expressed during *in vivo* synthesis which will aide in the development of new recombinant strains to realize the formation of taxol in a production host.

**FIGURE 22.3** The major metabolites of taxol biosynthesis discussed are shown along where the enzymatic steps are encoded by TS, taxadiene synthase; THY5a, cytochrome P450 taxadiene 5α-hydroxylase; TAT, taxadienol 5α-O-acetyl transferase; THY10b, taxoid 10β-hydroxylase. The final taxol molecule is also shown.
22.4 Specialized Fine Chemicals from Microorganism Biosynthesis

The following section details a number of efforts undertaken to synthesize some of the more industrially specialized chemicals through microbial biosynthesis. These specialty chemicals have high market value in the food industry, pharmaceuticals, cosmetics and other niche areas of consumer goods, particularly where “natural” products are an important concern among consumers. Many of the following chemicals to be discussed have been fully realized through microbial production while others are still in the development phases.

22.4.1 Polyketides

Polyketides form a large class of natural products with interest in both pharmaceutical and agricultural applications. The synthesis of these complex molecules originates from simple building blocks such as acetyl-CoA, malonyl-CoA, methylmalonyl-CoA and propionyl-CoA and is carried out by the action of polyketide synthases (PKSs). In traditional chemical polyketide synthesis, rapid polymerization forming unwanted products and proper site directed synthesis can be troublesome to overcome. As such, microbial biosynthesis can overcome these bottlenecks to improve yields of desired stereochemistries, yet there are issues such as natural microbial resistance still left to be solved. Since many microorganisms actively express unique PKSs responsible for the folding and post translational modifications needed, development of an efficient production platform requires simple overexpressions in the host organism. To engineer an *E. coli* polyketide over producer, PKS expression is accomplished primarily by enzyme mutations and plasmid transformations. In addition to expression, increasing the availability of the small precursor metabolites is a priority, particularly in high cell density conditions where basic building blocks are depleted rapidly. Compounding the challenge, the issues of expressing PKSs and precursor availability must be resolved independently and simultaneously, so that a well-synchronized metabolic system can be developed (Pfeifer *et al.*, 2001). Recently the generation of microbial production strains for a number of different polyketide antibiotics has been achieved using a variety of engineering and biochemical techniques.

Erythromycin is a potent antibiotic synthesized by the soil bacterium *Saccharopolyspora erythrea* where the macrolytic-antibiotic core is synthesized by the large modular PKSs. By incorporating genes into the *E. coli* chromosome for the co-expression of an array of PKSs, the synthesis of 6-deoxyerythronolide B (6dEB), the core molecule, was achieved. 6dEB is formed from one propionyl-CoA unit and subsequent elongation of six (2S)-methylmalonyl-CoA by the enzyme deoxyerythronolide B synthase (DEBS). DEBS is a three-subunit enzyme (\(\alpha_2\beta_2\gamma_2\)), comprising of two sets of 28 distinct active sites, seven of which are modified after translation by pantetheinylation. Due to the complex chemical structure hampering the total chemical synthesis of the antibiotic, any large-scale production relies on fermentation technologies for part of the synthesis steps. Expression of DEBS genes has been achieved in recombinant *Streptomyces coelicolor* (Kao *et al.*, 1994), but due to the challenges in developing a scalable fermentation process of *Actinomyces*, researchers have been seeking to create a production platform in recombinant *E. coli*. For that reason, the three subunits of DEBS were cloned individually into the *E. coli* expression vector pET21c. In order to facilitate pantetheinylation of the recombinant DEBS and synthesis of propionyl-CoA, a phosphopantetheinyl transferase gene (*sfp*) and propionyl-CoA synthase (*prpE*) were also inserted into *E. coli* by integration into the *prpRBCD* operon within the *E. coli* genome (Pfeifer *et al.*, 2001). Disruption of the *prp* operon, which is responsible for propionate metabolism, was intended to allow optimum conversion of exogenously supplemented propionate into propionyl-CoA by the *prpE* gene product. Furthermore, the two-subunit propionyl-CoA carboxylase (*pcc*) and the biotin ligase carrier protein (*birA*) were also introduced into the recombinant strain mediated by a coreplicable plasmid. Introduction of the carboxylase gene allowed the conversion of propionate into (2S)-methylmalonyl-CoA, which served
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as an extender unit of the recombinant DEBS. Fermentation of the highly engineered recombinant strain in propionate supplemented media yielded 0.1 mmol of 6dEB per gram of cellular protein per day, which is superior to wild-type S. erythraea, and compatible to a modified strain for industrial 6dEB production (Pfeifer et al., 2001). More recently, the DEBS genes have been inserted directly onto the chromosome of E. coli, although only minimal production was found (Wang and Pfeifer, 2008). An interesting study illustrated microbial production’s ability for site directed biosynthesis while producing a modified form of erythromycin in combining two widely used aspects of metabolic engineering: alteration of genetic elements and heterologous gene introduction. First the functional unit of the erythromycin PKS was replaced by removing the methylmalonate-specific acyltransferase domain responsible for formation of the methyl side chain at C6 with an ethylmalonyl-specific acyltransferase used for niddamycin biosynthesis. This produced an erythromycin-like product, but only after further expression of a gene encoding crotonyl-CoA reductase was the recombinant strain able to produce the desired 6-ethylerythromycin product (Stassi et al., 1998).

