

Filamentous Fungi for Production of Food Additives and Processing Aids

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Abstract Filamentous fungi are metabolically versatile organisms with a very wide distribution in nature. They exist in association with other species, e.g. as lichens or mycorrhiza, as pathogens of animals and plants or as free-living species. Many are regarded as nature's primary degraders because they secrete a wide variety of hydrolytic enzymes that degrade waste organic materials. Many species produce secondary metabolites such as polyketides or peptides and an increasing range of fungal species is exploited commercially as sources of enzymes and metabolites for food or pharmaceutical applications. The recent availability of fungal genome sequences has provided a major

opportunity to explore and further exploit fungi as sources of enzymes and metabolites. In this review chapter we focus on the use of fungi in the production of food additives but take a largely pre-genomic, albeit a mainly molecular, view of the topic.

Keywords Acids · Enzymes · Food · Fungus · Vitamins

1

Introduction

Filamentous fungi have long been used in the production of foods and beverages. Although only a small fraction of all fungal species is either eaten or used as a source of food additives or processing aids, there are still many species used for those purposes and there is considerable experience of handling those species at the commercial scale. We focus here on the fungal products used in food production and these include enzymes and smaller metabolites such as fatty acids, other organic acids, vitamins and flavour compounds. In some cases, the fungal products that are important parts of food manufacture are provided in situ rather than added exogenously. Examples of such foods include many fermented soy-based foods, and many mould-ripened foods where the fungus provides flavour. For the purposes of this chapter we adopt the current European Commission definitions of “food ingredient”, “food additive” and “processing aid”. A food ingredient is a component of the food that characterises that food. Some fungal products are classed as “additives”. In that case, the additive is not a component that particularly characterises the food but, rather, the additive is provided for a specific purpose such as manufacture, processing, colouration or preservation of the food and the additive remains in the food. Many fungal products fall into this category and are discussed in this chapter. Several fungal enzymes are used in food processing but, in the main, they do not class as additives because they do not generally remain in the food with functionality – rather, they remain as residues from an early stage in the food manufacture. In that case, they are processing aids rather than additives. We include discussion of fungal enzymes in this chapter and the title is therefore widened to include processing aids.

2

Enzymes from Fungi

The range of native fungal enzymes used in foods is impressive and the enzymes have a large variety of applications. As reviewed previously [1, 2], fungi probably account for ca. 40% of available commercial enzymes and there are many published lists of enzymes and their applications [3, 4]. As more fungal genomes are sequenced and annotated, an even broader range of fungal enzymes with food applications will emerge. In addition, fungi are promising

Table 1 Fungal enzymes approved for use in food production (enzyme list taken from <http://www.amfep.org>). Note that the source fungus is provided even though, in some cases, the enzyme might be produced in recombinant form from a different fungus

Enzyme	Fungal genus	Typical application
Aminoacylase	<i>Aspergillus</i>	Synthesis of L-amino acids
Aminopeptidase	<i>Aspergillus</i> <i>Rhizopus</i>	Protein hydrolysis: flavour enhancement, debittering
α -Amylase	<i>Aspergillus</i> <i>Thermomomspora</i>	Starch liquefaction, alcohol production
Arabinanase	<i>Aspergillus</i>	Lignocellulose degradation, e.g. for vegetable processing and bioethanol
Asparaginase	<i>Aspergillus</i>	Reduction of acrylamide formation: applications in baking
Carboxypeptidase	<i>Aspergillus</i>	Protein hydrolysis: flavour enhancement, debittering
Catalase	<i>Aspergillus</i>	Removal of hydrogen peroxide, used together with glucose oxidase to improve storage of foods and to produce gluconic acid from glucose
Cellulase	<i>Aspergillus</i> <i>Humicola</i> <i>Penicillium</i> <i>Trichoderma</i>	Lignocellulose degradation, e.g. for vegetable processing, malting and brewing, and production of bioethanol
Dextranase	<i>Chaetomium</i>	Used in sugar industry and as stabilizers
Esterase	<i>Rhizomucor</i>	Feruloyl esterases are used in lignocellulose degradation for a variety of applications
α -Galactosidase	<i>Aspergillus</i>	Raffinose hydrolysis; used in brewing and to reduce bloating
β -Galactosidase	<i>Aspergillus</i>	Lactose hydrolysis; used in some milk products, whey utilisation
β -Glucanase	<i>Aspergillus</i> <i>Disporotrichum</i> <i>Humicola</i> <i>Penicillium</i> <i>Trichoderma</i>	Lignocellulose degradation; used in brewing, juice clarification
Glucoamylase	<i>Aspergillus</i> <i>Rhizopus</i>	Starch degradation for release of glucose as substrate for fermentations; used in starch and brewing industries
Glucose oxidase	<i>Aspergillus</i> <i>Penicillium</i>	Removal of glucose. Often used with catalase, e.g. to produce gluconic acid from glucose
α -Glucosidase	<i>Aspergillus</i>	Starch processing enzyme
β -Glucosidase	<i>Aspergillus</i> <i>Penicillium</i>	Lignocellulose degradation and also flavour enhancement in wines
Glucosyltransferase	<i>Aspergillus</i>	Production of oligosaccharides with food-relevant properties, e.g. low calorie glycans

Table 1 (continued)

Enzyme	Fungal genus	Typical application
Hemicellulase	<i>Aspergillus</i>	Lignocellulose degradation, and applications in starch and baking industries
Inulase	<i>Aspergillus</i>	Production of fructose and oligomers. May be used as a prebiotic
Laccase	<i>Myceliophthora</i> <i>Polyporus</i> <i>Thielavia</i> <i>Trametes</i>	Lignocellulose degradation; used in brewing and other beverage industries
Lipase	<i>Aspergillus</i> <i>Penicillium</i> <i>Mucor</i> <i>Rhizomucor</i> <i>Rhizopus</i>	Various lipase specificities, e.g. in cheese manufacture
Mannanase	<i>Aspergillus</i> <i>Trichoderma</i>	Lignocellulose degradation
Pectin lyase	<i>Aspergillus</i> <i>Penicillium</i> <i>Rhizopus</i> <i>Trichoderma</i>	Degradation and modification of pectins; used in fruit and wine industries
Pectin methylesterase	<i>Aspergillus</i> <i>Penicillium</i> <i>Rhizopus</i> <i>Trichoderma</i>	Degradation and modification of pectins; used in fruit and wine industries
Pentosanase	<i>Aspergillus</i> <i>Humicola</i> <i>Trichoderma</i>	Breakdown of pentosan for reduction in viscosity; used in baking
Phosphatase	<i>Aspergillus</i>	Dephosphorylation, e.g. in legume processing and baking
Phosphodiesterase	<i>Leptographium</i> <i>Penicillium</i>	Used as a flavour enhancer
Phospholipases A and B	<i>Aspergillus</i> <i>Trichoderma</i>	Processing aid, used mainly in the cheese industry
Phytase	<i>Aspergillus</i>	Dephosphorylation, e.g. in legume processing and baking
Polygalacturonase	<i>Aspergillus</i> <i>Penicillium</i> <i>Trichoderma</i>	Degradation and modification of pectins; used in fruit and wine industries
Protease	<i>Aspergillus</i> <i>Cryphonectria</i> <i>Penicillium</i> <i>Rhizomucor</i> <i>Rhizopus</i>	Variety of applications including the baking and cheese (chymosin) industries
Pullulanase	<i>Trichoderma</i>	Used in starch (amylopectin) processing
Rhamnosidase	<i>Penicillium</i>	Used in flavour development (e.g. in wines) and debittering

Table 1 (continued)

Enzyme	Fungal genus	Typical application
Tannase	<i>Aspergillus</i>	Use in tea products and to enhance anti-oxidant properties
Transglutaminase	<i>Streptoverticillium</i>	Used to cross-link proteins in a variety of applications, e.g. in meat and cheese industries
Xylanase	<i>Aspergillus</i> <i>Disporotrichum</i> <i>Humicola</i> <i>Penicillium</i> <i>Trichoderma</i>	Xylan degradation, e.g. in brewing

hosts as cell factories for the production of heterologous enzymes [5–8] and many of those enzymes have food applications. In addition to being added exogenously during food manufacture, the provision of fungal enzymes is a key component in the manufacture of fermented soy products [9, 10] and we therefore discuss in this chapter enzymes that are synthesised in situ or added separately. There is a distinction made between enzymes used as processing aids (the majority of food enzymes) and those approved as food additives (e.g. invertase in soft-centered chocolates). For approval of food ingredients as additives, a history of safe use has been a guiding principle, although the addition of new compounds to approved lists requires evaluation governed by strict procedures. In Europe, the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) provides a catalogue of enzymes approved for use in food within Europe (<http://www.amfep.org>). Filamentous fungal-derived approved enzymes, taken from AMFEP, are listed in Table 1. Their data includes both native and recombinant fungal enzymes, and a subset of the recombinant (heterologous) enzymes produced by fungi was tabulated in a recent review [11]. In the US, the Enzyme Technical Association (<http://www.enzymetechnicalassoc.org>) provides a list of enzymes approved as food additives either for specific uses or as generally regarded as safe (GRAS) substances. Most enzymes are used in practice, however, as processing aids.

2.1

Native Enzymes Expressed by Filamentous Fungi

2.1.1

Submerged Liquid Culture

Enzymes are produced from fungi grown in a variety of formats and these mainly involve submerged liquid culture. The use of solid or semi-solid sub-

strates is discussed separately in the coming sections. Because most of the fungal species used to supply industrial enzymes are naturally adept at degrading polymeric material, the spectrum of enzymes produced from fungi is dominated by hydrolytic enzymes for the degradation of proteins and the major plant-derived polysaccharides that include cellulose, hemicellulose, pectin and starch. The other major carbonaceous polymer is lignin, which is impervious to hydrolytic degradation but its degradation by some species of fungi is also possible through the action of peroxidases and oxidases. The genome sequence of the lignin-degrading "white-rot" Basidiomycete *Phanerochaete chrysosporium* is now publicly available [12] and is likely to lead to a better knowledge of the range of enzymes available for degradation of lignin. These enzymes are primarily used in applications such as waste treatment and environmental clean-up, which are beyond the scope of this chapter (for further details of lignin degradation using fungal enzymes see [13, 14]).

The degradation of plant cell wall polysaccharides, and the ferulic acid-containing cross links between the polysaccharides, by enzymes from *Aspergillus* spp. has been described recently in detail by de Vries and Visser [15]. This review provides a detailed description of the *Aspergillus* enzyme systems which degrade cellulose, different hemicelluloses (with a focus on the xylans and (galacto)glucomannans) and the pectins. Recent genome sequence data can also predict genes likely to encode enzymes that degrade plant cell wall polymers [16]. Indeed, the number of available fungal genome sequences is increasing (<http://www.broad.mit.edu/annotation/>, and other sites) and several genome sequences have been annotated and published (e.g. [17, 18]). In addition to encoding enzymes capable of degrading plant cell wall polymers, *Aspergillus* spp. are sources of starch-degrading enzymes [19, 20]. *Aspergillus* spp. are not alone in secreting industrially useful polysaccharide-degrading enzymes and, in particular, *Trichoderma* [21, 22] and *Penicillium* [23, 24] are genera that include species noted for production of commercial levels of cellulases and xylanases [22–25], and *Rhizopus* spp. [20, 26, 27] join *Aspergillus* spp. as important sources of commercial glucoamylase, lipase and pectinases. Plant cell wall polysaccharides are linked through both covalent (e.g. ferulic acid links) and non-covalent bonds (especially hydrogen bonds). Fungal ferulic acid esterases have been summarised elsewhere [15, 28, 29] and another enzyme activity, glucuronyl esterase, may cleave susceptible links between lignin and hemicellulose [30]. Recently, a protein was described from *Trichoderma reesei* that has similarity to plant expansins which have a role in disrupting hydrogen bonds between plant cell wall polymers without itself causing hydrolysis. The fungal protein, which had a cellulose-binding domain at the N-terminus, was named swollenin [31] and it remains to be seen if this and other related proteins will find applications in food processing. Already, a fusion protein of swollenin and feruloyl esterase has been constructed and used [32]. Many of the plant cell wall-degrading enzymes from fungi find a wide variety of applications as aids during the processing

of fruits and vegetables [15]. For example, xylanases are used to improve the quality of bread doughs, in clarifying juices and in the production of xylose and oligomers of xylose used as food additives. Similarly, pectinases are used in juice clarification and for production of monomeric components of pectins that serve as the starting materials for conversion to other products (e.g. galacturonic acid to ascorbic acid). The use of fungal amylases is important in the starch processing industries to provide glucose syrups used in many fermentation processes. As well as being sources of enzymes that degrade plant cell wall polymers, fungi provide many other enzymes used as food processing aids and these include galactosidase, catalase, glucose oxidase, lactase, lipase, phospholipase, phytase and, importantly, proteases where *Aspergillus* and *Rhizopus* spp. provide the bulk of these commercial enzymes.

