

The microbial cell factory

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Microorganisms have been used for decades as sources of antibiotics, vitamins and enzymes and for the production of fermented foods and chemicals. In the 21st century microorganisms will play a vital role in addressing some of the problems faced by mankind. In this article three of the current applications in which microbes have a significant role to play are highlighted: the discovery of new antibiotics, manufacture of biofuels and bioplastics, and production of fine chemicals *via* biotransformation.

Introduction

Microorganisms have been used for centuries as a means of production, long before Robert Hooke's and Antoni van Leeuwenhoek's first observations of the microbial world. Industrial microbiology became very well developed in the 20th century, and there are many textbook examples of microbial applications and products (Table 1). Furthermore, the emergence of molecular biology has further increased the productivity of microorganisms (through mutation), and enabled them to be used as hosts for the manufacture of non-microbial products (*e.g.* mammalian proteins such as insulin). In this paper, examples of contemporary applications of microorganisms as producers of commodity and fine chemicals are presented.

Antibiotics

The screening of microorganisms, particularly *Streptomyces* and *Bacillus* bacteria, and fungi belonging to *Aspergillus* and *Penicillium*, undertaken after Flemming's discovery of penicillin, has resulted in the discovery of over 20 000 antibiotics.¹ Unfortunately, only a fraction of these are clinically useful owing to toxicity problems. The number of new antibiotics, which were discovered mainly based on the ability of culture extracts to inhibit other microorganisms, has declined sharply since the mid-1980s.² Despite the reduced number of new antibiotics being reported, there is evidence that many more antibiotics remain to be discovered,³ and researchers in this area are exploring alternative methods to uncover these at a time when new antimicrobial compounds are increasingly needed owing to resistance to the currently used drugs.

Genome mining

It was established at an early stage that culture conditions affected antibiotic production and that altering a range of physicochemical parameters resulted in the production of new

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Table 1 Examples of common microbial products^a

Microorganism	Product
<i>Saccharomyces cerevisiae</i>	Alcohol, bread
Lactic acid bacteria	Cheese, yoghurt
<i>Penicillium chrysogenum</i>	Penicillin
<i>Leuconostoc</i> spp.	Dextrans
<i>Corynebacterium glutamicum</i>	Glutamic acid
<i>Propionibacterium freudenreichii</i>	Vitamin B12
<i>Aspergillus niger</i>	Citric acid
Numerous bacteria and fungi	Enzymes (amylase, protease, lipase, cellulase, etc)

^a For the purposes of this paper, 'product' refers to microbial metabolites, enzymes and biotransformation compounds.

bioactive secondary metabolites from known antibiotic-producing strains (one strain, many compounds⁴). Increasing sophistication of molecular biology tools led to the identification of biosynthetic gene clusters coding for the enzymes necessary to catalyse the biochemical reactions leading to antibiotics, and opened a new avenue in the search for new bioactive compounds.

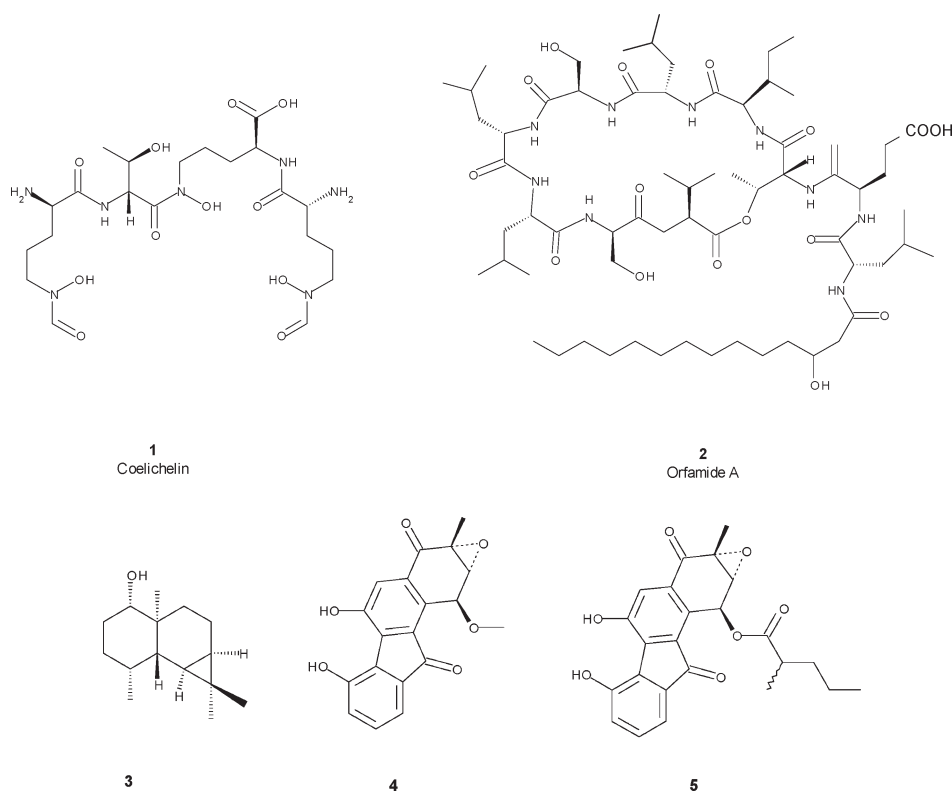
The first antibiotic-producing bacterium to have its genome sequenced was *Streptomyces coelicolor* A3(2),⁵ from which the secondary metabolites actinorhodin, undecylprodigiosin, calcium-dependent antibiotic (CDA) and methylenomycin were previously isolated. The genome sequence revealed other biosynthetic gene clusters for secondary metabolites that had not been previously isolated (so-called 'cryptic' secondary metabolites). Early sequencing data led to the discovery of the *cch* cluster coding for a trimodular non-ribosomal peptide synthase (NRPS), of which the corresponding peptide was not known. Using predictive models of the adenylation (A) domains, the amino acids L- δ -N-formyl- δ -N-hydroxyornithine (L-hfOrn), L-threonine (L-thr) and L- δ -N-hydroxyornithine (L-hOrn) were proposed as the amino acids comprising the new peptide coelichelin **1**.⁶ Subsequent purification of coelichelin from iron-limited supernatants of *S. coelicolor* permitted thorough structural analyses and revealed that the compound was a tetrapeptide (D-hfOrn-D-*allo*-thr-L-hOrn-D-hfOrn).⁷

