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Review

Metabolic regulation and overproduction of primary metabolites

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Summary

Overproduction of microbial metabolites is related to developmental phases of microorganisms. Inducers, effectors, inhibitors and various signal molecules play a role in different types of overproduction. Biosynthesis of enzymes catalysing metabolic reactions in microbial cells is controlled by well-known positive and negative mechanisms, e.g. induction, nutritional regulation (carbon or nitrogen source regulation), feedback regulation, etc. The microbial production of primary metabolites contributes significantly to the quality of life. Fermentative production of these compounds is still an important goal of modern biotechnology. Through fermentation, microorganisms growing on inexpensive carbon and nitrogen sources produce valuable products such as amino acids, nucleotides, organic acids and vitamins which can be added to food to enhance its flavour, or increase its nutritive values. The contribution of microorganisms goes well beyond the food and health industries with the renewed interest in solvent fermentations. Microorganisms have the potential to provide many petroleum-derived products as well as the ethanol necessary for liquid fuel. Additional applications of primary metabolites lie in their impact as precursors of many pharmaceutical compounds. The roles of primary metabolites and the microbes which produce them will certainly increase in importance as time goes on. In the early years of fermentation processes, development of producing strains initially depended on classical strain breeding involving repeated random mutations, each followed by screening or selection. More recently, methods of molecular genetics have been used for the overproduction of primary metabolic products. The development of modern tools of molecular biology enabled more rational approaches for strain improvement. Techniques of transcriptome, proteome and metabolome analysis, as well as metabolic flux analysis. have recently been introduced in order to identify new and important target genes and to quantify metabolic activities necessary for further strain improvement.

1. Introduction

Primary metabolites are microbial products made during the exponential phase of growth whose synthesis is an integral part of the normal growth process. They include intermediates and end-products of anabolic metabolism, which are used by the cell as building blocks for essential macromolecules (e.g. amino acids, nucleotides) or are converted to coenzymes (e.g. vitamins). Other primary metabolites (e.g. citric acid, acetic acid and ethanol) result from catabolic metabolism; they are not used for building cellular constituents but their production, which is related to energy production and substrate utilization, is essential for growth. Industrially, the most important primary metabolites are amino acids, nucleotides, vitamins, solvents and organic acids. These are made by a diverse range of bacteria and fungi and have numerous uses in the food, chemical and nutriceutical industries. Many of these metabolites are manufactured by microbial fermentation rather than chemical synthesis because the fermentations are economically competitive and produce biologically useful isomeric forms. Several other industrially important chemicals could be manufactured via microbial fermentations (e.g. glycerol and other polyhydroxy alcohols) but are presently synthesized cheaply as petroleum by-products. However, as the cost of petroleum has

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skyrocketed recently, there is now renewed interest in the microbial production of ethanol, organic acids and solvents.

Living cells derive energy through metabolism employing reduction and oxidation (redox) reactions (Garcia-Vallve, 2004). The oxidation of carbon sources, e.g. glucose, and the transfer of electrons involve two paths: biosynthesis and energy metabolism. Only a small part of the electrons are used in reduction reactions to supply new cellular material (i.e. biosynthesis). Most are passed to terminal electron acceptors either directly or via a pathway of redox reactions. Terminal electron acceptors are necessary to maintain a redox balance in the cell. In aerobes, oxygen is the ultimate electron acceptor yielding water as product. For the anaerobes, a large number of acceptors are used producing many products (alcohols, fatty acids, H_2). Anaerobes cannot synthesize an O_2-linked energy conversion system and thus cannot use O_2 as the terminal electron acceptor. They also show a wide range of sensitivity to oxygen, some being killed by exposure to even traces of O_2.

Bacteria such as streptococci and clostridia have no respiratory chain but possess complexes of integral membrane proteins and freely diffusible molecules that shuttle electrons from one complex to the next. Thus, the reducing equivalents that are produced by carbon source catabolism cannot be reoxidized by oxygen or nitrate, i.e. external electron acceptors. Instead, organic intermediates of catabolism (like fumarate or succinate) are used and the reduced products are excreted. These are the primary metabolites of such cultures.

2. Regulation of primary metabolism

Microbial metabolism is a conservative process that usually does not expend energy or nutrients to make compounds already available in the environment, and does not overproduce components of intermediary metabolism. Coordination of metabolic functions ensures that, at any given moment, only the necessary enzymes, and the correct amount of each, are made. Once a sufficient quantity of a material is made, the enzymes concerned with its formation are no longer synthesized and the activities of preformed enzymes are curbed by a number of specific regulatory mechanisms such as feedback inhibition.

Transcription is the principal site for control of bacterial and eukaryotic expression and is dependent on transcription factors, i.e. proteins which bind near or at promoters, thus activating or repressing transcription initiation in response to extracellular signals. To initiate transcription in bacteria, RNA polymerase must associate with a particular sigma factor (σ). Sigma factors are small proteins that direct RNA polymerase to specific classes of promoter sequences (Woesten, 1998). In most bacteria, sigma A or sigma D, also known as sigma 70 (the major 'housekeeping' sigma factor) controls the major housekeeping functions and most RNA synthesis in the growth phase. However, there are additional sigma factors, which recognize different consensus sequences. These sigma factors not only allow the cell to carry out basal gene expression and exponential growth but also to respond to developmental or environmental signals. The number of sigma factors depends on the bacteria; thus *Escherichia coli* makes seven sigma factors whereas *Bacillus subtilis* makes seventeen. There are also anti-sigma factors which bind to and inhibit sigma factor function, thus preventing the interaction of the latter with RNA polymerases. There are even anti-anti-sigma factors, which are antagonists of anti-sigma factors (Mittenhuber, 2002). A wide range of cellular processes are regulated by anti-sigma factors, including bacteriophage growth, sporulation, stress response, flagellar biosynthesis, pigment production, ion transport and virulence expression.

The primary control of gene expression in eukaryotes is also at the level of transcription and is exerted by transcription factors. While prokaryotic transcription factors bind close to the gene to be transcribed, eukaryotic transcription factors often bind hundreds or thousands of base pairs upstream of the gene. Upstream of about 80% of eukaryotic genes is the TATA box (i.e. TATA is part of the sequence), which binds one type of transcription factor. Transcription factors include (i) helix–turn–helix structures, (ii) zinc fingers, (iii) leucine zippers, (iv) helix–loop–helix structures and (v) high-mobility groups as their binding mechanism. After binding to DNA, the factors interact with other factors or with RNA polymerase itself to modulate transcription either in the positive direction [transcription activation (the usual case)] or in the negative direction (transcription repression). The interaction is a function of other domains in the transcription factor, which have a high concentration of acidic amino acids, glutamine residues or proline residues. Transcription repression usually occurs when a repressive transcription factor binds to DNA and blocks the attachment or action of activating transcription factors. Control of the transcription factor itself occurs by regulating its activity by protein–protein interaction, phosphorylation or glycosylation.

RNA polymerase catalyses the sequential addition of ribonucleotides using the bases of one strand of DNA as template at a rate of 43 bases s^{-1} (Richardson, 1993). The elongation process is very stable requiring termination signals at the end of a gene or operon to prevent transcription of neighbouring genes. Sometimes, proteins such as rho factor are required for termination of certain elongation processes. Termination is also important in attenuation control and antitermination. In attenuation, a terminator sequence is present in the leader region

forming a termination structure in the mRNA and preventing transcription of the gene or operon (e.g. tryptophan in the case of the intrinsic terminator *trpA*). As a result, the terminator structure in the mRNA is not formed and the gene or operon is transcribed. This is often the case in amino acid biosynthetic operons. In anti-termination, a terminator is present but under certain conditions, it can be bypassed, thus allowing transcription. These terminators are upstream of the first gene of an operon and/or between genes of an operon. Often, the first gene of an operon encodes a regulatory RNA-binding protein, which binds to the terminator structure in mRNA and interferes with termination. Control of the operon is carried out by a metabolic signal such as an inducer.

2.1 Regulatory mechanisms involved in the biosynthesis of primary metabolites

2.1.1 Induction. This is a control mechanism by which a substrate (or a compound structurally similar to the substrate, or a metabolically related compound) 'turns on' the synthesis of enzymes, which are usually involved in the degradation of the substrate. Enzymes that are synthesized as a result of genes being turned on are called inducible enzymes and the chemical that activates gene transcription is called the inducer. Inducible enzymes are produced only in response to the presence of their substrate and, in a sense, are produced only when needed. In this way, the cell does not waste energy synthesizing unneeded enzymes. The inducer molecule combines with a repressor at the DNA level and thereby prevents the blocking of an operator by the repressor, leading to the transcription of the gene and translation of the messenger RNA encoding the enzyme. Although most inducers are substrates of catabolic enzymes, products can sometimes function as inducers. As examples, malto-dextrins can induce amylase, fatty acids induce lipase, urocanic acid induces histidase, and galacturonic acid induces polygalacturonase. Some coenzymes induce enzymes, as in thiamine induction of pyruvate decarboxylase. Substrate analogues that are not attacked by the enzyme ('gratuitous inducers') are often excellent inducers of enzyme synthesis.

The most thoroughly studied inducible enzyme system is that for lactose hydrolysis in *E. coli*, which provided the basis of a model system for negative control of protein synthesis (Jacob and Monod, 1961). Negative control means that a regulatory protein encoded by a regulator locus interferes with transcription. In the case of the *lac* operon in *E. coli*, about 10 molecules of repressor are made per regulator locus. The operator locus of the *lac* operon is 27 base pairs long. The *lac* repressor is a tetramer protein with a molecular mass of 150 000 containing 347 amino acid residues. In *Pseudomonas putida*, tryptophan synthetase is induced by indoleglycerophosphate and the entire tryptophan branch is induced by chorismate in *B. subtilis*.

Positive regulation of transcription by the regulator locus is another type of control mechanism. Here, the regulatory protein encoded by the regulator gene is necessary for transcription to occur. Binding of the inducer activates this regulatory protein. The complex binds at the operator region and turns on gene expression. Positive control occurs in *E. coli* for utilization of L-rhamnose, maltose and arabinose. Another induction system involving positive control is galactose utilization in *Saccharomyces cerevisiae*. The system consists of seven genes and no operons. Five of the pathway genes are regulated by galactose but not *gal5* (encoding phosphoglucomutase), which is constitutive. The system involves four different chromosomes. The GAL4 protein transcriptionally activates the other five genes. The GAL80 protein binds directly to GAL4 preventing its activating function. The inducer, formed from galactose by the seventh gene, *gal3*, inactivates GAL80 thus allowing GAL4 to activate transcription of the five pathway genes. Induction in filamentous fungi such as *Aspergillus nidulans* is mainly of the positive control type.

2.1.2 Carbon source regulation. Like enzyme induction, carbon source regulation [more commonly known as carbon catabolite repression (CCR)] is one of the conservative mechanisms which safeguards against wasting a cell's protein-synthesizing machinery, and operates when more than one utilizable substrate is present in the environment. The cell produces enzymes to catabolize the most rapidly assimilated carbon source while synthesis of enzymes utilizing other substrates is repressed until the primary substrate is exhausted. The repressed enzymes are usually inducible. Carbon catabolite repression is a phenomenon usually caused by glucose, but in different organisms, other rapidly metabolized carbon sources can cause repression and, indeed, sometimes repress catabolism of glucose. An example of this occurs in *Pseudomonas aeruginosa*, where citrate is the preferred carbon source over glucose (Ng and Dawes, 1973). In *Pseudomonas*, there are up to five overlapping CCR systems coordinating carbon utilization (Rojo and Dinamarca, 2004) and even different CCR systems modulate catabolite repression simultaneously (Del Castillo and Ramos, 2007).

Several mechanisms for CCR have been reported in microorganisms. One involves the phosphoenolpyruvate-:phosphotransferase system (PTS) which utilizes a protein phosphoryl transfer chain to transport and phosphorylate its sugar substrates.

In *E. coli*, PTS consist of four high-energy phosphoprotein intermediates and five protein domains. One of these

proteins, $EIIA^{glc}$, is phosphorylated by a heat-stable phosphoprotein (HPr). In this form, $EIIA^{glc}$~P transfers its phosphate to high-affinity protein EIIB/C. For this purpose, $EIIA^{glc}$ contains two histidines (His75 and His90). His90 is the acceptor for the phosphate group from HPr and His75 is important for its transfer to a high-affinity enzyme IIB/C. Enzyme IIB/C occurs in the membrane as a homodimer. The amino acid chain of domain IIC crosses the membrane eight times harbouring the sugar binding site. The hydrophilic domain IIB transfers the phosphate group from $EIIA^{glc}$~P to the glucose, producing glucose 6-phosphate.

Besides transferring the phosphate group, $EIIA^{glc}$~P activates adenylate cyclase. Activated adenylate cyclase synthesizes cyclic 3′,5′-adenosine monophosphate (cAMP), which has been defined as a second messenger. This nucleotide is necessary for synthesis of inducible enzymes and its intracellular levels mediate carbon catabolite repression. To activate transcription, cAMP binds to the DNA promoter region via a specific binding protein (cAMP receptor protein or CRP), a dimer of identical subunits and two separate domains. Each CRP subunit finds one cAMP molecule and after binding, undergoes an allosteric transition to an active state in which it binds to specific portions of promoter DNA. The N-terminal attaches to cAMP and the C-terminal to DNA thus increasing the affinity of RNA polymerase to that particular promoter and thus the frequency of transcription (Botsford and Harman, 1992). The consensus sequence to which CRP binds in the presence of cAMP [aa-TGTGA(N_7)CACa-t] occurs at a variety of locations in the promoter relative to the start site for transcription (Gottesman, 1984). As promoters of different operons have different affinities for the complex (Piovant *et al.*, 1975), not all promoters are binding the complex and undergoing transcription initiation at the same time. In the presence of glucose, the sugar is transported into the cell and concomitantly phosphorylated. This event causes dephosphorylation of $EIIA^{glc}$~P, mediates inducer exclusion and deactivates adenylate cyclase (Stewart, 1993). Inactivation of adenylate cyclase causes the cytoplasmic cAMP concentration to diminish and promotes dissociation of the cAMP–CRP complex from the DNA and deactivation of transcriptional initiation. In its phosphorylated form (no glucose present), $EIIA^{glc}$ has no activity to exclude inducers and activates adenylate cyclase (De Reuse and Danchin, 1991). The gene for $EIIA^{glc}$ is called *crr*, because mutants of *E. coli* lacking this gene are not subject to CCR.

During glucose assimilation, the intracellular concentration of cAMP is depressed 1000-fold, whereas metabolism of a non-repressive carbon source has little effect on cAMP levels. cAMP reverses CCR of many enzymes in *E. coli*. Mutants that cannot make CRP or adenylate cyclase fail to grow, or grow poorly, on lactose, glycerol and other carbon sources, whereas mutants lacking cAMP phosphodiesterase (which degrades cAMP to AMP) are insensitive to CCR (Monard *et al.*, 1969). Transport systems known to inhibit adenylate cyclase include those of the PTS (glucose, mannitol), proton symport (lactose) and facilitated diffusion (glycerol). Protein kinase in *E. coli* is independent of cAMP (Dadssi and Cozzone, 1985).

Carbon catabolite repression occurs in other organisms such as *Bacillus* species, *P. aeruginosa*, *Arthrobacter crystallopoietes*, *Rhizobium meliloti* and anaerobic bacteria, e.g. *Bacteroides fragilis*. However, in some of these microorganisms, cAMP has not been detected, nor has it been shown to play a role in CCR. cAMP was found in *B. subtilis* but only when grown with oxygen limitation (Mach *et al.*, 1984). Adenylate cyclase and phosphodiesterase were also found under these conditions. cAMP was found in *Bacillus circulans* but only in media rich in glucose. Furthermore, its addition repressed the formation of xylanase (inducible) and 1,3,β-D-glucanases as did glucose (Esteban *et al.*, 1984). It appears that cAMP is a negative effector in this strain. Other strains of *B. circulans* and other *Bacillus* species (*megaterium* and *cereus*) do not contain cAMP.

In Gram-positive bacteria, carbon source utilization is regulated by carbon catabolite repression. Most of the knowledge on this regulatory mechanism has been obtained with *B. subtilis*. Here, CCR is due to a complex of two proteins acting at a *cis*-acting locus, upstream of catabolite repressible genes (Hueck and Hillen, 1995). The two proteins are a PTS-carrier protein (Hpr) and a catabolite control protein A (CcpA). It is known that uptake of a rapidly utilized sugar is effected by the PTS. Uptake leads to a build-up of glycolytic intermediates, which results in phosphorylation of protein HPr at Ser-46. The catalyst is an ATP-dependent protein kinase activated by fructose-1,6-diphosphate and other glycolytic intermediates. The phosphorylated HPr interacts with CcpA before binding, as a specific ternary complex, to the *cis*-active operator DNA sequence called *cre*, present in the promoter or the 5′ region of at least 29 genes, thus interfering with their expression. The complex consists of two molecules of HPr(Ser-P), a CcpA dimer and the *cre* sequence (Reizer and Reizer, 1996; Jones *et al.*, 1997). CcpA is composed of a helix–turn–helix DNA-binding domain and a C-terminal domain which binds to HPr(Ser-P) but not to unphosphorylated HPr. It causes repression of a number of enzymes such as α-amylase, gluconate kinase, β-glucanase, glucitol dehydrogenase, lichenase, mannitol-1-phosphate dehydrogenase and mannitol-specific PTS permease. In addition, it affects several operons like the xylose operon, the gluconate operon and the histidine-utilization operon. When glucose is low, a phosphatase inactivates Hpr(Ser-P) by dephosphoryla-

tion and carbon catabolite repression is relieved. Transcriptional profiling of *B. subtilis* in response to glucose revealed that: (i) the transcriptional regulator CcpA represses genes involving utilization of secondary carbon sources, (ii) glucose induces glycolytic enzymes, the genes involved in conversion of pyruvate to acetate with concomitant phosphorylation, and (iii) excess glucose represses genes required for complete oxidation of glucose [i.e. those of the tricarboxylic acid (TCA) cycle, and terminal respiration] (Blencke *et al*., 2003). Pentose phosphate cycle genes are unaffected by glucose.

