Engineering primary metabolic pathways of industrial micro-organisms

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Abstract

Metabolic engineering is a powerful tool for the optimisation and the introduction of new cellular processes. This is mostly done by genetic engineering. Since the introduction of this multidisciplinary approach, the success stories keep accumulating.

The primary metabolism of industrial micro-organisms has been studied for long time and most biochemical pathways and reaction networks have been elucidated. This large pool of biochemical information, together with data from proteomics, metabolomics and genomics underpins the strategies for design of experiments and choice of targets for manipulation by metabolic engineers. These targets are often located in the primary metabolic pathways, such as glycolysis, pentose phosphate pathway, the TCA cycle and amino acid biosynthesis and mostly at major branch points within these pathways. This paper describes approaches taken for metabolic engineering of these pathways in bacteria, yeast and filamentous fungi.

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1. Introduction

In the past, improvement of microbial and cellular processes was achieved mainly by evolutionary (classical) breeding methods or repeated rounds of mutagenesis and selection of a desired phenotype. These methods are still very useful (Sauer, 2001; Sonderegger and Sauer, 2003; van Maris et al., 2004b), but since the introduction of recombinant DNA technology a more rational approach for biotechnological process development and optimisation became obvious. Bailey defined metabolic engineering as an “Improvement of cellular activities by the manipulation of enzymatic, transport and regulatory functions of the cell with use of recombinant DNA technology” (Bailey, 1991), which was generalized to “purposeful modification of the intermediary metabolism using recombinant DNA technology” (Cameron and Tong, 0168-1656/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
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1993) or “genetic modification of cellular biochemistry to introduce new properties or to modify existing ones” (Jacobsen and Khosla, 1998).

Today the main goals of metabolic engineering can be summarized in the following four categories: (1) improvement of yield, productivity and overall cellular physiology, (2) extension of the substrate range, (3) deletion or reduction of by-product formation and (4) introduction of pathways leading to new products. Commonly these goals can be achieved by a three-step procedure. Firstly, a genetic modification is proposed, based on metabolic models. After genetic modification, the recombinant strain is analysed and the results are then used to identify the next target for genetic manipulation, if necessary. Thus, the construction of an optimal strain involves a close interaction between synthesis and analysis, usually for several consecutive rounds. The rapid development and frequent success in this field is demonstrated by the large number of reviews about the theoretical and practical aspects of metabolic engineering (Cameron and Chaplen, 1997; Cameron and Tong, 1993; Nielsen, 1998, 2001; Ostergaard et al., 2000; Stephanopoulos, 1994; Stephanopoulos, 1999; Stephanopoulos and Sinskey, 1993; Stephanopoulos and Vallino, 1991). Knowledge of cellular and microbial physiology (Nielsen and Olsson, 2002), as well as the underlying metabolic networks or enzymes, is an important prerequisite for successful engineering. The need for readily available information on these topics has been addressed by the creation of several databases, such as KEGG (Kanehisa, 1997; Kanehisa and Goto, 2000), BRENDA (Schomburg et al., 2002) and MetaCyc (Krieger et al., 2004). Software tools, e.g. FluxAnalysyer (Klamt et al., 2002), MetaFluxNet (Lee et al., 2003), OptKnock (Burgard et al., 2003; Pharkya et al., 2003) and MetaboLogic (Zhu et al., 2003) link experimental data with database knowledge. Recently, a computational approach for the identification of every possible biochemical reaction from a given set of enzyme reaction rules was reported (Hatzimanikatis et al., 2005). This allows the de novo synthesis of metabolic pathways composed of these reactions and the evaluation of these novel pathways with respect to their thermodynamic properties.

A new term, ‘Inverse Metabolic Engineering’ (IME) has been coined to encompass the construction of strains with a particularly desirable physiological phenotype, e.g. enhanced production of heterologous protein (Bailey et al., 2002; Gill, 2003; Nielsen, 1998, 2001). This is a challenging approach to identify the genes that confer a prominent phenotype when the responsible mutation(s) are anywhere on the genome, but not at the place of the coding gene itself. For example, over-expression of certain sigma factors enhanced protein production in stationary phase (Weikert et al., 2000). Screening of libraries to detect such changes has been substantially superseded by analysis of transcriptomic arrays to detect up- or down-regulation of genes, if genome data were available. This is an interesting alternative to the classic approach for detection of rate-limiting steps, where it is necessary to have extensive knowledge about the pathway and the reaction kinetics of the enzymes involved.

Metabolic engineers have access to a vast array of genetic tools to design new intriguing strains. Existing platforms include suitable and safe laboratory strains, transformation systems, auxotrophic and dominant markers and also constitutive as well as tightly-regulated promoters. Simultaneous over-expression of several enzymes using only strong promoters might not improve existing pathways but instead stress the organisms by increasing the metabolic burden (Mattanovich et al., 2004). Furthermore, high levels of several or all enzymes of a pathway may lead to undesirable changes in metabolite levels and subsequent down-regulation of some enzymes. The rigid control of the fluxes in, especially, the central carbon metabolism has been demonstrated already in yeast. The genes for eight different enzymes were placed on multi-copy vectors. Separately or pairwise overproduction of these glycolytic enzymes did not result in higher rate of ethanol formation or modified levels of key metabolites, compared to a wild-type strain (Schaaff et al., 1989). Balanced and coordinated expression of enzymes of a metabolic pathway therefore requires sets of, possibly artificial, promoters (Alper et al., 2005; Jensen and Hammer, 1998; Mijakovic et al., 2005; Solem and Jensen, 2002). Synthetic promoters have been used to study the control of the glycolytic flux in E. coli by measuring the ATP demand (Koebmann et al., 2002).

Literature and database knowledge, combined with experimental data and mathematical modelling, can be used to identify metabolic networks (Pedersen et al., 2000). Models for metabolic flux distribution are needed to improve the production of a desired metabolite. Once the network is known, such models include
the respective fluxes and how they are controlled. Metabolic flux analysis (MFA), metabolic control analysis (MCA) and the biochemical systems theory (BST) are most commonly used techniques to create mathematical models.

Depending on the method, certain principles need to be taken into account (Voit, 2003). Metabolic flux quantification is a crucial prerequisite to direct as much carbon as possible to the desired product. In the simplest concept of metabolite balancing or stoichiometric MFA, material balances are set up for each metabolite in the network. Assuming the metabolite concentrations are in steady state, a set of algebraic equations relating the fluxes is obtained. Linear programming is then used to calculate the fluxes through the branches of the network (Ren et al., 2003; Stephanopoulos et al., 1998). Further information is needed to complement the flux data to derive a more accurate model. The use of $^{13}$C-labelled glucose and measurement of the labelling patterns of the intracellular metabolites (e.g. proteinogenic amino acids) by NMR or MS techniques (GC–MS, LC–MS, MALDI-TOF-MS) provides an additional set of data to augment the metabolite balances (Wiechert, 2001). This additional information then allows MFA to be extended to other useful applications: identification of branch points (nodes) in cellular pathways or the existence of different pathways, calculation of non-measured extracellular fluxes and most importantly the calculation of maximum theoretical yields. There are numerous recent examples for the use of NMR or GC–MS techniques for metabolic flux analysis, although in most cases GC–MS is the preferred method because it is usually faster and more sensitive (Blank et al., 2005; Christensen and Nielsen, 1999; Fredlund et al., 2004; Raghevendran et al., 2004; Thykaer et al., 2002; Van Dien et al., 2003).

