

Metabolism of non-growing cells and their application in biotransformation

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Abstract

Growing cells is the typical mode of operation in many aspects of biotechnology and metabolic engineering. This comes about due to cell growth processes creating a driving force that pull metabolic flux along different metabolic pathways, that indirectly help move substrate to product. But, there is an alternative mode of operation that uses resting (non-growing) cells to achieve similar or even higher productivities. In general, resting cells are provided with carbon substrates for biocatalytic reactions but starved of nitrogen or phosphorus. Such resting cells have been usefully employed in many forms of biocatalysis and biotransformation, with or without cofactor regeneration. However, much remains unknown about the transcriptome and metabolome of resting cells in biotransformation settings. This short writeup provides the backdrop of resting cells in biocatalysis, documents their use in biotransformation with application examples, and identifies research gaps that could be filled with contemporary RNA-seq and mass spectrometry proteomics technology. Overall, utility of resting cells in biocatalysis and the extant knowledge gap in their fundamental physiology are highlighted in this resource.

Keywords: resting cells, biocatalysis, cofactor regeneration, transcriptome, biotransformations,

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Non-growing (resting) cells in biotransformation

Biotransformations sought the use of enzymes and whole cells for the conversion of substrates into desired products. Typically, whole cell biocatalysts are used given problems with instability and purification of pure enzymes. However, whole cell biocatalysis is not without its problems. For example, use of growing cells divert precious metabolic energy away from product formation and into biomass formation [1]. Another problem comes from the depletion of NADPH during growth [1, 2]. Thus, non-growing cells (or resting cells) has been an alternative strategy for achieving higher productivity during biotransformation [2-5]. In this approach, cells are grown to a desired concentration, harvested and resuspended in a buffer supplemented with a substrate but not nitrogen source [1, 5]. Besides increased productivities of product, possible reuse of the non-growing cells in multiple biotransformation and reduced consumption of NADPH in anabolic reactions also makes the approach appealing [1, 6-8]. Downstream product separation could also be made easier [6]. But biocatalyst stability is a

limiting factor [1]. Additionally, substrate and product inhibition have also been reported to limit resting cell biotransformation [1, 6]. Another approach of using non-growing cells incorporate a two step biotransformation method where there are separate steps of growing and non-growing cells biotransformation [6].

Starved of nitrogen and unable to synthesize protein, cells enter a non-growing state whose metabolic network, transcriptomes, and metabolome remain poorly understood, at least for important microbial chassis of biotechnological importance such as *Escherichia coli*, and *Saccharomyces cerevisiae*. Another approach for inducing non-growing cells utilize phosphate starvation, which has been shown to maintain glucose metabolism for several days and helps NADH production where cells liberate Pi from phosphorylated compounds [9]. However, relatively high intracellular concentrations of inorganic phosphate at 13 and 8 mM after 20 min and 3 hour of stationary phase growth revealed difficulty in bringing cells to a non-growing state [9]. In addition, production of reactive oxygen species is another problem afflicting the approach [9]. Another study, however, reported possible interference with NADPH generation in carbon and phosphate-starved cells [5].

A recent study on a non-biotechnological chassis, *Rhodopseudomonas palustris*, revealed that its transcriptome as profiled by RNA-seq exhibited distinct features of activation of nitrogen scavenging genes and deactivation of other metabolic processes [3]. However, the genes profiled fell into a rather narrow group of ribosomal proteins, photosynthetic proteins and those of the central metabolic pathway [3]. To examine the question of why metabolic flux channel from acetate to CO₂, ¹³C metabolic flux analysis was conducted and revealed that the tricarboxylic acid cycle (TCA) was in the “on” state in nitrogen-starved cells fed with glucose [3]. However, over-activation of the TCA cycle might produce excessive reducing power. Another study assessed the productivity of styrene oxide from recombinant *E. coli* expressing *StyAB* through metabolic flux balance analysis of the genetically perturbed metabolic network [1]. Metabolic flux analysis has been employed for the estimation of NADH regeneration rates and the metabolic choke points preventing NADH synthesis [10]. Results revealed almost exclusive use of pentose phosphate pathway at high rates of glucose catabolism [10]. This is corroborated by another analysis of the metabolic network of engineered *E. coli*, where it was found that pentose phosphate pathway, TCA and proton-translocating transhydrogenase, PntAB are major contributors of NADPH [8]. In another analysis, it was found that NADPH could be regenerated from the oxidative part of the pentose phosphate pathway [2]. Finally, transfer of reducing equivalents between NADH and NADPH may not be efficient [8].

The need for cofactor regeneration

Whether growing or non-growing cells, use of NADH or NADPH dependent enzymes such as oxidoreductase in biotransformations necessitate the regeneration of cofactors. Specifically, NADH is needed for catabolic reactions while NADPH supports anabolic

reactions. Approaches for regenerating cofactors include direct electrochemical regeneration and photochemical regeneration, but they suffer from high overpotential and low activity, respectively [11]. For example, an artificial hydrogen-dependent, water soluble iridium catalyst could regenerate NADH, but the turnover frequency was only 36 h^{-1} [11]. Direct supplementation of cofactors to fermentation is another possibility, use of whole cells for regenerating cofactors is more commonly used [2]. To this end, three main approaches used in the industry for cofactor regeneration are the (i) feeding of different carbon substrates to cells, (ii) use of enzymes for the regeneration of cofactors (i.e., coupled enzyme approach), and (iii) use of an enzyme that perform the biotransformation concomitant with the regeneration of cofactors (i.e., coupled substrate approach). For example, intracellular NADH levels increased with the duration of biotransformation in non-growing cells [1]. However, despite efforts aimed at increasing cofactor regeneration, permeabilization of bacterial membranes or inhibition from metabolic activity could reduce NADH availability [1].

