Synthetic Biology for Cellular Remodelling to Elicit Industrially Relevant Microbial Phenotypes

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Contents

Abstract

Industrial microbiology is proposing an increasing number of bio-based processes that are ready to move from the validation to the demonstration step, with the industrial world being more open to this opportunity for a change. The challenge is therefore to make such processes viable and competitive. When moving from the lab to the industrial scale, the degree of complexity is

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increasing, and the engineered cell factories very often display emerging properties that can be explained only from a systems perspective. Unfortunately, cellular rewiring often leads to a lower accumulation of the desired product. Synthetic biology is willing to take advantage from the knowledge on mechanisms involved in cellular homeostasis and, thanks to the principles of abstraction, modularity and standardisation, translate them into more efficient cell factories. Indeed, this novel approach to potentiate the power of metabolic engineering can be applied not only to a specific metabolic pathway but can be extended to networks indirectly connected to the pathway of interest. In this chapter, some of the principal synthetic tools developed to regulate or redirect the remodelling of cell factories, from genomic to metabolic level, with the aim to obtain higher titers, yield and productivity of bio-based products will be described and commented.

5.1 Introduction

Industrial microbiology is moving from a promising field of application to a real alternative to chemical synthesis, as witnessed by the increasing number of processes emerging at industrial scale [see, for a recent review, Porro et al. (2014)]. The majority of the latest announcements relate to recombinant productions. This fact highlights at least two important aspects: (1) the efforts in improving metabolic engineering approaches and tools turn out to be more and more successful and (2) the industrial world seems to be more open than in the past for a change. Indeed, despite all the optimisation strategies for development of an effective cell factory that can be imagined at lab scale, the real viability test of a process is only possible approaching industrial scale. Therefore, industries need to open up to the possibility to test the new biocatalysts and eventually to consider how to combine operative conditions with biological needs and vice versa. For industrial use, the biocatalyst is forced to produce a non-natural product (or, even if natural, to accumulate it up to non-physiological titer) in a non-natural environment (where the catalyst might tend to be metabolically inactive). Notably, the coexistence of these two inappropriate conditions triggers different (and possibly coordinated) cellular responses that involve the cell as a whole, having as a consequence an effect on metabolism and therefore on production. Despite the increasing number of studies on single responsive elements as well as on genome-scale networks, especially devoted to well-established cell factories such as *Escherichia coli* or *Saccharomyces cerevisiae*, it is still hard to predict how the cells will reprogramme their state as a response to the process conditions. In line with that, a significant number of reports are proposing adaptive laboratory evolution (ALE) as an efficient strategy to select the desired phenotype [for recent reviews, see Dragosits and Mattanovich (2013) and Winkler and Kao (2014)]. This is not in contrast with strategies proposing a rational design, since the analysis of the novel set-up of the evolved

cell factory can contribute to a better understanding of the whole system and/or to shed light on still uncovered responsive elements. As a remark, it has to be said that the efficacy of ALE is strongly related to the conditions applied for strain selection, which should be intended to mimic as much as possible the relevant process conditions.

Considering that, would it imply that the role of metabolic engineering or, more generally, of a tailored manipulation is limited to the initial stage of strain construction?

The ultimate idea is to come up with a synthesis of the two strategies, resulting in the construction of synthetic cells (as reported in Gibson et al. (2010) and within the programme Synthetic Yeast 2.0, http://syntheticyeast.org), specifically dedicated to a particular production or function. The outcomes of these projects will reverberate not only in applications with social impact but also in novel insights at the fundamental level of knowledge, opening the possibility to extend the concepts from single-cell organisms to more complex systems, such as cellular communities, possibly organised in differentiated organs or pluricellular organisms.