Metabolic flux analysis is a valuable tool to study pathway interactions and the quantification of flux distribution around branch points, but it does not provide insight in flux control, which describes the relationship between the flux in a pathway and the activity of its enzymes. This relation is defined by MCA (Heinrich and Rapoport, 1974; Kacser and Burns, 1973) in terms of the flux control coefficient (FCC), which represents the percentage change in flux divided by the percentage change in activity of an enzyme that was responsible for that flux change. According to this concept, an enzyme with a flux control coefficient close to 1 could be defined as “rate-limiting”. However, quantitative measurements of flux control coefficients showed that such values are unusual (Fell, 1992, 1998) and that flux control is distributed over all steps in a pathway, with some steps having a higher flux control than others. The fact, that metabolic fluxes exert control at several levels, e.g. transcriptional control (Zaslaver et al., 2004), translational control, activation-inactivation or allosteric control of enzymes, makes it especially challenging to predict the consequences of genetic modifications. An alternative modelling approach, that does not require the vast amounts of information needed for MCA was presented by (Visser et al., 2000). Their approach, termed “tendency modelling”, minimises the number of model parameters and the mathematical effort, but only aims to predict flux tendencies.

Biochemical systems theory (Savageau, 1969), which is based on kinetic models and accounts for regulatory signals, e.g. feedback inhibition allows new kinetic models to be set up to quantify fluxes through pathways, not only at steady state but also under transient conditions.

The so-called ‘–omics’ technologies, mainly functional genomics, proteomics and metabolomics contribute significantly towards metabolic engineering as large amounts of data are produced, which can be used to gain a better understanding of flux control and consequently lead to improved models of metabolic pathways. DNA microarrays (Dharmadi and Gonzalez, 2004) are used to study the transcriptional responses of an organism to genetic and environmental changes (Boer et al., 2003; Featherstone and Broadie, 2002; Giaever et al., 2002; Oh and Liao, 2000a, 2000b). This genomic approach has allowed the identification of regulatory networks. A reduction of the levels of negative regulators or increase of positive regulators of gene expression then should enable a uniform and balanced increase of enzyme activities of the target metabolic pathway. Processing and normalization of the microarray data obtained is still challenging. In an attempt to reduce this problem, (Hyduke et al., 2003) developed a software package to estimate gene-specific confidence intervals for each gene in a cDNA microarray data set.

Proteome analysis allows profiling of a large number of proteins on a two-dimensional polyacrylamide gel by systematic separation, identification and quantification (Han et al., 2001; Kim et al., 2004b). Thereby, changes in the levels of protein expression
of different mutants or under different environmental conditions can be determined and used to define target enzymes/proteins for further manipulation (Han and Lee, 2003). Clearly, proteome analysis is not limited to 2D PAGE and protein quantification but also allows the identification of post-translational modifications (Kirkpatrick et al., 2005; Peng et al., 2003) which are potent regulators of protein function (Mesojednik and Legisa, 2005).

Metabolomics covers the quantification of intracellular and extracellular metabolite concentrations using analytical devices in appropriate time scales (Buchholz et al., 2002; Burja et al., 2003). Metabolome data can also be used to identify a silent phenotype in terms of growth rate or other fluxes by quantification of changes in certain metabolite concentrations relative to the concentration of a selected metabolite. These “metabolic snapshots” allow identification of functions of deleted genes (Raamsdonk et al., 2001).

1.1. Yeast

Although there are numerous technological applications for non-conventional yeasts, e.g. Pichia pastoris, Schizosaccharomyces pombe or Hansenula polymorpha (Pichia angusta) (Spencer et al., 2002), metabolic engineering has been performed almost exclusively with Saccharomyces cerevisiae. Nevertheless, considerable metabolic information about non-conventional yeasts has been gathered lately (Blank et al., 2005; Ren et al., 2003). The physiological properties of non-conventional yeasts are interesting and presumably more applications in metabolic engineering will follow (Branduardi et al., 2004). Metabolic flux profiles of the yeasts P. stipitis (Fiaux et al., 2003) and P. pastoris (Sola et al., 2004) have recently been determined using NMR spectroscopy. However, the most notable progress in engineering of central metabolism has been reported for S. cerevisiae.

The extension of substrate range for fermentation is one of the most active fields, because this is an important prerequisite to make the production of bulk products, mainly ethanol, economically feasible. Cellulosic biomass is an attractive feedstock since it is available as a waste product in large amounts. The hydrolysates of these complex carbohydrate polymers contain different hexoses and pentoses, including glucose, galactose, mannose, arabinose and xylose, the last being the most abundant hemicellulose sugar. This fact lead to an huge amount of research devoted to fermentation of xylose and other pentoses (Jeffries and Jin, 2004).

Wild-type strains of S. cerevisiae, while basically capable of growing on xylose are not capable of fermenting this compound anaerobically. As the costs of aerating bioreactors for ethanol production is significant, this presents a major obstacle for industrial application.

The introduction of the XYL1 and XYL2 genes from the xylose-metabolizing yeast P. stipitis, encoding the NAD(P)H dependent xylose reductase (XR) and the NADH dependent xylitol dehydrogenase (XDH), as well as over-expression of the endogenous XKS1 gene, encoding a xylulokinase (XKS), allowed anaerobic growth and ethanol fermentation in the S. cerevisiae strain TMB3001 (Eliasson et al., 2000; Toivari et al., 2001) Over-expression of the XKS1 gene leads to increased xylose utilisation and lowered ATP/ADP ratios (Toivari et al., 2001). Inclusion of glucose in the media was still necessary and xylose consumption and ethanol production were low. Nevertheless, this approach showed potential for further improvement, despite the restrictive effects of the anaerobic redox imbalance and extensive by-product formation (e.g. xylitol). Expression of the xylose utilisation pathway described above in combination with evolutionary engineering, improved the xylose catabolism of TMB3001 and resulted in the TMB3001C1 strain (Sonderegger and Sauer, 2003). This was the first report of a strain capable of growing on xylose as sole carbon source under strictly anaerobic conditions. This strain produced 19% more ethanol than the parent strain, but still was hampered by slow growth (0.119 h⁻¹) and high xylitol production. Expression of redox metabolism genes was altered in a way that more NADH was reoxidized and more NADPH was formed, allowing faster conversion of xylose to xylulose. Nevertheless, the imbalance of cofactors was still growth limiting, as addition of acetoin, an NADH oxidizing compound, increased anaerobic growth rate (Sonderegger et al., 2004a). Cofactor balances calculated from flux analysis revealed a constant specific NADPH production rate in the cytosol and increased production of ATP. Whether higher NADPH production could improve the growth rate further is still an open question. However, the results from the evolved
strain demonstrated that xylose metabolism is not linked to respiration. The authors concluded, that the rate of ATP production is the actual limiting factor for anaerobic growth on xylose, and not the redox balance as such, which still limits the ATP production rate. Several approaches to alleviate this imbalance to improve ethanol production were reported, leading to the conclusion that the branch point between glycolysis and PPP is a hot spot for engineering. Jeppsson et al. (2002) reduced the flux through the NADPH-producing pentose phosphate pathway (PPP) by disruption of either the \textit{GND1} gene, coding for an isoform of 6-phosphogluconate dehydrogenase, or the \textit{ZWF1} gene, encoding glucose 6-phosphate dehydrogenase. Disruption of the \textit{GND1} gene prevented conversion of 6-phosphogluconate to ribulose 5-phosphate. Consequently both strains showed increased ethanol yields and lower xylitol production. Disruption of the \textit{ZWF1} gene resulted in the highest ethanol and lowest xylitol yield (0.41 and 0.05 g g\textsuperscript{-1}, respectively) among the tested strains. Acetate production was increased and the rate of xylose consumption was lowered by 84% due to reduced NADPH-dependent reduction of xylose to xylitol. Over-expression of the xylitol reductase (XR), which is responsible for this conversion, significantly elevated the xylose consumption rate, but with the drawback of increased glycerol yields. This is due to DHAP conversion by XR. The depletion of this precursor for glycerol production limited the usefulness of increased XR activity (Jeppsson et al., 2003). Over-expression of a NADP\textsuperscript{+}-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) from \textit{Kluyveromyces lactis}, whose function is not coupled to wasteful \textit{CO}_{2} formation, in a \Delta zwf1 \textit{S. cerevisiae} strain also increased rate and yield of ethanol production and decreased xylitol production. This strategy allowed the reduction of the \textit{CO2/ethanol} ratio of 2.5 (mol/mol) in the control strain to 1.3 in the recombinant strain, thereby improving the ethanol yield (Verho et al., 2003).

