Industrial revolution with microorganisms

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Mankind has used microbes from the dawn of history to perform services and produce useful chemicals and bioactives. Mixed complex communities, which are resilient over time, preserved food, made alcoholic beverages and treated wastes, all in the absence of an understanding of the underlying biological processes. Moving to single microbial transformation systems led to high-level production of primary (amino acids, nucleotides, vitamins – used as flavour-enhancing agents, nutritional supplements and pharmaceuticals – solvents and organic acids, including biofuels) and secondary (pharmaceuticals, enzyme inhibitors, bio-herbicides and pesticides, plant growth regulators) metabolites and bioactives (including bacteriocins and enzymes). Several hallmark discoveries in microbiology and other sciences over the last 60 years transformed our ability to discover, manipulate, enhance and derive commercial benefit from industrial applications of microorganisms. This article attempts to capture some of the key discoveries that revolutionised industrial microbiology and speculates about where the "omics" revolution will take us next.

Evolution before revolution

Brewing is probably mankind's oldest biotechnological process, given archaeological evidence of this art was found on Sumarian tablets dating back to *circa* 1,800 BC, and possibly having origins as long as 10,000 years ago[1]. Although the Sumarian goddess of brewing, Ninkasi, may have assisted the fermentations, it was the skill of the brewers in maintaining an active brewing agent that produced a reliable and acceptable product, in the absence of knowing the agent was a living, mixed microbial community dominated by yeast. It represents one of the many ancient technologies that employed microbial consortia in preserving food and enhancing food traits by altering flavour, aroma and texture. While defending their habitats against other microbes, members of the consortia improved food safety by decreasing, or increasing, pH and by producing growth-inhibiting metabolites (ethanol, acetic and lactic acids, other organic acids) or other antimicrobials, including bacteriocins (like nisin or lactain F) and enzymes (including proteinases and lysozyme). Across the globe and across time, fermentation was employed in different forms to make a plethora of foods and beverages based on locally available plants and animals, using:

- milk (from different animal species – cow, mare, sheep, camel – on different continents, making beverages like kefir and curded products including cheese and yogurt)
- vegetables (usually lactic and acetic acid fermentations of raw vegetables, producing sauerkraut, kimchi, pickles, olives), roots and tubers (beverages, pastes, confectionary particularly in African, Asian and Pacific countries)
- beans (soy sauce, stinky tofu, miso, cocoa, coffee, vanilla)
- cereals (in bread-making; beverages – sake, rice wine, malt whisky, vodka)
- fruits (wine, vinegar, cider)
- meat and fish (salami and related fermented sausages; fish sauces; shrimp paste; pickled herrings and anchovies)[2,3].

Complex communities of microbes also produced silage, made compost for soil improvement and, since the end of the 19th century, treated waste water and sewage[4]. Whether in food production or environmental services, a feature common to these systems was the ability of communities to adjust to changing inputs, including fluctuations in nutrient sources or changing availability of these over time, the presence of transient inhibitors and uncontrolled temperatures. The robustness of

natural microbial consortia is predicated on the cooperative metabolism across species so they can handle multiple nutrient sources and undertake catabolism not possible by pure cultures. This produces outcomes that are predictable despite changes in the make-up of microbial species within the ecosystem, as the ecology is determined by the physical environment plus the chemistry of the environment created by the consortium to exclude other microbes. This resilience of the biological systems and history of their use has meant the retention of mixed cultures in industrial processes that include traditional foods, biogas production, biological soil remediation and wastewater treatment[2]. However, the key transforming discoveries in the 17th to 19th centuries underpinned the shift to pure culture fermentations and the emergence of the industrial revolution using microorganisms in the 20th century.