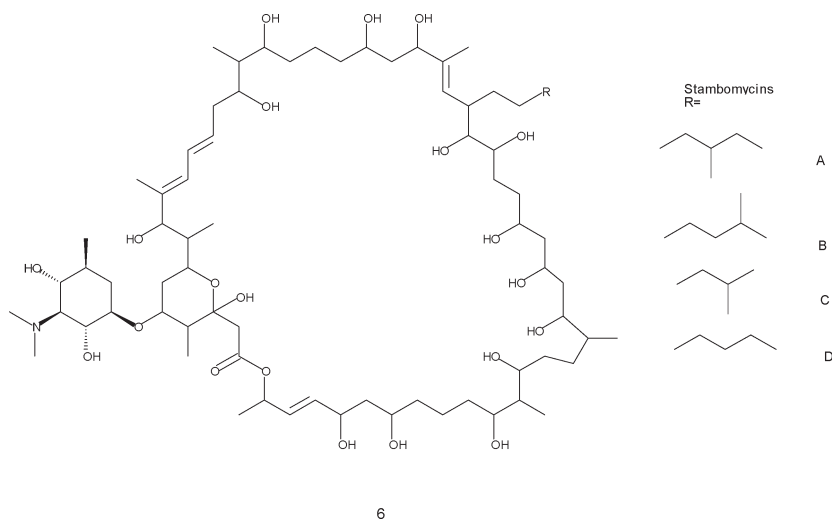
The signature sequences of adenylation domains in NRPS were exploited by Gross *et al.*⁸ to isolate a putative non-ribosomal peptide from *Pseudomonas fluorescens* Pf5 by employing a 'genom isotopic' approach. The genome sequence of this bacterium revealed novel biosynthetic clusters, one of which encoded the biosynthesis of the cyclic lipopeptide, orfamide A **2**. Based on the translated sequence, this compound contains four leucine residues, thus, [¹⁵N] leucine was added to the culture enabling

fractionation of the metabolite to be guided by ¹H-¹⁵N-HMBC NMR.

New compounds have been isolated *via* heterologous expression of clusters identified from genome sequencing, for example the sesquiterpene avermitilol **3**, of which the biosynthesis is encoded on the *sav76* gene of the *S. avermitilis* genome, but which was not known until *sav76* was heterologously expressed in *E. coli*.⁹ Metagenomics has provided an additional route to antibiotic genes for heterologous expression, namely genes from unculturable strains in the environment. However, the size of the complete gene cluster is often too large to be easily expressed in a bacterial vector. Feng *et al.*¹⁰ screened a megalibrary of eDNA from desert soil for type II PKS genes and identified overlapping cosmid clones. The complete biosynthetic gene sequence was reassembled in *S. cerevisiae* *via* transformation-assisted recombination to yield a bacterial artificial chromosome (BAC) containing a continuous stretch of 70 kb comprising a novel biosynthetic gene cluster; fluostatin F **4**, and the stereoisomers fluostatin G and H **5** were produced by recombinant *S. albus* containing the BAC.

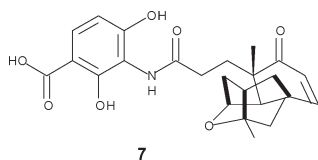
Of considerable interest is the activation of orphan pathways *in situ*, by expressing a regulatory gene present in the cluster and by-passing the required environmental trigger for expression. A giant type I modular PKS was identified in the *S. ambofaciens* ATCC23877 genome by Laureti *et al.*,¹¹ and one of the genes in the cluster was predicted to encode a regulator protein belonging to the LuxR family (LAL). This gene (*samR0484*) was inserted into the vector pIB139 putting it under the control of the strong *ermE* promoter, and introduction of the plasmid into *S. ambofaciens* resulted in constitutive expression of *samR0484* leading to the expression of the PKS genes and production of previously unidentified stambomycins **6**.





Screening methodologies

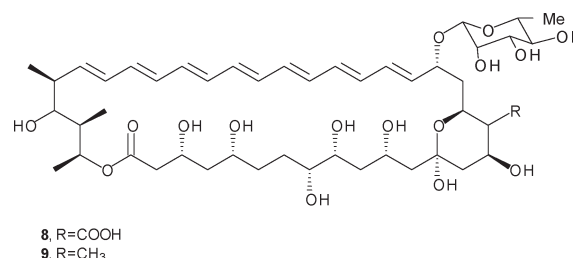
To overcome the toxicity problems associated with whole cell assays, researchers have examined other methods to screen microbial extracts for antibiotic activity, which targets proteins that are exclusively related to pathogens. Young *et al.*¹² developed a two-plate assay incorporating *Staphylococcus aureus* expressing antisense RNA (AS RNA) targeting the expression of two key enzymes of bacterial fatty acid biosynthesis, FabH (initiation condensing) and FabF (elongation). Expression of the AS RNA was induced by xylulose, thus agar plates without added xylulose that were inoculated with *S. aureus* resulted in no reduction of FabH/FabF expression, and were used as controls. *S. aureus* growing on plates containing xylulose expressed reduced levels of FabH/FabF thus were more susceptible to antibiotics that specifically targeted these enzymes. The usefulness of this assay was demonstrated with its application in screening 250 00 extracts leading to the discovery of platensimycin **7** from *Streptomyces platensis* recovered from a South African soil sample.¹³ This natural product, which is, crucially, representative of a new class of antibiotic, was shown to be selective in its inhibition of bacterial fatty acid biosynthesis, inhibited Gram positive bacteria, and displaying no toxicity *in vivo*.