In *S. cerevisiae*, cAMP is not thought to be a mediator of carbon catabolite repression (Eraso and Gancedo, 1984). In this yeast, glucose repression is mediated by both hexokinases (P1 and P2) but not by glucokinase. Hexokinase PII appears to be the repressor protein of glucose repression in *Saccharomyces carlsbergensis*, an organism which has three hexokinases (hexokinase PI and PII and glucokinase). Mutants in hexokinase PII are resistant to CCR of α-glucosidase and invertase. High glucose leads to increased hexokinase PII whereas glucose limitation leads to decreased hexokinase PII. Addition of xylose to high glucose led to 98% inactivation of hexokinase PII and derepression of invertase (Fernandez *et al*., 1985).

It is doubtful that cAMP plays any role in carbon catabolite repression in molds such as *A. nidulans* (Arst and Bailey, 1977). The gene for carbon catabolic repression in *A. nidulans* and *Aspergillus niger* is *creA* (Drysdale *et al*., 1993). It encodes a DNA-binding protein with two zinc-finger domains of the C_2H_2 class. The sequence 5′-SYGGRG-3′ has been proposed as the DNA consensus for Cre-A binding (Cubero and Scazzochio, 1994). A similar protein, Cre1, mediates glucose repression in *Trichoderma reesei* (Strauss *et al*., 1995). Similarity in amino acid sequence is 55% between Cre1 and CreA. The DNA sequence to which CreA binds is 5′-GCGGAG-3′ which matches well with the sequence of the above CreA binding site. These genes act by positive control. Genes *creB* and *creC*, possibly encoding membrane proteins, are also involved in carbon source regulation in *A. nidulans* in that their mutation leads to carbon source derepression of carbon controlled enzymes (Hynes and Kelly, 1977).

2.1.3 Nitrogen source regulation. Nitrogen can be assimilated from inorganic or organic sources. Its assimilation from inorganic sources requires reduction to ammonia, followed by incorporation into intracellular metabolites. The appropriate distribution of nitrogen among various pathways usually involves specific or local regulatory mechanisms, such as end-product inhibition or end-product-mediated transcriptional control. In addition, some global regulators control the expression of genes from several pathways and thereby coordinate metabolism. The ability to assimilate particular inorganic or organic nitrogen sources depends on the particular organism. Organic nitrogen sources are usually monomeric units of macromolecules (e.g. amino acids or nucleobases) or compounds derived from them (e.g. agmatine or putrescine). Ammonia usually supports the fastest growth rate and is therefore considered the preferred nitrogen source for *E. coli*. The biochemical basis of this 'ammonium preference' is explained by the repression of enzymes acting on the alternative nitrogenous substrates present in the culture medium. Nitrogen source regulation (NSR) is known by many other names such as nitrogen metabolite repression, nitrogen catabolite repression and ammonia repression. Enzymes typically under such control are proteases, amidases, ureases and those that degrade amino acids.

Key enzymes that are involved in the mechanism of NSR are those of ammonium assimilation such as NADP-glutamate dehydrogenase (GDH), glutamine synthetase (GS or GSI), glutamate synthase (GOGAT) and alanine dehydrogenase (ADH). It appears that they are not involved as regulatory proteins but simply as catalytic proteins; in the latter case, the pool sizes of one or more substrates and/or products of these enzymes, e.g. glutamine, glutamate or alanine, are critical in bringing about repression. In enteric organisms, the two enzymes which mainly account for NH_3 incorporation are: (i) GSI and (ii) GOGAT. They constitute a single system whereby GSI converts glutamate and NH_3 to glutamine and GOGAT converts one mole of glutamine and one mole of α-ketoglutarate to two moles of glutamate. The overall reaction produces glutamate from α-ketoglutarate and NH_3. GSI contains 12 identical subunits. GSI from enteric organisms and streptomycetes are post-translationally modified by adenylylation but those from *Bacillus* and *Clostridium* are not. The most active form of GSI is the unmodified form. The least active contains an adenylate molecule on each subunit. Nitrogen sufficiency leads to adenylylation whereas nitrogen deficiency leads to deadenylylation. Adenylylation is catalysed by adenylyl transferase (ATase). Adenylylation by ATase is promoted by deuridylated P_{II} which is produced by UR action on $P_{II}(UMP)_3$ under nitrogen sufficiency (high glutamine/α-ketoglutarate ratio). Deadenylylation by ATase is promoted by $P_{II}(UMP)_3$ formed by UTase action on P_{II} under nitrogen limitation (low glutamine/α-ketoglutarate ratio).

A regulatory gene (*glnG*) in *E. coli* and other enteric bacteria encodes nitrogen regulator I (NR_I), a dimer protein with a subunit weight of 54 000. NR_I is produced at a high level (90 molecules per cell) under nitrogen limitation and at a low level (5 molecules per cell) under nitrogen excess. It activates transcription of *glnA*, encoding glutamine synthetase, under nitrogen limitation and

represses it under conditions of excess nitrogen. NR_I binds to DNA at or near the promoter of *glnA* and is thought to regulate production of all nitrogen-regulated systems (Shiau *et al.*, 1992). Furthermore in enteric organisms, glutamine synthetase and the other enzymes are regulated in positive and negative directions by three genes (*ntrA*, *ntrB*, *ntrC*). Two of these, *ntrB* and *ntrC*, are located next to the glutamine synthetase structural gene; *ntrA* is at a distance. NtrA is a sigma factor also known as RpoN or σ^{54} (Elderkin *et al.*, 2005). The nitrogen regulation (ntr) system in enteric bacteria is responsible for activation of the glutamine synthetase operon (*glnAntrBC*), the uptake systems for glutamine (*glnHPQ*), arginine (*argT*) and histidine (*hisJQMP*), nitrate and nitrite assimilation (*nasFEDCBA*), and nitrogen fixation (*nifLA*). The system is complex (Merrick and Edwards, 1995) and responds to the sufficiency or limitation of the intracellular nitrogen pool. It is composed of four proteins: (i) response regulator NtrC, (ii) sensor histidine protein kinase NtrB, (iii) P_{II}, a small protein encoded by *glnB*, and (iv) uridyltransferase/uridyl-removing enzyme (Utase/UR) encoded by *glnD* (Fig. 1). For nitrogen-controlled genes to be turned on, they need phosphorylated NtrC. NtrC is the response regulator of the signal transduction system; NtrB is its partner sensor kinase. In its phosphorylated state (NtrC-P), it activates transcription of the nitrogen-regulated genes. It binds to DNA having a helix–turn–helix motif in its C-terminal domain. NtrB, the sensor protein kinase, catalyses its own phosphorylation and then NtrC phosphorylation under conditions of nitrogen deficiency. On the contrary, NtrC dephosphorylation occurs under nitrogen sufficiency. NtrB is cytoplasmic and dephosphorylates NtrC-P only when it interacts with protein P_{II} and can only phosphorylate NtrC when it interacts with $P_{II}(UMP)_3$. Protein P_{II} can either be in its native state (P_{II}) or its uridylated state [$P_{II}(UMP)_3$]. The uridylation reaction is carried out by Utase under conditions of nitrogen deficiency. The deuridylation reaction is catalysed by UR under conditions of nitrogen sufficiency. This uridylation/deuridylation system responds to the glutamine/α-ketoglutarate ratio. Low ratios indicate nitrogen limitation leading to P_{II}-uridylation and hence NtrC-P, whereas high ratios indicate nitrogen sufficiency leading to P_{II} deuridylation and NtrC.

In *P. aeruginosa*, glutamine appears to be the effector of nitrogen source repression. In a GS-negative mutant, urease and histidase are derepressed when growth was limited for glutamine; addition of ammonia or glutamate has no effect. Addition of glutamine does cause repression of these two enzymes as well as NADP-GDH (Janssen *et al.*, 1981). In *B. subtilis*, there are two transcriptional regulators of nitrogen metabolism, GlnR and TnrA (Fisher, 1999). They are very similar proteins, binding to the same DNA consensus sequence but are

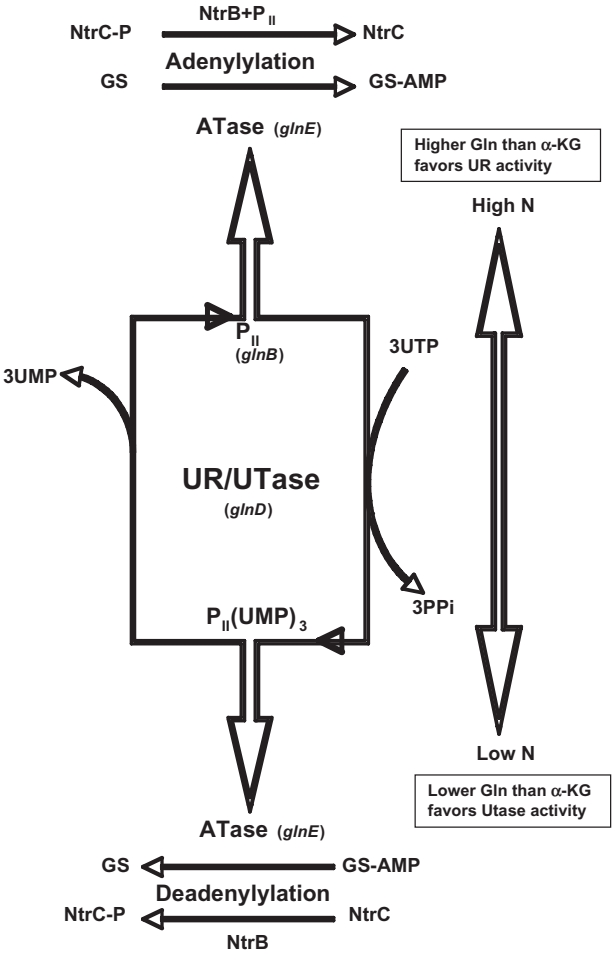

Fig. 1. Schematic model for the regulation of the GS activities and NtrC protein in response to nitrogen status. UTase (*glnD* product) catalyses the uridylylation of P_{II} (*glnB* product). UR activity of UTase catalyses P_{II} deuridylylation. Adenyltransferase (Atase: *glnE* product) catalyses the adenylylation and deadenylylation of GS. NtrB protein kinase catalyses the phosphorylation and dephosphorylation of NtrC, a DNA-binding response regulator.

active under different nutritional growth conditions. The activities of both proteins are regulated by GS. In the case of TnrA, the regulation involves a protein–protein interaction. TnrA is active only under conditions of nitrogen limitation. In nitrogen excess, TnrA becomes bound to the feedback inhibited form of GS and is unable to bind to DNA. When nitrogen becomes limiting, TnrA is released and can then bind to DNA. Gene *glnA* is part of a *glnRA* operon. Expression of *glnRA* is repressed by GlnR in media with high nitrogen (Fisher and Sonenshein, 1991). GlnR is small (125 residues) and dimeric, and binds to two operators upstream of *glnRA* to inhibit transcription (Gutowski and Schrier, 1992). Enzymes under N control are glutamine synthetase I, aspartase, asparaginase, urease, GABA permease but only GSI is regulated by GlnR; an unknown system(s) must regulate the others.

Nitrogen regulation in the yeast *S. cerevisiae* involves three main elements: (i) the enzymes catalysing the synthesis and interconversion of nitrogenous compounds, (ii) the permeases for uptake of nitrogenous compounds, and (iii) the transcription factors and membrane trafficking proteins which control the activity of the enzymes and permeases (Magasanik and Kaiser, 2002). Preferred nitrogen sources are glutamine, asparagine; those non-preferred are proline, α-aminobutyrate, ornithine, allantoin and urea. The expression of nitrogen-regulated genes is activated by transcription factors Gln3p and Ni11p with intracellular glutamine and glutamate acting as signals preventing such activation.

The gene controlling NSR in *Aspergillus* is *areA* (Marzluff, 1981) named *Nre* (for nitrogen regulator) in other fungi. The gene encodes a regulatory protein (AREA) exerting positive control on transcription. The regulatory protein is active under conditions of derepression (e.g. low ammonium supply). The intracellular effector appears to be glutamine rather than ammonia but more work is needed on this point. Mutations can be of the type *areA*^{r} in which a large variety of nitrogen sources can no longer be utilized for growth and the enzymes catalysing their usage cannot be derepressed, or another type, *areA*^{d} in which they cannot be repressed by ammonia; all still require inducer. Some *areA*^{d} type mutants produce more enzyme than their parents. There has been considerable controversy as to whether protein elements such as NADP-GDH or GS also play a direct role but, at present, the data favour glutamine itself as being the master effector in fungi. Glutamine produced in the presence of high ammonium, in some unknown fashion, causes nitrogen metabolite repression. Glutamine does not appear to bind directly to the AREA protein. In *Aspergillus*, factor NMR-A (similar to NMR found previously in *Neurospora*) acts negatively. Upon ammonium limitation, glutamine concentration would drop, the regulatory protein would assume an active conformation, and bind at the recognition sites of the structural genes. Nitrogen sources that are more repressive than ammonia are probably more easily converted to glutamine; that is, they may not have to be converted to ammonia before exerting repression. The stability of the AREA mRNA is controlled. It turns over rapidly during conditions of nitrogen repression but is more stable when nitrogen sources are limiting.

In *Neurospora crassa*, NSR is exerted by ammonium, glutamine and glutamate. Ammonium and glutamate may act via glutamine formation. These compounds repress the formation of the *nit-2* gene product which acts as a positive control agent for use of poorer nitrogen sources, i.e. the *nit-2* gene product is a positive effector for expression of structural genes encoding enzymes involved in the utilization of secondary nitrogen sources. One such repressible enzyme is an extracellular L-amino acid deaminase. Its expression requires inducer (one of many amino acids), lifting of nitrogen metabolite repression and the presence of the *nit-2* gene product. Glutamine does not appear to act directly to repress *nit-2*. Instead, another gene, *nmr-1*, binds to two regions of NIT2 and inhibits its function. NMR is not believed to be activated directly by glutamine, but another, still unknown, factor must bind glutamine and lead to nitrogen repression. Thus, mutations of *nmr-1* allow production of these enzymes in the presence of glutamine, NH_4 or glutamate (DeBusk and Ogilvie, 1984). In addition to the positively acting *nit-2* nitrogen control gene of *N. crassa*, a negatively acting *nmr* control gene exists. Unlike the situation in *Aspergillus*, the NIT2 mRNA is relatively stable under both nitrogen limitation and nitrogen repression conditions. An interaction between the NIT2 protein and the pathway-specific NIT4 protein is required for optimal expression of nitrate-inducible genes, e.g. *nit-3* which encodes nitrate reductase. It is also interesting that *Neurospora* possesses several additional GATA factors with DNA binding activities identical to or very similar to that of NIT2. It now appears that specific control is exerted in each case by interactions with proteins restricted to the distinct area of metabolism (Feng and Marzluff, 1998; Feng *et al*., 2000).

2.1.4 Phosphorus source regulation. In natural environments, inorganic phosphorus is commonly the major growth-limiting nutrient. Thus, biological systems have evolved a variety of responses to modulate their phosphorus requirement or to optimize its utilization. In *E. coli*, over 30 genes are part of the phosphate regulon (*Pho* regulon) and are transcriptionally activated by phosphorylated PhoB when the cell finds itself in low phosphate (Shinagawa *et al*., 1994). These genes encode proteins involved in uptake and utilization of phosphorus compounds. PhoR promotes the phosphorylation of PhoB under limiting phosphate conditions and dephosphorylation of PhoB in excess phosphate. PhoR and PhoB are thus a two-component signal transduction system. PhoR autophosphorylates and transfers the phosphate to PhoB. The environmental concentration of phosphate is monitored by the periplasmic phosphate-binding protein PstS, which transmits the signal for excess phosphate across the cytoplasmic membrane via PstC, PstA, PstB, PhoU to PhoR. Phosphorylated PhoB binds to the promoters of 31 genes containing *pho* boxes and interacts with RNA polymerase allowing initiation of mRNA synthesis.

Nucleases and phosphatases are usually repressed by phosphate in fungi. In addition, phosphate represses proteases, isocitrate lyase, fructose diphosphate aldolase, NADP isocitrate dehydrogenase and malate dehydrogenase in *Neurospora*. Phosphate also suppresses the production of riboflavin by *Eremothecium ashbyii* (Mehta and

Modi, 1981). Phosphate-derepressed mutants can be selected by growth with a phosphate ester (e.g. β-glycerol phosphate) as the sole source of carbon in the presence of high phosphate (Torriani and Rothman, 1961).