The search is always on for enzymes with new properties (e.g. in terms of pH optimum, salt tolerance, proteolytic resistance or thermal stability) and for strains that produce enzymes at improved yield or, at least, under production conditions where the cost is lowered (which might include the use of cheaper substrates for growth or improved bioreactor formats). In addition, the availability of fungal genome sequences is providing a resource for the identification of genes that may encode interesting new activities. So, for example, many cellulose-binding and starch-binding domains have sequence similarities that encourage a search of databases for genes encoding familial enzymes. This type of strategy can be taken to levels of greater detail and ingenuity, dictated only by knowledge of key sequence motifs or functional domains in target enzymes (e.g. in the cellulases and xylanases [33–35]) and also by availability and access to fungal genome sequences. The approach is also tempered by the knowledge that sequence is not a guarantor of functionality, therefore it is necessary to clone and express genes of interest so that the encoded enzymes can be characterised. A recent example of this approach is provided by the cloning of the gene encoding a prolidase (a protease) from *Aspergillus nidulans* [36]. The approach of purifying enzyme activities and then resorting to reverse genetics for gene cloning has been the principal recombinant DNA-based strategy alongside classical strain improvement by mutagenesis and screening. Although a flurry of genome sequence data mining is inevitable in the immediate future and mid-term, and likely to be productive, the more classical approaches should not be overlooked. Xylanases have been purified and characterised from several fungi (especially species of *Aspergillus*, *Trichoderma* and *Agaricus*) and, in some cases, the encoding genes have been cloned and aspects of their transcriptional regulation studied [15, 37–41]. In addition to xylanases from *Penicillium funiculosum*, xylanases and their genes have been described from *Penicillium purpurogenum* (see [24] and references therein).

The transcriptional regulation of the main groups of genes encoding hydrolytic enzymes from fungi has been well described [42, 43] and will not be covered in detail again here. The cellulolytic and xylanolytic genes from

fungi are, for example, subject to transcriptional regulation through substrate induction and carbon catabolite repression, and there is interplay between the two systems. The roles of the transcriptional activator XlnR and the carbon catabolite repressor protein CreA, in *Aspergillus* spp. have been described recently in detail [15, 44]. XlnR (thought originally to be a specific regulator of xylanase-encoding genes but now known to regulate other genes including some cellulases) binds to a UAS (upstream activator sequence) with the core consensus GGCTAA whereas CreA binds to a URS (upstream repressing sequence) with the consensus SYGGRG (where S = C or G, Y = C or T, R = A or G). A summary of inducing and repressing mechanisms of fungal cellulases has recently been provided [45] and the expression of ten hemicellulases from *T. reesei* grown in the presence of various di- and monomeric sugars as well as some polymeric carbon sources was also reported [46]. The role of XynR as a regulator of genes encoding xylanases and cellulases in *Aspergillus oryzae* has also recently been reported [41, 47]. We continue to learn more of the mechanisms of both induction and repression of the fungal cellulases. For example, it has recently been shown that phosphorylation of Cre1 is necessary for binding to its recognition site and subsequent action as the mediator of carbon catabolite repression in *T. reesei* [48]. The isolation of putative transcriptional regulators (in addition to the Cre1 repressor) of cellulase-encoding genes in *T. reesei* has recently been successful with the description of two genes *ace1* [49] and *ace2* [50] that encode transcriptional regulators that contribute to the transcriptional regulation of some cellulase- and xylanase-encoding genes. It has been hypothesised that regulation of the xylanase gene *xyn1* in *T. reesei* is orchestrated by an interplay of the Cre1 and Ace1 regulators with Xyr1 (homologue of the *A. niger* XlnR and both regulators have closely related consensus binding motifs) acting as a transactivator [51]. Not all enzymes involved in the complete degradation of cellulose to glucose need be extracellular as demonstrated by the description of an intracellular β -glucosidase in *T. reesei* [52] and prediction of intracellular hydrolases in *A. nidulans* [53]. The induction of genes encoding pectinases and ferulic acid esterases [15] requires the presence of substrate and, in the case of pectinases for example, it appears that D-galacturonic and/or glucuronic acids may be the key components that lead to transcriptional induction via a mechanism yet to be described. Expression profiling of the known pectinase- and ferulic acid esterase-encoding genes in *A. niger* confirmed the importance of D-galacturonic acid (or a derived metabolite) as a general regulator of pectinase gene expression [54]. This study went further and showed that, in addition, subsets of genes encoding pectinolytic activities respond to L-arabinose, L-rhamnose or ferulic acid. Regulation of pentose catabolic pathway genes has been described further recently [55].

The secreted production of cellulases to high yields [22] by *T. reesei* is well known and underpins commercial activities with the system. The cellulases secreted by *T. reesei* are glycosylated and a combined approach of metabolic

labelling and proteomics has enabled a kinetic analysis of the assembly and secretion of these glycoproteins [56]. The heterogeneity and kinetics of secretion of cellobiohydrolase I (Cbh1) with respect to glycosylation during its assembly within the secretory pathway was demonstrated. While it took on average about 4 min to synthesise a Cbh1 glycoprotein, it took about 11 min for its secretion. This type of analysis may prove to be useful in identifying bottlenecks in secreted production of an enzyme and may be particularly useful when applied to heterologous proteins. Global analyses of protein production using proteomic approaches with *T. reesei* are proving useful and use can also be made of cDNA microarrays in global analyses of transcriptional regulation [57]. Studies with *T. reesei* have also shown that O-linked glycosylation of cellulases during the secretory pathway has an impact on secreted yields [58], i.e. affects the passage of glycoproteins.

Fungi (especially *A. niger*, *A. oryzae* and *Rhizopus* spp.) are used to produce proteases for use as processing aids and proteases are important activities in the solid state koji fermentation (see below). As an example, *A. niger* encodes a very large number of different proteases and, to date, these have been mainly studied as purified enzymes or in mutagenised strains that have lost an activity [59–61]. Not all of these proteases are secreted or lend themselves to commercial applications. Even so, removal of a contaminating protease activity may be just as useful, depending on the application, as overproducing a target protease so continued exploration of fungal proteases and, in particular, the regulation of their expression is warranted. Protease-encoding genes are subject to transcriptional regulation in response to nitrogen and carbon sources (reviewed in [59]) and pH is also a potent regulator of proteases and mediated by the PacC regulator [62]. The pH response and regulatory system appears to operate with close similarity in many fungal species (reviewed in [63]) and leads to transcriptional regulation of a wide variety of genes used in food processing, e.g. xylanases and pectinases [15].

Fungi are sources of commercial quantities of enzymes for degradation of starch. These include both *endo*-acting and *exo*-acting amylases, and some with de-branching activity. In order to explain the mechanism of induction (e.g. by starch or maltose) and repression (e.g. by glucose or xylose) of the starch-degrading enzymes, there has been extensive analysis of promoters through deletion studies [64, 65]. Overexpression studies have suggested that the various amylase-encoding genes share common transcriptional regulators and have shown that titration of transcription factors can occur [66–71]. The CreA protein that mediates carbon catabolite repression of cellulases also operates to repress transcription of amylases in fungi [44, 70]. Transcriptional activation is mediated through the AmyR zinc-finger protein [71, 72]. A review summarises the functions of both the XlnR and AmyR transcriptional activators as well as the role of the CCAAT-binding protein complex in enhancing promoter activity of many fungal genes, including the genes encoding cellulose- and starch-degrading enzymes in fungi [73].

2.1.2

Solid State Culture

The growth of microorganisms on surfaces can lead to growth characteristics and products formed that differ from those of the same cultures grown in submerged liquid culture [74, 75]. The capacity of some species of filamentous fungi to secrete degradative enzymes is exploited in solid state fermentations (SSF) for production of fungal biomass (i.e. edible mushrooms) and a variety of foods and beverages [9, 61, 76]. The manufacture of products from fermented soy, rice and other plant materials is widespread in far Eastern countries where species of *Aspergillus* and *Rhizopus* are most commonly exploited to initiate the initial breakdown of complex organic matter [9, 76]. Increasingly, SSF is being used in Europe and the USA for the manufacture of fermented products. SSF is seen as a means of making a range of valuable products in a more cost effective manner as it enables the synthesis of valuable products from substrates which have a low or even a negative value, e.g. waste agricultural products which incur a high cost for either transport and disposal or for conversion into animal feed. The fungi are particularly well-adapted for SSF as they have a high capacity for the secretion of hydrolytic enzymes necessary for the degradation of plant cell walls and storage carbohydrates. Secondary fermentations can then be carried out by yeasts and bacteria, which utilise the partially degraded organic matter produced by the action of fungal enzymes.

Several comparisons have been made between fungal strains grown in SSF and liquid cultures (reviewed in [74, 75]). For example, depending on the substrate used, secretion of specific degradative enzymes such as amylases, phytases and pectinases is commonly higher in SSF than in liquid cultures [77–79]. The differential expression of two glucoamylase-encoding genes by *A. oryzae* is particularly marked with the *glaB* gene being strongly up-regulated in SSF [80, 81]. In addition, SSF-grown *Aspergillus* is reported to be less sensitive to carbon catabolite repression than when grown in liquid culture [82]. The synthesis of non-enzymic metabolites by fungi can also be enhanced in SSF (e.g. [83]).