The commercial production of tylosin, a complex polyketide antibiotic, was recently accomplished where the concept of DNA shuffling was adapted to shuffle the DNA of an entire genome, that of the bacterium Streptomyces fradiae (Zhang et al., 2002). In order to generate a new tylosin over-producer, the wild-type S. fradiae strain was subjected to one round of chromosomal random mutagenesis through cellular exposure to a nitrosoguanidine mutagen. Upon screening of 22,000 individual mutants, 11 strains producing more tylosin than the wild-type were isolated. To generate a genome-shuffled library, protoplasts of the 11 strains were mixed in equal proportion and recursively fused. One thousand clones were screened from the first round of genome shuffling, and seven identified superior strains were used as the parental strains for the next shuffling cycle. Similarly, another 1,000 new colonies were screened and seven strains with further improvement of tylosin production were isolated. Analysis of two over-producer strains from those isolated showed tylosin titers nine-fold higher compared to the wild-type S. fradiae. It is compelling that the development of a similar overproducer strain using various mutagens took place in 20 years, requiring 1 million assays while application of the genome shuffling method achieved the creation of an overproducer strain in the course of 1 year with only 24,000 assays.

In some cases the application of a few point mutations within the enzymatic coding sequence will result in radical changes of the enzyme’s catalytic properties, as has been the case for mutations of the plant type III PKSs. These key enzymes are responsible for the biosynthesis of structurally diverse valuable natural products found in plants (extensively reviewed in Austin and Noel, 2003) and include such enzymes as benzalacetone synthase (BAS) which catalyzes a condensation reaction of 4-coumaroyl-CoA with one malonyl-CoA to form benzalacetone. This critical molecule is the major precursor of the anti-inflammatory lindleyin found in rhubarb, the chemicals gingerol and curcumin found in ginger plants, and the characteristic chemical conferring the raspberry aroma, raspberry ketone. With the availability of amino acid sequences for various plant type III PKSs, for example, chalcone synthase, the resveratrol producing enzyme stilbene synthase (STS), 2-pyrene synthase (2-PS), and acridone synthase (ACS), bioinformatic studies have highlighted critical differences within the catalytic region of these enzymes. After sequence alignment, it was shown that the conserved amino acid residue Phe-215, thought to be a crucial integral of the catalytic activities of CHS, is not present in the BAS gene. By replacing the amino acid Leu-215, together with its adjacent Ile-214 with Phe-215 and Leu-214, respectively, it was shown to confer CHS activities to an enzyme naturally exhibiting BAS activity. The mutations of the BAS sequence resulted in chalcone-forming properties, in which the chalcone naringenin, along with other byproducts were generated from incubation with appropriate substrates (Abe et al., 2003). These bioinformatic endeavors are vital to optimization efforts of microbial production cell lines for polyketides as well as the array of fine chemicals available for production by microorganisms.

22.4.2 Microbial Synthesis of Chain Molecules

The microbial production of polyunsaturated fatty acids (PUFAs) is beginning to reestablish itself as a cost-effective production method, especially since microbial oils, or single cell oils (SCOs), where
found to be high in PUFA content. PUFAs do not occur to any extent in plant extracted oils generated by agriculture and until now could only be generated from marine animal sources (Ratledge, 2004). Clear clinical evidence has shown PUFAs to be highly important to protect infants from cardiovascular disease and to be beneficial in the development of brains and retina functions to achieve improved memory and eyesight (Damsgaard et al., 2006; von Schacky and Dyerberg, 2001). Their prominence in mother’s milk and absence from cow’s milk and infant formulas has only set to reinforce the importance of these critical molecules for human development. Today a number of different strains are being used to produce some of the more important fatty acids.

PUFAs are long chains of 16 or more carbon atoms containing two or more double bonds along the chain. Though the needed amounts of PUFAs differ among species, they are required by all for normal cellular functioning as they are responsible for membrane fluidity and act as signaling molecules in some species. In general, most fatty acids necessary for cell survival are synthesized by cellular metabolic pathways. However the synthesis of some natural fatty acids, such as linoleic acid (18:2ω6), and ω-3, ω-6, and ω-9 fatty acids, can not occur in mammals but are essential parts of diets for normal growth and development (Chemler et al., 2006). The formation of PUFAs requires the expression of a number of chain lengthening elongases as well as a variety of desaturases used to introduce double bonds (i.e., Δ5-, Δ6-, Δ12-). Although all living organisms must synthesize some lipids for membranes, few microorganisms are able to accumulate lipid levels of greater than 20% of the cell mass, but some yeast (along with a few species of algae and fungi) are able to naturally synthesize large amounts of lipids, making yeast an ideal host for microbial production of PUFAs (Figure 22.4).