Of great interest is inorganic polyphosphate (poly P) which is a linear polymer of many tens or hundreds of orthophosphate (P_i) residues linked by high-energy phosphoanhydride bonds. Poly P is found in cells of all bacteria, archaea, fungi, protozoa, plants and animals and is prominent in many organisms, especially so in the vacuoles of yeast, where it may represent 10–20% of the cellular dry weight. It is produced by polyphosphate kinase (PPK), which catalyses the reversible transfer of the terminal phosphate of ATP to form a long-chain poly P (Ahn and Kornberg, 1990). Paradoxically, despite the huge amounts of poly P in yeast, PPK-like activity has not been found in whole-cell lysates, although a feeble activity has been extracted and partially purified from a vacuolar preparation. The *E. coli* gene (*ppk*) encoding PPK has been cloned, sequenced and overexpressed (about 100-fold). The gene possesses an open reading frame for 687 amino acids (mass of 80 278 Da). Polyphosphate kinase has been purified from overproducing cells after release from attachment to the cell outer membrane; the purified soluble PPK reassociates with cell membrane fractions. About 850 molecules of PPK are found in a wild-type cell. The poly P roles can be summarized as follows: substitute for ATP and energy source, reservoir for inorganic phosphate, chelator of metal ions, channel for DNA entry, regulator for stress and survival, and regulator of development. However, the most widely used and most significant roles of polyphosphate are probably regulatory control in nutritional stringencies, environmental stresses, stationary-phase adaptations and development (Kornberg *et al*., 1999).

2.1.5 Sulfur source regulation. Sulfatases are regulated by sulfate and sulfur amino acids. In addition to a variety of nutrients used to maintain continuous growth, bacteria require a source of sulfur. As for other nutrients, the use of sulfur is controlled by one or a few pleiotropic transcriptional regulatory proteins. Thus, in *E. coli* and the closely related *Salmonella typhimurium*, sulfur metabolism is controlled by the CysB transcriptional activator (Kredich, 1992). The cysteine regulon includes most of the genes required for synthesis of cysteine and genes for uptake of sulfur sources such as L-cystine, sulfate, thiosulfate and taurine. Transcriptional activation of these genes requires CysB, the inducer *N*-acetyl-L-serine and conditions of sulfur limitation. CysB is a tetrameric LysR-type regulator with an N-terminal DNA-binding domain, a central inducer-binding domain and a C-terminal oligomerization domain that is essential for stability (Lochowska *et al*., 2001). Its activity is regulated by an efflux pump specific for cysteine metabolites. CysB is also an autorepressor, preventing expression of its own structural gene, *cysB*. In *E. coli* and *S. typhimurium*, *cysB* mutations influence carbon oxidation and carbohydrate fermentation, and possibly carbon utilization. Both effects are at the transcriptional level and are partially reversed by exogenous cAMP or a sulfur source such as cysteine or djenkolate. The effect seems to be via the regulation of the cAMP biosynthetic enzyme, adenylate cyclase, which is activated by the IIA^{Glc} protein of the PTS. Cysteine inhibits inducer synthesis, resulting in maximal repression of the sulfur regulon. Growth with poor sulfur sources such as glutathione results in maximal derepression of the sulfur regulon.

Little is known about the genes and enzymes involved in sulfur assimilation in *B. subtilis*, or about the regulation of their expression or activity. Study of a wild-type strain grown with either sulfate or glutathione as sole sulfur source revealed that the synthesis of 15 proteins is modified under these two conditions (Coppee *et al*., 2001). In the presence of sulfate, an increased amount of proteins involved in the metabolism of C1 units (SerA, GlyA, FolD) and in the biosynthesis of purines (PurQ, Xpt) and pyrimidines (Upp, PyrA, PyrF) was observed. In the presence of glutathione, the syntheses of two uptake systems (DppE, SsuA), an oxygenase (SsuD), cysteine synthase (CysK) and two proteins of unknown function (YtmI, YurL) were increased. The *ytmI* gene is part of a locus of 12 genes which are co-regulated in response to sulfur availability. This putative operon is activated by a LysR-like regulator, YtlI. This is the first regulator involved in the control of expression in response to sulfur availability to be identified in *B. subtilis*.

In *N. crassa*, sulfate uptake is an important point of regulation of sulfur metabolism (Tao and Marzluf, 1998). Sulfate uptake is subject to sulfur (metabolite) repression in which excess sulfate turns off the expression of sulfate permease-encoding genes. Also, structural genes coding for aryl sulfatase, choline sulfatase, sulfate permeases I and II, a high-affinity methionine permease and an extracellular protease are turned on when sulfur becomes limiting. These unlinked genes are regulated by the *cys-3* gene, a positively acting master sulfur regulatory gene whose product activates their expression. This gene product is a 236-amino-acid residue protein containing a leucine zipper element in its basic region. The leucine zipper is characteristic of DNA-binding proteins (a leucine zipper is a protein sequence in which a leucine or methionine occurs at exactly every seventh position). The CYS-3 protein binds to DNA at the 5′-upstream portion of *cys-14* (encoding sulfate permease). It also binds to an upstream sequence of the *cys-3* gene itself suggesting the possibility of autoregulation. Mutation in the basic region eliminates DNA binding (Fu and Marzluf, 1990). Another regulatory gene, *scon*, controls the expression of

cys-3 in a negative fashion, i.e. *scon* mutants are insensitive to sulfur source repression and produce the enzymes constitutively.

2.1.6 Feedback regulation. The most important mechanism responsible for regulation of the enzymes involved in biosynthesis of amino acids, nucleotides and vitamins is not induction or nutrient repression, but feedback regulation. This category of regulation functions at two levels: enzyme action (feedback inhibition) and enzyme synthesis (feedback repression and attenuation).

In feedback inhibition, the final metabolite of a pathway, when present in sufficient quantities, inhibits the action of the first enzyme of the pathway to prevent further synthesis of intermediates and products of that pathway. Feedback repression involves the turning off of enzyme synthesis when sufficient amounts of the product have been made and it starts to accumulate. The end-product of the pathway acts as a co-repressor. The aporepressor specified by the regulator locus is inactive in the absence of its co-repressor and is unable to bind to the operator. However, in the presence of co-repressor, an active repressor is formed which binds to the operator to prevent transcription by RNA polymerase and hence prevents enzyme synthesis.

Many of the amino acid biosynthetic pathways are regulated not by the amino acids themselves but by their charged tRNA molecules. Thus, whereas feedback repression is effected by the amino acid end-products acting as co-repressors interfering with transcription initiation, another type of control called attenuation (transcription termination control) involves charged tRNA and transcription termination. Unless a significant number of the intracellular tRNA molecules for a particular amino acid are in the uncharged state, the genes coding for that amino acid's biosynthetic enzymes cannot be efficiently transcribed. In the presence of an excess of charged tRNA, transcription is initiated but terminated between the operator and the first structural gene (Kolter and Yanofsky, 1982). Attenuation is known to control certain bacterial amino acid biosynthetic operons, e.g. threonine, isoleucine, valine, leucine, phenylalanine, histidine. Unlike these operons, the tryptophan operon is regulated by both repression and attenuation, whose combined action permits a level of expression over a 600-fold range. Repression is responsible for an 80-fold range and attenuation for a sevenfold range. Repression responds to the level of tryptophan in the cell and attenuation to the level of charged $tRNA^{trp}$. The two mechanisms act at different degrees of tryptophan deprivation. Repression acts first, i.e. when the tryptophan level drops to that of moderate starvation, whereas attenuation acts in the moderate to severe tryptophan starvation range (Yanofsky *et al.*, 1984).

In *S. cerevisiae*, the *leu3* gene of the leucine biosynthetic pathway appears to be the master regulatory gene of specific control of branched amino acid biosynthesis. It encodes an 886-amino-acid polypeptide that is produced upon leucine starvation. This positively acting DNA binding protein (LEU-3) binds to a decapeptide palindromic sequence (CCGG pur pur CCGG) in the promoters of at least four genes of leucine biosynthesis and one gene of isoleucine-valine biosynthesis (Friden and Schimmel, 1988) and activates their transcription. This control probably acts on more than these five genes, i.e. on many genes of the branched amino acid pathway, all of which would have this decanucleotide sequence in their promoter. The site of this decanucleotide is within 130–376 nucleotides upstream of the transcriptional start site and defines a leucine-sensitive upstream activation sequence.

Feedback repression of purine nucleotide biosynthesis in *E. coli* is exerted by binding of the co-repressor, hypoxanthine or guanine, to the product of the *purR* gene (Rolfes and Zalkin, 1990). Hypoxanthine and guanine act cooperatively to change the conformation of PurR, thus enhancing its binding to DNA.

2.1.7 Additional types of regulation. Other types of regulation include metabolic interlock (Jensen, 1969), stringent control (Cashel *et al.*, 1996) and regulatory inactivation (Switzer, 1977). The effector of stringent control is the alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp) (Laurie *et al.*, 2003). It binds to the core of RNA polymerase resulting in activation or repression of gene expression. It is produced by ribosomes via ppGpp synthetase ('stringent factor') encoded by *relA*. Synthesis occurs via binding of uncharged tRNA to the ribosome A site. Control by ppGpp involves the effect of amino acid deficiency on a large number of physiological activities in bacteria (Gallant, 1979). Deficiency of any amino acid leads to production of ppGpp from GTP. This intracellular effector redirects the cells activities to correction of the amino acid deficiency. In stringent control, ppGpp shuts off stable RNA synthesis, i.e. rRNA and tRNA as well as mRNA for ribosomal proteins. The rate of total mRNA synthesis drops only modestly however. This shut-off acts at the level of transcription. It appears that ppGpp interferes with the binding of RNA polymerase to promoters of stringently controlled operons. The operons controlling threonine and isoleucine biosynthesis are positively regulated by ppGpp

Regulatory inactivation refers to the selective inactivation of enzymes (Switzer, 1977) by two different mechanisms. In modification inactivation, the enzyme remains intact but its physical state is changed or it is covalently modified. Covalent modifications include phosphorylation of a specific serine or threonine residue, nucleotidylation of a specific tyrosine residue, ADP ribosylation of an

arginine residue, methylation of a glutamate or aspartate carboxyl group, acetylation of an ε-amino group of a lysine residue or tyrosinolation of a protein terminal carboxyl group (Chock *et al*., 1980). In degradative inactivation, at least one peptide bond is broken; it may represent the first step in protein turnover. It is carried out by proteases which are restricted from non-selective action by confinement in vacuoles or by protease inhibitors. Regulatory inactivation usually occurs after the exponential phase of growth, especially after exhaustion of a source of carbon or nitrogen. This inactivation serves to prevent futile cycles of metabolism, to destroy enzymes no longer needed and to divert branch point metabolism from one branch to another.

3. Approaches to strain construction

3.1 Mutation and screening or selection

Organisms used today for industrial production of primary metabolites have been developed by programmes of mutation followed by selection or screening. Such efforts often start with organisms having some capacity to make the desired product but which require multiple mutations leading to deregulation in a particular biosynthetic pathway before high productivity can be obtained. The sequential mutations ensure that nutrients are channelled efficiently to the appropriate products without significant deviation to other pathways. These mutations presumably involve not only release of feedback controls but also enhancement of the formation of pathway precursors and intermediates. This approach to strain improvement has been remarkably successful in producing organisms that make industrially significant concentrations of primary metabolites. However, some of the problems with this 'brute force' approach include (i) the necessity of screening large numbers of mutants for the rare combination of traits sequentially obtained that lead to overproduction, and (ii) the weakened vigour of the producing strain following several rounds of mutagenesis.

3.2 Genetic engineering

More recent approaches utilize the techniques of modern genetic engineering to develop strains overproducing primary metabolites. This rationale for strain construction relies largely on the same principles of regulation discussed in the previous sections, but aims at assembling the appropriate characteristics by means of *in vitro* recombinant DNA techniques. This is particularly valuable in organisms with complex regulatory systems, where deregulation would involve many genetic alterations.

Production of a particular primary metabolite by deregulated organisms may inevitably be limited by the inherent capacity of the particular organism to make the appropriate biosynthetic enzymes, i.e. even in the absence of repressive mechanisms, there may not be enough of the enzyme made to obtain high productivity. One way to overcome this is to increase the number of copies of structural genes coding for these enzymes by genetic engineering. Another way often used in combination with this strategy is to increase the frequency of transcription, which is related to the frequency of binding of RNA polymerase to the promoter region (Rosenberg and Court, 1979). The former can be achieved by incorporating the biosynthetic genes *in vitro* into a plasmid which, when placed in a cell by genetic transformation, will replicate into multiple copies; some 'amplifiable' plasmids, such as pBR322, can exist at a level of 50 copies per cell. Increasing the frequency of transcription involves constructing a hybrid plasmid *in vitro*, which contains the structural genes of the biosynthetic enzymes but lacks the regulatory sequences (promoter and operator) normally associated with them. Instead, the structural genes are placed next to an efficiently and frequently read promoter and operator, and are now subject to regulation by these sequences. The ideal plasmid for metabolite synthesis would contain a regulatory region with a constitutive phenotype, preferably not subject to nutritional repression.

One of the major problems in using strains in which the desired characteristics are encoded by a plasmid is the difficulty in maintaining the plasmids during fermentation. Plasmid instability in the absence of selective pressure leads to a dilution of the plasmid in the population and loss of the desired phenotype. One solution is to use antibiotic pressure during fermentation so that only organisms resistant to the antibiotic (due to the presence of a plasmid-borne resistance gene) can survive. Plasmid stabilization was also accomplished by cloning the valyl-tRNA synthetase gene in a plasmid which was transformed into *E. coli* carrying a temperature-sensitive mutation in the chromosomal valyl-tRNA synthetase gene. At the non-permissive temperature, growth was dependent on the plasmid. The plasmid was stabilized for at least 150 generations (Nilsson and Skogman, 1986). Effective recombinant DNA plasmid construction in *E. coli* can lead to 20% of the total cell protein being that of a single protein.

Combinations of deregulation and plasmid amplification can yield a synergistic effect. For example, a chromosomal regulatory gene mutation in *E. coli* yielded fivefold overproduction of phosphatidylserine synthetase. Recombinant DNA technology using a plasmid containing the structural gene resulted in 10-fold overproduction. Putting both in the same strain led to 50-fold overproduction (Sparrow and Raetz, 1983).

3.3 Novel genetic technologies

'Genome-based strain reconstruction' achieves the construction of a superior strain which only contains mutations crucial to hyperproduction, but not other unknown mutations which accumulate by brute-force mutagenesis and screening (Ohnishi *et al*., 2002). This approach was used to improve lysine production (see Section 4.1.2).

The directed improvement of product formation or cellular properties via modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology is known as 'metabolic engineering' (Stephanopoulos, 1999; Nielsen, 2001). Analytical methods are combined to quantify fluxes and to control them with molecular biological techniques in order to implement suggested genetic modifications. Different means of analysing flux are (i) kinetic based models, (ii) control theories, (iii) tracer experiments, (iv) NMR magnetization transfer, (v) metabolite balancing, (vi) enzyme analysis and (vii) genetic analysis (Eggeling *et al*., 1996). The overall flux through a metabolic pathway depends on several steps, not just a single rate-limiting reaction (Kacser and Acerenza, 1993). Amino acid production is one of the fields with many examples of this approach (Sahm *et al*., 2000). Other processes improved by this technique include vitamins, carotenoids, organic acids, ethanol and 1,3-propanediol (see Sections 4.3, 4.4, 4.5.1 and 4.5.3).

A genome-wide transcript expression analysis called 'massive parallel signature sequencing' (Brenner *et al*., 2000) was successfully used to discover new targets for further improvement of riboflavin production by the fungus *Ashbya gossypii* (see Section 4.3.2). These recent technologies and mathematical approaches will all contribute to the generation and characterization of microorganisms able to synthesize large quantities of commercially important metabolites. Ongoing sequencing projects involving hundreds of genomes, the availability of sequences corresponding to model organisms, new DNA microarray and proteomics tools, as well as the new techniques for mutagenesis and recombination described above, will accelerate strain improvement programmes. The development and combined application of these technologies will help to develop what was already succinctly described several years ago as 'inverse metabolic engineering' (Bailey *et al*., 1996), i.e. a method to identify, construct or calculate a desired phenotype, identify the molecular basis of that desirable property, and incorporate that phenotype into another strain or other species by genetic and environmental manipulations.

Molecular breeding techniques such as 'DNA shuffling' come closer to mimicking natural recombination by allowing *in vitro* homologous recombination (Ness *et al*., 2000). These techniques not only recombine DNA fragments but also introduce point mutations at a very low controlled rate (Stemmer, 1994; Zhao and Arnold, 1997). Unlike site directed mutagenesis, this method of pooling and recombining parts of similar genes from different species or strains has yielded remarkable improvements in enzymes in a very short amount of time (Patten *et al*., 1997). 'Whole genome shuffling' is a novel technique for strain improvement combining the advantage of multiparental crossing allowed by DNA shuffling with the recombination of entire genomes. Such recursive genomic recombination has been used to improve acid tolerance of a commercial lactic acid-producing *Lactobacillus* sp. (Patnaik *et al*., 2002).