Novel antibiotics via metabolic engineering

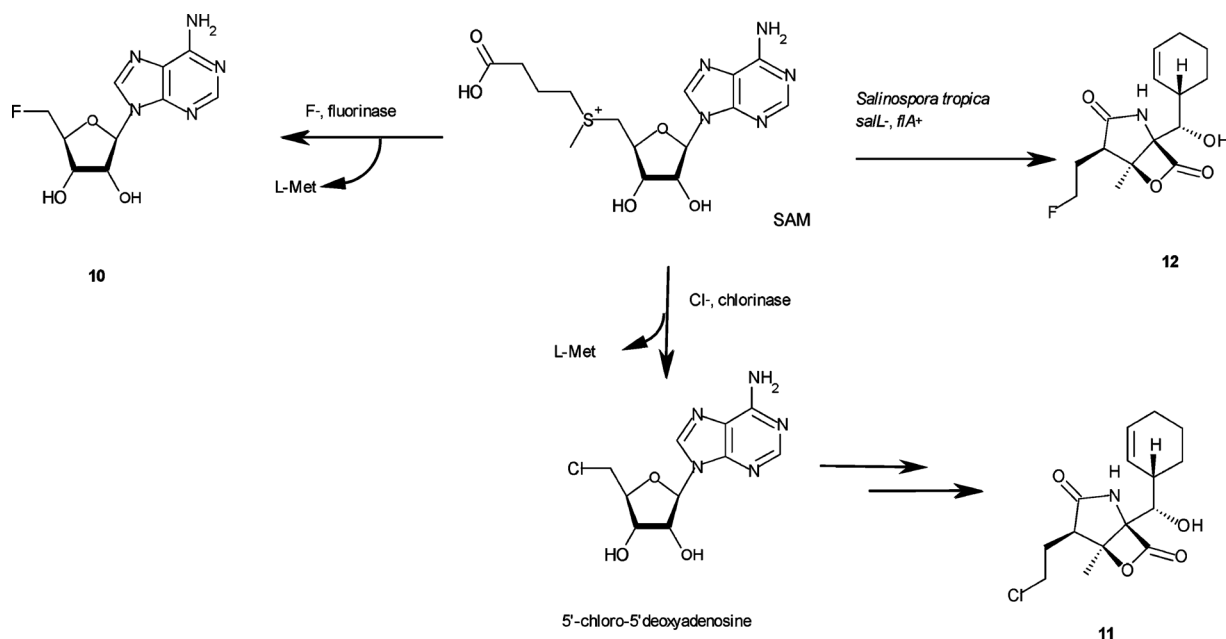
The addition or removal of biosynthetic genes has resulted in the generation of novel antibiotics with altered biological properties, such as improved antimicrobial activity or reduced toxicity. Amphotericin B **8** is a polyene macrolide with antifungal, antiviral and anti-prion activities, and is synthesised by *Streptomyces nodosus*; however, its clinical application is restricted by nephrotoxicity. Chemical modification of the exocyclic carboxylic acid group reduces the toxicity of amphotericin B, and it was

demonstrated by Carmody *et al.*¹⁴ that the targeted deletion of the gene responsible for oxidation of the C-16 methyl, *amphN*, which codes for a cytochrome P450, resulted in a mutant that produced 16-descarboxyl-16-methylamphotericin B **9**. This compound had comparable antifungal activity to amphotericin B, but was 10-fold less hemolytic.



Halogenation of antibiotics can alter their activity profile, and in the last decade our understanding of biological halogenations has dramatically advanced. One of the most interesting discoveries was the first wild-type fluorinating enzyme (fluorinase) in *Streptomyces cattleya*,¹⁵ and its corresponding gene *flA*. The reaction catalysed by the fluorinase is an S_N2 displacement of methionine from *S*-adenosylmethionine by fluorine, yielding 5'-fluoro-5'-deoxyadenosine **10** (Scheme 1). An analogous reaction was uncovered in the biosynthesis of salinosporamide A **11** in the marine bacterium *Salinospora tropica*, although in this case, chloride was the halide. Salinosporamide A is a 20S proteasome inhibitor currently undergoing phase I trials as a treatment for multiple myeloma. The cytotoxicity of this compound is known to be related to the presence of a halogen, and recently the chlorinase gene *salL*, was replaced with *flA*¹⁶ resulting in the production of fluorosalinosporamide A **12**, which has reduced cytotoxicity (Scheme 1).