However, this scenario is still far from being real, while the need of a turn for establishing a viable bioeconomy is more and more urgent in our society. This is how synthetic biology can also come to the aid of industrial biotechnology. To make a complex story as simple as possible, a recombinant cell factory is usually genetically modified to (over)express endogenous or heterologous genes and/or to impair endogenous gene(s) function, to obtain the desired product. These modifications, per se, imply a rewiring of (genetic) circuits, necessary to cope with the newly imposed set-up. In addition, when cell factories are cultivated in the production medium and particularly under industrially relevant conditions, they have to cope with constantly fluctuating and often limiting external and internal parameters along the fermentation time. This will also have an effect on the rewiring of circuits, which is partially dependent on the strain's potential and partially on the severity of process constraints. Unfortunately, the ultimate result of the cellular adaptation is very often not optimal for process success. Synthetic biology, through the study of master regulator key elements and the reconstruction of such elements as parts, could allow us to regulate or redirect some of the cellular rewiring. This can turn into higher production, yield and productivity (Van Dien 2013), which matches with our need to reduce time-to-market.

Here we recapitulate the principles, together with the description of some synthetic tools and approaches (with a preference for *S. cerevisiae* and *E. coli* as chassis, due to the availability and applicability of tools), which have been developed to elicit cellular phenotypes to be selected for matching with specific purposes.

5.2 Rewiring the Cellular Set-Up at Genomic Level

Based on currently available information, the largest number of manipulations for the construction of a cell factory is targeted at genomic level. In this context, synthetic biology is developing combinatorial approaches, often with the final aim of streamlining genomes, and consequently transcriptomic responses. Here

we recapitulate some of the most promising approaches, which follow the principles of modularity and standardisation.

The perspective is that in a near future, the achieved knowledge, at least regarding the most used cell factories, will allow a tailored design, where predictions can be translated into results with less dependence on vast use of screening procedures.

5.2.1 Towards Minimal Cell, If Ever

In addition to the above-mentioned project on *S. cerevisiae* 2.0, genome reduction has already been translated into a desired phenotype by manipulating *E. coli* cells. This is, for example, the case for strain MDS42, tested as cell factory for lentiviral expression after removal of about 40 insertion sequences (Csörgo et al. 2012). Interestingly, a further reduction was targeted to the genes encoding error-prone DNA polymerases: this loss-of-function resulted into low-mutation-rate strains, which means genetic stability. Indeed, the maintenance of the desired genome over the whole process is definitely a must for standardisation and quality of production, especially if the cell factory is the result of desired and tailored recombinant manipulations. However, it is also interesting to notice that variations on genome structures elicited by detrimental process conditions and often observed at the end of fermentation processes [duplications, mutations, aneuploidy, etc.; see as example Puig et al. (2000)] can be considered as precious source of information due to our still poor knowledge about cellular intrinsic potential.

What is very interesting in examining the literature is that the "minimal" cell can be tailored only in connection with defined environmental conditions: referring to the previous example, it is clear that a strain manipulated as such very likely turns out with a different fitness if compared and in competition with natural strains. Following the same line of thoughts, the lack of genes encoding for biofilm structural components (May and Okabe 2011) can be a way of reducing genomic functions, but only if this loss-of-function is not going to compromise the final performance of the strain. The multiple techniques for genome reduction currently under development are therefore highly relevant [see, as recent examples, Xue et al. (2014) and Suzuki et al. (2015)], but most of them remain to be tested at production level.

5.2.2 Genome Engineering and Combinatorial Libraries for Tuned (Recombinant) Expression

Experimental evolution can be an efficient strategy to obtain a desired phenotype, as demonstrated by many recent applications of adaptive laboratory evolution (ALE) [see, as examples, Marietou et al. (2014); Patzschke et al. (2015) and Tilloy et al. (2014)]. However, at laboratory scale, the natural variation is limited, such as the timescale of such experiments to achieve a profound rearrangement of the

genome is limited. Considering literature data about cell factory and selective conditions, an average of 0.97 SNPs in an adapting genome for every 100 generations of evolution was recently calculated (Dettman et al. 2012). It can be estimated that cultivations for ALE typically last from 200 to 1000 generations. Therefore, considering the average of mutation frequency and genome dimension, this can result into 4–20 independent mutations per population (see Pál et al. (2014) and references therein). This is well exemplified by the experiments by Barrick and coauthors, showing that an *E. coli* strain acquired only 45 mutations over 20,000 generation of adaptation to glucose minimal medium (Barrick et al. 2009) and further pointing out a complex relation between genomic and adaptive evolution. Moreover, it is very difficult to establish an appropriate control for mutational processes and to distinguish beneficial mutations from neutral ones.