4. Microbial processes

4.1 Amino acid production processes

Among the amino acids, L-glutamate and L-lysine, mostly used as feed and food additives, respectively, represent the largest products in this category. Produced by fermentation are 1.5 million tons of L-glutamate and 850 000 tons of L-lysine-HCl. Table 1 shows the annual production of amino acids. The total amino acid market was about 4.5 billion dollars in 2004 (Leuchtenberger *et al*., 2005).

Top fermentation titres reported in the literature are shown in Table 2. Genetic engineering has made an impact by use of the following strategies: (i) amplification of a rate-limiting enzyme of pathway, (ii) amplification of the first enzyme after a branch point, (iii) cloning of a gene encoding an enzyme with more or less feedback

Table 1. Worldwide production of selected amino acids.

Example	Production (tons)	Method
L-Alanine	500	E
L-Aspartic acid	10 000	E
L-Arginine	1 200	F
L-Cysteine	4 000	C, E
L-Glutamate	1 500 000	F
L-Glutamine	2 200	F
Glycine	22 000	C
L-Histidine	400	F
L-Isoleucine	400	F, Ex
L-Leucine	500	F, Ex
L-Lysine	850 000	F
DL-Methionine	500 000	C
L-Phenylalanine	13 000	F, C
L-Proline	350	F
L-Serine	300	F
L-Threonine	70 000	F
L-Tryptophan	3 000	F, E
L-Tyrosine	170	F
L-Valine	500	F

C, chemical synthesis; E, enzymatic; Ex, extraction; F, fermentation. Ikeda (2003); Pfefferle *et al.* (2003); Kraemer (2004); Kroemer *et al.* (2004); Business Communications Company (2005); Wendisch (2005); Leuchtenberger *et al.* (2005); Wada and Takagi (2006); Li *et al.* (2007).

Table 2. Some examples of amino acids levels produced by fermentation.

Amino acid	Titre (g l^{-1})	Microorganism	Reference
L-Alanine	75	*Arthrobacter oxydans*	Hashimoto and Katsumata (1998)
L-Arginine	96	*Serratia marcescens*	Ikeda (2003)
L-Glutamate	88	*Brevibacterium lactofermentum*	Das (1995)
L-Glutamine	49	*Corynebacterium glutamicum*	Li *et al.* (2007)
L-Histidine	42	*Serratia marcescens*	Sugiura *et al.* (1987)
L-Isoleucine	30	*C. glutamicum*	Eggeling *et al.* (1997)
L-Leucine	34	*B. lactofermentum*	Tsuchida and Momose (1986)
L-Lysine-HCl	170	*C. glutamicum*	Kraemer (2004)
L-Methionine	25	*Brevibacterium heali*	Mondal and Chatterjee (1994)
L-Phenylalanine	51	*Escherichia coli*	Ikeda, 2003
L-Proline	108	*Clostridium acetoacidophilum*	Nakanishi *et al.* (1987)
L-Serine	65	*Methylobacterium* sp.	Ikeda (2003)
L-Threonine	100	*E. coli*	Debabov (2003)
L-Tryptophan	58	*C. glutamicum*	Ikeda and Katsumata (1999)
L-Tyrosine	26	*C. glutamicum*	Ikeda and Katsumata (1999)
L-Valine	99	*C. glutamicum*	Ikeda (2003)

regulation, (iv) introduction of a gene encoding an enzyme with a functional or energetic advantage as replacement for the normal enzyme, and (v) amplification of the first enzyme leading from central metabolism to increase carbon flow into the pathway followed by sequential removal of bottlenecks caused by accumulation of intermediates. Transport mutations have also become useful, i.e. a mutation decreasing amino acid uptake allows for improved excretion and lower intracellular feedback control. This has been especially useful in production of tryptophan and threonine. In cases where excretion is carrier-mediated, increase in activity of these carrier enzymes increases production of the amino acid. Exporter genes in *Corynebacterium glutamicum* are known for lysine, isoleucine and threonine.

Amino acids produced by microbial process are the L-forms. Such stereospecificity makes the process advantageous as compared with synthetic process. Microbial strains employed in microbial process for amino acid production are divided into four classes, i.e. wild-type strains, auxotrophic mutants, regulatory mutants and auxotrophic regulatory mutants. Using bacterial mutants, all the essential amino acids can be produced by 'direct fermentation' from cheap carbon sources such as carbohydrate materials or acetic acid.

Plasmid vector systems for cloning in *C. glutamicum* were established and amino acid production by *C. glutamicum* and related strains has been improved by gene cloning (Jetten and Sinskey, 1995; Kirchner and Tauch, 2003). Extensive research has been performed on sequencing the genome of *C. glutamicum* and to investigate its genetic repertoire (Moeckel *et al*., 1999). The genome was sequenced by Kyowa Hakko scientists (Ikeda and Nakagawa, 2003) and also by a collaboration of German workers (Kalinowski *et al*., 2003). The latter group reported a single circular chromosome with 3 282 708 base pairs, 3002 protein-coding genes of which 2489 could be assigned functions. The whole-genome sequence of *C. glutamicum* has been deposited in the DDBJ/GenBank/EMBL database under the Accession No. BA000036. The genome of the closely related glutamate-overproducing species, *Corynebacterium efficiens*, has also been sequenced (Nishio *et al*., 2003).

One of the key tasks in targeted strain optimization is the identification of genetic modifications that lead to improved strain characteristics. The experience of the past clearly shows that a detailed quantitative knowledge of metabolic physiology is required for the rational design of superior production strains. Metabolic reconstruction via functional gene annotation revealed fascinating insights into *C. glutamicum*, including functional predictions for over 60% of the identified genes (Ikeda and Nakagawa, 2003). Gene expression (transcriptome) analysis has been performed by the development of specific DNA microarrays which are being used to investigate gene expression during the growth of *C. glutamicum* (Wendisch, 2003). Expression profiles of selected genes of central metabolism (Loos *et al*., 2001) and amino acid production (Glanemann *et al*., 2003) have been determined. For proteome analysis, two-dimensional gel electrophoresis was used to identify different proteins and to study the influence of nitrogen starvation on the proteome (Schmid *et al*., 2000). An excellent review of proteomics in this organism has been published (Schaffer and Burkovski, 2005). For the quantification of metabolic fluxes (the 'fluxome'), comprehensive approaches combining ^{13}C tracer experiments, metabolite balancing and isotopomer modelling have been developed and applied to *C. glutamicum*. They involve comparative fluxome analysis during growth on different carbon sources, and glutamate and lysine production in batch cultures by different mutants (Kiefer *et al*., 2004). Reviews on metabolic engineering of amino acid producers include those of Sonntag and colleagues (1995), Eggeling and colleagues (1996)

and Ikeda (2003). A useful review of the amino acid fermentation field is Kraemer (2004).

4.1.1 L-Glutamic acid. Monosodium glutamate (MSG) is a potent flavour enhancer made by fermentation. The glutamic acid fermentation was discovered in Japan by Kinoshita, Udaka and Shimono in 1957 (Kinoshita *et al*., 1957). Although many genera and species are included in the group of glutamate overproducers, e.g. species of *Micrococcus*, *Corynebacterium*, *Brevibacterium* and *Microbacterium*, all are taxonomically similar and *Brevibacterium lactofermentum* and *Brevibacterium flavum* are now classified as *C. glutamicum* ssp. *lactofermentum* and ssp. *flavum* respectively. These organisms were shown to possess the Embden-Meyerhof glycolytic pathway (EMP), the pentose monophosphate pathway, the TCA cycle and the glyoxylate bypass (Kinoshita, 1985). The TCA cycle requires a continuous replenishment of oxaloacetate in order to replace the intermediates withdrawn for the synthesis of biomass and amino acids. This anaplerotic function is fulfilled by phosphoenolpyruvate carboxylase (Ozaki and Shiio, 1969) and a pyruvate-carboxylating enzyme (Tosaka *et al*., 1979). An excellent review of the metabolism of *C. glutamicum* has appeared (Wendisch, 2006).

Glucose is the preferred C source for *C. glutamicum* (Georgi *et al*., 2005). Fructose and sucrose are as good as glucose for glutamate production but not as good for lysine production (Kiefer *et al*., 2002). Glucose does not exert catabolite repression on use of other C sources except for L-glutamate (Wendisch, 2006).

Normally, glutamic acid overproduction would not be expected to occur due to feedback regulation. Glutamate feedback controls include repression of PEP carboxylase, citrate synthase and NADP-GDH; the last-named enzyme is also inhibited by glutamate. However, by decreasing the effectiveness of the cell barrier to outward passage, glutamate can be pumped out of the cell thus allowing its biosynthesis to proceed unabated. The excretion of glutamate frees the glutamate pathway from feedback control until excessive levels are accumulated.

Glutamate excretion is intentionally effected by various manipulations. Limitation of biotin was the first means discovered to bring about glutamate overproduction in *C. glutamicum*. All glutamate overproducers are natural biotin auxotrophs. Biotin is a cofactor of acetyl-CoA carboxylase which is essential for biosynthesis of fatty acids. The surprising report (Somerson and Phillips, 1961) that the addition of penicillin to cells grown in high biotin resulted in excretion of glutamic acid led Shiio and colleagues (1962) to postulate: (i) that growth of the glutamate-overproducing bacterium in the presence of non-limiting levels of biotin results in a cell envelope permeability barrier restricting the outward passage of intracellular amino acids out of the cell, and (ii) that inhibition of cell wall biosynthesis by penicillin alters the permeability properties of the cell envelope and allows glutamate to pass out of the cell. The commonality in the various manipulations that had been found to bring about high-level production of L-glutamic acid, i.e. limitation of biotin, addition of penicillin or fatty acid surfactants (e.g. tween 60) to exponentially growing cells, was recognized and the permeability mechanism was strongly supported (Demain and Birnbaum, 1968; Demain, 1971). Apparently all of these manipulations result in an altered lipid composition of the cell envelope, which favours active exit of glutamate from the cell. This view was further supported by the discoveries that oleate limitation of an oleate auxotroph (Kitano *et al*., 1972) and glycerol limitation of a glycerol auxotroph (Nakao *et al*., 1972) also brought about glutamate excretion. Both oleate and glycerol are precursors of phospholipids. Glutamate-excreting cells were later found to have a major decrease in cell lipids especially phospholipids (Laneelle and Clement, 1986). It thus became clear that high-level glutamate excretion required (i) growth inhibition in the presence of unlimited carbon and energy sources, and (ii) a change in strain on the envelope caused by deficiency of biotin, oleate or glycerol or addition of certain agents.

The cell envelope of *C. glutamicum* is very different from most Gram-positive bacteria and resembles those of Gram-negative bacteria (Schluesener *et al*., 2005). It contains the following layers: (i) plasma membrane which is mainly phosphatidylglycerol, (ii) peptidoglycan covalently attached to arabinogalactan esterified with mycolic acids, (iii) free mycolic acids and (iv) a crystalline protein layer known as the S layer. The permeability of the cell envelope is affected by lipid composition (Puech *et al*., 2000) and by specific import and export systems.

Despite the above evidence, the leaky envelope hypothesis was discounted (Hoischen and Kraemer, 1989), in favour of an efflux system specific for glutamate which was regulated by the energy state of the cell. However, the criticism was not absolute as these authors acknowledged the change in the composition of the cell membrane and stated that this membrane lipid alteration was an essential (but not sufficient) requirement for effective glutamate secretion. The action of biotin was attributed to effects on intermediary metabolism, correlating with the activity of fatty acid synthetases. Lambert and colleagues (1995) claimed that a change in membrane fluidity or general leakiness was not involved in glutamate excretion.

Studies of Kawahara and colleagues (1997), Kimura and colleagues (1999), Nakamatsu (2001) and Shimizu and colleagues (2003) led to another possibility, i.e. attributing glutamate overproduction to a decrease in the activity of α-ketoglutarate dehydrogenase (ODHC). In

addition to metabolic flux studies, a *C. glutamicum* mutant with a deletion of the *odhA* gene, encoding the E1 subunit of ODHC, was found to excrete high levels of glutamate without any of the above-mentioned triggers (biotin limitation or addition of penicillin), supporting that a change in the metabolic flux alone is sufficient to cause glutamate secretion (Asakura *et al*., 2007). This complex was shown to be regulated by a mechanism that involves a 15 kDa protein named OdhI and a serine/threonine protein kinase G (PknG). In its unphosphorylated state, OdhI binds to the E1 subunit (OdhA) of ODHC and, thereby, inhibits its activity. Inhibition is relieved by phosphorylation of OdhI at threonine residue 14 by PknG under conditions requiring high ODHC activity (Schultz *et al*., 2007).

In 2001, however, the permeability modification hypothesis was further supported. The various manipulations leading to glutamate overproduction were shown to cause increased permeability of the mycolic acid layer of the cell wall (Eggeling and Sahm, 2001). The glutamate-overproducing bacteria are characterized by a special cell envelope containing mycolic acids which surrounds the entire cell as a structured layer and is thought to be involved in permeation of solutes. The mycolic acids esterified with arabinogalactan and the non-covalently bound mycolic acid derivatives form a second lipid layer, the cytoplasmic membrane being the first. As stated by these authors, 'The concepts of "permeability of the cell wall" as originally used in the very first work on L-glutamate production more than forty years ago now takes on a new meaning'. Nampoothiri and colleagues (2002) provided evidence that overexpression or inactivity of genes involved in lipid synthesis changes glutamate efflux dramatically, alters the chemical and physical properties of the cytoplasmic membrane, and that this was necessary to achieve efflux of L-glutamate. Indeed, the authors state 'that altering the phospholipid content alone is sufficient to enable L-glutamate efflux'. Burkovski and Kraemer (2002) further stated that 'There is no doubt that stimulation of glutamate excretion in *C. glutamicum* is directly or indirectly related to membrane and/or cell wall integrity'. Further support to this view came from Radmacher and colleagues (2005) who showed that ethambutol (EMB), an anti-*Mycobacterium tuberculosis* agent, caused L-glutamate efflux by targeting the arabinosyltransferase of *C. glutamicum*. The consequence of EMB addition was a marked disorder of the cell envelope, due to less arabinan deposition in the cell wall arabinogalactan, and a reduced content of the cell wall-bound mycolic acids. Also, a mechanosensitive channel homologue has been found to induce glutamate production (Nakamura *et al*., 2007). It thus appears that either an increase in cell envelope permeability or a decrease in α-ketoglutarate dehydrogenase can elicit overproduction and excretion of glutamate.

L-Glutamate titres over 80 g l^{-1} have been described in the literature (Das *et al*., 1995; Delaunay *et al*., 1999). Metabolic engineering approaches have been used to analyse the fermentation (Sonntag *et al*., 1995; Eggeling *et al*., 1996; Takac *et al*., 1998; Kimura, 2003).

4.1.2 L-Lysine. The bulk of the cereals consumed in the world are deficient in the amino acid, L-lysine. This is an essential ingredient for the growth of animals, and is an important part of a billion-dollar animal feed industry. Lysine supplementation converts such cereals into balanced food or feed for animals for poultry, swine and other livestock. In addition to animal feed, lysine is used in pharmaceuticals, dietary supplements and cosmetics. It has also been shown to be useful in the prevention of atherosclerosis and for treatment of herpes simplex virus infections.

Lysine is a member of the aspartate family of amino acids. It is made in bacteria by a branched pathway that also produces methionine, threonine and isoleucine. This pathway is controlled very tightly in an organism like *E. coli*, which contains three aspartate kinases, each of which is regulated by a different end-product. In addition, after each branch point, the initial enzymes are inhibited by their respective end-products and no overproduction usually occurs. However, in lysine fermentation organisms (*C. glutamicum* and its relatives), there is a single aspartate kinase which is regulated via concerted feedback inhibition by threonine plus lysine. Molar fluxes through various pathways leading to glutamate or lysine have been measured (Sonntag *et al*., 1995). Whereas the pentose phosphate pathway only contributes 20% of the total carbon flux for glutamate formation, it contributes 60–70% for lysine overproduction (Ishino *et al*., 1991). This is evidently due to the high level of NADPH required for lysine formation. Use of rDNA technology has shown that the major limiting factors in lysine overproduction are: (i) feedback inhibition of aspartokinase by lysine plus threonine, (ii) the low level of dihydrodipicolinate synthase, (iii) the low level of PEP carboxylase and (iv) the low level of aspartase. Much work has been done on auxotrophic and regulatory mutants of the glutamate-overproducing strains for the production of lysine. By genetic removal of homoserine dehydrogenase, a glutamate-producing wild-type *Corynebacterium* strain is converted into a lysine-overproducing mutant that cannot grow unless methionine and threonine are added to the medium. As long as the threonine supplement is kept low, the intracellular concentration of threonine is limiting and feedback inhibition of aspartate kinase is bypassed, leading to excretion of over 70 g l^{-1} lysine in culture fluids. In some strains, addition of methionine and isoleucine to the medium increases lysine overproduction. Selection for S-2-aminoethylcysteine (AEC; thialysine) resistance

blocks feedback inhibition (up to 1 mM L-lysine plus 5 mM L-threonine) of aspartate kinase (Sahm, 1996). Other antimetabolites useful for deregulation of aspartate kinase include a mixture of α-ketobutyrate and aspartate hydroxamate. Leucine auxotrophy can also increase lysine production. A production strain (B-6) of *C. glutamicum* of Kyowa Hakko produces about 100 g l^{-1} L-lysine (Hayashi *et al*., 2006). Transcriptome analysis revealed that B-6, as compared with the wild type, is upregulated in the pentose–phosphate path and amino acid biosynthetic genes and downregulated in TCA cycle genes. L-Lysine titres are known to be as high as 170 g l^{-1} (Kraemer, 2004).