A recent review of the koji process [9], that employs *A. oryzae* enzymes secreted from the fungus during growth on the solid substrate (e.g. soy or cereals), updates some earlier reviews (e.g. [76]) and provides a clear analysis of the process and the key role of fungal enzymes in degrading the plant materials prior to subsequent fermentation by other microorganisms. The recently published genome sequence of *A. oryzae* [84] offers major promise for further development of *A. oryzae* in solid-substrate fermentations (SSF) [10]. The expectation is that a combination of genome sequence, gene microarrays and proteomic studies will provide the scientific community and commercial users of *A. oryzae* with a major resource for understanding the science of solid state fermentations [85] in relation to the transcription of genes en-

coding enzymes important for the koji process. Analogous facilities will also be available for other key industrial enzyme-producing fungi. The *A. oryzae* genome sequence should aid investigations into the molecular basis of regulatory processes that are specific to solid state cultivation of the fungus. At present, as mentioned previously, we know that solid state cultivation leads to altered patterns of transcription compared to that when the same fungus is grown in submerged culture, and we already have examples of SSF-specific promoters that have been used in the construction of new hybrid promoters [86]. The lower water activity in solid state cultivation may be the trigger to altered transcriptional regulation [9, 10] but we are far from explaining at the molecular level all the differences in morphology and transcriptional regulation in *A. oryzae* grown under the two different cultivation conditions. The solid state system is characterised by low water activity, the presence of surfaces and also gradients in nutrients [74, 75, 87] that combine to present a highly complex system. Unravelling the system will require its physical characterisation alongside the use of genomic approaches. A recent comparison between SSF (on wheat bran) and submerged cultivation (using a wheat bran extract to support growth) of *A. oryzae* [88] used subtractive cloning of genes differentially expressed under the two different regimes. Sequences were compared with the *A. oryzae* EST database as well as other gene databases in order to identify putative genes that were specifically transcribed or repressed in SSF compared to submerged culture. The database generated by this approach will require further analysis but it is clear that SSF leads to the specific expression of a large number of genes. *A. oryzae* has a further attraction for being the target of detailed genomic and proteomic studies [10]. Not only is it a species used widely in solid state fermentations but it is also very closely related to other food-use fermentative species (*Aspergillus sojae*) and aflatoxigenic species (*Aspergillus flavus* and *Aspergillus parasiticus*) [89–91]. This is not to suggest that koji moulds are likely to synthesise aflatoxins because they do not, and there are known gene-based reasons underlying the lack of toxin synthesis by *A. sojae* [92] and *A. oryzae* [93]. But, as a group of species that are important for both their enzymes and their capacity for secondary metabolite production, the genomic and proteomic approaches now available present us with unparalleled opportunities for detailed mechanistic studies.

2.2

Heterologous Proteins Expressed by Filamentous Fungi

Fungal species that have been used successfully as cell factories for commercial enzymes that are used in food manufacture appear to be attractive hosts for production of any protein, whether it be encoded by a native or heterologous gene. The fungi lend themselves to commercial-scale operations (i.e. the fungi are industrially friendly and produce profitable yields of target

enzymes) and produce enzymes in formulations that have a history of safe use. Most of the enzymes produced in this way are secreted so there is no recourse to mycelial breakage and, for many formulations, the mycelium is simply removed from the culture to leave a saleable product. It has not, however, been so straightforward to develop fungi as hosts for the production of heterologous enzymes on the commercial scale. This chapter is concerned with fungal products used in foods so, in the case of enzymes, we are dealing primarily with a bulk process where there is no commercial sense in secreting a poor yield of a target protein (unlike in some clinical applications where a high price for the enzyme might mitigate against a low yield). In the main, and despite some success stories, e.g. chymosin from *Aspergillus awamori* [94] and lactoferrin from *A. oryzae* [95], heterologous protein production from fungi has led to strains that secrete far lower (often 1–3 orders of magnitude) levels of heterologous enzymes than can be achieved with native enzymes. The reasons underlying this lack of success have been evaluated in detail in recent reviews [1, 7, 8] so will only be summarised here.

The first major success story was the secreted production of chymosin from *A. awamori* which has been summarised [94]. The approaches used during this work to produce a production strain have been adopted in most of the subsequent, and less successful, attempts to produce heterologous proteins at high secreted yields. The key elements to the success with chymosin were the use of a strong native fungal promoter (from the glucoamylase gene, *glaA*) driving the expression of a translational fusion of the pro-chymosin gene with the gene encoding glucoamylase (as a carrier protein), deletion of the gene encoding the major aspartyl protease (aspergillopepsin, *pepA*) and then rounds of mutagenesis and screening (including selection for resistance to deoxyglucose). Pro-chymosin, being a protease, was able to cleave the glucoamylase-pro-chymosin fusion protein post-secretion to release mature chymosin. This, “fusion strategy” has been widely adopted and modified somewhat (summarised in [1, 5–7]) for use with target proteins that are not proteases. In those cases, an endoproteolytic dibasic amino acid cleavage site that is recognised by the intracellular KEX2-like protease [96, 97] is incorporated during construction of the expression construct at the fusion site between the coding regions for the carrier protein and target protein – incorporated, for example, in the high level expression of lactoferrin from *A. oryzae* [98]. The dibasic amino acid cleavage sites are generally, but not always [99, 100] cleaved faithfully during secretion to release the target protein. As many fungal species are excellent protease producers, it is not surprising that deletion of protease activity, either by mutagenesis or targeted gene disruption, can lead to improvements in the yields of protease-sensitive proteins. The use of a strong fungal promoter and optimised gene copies (this does not necessarily mean highest number [66]) has also been adopted quite generally as a strategy, although there have been few attempts to tailor promoters for particular uses. One recent and welcome addition to the list of

available promoters has been the construction of a strain of *A. niger* able to use the *A. nidulans alcA* promoter [101], a strong and tightly regulated promoter which is increasingly well understood in terms of the mechanism of transcriptional regulation [102]. With the exception of fungal genes expressed “heterologously” in a related fungal species, it is sobering to note that there has been no significant advance that has led to the reliable secreted production at high yield of all heterologous proteins in fungi. Some target proteins are more easily expressed and secreted than others, so the nature of the protein itself is important. There are issues relating to codon usage and mRNA stability that are important for the expression of some heterologous proteins but it appears that many problems are post-translational and most probably reside in the secretory pathway [1, 5–7]. Therefore, in those cases, it will be necessary to understand the very nature of what makes the target protein appear as foreign to the fungal secretory system before strategies to overcome the difficulties can be devised. It may therefore be necessary to tailor the fungus to the particular target protein although it is hoped that new understanding will be generally applicable.

Most current studies aimed at improving the secreted yield of heterologous proteins from fungi are with target proteins that are not destined for food use. It is likely though that knowledge gained in those systems will be advantageous in the production of food-use proteins. The secretion process itself, involving protein folding, glycosylation and vesicular transport to the hyphal tip, is a major target of current research effort [5, 7]. Accordingly, the morphology of the producer strain is likely to be a key issue in optimising secreted yields of proteins, especially under production conditions where physical properties of a mycelium are important. Folding of secretory proteins takes place within the lumen of the endoplasmic reticulum (ER) and is assisted by foldases and chaperones that are resident in the lumen. Activation of the unfolded protein response (UPR) [103, 104] by heterologous proteins indicates that the luminal environment is not conducive to folding that particular target protein with the kinetics expected by the fungus (i.e. compared to its native proteins). The UPR leads to the transcriptional up-regulation of a wide range of genes (e.g. in *Saccharomyces cerevisiae* [105], *T. reesei* [106] and *A. niger* [107]), some of which encode chaperones and foldases that are resident in the lumen of the endoplasmic reticulum, i.e. a stress response mechanism within the cell to overcome a detected difficulty in protein folding. The UPR also leads to proteolytic degradation (via the proteasome) of poorly folding proteins within the ER: this is termed ERAD (endoplasmic reticulum associated protein degradation) [108]. Current knowledge on the detail of the UPR in filamentous fungi has recently been summarised [5, 7]. An issue relevant to the discussion in this chapter is whether, and how, knowledge of the UPR has helped to improve the folding of heterologous proteins in the ER. One strategy, for example, is to deliberately induce the UPR in the expectation

of a folding problem when expressing a heterologous protein. General up-regulation of the UPR through overexpression of the mediating transcription factor Hac showed some promise with some proteins [109] and there have been various examinations of altered expression of individual chaperones or foldases [110–113]. This approach has not generally been as useful as anticipated although there are some indications that it can work for some chaperone (or foldase)/heterologous protein combinations. Few such studies have been with food-use proteins, a notable exception being the expression of the sweet protein thaumatin from *A. awamori* [114]. Thaumatin is a 22 kDa plant-derived protein that contains eight disulphide bonds and can be expressed in *A. awamori* (a close relative of *A. niger*). Co-expression of additional copies of *pdiA* (encoding protein disulphide isomerase) was studied and an optimal level of PdiA protein (between two and four-fold compared to the natural level) was shown to improve the secreted yield of thaumatin by five-fold whereas the secreted levels of two native proteins were unaffected [114]. In contrast, lysozyme is a smaller (ca. 14 kDa) protein containing four disulphide bonds but co-expression of additional *pdiA* gene copies did not affect secreted lysozyme levels [110]. Lysozyme is an interesting target protein for food applications as an antimicrobial [115–117] and, like the antimicrobial lactoferrin [95, 118, 119] has been successfully produced from *Aspergillus* spp. [120, 121]. In both cases, the structures of recombinant lysozyme [120] and lactoferrin [122] have been shown to be authentic. In an interesting aside, variant lysozymes have also been secreted from *A. niger* and there has been a recent focus on variant forms of lysozyme that form amyloids that are associated with disease and death in humans [123]. Thus, the production system for variant forms of lysozyme [124, 125] underpins studies of the key protein folding events that lead to a variety of amyloid diseases [126].

3

Organic Acids

Filamentous fungi produce a number of organic acids that are used as additives in the food industry (Table 2) and [127, 128]. Although the basic biochemical pathways for their synthesis are known, the mechanisms by which overproduction is achieved in industrial applications are still under investigation in many cases. Acidogenesis is considered to be one energy-efficient way of dealing with excess carbon when other nutrients, particularly nitrogen, are limiting [129]. Another strategy is to divert excess carbon to the biosynthesis of fatty acids and storage lipids [130]. Several fungi are rich sources of fatty acids (Table 2), especially organisms that produce high yields of triacylglycerol (TAG) oil, the so-called oleaginous fungi [131, 132]. In this section, production of organic acids will be reviewed and recent genetic and molecu-

Table 2 Organic acids, including fatty acids, produced by filamentous fungi for use, or with potential use, as food additives

Product	Use(s)	Fungal source
Citric acid	pH regulator; acidulant; acid flavour; preservative; lipid antioxidant; emulsifier	<i>A. niger</i>
Gluconic acid (derivatives and δ -gluconolactone)	Chelating agent (for mineral enrichment); slow-acting acidulant; preservative	<i>A. niger</i> ; <i>Penicillium</i> spp.
L-Malic acid	Acidulant; preservative; “smooth” acid flavour	<i>Schizophyllum commune</i> ; <i>Paecilomyces varioti</i>
Lactic acid	Acidulant; preservative	<i>Rhizopus oryzae</i>
Fumaric acid ^a	Acidulant; long-lasting “strong” acid flavour	<i>Rhizopus</i> spp.
Succinic acid ^a	Flavouring agent	<i>Rhizopus</i> spp. (mixed fermentation with bacteria)
Tartaric acid ^a	Flavouring agent	<i>Aspergillus</i> spp.; <i>Penicillium notatum</i>
iso-Ascorbic acid ^a	Preservative	<i>Aspergillus</i> spp.; <i>Torula</i> spp.; <i>Penicillium</i> spp.; <i>Fusarium</i> spp.
Kojic acid ^b	Antibrowning agent; antioxidant; preservative	<i>A. oryzae</i> ; <i>A. sojae</i> ; <i>Penicillium</i> spp.
γ -Linolenic acid ^{c,d}	Nutritional supplement	<i>Mucor circinelloides</i> ; <i>Mortierella isabellina</i>
Arachidonic acid ^{c,e}	Baby milk supplement	<i>Mortierella alpina</i>