Improving antibiotic yield is also an important consideration in industrial microbiology. In some cases antibiotic production is limited by the concentration of a precursor. This can be addressed most easily by the addition of the precursor to the culture medium, for example, the production of the polyenyl pyrrole rumbrin **13** in *Auxarthron umbrinum* can be increased 5-fold by the addition of 10 mM pyrrole-2-carboxylate, which is the biosynthetic starter unit.¹⁷ However, if access to the putative



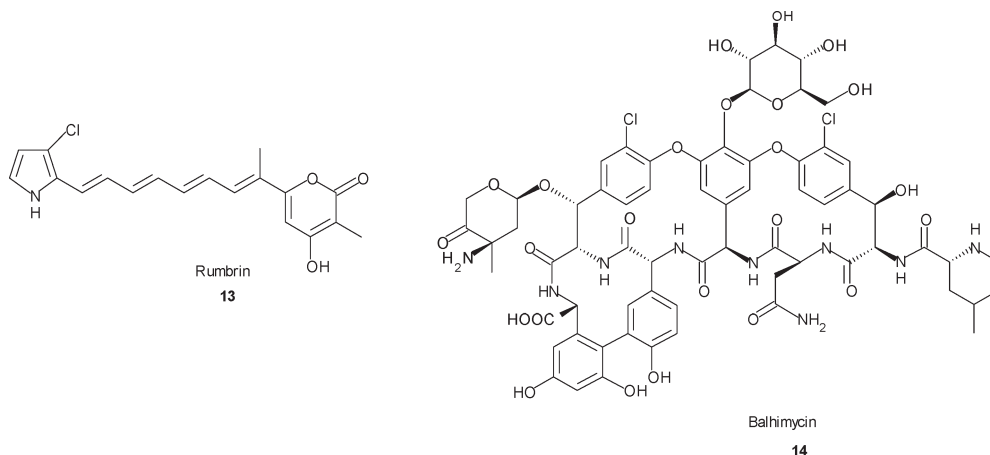
Scheme 1 Metabolites from halogenation of *S*-adenosylmethionine (SAM).

precursor is limited, or particularly expensive, then this approach is not practical. One solution is metabolic engineering to improve precursor supply. One example of this is the production of the vancomycin-like antibiotic balhimycin **14**, which is produced by *Amycolatopsis balhimycina*. Analysis of the genes bordering the balhimycin biosynthetic gene cluster revealed the presence of two genes coding for enzymes normally associated with primary metabolism: 3-deoxy-D-arabinoheptulosonate synthase (*dhap*) and prephenate dehydrogenase (*pdh*). Both enzymes are involved in the shikimate pathway, the intermediates and precursors of which are either directly or indirectly required for the biosynthesis of the amino acids comprising the glycopeptides. Analysis of the genome revealed that other copies of the genes were present (presumably for primary metabolism), thus it was reasoned that the *dhap* and *pdh* genes bordering the cluster play a role in balhimycin biosynthesis by ensuring precursor supply.¹⁸ To investigate this, strains were constructed that expressed an additional copy of one or both of the genes, and it was found that for *A. balhimycina* strains that had an additional copy of *dhap* or both *dhap* and *pdh*, the production of balhimycin increased 250%.

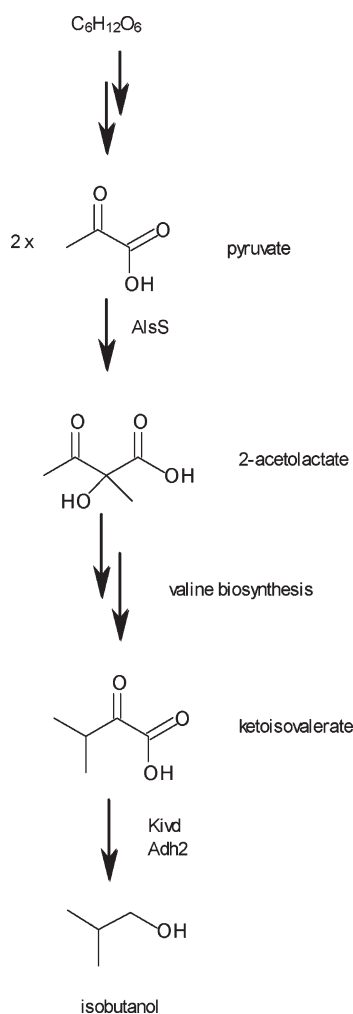
Biofuels and bioplastics

The desire to replace non-renewable energy sources has driven research in renewable fuels using microorganisms to produce alcohols, hydrocarbons, fatty acids, *etc.* directly from carbon dioxide or plant material. The fermentation of glucose in the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* has been exploited for the production of ethanol for use as a fuel. For this process to be economical it is necessary to employ non-edible carbohydrates, typically lignocelluloses, and the challenge for fermentative microbes is to break down the polymer to yield the fermentable glucose monomers. Pre-treatment of the feedstock with enzymes such as cellulases increases the costs, thus the ideal microbe for ethanol production should have the ability to degrade the feedstock and ferment the glucose to ethanol. This consolidated bioprocessing can be achieved by heterologously expressing cellulases and related enzymes in either *Z. mobilis*¹⁹ or *S. cerevisiae*.²⁰

The obligately anaerobic microorganisms belonging to the *Clostridium* genus can produce isopropanol, n-butanol and



n-hexanol, which are more attractive fuels than ethanol, as fermentation products. Industrial-scale fermentations are complicated by *Clostridium* spp. requirement for anaerobic conditions and their slow growth rates, thus expression of clostridial genes in *E. coli* and *S. cerevisiae* has been attempted to improve production.^{21,22} However, the concentrations of alcohols produced are low, possibly due to the cytotoxic effects of the products, redox imbalance created by the increased requirements for reduced coenzymes involved in fermentation, and poor enzyme activity. Atsumi *et al.*²³ employed a non-fermentative strategy to produce higher alcohols by engineering *E. coli* to express 2-ketoacid decarboxylase (Kivd from *Lactococcus lactis*) and alcohol dehydrogenase (Adh2), thus converting the 2-ketoacid intermediates of amino acid biosynthesis into alcohols (Scheme 2). The strain was further engineered to increase the amount of pyruvate available for conversion to 2-ketoisovalerate by deleting genes involved in transforming pyruvate to other by-products, and included a *Bacillus subtilis* gene (*alsS*), which codes for acetolactate synthase and has a higher affinity to pyruvate than the *E. coli* IlvH that is involved in the conversion of pyruvate to 2-ketoisovalerate. These modifications resulted in yields of isobutanol reaching 22 g L⁻¹, which compares



Scheme 2 Non-fermentative biosynthesis of higher alcohols as biofuels.

favourably to the production in *Saccaromyces cerevisiae* (3 g L⁻¹),²⁴ which produces this alcohol *via* valine metabolism.