Excretion of lysine by *C. glutamicum* is by active transport reaching a concentration of several hundred millimolar in the external medium. Lysine, a cation, must be excreted against the membrane potential (outside is positive) and excretion is carrier-mediated (Fig. 2). It uses a 2 OH^-/lysine symporter (Broer *et al*., 1993) and is catalysed specifically by a dipeptide uptake system (Erdmann *et al*., 1993). The system is dependent on electron motive force, not ATP.

Genome-based strain reconstruction was used to improve the lysine production rate of *C. glutamicum* by comparing high-producing strain B-6 (production rate slightly less than 2 g l^{-1} h^{-1}) with a wild-type strain (Ohnishi *et al*., 2002). Comparison of 16 genes from strain B-6, encoding enzymes of the pathway from glucose to lysine, revealed mutations in five of the genes. Introduction of three of these mutations (*hom*, *lysC* and *pyc* encoding homoserine dehydrogenase, aspartokinase and pyruvate carboxylase respectively) into the wild type created a new strain which produced 80 g l^{-1} in 27 hours, at a rate of 3 g l^{-1} h^{-1}, the highest rate ever reported for a lysine fermentation. An additional increase (15%) in L-lysine production was observed by introduction of a mutation in the 6-phosphogluconate dehydrogenase gene (*gnd*). Enzymatic analysis revealed that the mutant enzyme was less sensitive than the wild-type enzyme to allosteric inhibition by intracellular metabolites. Isotope-based metabolic flux analysis demonstrated that the *gnd* mutation resulted in 8% increased carbon flux through the pentose phosphate pathway during L-lysine production (Ohnishi *et al*., 2005).

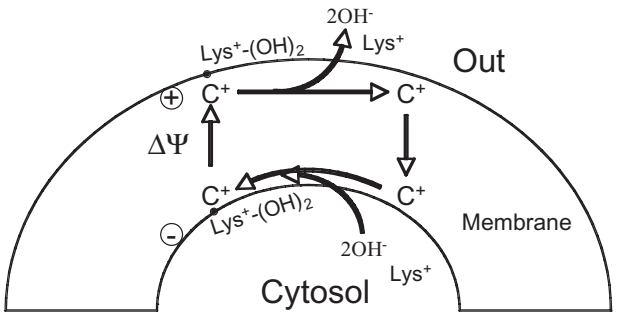

Fig. 2. Mechanism of L-lysine excretion. The carrier is loaded at the cytosolic side, translocates the substrate from the inner side and releases it outside the cell, and then the carrier is reconstituted. ΔΨ, electron motive force.

Metabolic engineering has been used in *C. glutamicum* to improve L-lysine production (Sahm *et al*., 2000). Metabolic flux studies of wild-type *C. glutamicum* and four improved lysine-producing mutants available from the ATCC showed that yield increased from 1.2% to 24.9% relative to the glucose flux.

4.1.3 L-Threonine. This amino acid is the second major amino acid used for feeding of pigs and poultry. The pathway of threonine biosynthesis is similar in all microorganisms. Starting from L-aspartate, the pathway involves five steps catalysed by five enzyme activities: aspartokinase (AK), aspartate-semialdehyde dehydrogenase (ASA-DH), homoserine dehydrogenase (HDI), homoserine kinase (HK) and threonine synthetase (TS).

Production of L-threonine has been achieved with the use of several microorganisms. In *Serratia marcescens*, Komatsubara and colleagues (1979) reported construction of a high threonine producer by transductional crosses which combined several feedback control mutations into one organism. Three classes of mutants were obtained from the parental strain as the source of genetic material for transduction: (i) strain HNr2l, in which both the threonine-regulated AK and HD were resistant to inhibition by threonine. It was selected on the basis of β-hydroxynorvaline resistance, (ii) strain HNr59, also selected for β-hydroxynorvaline resistance, in which HDI was resistant to both inhibition and repression and the threonine-regulated AK was constitutively synthesized, and (iii) strain AECrl74, which was resistant to thialysine, in which the lysine-regulated AK was resistant to feedback inhibition and repression. As at least one of the three key enzymes in threonine synthesis was still subject to regulation in these strains, each produced only modest amounts of threonine (4.1–8.7 g l^{-1}). Recombination of the three mutations by transduction yielded a strain which produced higher levels of threonine (25 g l^{-1}), had AK and HD activities which were resistant to feedback regulation by threonine and lysine, and was also a methionine bradytroph (leaky auxotroph). Another six regulatory mutations derived by resistance to amino acid analogues were combined into a single strain of *S. marcescens* by transduction. These mutations led to desensitization and derepression of AKs I, II and III and HDIs and II. The resultant transductant produced 40 g l^{-1} threonine (Komatsubara *et al*., 1983). The amino acid-overproducing *S. marcescens* strains were further improved by recombinant DNA technology. A mutant overproducing PEP carboxylase made 63 g l^{-1}

threonine, a 21% increase (Sugita and Komatsubara, 1989).

In *E. coli*, threonine production was increased to 76 g l^{-1} by conventional mutagenesis and selection/screening techniques. Of major importance were mutations to decrease regulation of the pathway and of degradation of the amino acid (Furukawa *et al.*, 1988). Recently, a comparative analyses of transcriptome, proteome and nucleotide sequences between a prototrophic (W3110) and an L-threonine-producing *E. coli* TF5015 was carried out (Lee *et al.*, 2003). The latter strain required both L-methionine and L-isoleucine for growth and showed resistance to various chemical analogues. Expression patterns of the genes and proteins were investigated for both strains by using DNA macroarrays, containing virtually every gene of *E. coli*, and two-dimensional gel electrophoresis. The profiles were analysed in terms of the accumulation of L-threonine and physiological consequences in the mutant strain. Upregulation of the *thr* operon in TF5015 was confirmed by both transcriptome and proteome analyses. Comparison of gene expression profiles between W3110 and TF5015 showed that only 54 of 4290 genes (1.3%) exhibited differential transcript expression patterns. This was an interesting result as TF5015 produces a much higher level of threonine compared with the parent strain W3110. DNA sequencing of the *thr* operon revealed a replacement of serine with phenylalanine at position 345 in the *thrA* product, AK I-HDI, of TF5015. Enzyme assay of mutated AK showed that the activity of AKI was not inhibited by threonine. Genetic analysis revealed that the mutation of *thrA* resulted in a release of feedback inhibition of AKI-HDI by threonine in TF5015 rather than a deregulation of feedback repression by threonine plus isoleucine. Therefore, the authors suggested that the mechanism of L-threonine production by TF5015 probably results from releasing feedback regulation and blocking carbon flow into undesirable by-products. An *E. coli* fed-batch process was devised with methionine and phosphate feeding which yielded 98 g l^{-1} L-threonine at 60 h (Lee, Lee *et al.*, 2004). Another *E. coli* strain has been developed via mutation and genetic engineering and optimized by inactivation of threonine dehydratase (TD) resulting in a process yielding 100 g l^{-1} in 36 h of fermentation (Debabov, 2003).

Threonine excretion by *C. glutamicum* is mainly (>90%) effected by a carrier-mediated export mechanism dependent on membrane potential but not on the presence of sodium ions (Palmieri *et al.*, 1996). It is probably an antiport system against protons. Passive diffusion accounts for less than 10%. Threonine uptake is by a separate transport mechanism involving a carrier in symport with Na ions. Cloning in extra copies of threonine export genes into an *E. coli* strain producing threonine led to increased production (Kruse *et al.*, 2002). Also increased was resistance to toxic antimetabolites of threonine. Another means of increasing threonine production is reduction in the activity of serine hydroxytransferase which breaks threonine down to glycine (Simic *et al.*, 2002).

In *C. glutamicum* ssp. *lactofermentum*, threonine production reached 58 g l^{-1} when a strain producing both threonine and lysine (isoleucine auxotroph resistant to thialysine, α-amino-β-hydroxyvaleric acid and S-methylcysteine sulfoxide) was transformed with a recombinant plasmid carrying its own *hom* (encoding HD), *thrB* (encoding HK) and *thrC* (encoding TS) genes (Ishida *et al.*, 1994). Medium modifications such as addition of complex nutrients, thiamine, biotin and NaCl were necessary for plasmid stability and solving growth lag problems.

4.1.4 L-Isoleucine. Isoleucine is of commercial interest as a food and feed additive and for parenteral nutrition infusions. This branched-chain amino acid is currently produced both by extraction of protein hydrolysates and by fermentation with classically derived mutants of *C. glutamicum.* The biosynthesis of isoleucine with *C. glutamicum* involves eleven reaction steps, of which at least five are controlled with respect to activity or expression. L-Isoleucine synthesis shares reactions with the lysine and methionine pathways. In addition, threonine is an intermediate in isoleucine formation, and the last four enzymes also carry out reactions involved in valine, leucine and pantothenate biosynthesis. Therefore, it is not surprising that multiple regulatory steps identified in *C. glutamicum*, as in other bacteria, are required to ensure the balanced synthesis of all these metabolites for cellular demands. In *C. glutamicum*, flux control is exerted by repression of the *homthrB* and *ilvBNC* operons. The activities of AK, HD, TD, and acetohydroxy acid synthase (AHAS) are controlled by allosteric transitions of the proteins to provide feedback control loops, and HK is inhibited in a competitive manner (Sahm, 1995). Isoleucine increased the Km of TD from 21 to 78 mM whereas valine reduced it to 12 mM. The AHAS was 50% feedback inhibited by isoleucine plus valine plus leucine.

Isoleucine processes were devised in various bacteria such as *S. marcescens*, *C. glutamicum* ssp. *flavum* and *C. glutamicum*. In *S. marcescens*, resistance to isoleucine hydroxamate and α-aminobutyric acid led to derepressed L-threonine deaminase (TDA) and AHAS and production of 12 g l^{-1} isoleucine (Kisumi *et al.*, 1977). Further work by Komatsubara and colleagues (1979) involving transductional crosses into a threonine overproducer yielded isoleucine at 25 g l^{-1}. The *C. glutamicum* ssp. *flavum* work employed resistance to α-amino-β-hydroxyvaleric acid and the resultant mutant produced 11 g l^{-1} (Shiio *et al.*, 1973). D-Ethionine resistance was used by Ikeda and colleagues (1976) to yield a mutant producing 33 g l^{-1} in a fermentation continuously fed with acetic acid.

A threonine-overproducing strain of *C. glutamicum* was sequentially mutated to resistance to thiaisoleucine, azaleucine and α-aminobutyric acid; it produced 10 g l^{-1} isoleucine (Kase and Nakayama, 1977). Metabolic engineering studies involving overexpression of biosynthetic genes were useful in improving isoleucine production by this species. Colon and colleagues (1995) obtained an isoleucine-producing strain by cloning multiple copies of *hom* (encoding HDI), and wild-type *ilvA* (encoding TD) into a lysine overproducer, and by increasing HK (encoded by *thrB*); 15 g l^{-1} isoleucine was produced. Independently, Morbach and colleagues (1995) cloned three copies of the feedback-resistant HD gene (*hom*) and multicopies of the deregulated TD gene (*ilvA*) in a deregulated lysine producer of *C. glutamicum*, yielding an isoleucine producer (13 g l^{-1}) with no threonine production and reduced lysine production. Application of a closed loop control fed-batch strategy raised production to 18 g l^{-1} (Morbach *et al.*, 1996). Further metabolic engineering work involving amplification of feedback inhibition-insensitive biosynthetic enzymes converted lysine overproducers and threonine overproducers into *C. glutamicum* strains yielding 30 g l^{-1} isoleucine (Sahm *et al.*, 1999).

4.1.5 L*-Proline.* The primary precursor for proline biosynthesis in bacteria is glutamate. Bacterial proline synthesis from glutamate occurs via three enzymatic reactions, catalysed by γ-glutamyl kinase (GK, the *proB* product), γ-glutamyl phosphate reductase (GPR) (*proA* product) and Δ^1-pyrroline-5-carboxylate reductase (P5C) (*proC* product). For the majority of bacteria, the *proB* and *proA* genes constitute an operon, which is distant from *proC* on the chromosome. For both prokaryotic and eukaryotic systems, proline synthesis from glutamate is regulated by feedback inhibition of the first enzyme in the pathway. Studies on purified enzymes suggest that in addition to proline-mediated inhibition, the γ-glutamyl kinase activities of GK and P5CS are also modulated to a lesser extent by glutamate and ADP, thereby tuning proline synthesis to cellular substrate and energy availability.

Proline-hyperproducing strains of bacteria, exhibiting reduced proline-mediated feedback inhibition of GK activity (a result of single-base-pair substitutions in the bacterial *proB* gene-coding region), have been isolated based on their resistance to toxic proline analogues (L-azetidine-2-carboxylic acid and 3,4-dehydro-DL-proline), compounds that inhibit GK activity while not interfering with protein synthesis. Cloning of the three genes of proline biosynthesis in *E. coli* on multicopy plasmids and selection of mutants of such plasmid-containing strains to resistance to 3,4-dehydroproline led to a process producing 20 g l^{-1} proline (Bloom *et al.*, 1984).

A sulfaguanidine-resistant mutant of *C. glutamicum* ssp. *flavum* produced 35 g l^{-1} proline (Tsuchida *et al.*, 1986). When a glutamate-producing strain of *C. glutamicum* was grown under modified conditions, it made 48 g l^{-1} (Nakanishi *et al.*, 1973). A strain of *Corynebacterium acetoacidophilum* produced 108 g l^{-1} proline when grown in the presence of glutamate (Nakanishi *et al.*, 1987).

A mutant of *S. marcescens* resistant to 3,4-dehydroproline, thiazolidine-4-carboxylate and azetidine-2-carboxylate and unable to utilize proline produced 50–55 g l^{-1} L-proline (Sugiura *et al.*, 1985a). Cloning of a gene bearing the dehydroproline-resistance locus on a plasmid yielded a recombinant strain of *S. marcescens* producing 75 g l^{-1} L-proline (Sugiura *et al.*, 1985b). Further development work increased production to over 100 g l^{-1} (Masuda *et al.*, 1993).

4.1.6 Aromatic amino acids. In *C. glutamicum* ssp. *flavum*, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (DAHPS) is feedback inhibited concertedly by phenylalanine plus tyrosine and weakly repressed by tyrosine. Other enzymes of the common pathway are not inhibited by phenylalanine, tyrosine and tryptophan but the following are repressed: shikimate dehydrogenase (SD), shikimate kinase (SK) and 5-enolpyruvylshikimate-3-phosphate synthase. Elimination of the uptake system for aromatic amino acids in *C. glutamicum* results in increased production of aromatic amino acids in deregulated strains (Ikeda and Katsumata, 1994). Cloning of relevant genes in various bacteria resulted in increases in aromatic amino acid production over those titres obtained by conventional mutagenesis and selection. Overproduction of aromatic amino acids and derivatives has been improved by metabolic engineering (Bongaerts *et al.*, 2001).

4.1.6.1 L*-Tryptophan.* L-Tryptophan has application as a supplement in animal feed. A tryptophan process was improved from 8 g l^{-1} to over 10 g l^{-1} by mutating the *C. glutamicum* ssp. *flavum* producer to azaserine resistance (Shiio *et al.*, 1982). Azaserine is an analogue of glutamine, the substrate of anthranilate synthase (AS). The mutant was two- to threefold derepressed in DAHPS, dehydroquinate synthase (DQS), SD, SK and chorismate synthase (CS). A further mutant (sulfaguanidine-resistant) showed additional increases in DAHPS and DQS and tryptophan production (Shiio *et al.*, 1984). The reason sulfaguanidine was chosen as the selective agent involves the next limiting step after derepression of DAHPS, i.e. conversion of the intermediate chorismate to anthranilate. Chorismate can also be undesirably converted to *p*-aminobenzoic acid (PABA) and sulfonamides are PABA analogues. Sulfaguanidine-resistant mutant S-225 was obtained from *C. glutamicum* ssp. *flavum* A-100 and production was increased from 10 g l^{-1} tryptophan to 19 g l^{-1}.

The sulfaguanidine-resistant mutant was still repressed by tyrosine but showed higher enzyme levels at any particular level of tyrosine (Sugimoto and Shiio, 1985).

Gene cloning of the tryptophan branch and mutation to resistance to feedback inhibition yielded a *C. glutamicum* strain producing 43 g l^{-1} L-tryptophan (Katsumata and Ikeda, 1993). The genes cloned were those that encoded AS, anthranilate phosphoribosyl transferase, a deregulated DAHPS, and other genes of tryptophan biosynthesis. However, sugar utilization decreased at the late stage of the fermentation and plasmid stabilization required antibiotic addition. Sugar utilization stopped due to killing by accumulated indole. By cloning in the 3-phosphoglycerate dehydrogenase gene (to increase production of serine which combines with indole to form more tryptophan) and by mutating the host cells to deficiency in this enzyme, both problems were solved (Ikeda *et al*., 1994). The new strain produced 50 g l^{-1} tryptophan with a productivity of 0.63 g l^{-1} h^{-1} and a yield from sucrose of 20%. Further genetic engineering to increase the activity of the pentose phosphate pathway increased production to 58 g l^{-1} (Ikeda and Katsumata, 1999).