Engineering the synthesis of PUFAs in yeast has progressed in recent years through the functional expression of enzymes from a variety of exogenous sources. For example, accumulation of γ-linolenic acid (18:3ω6) was achieved by the expression of Δ6-desaturase from *Mucor rouxii* in yeast. After sequencing and cloning of the encoding gene, it was transformed into the strain using vector pYES2 downstream of a GAL1 promoter. Fermentations resulted in yields of approximately 7% of the total fatty acids as γ-linolenic acid when fed with the precursor linoleic acid (18:2ω6) (Laoteng et al., 2000). A similar study introduced Δ6-desaturase and Δ12-desaturase from *Mortierella alpine*, where the resulting γ-linolenic acid (18:3ω6) accumulation was as high as 8% of total fatty acid content for the coexpression in *S. cerevisiae* (Huang et al., 1999). While this is only the beginning of expressing the synthesis of PUFAs in microorganisms, further efforts have uncovered PKS used in the *Shewanella* bacteria that are similar to the fatty acid synthase (FAS) used by *E. coli*. Eight PKS domains were found to lead to PUFA biosynthesis, thus identifying the mechanism used for long chain fatty acid synthesis in *Thraustochytrids* where the fatty acid chain remains unsaturated as the chain continues to be lengthened (Metz et al., 2001). This is unlike conventional eukaryotic synthesis where the chain is completely saturated while growing, thus providing easier access to longer chain PUFAs through possible plasmid transformations into recombinant hosts.

Wax esters are long chain carbon molecules with lengths from 38 to 44 carbons and are composed of mainly 20:1 fatty acids and 20:1 and 22:1 fatty alcohols. They are primarily utilized as lubricants but also in medicine, cosmetics, and the food industry. A recent study used recombinant *E. coli* to produce wax esters similar to those found in the Jojoba plant (*A. baylyi*), from which plant wax is extracted at a high cost. Only with the coexpression of plant acyl-CoA reductase Acr1 using an ampicillin selective plasmid and a kanamycin selective plasmid harboring *A. baylyi* ADP1 wax ester synthase/acyl-CoA-diacylglycerol acyltransferase (WS/DGAT) the synthesis of a mixture of wax esters was accomplished, predominantly containing palmitoyl oleate (C34:1) (Kalscheuer et al., 2006). Bacterial WS/DGAT was shown in an earlier study to have a highly unspecific acyltransferase activity and was capable of accepting a broad range of alcohols as substrates from long chain fatty alcohols to the short chain alcohols of ethanol (Kalscheuer et al., 2004).

### 22.4.3 Pigments, Flavor, and Fragrance

While many previously mentioned compounds can be classified as color or flavor compounds, the following are typically utilized for their properties of exhibiting color and/or imparting flavor and
fragrance. The following sections contain molecules derived from precursors of one or more molecular classes to create large complex molecules that pose difficult challenges when using chemical synthesis. As such, interest in microbial biosynthesis and biocatalysis has grown as it provides an ample means of production for many of the critical flavors, colors and scents used in food, perfumes, and cosmetics. Additionally, a number of these compounds have been shown to have medicinal benefits either in their natural state or in a modified form of their natural product.

22.4.3.1 Flavors

Development of flavors to be used in food products, especially cheese, wine, and fermented sausages, as well as aromas has recently undergone a tremendous “back-to-nature” demand. This phenomenon is ever present in today’s food markets where consumers’ preference for ‘naturally’ flavored products instead of the synthetic (chemically synthesized) flavors is growing (Demyttenaere and van Ruth, 2001). Recent legislation in Europe and the United States has defined “natural” products as those synthesized by native enzymes, and with consumer interest in natural labeled products high, it has resulted in a push toward the development of flavors in microbial synthesis. A large amount of development has occurred in starter cultures used to provide the enzymes for dairy products, most notably cheeses. The important organisms used in these cultures include Lactococcus lactis, Lactobacillus, Streptococcus, and Propionibacterium, and other lactic acid bacteria, impart cheeses with their characteristic flavors due to
the organisms’ ability to synthesize the required peptides and amino acids making up the volatile aroma compounds needed. Lactic acid bacteria are efficient synthesizers of the branched-chain amino acids (Leu, Ile, Val) used for malty and fruity flavors, the aromatic amino acids (Phe, Tyr, Trp) responsible for floral and chemical flavors and the sulphuric amino acids (Met, Cys) that create the cabbage, meat, and garlic flavors. Much interest has also been paid to the development of high throughput screening methods for product analysis from flavor-forming organisms’ fermentations since these products are critical in the formation of various flavors (Smit et al., 2004, 2005). In addition to cheeses, bioflavors are becoming increasingly important as additives in beer and other carbonated beverages as alternatives to chemically synthesized analogs which are coming under increased scrutiny due to possible negative health and environmental effects (Vanderhaegen et al., 2003).