Engineering of ammonium assimilation has been shown to be another good method to overcome the obstacle of redox cofactor imbalance. The \textit{GDH1} gene encodes an NADPH-dependent glutamate dehydrogenase, which catalyses the assimilation of ammonium to glutamate by reaction with 2-oxoglutarate. Its deletion renders \textit{S. cerevisiae} unable to assimilate ammonium in a NADPH-dependent manner. Instead, NADH was redirected for ammonia assimilation at the cost of glycerol production. Increased ethanol and decreased glycerol yields were achieved, although the overall ethanol productivity was severely affected since growth was reduced. Alternative reactions for ammonia assimilation were provided by over-expression of the \textit{GDH2} gene (NADH-dependent glutamate dehydrogenase) or the GS-GOGAT system (cooperative action of glutamate synthase (\textit{GLT1}) and glutamine synthetase (\textit{GLN1})) in the \Delta gdh1 strain to successfully overcome the growth problem (Nissen et al., 2000). After this proof of principle, the same strategy was used in a xylose consuming strain, resulting in reduced xylitol excretion in combination with a 15% higher xylose fermentation rate and an increased yield of ethanol (Roca et al., 2003). Under aerobic conditions these modifications reduced flux through the pentose phosphate pathway and increased flux through the TCA cycle (Moreira dos Santos et al., 2003). Unfortunately, the applicability of this strategy for NADH reoxidation by reduction of xylitol excretion is limited, due to the coupled requirement for anabolic ammonium. Sonderegger et al. (2004b) introduced the phosphoketolase pathway in their mutant strain, TMB3001C1, thereby engineering the redox metabolism rationally. Addition of a functional phosphoketolase...
pathway, consisting of a phosphoketolase (PK), phosphotransacetylase (PTA) and an acetaldehyde dehydrogenase (ACDH), theoretically leads to a net reoxidation of one NADH per xylose converted to ethanol. PK’s convert xylulose-5-P to glyceraldehyde-3-P and acetyl-P, PTA catalyses the formation of acetyl-CoA from acetyl-P followed by ACDH-catalysed formation of acetaldehyde (see Fig. 1). This engineering strategy improved the yield of ethanol by about 25% without affecting the anaerobic fermentation rate. The authors also identified acetate as a strong inhibitor of xylose fermentation and disruption of the **ALD6** gene, encoding the NADPH-dependent aldehyde dehydrogenase, increased the xylose fermentation rate by 50%. The next logical step, namely combination of reduced acetate formation with the phosphoketolase pathway, further improved the fermentation and production features of the resulting strain in comparison to the control. This alternative route of carbon to ethanol obviously did not interfere with other metabolic reactions, which is a great advantage over other strategies, which usually resulted in increased ethanol yield at the cost of the fermentation rate or vice versa. Further improvements of this strategy, by either rational or evolutionary methods, seem possible.

Fermentation of xylose under anaerobic conditions is made feasible by a completely different approach by expression of a heterologous xylose isomerase (XI), which directly converts xylose into xylulose in a redox-neutral manner. This strategy circumvents the problem of cofactor imbalance. However, most attempts were unsuccessful because the introduced xylose isomerases were not expressed well, an exception being the xylose isomerase from the anaerobic fungus *Piromyces sp.* (Kuyper et al., 2003, 2004). Expression of this enzyme resulted in anaerobic growth on xylose with a specific growth rate of 0.005 h⁻¹, which was improved significantly by evolutionary breeding methods to 0.03 h⁻¹, accompanied by an ethanol yield of 0.42 g g⁻¹(xylose)⁻¹ (Kuyper et al., 2004). Most recently, over-expression of all structural enzymes involved in conversion of xylose further enhanced the growth rate to 0.09 h⁻¹ (Kuyper et al., 2005).

All of these improvements were achieved in laboratory strains, as industrial strains of *S. cerevisiae* are usually diploid or polyploid and lack auxotrophic markers. (Wang et al., 2004) have introduced the xylose utilisation pathway (XR, XDH and XKS) in an industrial strain. Ethanol yield was increased in conjunction with high xylitol excretion due to the redox imbalance described above. This demonstrated that the results from laboratory strains can be extrapolated to industrial strains, as expected.

Arabinose is another widespread pentose for ethanol production. Early attempts to express either the bacterial (Sedlak and Ho, 2001) or fungal arabinose-utilisation pathway (Richard et al., 2002, 2003) in *S. cerevisiae* were not extremely successful due to problems with arabinose uptake. In addition, a redox cofactor imbalance resulted in minimal production of ethanol. Recently an NADH-dependent reductase was identified from the yeast *Ambrosiozyma monospora* (Verho et al., 2004). This enzyme converts l-xylulose, which is an intermediate of arabinose-utilisation, and may help to circumvent, at least partially, the imbalance. Over-expression of enzymes assembling a bacterial arabinose-utilisation pathway, consisting of *Bacillus subtilis* AraA, *E. coli* AraB and AraD as well as over-expression of the arabinose-transporting galactose permease from yeast, allowed the development of an arabinose-utilising strain. A *B. subtilis* gene was chosen because the corresponding *E. coli* enzyme was not expressed well. However, the doubling time of the transformants was very low and strains could not grow on arabinose as sole carbon source. To improve the growth rate, transformants were subjected to serial transfer under restrictive conditions to apply selective pressure and a doubling time of about 7.9 h was reached for anaerobic growth on arabinose with an ethanol yield of 0.08 g g⁻¹(cell dry mass)⁻¹ (Becker and Boles, 2003). Analysis by DNA microarrays revealed that the main reasons for improved growth were a low l-ribulokinase activity and increased transaldolase activities. Both results are consistent with earlier data. Pathways with an initial ATP-requiring reaction, such as the l-ribulokinase reaction, which finally produce a surplus of ATP, need tight control of the respective enzyme. Under conditions for which ATP consumption and production are equal, accumulation of pathway intermediates may continue because the ATP level does not change. Therefore, the initial reaction is not limited by the failure of the remaining pathway reactions (Teusink et al., 1998). A few years earlier Walfridsson et al. (1995) showed that the endogenous transaldolase activity of *S. cerevisiae* was too low for efficient utilisation of PPP metabolites, because over-expression
of the gene encoding transaldolase enhanced growth on D-xylose significantly.

Redirection of carbon fluxes through alternative pathways also seems to be a suitable application for wine production. Depending on the strain and the composition of must, higher levels of acetic acid are sometimes produced, affecting the quality of product. The concentration of acetic acid in fermented products, such as wine and beer, must remain low. (Remize et al., 2000) investigated the effects of reduced excretion of acetate by S. cerevisiae via engineering of the “pyruvate dehydrogenase bypass”. Pyruvate decarboxylase (encoded by three structural genes: PDC1, PDC5 and PDC6), acetaldehyde dehydrogenase, encoded by the ALD gene family and acetoacetyl-CoA synthetase (ACS) are responsible for this bypass. Reduction of pyruvate decarboxylase activity did not result in reduced levels of acetate, whereas ALD6 disruptants, exhibiting 60 and 30% of wild-type acetaldehyde dehydrogenase activity, showed a substantial reduction in yield of acetate (75 and 40%, respectively). This effect was associated with a rerouting of carbon flux towards succinate, butanediol and glycerol. The regulatory capability of the ALD6 disruptant was further used for the overproduction of glycerol, which is employed for synthesis of many different products, e.g. cosmetics. In S. cerevisiae, glycerol is synthesised from DHAP, a glycolytic intermediate, in a two-step reaction: reduction by glycerol 3-phosphate dehydrogenase (GPD) and consecutive dephosphorylation by glycerol 3-phosphatase (GPP). Over-expression of a glycerol 3-phosphate dehydrogenase isoenzyme, encoded by GPD2, is known to increase production of glycerol and lower production of ethanol, but this is accompanied by increased levels of acetic acid.