4.1.6.2 L-Phenylalanine and L-tyrosine. A deregulated strain of *E. coli* in which feedback inhibition and repression controls were removed made 11 g l^{-1} phenylalanine in a fed-batch culture and 9 g l^{-1} in continuous culture (Choi and Tribe, 1982). Production of phenylalanine amounted to 28 g l^{-1} when a plasmid was cloned into *E. coli* containing a feedback inhibition-resistant version of the CM-prephenate dehydratase (PD) gene, a feedback inhibition-resistant DAHPS and the O_RP_R and O_LP_L operator-promoter system of lambda phage. Control of plasmid expression was by temperature manipulation (Sugimoto *et al*., 1987). Further process development of genetically engineered *E. coli* strains brought phenylalanine titres up to 46 g l^{-1} (Konstantinov and Yoshida, 1992). Independently, genetic engineering based on cloning *aroF* and feedback-resistant *pheA* genes created an *E. coli* strain producing 50 g l^{-1} (Backman *et al*., 1990).

A *C. glutamicum* ssp. *lactofermentum* culture, obtained by selection with *m*-fluorophenylalanine, produced 5 g l^{-1} phenylalanine, 7 g l^{-1} tyrosine and 0.3 g l^{-1} anthranilate and contained a desensitized DAHPS and PD. DHAPS in the wild-type was inhibited cumulatively by phenylalanine and tyrosine whereas PD was inhibited by phenylalanine. Cloning of the gene encoding PD from a desensitized mutant and the gene encoding desensitized DAHPS increased the enzyme activities and yielded a strain producing 18 g l^{-1} phenylalanine, 1 g l^{-1} tyrosine and no anthranilate (Ito *et al*., 1990a). Further cloning of a recombinant plasmid expressing desensitized 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase increased production to 26 g l^{-1} phenylalanine (Ito *et al*., 1991). Similarly, *C. glutamicum* strains have been developed producing 19 (Ozaki *et al*., 1985), 23 (Ikeda *et al*., 1993) and 28 g l^{-1} phenylalanine (Ikeda and Katsumata, 1992).

When SK was cloned into a tyrosine-producing *C. glutamicum* ssp. *lactofermentum* strain, tyrosine production increased from 17 g l^{-1} to 21 g l^{-1} (Ito *et al*., 1990b). Cloning of desensitized genes encoding DAHPS and CM from a deregulated phenylalanine-producing *C. glutamicum* strains into the deregulated tryptophan producer, *C. glutamicum* KY 10865 (CM-deficient strain, phenylalanine and tyrosine double auxotroph with a desensitized AS) shifted production from 18 g l^{-1} tryptophan to 26 g l^{-1} tyrosine (Ikeda and Katsumata, 1992).

An enymatic bioconversion of phenol, pyruvate, pyridoxal phosphate and ammonium chloride to L-tyrosine utilizes a thermostable and chemostable tyrosine phenol lyase from *Symbiobacterium toebii* (Kim *et al*., 2007). The process yields 130 g l^{-1} in 30 h with continuous feed of substrate.

4.2 Production processes for purines and pyrimidines, their nucleosides and nucleotides

Commercial interest in nucleotide fermentations is due to the activity of two purine ribonucleoside 5′-monophosphates, namely guanylic acid (guanosine 5′-monophosphate; GMP) and inosinic acid (inosine 5′-monophosphate; IMP), as flavour enhancers. It is quite impressive that a 1:1 mixture of MSG with IMP or GMP gives flavour intensity 30 times stronger than MSG alone. A review of the production of flavour enhancers for the food industry is Elhariry and colleagues (2004). Over 15 000 tons of IMP and GMP were produced in 2003 (Ishige *et al*., 2005).

4.2.1 Purines and derivatives. The purine residue of IMP is built up on a ribose ring in eleven enzymatically catalysed reactions. Ribose phosphate pyrophosphokinase is the first pathway enzyme and catalyses the conversion of α-D-ribose-5-phosphate (R5P) and ATP to 5-phosphoribosyl-α-pyrophosphate (PRPP). Adenosine-5′-monophosphate (AMP) and GMP are synthesized from IMP. AMP formation involves participation of two enzymes, adenylosuccinate synthetase and adenylosuccinase. GMP synthesis requires the participation of IMP dehydrogenase and GPM synthetase. PRPP synthetase is feedback inhibited by AMP, GMP and IMP. Adenylosuccinate synthetase is inhibited by AMP. IMP dehydrogenase is inhibited by xanthosine-5′-monphosphate (XMP) and GMP. The genes encoding the enzymes of IMP biosynthesis in *B. subtilis* constitute the *pur* operon, whereas the genes encoding the GMP biosynthetic enzymes, *guaA* (GMP synthetase) and *guaB* (IMP dehydrogenase), and the *purA* gene encoding adenylosuccinate (sAMP)

synthetase all occur as single units. The *purB* gene encodes an enzyme involved in both IMP and AMP biosynthesis and is located in the *pur* operon. The levels of purine biosynthetic enzymes (except for GMP synthetase) are repressed in cells grown in the presence of purine compounds. Transcription of the *pur* operon is regulated negatively by adenine and guanine compounds including ATP and guanine (or hypoxanthine).

Techniques similar to those described above for amino acid fermentations have yielded IMP titres of 27 g l^{-1} (Kuninaka, 1996). As only low levels of GMP have been produced by direct fermentation, it is usually made by bioconversion of XMP. Genetic modification of *Corynebacterium ammoniagenes* involving transketolase (an enzyme of the non-oxidative branch of the pentose phosphate pathway) resulted in the accumulation of 39 g l^{-1} XMP (Kamada *et al*., 2001). This work demonstrates the need for high levels of pentose (ribose) for nucleotide and nucleoside biosynthesis and overproduction.

The key to effective accumulation of purines and their derivatives is limitation of intracellular AMP and GMP. This limitation is best effected by restricted feeding of purine auxotrophs. Thus, adenine-requiring mutants lacking adenylosuccinate synthetase accumulate hypoxanthine or inosine that results from breakdown of intracellularly accumulated IMP. Certain adenine auxotrophs of *B. subtilis* excrete over 10 g l^{-1} inosine. These strains are still subject to GMP repression of enzymes of the common path. To minimize the severity of this regulation, the adenine auxotrophs are further mutated to eliminate IMP dehydrogenase. These adenine-xanthine double auxotrophs show a twofold increase in specific activity of some common-path enzymes and accumulate inosine up to 15 g l^{-1} under conditions of limiting adenine and xanthine (or guanosine). Further deregulation is achieved by selection of mutants resistant to purine analogues. Thus, mutants resistant to azaguanine with requirements for adenine and xanthine produce over 20 g l^{-1} inosine. Insertional inactivation of the IMP dehydrogenase gene in a *B. subtilis* strain yielded a culture producing inosine at 35 g l^{-1} (Miyagawa *et al*., 1989).

Cloning of IMP dehydrogenase has been used to improve guanosine production in *B. subtilis*. The donor strain, NA7821, produced a low level of purine nucleosides but the distribution was in favour of guanosine (1 g l^{-1} inosine versus 9 g l^{-1} guanosine). The recipient strain, NA6128, was auxotrophic for adenine, lacked GMP reductase and purine nucleoside phosphorylase, and was resistant to 8-azaguanine, adenine and adenosine; it produced 19 g l^{-1} inosine and 7 g l^{-1} guanosine. Cloning of IMP dehydrogenase from strain NA7821 into NA 6128 yielded recombinant NA 6128 (pBxl21), which produced 5 g l^{-1} inosine and 20 g l^{-1} guanosine (Miyagawa *et al*., 1986). Other *B. subtilis* mutants produce as much as 30 g l^{-1} guanosine (Qian *et al*., 2006). Nucleosides such as inosine and guanosine are then converted to their active nucleotide derivatives chemically, microbiologically or enzymatically (Mori *et al*., 1997). IMP is produced at 156 g l^{-1} from inosine with a molar yield of 79% in 24 h (Mihara, 2004).

4.2.2 Pyrimidines and derivatives. The *de novo* pyrimidine biosynthetic pathway involves five enzymes and results in uridine-5′-monophosphate (UMP) production. Aspartate transcarbamoylase, the first pathway enzyme committed to pyrimidine biosynthesis, catalyses the conversion of aspartate and carbamoylphosphate to carbamoylaspartate. The subsequent biosynthetic pathway enzymes are dihydroorotase, dihydroorotate dehydrogenase, orotate phosphoribosyltransferase and orotidine-5′-monophosphate (OMP) decarboxylase. Uridine triphosphate (UTP) is produced from UMP by the sequential actions of two nucleoside kinases. Cytidine triphosphate (CTP) is formed by amination of UTP by CTP synthetase. The pyrimidine biosynthetic pathway is regulated at the level of gene expression in several species of bacteria. In addition, the regulation of aspartate transcarbamoylase activity determines the rate of pyrimidine nucleotide synthesis. The pyrimidine nucleotide biosynthesis (*pyr*) operon in *B. subtilis* contains 10 cistrons. The first gene in the operon encodes PyrR, which is the regulatory protein for the *pyr* operon. The PyrR protein binds in a uridine nucleotide-dependent manner to three attenuation regions located in the 5′-leader region (binding loop 1, BL1), the *pyrR–pyrP* intercistronic region (BL2) and the *pyrP–pyrB* intercistronic region (BL3) of *pyr* mRNA. PyrR recognizes conserved RNA sequences, but only if they are properly positioned in the correct secondary structure (Bonner *et al*., 2001). The second gene in the operon encodes PyrP, which is a uracil permease. Uridine-5′-monophosphate kinases from *E. coli* and *B. subtilis* are activated by GTP and inhibited by UTP (Gagyi *et al*., 2003).

Selection for antimetabolite resistance has proven to be successful in development of nucleotide and nucleoside fermentations (Demain, 1978). Cytidine production by a *B. subtilis* cytidine-deaminase-deficient mutant with resistance to fluorocytidine amounted to 10 g l^{-1}. Further mutation to 3-deazauracil resistance increased production to 14 g l^{-1}. By introducing a gene encoding a feedback-resistant carbamyl phosphate synthase, cytidine production was increased to 18 g l^{-1}. Homoserine dehydrogenase (HSD) deficiency in *B. subtilis* increased cytidine production in a deregulated mutant from 9 g l^{-1} to 23 g l^{-1} (Asahi *et al*., 1996). Increasing the glucose concentration raised production to 30 g l^{-1}. Uridine production by mutants of *B. subtilis* resistant to pyrimidine antimetabolites can produce 55 g l^{-1} uridine (Doi *et al*., 1989).

4.3 Vitamin production processes

More than half of vitamins produced commercially are fed to domestic animals (Stahmann, 2002). The vitamin market is several billion dollars per year. Microbes produce five vitamins commercially: vitamin B_{12} (cyanocobalamin), ascorbic acid (vitamin C), riboflavin (vitamin B_2), pantothenic acid (vitamin B_5) and biotin. Some vitamin processes have been improved by metabolic engineering (Sybesma *et al.*, 2004)

4.3.1 Vitamin B_{12}. Bacterial formation of vitamin B_{12} by bacteria has been going on for a long time. The anaerobic pathway is about 4 billion years old whereas the aerobic pathway evolved when our atmosphere became enriched with oxygen about 2 billion years ago (Scott and Roessner, 2002). Vitamin B_{12} is produced commercially at about 10 tons per year (Martens *et al.*, 2002). Fermentations have to be run under complete or partial anaerobiosis when using species of *Pseudomonas* or *Propionibacterium*. *Propionibacterium freudenreichii* can produce 206 mg l^{-1} although the major industrial organisms are *Pseudomonas denitrificans* and *Propionibacterium shermanii.* Conventional strain improvement has yielded *P. denitrificans* strains producing 150 mg l^{-1} (Spalla *et al.*, 1989).

Increasing the activity of S-adenosyl-L-methionine: uroporphyrinogen III methyltransferase (SUMT) by cloning in a DNA fragment containing this gene (*cobA*) in *P. denitrificans* increased vitamin B_{12} production by 100% (Crouzet *et al.*, 1993). SUMT is at the branch point of the haem and B_{12} biosynthetic pathways. Cloning of the gene *cob1* increased S-adenosyl-L-methionine:precorrin-2-methyltransferase (SP_2MT) and B_{12} production by 30%. SP_2MT is at the branch point of the siroheme and B_{12} pathways. On the other hand, cloning the δ-aminolevulinate synthase (ALAS) gene increased ALAS activity but not B_{12} biosynthesis.

Vitamin B_{12} biosynthesis in *S. typhimurium* involves three closely located operons: CobI, CobII and CobIII mapping at 41 min, and another locus CobA at 34 min (Escalante-Semerena *et al.*, 1990). CobI is involved in biosynthesis of cobinamide. CobII deals with 5,6-dimethylbenzimidazole (DMB) biosynthesis; CobIII involves the linking of the two moieties to form cobalamine; and CobA is involved in adenosylation of an early precursor of the corrin ring. Transcription of the CobI operon genes does not occur under aerobiosis (Anderson and Roth, 1989). In addition, this operon is repressed by the ultimate end-product, cobalamin. It is not oxygen itself that causes the repression but the lack of a reducing environment in the cell.

4.3.2 Riboflavin. Annual production of riboflavin is 4000 tons per year (Vandamme and Soetaert, 2006). Riboflavin overproducers include two yeast-like molds, *E. ashbyii* and *A. gossypii*, which synthesize riboflavin in concentrations higher than 20 g l^{-1}.

In *A. gossypii*, riboflavin production is stimulated three- to fourfold by the precursors glycine and hypoxanthine (Kaplan and Demain, 1970; Monschau *et al.*, 1998). The level of production, which occurs after growth rate declines, is determined by the activity of the promoter of gene *RIB3.* This gene encodes 3,4-dihydroxy-2-butanone-4-phosphate (DHBP) synthase, the first enzyme of the pathway (Schloesser *et al.*, 2001). Mutation of *A. gossypii* to resistance to aminomethylphosphonic acid (a glycine antimetabolite) yielded improved producers. Isocitrate lyase (ICL) is important for use of fatty acids for riboflavin production (Schmidt *et al.*, 1996). Itaconate, an inhibitor of ICL, eliminated the yellow colour of *A. gossypii* colonies. A mutant that was yellow on itaconate-containing agar produced 15% more enzyme and 25-fold more riboflavin. When grown in glucose, ICL-specific activity dropped by 33% in the mutant but riboflavin production increased eightfold. The mutation appears to be a regulatory mutation affecting ICL-specific activity. A genome-wide transcript expression analysis, i.e. massive parallel signature sequencing (Brenner *et al.*, 2000), was successfully used for further improvement of riboflavin production by *A. gossypii* (Karos *et al.*, 2004). The authors identified 53 genes of known function, some of which could clearly be related to riboflavin production. This approach also allowed the finding of sites within the genome with high transcriptional activity during riboflavin biosynthesis that are suitable integration loci for the target genes found.

Processes using recombinant *B. subtilis* strains that produce 30 g l^{-1} riboflavin have been developed. In this microorganism, riboflavin formation is regulated by feedback repression, not inhibition (Bresler *et al.*, 1973). An aporepressor encoded by *ribC*, whose effectors are riboflavin, FMN and FAD, is responsible for this effect. Mutations of *ribC* led to riboflavin overproduction. Sequential selection for resistance to 8-azaguanine, decoyinine, methionine sulfoxide and roseoflavin plus multiple copies of the riboflavin biosynthetic *rib* operon yielded overproducing mutants (Perkins *et al.*, 1999). Further improvement was achieved when an extra copy of the *ribA* gene was introduced into the culture. This gene encodes both GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase, both of which act to commit precursors GTP and ribulose-5-phosphate to riboflavin biosynthesis.

A *Candida famata* (*Candida flareri*) strain produced 20 g l^{-1} in 200 h. It was obtained by mutation and selection for resistance to 2-deoxyglucose (DOG), iron, tubercidin (a purine analogue) and depleted medium, plus protoplast fusion (Heefner *et al.*, 1992). The process

depends on the addition of glycine and hypoxanthine. Selection for resistance to the adenine antimetabolite 4-aminopyrazolo (3,4-d) pyrimidine) improved production (Heefner *et al*., 1993). Threonine showed a ninefold stimulation in a strain with a cloned threonine aldolase, which converts threonine to glycine. Having the cloned gene, but without added threonine, resulted in no stimulation, presumably due to the low level of internal threonine resulting from aspartokinase feedback inhibition.