22.4.3.1 Precursor Biosynthesis Is a Vital Step in Flavor Production

Biosynthesis of secondary products creates a large drain on microbial metabolism, particularly in production of the critical precursor metabolites needed for flavor compounds, as such, the development of strains unaffected by such metabolic demands has garnered a lot of interest. While the elucidation of the complex pathways leading to the formation of the many flavor compounds has just started, the catabolism of small molecule precursors and the important character imparting amino acids has been known for some time (Ardo, 2006). The biosynthesis of all aromatic amino acids begins with the shikimate pathway, encoded by the _aro_ gene cluster, in which the glycolysis and pentose precursors are reacted to from the branching molecule chorismate via shikimate. In the first step, erythrose 4-phosphate and phosphoenolpyruvate are converted to 3-dehydro-shikimate by 2-dehydro-3-deoxyphosphoheptonate aldolase (_aroFGH_), 3-dehydroquinate synthase (_aroB_) and 3-dehydroquinate dehydrate (_aroD_). Shikimate, shikimic acid, is then formed using NAD or NADP by shikimate dehydrogenase (the two isoforms are encoded by _aroE_ or _ydiB_, respectively). The remained of the gene cluster then converts shikimate using ATP to chorismate by shikimate kinase I or II (_aroK_ or _aroL_), 3-enolpyruvylshikimate-5-phosphate synthetase (_aroA_), and chorismate synthase (_aroC_). Chorismate can then branch into phenylalanine and tyrosine synthesis, tryptophan synthesis. (Figure 22.5)

For the final three steps of phenylalanine biosynthesis, chorismate in _E. coli_ is acted on by a bifunctional enzyme encoded by _pheA_ in which chorismate mutase is encoded on the N-terminus and prephenate dehydrase on the C-terminus. This bifunctional enzyme generates phenylpyruvate via prephenate, the branching molecule for tyrosine synthesis. Phenylpyruvate is then converted to phenylalanine using glutamate by an aminotransferase (_aspC_). Tyrosine on the other hand synthesized from prephenate through the action of NAD-dependent prephenate dehydrogenase, encoded by _tyrA_, to form β-hydroxyphenylpyruvate. Then a transaminase encoded by _tyrB_ makes the final conversion with glutamate to form L-tyrosine. L-tryptophan biosynthesis begins back at chorismate using glutamine to generate indole through a series of reactions controlled by enzymes of the _trp_ operon. The enzymes encoded by _trpA_, _trpC_, _trpD_, and _trpE_ are sometimes found in bifunctional enzymes, such as the _trpDE_ complex in _E. coli_. Indole is finally converted to L-tryptophan by tryptophan synthase (_trpB_).

Of particular importance is L-phenylalanine, an essential amino acid, as it has wide interest for use as a feed source for a number of aromatic compounds, such as raspberry ketone (see below). It is predominantly produced as the starting chemical for the low-calorie sweetener aspartame, created by the Nutrasweet process (Bongaerts et al., 2001). The major microorganisms used to synthesize phenylalanine include strains of _E. coli_, _C. glutamicum_, and _Brevibacterium flavum, lactofermentum_ and _linens_ (Boyaval et al., 1983; Ito et al., 1990; Wu et al., 2003). Engineering _C. glutamicum_ resulted in an increased production of phenylalanine through the introduction of feedback resistant variants of D-arabinohexulononate 7-phosphate (DHAP) synthase, chorismate mutase, and prephenate dehydratase, three important genes along the pathway for aromatic amino acid synthesis. The genes were all cloned onto one coreplicable vector and transformed to the bacterium for expression. Transformants were isolated and screened for altered carbon flows where one such strain produced up to 26 g/L of phenylalanine (Ikeda and Katsumata, 1992).
Various groups have engineered *E. coli* for phenylalanine biosynthesis using an array of heterologous genes with various levels of success (Backman et al., 1990; Konstantinov et al., 1991; Miller et al., 1987). Miller and colleagues inserted a wild-type DHAP synthase (*aroF*) along with a feedback resistant chorismate mutase/prephenate dehydratase (*pheA*) to perform the phenylalanine synthesis (Miller et al., 1987). It was later published that when the strain was combined with an optimized fermentation process, titers of 50 g/L for 36 hr fermentations where obtained with production levels of 0.23 g of L-phenylalanine per gram of glucose fed (Backman et al., 1990). In a similar study, feedback resistant forms of DHAP synthase and chorismate mutase/prephenate dehydratase where cloned onto a temperature-controlled expression vector enabling the production levels of up to 16.9 g/L, with additional process development eventually resulting in process titers of 46 g/L with a productivity of 0.85 g/L/hr (Konstantinov et al., 1991; Takagi et al., 1996).