Third and fourth generation biofuels refer to those that are produced from CO₂ by algae; third generation fuels are extracted directly from algal biomass, and fourth generation fuels are produced by genetically modified algae. Dexter and Fu²⁵ engineered the cyanobacterium *Synechocystis* sp. PCC6803 to express the pyruvate dehydrogenase (*pdh*) and alcohol dehydrogenase (*adh*) genes from *Zymomonas mobilis*, under the control of the light-driven promoter *psbAII*. The resulting strain was able to produce 5.2 mmol ethanol OD₇₃₀ unit⁻¹ L⁻¹ day⁻¹ in an autotrophic bioreactor. Autotrophic butanol production has been recently demonstrated by Lan and Liao,²⁶ who expressed genes from the CoA pathway, which converts acetyl CoA to butanol (up to 14.5 mg L⁻¹), in the cyanobacterium *Synechococcus elongatus* PCC7942. One particular challenge to this approach is the expression of genes that code for enzymes that are O₂-sensitive in an oxygenic phototroph. It was suggested that expression of these genes in anoxygenic photosynthetic bacteria, such as green and purple sulphur bacteria, which use an alternative electron donor to O₂, may be a solution to this.

Petroleum-based plastics are extensively used, yet the depletion of oil and disposal concerns have encouraged research into bioplastics which can be produced microbially from waste, are biodegradable and biocompatible, and are produced commercially by several companies (*e.g.* Metabolix in the US, Mitsubishi GAS in Japan). Production of polyhydroxyalkanoates (PHAs, bioplastic) has been known for decades and is observed in numerous bacteria, in which these polyesters of *R*-hydroxyalkanoic acids are used as a store for carbon and energy, and typically accumulate under nutrient-limiting (N or P) conditions, forming intracellular granules (Fig. 1). PHAs are categorised as either short chain length, containing 3–5 carbon atoms in the monomeric unit (*e.g.* poly(3-hydroxybutyrate)) or medium chain length, containing 6–14 carbons in the monomer (*e.g.* poly(3-hydroxyoctanoate)); the size of the PHA typically ranges from 50–1000 kDa. Despite the attractiveness of bioplastics as an

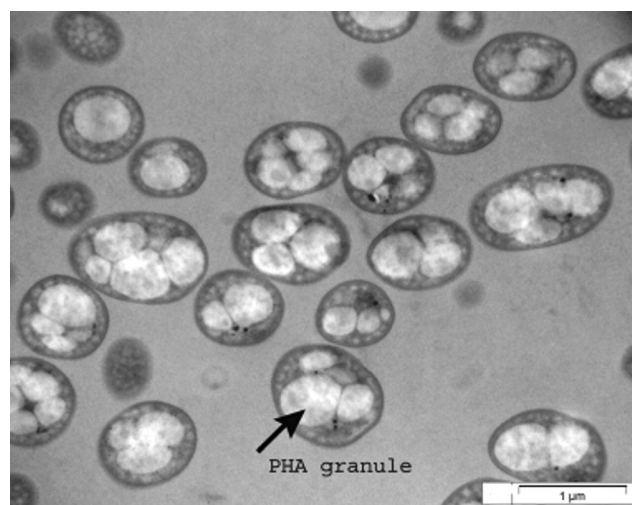


Fig. 1 Electron micrograph showing the accumulation of PHA in *P. putida* cultivated on styrene.²⁹ Reproduced with permission from the American Society for Microbiology.

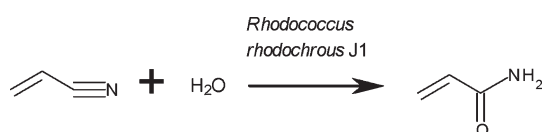
alternative to petroleum-based plastics, there are some factors that frustrate their application, in particular yield, recovery and physicochemical properties. Employing an inexpensive medium for culturing the PHA-producing strains has been widely investigated, and an attractive strategy is to use waste, for example from food processing, to culture the PHA-producing bacteria. An innovative approach for PHA production has been employed by O'Connor *et al.*^{27–29} who use environmentally-damaging xenobiotic compounds such as styrene, polystyrene and polyethylene terephthalate (PET) to produce PHA in *Pseudomonas putida*. A range of recovery methods have been investigated including solvent extraction (conventional solvent and supercritical CO₂), cell disruption by chemical, biochemical or mechanical means, and spontaneous liberation of PHA through autolysis.³⁰

Biotransformation for fine chemical production

Employing whole-cell or cell-free enzyme preparations (as a crude extract or purified system) to catalyse industrially important reactions is highly attractive. Enzyme-catalysed reactions are characterised by strict regio- and enantio-selectivity, result in few by-products and can be conducted under mild conditions. Purified enzymes tend to be less stable than whole-cell biocatalysts, and any coenzyme requirement can make the reaction prohibitively expensive; on the other hand, whole-cell biocatalysts can regenerate cofactors, but may also catalyse further unwanted transformations. Despite the obvious advantages of biocatalysts, and their historical use, which stretches back to 5000 BC when *Acetobacter* was used to produce vinegar from ethanol, their use in fine chemical production is minor compared with classical synthetic chemistry. Delivery times in particular, compel companies to employ established organic chemical methods, rather than develop a biocatalytic method, thus, in most instances biocatalysis is not considered unless there is no other alternative.³¹ Nevertheless, there are some highly successful biotransformations that are employed by industry, which deliver product on a multi-tonne scale, some examples of which are given below.