4.3.3 Vitamin C. Vitamin C has a global production of 110 000 tons per year (Macauley *et al*., 2001; Deppenmeier *et al*., 2002), selling for $6–8 kg. It is used for nutrition of humans and animals as well as a food antioxidant. The otherwise chemical seven-step Reichstein process includes one bioconversion reaction, the oxidation of D-sorbitol to L-sorbose by *Gluconobacter oxydans*, as the first step in ascorbic acid production. The biotransformation proceeds at the theoretical maximum, i.e. 200 g l^{-1} D-sorbitol can be converted to 200 g l^{-1} L-sorbose, when using a mutant of *G. oxydans* selected for resistance to a high sorbitol concentration. The biconversion is used rather than a chemical reaction as the latter produces unwanted D-sorbose along with L-sorbose. An excellent fed-batch bioconversion process uses a starting concentration of 100 g l^{-1} of D-sorbitol and achieves production of 280 g l^{-1} L-sorbose in 16 h with a productivity of 17.6 g l^{-1} h^{-1} (Giridhar and Srivastava, 2002). The Reichstein process converts glucose to 2-keto-L-gulonic acid (2-KLGA) in five steps with a yield of 50%. Then, 2-KLGA is chemically converted to L-ascorbic acid in two more steps.

The Reichstein process has provided ascorbic acid for 70 years but is being threatened now by fermentation processes (Bremus *et al*., 2006). A mixed culture of *G. oxydans* strain DSM4025 (which converts L-sorbose to 2-KLGA) and *Gluconobacter suboxydans* IFO 3255 (which converts D-sorbitol to L-sorbose) was able to convert 138 g l^{-1} D-sorbitol to 112 g l^{-1} 2-KLGA, with a molecular conversion yield of 75% in 2 days (Hoshino, 2000). A recombinant strain of *G. oxydans* containing genes encoding L-sorbose dehydrogenase and L-sorbosone dehydrogenase from *G. oxydans* T-100 was able to produce 2-KLGA effectively from D-sorbitol (Saito *et al*., 1997). Mutation to suppress the L-idonate pathway and improvement of the promoter led to production of 130 g l^{-1} 2-KLGA from 150 g l^{-1} D-sorbitol.

Another development was the metabolic engineering of *Pantoca citrea*, a Gram-negative bacterium capable of producing keto sugars. By mutations eliminating the conversion of glucose to glucose 6-phosphate and the conversion of gluconate to gluconate 6-phosphate, a culture was prepared which converted 97% of the glucose fed to 2-KLGA (Sanford *et al*., 2004).

4.3.4 Biotin. The annual production of biotin amounts to about 30 tons per year. The repressor of the biotin biosynthetic pathway is the enzyme acetyl-CoA carboxylase biotin holoenzyme synthetase that catalyses attachment of biotin from biotin-5′-adenylate to acetyl-CoA carboxylase (Barker and Campbell, 1981). The co-repressor is biotin-5′-adenylate.

Strains of *S. marcescens* obtained by mutagenesis and cloning produce 500 mg l^{-1} biotin plus 100 mg l^{-1} desthiobiotin. High concentrations of sulfur and ferrous iron increased biotin production by the *S. marcescens* recombinant strain to a level of 600 mg l^{-1} (Masuda *et al*., 1995). A process using an *E. coli* mutant resistant to β-hydroxynorvaline (a threonine antimetabolite) yielding 970 mg l^{-1} has been patented (Matsui *et al*., 2001). Biotin was further increased by using a *B. subtilis* strain resistant to 5-(2-thienyl) pentanoic acid (a biotin analogue) and overexpressing several *bio* genes to values over 1 g l^{-1} (Bower *et al*., 2001). Although the above mentioned biotin titres seem to be useful, none of the patented technologies is cost-effective enough to reach the production line. According to Hong and colleagues (2006), the limitation in the microbial production processes seems to be due to several factors of the fermentation processes such as expensive biotin precursors, plasmid instability, possible toxic side-effects of some metabolites, and finally, the costly purification process.

4.3.5 Other vitamins. Recombinant *E. coli*, transformed with genes encoding pantothenic acid (vitamin B_5) biosynthesis, and resistant to salicylic and/or other acids, produce 65 g l^{-1} D-pantothenic acid from glucose using β-alanine as precursor (DeBaets *et al*., 2000). Seven thousand tons per year are made chemically and microbiologically. Thiamine (vitamin B_1) is produced synthetically at 4000 tons per year. Pyridoxine (vitamin B_6) is made chemically at 2500 tons per year. The vitamin F (polyunsaturated fatty acids) processes of *Mortierella isabellina* or *Mucor circinelloides* yield 5 g l^{-1} γ-linolenic acid.

Carotenoid production processes have been extensively studied (Johnson and Schroeder, 1995) but none has reached the stage to economically challenge chemical methods. Processes in development include those yielding β-carotene, lycopene, zeaxanthin and astaxanthin. Some have been improved by metabolic engineering and directed evolution (Barkovich and Liao, 2001; Lee and Schmidt-Dannert, 2002; Tao *et al*., 2005).

4.4 Organic acid production processes

Citric, gluconic, itaconic and lactic acids are the main organic acids with commercial application as chemicals (Magnuson and Lasure, 2004). Production of organic acids has been improved by classical mutation and

screening/selection techniques as well as by metabolic engineering (Kraemer *et al.*, 2003).

4.4.1 Acetic acid. Over 7 million tons of acetic acid are made worldwide, over half by microbial methods (Causey *et al.*, 2003). Vinegar has been produced microbiologically as far back as 4000 BC. Vinegar fermentation is best carried out with species of *Gluconacetobacter* and *Acetobacter* (Deppenmeier *et al.*, 2002). A solution of ethanol is converted to acetic acid in which 90–98% of the ethanol is attacked yielding a solution of vinegar containing 12–17% acetic acid.

Acetate excretion is not merely 'overflow' metabolism but allows the cell to grow faster and to reach higher cell densities. Metabolic engineering studies have been carried out (El-Mansi, 2004). Titres of acetic acid have reached 53 g l^{-1} with genetically engineered *E. coli* (Causey *et al.*, 2003), 83 g l^{-1} with a *Clostridium thermoaceticum* mutant (Parekh and Cheryan, 1994) and 97 g l^{-1} with an engineered strain of *Acetobacter aceti* subsp. *xylinium* (Beppu, 1993).

4.4.2 Citric acid. Production of citric acid by *A. niger* and yeasts amounts to about 1 million tons per year (Magnuson and Lasure, 2004). Annual sales have reached $2 billion. The best strains of *A. niger* make over 200 g l^{-1} citric acid from 250 g l^{-1} glucose or sucrose (Al-Obaidy and Berry, 1979). Keys to the fermentation are excess carbon source, low pH, dissolved oxygen and limited concentrations of certain trace metals and phosphate (Roehr, 1998).

Glucose is converted to pryuvate by the Embden-Meyerhof-Parnas (EMP) pathway, then to acetylcoenzyme A which enters the TCA cycle by condensing with oxaloacetate to form citric acid. Citric acid production is stimulated by growing this fungus in a high sucrose concentration (10–20%). This is probably the result of the sugar's ability to cause the intracellular accumulation of fructose 2,6-bisphosphate which activates glycolysis (Kubicek-Pranz *et al.*, 1990). Fructose 2,6-bisphosphate is the product of phosphofructokinase II. Another key to a successful citric acid fermentation by this fungus is a deficiency of Mn^{2+}. Also important is the restriction of the activity of isocitrate dehydrogenase, while maintaining an active citrate synthase. This prevents oxidative decarboxylation of isocitrate to α-ketoglutarate. As the equilibrium of aconitase, which converts citric acid to isocitric acid, is markedly in favour of citrate, citric acid accumulates. Two isocitrate dehydrogenases, mitochondrial and $NADP^+$ specific, are inhibited by citrate. As the cofactor of this enzyme is Mg^{2+} or Mn^{2+}, citrate's ability to chelate these metals restricts enzyme activity; thus citrate inhibits its own degradation. Citrate inhibition of isocitrate dehydrogenase is in direct proportion to citric acid yield.

In addition to Mn^{2+}, the metal deficiencies necessary for good citric acid production in different media and by different strains of *A. niger* are Fe^{2+} and Zn^{2+}. However, the major required limitation is that of Mn^{2+}. The principal regulatory control site in the reactions from glucose to citrate is phosphofructokinase. This enzyme is inhibited by citrate, an event that would not be favourable for overproduction of citric acid. However, Mn^{2+} deficiency slows down growth, leading to degradation of intracellular nitrogenous macromolecules and a fivefold increase of NH_4^+ in mycelia. The high ammonium concentration reverses citrate inhibition of phosphofructokinase, thus assuring the continued conversion of glucose to citrate. Mutants whose phosphofructokinase I is partially desensitized to citrate inhibition are less dependent on low Mn^{2+} for high citric acid production (Schreferl *et al.*, 1986). Citrate inhibition of phosphofructokinase is also reversed by fructose 2,6-diphosphate and AMP (Kubicek, 1998).

The optimum pH for citric acid production by *A. niger* is 1.7–2.0. At pH values higher than 3.0, oxalic and gluconic acids are produced instead. Low pH inactivates glucose oxidase and prevents gluconate production (Kubicek and Roehr, 1986). Mutants of *A. niger* with greater resistance to low pH are improved citric acid producers. Other selective tools include resistance to high concentrations of citrate (Leopold, 1959), and sugars (Schreferl-Kunar *et al.*, 1989).

Yeasts of the *Candida* genus also excrete large amounts of citric acid and isocitric acid. The key of the yeast citrate process appears to be a sharp drop in intracellular AMP following nitrogen depletion, inhibiting the AMP-requiring isocitrate dehydrogenase. *Candida guilliermondii* excretes large quantities of citric acid without the undesirable isocitric acid when cultured in the presence of metabolic inhibitors (such as sodium fluoracetate, *n*-hexadecylcitric acid or *trans*-aconitic acid). These inhibitors block the TCA cycle at the aconitase step. Mutation of *Candida lipolytica* to aconitase deficiency is also effective (Akiyama *et al.*, 1973). The optimum pH for the yeast citrate process is above 5.0. Lower pH values lead to production of polyhydroxy compounds such as erythritol and arabitol (Tabuchi *et al.*, 1973). The yeast process yields a conversion of 140–150% based on hydrocarbon used, a productivity of 1.4 g l^{-1} h^{-1} and a broth concentration as high as 225 g l^{-1} (Kubicek and Roehr, 1986). High concentrations of citric acid are also produced by *Candida oleophila* from glucose (Anastassiadis *et al.*, 2002). In chemostats, 200 g l^{-1} can be made and more than 230 g l^{-1} can be produced in continuous repeated fed-batch fermentations. This compares to 150–180 g l^{-1} by *A. niger* in industrial batch or fed-batch fermentation in 6–10 days. The key to the yeast fermentation is nitrogen limitation coupled with an excess of glucose. The citric acid is secreted by a specific energy-dependent transport

system induced by intracellular nitrogen limitation. The transport system is selective for citrate over isocitrate. *Yarrowia lipolytica* produces up to 198 g l^{-1} citric acid in fed-batch fermentations on sunflower oil with a very low production of isocitric acid (Aurich *et al*., 2003).

4.4.3 Lactic acid. The global market for lactic acid is about 250 000 tons per year (Industrial Biotechnology and Sustainable Chemistry, 2004). *Rhizopus oryzae* is favoured for production as it makes stereochemically pure L(+)-lactic acid whereas lactobacilli produce mixed isomers; furthermore, lactobacilli require yeast extract. However, a mutant strain of *Lactobacillus lactis* has been developed which produces 195 g l^{-1} L-lactic acid from 200 g l^{-1} glucose (Bai *et al*., 2004) and a productivity of 1.76 g l^{-1} h^{-1}. A better productivity (2.14 g l^{-1} h^{-1}) was achieved with an exponential fed-batch process using *Lactobacillus casei* which yielded 180 g l^{-1} (Ding and Tan, 2006). *Rhizopus oryzae* normally converts 60–80% of added glucose to lactate, the remainder going to ethanol. By increasing lactic dehydrogenase levels via cloning, more lactate and less ethanol were produced (Skory, 2004). Mutation of wild-type *R. oryzae* led to L(+)-lactic acid production of 131–136 g l^{-1}, a yield from glucose of 86–90% and a productivity of 3.6 g l^{-1} h^{-1} (Ge *et al*., 2004). This was a 75% improvement over the wild-type strain. The final strain was the result of a six-step mutation sequence. A transgenic wine yeast genetically engineered to contain six copies of the bovine L-lactate dehydrogenase gene produces L-(+)-lactate at 122 g l^{-1} (Saito *et al*., 2005). Whole genome shuffling has been used to improve the acid tolerance of a commercial lactic acid-producing *Lactobacillus* sp. (Patnaik *et al*., 2002).

A recombinant *E. coli* strain has been constructed that produces optically active pure D-lactic acid from glucose at virtually the theoretical maximum yield, e.g. two molecules from one molecule of glucose (Zhou *et al*., 2003). The organism was engineered by eliminating genes of competing pathways encoding fumarate reductase, alcohol/aldehyde dehydrogenase and pyruvate formate lyase, and by a mutation in the acetate kinase gene. D-Lactic acid has been produced at 61 g l^{-1} by a recombinant strain of *S. cerevisiae* containing the D-lactic dehydrogenase gene from *Leuconostoc mesenteroides* (Ishida *et al*., 2006).

Products in development are the non-chlorinated solvent, ethyl lactate, and the bioplastic, polylactide. Polylactide is made by converting corn starch to dextrose, fermenting dextrose to lactic acid, condensing lactic acid to lactide, and polymerizing lactide.

4.4.4 Pyruvic acid. A recombinant strain of *E. coli* which is a lipoic acid auxotroph and defective in F_1ATPase produces 31 g l^{-1} pyruvic acid from 50 g l^{-1} glucose (Yokota *et al*., 1994). The lowering of the energy level in the cell by the F_1ATPase deletion increases glucose uptake and glycolysis rate, thereby leading to an increase in pyruvate production. An improved fermentation has been developed using *Torulopsis glabrata* yielding a pyruvic acid concentration of 77 g l^{-1}, a conversion of 0.80 g g^{-1} glucose and a productivity of 0.91 g l^{-1} h^{-1} in 85 h (Li *et al*., 2002). By disrupting the gene encoding pyruvate decarboxylase, an improved strain of *T. glabrata* was obtained which produced 82 g l^{-1} pyruvate in 52 h (Wang *et al*., 2005). By directed evolution in a chemostat, a mutant *S. cerevisiae* strain was made which produces from glucose 135 g l^{-1} pyruvic acid at a rate of 6 to 7 mmol per gram biomass per hour during exponential growth with a yield of 0.54 g of pyruvate per gram of glucose (van Maris *et al*., 2004).

4.4.5 Fumaric acid. Fumaric acid is utilized by the plastics industry in polyester and alkyd resins, and the remainder goes to lesser volume uses such as rosin adducts, varnishes and foods. *Rhizopus arrhizus* can produces large amounts of fumaric acid when grown in the presence of glucose. From 120 g l^{-1} glucose, *R. arrhizus* can produce 97 g l^{-1} fumaric acid (Kenealy *et al*., 1986). The molar yield from glucose is 145% and involves CO_2 fixation from pyruvate to oxaloacetate and the reductive reactions of the TCA cycle. The use of an integrated system of simultaneous fermentation–adsorption for the production and recovery of fumaric acid from glucose enhanced the fermentation rate, and sustained cell viability (Cao *et al*., 1996).

4.4.6 Other acids. Succinic acid is made chemically at 15 000 tons per year for commercial use as (i) a surfactant/detergent extender/foaming agent, (ii) an ion chelator in electroplating to prevent metal corrosion and pitting, (iii) an acidulant/pH modifier/flavouring agent/antimicrobial agent for food and (iv) a chemical in the production of pharmaceuticals (Zeikus *et al*., 1999). Market size is $400 million per year. Production by fermentation with *Actinobacillus succinogenes* amounts to 40 g l^{-1}, a productivity of 7 g l^{-1} h^{-1}, and a 76% yield from glucose (Urbance *et al*., 2004). Bioconversion from fumarate yields 85 g l^{-1} succinate after 24 h (Kang and Ryu, 1999). An *A. succinogenes* mutant resistant to flouroacetate reached 105 g l^{-1} and a productivity of 1.34 g l^{-1} h^{-1} (Guettler *et al*., 1996). Metabolic engineering of *Mannheimia succiniciproducens* led to a strain which produces 52 g l^{-1} succinic acid at a yield of 1.16 mol per mol glucose and a productivity of 1.8 g l^{-1} h^{-1} in fed-batch culture (Lee *et al*., 2006). A titre of 99 g l^{-1} has been reached with recombinant *E. coli* yielding a productivity of 1.3 g l^{-1} h^{-1} (Vemuri *et al*., 2002).

Shikimic acid is the starting point for chemical synthesis of Tamiflu, an antiviral agent. Metabolic engineering of *E. coli* yielded an overproducer making 84 g l^{-1} shikimic acid with a 0.33 molar yield from glucose (Chandran *et al.*, 2003). Shikimic acid (50–90 g l^{-1}) can be produced in 30% (mol mol^{-1}) yield from glucose with an *E. coli aroL* and *aroK* mutant which overexpresses *aroF*, *aroB* and *aroE*, and *tktA* and *ppsA* of central metabolism (Chandran *et al.*, 2003; Kraemer *et al.*, 2003). Dehydroshikimic acid is an antioxidant used for preservation of food, feed and other oxidative sensitive products, over extended periods of time and at elevated temperatures. This compound is produced at 69 g l^{-1} in 30% yield (mol mol^{-1}) from glucose using an *E. coli aroE* mutant overexpressing two genes: *tktA* encoding transketolase and feedback-insensitive *aroF* encoding DS (Li *et al.*, 1999).