Synthetic biology approaches are implementing genome engineering, with at least two different aims: (1) to increase the number of variants generated in a limited time scale and (2) to facilitate the traceability of such mutations, to consequently facilitate the description (if not the comprehension) of the results. Remarkably, genome engineering can generate modifications that have never been explored in nature or that might have occurred in ancient time, when divisions among the three cellular lines were still at the very beginning and the incidence of horizontal gene transfer was more relevant.

Multiplex automated genome engineering (MAGE) allows the targeted editing of many different locations mediated by the replacement of the natural sequences with generated oligonucleotides that can operate an allelic exchange (Wang et al. 2009). This recombination, which can be iteratively repeated in cycles, generates a combinatorial genomic diversity. This technique was efficiently applied on *E. coli* to optimise the production of lycopene, an industrially relevant carotenoid. Recently, the MAGE technology has been implemented based on the exploitation of suicide plasmids, to make it applicable not only to few laboratory strains but also to industrial bacterial strains (Ryu et al. 2014).

MAGE can be conceivably combined with CAGE (conjugative assembly genome engineering), a method of large-scale transfer of engineered (and marked) genomic modules to assemble them into a single genome, which is based on the appropriate positioning of *oriT* conjugational sites and selection markers (Ma et al. 2014). The combination can allow reconstructing a (whole) genome where the genetic code has been modified, or expanded, for example, for the production of proteins including non-natural amino acids.

As mentioned before, the traceability of the introduced variation(s) is highly desirable. Trackable multiplex recombineering (TRMR) is such a method where DNA barcodes, detectable by microarray analyses, have been included in the recombineering cassettes (Mansell et al. 2013).

In view of evoking unravelled cellular potential to accomplish industrially relevant features, Alper et al. (2006) have proposed a strategy for reprogramming the transcriptional set-up by mutagenesis applied on key transcriptional elements. This technique, named global transcription machinery engineering (gTME), is indeed based first on the identification of transcription factors (TFs) that are

known as master regulators of general rewiring events in the cell, second in the construction of a library of mutated versions of said TFs (by error-prone PCR), third in the transformation of this library into the target cell factory, and finally in the selection under desired conditions of the strain(s), where the mutation(s) will evoke the desired novel transcriptional set-up. Eventually, the selected strains are investigated both for the mutations occurring at the TF level and more generally at the transcriptomic level, to possibly transfer the identified perturbations into the parental strain.

This technique has been first applied to *S. cerevisiae* to improve ethanol tolerance and volumetric productivity (Alper et al. 2006), followed by another example in *E. coli* (Alper and Stephanopoulos 2007), where the mutagenesis applied on a sigma factor also elicited different industrially relevant phenotypes, namely, ethanol tolerance, lycopene overproduction and an example of acquisition of multiple phenotypes (specifically, ethanol and sodium dodecyl sulfate tolerance). Different selection approaches are then proposed, combining the more classical growth assays on plates with chemostat cultivations. A number of different reports have been published, exploiting the principle of the strategy to accomplish a complex phenotype rearrangement [see as recent examples Tan et al. (2015) and Zhao et al. (2014)].

Similarly, but taking advantage of a different mechanism, a transposon-mediated mutant library has been used to identify strains with novel traits (Kim et al. 2011). Given their relatively simple design and their high compatibility at transducing genetic material, transposons are highly attractive to be used as parts in synthetic biology.