Acrylamide production by nitrile hydratase

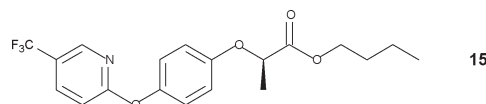
Acrylamide is an important commodity chemical that is used in the production of polymer flocculants in paper manufacture and oil recovery. Production of acrylamide can be achieved chemically by oxidising acrylonitrile using a copper catalyst and heating to 100 °C. The Japanese manufacturer DiaNitrix, a subsidiary of Mitsubishi Rayon, employs a biocatalyst for the production of acrylamide (95 000 t annually). The biocatalyst is a bacterium, *Rhodococcus rhodochrous*. J1, that produces an enzyme, nitrile hydratase, which converts acrylonitrile to acrylamide under very mild conditions (Scheme 3) and removes the need to regenerate the catalyst. This biocatalytic process is more favourable than the chemical production because (i) there is no



Scheme 3 Biotransformation of acrylonitrile to acrylamide.

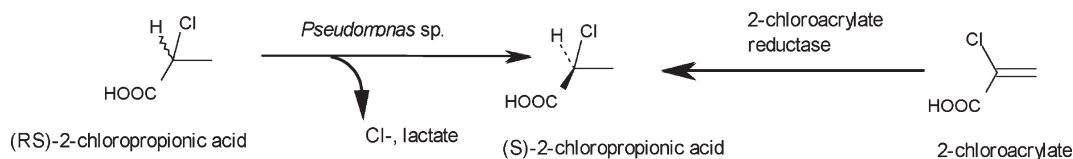
acrylic acid produced (ii) there are no by-products (iii) less energy is consumed (iv) the production costs are lower.³²

R. rhodochrous J1 produces two nitrile hydratases with different molecular weights. The 'high' molecular weight enzyme (520 kDa) efficiently transforms acrylonitrile (K_m 1.89 mM) to acrylamide, and is a cobalt-containing enzyme.³³ Inclusion of urea in the culture medium as an inducer results in >50% of protein in cell extracts as this enzyme. Furthermore, the enzyme is more heat stable than the nitrile hydratase of *P. chlororaphis*, which was the original biocatalyst employed, and is more resistant to the inhibitory effects of both substrate and product.³⁴ When manufacture of acrylamide was switched to the new biocatalyst, the productivity went from 6 000 t year⁻¹ to >30 000 t year⁻¹.³⁵ For industrial use the bacterial cells are immobilised in a cationic acrylamide polymer gel lattice, by mixing cells suspended in potassium phosphate buffer with the monomer solution (acrylamide/dimethylaminoethyl methacrylate/*N,N'*-methylenebisacrylamide). Polymerisation is initiated by β -dimethylaminopropionitrile and ammonium persulfate. Immobilisation helps prevent the elution of impurities from cells, eliminates the need to separate cells from the reaction mixture and increases the stability of the enzyme towards the substrate and product.³⁶ Considerable work has been conducted on the genetics of nitrile hydratase, and this has led to the development of a biocatalyst that has a greater heat resistance, which enables the catalyst to be used for longer. Site-directed mutagenesis studies have determined that there are several residues on both the α and β -subunits of the enzyme that can be replaced, resulting in improved heat resistance, in particular I β 88F and the double mutant E β 93G, E β 103D, which retained 89% activity after heating at 55 °C for 30 min, compared with 40% activity retained by the wild-type enzyme.³⁷

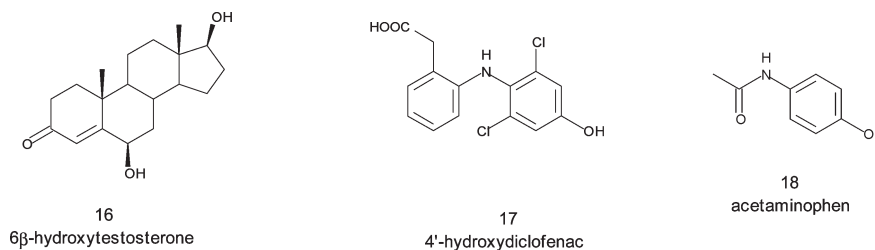


Production of (*S*)-2-chloropropionic acid

(*S*)-2-Chloropropionic acid is an important synthon in the manufacture of phenoxypropionic acid herbicides such as Fusilade® 15, and can be synthetically prepared from lactic acid, which is esterified and chlorinated with thionyl chloride. A biotransformation route was developed by ICI in the 1980s using racemic 2-chloropropionic acid as the starting material and employing a microbial dehalogenase enzyme that specifically degraded the (*R*)-enantiomer. This approach has been used successfully by Avecia to produce 2 000 tonnes year⁻¹ of (*S*)-2-chloropropionic acid (Scheme 4). One obvious drawback to this approach is that the maximum possible yield is 50%, thus other methods of (*S*)-2-chloropropionic acid production have been investigated. For example, a novel bacterial reductase capable of reducing 2-chloroacrylic acid to 2-(*S*)-chloropropionic acid was identified in *Burkholderia* sp. W2.^{38,39} In the wild-type strain the 2-(*S*)-chloropropionic acid was eventually dechlorinated, thus the gene coding for the 2-haloacrylate reductase was cloned and expressed in *E. coli*, along with a glucose dehydrogenase gene from *Bacillus subtilis*, which allowed the regeneration of the coenzyme NADPH.⁴⁰ The recombinant strain produced 346 mM (*S*)-2-



Scheme 4 Biological production of (*S*)-2-chloropropionic acid *via* dehalogenation of (*RS*)-2-chloropropionic acid and reduction of 2-chloroacrylate.



chloropropionic acid (99% e.e.) from 385 mM 2-chloroacrylate (90% yield).