Efforts to increase tyrosine production in microorganisms have been just as vast using some of the same biochemical techniques and strains. Increasing the central carbon flows toward aromatics has been a common approach among all the aromatics, and has been shown to lead to increased levels of tyrosine production in *C. glutamicum* (Bongaerts et al., 2001). Additionally, by overexpressing the homologous transketolase of the pentose phosphate pathway, *C. glutamicum* strains were able to have 10–50% increases in product titers (Ikeda, 2006).

The development of tryptophan overproducers has been driven by the increased market interest in tryptophan itself, as well as the possibility of a route for production of indigo, an industrial blue dye, from indole. Alterations of the aromatic pathway, increases to precursors and regulation of pathway enzymes have all been attempted in increasing production (Bongaerts et al., 2001). A simple hypothesis for tryptophan over producers is to delete *pheA* and *tyrA* leading to phenylalanine and tyrosine production, respectively. However, this has been shown to cause autotrophic shock and actually lead to reduced production levels. Thus, it was later shown that the simple overexpression of the first step in tryptophan synthesis from chorismate, anthranilate synthase (*trpE*), resulted in only small carbon fluxes away from tryptophan. This is a result of anthranilate synthase’s higher affinity for chorismate (Bongaerts et al., 2001; Ikeda, 2006).

An important aspect of precursor production is to have at hand an efficient extraction method after synthesis. One study has developed a method for the continuous extraction of the precursors in a bioreactor by investigation the production ester precursors (isobutyl acetate, ethyl acetate, and propyl acetate) and the terpenoid precursors of citronellol and geraniol (Bluemke and Schrader, 2001). Since aroma compounds tend to pose strong inhibitory effects on microorganism growth even at low levels, an integrated bioprocess (IBP) was designed using pervaporation to separate the aroma products during
continuous fermentation. Using the IBP, the product mass increased from 50% to 413% depending on the component cultivated from *Ceratocystis moniliformis*. IBP was able to produced increased amounts of aroma compounds in highly concentrated aroma mixtures free from the culture broth and thus making further purification easier (Bluemke and Schrader, 2001).

### 22.4.3.1.2 Vanillin: A Major Flavor

The use of higher fungi, particularly white-rot basidiomycetes, has been explored for the biosynthesis of attractive odors via *de novo* synthesis in native metabolic channels. Living on dead or live timber, these fungi are able to completely degrade lignin, a polymer of substituted alcohols, and metabolize the resulting monomers into aromatic compounds of interest (Lomascolo et al., 1999). One of the most important compounds from these organisms is vanillin (3-methoxy-4-hydroxybenzaldehyde), a compound widely used in food preparation and fragrances. While production of vanillin through chemical synthesis (~US$15/kg) is possible, it is considerably more profitable when coming from a “natural” production mechanism.

Vanillin synthesis in white-rot fungi begins with the metabolism of lignin to form the precursor ferulic acid. This is then converted into vanillic acid by the degradation of the propenoic acid chain residue and either further reduced to form vanillyl alcohol via vanillin by aryl aldehyde dehydrogenase or oxidized to from methoxyhydroquinone (Lomascolo et al., 1999). This process for vanillin production yielded only 64 mg/L after a seven day culture (with a 27.5% molar yield) using the fungi *Pycnoporus cinnabarinus* with ferulic acid substrate, and has been patented (Lesage-Meessen et al., 1996; Sun, 2005). To improve this, a two-step, patented process was also developed that uses *Aspergillus niger* to transform ferulic acid to vanillic acid at an 88% molar yield and the use of *P. cinnabarinus* in the second step for synthesis of vanillin (Lesage-Meessen et al., 1996; Sun, 2003).