Itaconic acid production by the basidiomycete *Pseudozyma antarctica* grown under N-limitation reached a level of 30 g l^{-1} from 80 g l^{-1} glucose (Levinson *et al.*, 2006). The same titre is produced with *A. niger* with a yield from sucrose in molasses of 70%. The market amounts to 17 000 tons (Magnuson and Lasure, 2004).

Production of gluconic acid amounted to 150 g l^{-1} from 150 g l^{-1} glucose plus corn steep liquor in 55 h with *A. niger* (Znad *et al.*, 2004). Production level is 50 000–60 000 tons per year (Industrial Biotechnology and Sustainable Chemistry, 2004; Anastassiadis *et al.*, 2005). *Aureobasidium pullulans* can produce 504 g l^{-1} in fed-batch fermentation and over 400 g l^{-1} in continuous fermentation (Anastassiadis *et al.*, 2003). A mutant of *G. oxydans* in which membrane-bound gluconate-2-dehydrogenase was inactivated, thus eliminating 2-ketogluconate production, produced 5-ketogluconic acid directly from glucose in 84% yield (Elfari *et al.*, 2005). 5-Ketogluconate is a precursor of L-(+)-tartaric acid used in the food and textile industries.

Cloning of fumarase in *S. cerevisiae* remarkably improved the malic acid bioconversion from fumaric acid from 2 g l^{-1} to 125 g l^{-1} (Neufeld *et al.*, 1991). Conversion yield was near 90%.

Kojic acid production by *Aspergillus oryzae* was improved to 41 g l^{-1} by NTG mutagenesis of conidia followed by UV mutagenesis of protoplasts (Wan *et al.*, 2004). The acid is used as an anti-inflammatory drug, a precursor of flavour enhancers, an anti-browning agent in foods, and a whitening agent and UV protectant in skin care products.

Adaptation of *Propionibacterium acidipropionici* to a fibrous-bed bioreactor allowed production of 72 g l^{-1} propionic acid from glucose, as compared with 52 g l^{-1} in a fed-batch fermentation (Suwannakham and Yang, 2005). The new process also decreased acetate and succinate production.

4.5 Ethanol and related compounds

4.5.1 Ethanol. Ethyl alcohol is produced in Brazil from cane sugar at over 4 billion gallons per year and is used either as a 22–25% blend or as a pure fuel. In the USA, 20 million barrels of petroleum are used daily; 60% of this is imported (Gray *et al.*, 2006). Liquid fuels such as gasoline, diesel and jet fuel, all used for transportation, constitute 70% of the total. Ethanol was produced in the USA at over 4 billion gallons with the rest of the world producing about 8 billion gallons. The current market amounts to $15 billion. The USA uses corn and could produce up to 13 billion gallons per year from this source. It is chiefly used as an oxygenate added to gasoline to reduce CO_2 emissions by improving overall oxidation of gasoline. The steady increase in consumption is due in part to phasing out of the use of methyl tert-butyl ether (MBTE) as gasoline oxygenate, as ruled by many state legislatures in the USA.

Ethanol is a primary metabolite produced by fermentation of sugar, or of a polysaccharide that can be depolymerized to a fermentable sugar. *Saccharomyces cerevisiae* is used for the fermentation of hexoses, whereas *Kluyveromyces fragilis* is employed for lactose utilization. *Pichia stipitis* or *Candida* species can be used if a pentose is the substrate. *Saccharomyces cerevisiae* produces as high as 96.7 g l^{-1} ethanol in 96 h fermentation on sucrose (Caylak and Vardar, 1996), 70 g l^{-1} on sugar cane molasses in 30 h (Navarro *et al.*, 2000) and 53 g l^{-1} on beet molasses in 192 h (Roukas, 1996).

Under optimum conditions, approximately 10–12% ethanol by volume can be obtained from sugar within 5 days. Such a high concentration slows down growth and the fermentation ceases. With special yeasts, the fermentation can be continued to produce alcohol concentrations of 20% by volume, but these concentrations are attained only after months or years of fermentation. At present, all beverage alcohol is made by fermentation. Industrial ethanol is mainly manufactured by fermentation, but some is still produced from ethylene by the petrochemical industry.

Further increases in ethanol production will have to come from biomass. Available biomass reserves are about 200 million dry tons per year. This could yield 16 billion gallons of ethanol based on an overall yield of 80 gallons per dry ton.

Bacteria such as clostridia and *Zymomonas* are being re-examined for ethanol production after years of neglect. *Clostridium thermocellum*, an anaerobic thermophile, can convert waste cellulose (i.e. biomass) and crystalline cellulose directly to ethanol (Demain *et al.*, 2005). If waste cellulose could be efficiently converted to ethanol, the available cellulosic feedstocks in the USA could supply 20 billion gallons of ethanol in comparison with the 5 billion

gallons currently made from corn. This would be more than enough to add 10% ethanol to all gasoline used in the USA (Lynd *et al*., 2002). The US Department of Agriculture and US Department of Energy have estimated that 1 billion tons of biomass could be produced annually from this substrate which could yield 80 billion gallons of bioenergy, about 30% of current usage (Gray *et al*., 2006). Other clostridia produce acetate, lactate, acetone and butanol, and will be used to produce these chemicals when the global petroleum supplies begin to become depleted.

Fuel ethanol produced from biomass would provide relief from air pollution caused by the use of gasoline and would not contribute to the greenhouse effect. *Escherichia coli* has been converted into an excellent ethanol producer (43% yield, v/v) by recombinant DNA techniques. By cloning and expressing the alcohol dehydrogenase and pyruvate decarboxylase genes from *Zymomonas mobilis* in *Klebsiella oxytoca*, the recombinant strain was able to convert crystalline cellulose to ethanol in high yield when fungal cellulase was added (Doran and Ingram, 1993). Per cent of maximum theoretical yield was 81–86% and titres as high as 47 g l^{-1} ethanol were produced from 100 g l^{-1} cellulose. Other genetically engineered strains of *E. coli* can produce 60 g l^{-1} ethanol (Yomano *et al*., 1998). Ethanol production has been further increased by metabolic engineering (Nissen *et al*., 2000).

Most recombinant strains of *E. coli*, *Zymomonas* and *Saccharomyces* convert corn fibre hydrolysate to 21–35 g l^{-1} with yields of 0.41–0.50 ethanol per gram of sugar consumed (Bothast *et al*., 1999; Dien *et al*., 2000). For a recombinant *E. coli* strain making 35 g l^{-1}, time was 55 h and yield was 0.46 g of ethanol per gram of available sugar, which is 90% of maximum attainable. Corn fibre contains 70% by weight of carbohydrate, made up of cellulose and hemicellulose. It is produced at 3.4 million dry tons per year which could yield up to 4 billion gallons of ethanol, assuming an 80% conversion. The best pretreatment of corn fibre appears to be dilute acid, which avoids production of inhibitory compounds such as furfural or 5-hydroxymethyl furfural acid from lignin. Addition of cellulase and β-glucosidase yielded 85–100% of theoretical yield of monomeric sugars (Saha and Bothast, 1999).

4.5.2 Glycerol. Glycerol has uses in the drug, food, cosmetics, paint and many other industries. Production of glycerol is usually performed by extraction of materials from the fat and oil industries, or by chemical synthesis from propylene, but good fermentations using *S. cerevisiae* and osmotolerant yeasts are available (Wang *et al*., 2001; Taherzadeh *et al*., 2002). Six hundred thousand tons of glycerol are produced annually. A number of studies are being performed using physiological control and genetic engineering in the hopes of making the fermentation process competitive. *Saccharomyces cerevisiae* can produce up to 230 g l^{-1} glycerol (Kalle and Naik, 1985; Vikar and Panesar, 1987). Osmotolerant yeast strains (*Candida glycerinogenes*) can produce 137 g l^{-1} with yields of 63–65% and a productivity of 32 g l^{-1} day^{-1} (Zhuge *et al*., 2001). *Candida magnoliae* produces 170 g l^{-1} in a fed-batch fermentation (Peterson *et al*., 1958) and in a similar type of process, *Pichia farinosa* can produce up to 300 g l^{-1} (Vijaikishore and Karanth, 1986).

4.5.3 1,3-Propanediol. A strain of *Clostridium butyricum* converts glycerol to 1,3-propanediol (PDO) at a yield of 0.55 g per gram of glycerol consumed (Papanikolaou *et al*., 2000). In a two-stage continuous fermentation, a titre of 41–46 g l^{-1} was achieved with a maximum productivity of 3.4 g l^{-1} h^{-1}. At lower dilution rates, butyrate was produced, and at higher dilution rates, acetate was made. Recent metabolic engineering triumphs have included the development of an *E. coli* culture that grows on glucose and produces PDO at 135 g l^{-1}, with a yield of 51% and a rate of 3.5 g l^{-1} h^{-1} (Sanford *et al*., 2004). To do this, they introduced eight new genes to convert dihydroxyacetone phosphate (DHAP) into PDO. These included yeast genes converting dihydroxyacetone to glycerol and *Klebsiella pneumoniae* genes converting glycerol to PDO. They improved production in the recombinant by modifying 18 *E. coli* genes, including regulatory genes. PDO is the monomer used to chemically synthesize industrial polymers such as polyurethanes and the polyester fibre Sorono™ by DuPont. This new bioplastic is polytrimethylene terephthalate (3GT polyester) made by reacting terephthalic acid with PDO (Nakamura and Whited, 2003). PDO is also used as a polyglycol-like lubricant and as a solvent.

4.5.4 Erythritol. The non-cariogenic, non-caloric and diabetic-safe sweetener erythritol is made by fermentation. It has 70–80% the sweetness of sucrose. Osmotic pressure increase was found to raise volumetric and specific production, but to decrease growth of *Triginopsis variabilis*, the producer (Kim *et al*., 1997). By growing cells first at a low glucose level, i.e. 100 g l^{-1} and then adding 200 g l^{-1} glucose at 2.5 days, erythritol titre was increased to 45 g l^{-1} as compared with a single-stage fermentation with 300 g l^{-1} glucose which yielded only 24 g l^{-1}. In both cases, 150 g l^{-1} glucose remained at the end. Production of erythritol by a *C. magnoliae* osmophilic mutant yielded a titre of 187 g l^{-1}, a rate of 2.8 g l^{-1} h^{-1} and 41% conversion from glucose (Ryu *et al*., 2000). Other processes have been carried out with *Aureobasidium* sp. (165 g l^{-1} from glucose with a 48% yield) (Ishizuka *et al*., 1989) and the osmophile *Trichosporon* sp. (188 g l^{-1} with a

productivity of 1.18 g l^{-1} h^{-1} and 47% conversion (Park *et al*., 1998). Erythritol can also be produced from sucrose by *Torula* sp. at 200 g l^{-1} in 120 h with a yield of 50% and a productivity of 1.67 g l^{-1} h^{-1} (Kim *et al*., 2000).

4.5.5 Dihydroxyacetone. Dihydroxyacetone (DHA) is used as a cosmetic tanning agent and as an intermediate for production of chemicals and surfactants (Deppenmeier *et al*., 2002). It is produced from glycerol by *Gluconobacter* species. A 90% conversion from 200 g l^{-1} glycerol has been obtained. Overexpression of the gene coding for the glycerol dehydrogenase (sldAB) increases glycerol oxidation and improves the DHA formation rate, as well as the final DHA concentration (Gätgens *et al*., 2007).

4.5.6 Mannitol. D-Mannitol is a naturally occurring polyol, widely used in the food, chemical and pharmaceutical industries. About 40 000 tons are produced annually. It is only poorly metabolized by humans, is about half as sweet as sucrose and is considered to be a low-calorie sweetener. It is produced mainly by catalytic hydrogenation of glucose/fructose mixtures but 75% of the product is sorbitol, not mannitol. For this reason, fermentation processes are being considered. Several heterofermentative lactic acid bacteria from the genera *Lactobacillus* and *Leuconostoc* have been reported to produce mannitol from fructose (Yun and Kim, 1998). An inexpensive medium containing molasses, fructose syrup, soy peptone and corn steep liquor was developed for production of 105 g l^{-1} mannitol by *Lactobacillus intermedius* (Saha, 2006b). Manganese added at 0.033 g l^{-1} allowed this microorganism to convert 300 g l^{-1} fructose to 201 g l^{-1} mannitol and 62 g l^{-1} lactic acid (Saha, 2006a). Recombinant *E. coli* produced up to 91 g l^{-1} mannitol (Kaup *et al*., 2004) and *Leuconostoc* sp. up to 98 g l^{-1} (von Weymarn *et al*., 2002). Mannitol production reached 223 g l^{-1} using *C. magnoliae* with Ca^{2+} and Cu^{2+} supplementation (Lee *et al*., 2007).

4.5.7 Sorbitol. This polyol, also called D-glucitol, is 60% as sweet as sucrose and has use in the food, pharmaceutical and other industries. Its worldwide production is estimated to be higher than 500 000 tons per year and it is made chemically by catalytic hydrogenation of D-glucose. Several microorganisms have been tested for the production of sorbitol, but only a few of them have been suggested as potential sorbitol producers, including three yeast strains and the ethanol-producing bacterium *Z. mobilis*. Toluenized (permeabilized) cells of *Z. mobilis* produce 290 g l^{-1} sorbitol and 283 g l^{-1} gluconic acid from a glucose and fructose mixture in 16 h with yields near 95% for both products (Chun and Rogers, 1988). This and other potential processes have been reviewed by Silveira and Jonas (2002). Metabolic engineering of *Lactobacillus plantarum* for high sorbitol production was successfully achieved by a simple two-step strategy [overexpressing the two sorbitol 6-phosphate dehydrogenase genes (srlD1 and srlD2) identified in the genome sequence]. However, the use of *L. plantarum* as a cell factory for polyol production requires further optimization of conversion efficacy (Ladero *et al*., 2007).

4.5.8 Xylitol. Xylitol is a naturally occurring sweetener with anticariogenic properties and use in some diabetes patients. It can be produced chemically by chemical reduction of D-xylose. A mutant of *Candida tropicalis* produces 40 g l^{-1} from D-xylose with over a 90% yield (Gong *et al*., 1981). Better xylitol production (150 g l^{-1}) was obtained with *C. guilliermondii* 2581 at pH 6.0 and shaking at 60 r.p.m. (Zagustina *et al*., 2001). Under these conditions, the substrate concentration (150 g l^{-1}) was totally consumed.

4.5.9 Acetone/butanol. Early in the 19 century, the acetone-butanol fermentation process was a commercial operation but was later replaced by chemical synthesis from petroleum because of economic factors. These included the low concentration of butanol in the broth (1%) and the high cost of butanol recovery. Early work has been reviewed by McNeil and Kristiansen (1986).

Clostridium beijerinckii and *Clostridium acetobutylicum* are the organisms of choice for the fermentation. The latter was isolated by Chaim Weizmann in England due to the need during World War I for acetone to be used in explosives. Weizmann was commissioned by Winston Churchill, the First Lord of the Admiralty, to develop a microbial acetone process. The organism mainly produces butanol and acetone but also smaller amounts of acetate, butyrate and ethanol. After the war, the fermentation was used to supply butanol, an excellent solvent to act as a quick-drying lacquer for car bodies in a rapidly expanding US automotive industry. The fermentation became important again at the start of World War II as a source of acetone for manufacture of munitions. Weizmann became the first President of the new State of Israel in the late 1940s. However, in the 1950s, the process was replaced by production from petroleum. Problems included the cost of substrate, the cost of recovery and the toxicity of butanol to the microbe. At 13 g l^{-1}, growth essentially stopped.

Despite the above, research on this fermentation has continued over many years, dealing with process engineering, mutation and metabolic engineering (Mermelstein *et al*., 1993). Butanol-resistant mutants showed increased production of butanol and acetone (Hermann *et al*., 1985). Biochemical engineering modifications were able to increase total acetone, butanol and ethanol

production (ABE) to 69 g l^{-1} (Qureshi *et al*., 1992). A mutant in the presence of added acetate was able to produce from glucose almost 21 g l^{-1} butanol and 10 g l^{-1} acetone from glucose (Chen and Blaschek, 1999). Acetate both stimulates production and helps stabilize the culture. The parent culture was known to be highly unstable (Kashket and Cao, 1995). A continuous flow process using degermed corn has been developed recently (Ezeji *et al*., 2007).

Biobutanol is looked upon as a more favourable future fuel than bioethanol. Butanol has one-third higher energy content than ethanol and automobile engines do not require modification until the percentage of butanol reaches 40% of the total; ethanol requires modification when it reaches over 15% of the total fuel mixture (Schwarz and Gapes, 2006).

4.6 Glucosamine

Glucosamine is used for osteoarthritis and is made by acid hydrolysis of chitin from shellfish waste. As many patients have shellfish allergies, a fermentation source is desirable. Metabolic engineering of *E. coli* yielded a process producing 17 g l^{-1} glucosamine (Deng *et al*., 2005). In this strain, glucosamine synthase was overexpressed, glucosamine degradative genes were inactivated, and the inhibition of glucosamine synthase by glucosamine was decreased by mutational modification of the enzyme via error-prone PCR. Overexpression of a heterologous glucosamine-6-P-N acetyltransferase yielded a strain making 110 g l^{-1} *N*-acetylglucosamine which is easily converted to glucosamine by mild acid hydrolysis.

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