^a Produced primarily by chemical synthesis

^b Use in foods now banned because of mycotoxin properties

^c Produced as a fatty acid component of triacylglycerol oil

^d Produced now from plants

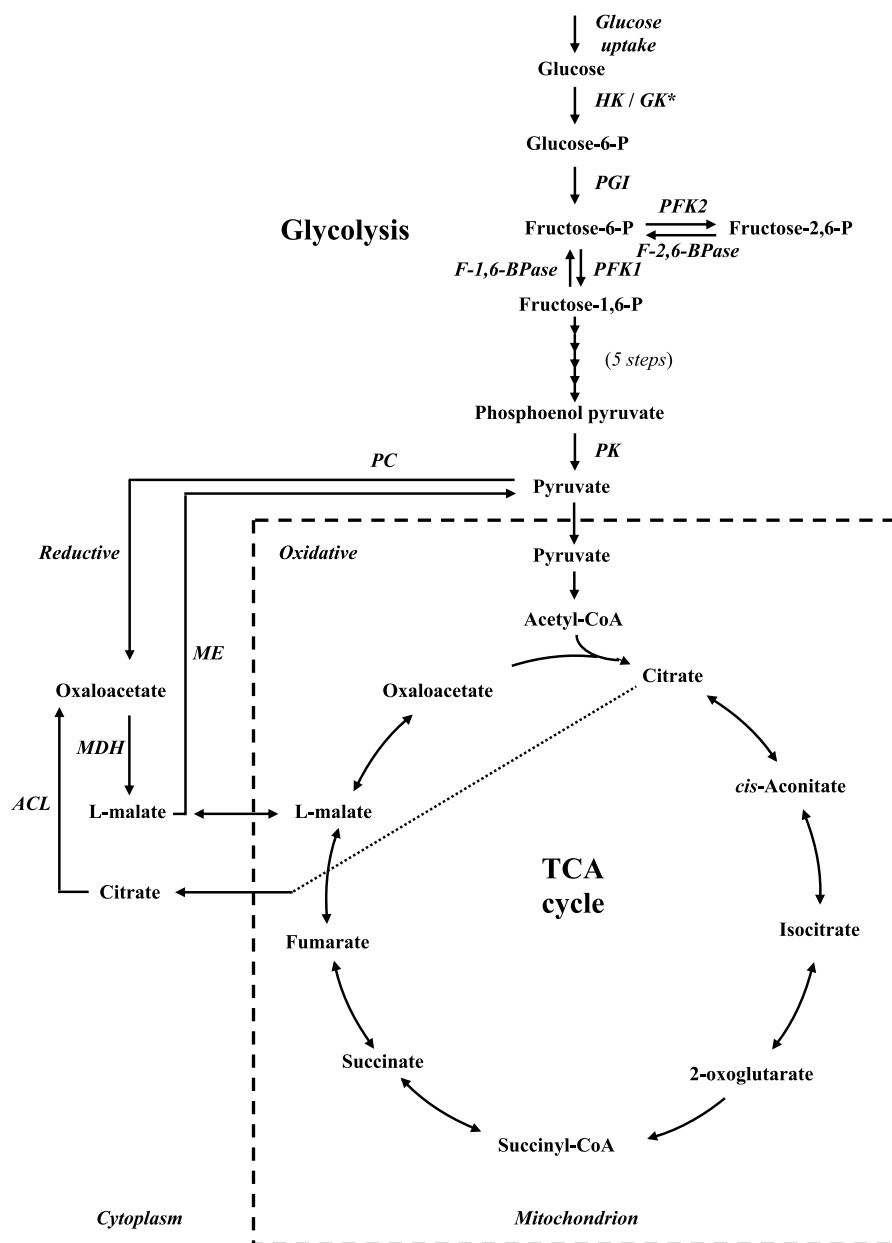
^e Blended with an algal docosahexaenoic-rich oil

lar biological approaches to understand and manipulate these pathways will be discussed where applicable.

3.1

Citric Acid

Many of the organic acids excreted by filamentous fungi are intermediates of the tricarboxylic acid (TCA) cycle (Fig. 1). Their efflux into the growth medium is considered an “energy-spilling” phenomenon which avoids the synthesis of excess NADH from TCA cycling. Also termed “overflow metabolism”, the fungi in question are able to uncouple catabolic and anabolic processes in order to maintain a high flux of carbon when the car-



bon source is in excess and some other factor is limiting [133]. Organic acid excretion can reduce the pH of the culture medium to below 2.0 which is tolerated by these fungi because of their extremely efficient intracellular pH-homeostatic system [134]. Citric acid, by far the most important econom-

- ◀ **Fig. 1** Schematic diagram of glycolysis, the tricarboxylic acid (TCA) cycle and associated pathways showing the biosynthesis of many of the important organic acids excreted by fungi (modified from [127, 130, 154]). The following glycolytic and associated enzymes are abbreviated: *HK*, hexokinase; *GK*, glucokinase (* less active during citrate production [151]); *PGI*, phosphoglucose isomerase; *PFK1*, 6-phosphofructo-1-kinase; *PFK2*, 6-phosphofructo-2-kinase; *F-1,6-BPase*, fructose-1,6-bisphosphatase; *F-2,6-BPase*, fructose-2,6-bisphosphatase; *PK*, pyruvate kinase; *PC*, pyruvate carboxylase; *MDH*, malate dehydrogenase; *ME*, malic enzyme (malate decarboxylase); *ACL*, ATP:citrate lyase

ically, is used as a pH regulator and acidulant that imparts acid flavour in a wide variety of foods ranging from carbonated soft drinks to processed meats [127]. The chief commercial source of citric acid used in the food industry is *A. niger*, accounting for an estimated 0.4 million tonnes worldwide worth £0.5 billion in 2001 [135–137], with demand continuing to increase by about 5% annually. This acid has GRAS status, being regarded as an extremely safe food additive, and is used as the standard against which other acidulants are measured in food formulations. The favoured production method is large-scale submerged fermentation using pure sugar or molasses as the carbon source [138]. In the past, a small proportion was made in Japan by solid state koji fermentation on substrates such as wheat bran or sweet potato waste pulp [139, 140] but this method is now uneconomic. Improvements in citric acid yield by *A. niger* have been achieved by both strain development, using standard mutagenesis and selection, and optimising culture conditions [127, 141, 142]. Metabolic engineering to improve strains by recombinant DNA technology is less advanced but offers future rewards once the key enzymes that regulate flux through the pathway have been identified [128].

The attainment of high citric acid yields in industrial fermentations is associated with a number of interacting nutritional and physiological factors, including high sugar and oxygen concentrations, low pH and deficiency in manganese and iron ions [127, 135, 143]. Of these parameters, sugar concentration is thought to be one of the most important because of the induction of a low-affinity glucose transporter and the subsequent phosphorylation of glucose by hexokinase, two significant flux control points at the start of the glycolytic pathway [143] and (Fig. 1). A number of biochemical studies on the control of citric acid production have identified regulatory roles for phosphofructokinase I, pyruvate kinase, pyruvate carboxylase, citrate synthase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase [127, 129]. During overproduction of citric acid, it was believed that the TCA cycle was interrupted at one or more steps after citrate biosynthesis but this is in dispute [129, 144]. However, several of the TCA cycle enzymes are subject to tight regulation, one example being inhibition of the irreversible 2-oxoglutarate dehydrogenase step. A recent investigation of this reaction by flux analysis has implicated a 4-aminobutyrate (GABA) shunt moving carbon from glutamate to succinate rather than via 2-oxoglutarate [145]. During acidogenesis, GABA

levels increased by about four-fold and flux from glutamate to succinate was greatly reduced. This therefore represents another flux control point at which genetic modification could elevate citric acid yields. If the fermentation process is not strictly controlled during citric acid biosynthesis, oxalic acid may occur as an unwanted, toxic by-product from the breakdown of oxaloacetate. A mutant strain of *A. niger* has been isolated that is defective in both glucose oxidase and oxaloacetate acetylhydrolase (OAH), the latter being responsible for oxalate accumulation, and interestingly, this mutant produced citric acid in a medium with a much higher pH and manganese ion concentration than normal [146]. The basis for this phenomenon has yet to be established but it confirms that the regulation of citric acid production can be modified. As already discussed, industrial citric acid-producing strains of *A. niger* have undergone several rounds of mutagenesis and many are defective in OAH activity, making them oxalate-negative strains.

To date, molecular genetic strategies to increase overall yields of citric acid or productivity rates have proved largely unsuccessful. The gene encoding citrate synthase (*citA*), which forms citrate from oxaloacetate and acetyl-CoA and which in the past has been considered a key step in the citric acid cycle, has been cloned from *A. niger* and overexpressed in this fungus with no apparent effect on citrate accumulation or on the levels of any other TCA cycle intermediate [147], which may or may not have been foreseen [148]. Similarly, overexpression of the *A. niger* phosphofructokinase I (*pfkA*) and pyruvate kinase (*pkA*) genes, either individually or together, did not increase the rate of citric acid production suggesting that these enzymes also do not contribute significantly to regulating flux through the pathway [149]. One genetic modification in *A. niger* that did have a beneficial effect on citric acid synthesis was the disruption of one of the two differentially regulated trehalose-6-phosphate synthase genes (*tpsA*, otherwise designated *ggsA*) whose metabolic product, trehalose-6-phosphate, was shown to inhibit hexokinase activity, albeit to differing degrees in vitro [150, 151], and therefore to have a role in regulating glycolytic flux in this organism. Increased initial rates of citric acid formation were only evident in the *tpsA* disruptant when sugar concentrations in the medium were $\geq 5\%$ (w/v), which would have resulted in sufficiently inhibitory cellular concentrations of trehalose-6-phosphate in the wild-type strain [150]. The final yield of citric acid produced was however unchanged. Interestingly, multicopy *hxA* transformants of *A. niger* have been created that produced 50 times the wild-type level of hexokinase but no data have yet been presented on citric acid biosynthesis in these strains [151]. *A. niger* possesses a second respiratory pathway that is catalysed by the alternative oxidase (AOX) in addition to the normal cytochrome pathway and this has been implicated in citric acid biosynthesis as a way of continuing glycolysis during reduced TCA cycle activity [129, 152]. The gene encoding AOX, *aox1*, has been cloned [153] but its regulation during citrate production is still under study. Mathematical modelling has recently highlighted three main

flux control points for citric acid production, viz. sugar uptake into the cell, mitochondrial transport of pyruvate and citrate and citrate excretion from the cell, which correlates well with current experimental evidence [154]. In order to optimise citric acid productivity, this study indicated that the levels of at least 13 biosynthetic enzymes and transport proteins would have to be modified for a significant increase in yield to be achieved.

3.2

Gluconic, Malic and Kojic Acids

Gluconic acid is added to food as a slow-acting acidulant but only to a limited extent and even then mainly as the biosynthetic precursor, δ -gluconolactone which is used in combination with sodium bicarbonate to control the leavening of baked products. Because of its effective chelating properties, gluconic acid and its derivatives are being increasingly used to enrich foods with minerals such as calcium or iron but its main, non-food uses are in cleaning applications and concrete admixtures. A large proportion of the approximately 45 000 tonnes of gluconic acid used annually worldwide [155] is produced by submerged fermentation using *A. niger* [127, 141] or more recently by an in vitro process utilising concentrated glucose solutions and glucose oxidase and catalase enzymes isolated from *A. niger* [156]. Biosynthesis of δ -gluconolactone from glucose is catalysed extracellularly at pH values >3.5 by glucose oxidase and subsequent gluconolactonase-mediated hydrolysis of this intermediate yields gluconic acid [157] which can itself act as a carbon source for the fungus [158]. Increased biosynthetic yields of gluconic acid have been achieved mainly by optimising fermentation parameters and by strain improvement in a similar fashion to citric acid production [127, 141, 159–162]. In both approaches, this is mainly achieved by increasing glucose oxidase levels. Gluconic acid can also be synthesised chemically by the direct electrochemical oxidation of glucose.

The use of malic acid as an alternative acidulant in food and drink has gained importance over the last 10–20 years with the increasing popularity of artificial sweeteners such as saccharin and aspartame. Malic acid provides a slower-acting acid taste compared with citric acid. When present either on its own or in combination with citric acid, it can impart a smoother, more palatable flavour without the aftertaste associated with the sweetener and can also reduce the amount of sweetener required [163, 164]. Although malic acid for the food industry is primarily made by chemical means, resulting in a DL-racemic mixture, fungal fermentation giving L-malic acid remains a possibility, especially with strains of *Schizophyllum commune* and *Paecilomyces varioti* which can produce relatively high yields of this TCA cycle intermediate [127]. Lactic and fumaric acids are two other organic acids used as food acidulants with different taste characteristics which can be excreted by fungi such as *Rhizopus* spp. [127, 165] but both are obtained from other sources at present.