The combination of over-expression of the GPD2 gene (glycerol 3-phosphate dehydrogenase) and ALD6 disruption (Eglinton et al., 2002) reduced production of acetic acid four-fold, while raising the concentration of glycerol in the medium from 13.4 to 16.3 g/l compared with control strain that did not have the ALD6 gene disrupted.

High glycerol yields have also been achieved in Δtpi1 mutants, lacking the glycolytic enzyme triose phosphate isomerase. Apparently accumulation of DHAP is prevented by its conversion to glycerol. These mutants are not capable of growth on glucose as sole carbon source. A quadruple mutant (Δtpi1 Δnde1 Δnde2 Δgut2) was constructed by Overkamp et al. (2002) to show that the growth defect was due to mitochondrial reoxidation of cytosolic NADH, thus rendering it unavailable for reduction of DHAP. NDE1 and NDE2 encode isoenzymes of extra-mitochondrial NADH dehydrogenase, while GUT2 encodes an enzyme of the glycerol 3-phosphate shuttle. The mutant strain grew on glucose, although rates were dependent on concentration of glucose in the medium. As conversion of glucose into glycerol and pyruvate is neutral in terms of ATP production, growth of the quadruple mutant on glucose seemingly depends on respiration for production of ATP. Expression of many enzymes involved in the respiratory dissimilation of pyruvate is subject to catabolite repression, explaining the sensitivity to glucose by the mutant strain. Serial transfer in batch cultures was performed and the growth rate was improved, but remained quite low, a fact that interestingly may contribute to the high yield of glycerol of over 200 g l⁻¹ (Overkamp et al., 2002).

Production of lactic acid has received a lot of attention due to its numerous applications in food, pharmaceutical and cosmetic industry as well as in the synthesis of biodegradable polymers. Although lactate is classically produced using bacterial cells, engineered yeast cells offer solutions for problems, such as low pH, inhibitory effects of the produced acid and purification procedures. The first attempts to produce lactate in yeasts by expression of a lactate dehydrogenase (LDH) (Adachi et al., 1998; Dequin and Barre, 1994; Porro et al., 1995; Skory, 2003) resulted in low yields and production of ethanol. Reduction of the pyruvate decarboxylase or alcohol dehydrogenase activity (Skory, 2003) did not improve the lactate production in a satisfactory manner. Alcoholic fermentation can be completely eliminated by deletion of the three genes for pyruvate decarboxylase (Hohmann, 1991), but is known to be associated with severe impairment of cellular growth (Flikweert et al., 1996; Pronk et al., 1996). Kluyveromyces lactis, which only possesses one PDC gene, was used as an alternative for S. cerevisiae (Porro et al., 1999). Deletion of the PDC1 gene and expression of a bovine LDH resulted in a total replacement of ethanol for lactic acid fermentation, without hindering growth, thereby increasing the lactic acid yield 2.7-fold in comparison with the heterolactic control strain. Despite the high LDH activity, the highest obtained yield of 1.19 is still below the maximum
theoretical value of 2 mmol of lactate per mmol glucose. van Maris et al. (2004a) attempted to elucidate the role of oxygen in metabolically engineered homofermentative lactate producing *S. cerevisiae* and analysed its behaviour under various aeration conditions. The authors concluded that the conversion of glucose to extracellular lactate does not yield ATP, most probably due to the need of energy for the product export, thereby also providing an explanation for the low lactate production rates under anaerobic conditions. Transgenic strains in which LDH genes were integrated into the *S. cerevisiae* genome under control of the *ADH1* promoter (Colombie et al., 2003) or the *PDC1* promoter (Ishida et al., 2005) brought no improvements. Increasing the copy number of integrated LDH genes yielded 122 g l \(^{-1}\) lactate with a high optical purity (>99.9%) on a cheap, cane-juice based medium (Saitoh et al., 2005), but the fundamental problems of lactate production in *S. cerevisiae*, such as growth depletion under anaerobic conditions or alcoholic fermentation have not yet been solved despite the efforts of numerous research groups.

### 1.2. Filamentous fungi

Filamentous fungi are an extremely diverse group of heterotrophic micro-organisms that are exploited for various biotechnological applications: they are used in the production of foods, beverages, organic acids, enzymes, polysaccharides, antibiotics and other pharmaceuticals. Fungi produce a vast array of secondary metabolites and some species have highly efficient protein secretion mechanisms that can be exploited to express homologous or heterologous gene products (O’Donnell and Peterson, 1992). Although there are several model organisms among filamentous fungi, such as *Neurospora crassa* and *Aspergillus nidulans*, they were mostly used for studying fungal physiology and genetics but not for metabolic engineering. In spite of the industrial importance of this group of micro-organisms, reports on the use of different techniques of metabolic engineering were published mostly by academic institutions. The papers deal predominately with the application of different mathematical models, such as: metabolic flux, metabolic control and metabolic networks analysis, to identify the strategic steps that could be improved by genetic manipulation in order to increase the productivity and/or yields. On the other hand, it seems that improvements of the fluxes by recombinant DNA techniques conducted by various industrial laboratories have remained largely unpublished. Therefore, papers dealing with the so-called constructive type of metabolic engineering (Bailey et al., 2002) are numerous while reports using the inverse metabolic engineering technique are infrequent.

As the filamentous fungi are obligate aerobes, no fermentative products are accumulated that would originate from the intermediates of glycolytic flux, but they may excrete some acids that are intermediates of the tricarboxylic acid cycle. Citric acid can be transported out of the cells of *Aspergillus niger*. This organism was therefore used for detailed studies of primary metabolite production (Ruijter et al., 2002). Information from the measurements of different metabolite levels was used by Torres (Torres, 1994a, 1994b) to prepare mathematical models that simulated production of citric acid. The authors used the BST method to propose a process optimisation solution. Data taken from batch fermentation were transposed to a metabolic model of carbohydrate metabolism, and the mechanistic model was translated subsequently in mathematical terms adopting the expression of an S-system representation within the framework of BST (Savageau, 1976).

It showed that, as the steady state was stable, sensitivity theory could be applied (Torres, 1994a). The flux and metabolite concentration control structure of the system that was derived indicated that substrate uptake was the main-rate-limiting step of the process (Torres, 1994b). Using this model, it was possible to use the linear programming to optimise the process. Maintaining the metabolite pools within narrow physiological limits and allowing the enzyme concentrations to vary within a 0.1- to 50-fold range of their basal values, would allow up to three-fold increase of the glycolytic flux and result in nearly 100% transformation of substrate into product (citric acid). To achieve these improvements, it would be necessary to modulate seven or more enzymes simultaneously (Torres et al., 1996). To test the model and to increase the glycolytic flux in *A. niger*, two genes coding for key regulatory enzymes have been overexpressed. However, no increase in glycolytic flux or citric acid accumulation was observed. It appeared that the fungus adapted to over-expression by decreasing the specific activity of the enzymes through reduction of the level of fructose-2,6-biphosphate, a potent effector of 6-phosphofructokinase (Ruijter et al., 1997). On the other hand, the metabolic control model of carbo-
hydrate metabolism proposed by Torres et al. (1996) was constructed using data obtained during the growth of A. niger in medium containing low concentrations of glucose. Today, it is accepted that this procedure leads to metabolic alterations and slower rate of accumulation of citric acid (Xu et al., 1989) At the concentrations higher then 400 mM, glucose enters the fungal cells on the basis of a simple diffusion model (Wayman and Mattey, 2000). Later, in another study 13C-NMR analysis revealed that the control of glycolysis during the cultivation of fungi in a medium containing a high concentration of sugar was shifted from the level of fructose-6-phosphate to that of glyceraldehyde-3-phosphate (Peksel et al., 2002). This puts a new light on further efforts for the optimisation of the process, either by traditional or recombinant techniques.

An A. niger mutant with a silenced aoh gene encoding oxaloacetate hydrolase was also evaluated by MFA (Pedersen et al., 2000). Oxalic acid is a toxic substance produced as a by-product by Aspergillus, which is used for the production of a variety of primary and secondary metabolites. Almost identical metabolic fluxes were recorded when the parental strain was compared with the mutant. This indicated that disruption of the aoh gene had no pleiotropic consequences.

