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Metabolic variability in bioprocessing: implications of microbial phenotypic heterogeneity

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Phenotypic heterogeneity is a major issue in the context of industrial bioprocessing. Stochasticity of gene expression is usually considered to be the main source of heterogeneity among microbial population, but recent evidence demonstrates that metabolic reactions can also be subject to stochasticity without any intervention of gene expression. Although metabolic heterogeneity can be encountered in laboratory-scale cultivation devices, stochasticity at the level of metabolic reactions is perturbed directly by microenvironmental heterogeneities occurring in large-scale bioreactors. Accordingly, analytical tools are needed for the determination of metabolic variability in bioprocessing conditions and for the efficient design of metabolic engineering strategies. In this context, implementation of single cell technologies for bioprocess monitoring would benefit from knowledge acquired in more fundamental studies.

Impact of microbial phenotypic heterogeneity on microbial bioprocesses

Until now, microbial phenotypic heterogeneity has generally only been explored in basic scientific research, and few studies have taken this important phenomenon into account in the context of industrial biotechnological applications [1,2]. The production of bio-based compounds is dependent on stochastic cellular mechanisms, leading to difficulties in controlling bioprocessing. It is thus of primary importance to increase our knowledge of the mechanisms involved in phenotypic diversification. One may expect, for example, that a biopharmaceuticals manufacturer would be most interested in variability in protein synthesis. However, the true situation is more complex because other mechanisms, such as stochasticity at the level of metabolic reactions, can also be involved. We first describe the main sources of microbial phenotypic heterogeneity, with special emphasis on metabolic reactions. Then we address the actual methodologies available for the characterization of microbial phenotypic heterogeneity. Finally, technologically relevant methodologies aimed at controlling microbial phenotypic heterogeneity will be considered.

What are the sources of microbial phenotypic heterogeneity?

Although stochasticity occurs at the level of gene expression [3], stochastic effects can be observed at the level of the metabolic pathways themselves (Figure 1A and Box 1). Metabolic reactions can be directly influenced by bioprocessing conditions, and thus deeper understanding of their influence of microbial phenotypic heterogeneity would lead to technical solutions that can be implemented in the design of more robust microbial cell factories. The fact that a clonal population of microbial cells can exhibit phenotypic diversification has attracted significant attention over the past decades. Most work carried out in this area has been at a fundamental level and has shown that gene expression is subject to noise, leading to phenotypic plasticity during microbial culture [4,5]. In the field of bioprocess operations, most of this phenotypic heterogeneity is due to epigenetic mechanisms [6] or stochasticity in biochemical reactions [4], in other words non-heritable traits, whereas mutations only become an issue on timescales longer than the typical timescales of batch and fed-batch processes [5]. Noise in gene expression generally results in a Gaussian distribution of protein content across different individuals of the same population. All these sources of phenotypic heterogeneity are known to affect bioprocess performance [1,2], but the respective contributions of these different components to phenotypic diversification are not known.

However, there is an accepted picture of the importance of microbial phenotypic heterogeneity. Lack of global productivity can often be attributed to a specific subpopulation of cells exhibiting a 'non-producer' or a 'low-producer' phenotype (Figure 1B). This phenomenon has been observed in various biotechnological fields, such as the production of solvent by *Clostridia* [7], the production of

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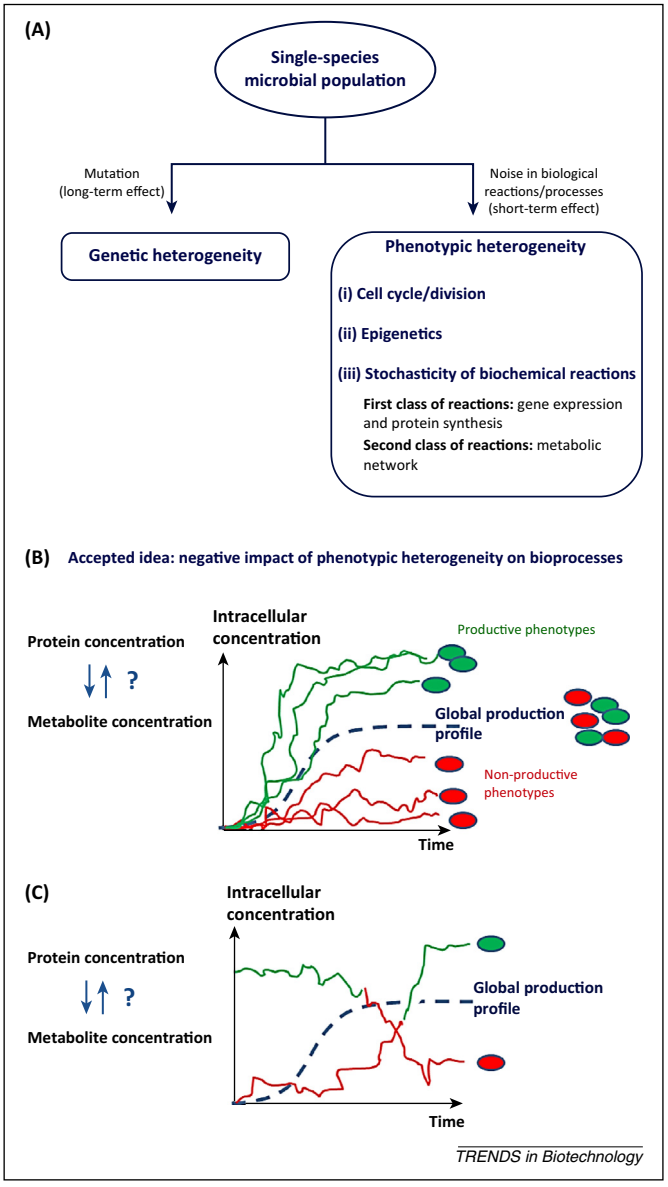

