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Cell factory engineering for improved production of natural products

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Natural product biosynthesis is inherently linked with the primary metabolism in terms of providing precursors and co-factors. Thus, even though it is important to improve the performance of natural product biosynthetic enzymes in building efficient cell factories, it is equally important to engineer the central carbon metabolism, such that this is adjusted to meet the biosynthetic demand of the desired product.

In more recent years this has been taken much further, resulting in refactoring of complex biosynthetic pathways in heterologous hosts, as illustrated by the recent reconstruction of a 23 step enzymatic pathway for production of hydrocodone in *S.cerevisiae*. Here there has been focus on a few different cell factory platforms for production of natural products: (1) the yeast *S. cerevisiae*, which is beneficial as it is easy to genetically engineer and enables expression of plant P450 enzymes; (2) the Gram-negative bacterium *Escherichia coli*, which allows for high-level expression of heterologous enzymes and has a flexible metabolism that can adjust easily to heterologous pathways; (3) the Gram-positive actinomycetes *Streptomyces coelicolor*, which produces several natural products and hence has the capacity to support production of many other natural products, in particular gene clusters from other actinomycetes. However, the list of cell factory platforms is expanding, and many would probably add a filamentous fungus to this list, but here there is less consensus, as both *P. chrysogenum* and various *Aspergillus* compete for this spot. The advantage of using cell factory platforms is that the central carbon metabolism can be tailored to production of a class of molecules that use the same precursor, e.g. acetyl-CoA often used for polyketide syntheses.

Here I will discuss some of the key points to consider when natural product biosynthesis has to be improved in a given microbial cell factory: (1) the central carbon metabolism as it provides the precursors and the co-factors for natural product biosynthesis; (2) choice of microbial cell factory; and (3) optimization of the enzymes in the biosynthetic pathway in order to avoid a proteome constraint issue.

2 Link between primary and secondary metabolism

Penicillin production by the filamentous fungus *P. chrysogenum* is a good example of how primary and secondary metabolism is closely linked. Penicillin is produced in a three-step
biosynthetic pathway. In the first step, the non-ribosomal peptide synthetase (NRPS) L-α-aminoadipoyl-L-cysteinyl-D-valine synthetase (ACVS) condenses L-α-aminoadipic acid, L-cystein, and L-valine into the tripeptide L-α-aminoadipoyl-L-cysteinyl-D-valine (ACV). In the next step, isopenicilllin N synthase (IPNS) converts ACV into isopenicillin (IPN) with the use of molecular oxygen as electron acceptor. In the final step, the L-α-aminoadipoyl side chain of IPN is cleaved off resulting in formation of 6-APA that can be further converted to penicillin V or penicillin G depending on whether the cells are fed with phenoxycetic acid or phenylacetic acid. Naturally, P. chrysogenum produces a minute amount of penicillin, and thus only ogenum phenoxyacetic acid or phenylacetic acid. Naturally, P. chrysogenum biosynthetic pathway. This led to increased NADPH supply for secondary metabolism. At the more general level, the key links between primary and secondary metabolism are (Fig. 1A): (1) precursor metabolites, with acetyl-CoA being the most notable as it is used as precursor for biosynthesis of polyketides as well as isoprenoids; (2) amino acids, that are used as precursors for production of non-ribosomal peptides; (3) aromatic amino acids, which are used as precursors for flavonoids and alkaloids. Many other intermediates of the central metabolism can serve as precursors for natural product biosynthesis, but those listed above represent important ones. Besides the precursor, there is also a strong link between central metabolism and natural product biosynthesis through the requirement for redox power, often in the form of NADPH, and Gibbs free energy, often in the form of ATP. These links are very well illustrated in studies where natural product pathways have been reconstructed in a heterologous host, and in order to achieve high level production it has been necessary to engineer central metabolism. Examples are the production by yeast of artemisinic acid, farnesene and resveratrol. Artemisinic acid is an anti-malarial drug and farnesene is a hydrocarbon that can be used as jet-fuel or for synthesis of squalene. Both chemicals are derived from farnesylpyrophosphate (FPP) by expressing a specific sesquiterpene synthase, and whereas farnesene is produced directly by one enzyme, artemisinic acid biosynthesis requires a few additional
biosynthetic steps. However, for production of both chemicals it is necessary to have efficient supply of FPP, and this required engineering of not only the mevalonate pathway leading to FPP, but also central carbon metabolism to ensure efficient provision of acetyl-CoA and NADPH. To ensure high-yields, a heterologous pathway involving phosphoketolase was expressed in yeast. This pathway takes xylose-5-phosphate, an intermediate of the pentose phosphate pathway, and converts it into acetyl-phosphate and glyceraldehyde-3-phosphate. Acetyl-phosphate can be converted directly to acetyl-CoA by another enzyme, and hereby a new pathway for providing acetyl-CoA was installed. For improving resveratrol biosynthesis, which is synthesized from phenylalanine or tyrosine as well as malonyl-CoA, it was necessary to engineer the aromatic amino acid biosynthetic pathway to allow for high flux, but also to improve the provision of malonyl-CoA. Also this pathway requires extensive NADPH, so steps to ensure efficient provision of this co-factor also had to be considered.