Xylose catabolism in Aspergillus species was studied by MCA (Prathumpai et al., 2003). In two A. nidulans and in one A. niger strain, catabolic flux exerted the main control at the level of polyol dehydrogenase, whereas when another strain was modelled, the conclusion was that the main control of flux control is at the first enzyme of the pathway, xylose reductase.

The primary metabolism of P. chrysogenum was analysed by 2D(13C,1H) COSY NMR measurements. Thus, the NADPH requirements for penicillin production were evaluated during growth in a chemostat with either ammonia or nitrate as the nitrogen source (van Winden et al., 2003). This advanced NMR method enabled the authors to measure a number of components of biomass. The data were used for new metabolic flux analyses in which the cofactor balances could be used or removed. The traditional non-oxidative pentose phosphate pathway was enhanced with additional transketolase and transaldolase genes. Glycolysis was enhanced with the fructose-6-phosphate aldolase/dihydroxyacetone gene or by adding the phosphoenolpyruvate carboxykinase gene. The results with the enhanced non-oxidative pentose phosphate pathway model showed that the transketolase and transaldolase reactions need not be as reversible to obtain a good fit of the 13C-labelling data. 13C-NMR tracer experiments and NMR analysis have been used for metabolic flux analysis of A. oryzae producing α-aminobutyrate (Schmidt et al., 1998). By growing this fungus on various nitrogen sources the authors showed that flux analysis can be performed on the basis of only well established stoichiometric equations and measurements of the labelling state of intracellular metabolites. There was no need for including NADH/NADPH balancing, nor assumptions for energy yield to determine intracellular fluxes of primary metabolism by NMR analysis.

A more advanced technique for metabolic flux analysis using NMR spectrometry for the detection of 13C-labelled isotopomers was employed for the construction of tentative model of central metabolism in Ashbya gossypii (de Graaf et al., 2000). The authors used 1H-NMR spectroscopy which enabled them to determine the complete isotopomer distribution in metabolites having a backbone consisting of up to at least four carbons. Thus, the isotopomer distribution of aspartate isolated from (1-13C) ethanol grown A. gossypii was determined.

Pentose phosphate fluxes of Penicillium chrysogenum during production of β-lactam antibiotics were described in two other studies. In the first stoichiometric model of P. chrysogenum, 61 internal fluxes were determined and 49 intracellular metabolite levels were measured. In addition, the uptakes of 21 amino acids, glucose, lactate and γ-aminobutyrate were taken into consideration and production of penicillin measured. The calculations showed that formation of penicillin is accompanied by a large flux through the pentose phosphate pathway due to an increased requirement for NADPH, which was needed for formation of cysteine. If cysteine was added to the media, the flux through the PPP was reduced (Jorgensen et al., 1995). In the second study of P. chrysogenum metabolism, different biosynthetic routes for generating cytosolic acetyl-CoA were shown to influence the theoretical values of ATP and NADPH requirements for cell biosynthesis (Henriksen et al., 1996). The importance of formation of cysteine in the cells of P. chrysogenum during production of penicillin was also confirmed by other authors (van Gulik et al., 2000). They analysed metabolic fluxes in high and low producing mycelium and compared the
stoichiometric models. It appeared that production of penicillin required significant changes in flux through primary metabolism. Four principal nodes of primary metabolism showed significant changes in flux partitioning and could be regarded as potential bottlenecks for increased productivity.

By feeding \(^{13}\text{C}\)-labelled glucose to a penicillin-overproducing strain, two novel pathways were discovered that might have an impact on product formation. First of all, degradation of phenoxyacetic acid (a side-chain precursor of penicillin) to acetyl-CoA by citrate lyase was proposed. Further experimental data suggested that the main activities of homocitrate synthase and \(\alpha\)-isopropylmalate synthase were located in the cytosol (Christensen and Nielsen, 2000). The degradation of another side-chain precursor, adipate, was evaluated using metabolic network analysis in a recombinant strain of \(P.\) chrysogenum. Chemostat cultures with and without adipate, showed that degradation of the precursor caused undesired consumption of adipate at the expense of formation of adipoyl-7-aminodeacetoxycephalosporanic acid (Thykaer et al., 2002).

Another approach was used for engineering of lovastatin producing \(Aspergillus\) terreus strains (Askenazi et al., 2003). The method referred to as association analysis served to reduce the complexity of profiling data sets to identify those genes whose expression was most tightly linked to secondary metabolite production. Transcriptional profiles were generated by genomic fragment microarrays from strains engineered to produce varying amounts of lovastatin. Metabolite detection methods were employed to quantify the production of secondary metabolites, lovastatine and (+)-geodine. Association analysis of combined metabolic and transcriptional data provided insight into the genetic and physiological control of product formation. This provided a tool for the improvement ofLovastatin production. Also promoters for a reporter-based selection system that was employed after classical mutagenesis were included.

A complex stoichiometric model of the central carbon metabolism was described recently, using information available on \(A.\) niger metabolism (David et al., 2003). The metabolic network was reconstructed by integrating genomic, biochemical and physiological information available for \(A.\) niger as well as other related fungi. In the model, 284 metabolites and 335 reactions were included of which 268 represent biochemical conversion. In addition, 67 transport processes between different intracellular compartments, the interior of the cell and surrounding medium were considered. The rationale of this work was to perform an \textit{in silico} characterisation of the behaviour of \(A.\) niger grown on different carbon sources. Thus, the metabolic capacities of \(A.\) niger under different genetic and environmental conditions were determined using the framework of metabolite balancing in combination with linear programming methods. This model predicts the optimal metabolic behaviour and upper limits for experimental data. However, the model requires further validation and optimisation using experimental data. Nevertheless, it can be used as a tool for the analysis, interpretation and prediction of metabolic behaviour and hence guide the design of improved production strains through metabolic engineering. Furthermore, this model could play a role in functional genomics. Metabolites or reactions for which there is no interconnectivity in the metabolic network imply that these metabolic reactions remain to be characterised.

2. Bacteria

2.1. \textit{Escherichia coli}

Intense studies on \textit{Escherichia coli} over the last 50 years have resulted in it becoming the prime prokaryotic genetic model. Work with this species was confined substantially to laboratory investigations until the advent of foreign protein production whereupon the utility of cloning methodologies for \(E.\) coli made it the pre-eminent host for ‘protein factories’. It was at this point that a troublesome characteristic of growth of \(E.\) coli, the production of acetate when grown on (cheap) glucose-based media (observed earlier (Bennett and Holms, 1975), ceased to become a curiosity and required a process optimisation solution. The problem is still being observed today (Rozkov et al., 2004).

Unwanted production of acetate was a waste of carbon (up to 1/3 of the glucose used could appear as acetate), caused pH control problems in the fermentation and the weak acetate anion could also interfere with bacterial energetics by dissipating the pH component of the membrane potential. A facile solution was to grow the cells on glycerol, which was much more expen-
sive, but this could also result in unwanted formation of product. Understanding how to grow *E. coli* efficiently, to high cell density, with good levels of production of foreign protein required a better understanding of the flux of primary metabolites.

As well as flux from the central pathways to acetate, there are other important factors: uptake of glucose, division of carbon flow at the level of glucose 6-phosphate (either to the EMP or to the PP pathway) and the interplay between phosphoenolpyruvate (PEP), pyruvate and intermediates of the TCA cycle (including the anaplerotic sequences that replenish TCA intermediates removed for growth or product formation). Glucose uptake is normally by the phosphotransferase system (PTS) that obligatorily consumes PEP and converts it to pyruvate while transferring the phosphate group to glucose so that the product of the transport process is glucose 6-phosphate – thus uptake and the end steps of glycolysis are usually linked.