5.3 The Long Road from mRNA to Proteins

Despite the critical regulation of gene expression at transcriptional level, all following events during gene expression are also crucial for reaching the desired phenotype. In this context, it is important to underline a general difference between production of pharma proteins and the production of chemicals, especially those implying different catalytic steps. In the first case, usually the heterologous gene is expressed at highest levels to maximise production (despite increasing evidence on the consequent metabolic burden suggests caution). In the second case, the relative level of all the enzymes involved in the metabolic pathway of interest has to be carefully balanced in terms of processing ability, allowing an optimal flux to the desired product, thereby avoiding the accumulation of intermediates, possibly toxic to the cells. Moreover, and this is true also for pharma-protein production, all the mechanisms responsible for translational events, sensing and repairing are of pivotal importance. The study of the structural elements present on mRNA molecules (Kwok et al. 2015; Mitchell and Parker 2014) and the implication of mRNA distribution, docking, storage and metabolism (Decker and Parker 2012; Walters and Parker 2014) are suggesting novel targets for controlling protein production.

To further extend these concepts, it has to be said that the classical dogma of the information flow from DNA to RNA to protein to direct cellular activities does not take epigenetics into account, neither noncoding RNAs, protein-protein interactions, nor post-translational modifications. These levels of regulation are currently being analysed by systems biology studies as emergent properties of the system but rarely targeted for synthetic approaches yet.

Nevertheless, synthetic biology is developing strategies to regulate these events, both with finite purpose and with the aim to elicit general responses and phenotypic configurations. Here we will focus on this second aspect.

5.3.1 Post-translational Regulation of Levels and Local Concentrations of Proteins

When using bacterial cell factories, the reconstruction of the desired metabolic pathway by grouping necessary genes in the form of an operon ensures coordination, but also a similar level of expression, since they are all under the control of the same promoter. However, this does not imply that the enzymatic levels are optimised for the production of interest. The possibility of a post-transcriptional tuning of protein levels by playing with the translation initiation region (TIR) present on mRNA molecules has been demonstrated for mevalonate production in *E. coli* (Pfleger et al. 2006). The authors demonstrated that by generating libraries of tunable intergenic regions (TIGRs), recombining various post-transcriptional control elements such as mRNA secondary structures, RNase cleavage sites and ribosome-binding site (RBS) sequences, it was possible to vary the relative expression of different genes of one or even two orders of magnitude. Remarkably, this can be translated into the possibility of fishing for strains where the protein levels are optimally concerted for the desired production.

Another level of general reorganisation that can be critical for recombinant productions is the localisation of the heterologous pathway. Substrate availability, local concentrations and subcellular compartmentalisation are all reasons in favour of a rational design implying the targeting of necessary enzymes on macromolecular scaffolds. In this view, the RNA role can be extended to the novel function of constituting a three-dimensional scaffold harbouring aptamers for docking the desired enzymatic functions, as it was demonstrated for hydrogen production in *E. coli* (Delebecque et al. 2011, 2012).

5.3.2 Rewiring Cellular Differentiation by Modulating Post-translational Modifications

Post-translational modifications of proteins, counting more than 600 different types in eukaryotic cells (as annotated in the RESID database, http://pir.georgetown.edu/resid/), are largely responsible for cellular responses and coordinated activities [see, for a review, Deribe et al. (2010)]. Very likely, one of the most abundant

modifications is phosphorylation, involved in signal transduction cascades and in coordinating external stimuli with cellular responses. The possibility to exert a control on such cascades can determine the success of a biotechnological process, as in the case of differentiation events. Indeed, one of the critical elements when working with sporulating bacteria is the possibility to control the triggering of this differentiation pathway. Vishnoi et al. (2013) pointed out the importance of the timing in the phosphorylation of the *Bacillus subtilis* master regulator of sporulation initiation, Spo0A. By controlling the induction of the kinase KinC, together with Spo0A, they could elicit sporulation even under ideal nutrient conditions, demonstrating the possibility to rewire even a fundamental cellular mechanism of survival. This study, as well as other similar, opens up the possibility to create synthetic relays and switches, which could be applied to industrial strains, when the triggering of sporulation can be critical for the stability of the production, as in the case of clostridial host organisms, very well known for acetone-butanol-ethanol fermentation (Patakova et al. 2013).