Kojic acid (5-hydroxy-2-hydroxymethyl- γ -pyrone) is produced by a number of fungi, particularly those of the *A. flavus*–*A. oryzae* group [166, 167]. This secondary metabolite has been associated in the past with several traditional Japanese koji-fermented foods and food products, such as *miso*, *shoyu* (soy sauce), *sake*, *amazake*, *shouchu* and *mirin*, but most *Aspergillus* strains used in koji have now been selected for their inability to produce this acid because of its mycotoxin properties [168]. Until recently, kojic acid was also added to a variety of processed foods as an antioxidant or antibrowning agent [169] and was promoted as a component of health foods. Although it has a long history of human consumption with no apparent medical problems, kojic acid is now considered to be a mycotoxin and recent evidence from animal studies has implicated it in the induction of thyroid cancers by affecting thyroid function [170]. The effect on thyroid function is reversible and is only associated with continuous high doses of kojic acid. Because of this concern, a number of foods have been screened for the presence of this metabolite and most proven to be negative [168, 171]. In addition, kojic acid has been shown to be approximately 90% inactivated by cooking [172]. Nevertheless, its use in foods has now been stopped even although it is not thought to present a major problem for human health [166]. The biosynthetic pathway is not understood completely, perhaps involving three separate routes, but the primary pathway is thought to be direct synthesis from glucose via reduction to gluconolactone and oxidation to 3-ketogluconic acid lactone. The latter then undergoes two dehydration steps resulting in the loss of two water molecules and the reduction of one double bond to form kojic acid. The genes encoding the kojic acid biosynthetic enzymes have yet to be identified. Recent studies have concentrated on increasing kojic acid yields from *A. oryzae* and *A. flavus* for non-food applications (e.g. in cosmetics, insecticides and biodegradable polymers). The approach has been to optimise fermentation parameters [173, 174] or improve strains by chemical mutagenesis [175].

3.3

Polyunsaturated Fatty Acids

In human nutrition, two polyunsaturated fatty acids (PUFAs), linoleic acid ($18:2n-6$) and α -linolenic acid ($18:3n-3$), are classed as essential fatty acids and are sometimes referred to as vitamin F, or the vitamin F group when some of their PUFA derivatives are included [176, 177]. Their absolute requirement for a healthy diet stems from the lack of fatty acid $\Delta 12$ -desaturase activity in humans (Fig. 2). Some filamentous fungi are used to produce fatty acids, particularly long-chain PUFAs [130, 179–181] that have importance both nutritionally and pharmacologically to humans. Long-chain PUFAs, arachidonic acid (ARA, $20:4n-6$) and docosahexaenoic acid (DHA, $22:6n-3$), are components of human milk and are incorporated into mem-

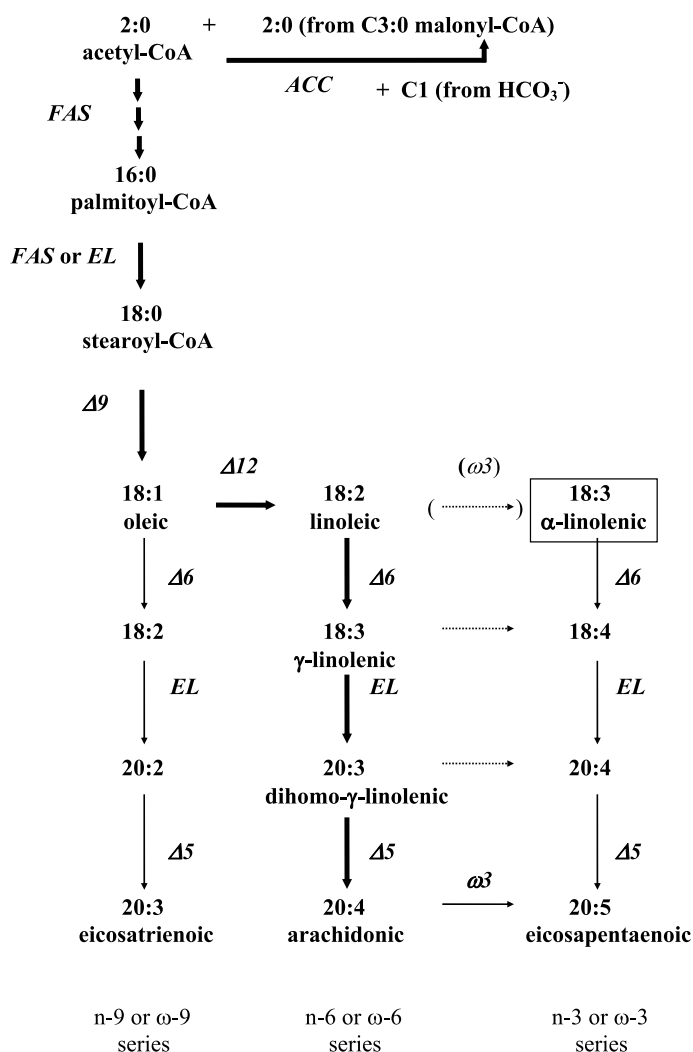


Fig. 2 PUFA biosynthetic pathway in *Mt. alpina*. Fatty acid biosynthetic pathway from acetyl-CoA (2 : 0) to polyunsaturated EPA (20 : 5n - 3), showing reactions catalysed by acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), fatty acid elongase (EL) and fatty acid desaturases (Δx or ωx). The major pathway of ARA biosynthesis is indicated by bold arrows whereas side reactions are shown by thinner arrows. Desaturation of ARA to EPA by $\omega 3$ -desaturase is stimulated by growth at low temperature. The $\omega 3$ -desaturation of 18 : 2n - 6 to 18 : 3n - 3 is bracketed as it occurs only at very low levels. The pathway for desaturation and elongation of exogenously supplied α -linolenic acid (boxed) to EPA is also shown. Pathway modified from [130, 131, 178]

branes of nerve cells and brain tissue. They are required for normal development and functioning of the central nervous system (CNS), especially in neonatal infants [182, 183], and are now added to a number of baby milk formulations. An ARA-rich oil supplement (ARASCO®) produced from the oleaginous fungus, *Mortierella alpina*, received GRAS approval in 2001 from the US Food and Drug Administration (FDA) for this purpose ([184] and <http://www.martekbio.com>). Oil from this fungus has been tested extensively for negative side effects and has been passed as safe [185–188]. FDA approval was also given to a DHA-rich oil (DHASCO®) [184] made by a heterotrophic marine alga, *Cryptocodinium cohnii* [189]. The ARA- and DHA-containing oils are blended together before addition to a range of baby milks. DHA is also produced by a group of marine microorganisms known as thraustochytrids that were once thought to be distantly related to filamentous fungi but which have now been reclassified [190, 191]. Oil from these organisms is not yet used in baby milk formulations but the whole cells are added to poultry feed to produce “ ω -3 enriched” eggs and to farmed fish feed [130]. Besides its role in CNS development and function, ARA, along with other long-chain PUFAs, γ -linolenic acid (GLA, 18 : 3 n – 6), dihomo- γ -linolenic acid (20 : 3 n – 6) and eicosapentaenoic acid (EPA, 20 : 5 n – 3), act as precursors to eicosanoid hormones (prostaglandins, leukotrienes and thromboxanes), some of which play an important role in combating or preventing a number of human diseases [192]. Interestingly, some species belonging to the Zygomycota, including *Mortierella* spp. and *Cunninghamella elegans*, also appear to synthesise some of these human hormones [193].

A GLA-rich oil was produced commercially by *Mucor circinelloides* as a human nutritional supplement but this has now been replaced by more competitively priced plant-derived products, evening primrose and borage oils, even although the fungal oil appeared to be a “healthier” product in terms of higher oleic acid (18 : 1 n – 9) and lower linoleic acid contents [130, 131]. As in most fungal biotechnological processes, PUFA production is carried out by submerged fermentation and many studies have reported improvements in oil yield and alterations in fatty acid composition by optimising and modifying culture parameters such as carbon source, nitrogen source, growth temperature, mineral concentrations and dissolved oxygen [194–203]. The production of PUFAs by fungi growing on solid substrates [204, 205] or immobilised in fluidised-bed fermenters [206] has also been described.

A simplified pathway for PUFA biosynthesis is outlined in Fig. 2. Typically, fungi synthesise saturated fatty acids with chain lengths of 16 and 18 carbon atoms up to a chain length of at least 26 carbon atoms, the latter albeit at very low levels, but most produce unsaturated fatty acids only up to 18 carbon atoms in length (18 : 2 n – 6 and traces of 18 : 3 n – 3) [132]. Several fungi make longer-chained, more unsaturated fatty acids up to 20 carbon atoms in length with up to five double bonds [131, 203, 207–209].

In most cases, these PUFAs are components of TAGs and some fungi can synthesise as much as 50% of their biomass as oil. A proportion of PUFAs are also components of membrane phospholipids and of more “exotic” lipids such as sphingolipids and cerebrosides that play important roles in membrane function [210]. Fatty acid synthesis is initiated by the formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACC) which is considered to be an important flux control point for fatty acid and lipid biosynthesis in many organisms [211, 212]. Subsequent elongations of the acyl moiety, each by two carbon units at a time, is carried out by the multi-functional fatty acid synthase (FAS) until palmitic (16 : 0) or stearic (18 : 0) acid is produced. At this stage the fatty acid is present in the fungal cytoplasm as the acyl-CoA ester which acts as the substrate for the ER-membrane bound fatty acid $\Delta 9$ -desaturase. Desaturation occurs on the cytoplasmic face of the ER membrane to produce primarily oleoyl-CoA (18 : 1 n - 9) in most organisms although palmitoyl-CoA (16 : 1 n - 7) may predominate in a few cases. The fatty acid then becomes incorporated into membrane phospholipids by the action of acyltransferases such as lysophosphatidylcholine:acyl-CoA acyltransferase (LPCAT) or glycerol-3-phosphate acyltransferase (GPAT) whose specificity determines at which position on the glycerol backbone the acyl group will be transferred [213, 214]. All subsequent desaturations indicated in Fig. 2 occur using the acyl moieties of phospholipids as substrates [213]. In relation to the elongation step prior to $\Delta 5$ -desaturation, the acyl group must first be removed from the phospholipid molecule by an as yet unknown mechanism, but most likely involving the formation of an acyl-CoA ester, before elongation can proceed [130]. Recent evidence has indicated that this elongation step is important in regulating the overall rate of ARA biosynthesis in *Mt. alpina* [215]. As stated above, there are at least two possible biosynthetic routes for the transfer of PUFAs to the final TAG storage oil product, with the specificity of each acyltransferase determining the complexity of the mix of TAGs formed. Phospholipids can be converted to diacylglycerols (DAGs), which are then used as substrates by acyl-CoA:diacylglycerol acyltransferase (DGAT) to form TAGs. Alternatively, de novo synthesis of DAGs by the Kennedy pathway starts with the synthesis of lysophosphatidic acid from glycerol-3-phosphate by GPAT, followed by the formation of phosphatidic acid by another acyltransferase, lysophosphatidic acid acyltransferase. Finally, phosphatidic acid is converted to DAG by the action of phosphatidic acid phosphohydrolase, summarised in [130, 180].