### 22.4.3.1.3 Raspberry Ketone and Raspberry Alcohol

The raspberry flavor widely used in soft drinks and other food products is the result of a combination of a number of different chemical components with raspberry ketone (4-(4-hydroxyphenyl)-butan-2-one) the most prominent effector. A part from the obvious native plant source raspberries, the ketone and its precursor alcohol (raspberry alcohol or betuligenol) have been identified in other berries as well as grapes, apples, and peaches. The enzymatic pathway for synthesis of raspberry ketone involves the β-glucosidase-catalyzed hydrolysis of the native betuloside to form betuligenol, which is finally converted to the ketone by microbial alcohol dehydrogenase. The enzymatic production process has been patented (Dumont et al., 1996; Falconnier, 1999). Alternative raspberry ketone production platforms have been developed that use cellular suspensions, such as plant cell cultures of *Rubus idaeus* (raspberry). Similarly to flavonoid synthesis, formation of raspberry ketone begins with the enzyme phenylalanine ammonia lyase converting phenylalanine to p-coumaric acid which then gains a hydroxyl group in forming p-coumaric acid by C4H. After the generation of p-coumaryl-CoA by 4CL, the pathway’s first committed enzyme, BAS condenses one molecule of malonyl-CoA with p-coumaryl-CoA to form benzalacetone. Raspberry ketone is finally synthesized by reducing benzalacetone with NADPH as the proton donor (Pedapudi et al., 2000). Cell suspensions of *Rubus idaeus* are able to produce the precursor and end metabolite in concentrations of 10–50 μM (Pedapudi et al., 2000).

While microbial production of raspberry ketone is still in its infancy, higher fungi have been used for production of the valuable metabolite. Specifically, the basidiomycete *Nidula niveo-tomentosa* was used for *de novo* synthesis of raspberry ketone in submerged cell cultures (Böker et al., 2001). Several approaches have also been described for improving *N. niveo-tomentosa’s* productivity. More specifically, growing cells in soy peptone media with glucose as a substrate and yeast extract as added nutrients resulted in raspberry ketone and raspberry alcohol production of 43.5–119 mg/L in combined titers over a range of cultivation periods (Böker et al., 2001). While other recent attempts have included the use of “green” biocatalytic oxidation (Kosjek et al., 2003), further elucidation of required enzymes involved in select synthesis steps could lead to the cloning and microorganism transformation, thus creating recombinant over producing strains for industrial production.
22.4.3.2 Pigments

With many of the synthetic dyes being attributed to adverse health and environmental effects, natural pigments have come under increased demand for use in a broad range of applications, such as (among others) food products, cosmetics, and animal feed. Many of these compounds also have alternative uses as pharmaceuticals and nutraceuticals or as starting materials for their development. The high-value pigment astaxanthin, which produces pink–orange hues, is one such case that is widely used as a pigmentation source for salmon and trout but, as it is a carotenoid, has been found to hold nutritional benefits as well (Guerin et al., 2003). In a study using the microalgae Haematoccus pluvialus, which naturally produces the pigment, chemostat cultures were performed at constant light irradiance and dilution rates with varying nitrate concentrations in the feed medium. Productivity levels were seen as high as 5.6 mg/L/day in optimal conditions where cells were growing and actively dividing in response to the limitation of nitrate availability (Del Rio et al., 2005).

Lee and Kim isolated and characterized the gene cluster responsible for astaxanthin biosynthesis in the marine bacterium Paracoccus haemundaensis, designated by crtW, crtZ, crtY, crtI, crtB, and crtE (see Figure 22.2). The cluster was PCR amplified and cloned onto and expression vector pCR-XL-TOPO and transformed into E. coli BL21(DE3) Codon Plus cells. The inserted gene cluster was used to synthesize β-carotene using crtE, crtB, crtI, and crtY which then is acted on by β-carotene ketolase (crtW) and β-carotene hydroxylase (crtZ), to form astaxanthin. Recombinant E. coli expressing astaxanthin biosynthesis where found to produce 400 mg/g of dry cell weight representing about 70% of the total carotenoids produced in the strain (Lee and Kim, 2006).

Naphthoquinones, generally derived from plants through extraction, are colored substances of red hues derived from phenylpropanoid and isoprenoid precursors. The chemicals have been used in diverse cultures as colorants for cosmetics, fabrics, and foods (Ballantine, 1969), and for medicinal applications, including antitumor, antiinflammatory, and antimicrobial agents (Papageorgiou, 1978). Production of naphthoquinones by the pathogenic fungus Cordyceps unilateralis BCC 1869 was investigated in shake flask cultures where cultivation conditions, including temperature, initial pH of medium, and aeration, were optimized to improve the yield of total naphthoquinones. The highest yield of naphthoquinones (3 g/L) was obtained from a 28 day culture grown in potato dextrose broth with an initial pH of 7.0, at 28°C with shaking-induced aeration at 200 rpm. An extraction process for isolation of the targeted naphthoquinone, 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone (3,5,8-TMON), from a culture of C. unilateralis, was also developed resulting in a yield of 1.2 g/L of 3,5,8-TMON or 40% of total naphthoquinones. The stability of 3,5,8-TMON was very high, even upon exposure to strong sunlight (70,000 lx), high temperature up to 200°C, and acid and alkali solutions at concentrations of 0.1 M (Unagul et al., 2005).