5.4 Metabolic Controls: How to Turn Them to Our Purposes and Not Against?

All the cellular mechanisms that have been mentioned up to this point need to be in strong connection and coordination with cellular metabolism. A cell operates its many and various functions through a flexible and efficient metabolism. As a consequence, knowing how the cellular metabolism is regulated and how it can be manipulated is of pivotal importance to optimise microbial cells for industrial bioprocesses. Metabolic engineering has provided several successful examples of de novo production of different classes of metabolites, optimisation of endogenous products and exploitation of many different (including nonnatural) substrates, among others (Table 5.1). However, it is clear that the designed metabolic pathways will unavoidably interfere with the native metabolism and with its control. Even more, the new metabolites produced will have an active role in the general novel set-up and response of the recombinant cell. To shortly list the principal effects, metabolite concentration can regulate enzymatic activity (different mechanisms), can lead to their covalent modifications and can influence translational and transcriptional regulation (at different levels), as recently reviewed by Wegner et al. (2014). All these responses may occur simultaneously, but acting at different levels, and with different timescales, both in the triggering and in the extinguishing of consequences. Noticeably, even the codon optimisation strategies started to take into account that there is not just a single cellular set-up that can be considered as an absolute reference. Generally speaking, codon frequency can differ, within the same cell, among genes encoding for enzymes belonging to different pathways (*i.e.* primary versus secondary metabolism). Even more, in *S. cerevisiae* it has been recently demonstrated that the levels of the different tRNA species vary along the cell cycle progression, contributing to the triggering and to the crossing of checkpoints (Frenkel-Morgenstern et al. 2012). Thanks to these studies, a

Table 5.1 Recent examples that exemplify the successful development of cell factories optimised for the production of some of the main bio-based chemicals

	Microorganism	Titer	Reference
Biofuels			
Ethanol (from lignocellulose)	*S. cerevisiae*	~60 g/L	Several studies, see review Sedlak and Ho (2004); Yamada et al. (2013)
n-Butanol	*E. coli*	30 g/L	Shen et al. (2011)
Isobutanol	*E. coli*	50 g/L	Baez et al. (2011)
Triacylglycerols (TAGs, to biodiesel)	*Yarrowia lipolytica*	25 g/L	Blazeck et al. (2014)
Fatty acid ethyl esters (FAEE)	*S. cerevisiae*	34 mg/L	Shi et al. (2014)
Chemicals			
1,3-Propanediol (1,3-PDO)	*E. coli*	135 g/L	Nakamura and Whited (2003)
Succinic acid	*Corynebacterium glutamicum*	146 g/L	Okino et al. (2008)
2,3-Butanediol (BDO)[a]	*Bacillus amyloliquefaciens*	132.9 g/L	Yang et al. (2013)
R-2,3-Butanediol (BDO)[a]	*S. cerevisiae*	100 g/L	Lian et al. (2014)
Styrene	*S. cerevisiae*	29 mg/L	McKenna et al. (2014)
Fine chemicals			
Amorphadiene	*S. cerevisiae*	40 g/L	Westfall et al. (2012)
Resveratrol	*S. cerevisiae*	391 mg/L	Sydor et al. (2010)
Artemisinic acid	*S. cerevisiae*	25 g/L	Paddon et al. (2013)
Omega-3 eicosapentaenoic acid (EPA)	*Y. lipolytica*	25 % DCW[b]	Xie et al. (2015)

[a]Also as fuel or fuel additive
[b]Calculated as percentage on cell dry weight (CDW)

condition-specific codon optimisation approach has been recently designed for improving heterologous productions in *S. cerevisiae* (Lanza et al. 2014).

The profound interconnection of metabolism with the other cellular functions makes it difficult to draw a line distinguishing specific techniques. However, the examples reported here started from metabolic master regulator for sensing and connection with metabolic fluxes as the main target for eliciting a cellular rewiring.