The connection between glucose uptake and PEP utilisation has been broken in an ingenious way. Strains that are defective in the glucose PTS are still capable of slow growth on glucose, via one of the galactose transporters (GalP), which uses a proton symport mechanism, after which the glucose is phosphorylated internally using ATP. Glucose does not act as an inducer of *galP*; therefore a constitutive mutant was needed. Selection in a chemostat gave further up-regulation of the transport activity and the eventual strain benefited from up-regulation of the glucose kinase (whose activity is normally redundant when the PTS is operating). Using such a strain, the conversion efficiency of glucose to 3-deoxy-d-arabinoheptulosonate 7-phosphate (DAHP), the first dedicated intermediate in aromatic amino acid biosynthesis was raised to 0.71 mol mol$^{-1}$, compared to 0.43 mol mol$^{-1}$ when the PTS was operational (Baez et al., 2001). By following the fate of $^{13}$C-labelled glucose, flux distributions have been confirmed (Flores et al., 2002). By over-expression of a combination of genes in a PTS-minus strain, (Baez-Viveros et al., 2004) were able to increase the $Y_{\text{PheGluc}}$ (the yield of phenylalanine per glucose consumed) nearly 57% compared to the PTS$^+$ strain.

The aromatic amino acid pathway has received attention due to renewed interest in the microbial production of shikimic acid. This intermediate of the aromatic pathway is a synthon for the neuraminidase inhibitor GS4104 (Tamiflu), an orally-active antiviral compound for prevention and treatment of influenza. Blocking the pathway, after the shikimate step, knocking out the shikimate importer to prevent re-absorbance of the product, and using a non-PTS uptake system resulted in a titre of 70 g/L and conversion efficiency from glucose of 0.24 (Kramer et al., 2003).

As with streptomycetes, the PP pathway in *E. coli* provides reducing power (NADPH) and carbon skeletons that are incorporated into nucleotides and aromatic amino acids, but carbon flux is diverted from the mainstream glycolytic pathway. Mutants in glucose 6-phosphate dehydrogenase (*zwf*) have a non-operational PP pathway but an increased TCA activity – consistent with the need to generate the ‘missing’ reducing equivalents by this route (Zhao et al., 2004). In another study, a $\Delta zwf$ mutant showed little change in overall physiology (glucose uptake, metabolite production) under glucose-limited conditions (flux was rerouted via the EMP pathway and the non-oxidative part of the PP pathway) whereas ammonia limitation resulted in extensive overflow metabolism accompanied by extremely low tricarboxylic acid cycle fluxes (Hua et al., 2003). This indicates that the environmental conditions can have a profound effect on the overall result. In the same study, a phosphoglucose isomerase (*pgi*) mutant was studied. This enzyme converts glucose 6-phosphate to fructose-6-phosphate and is effectively the first dedicated step of the EMP pathway. The *pgi* mutant used the PP pathway as the primary route of glucose catabolism, which was to be expected. Surprisingly, the glyoxylate shunt was active in this mutant, while the Entner-Doudoroff pathway played a minor role in glucose catabolism (Hua et al., 2003). Synthesis of plasmid-encoded proteins and plasmid-DNA replication often places a heavy metabolic burden on producing cells and usually lowers the growth rate. In contrast to studies where the glucose 6-phosphate dehydrogenase gene was disrupted in order to force more carbon down the glycolytic pathway, Flores et al. (2004) over-expressed *zwf* to overcome the problem of reduced growth rates.

The terminal stages of glycolysis involve complex interplays. PEP may give rise to pyruvate, either by pyruvate kinase (PK) or operation of the PTS, or it may give rise to oxalacetate via the anaplerotic reaction PEP carboxylase (*ppc*). Pyruvate may be converted in three ways: to lactate (oxidizing NADH), eventually to ethanol, or decarboxylated to form acetyl-CoA in
preparation for incorporation to the TCA cycle. There are two PK isofoms (pykA and pykF), so a double mutant had to be constructed (Emmerling et al., 2002). This mutant diverted carbon flux via Ppc and malic enzyme, which might be expected. Of course, pyruvate can also be produced in this PK-minus background by uptake of glucose and concomitant formation of pyruvate from PEP. The flux through Ppc was now greater than that previously through the PK step, indicating that glucose utilisation was increased. Flux through the PP pathway was reduced in concert during glucose-limitation but increased under ammonia limitation – further emphasising the role of environmental conditions on the outcome. These observations were substantially verified by GC–MS analysis of metabolite profiles (Fischer and Sauer, 2003)

The production of acetate can be substantially alleviated by mutation of acetate kinase and phosphotransacetylase, which catalyse formation of acetate from acetyl-CoA. However, this does decrease the cellular growth rate and often results in excretion of lactate from acetyl-CoA. However, this does decrease the cell growth rate and often results in excretion of lactate from acetyl-CoA. Interestingly, further de-repression of the glyoxylate shunt by the deletion of the arcA (phosphorylated ArcA is a dual transcriptional regulator of aerobic respiration control and represses transcription of the glyoxylate shunt operon (Spiro and Guest, 1991) did not significantly increase the succinate yield but did decrease the glucose consumption by 80%. Further, analysis of a ΔadhEΔldhAΔack-pta strain over-expressing a Bacillus subtilis NADH-insensitive citrate synthase gene increased succinate production to that seen in the ΔadhEΔldhAΔack-pta ΔiclR strain.

The capacity of metabolic networks to compensate for mutations is referred to as genetic robustness (Wagner, 2000). With redundancy ensured by gene duplication, the knockout of one gene is readily compensated either by one or more isoenzymes, or alternative pathways or genes with unrelated function becoming active and compensating for the loss of function. Recent transcript analysis of acetate-
grown *E. coli* compared with that grown on glucose highlighted the possible gene utilisation and the roles of converging pathways (Oh et al., 2002). For example, the recently characterised class I type of fructose-bisphosphate aldolase (*dhnA*; (Thomson et al., 1998)) was shown to be up-regulated compared with the known fructose-bisphosphate aldolase (*fba*) and the revelation of differentially regulated open reading frames that were poorly characterised consequently providing important information to predict their function in the future. The surprising induction of phosphoenolpyruvate synthase (*ppsA*), thought to be non-essential for gluconeogenesis during growth on acetate (Holms, 1996), together with both NAD-dependent (*sfcA*) and NADP-dependent (*maeB*) malic enzymes also highlights the dependency on genetic robustness. Further mutational investigation of these genes, as well as phosphoenolpyruvate carboxylase (*pckA*), showed that both enzymes served to provide the phosphoenolpyruvate pool. While single knock out mutations of *pckA* and *ppsA* still yielded growth on acetate, a double mutation did not, implying that PpsA, together with the malic enzymes, acts as an alternative pathway to PckA by delivering metabolites from the TCA cycle to the Embden-Meyerhoff pathway. Precision is the obvious key to successful metabolic engineering. The utility to predict gene function by microarray allows for the improvement of pathway information and the focus for future manipulation.

### 2.2. *Bacillus* sp.

*Bacillus* sp. are widely used for the production of vitamins and other products, including industrial enzymes, such as amylases, proteases and lipases. *Bacillus subtilis* has historically been an attractive host for the expression of protein on an industrial scale due to its ability to secrete foreign proteins directly into the culture medium (Doi and Wang, 1986; Priest, 1977). This therefore, e.g. minimises the need for extensive purification as seen for protein extracts from *E. coli*.

Anaplerotic reactions contribute to the flux through the TCA cycle, the main provider for metabolic precursor molecules, therefore the reactions at the interface between the lower part of glycolysis and the TCA cycle are an important metabolic subsystem (Blencke et al., 2003).

Pyruvate kinase mutants of *B. subtilis* showed a higher production of CO$_2$ from glucose in the TCA cycle than could be accounted to the remaining conversion of PEP to pyruvate by the phosphotransferase system. Zamboni et al. (2004) exposed the activity of the, normally gluconeogenic PEP carboxykinase as the reason for the increased flux towards the TCA cycle upon disruption of the actual anaplerotic reaction of pyruvate carboxylase. Interestingly, the mutations led to impaired growth in an industrial, riboflavin-producing strain, whereas the wild-type strain could not grow at all, most probably due to the unfavourable kinetics of ATP synthesis in this background.