In an example of reverse engineering, the gram-negative bacterium Ralstonia eutropha was not known to synthesize pigmentation. However, during a study of the 2-methylcitric acid cycle of R. eutropha in which its entire genome was cloned into E. coli, an open reading frame was discovered encoding biosynthetic enzymes for indigoids. Blue pigments of plant and bacterial origins, indigoids have been used as dyes and pharmaceuticals. A genomic library of R. eutropha was created and then inserted into pHC79, a vector capable of carrying large DNA fragments. Upon transfection of E. coli, blue color transformants were identified after spreading on selective agar plates. Because E. coli does not normally produce blue pigments, the pigment synthesis in the recombinant cells must be derived from the action of foreign enzymes from R. eutropha. Further isolation and subcloning experiments isolated an open reading frame of 1251 base-pairs deemed responsible for the blue color formation where further characterization identified the sequence to encode for a dehydrogenase having sequence similarity with known proteins (Drewlo et al., 2001).

22.5 Strategies and Trends

The previously presented examples of metabolic engineering of microorganisms clearly demonstrate that biocatalysis using unicellular organisms offers significant advantages for the production of fine
chemicals. Many of the metabolic engineering, biochemical, and bioinformatics techniques used for the generation of recombinant strains have been utilized with success; however, the quest for improved yields and the need for exploration of even larger metabolic and phenotypic spaces require the development of new experimental and computational tools that will eventually permit the construction of a phenotypic optimum. In this final section, we conclude with a look forward to a few of the novel strategies that have recently been developed for microbial biosynthesis.

22.5.2 New Approaches on Old Methods

Researchers have been able to insert fragments of DNA for some time through a number of different methods. The engineering of cellular function is commonly achieved by adopting foreign pathways or over expressing native enzymes, which requires a priori knowledge of the genetic framework in both the recombinant and native strains. Although this genetic insertion strategy has proven useful, it is limited to the availability of genome sequences and gene function information. Moreover, isolation, selection and culturing the enormous array of unknown organisms for the discovery of novel biosynthetic pathways are cumbersome and require more knowledge of the physiology of the organisms. Therefore, relying on the availability of characterized pathways is a bottleneck toward progress of novel enzyme or product discovery and recombinant synthesis. In the case where available genes are not known or data is unavailable, it has been possible to insert whole or random fragments of genomes followed by the creative selection of desirable traits with reverse engineering used to characterize unknown pathways of an end product. This method was used to express pigments unknown to the bacterium *R. eutropha* within in *E. coli* (as discussed above).

It is has been well established that both eukaryotic and prokaryotic cells use small, non-coding RNA sequences that can bind to RNA transcripts and prevent their subsequent translation. Such RNA molecules act as regulators in various signal transduction mechanisms (Gottesman, 2005) and in plasmid replication and copy number (Lacatena and Cesareni, 1981; Lacatena and Cesareni, 1983; Tomizawa and Itoh, 1981). Since producing fine chemicals in microorganisms is generally performed using cellular phenotypes under none native cellular conditions, RNA silencing (siRNA), or interference (RNAi) using artificial antisense RNA could be a powerful tool toward the goal of developing different genetic perturbations. Such an approach has been followed in the case of commercial production of two solvents, namely acetone and butanol using the gram-positive bacterium *Clostridium acetobutylicum*. To improve such solvent production, RNAi was employed for reducing the expression of enzymes in a competing pathway leading to butyrate formation. Two enzymes mediate butyrate synthesis, phosphotransbutyrylase (PTB) and butyrate kinase (BK) where PTB converts butyryl-CoA into butyryl phosphate with the subsequent reaction catalyzed by BK to produce butyrate. For the purpose of reducing the expression of BK, a synthetic oligonucleotide fragment was developed containing only ten codons of the original BK and its native putative ribosome binding site. To start and end the transcription of the antisense BK, the PTB promoter sequence and a rho-dependent termination sequence were also included in the construct (Desai and Papoutsakis, 1999). Repeating this approach, an antisense fragment for PTB was constructed by including a 567-bp PTB fragment, its putative ribosome binding site, and adc terminator. The *C. acetobutylicum* strain which expressed antisense butyrate kinase exhibited up to 90% lower BK synthesis and resulted in 50% and 35% higher final concentration of acetone and butanol, respectively. The strain which expressed the PTB antisense synthesized 70% lower PTB; however, acetone and butanol concentration were 96 and 75% lower, respectively, compared with that of the native strain (Desai and Papoutsakis, 1999).

22.5.2 Engineering the Genetic Machinery

In an effort to engineer optimal strains as high-value chemical production platforms, directed cell evolution, gene shuffling, in vitro recombination, and custom designed proteins are some of the few tools
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available to adjust the metabolic mechanisms of microbial biosynthesis. The strategies of directed evolution are inspired by the routes of evolution in nature, in which selective pressures lead to the accumulation of beneficial genetic mutations that confer metabolic fitness and thus the survival of the fittest cellular phenotypes (Koffas and Cardayre, 2005). The availability of structural studies is normally not a prerequisite to successful laboratory enzyme evolution, because directed evolution employs stochastic methods to generate mutant libraries. However, recently, deterministic methods that incorporate structural information have been described.