5.4.1 Mimicking the Dynamics of Metabolic Responses to Optimise Recombinant Productions

When active (growing, not resting) engineered cells are used for a commercially viable process of commodities production, a very high yield of production [at least 80 % of the theoretical yield according to the metrics of Van Dien (2013)] needs to be obtained. This means that the flux to the product has to be maximised, leading to an inescapable trade-off between growth and production. Channelling most of the substrate into the product can impair not only growth as final effect but can also lead to cofactor imbalance and toxic intermediate accumulation, and finally, it can reduce the cellular intrinsic resilience. A good compromise can be achieved by separating growth and production phase, which has been more easily obtained by modifying process conditions and not the expression pattern of the engineered cells. Synthetic biology is now offering the possibility to dynamically switch the metabolic configuration from "growth" to "production" by the design of genetic circuits, implying sensors and actuators (Michener et al. 2012).

A relevant number of genetic circuits are already available and continuously augmenting [see for a recent review Venayak et al. (2015)]. They can be divided in circuits with an on-off control of a two-stage fermentation process and circuits that can ensure a continuous control. The first approach utilises on-off methods that can involve physical parameters (such as temperature, pH) or chemicals (i.e. inducers) as triggers. Solomon and coauthors, for example (Solomon et al. 2012), optimised gluconate production by an engineered *E. coli* strain. Gluconate is a classical example of a product that depends on central carbon metabolism but at the same time is drawing flux from glycolysis. In this situation, tuning the expression of the endogenous competing enzymes can be the solution. To reach this goal despite the tight regulation distinguishing the glycolytic metabolism, the authors exogenously manipulated glucokinase (Glk) levels, either with an engineered antisense RNA or with an inverting gene circuit, inhibiting Glk activity by up to 25 % and 50 %, respectively. Using these techniques, gluconate production increased and acetate production decreased, as well as specific growth rate (up to 50 %), but with no difference in final biomass accumulation. This elegant and successful example of dynamic control would need to be verified at industrial scale, since not only the final titer but also the total fermentation time represents a crucial issue for commodities production.

The second approach makes use of dynamic sensors, so that the circuit can respond to environmental changes and control the metabolism accordingly. In the last few years, some examples have been described in the literature. Among them is a report on the dynamic control of lycopene production (Farmer and Liao 2000) where the lycopene biosynthetic pathway was under the positive control of a sensor responsive to acetyl-phosphate, a metabolite whose concentration indicates a high glycolytic flux.

A significant number of works relates with the cellular management of toxic compounds, which is a crucial point of viable industrial bioprocesses. Indeed, toxic compounds can derive from the starting substrate used as feedstock, or the toxicity

is determined by the accumulation of the product of interest. This is typically the case when alcoholic biofuels are produced, heavily interfering with many cellular components, starting from membranes. Dunlop and coauthors designed an in silico model, employing a search for a robust phenotype and considering the timing of cellular response, where solvent-resistant bacterial efflux pumps are under the control of a biofuel-responsive promoter, resulting in genetic architectures improved for biofuel production (Dunlop et al. 2010).

At the current stage of development, these systems have to be accurately customised, both in the construction as in the validation and demonstration steps: a future development can be envisaged in the combination of genetic circuits with fermentation strategies, aiming at an integrated contribution of systems and synthetic biology to guide successful industrialisation of bioprocesses.

5.4.2 Perturbing Central Regulatory Systems of the Principal Elements

The techniques just described underline the complexity of manipulating a native metabolism with the final aim of a complete redirection of the carbon flux to the desired product. An alternative way to overcome this limitation is to count on the cellular capability to reorganise when a central mechanism (also defined as non-pathway components, like transcription or translation) is perturbed. This was well described by McKee and coauthors: by perturbing the carbon storage regulator (Csr) system, previously described to cause a profound reorganisation of *E. coli* central metabolism (Edwards et al. 2011), they were able to obtain a twofold improvement of n-butanol production with a concomitant acetate decrease (McKee et al. 2012). The basis for these results remains to be understood, since the prominent effect of the perturbation was an increase in amino acid levels. However, the approach appears intriguing as it could be applied to other prokaryotic cells, being Csr a conserved system.