An intrinsic property and a critical process variable in fed-batch fermentations with slow-growing cells is the non-growth associated maintenance energy coefficient (Russell and Cook, 1995; Zamboni et al., 2003). Alternating environmental conditions and by redirection of electron flow to more efficient proton pumping branches within respiratory chains, it is possible to optimise this maintenance metabolism – where the organism remains in a viable state without growth but with optimised energy generation. *B. subtilis* possesses a branched respiratory chain consisting of both quinol (cyd, qox, yth operons) and cytochrome c (ctaCDEF) terminal oxidases (Calhoun et al., 1993; Neijssel and Teixeira de Mattos, 1994; Richardson, 2000). By increasing the efficiency of energy generation using a cytochrome *bd* oxidase mutation, the rate of maintenance metabolism was reduced by 40% (Zamboni et al., 2003). The possible route for improvement was the translocation of two protons per transported electron via the remaining cytochrome *aa$_3$* oxidase, instead of only one proton via the *bd* oxidase, increasing riboflavin production. Zamboni and Sauer (2003) added to this by indicating that efficient ATP generation is not necessary for exponential growth in batch culture. There was no apparent phenotypic difference in the *bd* oxidase mutation, and although the *aa$_3$* oxidase mutation caused severe disruption to the TCA flux with increased metabolic flow, no change in the product-corrected biomass yields was observed between the mutant and the parental strain.

### 2.3. *Streptomycetes*

The order *Actinomycetales* comprises a plethora of remarkable prokaryotic organisms. Genetically
diverse, they have a profound influence on our daily lives. While some cause disease, e.g. tuberculosis (Mycobacterium tuberculosis) and leprosy (Mycobacterium leprae), others are of great industrial importance, such as Corynebacterium glutamicum, which is a significant producer of amino acids (Ikeda, mitilis (Omura et al., 2001) and the genetically recently: the industrial-important Streptomyces aver-

which is a significant producer of amino acids (Ikeda, mitilis (Omura et al., 2001) and the genetically recently: the industrial-important Streptomyces aver-

Importance of the genus Streptomyces that makes them renowned.

Two streptomyces genomes have been sequenced recently: the industrial-important Streptomyces aver-

mitilis (Omura et al., 2001) and the genetically well-characterised Streptomyces coelicolor A3(2) (Bentley et al., 2002). At more than 8.66 Mb, the S. coelicolor genome encodes pathways for the production of over 20 natural secondary metabolites.

Secondary metabolic pathways have been the obvious choice for investigation of strain manipulation and yield improvement in Streptomyces. Until recently, the enzymes controlling the pathways of primary metabolism have been substantially ignored. Manipulation would allow greater harnessing of energy and reducing power that in turn could be used to synthesise cellular building blocks and those for the valuable metabolites.

Relatively little is known about the intermediary metabolic pathways of most streptomycetes. An extensive review on primary metabolism by Hodgson (2000) reported that the Embden-Meyerhof-Parnas (EMP), the pentose phosphate (PP) and TCA cycle pathways were present in a number of Streptomyces species. About 60% of the proteins identified in an early study of the S. coelicolor proteome were associated with primary metabolism (Hesketh et al., 2002). Interestingly, the genome sequence has identified many enzyme iso-

forms for some of the metabolic steps, which differ in temporal expression. Till date, this observation has not been exploited fully by manipulating the genes for such enzymes.

The precursors for polyketide secondary metabolites are derived primarily from the intermediates, acetyl-CoA and malonyl-CoA, peptide antibiotics are derived from amino acids, sugars, shikimate and nucleotides (Hodgson, 2000). Flux distributions calculated for production of actinorhodin (ACT, polyketide) and ‘calcium-dependent antibiotic’ (CDA, oligopeptide) in S. coelicolor (Kim et al., 2004a; Naemipoor and Mavituna, 2000), demonstrated the scope for re-programming. Precursor-directed biosynthesis with mutated strains, by metabolic flux rebalancing, provided novel products with improved properties (Wohleben and Pelzer, 2002). Fluxes associated with prephenate and oxoglutarate, succinate, nitrogen assimilation and flavin adenine dinucleotide (FAD) production were fundamental for CDA production, and correlated with direction of glucose flux to the oxidative PP pathway (Kim et al., 2004a).

Distribution of carbon flux distribution in chemostat cultures of S. lividans grown on glucose or glucone showed increased carbon flux through glycolysis and the PP pathway with increased growth rate, whilst the synthesis of both ACT and the pigmented antibiotic undecylprodigiosin (RED) was inverse to flux through the PP pathway (Avignone Rossa et al., 2002). Deletions of either of two genes (zwf1 and zwf2) encoding the isozymes of glucose-6-phosphate dehydrogenase, which is the initial enzyme of the PP pathway, resulted in enhanced production of the antibiotics ACT and RED (Butler et al., 2002). Interestingly, glucose was utilised more efficiently via glycolysis, with no decrease in concentration of NADPH. Similar results were achieved with the ΔdevB strain, mutated in 6-phosphogluconolactonase, the next enzyme of the PP pathway. However, a Δzwf1Δzwf2ΔdevB triple mutant produced reduced levels of ACT and RED, suggesting that NADPH is essential, either directly or indirectly, in antibiotic production.

Metabolic flux analysis, through following the distribution of labelling in precursor pools, is fundamental for the determination of influential pathways. Li et al. (2004) established that crotonyl-CoA reductase (CCR) had a significant role in provision of methylmalonyl-CoA for monensin biosynthesis in oil-based, 10-day fermentations of S. cinnamomensis. Labelling of the coenzyme A pools, and analysis of intracellular acyl-CoAs in high-titre production strains, demonstrated that ccr mutants had lower levels of the monensin precursor methylymalonyl-CoA, while expression of a heterologous ccr gene from S. collinus fully restored production of monensin. Extended oil-based fermentation with S. cinnamomensis C730.1 determined that ethyl [3,4-13C2]acetoacetate was incorporated intact into both the ethylmalonyl-CoA- and methylmalonyl-CoA-derived positions of monensin, whereas no labelling of the malonyl-CoA-derived positions was observed. A Δccr strain had reversed carbon
A flux from an acetoacetyl-CoA intermediate – a result contrasted in fermentations on glucose-soybean medium, which provided ethylmalonyl-CoA but not methylmalonyl-CoA. Production of precursors for secondary metabolites is therefore influenced substantially by fermentation conditions.

Engineering of unusual precursor pathways can result in increased polyketide production (Rodriguez et al., 2004). A tylosin-overproducer of *S. fradiae* was chosen as it made high levels of malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA precursors. When a fourth pathway, for methoxymalonyl-ACP synthesis from *S. hygroscopicus*, was introduced into a strain mutated in the tylosin polyketide synthases (PKS) genes, the resultant recombinant produced at least 1 g/l of a midecamycin analog. Such an approach could also be used for creating novel products.

*In silico* studies of metabolic flux distribution, linked to sensitivity analyses during various phases of the batch culture, is a fundamental tool in the re-engineering of pathways involved in primary metabolism. Whereas genetic amplifications create a wide range of flux values, deletion of a gene results in zero flux at that step. Specific routes should be investigated with care so that pathways fundamental to cellular biosynthesis or productivity are not impinged, i.e. as seen with the *S. coelicolor* mutant for citrate synthase, ΔcitA. This enzyme, which acts at the junction of the glycolytic and TCA pathway, is ideally situated to maintain physiological balance by decreasing glycolytic flux or increasing TCA cycle flux. However, the ΔcitA strain was unable to produce aerial mycelium or the antibiotics ACT and RED. Although it consumed glucose at a higher rate, it was unable to oxidize glycolytic products via the TCA cycle – a defect suppressed by the removal of glucose from the medium or genetic manipulations of glucose kinase, preventing the utilisation of glucose (Viollier et al., 2001a). Similarly, aconitase mutants had fundamental physiological defects (Schwartz et al., 1999; Viollier et al., 2001b).