In oleaginous fungi, the enzymes considered to be key in determining high lipid yields are ACC, ATP:citrate lyase (ACL) and malic enzyme (ME) [130, 216–218] and (Fig. 1). A “lipogenic metabolon” has been proposed where several of the enzymes involved in lipogenesis are intimately associated to effect tighter channeling of particular substrates [130]. In this model, ME is thought of as being the most important enzyme in supplying the reducing

power of NADPH for fatty acid synthesis by FAS. To date, there is no direct evidence for these protein–protein interactions, which might be quite weak but the yeast two-hybrid system might be a useful tool to test the metabolon hypothesis [219]. Nevertheless, overexpressing either the *Mc. circinelloides* or *Mt. alpina* genes that encode the fatty acid biosynthetic ME isoform in *Mc. circinelloides* resulted in a 150% increase in lipid accumulation, which strongly suggests that such an interaction occurs in vivo [220].

Initially, much of the PUFA biosynthetic pathway was elucidated from the isolation of mutant strains defective in a particular step and by the use of fatty acid desaturase inhibitors ([221] and Table 3). Some of the mutant strains have indeed proved useful in producing fungal oils with modified PUFA compositions, particularly some double or triple mutants. One drawback, however, with the use of mutants is that many are “leaky” in nature when the block in the pathway may not always be 100% effective. In cases where more defined blocks in the pathway or the overproduction of a particular enzyme activity are required, manipulation of the gene in question is more desirable.

The ultimate aim in most biotechnological applications is to improve productivity through a combination of metabolic engineering and strain improvement by mutagenesis and screening. With this in mind, much effort has been spent in isolating and characterising as many fungal genes as possible whose gene products act in the PUFA biosynthetic pathway and whose manipulation could lead to increased oil yields or modified TAG fatty acid composition for particular applications. Most of these genes are listed in Table 4, although not all are from oleaginous fungi and some, including those encoding LPCAT and other acyltransferases with distinct specificities, await isolation. The ACC-catalysed synthesis of malonyl-CoA has been manipulated in some organisms, including plants, to improve overall fatty acid and lipid yields [264]. To date, although the gene encoding ACC has been cloned from *A. nidulans* and its expression analysed [238], little if anything has been done to enhance lipid accumulation by manipulating ACC gene levels in fungi. All the fatty acid desaturase genes isolated so far from fungi encode proteins with similar characteristics to the membrane bound enzymes from other eukaryotes [265, 266]. Their enzymic activities have been confirmed in vivo by introducing each gene concerned into *S. cerevisiae* or *A. oryzae*, sometimes previously transformed with fatty acid desaturase genes acting earlier in the PUFA biosynthetic pathway or supplemented with the appropriate fatty acid substrate. Similarly, the in vivo function of the GLA elongase condensing subunit (GLELO) from *Mt. alpina* was confirmed in yeast transformants that were supplemented with GLA [261]. The ability to specifically redesign fatty acid desaturases to alter their substrate specificities and desaturation sites [267, 268] increases the feasibility of creating novel PUFAs, which may not occur naturally. Moreover, the bifunctionality of some fungal fatty acid

Table 3 Altering fatty acid composition in vivo using chemical inhibitors or genetic mutations in *Mt. alpina*

Treatment	Fatty acid desaturase(s) or other enzyme affected ^a	PUFA composition ^b	Refs.
Mut T4 ^c	$\Delta 9$	18 : 0 $\uparrow$; all PUFAs $\downarrow$	[222]
Mut48 ^c	$\Delta 12$	18 : 1 $n - 9 \uparrow$; 20 : 3 $n - 9 \uparrow$; all $n - 6$ and $n - 3$ PUFAs $\downarrow$	[223]
	$\Delta 12$	20 : 5 $n - 3^d \uparrow$	[224]
Mut M226-9 ^c	$\Delta 12$ and $\Delta 5$	20 : 4 $n - 3^d \uparrow$	[225]
	$\Delta 12$ and $\Delta 5$	20 : 2 $n - 9 \uparrow$	[226]
Mut M209-7 ^c	$\Delta 12$; $\Delta 6$ (enhanced)	20 : 3 $n - 9 \uparrow$	[227]
Mut JT-180 ^c	$\Delta 12$; $\Delta 5$ and $\Delta 6$ (both enhanced)	20 : 3 $n - 9 \uparrow$	[228]
Mut49 ^c	$\Delta 6$	18 : 2 $n - 6 \uparrow$; 20 : 3 $n - 6 \downarrow$; 20 : 4 $n - 6 \downarrow$	[223]
	$\Delta 6$	20 : 3($\Delta 5$) $n - 6^e \uparrow$; 20 : 4($\Delta 5$) $n - 3^f \uparrow$	[229]
Mut44 ^c	$\Delta 5$	20 : 3 $n - 6 \uparrow$; 20 : 4 $n - 6 \downarrow$	[223]
Mut S14 ^c	$\Delta 5$	20 : 3 $n - 6 \uparrow$; 20 : 4 $n - 6 \downarrow$	[202]
	$\Delta 5$	20 : 4 $n - 3^d \uparrow$	[178]
Sesame non-oil fraction (sesamin)	$\Delta 5$	20 : 3 $n - 6 \uparrow$; (+/- 20 : 4 $n - 6 \downarrow$)	[188, 230]
	LPCAT ^g	20 : 3 $n - 6 \uparrow$ at <i>sn</i> -2 of <i>sn</i> -PtdCho ^h	[231]
Sesamin and episesamin	$\Delta 5$	20 : 3 $n - 6 \uparrow$; 20 : 4 $n - 6 \downarrow$	[232]
Curcumin	$\Delta 5$ (and $\Delta 6$)	20 : 3 $n - 6 \uparrow$	[233]
Alkyl gallate	$\Delta 5$ and $\Delta 6$	18 : 1 $n - 9 \uparrow$; 18 : 2 $n - 6 \uparrow$; 20 : 4 $n - 6 \downarrow$	[234]
Mut K1 ^c	$\Delta 5$ and $\omega 3$	All $n - 3$ PUFAs $\downarrow$	[235]
Aspirin	Cyclooxygenases	20 : 4 $n - 6 \uparrow$	[236, 237]

^a Unless stated, all activities were reduced or completely inhibited^b Major fatty acid levels showing an increase ($\uparrow$) or decrease ($\downarrow$), primarily in the TAG lipid fraction^c *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-derived mutant^d Grown with 18 : 3 $n - 3$ (linseed oil)^e Only when grown at 12–28 °C^f Only when grown at <24 °C or when supplemented with 18 : 3 $n - 3$ or 20 : 3 $n - 3$ ^g Lysophosphatidylcholine : acyl-CoA acyltransferase^h *sn*-Phosphatidylcholine

Table 4 Cloned filamentous fungal genes required for fatty acid biosynthesis and lipid accumulation

Gene	Encoded enzyme activity	Accession number(s) and references
<i>accA</i>	Acetyl-CoA carboxylase	Y15996 [238]
<i>acl1</i>	ATP:citrate lyase subunit 1	AJ243817 [239]
<i>acl2</i>	ATP:citrate lyase subunit 2	AJ243817 [239]
<i>fasA</i>	Fatty acid synthase α subunit	U75347 [240]
<i>fasB</i>	Fatty acid synthase β subunit	U75347 [240]
<i>ole1 (sdeA)</i>	Fatty acid $\Delta 9$ -desaturase I	Y18553 [241]; AF085500 [241]; AB015612 [242]; AF026401 [243]; AF510861 [244]
<i>ole2</i>	Fatty acid $\Delta 9$ -desaturase II	Y18554 [241]; AB195980 [245]
$\Delta 12$ (<i>odeA</i>)	Fatty acid $\Delta 12$ -desaturase	AB020033 [246]; AF110509 [247]; AF161219 [248]; AF262955 [249]
$\Delta 6 - 1$	Fatty acid $\Delta 6$ -desaturase I	AF110510 [247]; AB020032 [250]; AB052086 [251]; AY392409 ^a [252]
$\Delta 6 - 2$	Fatty acid $\Delta 6$ -desaturase II	AB090360 [251]; AF290983 ^b [253]
$\Delta 5$	Fatty acid $\Delta 5$ -desaturase	AF054824 [254]; AF067654 [255]
$\omega 3$	Fatty acid $\omega 3$ -desaturase	AB182163 [256]
$\omega 9$ (<i>scd3</i>)	Fatty acid $\omega 9$ -desaturase	AB196774 [245]; AJ278339 [257]
<i>cytb5</i>	Cytochrome b_5	AB022444 [258]
<i>cbr1</i>	NADH-cytochrome b_5 reductase I	AB020035 [259]
<i>cbr2</i>	NADH-cytochrome b_5 reductase II	NA ^c [260]
<i>glelo</i>	γ -linolenic acid elongase CS ^d	AF206662 [261, 262]
<i>maelo</i>	Fatty acid elongase CS ^{d,e}	AF268031 [262]
<i>dgat2A</i>	Diacylglycerol acyltransferase 2	AF391089 [263]
<i>dgat2B</i>	Diacylglycerol acyltransferase 2	AF391090 [263]
<i>malEM (maeA)</i>	Malic enzyme (NADP-malate decarboxylase)	AF529885 [Szewczyk E, unpublished]; EAA57954 [17]; DQ973624 [220]; DQ975377 [220]

^a Originally described as isoform II^b Originally described as isoform I^c Not available^d Condensing subunit^e Most likely elongates 16 : 0 to 18 : 0

desaturases adds further complexity to the range of unsaturated fatty acids that can be synthesised [269–271].

In order to genetically manipulate an organism, it is first necessary to develop an efficient DNA transformation system for gene deletion or gene copy number amplification. In only two cases has such a system been reported for oil-producing fungi, viz. that of *Mc. circinelloides* where an extremely efficient transformation system has existed for several years [272–274] and *Mt. alpina* [275–277]. In *Mt. alpina*, the levels of ARA in relation to total fatty acids were elevated by about 10–30% after overexpressing the endogenous GLELO fatty acid elongase gene by genetic transformation [277, 278]. Gene silencing by RNA interference technology, which is also dependent on an efficient transformation system was used to reduce expression of the $\Delta 12$ -desaturase gene to modify fatty acid composition in this fungus [279]. Although the genetic manipulation of PUFA biosynthesis in oleaginous fungi is yet in its infancy, use has been made of the fungal fatty acid desaturase and elongase genes in modifying fatty acid composition in a number of other organisms, especially oil-seed crop plants [280–285], and several patents now exist for this purpose [262, 286–290].

4 Vitamins

Vitamins are essential micronutrients in human and animal diets. These compounds cannot be synthesised by mammals but are required to maintain normal metabolic and physiological functions. Plants and microorganisms, including filamentous fungi, can require vitamins as essential components for metabolism but are also capable of synthesising some of these compounds that are required by humans. The production of vitamins in traditional fermented foods involving fungi clearly benefit from their versatile metabolic abilities, either through direct biosynthesis or through the provision of precursor molecules and culture conditions to assist vitamin synthesis by co-fermenting microbial species. Tempe, for example, is a traditional Indonesian food prepared by inoculating pre-cooked soybeans with the spores of *Rhizopus* species. The resulting solid substrate fermentation will generate nutritionally significant quantities of the water-soluble vitamins: vitamin B₆, riboflavin, nicotinic acid and nicotinamide [291]. In general, vitamins are produced on an industrial scale for use as food and feed additives in addition to their use in numerous health care and cosmetic products. Vitamins are mainly prepared by chemical synthesis but there is an increasing trend towards the efficient use of natural and environmentally sensitive production methods. Therefore there are examples where biotechnological production routes, either by fermentation or microbial/enzymatic transformation, are beginning to make a contribution.