5.5 Enhancing Production: A Matter of Quantity or of Quality State?

A great deal of focus in cell factory design was and still is devoted to ensure quantitative settings (e.g., in terms of gene copy number, protein levels and so on), as an essential prerequisite for high production levels. However, a relevant number of evidence suggest a physiological balancing as the more correct strategy to be pursued (see also some of the previous examples). Therefore, in the last years, growing attention is being directed to the quality state of macromolecules, instead of their quantitative state. This concept becomes even more central when the cell factory has to operate within a process of production, encountering detrimental situations that can exacerbate unstable cellular settings. Indeed, many classes of macromolecules are very often found to be profoundly impaired by limiting

situations, resulting in lipid peroxidation, protein carbonylation, reactive species accumulation and cofactor imbalance. In these conditions, the titer of the desired product might be augmented by increasing the copy number of genes directly responsible of its production and by modulating their expression, mRNA stability, number of influx and efflux transporters, but all these efforts are doomed if the macromolecular quality control is not ensured. This is then another aspect where systems biology can suggest strategies for the development of synthetic tools to be applied in supporting the mainstream of production and to ensure the functionality of the system as a whole.

5.5.1 Cellular Stress and Integrity of the Cellular System

Only a functional proteome allows the proper expression of a cellular being and a rapid and appropriate response to altered intracellular or extracellular conditions. A functional proteome is therefore a prerequisite for a stable phenotype of production when a cell factory is challenged during an industrial process. To cite Prof. Radman: "the proteome sustains and maintains life, whereas the genome ensures the perpetuation of life by renewing the proteome, a process contingent on a preexisting proteome that repairs, replicates, and expresses the genome" (Krisko and Radman 2013). Regrettably, very often exactly the conditions of an industrial process elicit damages to the proteome. In most cases, oxidative stress plays an important detrimental role, and even when the production is anaerobic, the biomass propagation phase is aerobic. More generally, the occurrence of free radicals is quite common in industrial process condition, triggering a cascade of negative effects on different cellular networks. There is a growing number of examples where engineering cellular components belonging to the general mechanisms of scavenging activities result in an improved phenotype. This is the case of *S. cerevisiae* strains engineered for ascorbic acid production, resulting in cells more robust against a series of stressors, among them organic acids (Martani et al. 2013), or overexpressing gene(s) of the glutathione pathway resulting in a less sensitive phenotype to toxic compounds released from lignocellulose pretreatment (Ask et al. 2013). Such modifications determine a complete reorganisation of the cells, influencing many pathways, not only directly related to the operated modification or to the desired productive pathway. Therefore, a combined systems and synthetic approach will possibly lead in a near future to easily customisable tools for augmenting cellular robustness against stressors. An important caveat recalls what has been previously mentioned about the dynamics of the systems, which refers both to the intracellular and to the extracellular environment. Therefore, it has to be taken into account that different stressors are acting on the cells over time, but not always the same stressors, not always with the same intensity.

5.5.2 Novel Signalling Functions for "Old" Molecules

The potential and versatility of RNA molecules has been exploited from nature since its appearance, probably even before the definition of cellular units. We are in a continuous process of discovery of the many and different RNA functions inside cells: the deeper our knowledge gets, the more synthetic strategies for applying the novel information can be designed and adapted to the cell factory of interest. This is the case with microRNAs (miRNAs), small noncoding RNAs that can participate in the modulation of protein expression [see, for a review, Inui et al. (2010)], can act as mediators among cells [see, for a review, Kohlhapp et al. (2015)] and were also suggested to have a role in conferring robustness to the cells (Ebert and Sharp 2012). In particular, this role has been described in cells facing environmental fluctuation during development (Li et al. 2009), and miRNAs have been also depicted as stabilisers of fluctuations in gene expression (Chang et al. 2011; Herranz and Cohen 2010). Moreover, recent findings suggest miRNAs as essential for lifespan determination in the model organisms, *Caenorhabditis elegans* and *Drosophila* [as reviewed by Kato and Slack (2013)]. Considering the importance of robustness, ageing and ageing-related issues for industrial processes, miRNAs appear as a very promising target for remodelling cell factories in this direction.