The existence of microscopic organisms was established by Robert Hooke and Antony van Leeuwenhoek between 1665 and *circa* 1676, the former publishing observations on a fungus in 1665 (a "hairy mould" on a mouldy piece of leather) and the latter reporting the existence of protozoa and bacteria, as "animalcules" in "pepper water" (pepper soaking in water to soften the spice so flavours could be extracted) that had been sitting for some weeks[5]. Microscopy formed the backbone of what emerged as microbiology over the next 200 years, a period that was typified by heated debate about spontaneous generation – a debate settled by Louis Pasteur[6]. Starting life as a chemist and crystallographer, Pasteur was recognised as a major contributor to the field of molecular chirality, discovering molecular dissymmetry (enantioselectivity) in 1848, and concluding later that certain forms of chiral molecules, such as the "biological" racemic form of tartaric acid could only be formed by living beings so that molecular chirality was a manifestation of life[6,7]. In 1856, he shifted his research into studying fermentations, developing the "germ theory" that linked microbes with causal outcomes in the fermentation industry and underpinned the development of large-scale, aseptic processes in brewing, wine-making and the food industry more generally. Importantly, the germ theory penetrated medical sciences by influencing opinions on the causes of contagious disease and their prevention. The litany of discovery thereafter to the start of the 20th century is staggering: Lister (antiseptic surgery), Koch (first proof of germ theory in disease, growth of bacteria on solid media, Koch's postulates), Gram (visualisation and differentiation of bacteria), Petri (invented the Petri dish), Ivanovski (discovered viruses) and Beijerinck (showed viruses depending on host cells for growth).

While large-scale industrial fermentations are largely a phenomenon of the 20th century, the history of producing biofuels and solvents was founded in mid-19th century, with the use of ethanol in the first ignition engine prototypes and automobiles[8]. Chaim Weizmann's isolation of *Clostridium acetobutylicum* and development of the acetone, butanol and ethanol fermentation as an industrial process in 1912 transformed the fermentation industry. This was timely, as conversion of starch into acetone for cordite (a propellant in munitions) manufacture filled a strategic need for supplying this chemical in World War 1[9]. Rescreening for strains with high yields of butanol (*Clostridium*

Table 1. Examples of technologies used to develop penicillin production and, later, other antibiotics and metabolites (based on Kardos *et al*., 2011)[10].

Stage of product development	Technology
Microbial strain selection	Biological detection assays High throughput screening for producer strains Biomining for specific traits, genes
Strain improvement	High-frequency mutagenesis methods Genetic recombination/protoplast fusion/transformation Genetic manipulation of bottlenecks in synthesis, relief of catabolite repression, product secretion
Biochemistry	Nutritional effects of precursors Regulatory effects of small molecules Mapping pathways of synthesis for target product and side products Cell-free biosynthesis Metabolomics, increasing metabolic flux by mapping carbon flow to product Proteomics, to identify carbon flux, new transporters and regulators as targets for genetic manipulation[14].
Chemical engineering	Submerged aerobic fermentation Large-scale aerobic fermentation Continuous culture Counter-current extraction of product Large-scale extraction, purification
Analytical and medicinal chemistry	Elucidation of structures of product and intermediates, side products Chemical modification

saccharobutylicum) saw large-scale facilities established in Japan, the USA and South Africa from the 1930s. After production peaked in the 1950s, the technology became unprofitable due to increasing costs of substrates, and falling prices of chemicals now derived from the petrochemical industry. Few manufacturing facilities survived after the 1960s, except in South Africa, possibly China and the Soviet Union[9]. Closing this era of industrial microbiology coincided with the emergence of the burgeoning industry based on natural products, following the discovery of penicillin by Fleming in 1928[10].

Revolution as transformation

Unlike primary metabolites, which are made during microbial growth as part of normal metabolism, secondary metabolites are not essential for growth and are made during and after the idiophase of cell growth – when growth rate slows and cells move into stationary phase. Regulation of metabolite synthesis is highly conservative, where cells coordinate functions so that only the required amount of necessary enzymes are made to utilise nutrients available in the environment or to synthesise their own. Large amounts of intermediary metabolites are not normally accumulated and secondary metabolites are made in trace amounts in nature. Energy is conserved and used to support growth or survival. Overproduction of metabolites must therefore be elicited through strain manipulation using traditional mutagenesis or genetic engineering, as well as developing appropriate process technologies to optimise metabolic capacity.