The development of microarray technology based on the *S. coelicolor* genome enables strain comparison based on the characterisation of expression of thousands of genes, which provides information for future manipulation for strain improvement. An approach for ‘reverse engineering’ of improved erythromycin producing strains of *Aeromicrobium erythreum* by tagged-mutagenesis, identified two genes, *mutB* and *cobA*, in the primary metabolic branch for methylmalonyl-CoA utilisation. Knockouts of these genes resulted in a permanent metabolic switch in the flow of methylmalonyl-CoA, from the primary branch into a secondary metabolic branch, enhancing erythromycin overproduction (Reeves et al., 2004).

### 2.4. Corynebacteria

Corynebacteria, mainly *C. glutamicum*, have been used for industrial amino acid production for several decades and extensive studies about physiology, genetics, a considerable number of patents and metabolic engineering approaches emphasise the commercial importance of these micro-organisms (Bott and Niebisch, 2003; Eggeling et al., 2005; Jetten et al., 1994; Jetten and Sinskey, 1995; Kirchner and Tauch, 2003; Patek et al., 2003; Sahm et al., 1996; Wendisch, 2003).

Knowledge of the genome sequence (Kalinowski et al., 2003) and development of robust microarray procedures (Loos et al., 2001) facilitated research on biotechnological applications (Hermann, 2003). In 2003, l-lysine production exceeded 600,000 tons per year (Pfefferle et al., 2003), all of which were produced by fermentation.

*C. glutamicum* exhibits high gluconeogenic activity in vivo via phosphoenolpyruvate carboxykinase (PEPCK), which is responsible for phosphoenolpyruvate formation from anaplerotically synthesised oxaloacetate during growth on glucose, thereby contributing to an apparently futile substrate cycling between oxaloacetate, phosphoenolpyruvate and pyruvate (Petersen et al., 2000). Deletion of the respective *pck* gene in l-lysine producing strain MH20-22B resulted in increased intracellular concentrations of l-aspartate, l-lysine, pyruvate, oxaloacetate and in a 60% enhanced flux towards l-lysine biosynthesis, whereas over-expression of *pck* led to a 20% decrease, without significant changes in growth or substrate uptake rate (Petersen et al., 2001). Increased CO₂ production in the *pck* over-expressing strain was found and further indicates elevated energy demand with increasing PEPCK activity versus reduced CO₂ production and energy demand in a Δ*pck* mutant. The counterproductive effect of PEPCK activity, which is
essential for gluconeogenesis in *C. glutamicum*, has also been shown for glutamate production. PEPCk deficient mutants showed four-fold higher glutamate production, whereas *pck* over-expression lowered production about three-fold (Riedel et al., 2001).

Over-expression of pyruvate carboxylase, catalysing the formation of oxaloacetate from pyruvate, therefore should have a similar effect on lysine production. However, Koffas et al. (2003) showed that over-expression of the *pyc* gene enhanced growth but reduced specific lysine productivity. Simultaneous expression of aspartate kinase, a key enzyme of the lysine production pathway, abolished this deficiency and yielded more than 250% increase of the specific productivity. Over-expression of *pyc* in a strain with different regulatory characteristics resulted in seven-fold higher glutamate production and increased lysine accumulation (Peters-Wendisch et al., 2001), a clear indication of the importance of this gene for strain improvement. “Genome–based strain reconstruction” confirmed the importance of *pyc* in lysine production (Ohnishi et al., 2002). Mutations in genes of interest, in this case the genes of the lysine biosynthesis pathway, were identified by comparative genomic analysis in a classically developed production strain. Beneficial mutations are then reassembled in a wild-type background. Point mutations in three genes, the homoserine dehydrogenase gene (hom*V59A*), the aspartokinase gene (lysCT311H) and the pyruvate carboxylase gene (pyc*P458S*) were identified. Introduction of one mutation at a time into a wild-type strain led to increased accumulation of lysine and the combination of all three mutations showed a synergistic effect on the production in the resulting AHP-3 strain. Recently, the authors turned their attention to the PPP and identified a mutation in the 6-phosphogluconate dehydrogenase gene (*gnd*) as useful for lysine production (Ohnishi et al., 2005). Introduction of this mutation, which resulted in lower sensitivity towards allosteric inhibition by intracellular metabolites, into the AHP-3 strain led to 15% increased lysine production and 8% higher carbon flux through the PPP. A thorough investigation of the influence of point mutations in several genes of the central metabolism and of amino acid production pathways on different carbon sources was published recently (Georgi et al., 2005). The effects of the mutations were miscellaneous with respect to lysine yields on the carbon sources but clearly certified positive effects of hom*V59A* and pyc*P458S*. Fructose-1,6-bisphosphatase activity was revealed as a limiting factor if sucrose was the sole carbon source while over-expression of malE had no effect on the lysine production with the tested carbon sources.

Two intermediates of the PPP, ribose 5-phosphate and erythrose 4-phosphate, are important precursors for biosynthesis of nucleotides and aromatic amino acids, highlighting the importance of this pathway for efficient production of desired metabolites. Transketolase, an enzyme of the non-oxidative branch of the PPP, was investigated as a possible target for metabolic engineering to increase the availability of ribose 5-phosphate in *C. ammoniagenes* for production of inosine and 5′-xanthyllic acid. A 10-fold increase of transketolase activity resulted in about 80% product yield, compared to the parental strain. Disruption of the *tkt* gene on the other hand, resulted in up to 30% more accumulation of product, proving that interception of the ribose 5-phosphate shunt back to glycolysis by transketolase allows the cells to redirect the carbon flow through the oxidative PPP towards the purine-nucleotide pathway (Kamada et al., 2001). Disruption of the glucose 6-phosphate dehydrogenase gene (*zwf*) resulted in a decrease in product yield of about 50%, indicating the importance of the oxidative branch of the PPP for precursor supply, although non-oxidative synthesis was possible, albeit at an insufficient level (Kamada et al., 2003).

As l-lysine production correlates with intracellular NADPH supply, Marx et al. (2003) constructed a phosphoglucose isomerase (*pgi*) null mutant of *C. glutamicum* to redirect the carbon flux through the PPP. l-Lysine production increased with simultaneous decrease of by-product formation and growth rate, a common feature of *pgi* mutants due to disturbed metabolism of NADPH. Redirection of the carbon flux towards PPP may also have a general applicability for metabolic engineering of biosynthesis of metabolites for which NADPH supply is necessary. Another possibility is the engineering of the cofactor specificity of the enzymes involved (Banta et al., 2002).

l-Phenylalanine, one of the essential amino acids for humans, is synthesised from chorismate in three steps, catalysed by enzymes which are subject to feedback inhibition. Liu et al. (2004) blocked the branch
pathway of l-tyrosine biosynthesis by disruption of the tyrA gene and integration of aroG and pheA from E. coli, key genes in l-phenylalanine biosynthesis in E. coli. Improved enzyme activity in the desired pathway and disruption of the pathway competing for precursors resulted in the expected outcome, namely 30% increased yield.

Recently, an attempt to enable C. glutamicum to utilise whey, as a substrate, has been made. The determinants for lactose (β-galactosidase and lactose permease) and galactose (galactose operon) utilisation were heterologously expressed in the lysine production strain C. glutamicum ATCC 21253.

The engineered strain was capable of slow growth on whey-based medium and produced 2 mg/ml lysine, a 10-fold increase compared to the parent strain. Another interesting finding was that the engineered strain retained its viability after extended incubation, whereas the viability of the parental strain dropped about 90% after 225 h (Barrett et al., 2004).

3. Conclusion

Many different pathways and regulatory features have been targets for metabolic engineers, be it increased yield of product or elimination of product, which in the end serves the same purpose. As has been discussed in the introduction, engineering the often metabolism often has unexpected results and can cause new problems, most often reduced growth rates. Evolutionary breeding has been shown to be an excellent method to overcome these obstacles, when rational solutions were not available or extremely complex technically – if at all possible.

On the other hand, metabolic analysis of mutant strains has provided invaluable insights into the underlying changes in metabolism, providing data for the next round of metabolic engineering.

References