3 Choice of cell factory

The second critical factor for optimizing the production of natural products is the chose the right cell factory. This can be very difficult, but the following points should be considered: (1) is the pathway compatible with the host, i.e. can the host deliver the precursors required for the biosynthetic pathway and if the pathway spans different compartments in its endogenous host, can this be reproduced in the heterologous host. An excellent example of pathway compatibility with the host is the production of adipoyl-7-ADCA mentioned earlier, as here the host already had a very efficient pathway for biosynthesis of adipoyl-6-APA. Often it is, however, more difficult to judge, but there some of the following points can probably assist; (2) is it easy to engineer the cell factory that is going to host the heterologous pathway? The answer is for sure yes for E. coli and S. cerevisiae, but these two cell factories are not always optimal for production of natural products. E.g. it may be better to use a Streptomyces species to express pathways discovered in Gram-positive actinomycetes and a fungus to express pathways discovered in other fungi. This is due to differences in G/C-content of the gene clusters but also compatibility of the promoter regions. Here the final choice may depend on which tools are available for genetic engineering of the host; (3) even though it can be an advantage of expressing pathways for fungal natural products in a fungus, there are drawbacks of this approach. Thus, most filamentous fungi that produce natural products, contain a large number of biosynthetic gene clusters, i.e. up to 50 clusters have been discovered through genome-sequencing of several Penicillia. This means that there is a risk that enzymes from other biosynthetic pathways are expressed in the chosen host, and these enzymes may interfere with the heterologous biosynthetic pathway. This holds true in particular for glycosyltransferases, but also for many other types of so-called tailoring enzymes that decorate the aglycone. This can of course be an advantage if one is aiming for product diversity, but it does complicate pathway discovery and will also complicate optimizing production of a specific molecule. Here use of hosts that have no or limited secondary biosynthesis capabilities, such as E. coli and yeast, have benefits, but the trade-off is poor pathway compatibility; (4) the ability to express functional pathway enzymes in the host is a very important factor. In some cases this is hard to judge at the outset, but if the pathway e.g. contains P450 enzymes it does not makes a lot of sense to use E. coli as a host, as this class of enzymes express poorly in bacteria, whereas they generally express well in yeast. This is mainly due to their membrane association, i.e. they can be expressed to function in the endoplasmic reticulum membrane of yeast. This, however, also means that it is important to consider the proteome constraints discussed below (see also Fig. 1B), as this may now apply not only to the total proteome, but the proteome of the specific membrane compartment where the enzyme(s) need(s) to be expressed; and (5) is the natural product toxic to the cell factory? This is of course a very important factor. Often natural producers also express a gene encoding for an enzyme or transporter that confer resistance towards the chemical, and this may then also be expressed in the cell factory of choice. However, it is not always the case, and this then becomes part of the cell factory and natural product biosynthesis compatibility evaluation.

4 Enzyme activity and flux control

The third critical factor for improving natural product biosynthesis is the catalytic efficiency of the biosynthetic enzymes. Enzymes of secondary metabolism generally have lower $k_{cat}$ values and are generally larger than enzymes of the primary metabolism. This generally means that it may be hard to increase the flux through the biosynthetic pathway leading towards the natural product for predominantly two reasons.

First, with low $k_{cat}$ values of the enzymes it is generally necessary to express these enzymes to very high levels in order to ensure sufficient enzymatic capacity required for a given flux. Even though this can easily be achieved through using strong promoters and multi-copy expression (as has occurred naturally in high penicillin producing strains of P. chrysogenum that carries multiple copies of the biosynthetic gene cluster), there is a trade-off with this approach. High-level expression of the biosynthetic enzymes, which generally have a high molecular weight as mentioned above, results in an increased requirement for allocation of proteome mass to natural product biosynthesis. As the cellular proteome is finite (see Fig. 1B), this means that there have to be reduced allocation of proteome for other cellular processes. As the majority of the proteome in a cell is normally allocated for central metabolism and protein biosynthesis, the consequence is that there is a reduction in proteome allocated for these processes. The result is a reduction in growth rate. It may be possible to accept this trade-off, but it is important to keep in mind, and as it is difficult to control which part of the proteome should be “sacrificed”, the trade-off may well result in unwanted phenotypes. In order to overcome this problem it is therefore important to quantify the $k_{cat}$ of the biosynthetic enzymes and evaluate how much of the proteome is required for supporting a certain flux through the pathway. If
5 Conclusions

I here argue that in connection with optimization of cell factories for production of natural products there are in particular three aspects that needs to be considered: (1) engineering of the central metabolism in order to ensure sufficient supply of precursors and co-factors; (2) choice of cell factory; and (3) improving the properties of the enzymes in the biosynthetic pathway, particularly to overcome the problem of a proteome constraint within the cell (or specific compartment where the pathway is expressed). Having all these three aspects in mind, it should be possible to design a strategy for developing an efficient cell factory for production of natural products.

6 Conflicts of interest

There are no conflicts to declare.

7 Acknowledgements

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8 References