The path between observing natural synthesis of a potentially valuable microbial metabolite to a commercially viable product is now well trodden and the story of penicillin provides the archetypical approach[10-13]. The original Oxford strain of *Penicillium notatum* isolated by Fleming produced 5 mg/L of penicillin. It was clear that nutritional manipulations were insufficient to improve yields, so strain improvement through mutagenesis was developed using UV and ionising radiation, or chemical mutagenesis, followed by screening survivors for desirable traits with new isolates of *Penicillium chrysogenum*. Although described as "brute force" genetics which was labour intensive[12], titres were improved to 70 g/L with production of penicillin increasing from 2,300 kg in 1945 (cost of $11,000/kg) to 45 million kg in 2000 (cost of $11/kg).

The aims of strain improvement were not only to increase product titre but also included strategies for process improvement, such as: improving ability to assimilate inexpensive raw materials during fermentation or using mixtures of sugars (alleviating catabolite repression); altering product ratios (important in antibiotic production, as some species produce several products as secondary metabolites) and eliminating side products; enhancing product excretion to aid down-stream processing and alleviate feedback inhibition or, conversely, retain normally secreted intermediates essential for product formation; and shortening fermentation duration[12]. Some of the technologies employed to improve yields of penicillin, and other natural microbial products, are summarised in Table 1. There are numerous targets that can be altered through traditional mutagenesis or by genetic manipulation (Table 2). Among the most effective strategies was altering the level of metabolite synthesis by deregulating synthesis of enzymes involved in key pathways, coupled with altering enzyme activity by improving substrate binding or altering feedback regulation by products and intermediates of pathways. Anti-metabolites were used in positive selection of mutants following mutagenesis to this end, as these structural analogues of end-products or intermediates are toxic unless cells can dilute the anti-metabolite by overproduction of the true

Table 2. Mutagenesis or genetic manipulation strategies that can be applied to specific gene targets to improve metabolite production (based on Adrio *et al.,* 2006)[13].

Class of gene	Strategy	
	Mutagenesis	Genetic engineering
Structural genes for product synthesis	Anti-metabolite resistance – alter substrate binding or enzyme regulation site	Increase expression of rate-limiting enzyme by: introducing tandem repeats; amplifying part or all of a pathway; amplifying genes at key blockage points under regulation of strong promoter.
Regulatory genes determining the onset and expression of structural genes	Anti-metabolite resistance – deregulate promoter to constitutive synthesis	Disrupt negative regulatory genes for global or pathway-specific regulators.
Resistance genes determining resistance to the antibiotic being produced or to the primary metabolite	Selection of mutants resistant to antibiotic or anti-metabolite	Introduce resistance by targeting specific mechanisms.
Permeability genes controlling entry and exit of products	Selection of mutants resistant to inhibitors if taken up; sensitivity to surface-acting agents (for example, lysozyme, detergents)	Eliminate or enhance specific transports to retain/remove intermediates needed for synthesis or inhibiting enzyme activity; targeted alteration in surface structures (for example, lipids, peptidoglycan).
Regulatory genes controlling pathways for precursors and cofactors	Auxotrophy or reversion of auxotrophy by screening after mutagenesis	Inactivation of specific genes at regulatory points for precursors or cofactors, or their secretion. Perturbing central metabolism.

compound. Combinations of classical approaches and molecular genetics have been used more recently in strain construction to increase yields of primary (Table 3) and secondary metabolites[10-13]. Strategies have included rational design of enzymes (based on a knowledge of protein structure), directed evolution (random redesign), molecular breeding techniques, including DNA and whole genome shuffling, and combinatorial biosynthesis[13]. Random design techniques have also been used to generate improved enzymes, with different pH and temperature profiles, modified enantioselectivity, substrate specificity and stability in organic solvents, with proteins produced from directed evolution on the market as early as 2000[13]. The market value of industrial enzymes used in food processing, feed production, paper and leather processing, detergent manufacture, and the chemical and pharmaceutical industries was over $2 billion in 2000[11] and was estimated as $3.3 billion in 2011[15].