4.1

Riboflavin (Vitamin B₂)

Eremothecium ashbyii and *Ashbya gossypii* are closely related filamentous hemiascomycetes that have been used for the industrial production of riboflavin [292–294]. Yields up to 15 g of riboflavin per liter of culture have been reported from aerobic submerged fermentation using growth media containing industrial grade molasses or plant oils as carbon sources. Alternative waste organic materials have also been investigated for their ability to support riboflavin production by *E. ashbyii* [295]. As early as 1940 *E. ashbyii* was used for the commercial production of riboflavin to be replaced in 1946 by *A. gossypii* [296–298]. Both of these fermentations were later to become redundant as synthetic chemistry routes became economically favourable. This remained the case until Merck in 1974 and BASF in 1990 recommenced riboflavin production using *A. gossypii* [294]. Several *Aspergilli* have also been evaluated for their ability to produce riboflavin [299, 300]. *A. niger* and *Aspergillus terreus* have been shown to produce riboflavin upon fermentation with various carbon sources albeit at significantly lower levels [301]. *A. niger* and *A. terreus*, in common with *E. ashbyii* and *A. gossypii*, and in contrast to yeast species, are able to produce riboflavin in fermentation without being adversely affected by iron concentrations required to support growth [302].

The non-specific precursors of riboflavin biosynthesis are guanosine triphosphate and ribulose-5-phosphate. Therefore the supplementary addition of GTP synthesis precursors (purine biosynthesis precursors such as hypoxanthine and glycine) to the growth media of *A. gossypii* and *E. ashbyii*, have been reported to increase the production of riboflavin [299, 303, 304]. Several other strategies have been employed to improve the production of riboflavin, these include the selection of mutants with improved performance in fermentation. These approaches require not only the initial mutant selection but also a reappraisal of the growth conditions to optimise riboflavin production, as reported for a UV-mutant of *E. ashbyii* [305]. However, the majority of this work has focussed on *A. gossypii* since *E. ashbyii* displays innate genetic instability [299]. To this end the genes encoding the enzymes necessary for riboflavin biosynthesis have been cloned from *A. gossypii* [306]. The utilisation of plant oils as carbon and energy sources requires the β -oxidation pathway of filamentous fungi to operate in order to supply acetyl-CoA for energy and to support biosynthetic needs. *A. gossypii* and *E. ashbyii*, like many other filamentous fungi, will induce the key enzymes of the glyoxylate bypass, isocitrate lyase and malate synthase [307, 308]. These enzymes are peroxisomally located, and it is in the peroxisome that malate is produced as precursor of GTP synthesis [309]. Itaconate is an inhibitor of isocitrate lyase. Itaconate resistant mutants of *A. gossypii* overcome the effects of this inhibitor by producing higher levels of isocitrate lyase, and the increased metabolic flux possible because of this is thought to enhance the yields of

riboflavin [310,311]. However, since riboflavin yields were also increased under conditions when isocitrate lyase was repressed the mutations are likely to exhibit pleiotropic effects beneficial to riboflavin production. To take a rational approach to metabolically enhance riboflavin production the genetic tools necessary to manipulate *A. gossypii* have been developed, including DNA-mediated transformation with integrative and replicative vectors [312–314]. Using these techniques, isocitrate lyase levels of *A. gossypii* were also increased by the introduction of a second copy of the *ICL1* gene leading to increased riboflavin production upon fermentation with soybean oil [315]. Similarly, overexpression of the *GLY1* gene led to a ten-fold increase in threonine aldolase, which allowed an increase in precursor GTP synthesis in response to threonine supplementation and thereby increased riboflavin production [316]. To reduce the metabolic flux from glycine to serine the *SHM2* gene, encoding a serine hydroxymethyltransferase, was targeted for disruption. The mutation resulted in a significant increase in riboflavin productivity. Evidence for elevated glycine levels in these mutant strains was obtained from ^{13}C -labelling experiments [317]. These studies showed that *SHM1* disruption (encoding the mitochondrial isozyme) had no detectable effect on serine labelling, but disruption of *SHM2* led to a decrease in serine (2–5%) and an increase in glycine (59–67%) labelling.

Riboflavin efflux from *A. gossypii* is an active process supported by a specific carrier, the activity for which is up-regulated in production strains [318]. However, riboflavin in these strains has been found to accumulate in vacuoles leading to unwanted product retention. To circumvent this problem the vacuolar ATPase was inactivated by disruption of the *VMA1* gene that encodes one of the enzyme subunits [319]. The resulting mutant excreted all the riboflavin into the culture supernatant to improve product recovery.

We can expect further improvements in the production of riboflavin in *A. gossypii* in the near future as the genetic mechanisms that control the synthesis components are elucidated. Targets for the metabolic engineering of *A. gossypii* have been highlighted using genome-wide transcript expression analyses to follow the staged development of commercial production strains [320]. Initial findings show that 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) synthase, the first enzyme in the biosynthetic pathway for riboflavin, increases co-ordinately with transcription of its structural gene *RIB3* during stationary phase culture [321]. Moreover phosphoribosylamine biosynthesis in *A. gossypii* has been reported to be repressed by extracellular purines, where ATP and GTP inhibit phosphoribosyl pyrophosphate (PRPP) amidotransferase as the first enzyme committed to the precursor purine biosynthesis pathway required for riboflavin production. The constitutive overproduction of a mutant form of PRPP amidotransferase that was no longer subject to feedback inhibition, could abolish adenine-mediated transcriptional repression and enhance metabolic flow through the purine pathway, and thereby increase the production of riboflavin [322].

4.2

Pantothenic Acid (Vitamin B₅)

Commercial production of D-pantothenate involves initial reactions that generate racemic pantolactone from isobutyraldehyde, formaldehyde, and cyanide. The optical resolution of this racemate to D-pantolactone is a costly step in the synthesis before condensation with alanine to form D-pantothenic acid or 3-aminopropanol to form D-pantothenyl alcohol. As a cost-effective alternative to expensive synthetic alkaloids that are used in the chemical process to resolve DL-pantolactone, a biotransformation with a fungal enzyme, lactonohydrolase, has been proposed to produce D-pantoic acid directly [323]. Fungi of the genera *Fusarium*, *Gibberella* and *Cylindrocarpon* have been shown to produce lactonohydrolase activities capable of performing the optical resolution with D-pantoic acid yields in 96% enantiomeric excess [324]. These enzymes catalyse the stereospecific hydrolysis of a variety of D-lactones, of which D-pantolactone is a favoured substrate leaving L-pantolactone unchanged to be racemized and recycled as substrate. *Fusarium oxysporum* cells contain sufficient enzyme activity to carry out the biotransformation. Once prepared, these cells are also sufficiently robust that they may be immobilised in calcium alginate gels, in which form they retain around 90% of their lactonohydrolase activity over a long period, and may be conveniently recovered and reused in the process [325].

4.3

β -Carotene (Pro-Vitamin A)

β -carotene is used as a natural vitamin additive, as a functional antioxidant and as an orange/red colorant in food, animal feeds and cosmetics. Vitamin A deficiency leading to blindness remains a problem in developing countries. Filamentous fungi are a rich potential source of carotenes suitable for most applications [326]. In fungi, carotenoids are synthesised from prenyl pyrophosphates that are generated from the mevalonate biosynthetic pathway. Three molecules of acetyl-CoA are utilised to make 3-hydroxy-3-methylglutaryl-CoA which is converted via mevalonate to isopentenylpyrophosphate (IPP). IPP is isomerised to dimethylallyl pyrophosphate, a common precursor of carotenoid biosynthesis that is condensed with IPP or an alternative homoallylic pyrophosphate to elongate the structure [327]. In several filamentous fungal species this addition is performed by a single enzyme, farnesyl pyrophosphate synthase [328]. Successive “head-to-tail” additions by this reaction form C₂₀ geranylgeranyl pyrophosphate, which then combines with itself in a “head-to-tail” addition catalysed by phytoene synthase to form C₄₀ phytoene [327]. It is from this colourless precursor that β -carotene is formed by four consecutive dehydrations and two cyclization reactions [329]. Genes encoding the phytoene synthase and carotene cyclase

activities have been cloned from a number of fungi, including *Neurospora crassa* [330], *Xanthophyllomyces dendrorhous* (the perfect, yeast state of *Phaffia rhodozyma*) [331], *Mc. circinelloides* [332], *Phycomyces blakesleeanus* [333] and *Fusarium fujikuroi* [334]. In all cases the phytoene synthase and carotene cyclase functions are performed by a single bifunctional protein, an arrangement that contrasts with bacterial and plant kingdoms where the enzymic activities are associated with two separate protein products encoded on separate genes [333, 335]. Again in contrast to bacteria and plants that possess two phytoene dehydrogenase enzymes, fungi maintain a single enzyme that is capable of performing all dehydrogenase steps [329]. The structural genes encoding phytoene synthase/carotene cyclase and phytoene dehydrogenase are closely linked as a gene cluster in *Mc. circinelloides*, *P. blakesleeanus* and *F. fujikuroi* but, although on the same linkage group, the genes are somewhat distant in *N. crassa*. More recently overexpression of the *crtYB* (encoding bifunctional phytoene synthase and carotene cyclase) and *crtI* (phytoene desaturase) genes from *X. dendrorhous* was demonstrated to be sufficient to enable carotenoid production in the yeast *S. cerevisiae* [336].

β -carotene production using the fungus *Blakeslea trispora* has been industrialised for many years in Russia to yield both purified β -carotene and β -carotene-enriched mycelium for use as an animal feed additive [337]. The fungus exists in two mating types (+) and (-), of which the (+) mating type produces trisporic acid, a precursor of β -carotene biosynthesis that stimulates the (-) mating type to produce β -carotene when they are brought together in an optimised ratio. β -carotene overproducing mutants have been selected on the basis of resistance to lovastatin and acetoanilide. Using these mutants in aerobic submerged batch fermentations has yielded up to 7 g/l of β -carotene extracted from mycelia after a 7-day fermentation.

4.4

Prospective Vitamin Production

Fungi produce analogues of the vitamin C molecule L-ascorbic acid based on the molecule D-erythroascorbic acid [338], which confer similar antioxidant properties to vitamin C in these organisms [339]. In this pathway D-erythroascorbic acid is synthesised from D-arabinose in a three-step process as observed for the phytopathogen *Sclerotinia sclerotiorum* [340]. It has been demonstrated that *S. cerevisiae* can synthesise L-ascorbic acid when furnished with non-physiological substrates such as L-galactonolactone and L-galactose [341], which has led to the speculation that yeast could be engineered to make L-ascorbic acid in a direct fermentation, and can thereby replace some of the chemical synthetic steps currently required for the Reichstein process of production [342, 343]. Given the breadth of substrates available to filamentous fungi and their versatile metabolism it can only be a matter of time until production of vitamin C from these sources is investigated.

Vitamin B₆ compounds, mainly pyridoxine and pyridoxal phosphate, are currently produced by chemical synthesis. The complete biosynthetic pathway of vitamin B₆ is not known for fungi but recent work with the phytopathogenic fungus *Cercospora nicotianae* has identified the first genes involved in the synthesis of pyridoxine that appear to be conserved in Eubacteria and the Archaea [344, 345]. The initial gene was isolated on the basis that it conferred resistance of the fungus to a singlet-oxygen-generating toxin, cercosporin, which it produces to parasitise plants. The gene was therefore given the name *SOR1* but it became evident that the gene could rescue pyridoxine auxotrophs of both *C. nicotianae* and *A. flavus* [344]. This observation was subsequently confirmed in *A. nidulans* and *N. crassa*, in which homologues of *SOR1* were identified as the structural genes for *PYROA* and *pdx-1*, respectively, mutants of which require pyridoxine for growth [346, 347]. Analysis of the complete and emerging genome sequences in which PDX1 homologues appear, reveals that they are clustered with a second highly conserved gene which has also been confirmed as a functional component of the pyridoxine biosynthetic pathways of *C. nicotianae* and *N. crassa* by the complementation of closely linked pyridoxine-requiring mutants [345, 347, 348]. The gene pair is therefore known as *PDX1* and *PDX2*. Studies on the products of these may well help to establish a common biosynthetic route for pyridoxine biosynthesis, which could then be exploited to enable biotechnological production.