A completely different perspective for seeing the signalling would be not to consider individual cells but the whole population. Indeed, if cells belonging to different subpopulations could be devoted to diverse functions, this would decrease their metabolic burden and simplify the construction of genetic circuits. However, the necessary assumption is that such subpopulations have to be able to coordinate their functions through regulated intercellular communication exchanges, which should also act at reducing the possible noise of random fluctuations. Intercellular crosstalk exists and is widely used in all the three biological kingdoms of life, therefore also in microbial systems, but exactly because of the fundamental and ancient origin of the system, its engineering is far from being trivial.

As a starting point, the synthetic reconstruction of natural communication systems can be considered: their application is now primarily intended to validate the possibility to translate them in controlled circuits, and mainly in bacterial hosts, despite the first applications are emerging together with the extension of the concept from unicellular to pluricellular eukaryotic systems.

The bacterial quorum sensing (QS) system was the first to be translated in synthetic circuits and in particular the one based on LuxR/LuxI from *Vibrio fischeri*, where acyl-homoserine lactone (AHL, synthetised by LuxI) mediates intercellular coupling by binding to LuxR: in turn, the complex controls the expression of target genes [LuxI as first (Waters and Bassler 2005)].

When expressed in *E. coli*, this system allowed the cellular density-controlled expression of the killer gene ccdB (You et al. 2004) to autonomously regulate cellular density, or of the heterologous invasin [from *Yersinia pseudotuberculosis* (Anderson et al. 2006)] to invade tumour cells after injection in such tissue, with the possibility to target a specific delivery or response. A more complex system, including the coordinated expression of a protease that negatively regulates LuxI

expression by catalysing the degradation of AHL, allowed the creation of a synthetic oscillator (Danino et al. 2010). Examples of how to further implement this technology for inducing synchronised oscillations between bacterial populations have been reported (Liu et al. 2011; Prindle et al. 2012). The system can be further modulated if the genetic components of the LuxI/LuxR QS are separated in two populations acting as "sender" and "receiver" of signal, respectively.

Despite being still not directly connected with industrial production processes, the implementation of circuits of communication among cellular (sub)populations could inspire novel strategies for the exploitation of microbial communities, considering the final aim of developing consolidated bioprocess-based biorefineries (Shong et al. 2012).

5.6 Conclusions

The still incomplete knowledge about genes and their products, and therefore about cellular regulatory networks and their connections, is currently limiting the prediction of the final results when a novel biocatalyst is genetically generated. The process conditions, which are often detrimental for the cells and always producing fluctuations in chemical-physical parameters, further contribute to evoke cellular answers very difficult to be predicted. Screening and selection can therefore be a strategy to retrieve the desired phenotype, but there are at least two *caveats*: (1) to reproduce an industrial production process including all the limiting factors at lab scale is not trivial, resulting in the possible selection of an undesired or suboptimal strain and (2) the evoked phenotype might be not stable enough to constitute a viable and robust biocatalyst. On the other hand, the innate capability of cellular systems to adapt, to express emergent properties, to possibly communicate at intercellular level and to profoundly rearrange their settings at different level is still largely underestimated and unexploited (Lajoie et al. 2013).

One important element that has been only accidentally mentioned is timing: transcriptional regulations can be finely tuned and regulated, but they will result in slow responses, and quite similar situations will be encountered by manipulating translation, since protein half-life is in the average of hours. Therefore, circuits acting at these levels are matching the need for responses acting on the same order of time. Diversely, post-translational control mechanisms are effective on the order of fraction of seconds and can therefore be efficaciously applied to follow metabolic fluctuations, which occur on the order of minutes.

The urgency for a real turn into bioeconomy and for the establishment of viable biorefineries, possibly integrated in the territory, can also accomplish the rising need of organic waste management, which in turn can determine a more equal availability of feedstock. The uneven nature of sustainable biomasses together with industrial process requirements makes the development of robust and versatile biocatalysts unavoidable.

Systems and synthetic biology are currently playing a pivotal role, by successfully tuning their activities in providing a novel array of customisable, robust and

versatile cell factories with defined and predictable properties, and are additionally promising to move from synthetic cells to synthetic consortia.

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