Drug metabolite synthesis

One of the main reasons why drug candidates fail to make it to market is the toxicity of one or more of their metabolites formed *via* transformation of the parent drug in the liver. The US Food and Drug Administration has recently issued guidelines (called 'Metabolites In Safety Testing', MIST), which recommends that any drug metabolite that is produced in quantities >10% of the original drug should undergo full toxicology testing. The predominant metabolism of drugs is *via* hydroxylation catalysed by cytochrome P450 (CYP), a reaction which is challenging to execute using classical organic synthesis, thus for the production of drug metabolites a biocatalytic solution appears to be the most attractive.

Some microorganisms, such as *Cunninghamella elegans*, *Streptomyces griseus* and *Bacillus subtilis*, have CYPs, and have been shown to transform drugs in an analogous fashion to humans,^{41–44} thus might potentially be used for the production of some drug metabolites. However, usually several metabolites are produced, some of which may not be required, and consequently the process is not highly efficient. Clearly human CYPs would be the best biocatalyst to apply to drug metabolite production; however, there are 57 CYP isozymes, thus selectivity is an issue if liver extracts or microsomes are employed to generate a particular metabolite.⁴⁵ Purified CYPs are labile and require a supply of electrons, which come from NAD(P)H plus cytochrome P450 reductase (CPR), thus a whole-cell system is most appropriate for metabolite production. Heterologous expression of human CYPs has been achieved in insect and mammalian cell cultures, which are commercially available and are appropriate for analytical scale metabolite production. For preparative drug metabolite production, scalability is an important consideration and therefore a microbial expression system is more desirable.

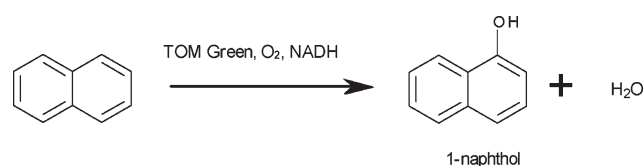
CYPs 3A4, 2C9 and 1A2 co-expression with CPR has been achieved in *E. coli* and used for the production of the main hydroxylated metabolites (**16–18**) of testosterone, diclofenac and phenacetin; purified metabolites were obtained in amounts up to 110 mg (for 4'-hydroxydiclofenac **17**).⁴⁶ Crucial to the

expression of the genes was a dissolved oxygen concentration of <1%, and optimisation of pH, temperature and glycerol concentration also impacted on the CYP activity. A collaboration between the University of Dundee and a number of pharmaceutical companies has resulted in high expression levels of 14 CYP isozymes in *E. coli* for the production of human metabolites. For the production of a given metabolite the recombinant CYPs are screened to identify the most appropriate one to be used for scale-up and production.⁴⁵

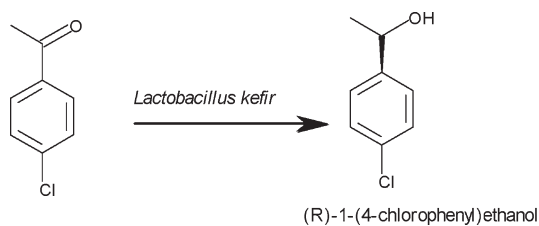
Yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have also been used as expression hosts for mammalian CYPs, which are intuitively the more appropriate microbial host as they are eukaryotic. Drăgan *et al.*⁴⁷ generated 2.8 g of 4'-hydroxydiclofenac **17** in 6 L of *S. pombe* expressing mammalian CYP2C9, considerably greater than the volumetric yield reported for other heterologous systems.^{46,48} Interestingly, when the yield of product was expressed with regard to time (mg L⁻¹ day⁻¹), the wild-type bacterium *Actinoplanes* sp. ATCC53711 was the best biocatalyst.⁴⁹ In this latter investigation the bacterium was immobilised in a cellulose-based hollow fibre tube and demonstrated 100% conversion of the starting material in <5 h and could be re-used up to seven times.

Biocatalysis in biphasic systems

A significant problem with biocatalysis, particularly in whole cells, is that either the substrate and/or the product are toxic, posing problems for the upscaling of the process. One method for avoiding this problem is the use of two-phase systems, in which an appropriate organic solvent (*i.e.* is biocompatible and has a high distribution coefficient for the product) is included in the bioreactor and acts as a sink for the toxic compounds. For example, 1-naphthol is a versatile industrial compound used in



Scheme 5 Biotransformation of naphthalene to 1-naphthol.