5 Flavour Compounds

Fermentation processes for the production of palatable foods has taken place since ancient times. Filamentous fungi of the genera *Aspergillus*, *Rhizopus*, *Mucor*, *Actinomucor*, *Amylomyces* and *Neurospora* are used in the manufacture of fermented foods from carbohydrate-rich but often otherwise bland staple cereal and vegetable crops. However, *Penicillium* species predominate in the ripening of richly flavoured cheeses and meats [349]. In these fermentations filamentous fungi often form part of a mixed microbial flora that give rise to the final organoleptic properties of the products. The additional microflora notwithstanding, it is clear that filamentous fungi produce a wide variety of taste and aroma active compounds that are of potential commercial value.

As noted in Sect. 3.1 above, citric acid is by far the largest volume flavour compound produced by filamentous fungi but the premium prices commanded by specialist flavour and fragrance ingredients have galvanised the search for new sources of these chemicals, which has included prospecting in the products of fungal metabolism [350]. Coupled with the customer demand for a wider variety of flavour compounds, there is a preference to produce these from natural sources or through natural processes [351]. For legislative

purposes “natural flavours” have a common definition in the European Union and the USA, being defined as compounds obtained from living cells, including food-grade microorganisms and their enzymes. This definition offers considerable opportunity for the biotechnological production of flavours in compatible microorganisms like filamentous fungi. In essence production may be accomplished through the recovery of aroma-active compounds from mixtures of fungal biosynthetic products (de novo synthesis) or by using fungi to perform a specific biotransformation/bioconversion process from an inactive or cheaper precursor.

5.1

De Novo Synthesis

The production of natural flavour compounds by fungi has been exploited for many years. Examples of recognised flavour chemicals produced de novo by filamentous fungi are presented in Table 5. However, the full biosynthetic

Table 5 De novo synthesis of flavour compounds in fungi

Source	Chemicals	Flavour notes
<i>Penicillium camemberti</i> <i>Penicillium caseicolum</i>	2-methoxy-3-isopropyl pyrazine	Earthy, nutty flavour of surface ripened cheeses – Brie [352]
<i>A. sojae</i>	2-hydroxy-3-6-substituted pyrazines	Roasted nutty, potato-flavour and soy sauce [353]
<i>Ceratocystis variospora</i> <i>Ceratocystis moniliformis</i> <i>Ceratocystis fimbriata</i> <i>E. ashbyii</i> <i>Penicillium roqueforti</i>	Acyclic terpenoids: geraniol and citronellol	Essential oils [354, 355]
<i>A. oryzae</i> <i>C. moniliformis</i> <i>Fusarium poae</i> <i>Tyromyces sambuceus</i> <i>Polyporus durus</i>	Methyl ketones γ -decalactone and other lactones	Blue cheese flavour [356, 357] Peach and other fruit flavours [358–360] Passion fruit [361] Coconut/pineapple [362, 363]
<i>Trichoderma</i> spp. <i>Agaricus</i> spp. <i>Polyporus</i> spp. <i>Pleurotus sapidus</i>	6-pentyl- α -pyrone Benzaldehyde	Coconut [364] Cherry and almond [365]
Basidiomycetes <i>Morchellaceae</i>	Octanols and octanals	Mushroom flavours [349, 366]

capacities of a number of species have not been realised in any commercial sense. This can be due to the low quantities of flavourants produced by fungi under the growth conditions used or problems of a technical nature relating to poor product recovery following fermentation. Species of the genus *Ceratocystis*, for example, are not only notable for their pathogenicity to trees but also for their ability to produce a wide range of flavour compounds, including aromas of peach, banana, pear, rose and citrus [354, 364, 367–370]. Typically, the headspace of these cultures contains the volatiles acetaldehyde, ethanol, isopropanol, ethyl acetate, ethyl isobutyrate, isoamyl acetate and ethyl-3-hexanoate. These cultures can also produce γ -decalactone when cultured in glycerol-urea medium or terpenoids such as citronellol, geraniol and nerol in response to galactose-urea [358]. *Ceratocystis fimbriata* and *Ceratocystis moniliformis* have been the focus of much of this research owing to their attractive growth rates and their ability to utilise agro-industrial wastes as substrates for aroma production [371]. Solid state fermentations of *C. fimbriata* with cassava bagasse, apple pomace or soya bean supported the production of strong fruity aromas, whereas amaranth or coffee husks produced a distinctive pineapple aroma [372, 373]. The yields of the flavour compounds from these fermentations are often limited because of the toxic nature of the compounds themselves. To recover these hydrophobic compounds and circumvent their inhibitory effects on growth, fermentation processes have been configured to allow in situ product removal (ISPR). To accomplish this, the fermentation vessel (bioreactor) was interlinked with a pervaporation membrane module to create an integrated bioprocess (IBP). Implementing an IBP with *C. moniliformis* and *C. fimbriata* resulted in higher growth yields of the fungi, and significantly more aroma compounds that were enriched in the permeates obtained from pervaporation, from which they could be conveniently recovered [374, 375].

5.2

Biotransformation and Bioconversion

Biotransformation and bioconversion processes are becoming commercially realistic alternatives to purely chemical synthesis routes for the production of chemicals. In particular the ability of biological systems to confer stereo- and regio-selectivity to synthetic routes is of great benefit. The use of biotransformation and bioconversion has a relatively long history in the development of flavours. As long ago as 1924 the synthesis of methyl ketones by moulds from triglycerides and fatty acid was examined with respect to formation of off-flavours in cocoa fat [376], and later with respect to the blue cheese flavours produced by *Penicillium roqueforti* [377]. In recent years there has been considerable progress in the use of precursor molecules to programme the synthesis of flavour compounds by microbes or their isolated enzymes. These studies are the subject of two recent treatises [355, 378]. Table 6 presents

Table 6 Production of flavour compounds by bioprocessing and biotransformation

Source	Precursor	Product	Flavour notes
<i>Penicillium digitatum</i> <i>Lasiodiplodia theobromae</i>	Limonene	Cyclic terpenoids: α -terpineol and jasmonate	Essential oils [355, 379]
<i>A. niger</i> <i>Penicillium chrysogenum</i> <i>Penicillium rugulosum</i> <i>P. roqueforti</i>	Geraniol	Linalool and α -terpineol	
	Fatty acids and short chain esters	Methyl ketones	Blue cheese flavour [377, 380]
<i>Aspergillus</i> spp.	Transformation of α -ionones	<i>trans</i> - and <i>cis</i> -3-hydroxy- α -ionone	Tobacco odours [381]
<i>Rhizopus</i> spp. <i>Aspergillus</i> spp.	Transformation of β -ionones	4- and 2-hydroxy- β - ionone	Floral – violet and rose-like [382, 383]
<i>A. niger</i> and <i>Pycnopus cinnabarinus</i>	Sugar beet pulp (ferulic acid)	Vanillin (3-methoxy-4- hydroxybenzaldehyde)	Vanilla flavour [384]
<i>A. niger</i> <i>Phanerochaete</i> <i>chrysosporium</i> <i>Cladosporium suaveolens</i> <i>Mucor</i> spp. <i>Polyporus</i> spp.	Castor oil or ricinoleic acid	4-decanolide	Fruit and creamy dairy flavours [385]
	Castor oil and lignin	Acetophenone	Orange blossom [386]

several examples of flavour chemicals produced by filamentous fungi from precursor molecules by biotransformation or bioconversion protocols. The use of two species of filamentous fungi in the development of a two-step process to produce the flavour compound vanillin is outlined below.

Vanillin or 3-methoxy-4-hydroxybenzaldehyde is used extensively in the food industry, most notably in ice cream and confectionery. The majority of the 12 000 tonnes consumed each year is the product of chemical syntheses, either from petrochemicals or lignin [386]. A small fraction of this production (0.5%) is extracted from the fermented pods of *Vanilla* orchids in association with a few additional compounds that add to the flavour intensity of natural vanilla. Natural vanillin attracts a price that is 80-fold or more that of the synthetic chemical product and it is this premium market that is the target for biotechnological production [387]. Several biotechnological routes leading to the production of vanillin have been investigated but in particular *A. niger* and *Pycnopus cinnabarinus* have been used in a two-step process to produce vanillin [384]. The process utilises *A. niger* to convert ferulic acid to vanillic acid and then a laccase deficient strain of *P. cinnabarinus* to reduce vanillic acid to vanillin. Use of the laccase deficient strain was adopted to prevent the formation of a deleterious ferulic acid polymer [331]. Cheap

sources of ferulic acid for this process are the agro-industrial waste products sugar beet pulp, maize bran and waste residues of rice bran oil [388–390]. However, the decarboxylation of vanillic acid to methoxyhydroquinone during the second step of the process limited the production of vanillin [391]. Addition of cellobiose was found to overcome this by channeling the vanillic acid into vanillin production [392]. *Phanerochaete chrysosporium* has been investigated as an alternative to *P. cinnabarinus* in the second step of the process but this fungus preferentially converted vanillic acid to vanillyl alcohol via vanillin. The implementation of ISPR for vanillin by selective adsorption with a polystyrenic resin was reported to yield 500 mg/l of vanillin [393]. Sensory evaluation of the vanillin made in this way identified that the vanilla aroma was accompanied a secondary pleasant chocolate odour [388]. It is only with natural sources of vanillin that such secondary organoleptic notes are perceived.

6

Conclusions

Filamentous fungi are metabolically versatile organisms that are exploited commercially as cell factories for the production of enzymes and a wide variety of metabolites. Although this chapter has focused on food applications for the fungus-derived products, many products find applications in the clinical area. Fungi are an immensely rich resource of enzymes and other natural products but it is a resource that is barely tapped because only a fraction of the world's fungal species is known to science. Many of those species that are known are not cultivable in the laboratory and, historically, this has restricted their use apart from harvesting natural populations of the fungus for culinary or medicinal purposes. This is about to change due to advances in genomic, proteomic and metabolomic (“omic”) technologies. The driver for exploitation of fungi in this way is primarily as sources of valuable clinical compounds but the principle of exploring the metabolic capacity of fungi using global approaches applies irrespective of the end-use of the product. Most lichen fungi, for example, are not readily cultivated in the laboratory and, in the wild, grow slowly. Although lichens are a major component of some ecosystems, and could be harvested for extraction of a product, there is potential in applying modern molecular approaches to clone pathway genes and express them in an amenable host [394]. Despite the potential, this is not an easy way forward but it serves to illustrate the point that emerging methodologies provide a potential that was not appreciated just a few years ago. Cloning and expressing pathway genes is aided by the clustering of many genes for secondary metabolite synthesis but their cloning and heterologous expression remains far more of a technical challenge than that for a single gene. For that reason, uncultivable (or poorly cultivable) fungi are likely to be a more immediate source

of genes for the production of novel enzymes and many of these could provide interesting activities for use as processing aids in foods.

We have taken an unashamedly molecular approach in this chapter and have paid scant attention either to the importance of the industrial process, i.e. the large-scale cultivation of fungi and down-stream processing, or to the regulatory aspects for approval of the use of additives in foods. In many respects, these facets of an industrial process will be the prime determinant of its success. We anticipate, however, the application of “omic” approaches in the area of processing to optimise the productive capacity of a chosen fungal cell factory in an industrial context. The ability to determine the flux of carbon, for example, into the desired product, and to identify and overcome bottlenecks in its production, will require a combination of bioreactor technology and global methods of analyses that are only now becoming possible. This capacity heralds the advent of new possibilities for fungi, because of their inherent metabolic diversity, in the provision of food additives.

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