DNA shuffling is based on an iterative processes of random mutation generation and selection for improvement of phenotypes toward a desired goal (Stemmer, 1994). In these methods, random point mutations are generated in the parental DNA sequences in order to introduce sequence diversity. Then, the redistribution of mutation locations is achieved through reassembly of the parental DNA pool by random selection of fragmented DNA where upon assembly the genetic library can be inserted into a tractable host, such as E. coli. Screening, through various high-throughput techniques that depend on the desired phenotype, clones with improved phenotypes are identified and used to isolate evolved genes responsible for improved fitness and consecutively used as the parental sequences in the next generation of evolved strains. Some strategies utilize a pool of homologous parental sequences derived from different species as a template for the recombination effort, in order to increase sequence diversity, thus increasing the protein sequence space (Crameri et al., 1998).

As a requirement of gene shuffling, the parental gene pool requires high sequence homology among the strains for successful reassembly of the fragmented DNA. Exploring novel crossover points in regions of low identity that would accelerate enzyme in vitro evolution is limited, since typical crossover points occur in DNA regions of high sequence identity, thus restricting the method’s impact ability (Bogarad and Deem, 1999). In response to this limitation, a method termed incremental truncation for the creation of hybrid enzyme (ITCHY) (Ostermeier et al., 1999b) was developed that allows such exploration by generating all possible fusions between two non-homologous genes (Ostermeier et al., 1999a). As a case study, ITCHY was implemented to create a glycinamide ribonucleotide (GAR) formyltransferase from E. coli and human DNA sequences, which only share 50% DNA sequence identity but do contain similar secondary structures and proper active sites. Taking the 5’-terminal of the E. coli sequence and the 3’-terminal of the human sequence, truncated unidirectional enzyme digests were performed to generate the ITCHY library to be used for construction. A full-length DNA library consisting of the E. coli and human fragments was assembled by adjoining the truncated sites from two randomly selected sequences which were then cloned into co-replicable plasmids for transfection of an E. coli strain deficient of GAR transformylase activity. Growing the E. coli mutant in the absence of purine led to the identification of the functional hybrid enzymes, where upon sequencing of the hybrid proteins, it was discovered that the sequences generated from ITCHY exhibited wider crossover distributions and as such, scanned a larger protein sequence space than a library generated from standard DNA shuffling (Ostermeier et al., 1999a).

Discovery of novel enzymatic activities has also been found by blindly recombining homologous and nonhomologous sequences to produce chimeric proteins, however the probability to isolate complete mutants with highly improved functions is low, and limited further by the prerequisite for good screening systems. In a more effective approach to protein evolution, native proteins are evolved based on their structure by swapping domains of structural similarity (Ostermeier and Benkovic, 2000; Ranganathan et al., 1999; Riechmann and Winter, 2000). The correct identification of interchangeable modules, freely swappable sequences, and the locations of safe crossover points are two essential objectives for successful structure-based recombination of DNA. In this effort, a measurement for the interactions between amino acid residues and levels of disruption resulting from the replacement of a subset of amino acids were developed in a computational algorithm called SCHEMA (Voigt et al., 2002). Locations of amino acids corresponding to a minimum level of disruption are used to identify the potential crossover points for swapping protein modules. The information obtained from SCHEMA was used to construct a structurally similar hybrid protein derived from β-lactamases (TEM-1) and PSE-4, two proteins that share
only 40% amino acid sequence homology. Domains of the two proteins were interchanged with the resulting hybrid sequences inserted into E. coli. To select proper functionality and activity of the hybrid β-lactamases, cultures grown under different antibiotic concentrations yielded several hybrid proteins exhibiting distinct modular combinations that corresponded to an increased antibiotic resistance by the host (Voigt et al., 2002).

While designer proteins have yet to be applied to fine chemical production, a recent construction allowed E. coli to secrete a black compound in response to light stimulus, thus functioning as a bacterial photograph. Phytocromes found in plants and some bacteria are two-component systems that consist of photoreceptor and response-regulator domains. The photoreceptor domain of phytochrome Cph1 derived from Synechocystis was fused with the EnvZ histidine kinase domain from E. coli. The bacterial photograph was created by introducing the chimera into E. coli containing a chromosomal insertion of lacZ reporter gene under the control of the OmpR-dependent ompC promoter, and two phycocyanobilin biosynthesis genes, hol1 and pcyA from Synechocystis. Phosphorylated histidine kinase acted as an activator for the lacZ transcription under conditions with no light excitation. In the presence of light, phycocyanobilin response inactivated the phosphorylation of the histidine kinase, hence the expression of lacZ was inactivated, producing a contrasting replica of the image on a lawn of E. coli (Levskaya et al., 2005) and making a first step in bacterial imaging.

References

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