Because of the value, market size and importance in controlling infection, biodiscovery mainly focused on anti-infectives until the 1970s, when secondary metabolite screening was broadened to detect bioactives that included enzyme inhibitors. Furthermore, "failed" antibiotics (not commercially viable as antibiotics), were rescreened for functionality, leading to alternative applications in medicine and agriculture. These secondary metabolites include: statins (enzyme inhibitors that lower cholesterol); immunosuppressants (important in organ transplantation); anti-tumour agents; anti-helmintics and growth promoters in animals; bioinsecticides, biopesticides and bioherbicides; and plant growth regulators. Multibillion dollar markets were in place for the human therapeutics by the end of the 2000s[11,12]. The annual anti-infectives market remains valuable, and is estimated at $66 billion currently, with β-lactam antibiotics representing approximately one-third of this market[12]. Although there are compelling arguments for continued biodiscovery of antibiotics, due to continued emergence of antibiotic-resistant microbial pathogens, emergence of new pathogens and treating microbes in biofilms[16], the relatively low unit value makes this unattractive for major pharmaceutical companies despite the opportunities presented though natural product screening and combinatorial chemistry. Fortunately, the hunt for new antibiotics has been picked up by biotechnology companies and academic laboratories.

The key discoveries that have underpinned the industrial revolution with microbes are many, and come from diverse fields of science and engineering. We automatically think of the key advances in molecular genetics, from the first report of recombination in *E. coli* in 1953[17], transferable plasmids in the 1960s and progression of our understanding of gene regulation in the same decade[18], the first use of restriction enzymes[19] and first expression of foreign genes in a bacterial host[20], moving from RNA and DNA manual sequencing in the early 1970s to Sanger's dideoxy-sequencing in 1977 to its later automation which facilitated the first human genome sequencing in 2001[21]. High-throughput sequencing and next generation sequencing technology platforms, in combination with many clever uses of PCR technology, have since opened up genome sequencing and gene characterisation at a much accelerated rate, with virtually all genomes of bacterial species of current medical and agricultural importance having been sequenced. Metagenomics hold the promise of recovering novel biomolecules and enzymes from uncultured species from a variety of environments, while metatranscriptomics and metaproteomics are developing to the point of utility in ecosystem analysis and monitoring microbial community evolution in response to environmental changes[22,23]. Proteomic analysis is already providing new strategies for improving production of primary metabolites in traditional industrial microbes, such as *Corynebacterium glutamicum*,

Table 3. Examples of primary metabolites, their use and commercial value[11].

Class of primary metabolite	Examples	Use	Organisms	Market value
Amino acids	Monosodium glutamate	Flavour enhancement	*Corynebacterium glutamicum and Brevibacterium* species	$2.1 billion
	Lysine	Cereal and feed supplement (essential amino acid)	As above	$1.5 billion
	Phenylalanine	Nutritional and food/feed supplement, aspartame, pharmaceutical use	Corynebacteria and *Escherichia coli*	$1 billion
Nucleotides	Guanylic (GMP) and inosinic (IMP) acids	Flavour enhancers	Corynebacteria	$360 million
Vitamins	Riboflavin (B_2)	Nutritional supplement, food colourant	*Eremothecium ashbyii, Ashbya gossypii, Candida* species	$150 million
	Cobalamin (B_{12})	Nutritional supplement, pharmaceutical use	*Propionibacterium shermanii, Pseudomonas denitrificans*	$105 million
Organic acids	Citric acid	Food flavour, acidulant, preservative, emulsifying agent; effervescence formulae in beverages; chelating agent in cleaning formulations, soaps, detergents; pharmaceutical formulations	*Aspergillus niger*	$2 billion