Figure 1. **(A)** Different sources of heterogeneity involved in microbial phenotypic diversification under bioprocessing conditions. **(B)** The accepted picture of the negative impact of microbial phenotypic heterogeneity on process performance. In the scheme a subpopulation with reduced protein synthesis capacity is shown that lowers the global yield of the bioprocess. **(C)** Microbial cells exhibit bistability, in other words they can switch to a producing state to a non-producing state, and vice versa [12,13].

lactobionic acid [8], and the production of recombinant proteins for biopharmaceutical applications [9,10]. Another interesting feature is the history-dependence of phenotypic heterogeneity. Several studies have shown that protein and metabolite production is dependent on the stage of the cell cycle, as observed for eukaryotes such as *Saccharomyces cerevisiae* [11] and *Pichia pastoris* [12], as well as for prokaryotic cells, for example in amino acid production by *Corynebacterium glutamicum* [13]. This phenomenon leads to microbial cell switching from a producer state to a non-producer state and vice versa (Figure 1C).

To better understand the mechanisms leading to heterogeneity in process conditions it will be necessary to integrate the phenotypic history of individual cells and also discriminate between the different sources of noise

Box 1. Does stochasticity applies for metabolic reactions?

Heterogeneities in metabolic activities are increasingly considered as a source of phenotypic heterogeneity. For example, phenotypic heterogeneity and microbial antibiotic resistance, a phenotype termed 'persistence', have been long interpreted as a consequence of stochasticity in gene expression [53]. More recently, new analyses have pointed out the role of metabolism in bacterial persistence [62]. Indeed, the entry into the persister phenotype depends mainly on the energy status of the cells, and is thus directly linked to their metabolic activity, the capacity to generate ATP, and the regeneration of cofactors (mainly shown for *Escherichia coli* and *Pseudomonas aeruginosa*). By contrast, cofactors engineering has been proposed as an efficient metabolic engineering strategy both for *E. coli* [49] and *Saccharomyces cerevisiae* [50]. One may ask if this improvement comes from a global improvement of the phenotypes or if single cell mechanisms are implicated. It is thus crucial, for a proper understanding of the impact of microbial phenotypic heterogeneity on bioprocess performances, to interpret correctly the source of stochasticity. Theoretically, two sources of noise or stochasticity in biochemical reactions can be considered:

Stochasticity in gene expression: in this case, the source of phenotypic heterogeneity is attributed solely to noise in gene expression. The term 'source' is important in this definition because gene regulation impacts upon enzymatic activities and nutrient transport inside cells, which in turn impact upon metabolic activities.

Stochasticity in metabolic reactions: generally, variability in metabolic reactions is attributed to variations in enzymes expression level, but ATP imbalance can also have a strong influence at this level. Another phenomenon that can be advanced to explain the stochasticity in biochemical reactions relates to the slow diffusion of reactants inside the cytoplasm, known as molecular crowding [63,64].

The two mechanisms of stochasticity can also occur simultaneously in microbial cells; in other words, stochasticity in gene induction has repercussions on the expression of metabolic enzymes, and this in turn induces variability at the level of metabolic reactions [21], the rates of metabolite production and consumption, as well as the rate of variation of cofactor and energy pools. It is also important to recognize that metabolic reactions can also have a significant impact on gene expression, reversing the classical flow of information [3].

(Figure 1A). Chemical engineers have integrated the fact that bioreactor scale-up induces the emergence of heterogeneous environmental conditions (e.g., in pH, nutrient, temperature, dissolved gas) which in turn induce phenotypic variability within the cultivated microbial population [1,14]. During the scaling-up procedure, mixing quality is lost to some extent, and the resulting environmental fluctuations induce specific responses at different levels of microbial physiology (transcriptional, translational, metabolic) [15,16]. In this context, classical stirred bioreactors can generate major perturbations during the scaling-up procedure, and are probably not the best choice for addressing the biological constraints [16]. Microbial cell factories exhibit reduced phenotypic heterogeneity during laboratory- and pilot-scale operations, leading to predictable and robust bioprocesses (unstable strains being discarded during the screening phase). However, variability generally occurs during large-scale operations, and it is well known that concentration gradients (e.g., spatial heterogeneities in substrate, dissolved oxygen, carbon dioxide, pH) exhibit a strong influence on cellular physiology [17]. Because microbial cells exhibit different circulation times in bioreactors, they are exposed to different concentration profiles, thereby leading to phenotypic segregation of the