Scheme 6 Asymmetric reduction of 4-chloroacetophenone.

the manufacture of a range of chemicals ranging from pharmaceuticals and agrochemicals to perfumes. A potential biocatalyst for the production of 1-naphthol from naphthalene is the toluene ortho-monooxygenase (TOM) variant TOM-Green, which is a V106A mutant arrived at *via* directed evolution (Scheme 5). As the enzyme requires NADH, the biotransformation is best conducted in whole cells to regenerate the coenzyme. However, the toxicity of both substrate and product precludes this as a biocatalyst for industrial scale production of 1-naphthol. Garikipati *et al.*⁵⁰ demonstrated the effectiveness of conducting the biocatalytic reaction in a biphasic system in alleviating the toxic effect. An *E. coli* strain expressing TOM-Green was employed and the reaction was evaluated in biphasic systems comprising either dodecane, dioctyl phthalate or lauryl acetate, with the latter being the best solvent to use. Optimisation of the organic phase ratio and substrate concentration led to a yield of 1.43 g L⁻¹ 1-naphthol, which is an eight-fold improvement in the production compared with the reaction in aqueous media.

The toxicity of organic solvents to whole cells led to the investigation of alternative co-solvents for biphasic biotransformations, and the discovery that water immiscible ionic liquids (ILs) could be employed instead.⁵¹ For example, when the asymmetric reduction of 4-chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol by *Lactobacillus kefir* (Scheme 6) was conducted in an aqueous system (50 g cells L⁻¹), the yield of product was 46.2% after 6 h and the enantiomeric excess (e.e.) was 98.1%. In comparison, when the biotransformation was conducted in a biphasic system with 1-*n*-butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (BMIM[*Tf*₂N], 20% v/v), the yield increased to 92.8% (e.e., 99.7%).⁵² Furthermore, the effect of the substrate in the biphasic system on the cell membrane integrity was minor (89% intact), in comparison to the effect of the substrate in buffer (almost zero intact). One drawback to ILs is the cost, which makes their large-scale application less attractive; however, it has been recently shown that 1-hexyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide, which was used as a co-solvent in the biphasic whole cell asymmetric reduction of 2-octanone by recombinant *E. coli*, could be recovered by distillation and re-used 25 times without any effect on the biotransformation.⁵³

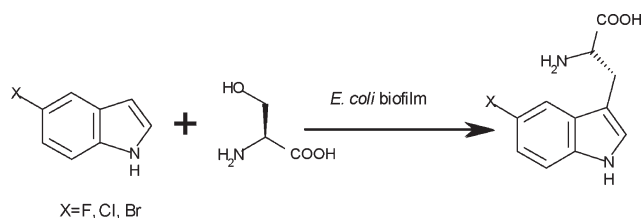
Biocatalyst immobilisation

Immobilisation has been widely employed to improve the longevity of biocatalysts, provide protection from the toxic effects of substrates/products and enable biotransformations to be conducted under more testing environmental conditions. A common method of immobilisation of microbial cells for

biotransformation is to drop a mixture of cells and sodium alginate into solution of CaCl₂, resulting in the entrapment of the cells in a bead of calcium alginate.⁵⁴ More recently microorganisms immobilised as biofilms have been evaluated as biocatalysts for the production of fine chemicals. Biofilms are microcolonies of bacteria encased in extracellular polymeric substances (EPS) and attached to surfaces. Much research on biofilms concerns their role in infection in cystic fibrosis patients, those associated with medical implants and catheters, and in biofouling of tubes and pipes.^{55,56} However, beneficial applications of biofilms have also been investigated in relation to bioremediation and biodegradation,^{57,58} and they have been applied to the production of bulk chemicals such as acetic acid and ethanol.⁵⁹ In fact, the same characteristics that make biofilms problematic in some areas, such as resistance to toxic compounds and long-term stability, are beneficial for biocatalysis and reports are now emerging of the application of biofilms in this area. Gross *et al.*⁶⁰ demonstrated that a mutant strain of the styrene degrader *Pseudomonas* sp. VLB120, that contained a knock-out mutation of the isomerase involved in the styrene degradation pathway, could transform styrene to (*S*)-styrene oxide in a tubular biofilm reactor, which was stable for 55 days. A biocatalyst biofilm of recombinant *E. coli* PHL644 expressing tryptophan synthase from *Salmonella enterica* sv. Typhimurium was generated by spin coating poly-L-lysine covered slides and used to produce halotryptophans from serine and 5-haloindole (Scheme 7).⁶¹ The yields of the halotryptophans were substantially improved in the engineered biofilm, for example there was 93% transformation of 5-fluoroindole to 5-fluorotryptophan in the engineered biofilm compared with 63% when cell-free lysate was employed. Furthermore, the stability of the enzyme immobilised in biofilm was much greater than planktonic cells, lysate or enzyme immobilised on an affinity column, and could be reused three times without loss of activity.

Outlook

Research on the application of microorganisms for the production of useful compounds is intense, however, the translation of the academic research into industrial and commercial processes is significantly more difficult to achieve. Many pharmaceutical companies have reduced R&D investment in natural products, just at a time when new antibiotics are desperately needed to counter resistance. With the development of new screening methods, the accessibility of genome sequences and decreasing costs of sequencing, companies might be encouraged to re-engage in antibiotic discovery.



Scheme 7 Biocatalytic conversion of 5-haloindole to 5-halotryptophan.

Although chemical production from biomass is worth over US \$50 billion year⁻¹,⁶² biotransformation is often employed in fine chemical production only as a last resort. One of the main issues is strain and enzyme availability,³¹ and the establishment of strain and enzyme databases, for example, Biocatcollection (www.biocatcollection.net), would facilitate easier screening and shorten development times. Further advances in other aspects of improving the biocatalytic process, such as alleviating toxicity and enabling reactions to be conducted in non-aqueous solvents (e.g. by using enzymes from extremophiles⁶³), will enable biocatalysts to play a more prominent role in chemicals production.

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