by identifying new target sites (such as transporters) for genetic manipulation[14]. Activity-based protein profiling, which can identify enzyme activity and inhibitors in proteomes, is well established and will help clarify protein function for uncharacterised proteins as well as identify cellular targets of natural products[24]. Handling large datasets and developing the algorithms for DNA and proteome analysis, and functional assignment, will remain a challenge as technology evolves. The contribution of mathematics and robotics has been profound in advancing molecular genetics.

Other analytical methods in chemistry were equally important in underpinning this revolution: the ability to separate small molecules was advanced by the development of gas chromatography in 1952, the first technology to separate isomeric forms of chemicals readily[25]. Rapid liquid chromatography, which later evolved into high-performance liquid chromatography, followed in the late 1960s[26]. Ion spectroscopy was first developed in the 1960s, using photoelectron spectroscopy to characterise intermediate size molecules. In the next decade, photodissociation was employed to analyse ions and the field developed over the next 30 years to underpin analysis of ions generated by several methods[27]. Electrospray ionisation was conceptualised in the 1970–80 period, although earlier work dated back to 1963: coupling the technology patented in 1985 to liquid chromatography stimulated interest from biologists in the early 1990s to apply this to determining the mass of ionised biological molecules, expanding the earlier utility of GC-MS in compound identification[28]. Mass spectrometry technology rapidly evolved into matrix-assisted, laser desorption/ionisation time-of-flight (MALDI-ToF) instrumentation, underpinning the mass and structural determination of peptides as well as proteomics. Again, construction of chemical data bases and computational tools for their analysis was equally important in providing the analytical tools for identification of biological molecules[29].

Revolution: turning of the cycle?

We are critically aware that microbial diversity is vast and that we are still discovering the metabolic capability of microbes that cannot be cultured, either from lack of knowledge of appropriate growth conditions or because of close symbiotic relationships that preclude isolating individuals within consortia. Co-cultures are of great interest in the production of natural product drugs[30] as well as for biofuels[31]. Co-culture in food production and environmental services is well understood and there are many examples in the literature where microbes collaborate to use substrates that cannot be degraded by any single strain but where significant biological and commercial outcomes occur[2]. Our own work on microbial degradation of polycyclic aromatic hydrocarbons (PAHs) demonstrated this phenomenon. After enriching for bacteria on individual PAHs from contaminated soils from Melbourne and Sydney, several pure cultures of bacteria were shown to degrade pyrene (4-ringed PAH) to CO_2 and water (mineralisation) but they could not grow on or significantly degrade 5-ring compounds (benzo[*a*]pyrene, BaP; dibenz [*a,h*] anthracene, DBA). However, some strains of *Stenotrophomonas maltophilia* could degrade BaP and DBA in the presence of pyrene, but intermediates of breakdown impaired total degradation of the 5-ringed compounds[32]. A consortium of a single fungal species, *Penicillium janthanellum*, and bacteria was isolated from a contaminated site in Western Victoria, and could grow on pyrene and other single or mixed substrates of lower molecular weight PAHs. The fungus was isolated and tested with the unrelated strains of *S. maltophilia*, in catabolism of pyrene and pyrene with BaP or DBA. Although the strains had not co-evolved and came from different locations, the co-cultures