Different carbon substrates such as glucose, succinate, and glycerol activate different metabolic pathways for the generation of necessary cofactors [12]. For example, glucose activates the glycolytic pathway while glycerol activates a mixture of gluconeogenesis and glycolysis. Another approach is the use of specific enzymes for the regeneration of cofactors. One possibility is glucose-6-phosphate dehydrogenase [2, 12] and formate dehydrogenase [13]. In particular, formate dehydrogenase is commonly used to couple NAD^+ reduction with the oxidation of formate to CO_2 . But the system suffers from low activity of the enzyme and solution acidification by carbon dioxide [11].

Synthesis of excess cofactor may prompt the cell to respond by activating metabolic processes that use the reducing equivalents, but details of this question remain unclear in the literature [8]. Possible avenues for the “dissipation” of cofactors include increased maintenance energy requirements, energy spilling, or uncoupling of NADH oxidation from substrate oxidation [8]. But, respiration remain a likely major contributor to usage of excess cofactors [8]. However, another study indicated that uncoupling, stress metabolism and energy spilling do not consume significant amounts of NADH in non-growing *E. coli* cells [1].

Model biotransformation reactions with resting cells

An example of non-growing cells biotransformation is the use of phenylacetone monooxygenase for the production of benzyl acetate where the NADPH-dependent enzyme requires an efficient system for cofactor regeneration [12]. Different carbon sources were chosen to help induce cofactor regeneration. However, glucose and succinate did not improve product yield while glycerol helped quadrupled the production of benzyl acetate likely due to efficient regeneration of NADPH [12]. Another example is the use of Baeyer-Villiger monooxygenase (cyclohexanone and cyclopentanone monooxygenase) in non-growing recombinant *E. coli* for the production of lactones [4]. Specifically, 20-fold higher volumetric

productivities were reported for non-growing cells compared to growing cells together with shorter reaction time, higher enantiomeric excess and the enabling of cleaner separations [4]. Higher transport rates of substrate into the cells was postulated to result from greater permeability of bacterial membrane of non-growing cells [4]. In another study, cyclohexanone monooxygenase was expressed in *E. coli* whole cells for the production of ε-caprolactone under non-growing conditions [14]. Substrate transport through the membrane was found to be the rate-limiting step while sufficient NADPH was detected on a stoichiometric basis giving a volumetric productivity 20 fold higher than that using growing cells [14]. However, the biotransformation yield was also limited by the relatively short half-life of the cyclohexanone monooxygenase [14]. Intracellular redox balance was maintained throughout the biotransformation and isocitrate dehydrogenase was postulated to supply most of the NADPH [14]. Finally, engineering flux through the pentose phosphate pathway was suggested to improve glucose utilization [14]. Vanillin production was also attempted in non-growing *E. coli* cells through the expression of feruloyl-CoA synthetase and feruloyl hydratase/aldolase from *Pseudomonas fluorescens* BF13 [7]. A response surface methodology was used in optimizing production conditions such as stage of cell growth, incubation temperature, biomass and substrate concentration [7]. Finally, while glucose is commonly used as auxiliary substrate, use of alternative substrates such as low-cost methanol for cofactor regeneration has been explored [15]. Specifically, methanol oxidation pathway of *Pichia pastoris* was utilized together with expression of formaldehyde dehydrogenase for enhanced recycling of NADH during the reduction of acetoin to 2,3-butanediol [15].

Possible research directions

Understanding the metabolic processes activated in non-growing cells relative to growing cells is critical to providing a compendium of genes and pathways suitable for engineering enhanced cofactor regeneration. To this end, RNA-seq and mass spectrometry proteomics at the population and single cell level could profile the transcriptomes of cells in non-growing and growing states, which would suggest a list of genes and pathways differentially activated in the non-growing state. Besides differential expression, it would also be interesting to understand the stresses (and, by extension, stress response) of non-growing cells. The goal is in the generation of strains of *E. coli* with optimized gene expression that help ensure a steady supply of NADH, NADPH and FADH₂ for promoting cofactor-dependent enzymatic processes. Doing so would require ¹³C labelling and tracing of the metabolic flux in non-growing and growing cells at various substrate incision points in *E. coli* central carbon metabolism. Another approach would be to inactivate cellular reactions that consume NADH, NADPH or FADH₂ without imposing unnecessary stress on the cell. Such a cofactor regeneration optimized strain may require the over-expression of particular cofactor regeneration enzymes under inducible control as well as deletion of regulatory motifs, but it should be ideally-suited for performing a variety of biotransformations involving oxidoreductase which constitutes about 25% of all known enzymes.

Conclusions

Without the need to divert metabolic energy and precursors to biomass formation, non-growing cells have been shown to enable higher volumetric productivities of product as well as offer possibility of reuse. However, issues such as enzyme stability and half-life, substrate transport, as well as substrate and product inhibition limit the application of non-growing cells in biotransformation. Relatively little is known about the global gene expression patterns of major biotechnology chassis such as *E. coli* in the non-growing mode, and this hampers further engineering of pathways for the efficient regeneration of cofactors necessary to support cofactor-dependent oxidoreductase role in biotransformation. RNA-seq and mass spectrometry proteomics could inform gene expression patterns in non-growing and growing cells at the global level, which provides the list of genes that could be targeted in metabolic engineering efforts to enhance cofactor regeneration. Carbon isotope tracing of metabolic flux would subsequently validate the enzymatic targets suitable for over-expression in biocatalysis and metabolic engineering.

Conflicts of interest

The author declares no conflicts of interest.

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