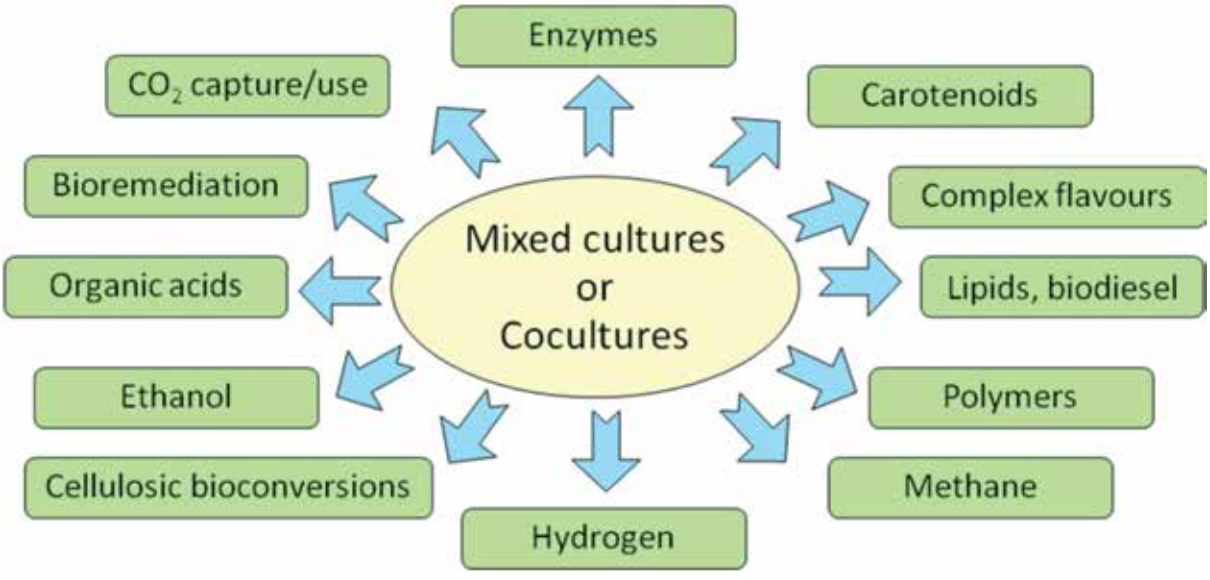

Figure 1. Areas where mixed cultures or co-cultures may impact on industrial microbiology in the future. Based on Bader *et al.* (2010)[2].

could cooperatively degrade and mineralise BaP[33]. Although the mechanism of co-degradation was not elucidated, it was clear that this only occurred due to ring-opening by either species, metabolism that alleviated inhibition by bacterial degradation products of BaP and enabled mineralisation[34]. The consortium could also degrade and detoxify PAHs in soil, demonstrating the stability of this cooperative system.

The potential of artificial consortia, as demonstrated in this PAH example, in biotechnology and the discovery of new unculturable species, particularly at the branch-points of microbial evolution, will be a rich area for future research that will identify new services, applications of consortia and novel bioactives (Figure 1). Of environmental interest is the future discovery of new fuels, elucidating how soil quality and functionality can be influenced by microbial consortia, how microbial ecosystems otherwise impact on CO_2 capture and cycling, and the utility of microbes in the global cycling of S, N and P. The next ambitious target will be the genetic manipulation of whole communities of microbes[23].

Koch's postulates may be sorely tested, as we go back to re-explore the ancient microbiology of consortia to create the next revolution in industrial microbiology.

> *... my work, which I've done for a long time, was not pursued in order to gain the praise I now enjoy, but chiefly from a craving after knowledge, which I notice resides in me more than in most other men. And therewithal, whenever I found out anything remarkable, I have thought it my duty to put down my discovery on paper, so that all ingenious people might be informed thereof.* Antony van Leeuwenhoek. Letter 12 June 1716.

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Prof. Demain pioneered discoveries in genetic and nutritional regulation of biosynthetic pathways leading to overproduction of primary and secondary metabolites and their scale-up in manufacturing processes. His distinguished career, following his time in industry with Merck and at MIT, continues as a Research Fellow in the Charles A. Dana Research Institute for Scientists Emeriti (RISE) at Drew University, NJ, USA.