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Rong, Yixin; Jensen, Sheila Ingemann; Woodley, John M.; Nielsen, Alex Toftgaard

Published in:
Biotechnology and Bioengineering

Link to article, DOI:
10.1002/bit.28791

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Rong, Y., Jensen, S. I., Woodley, J. M., & Nielsen, A. T. (2024). Modulating metabolism through synthetic biology: Opportunities for two-stage fermentation. *Biotechnology and Bioengineering*, *121*(10), 3001-3008. https://doi.org/10.1002/bit.28791

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Check for updates

Received: 20 October 2022 | Revised: 23 April 2024 | Accepted: 19 June 2024

DOI: 10.1002/bit.28791

REVIEW

Modulating metabolism through synthetic biology: Opportunities for two-stage fermentation

Yixin Rong[1] | Sheila Ingemann Jensen[1] | John M. Woodley[2] | Alex Toftgaard Nielsen[1]

[1]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark

[2]Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), Kongens Lyngby, Denmark

Correspondence
Alex Toftgaard Nielsen, The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, 2800, Denmark.
Email: atn@biosustain.dtu.dk

Funding information
Fermentation-based Biomanufacturing Initiative, Grant/Award Number: NNF17SA0031362; Novo Nordisk Foundation, Grant/Award Number: NNF20CC0035580

Abstract

Bio-based production of fuels, chemicals and materials is needed to replace current fossil fuel based production. However, bio-based production processes are very costly, so the process needs to be as efficient as possible. Developments in synthetic biology tools has made it possible to dynamically modulate cellular metabolism during a fermentation. This can be used towards two-stage fermentations, where the process is separated into a growth and a production phase, leading to more efficient feedstock utilization and thus potentially lower costs. This article reviews the current status and some recent results in application of synthetic biology tools towards two-stage fermentations, and compares this approach to pre-existing ones, such as nutrient limitation and addition of toxins/inhibitors.

KEYWORDS

bio-based manufacturing, growth decoupling, metabolic switches, synthetic biology tools, two-stage fermentation

1 | INTRODUCTION

The vast majority of chemicals produced today, ranging from commodity chemicals to pharmaceuticals, are produced using conventional petrochemical-based processes. Both in the United States and in the European Union, these processes account for 97% of the total chemicals produced (Burg et al., 2016; Spekreijse et al., 2019). In the past few decades, bio-based manufacturing processes have emerged as a more sustainable alternative to such processes, as they can make use of renewable feedstocks (Sheldon & Woodley, 2018). Nevertheless, broader industrial implementation of new bio-based processes has in the past often been hindered by having higher manufacturing costs compared to existing processes, which use low-cost raw materials (derived from crude oil) and have been well optimized during the many decades of deployment (Nattrass et al., 2016).

The cost of substrate makes up a substantial part of the operational costs in a bio-based process, typically around one-third. Depending on the specific product and process, it can be up to 71% of operational cost in particular cases (McClelland et al., 2021; Straathof, 2011). A main objective in optimizing a bio-based process is therefore to increase the yield of product per substrate; techno-economic analyses (TEA) of biorefineries show yield as the most important aspect of the fermentation step in reaching a competitive minimum sales price for the product (Bhagwat et al., 2021; Y. Li et al., 2021; McClelland et al., 2021). One method to achieve a higher yield is to run the fermentation in two stages (i.e., a two-stage fermentation or TSF); generally defined as having a growth phase that prioritizes biomass formation, and a subsequent production phase that prioritizes product formation (Burg et al., 2016) (Klamt et al., 2018). Figure 1 illustrates TSF as a process schematic, with a fermentation (growth) and a conversion (production) phase, possibly

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(a) Standard operation
CS
FS → Ferm → Conv → Cell sepn → DSP
Cells

(b) Cell recycle
CS
FS → Ferm → Conv → Cell sepn → DSP

FIGURE 1 Schematic representation of two-stage fermentation configurations with decoupled fermentation (ferm) and conversion (conv) in (a) standard operation and (b) with cell recycle. CS, conversion substrate; FS, fermentation substrate; DSP, downstream processing; Sepn, separation.

with separate substrates and/or cell recycling. A major advantage of having a fermentation process run primarily in the production phase is the reduction in substrate usage towards generating surplus biomass (Li et al., 2016) (Venayak et al., 2018). Being able to limit excess biomass formation can also be advantageous in regard to other cost contributors. High cell density also increases the viscosity and thus decreases the oxygen transfer rate, which can lead to the added cost factor of having to supply pure oxygen to the reactor (Klamt et al., 2018; S. Li et al., 2016; Venayak et al., 2018).

1.1 | Methods to limiting biomass formation

One method to achieve reduction of cellular growth in favor of production is to limit one of the nutrients that the cell needs to grow. The limiting nutrients may for example be primary nutrients such as the carbon source, nitrogen, phosphate or sulfate, or secondary nutrients such as trace elements or vitamins (Halka et al., 2018; Kundiyana et al., 2011; Perez-Zabaleta et al., 2019; Schuhmacher et al., 2014). Limiting a nutrient leads to changed gene expression levels in the cells, where the desired outcome is the downregulation of parts of metabolism not involved in product formation. The choice of which limitation is best can be governed by the product; carbon limitation may for example be used for secondary metabolite production, as it downregulates primary metabolism (Sun et al., 2007). Phosphate and sulfate limitation has been applied to improve yields in both primary and secondary metabolite production; e.g. in the production of L-tryptophan, as amino acid biosynthesis doesn't require phosphate (Schuhmacher et al., 2014) (Bruheim et al., 2002) (Li et al., 2016). Efforts have also been made in recent years to apply omics methods and in-silico modeling to better understand the effects of nutrient limitation on cellular metabolism (Lempp et al., 2019) (Irani et al., 2015). However, in most cases it is still difficult to predict the most effective nutrient limitation strategy, meaning that preliminary fermentation experiments have to be made for each new product. Furthermore, nutrient limitation can only affect the cellular metabolism through native regulatory mechanisms, leading to a limited set of possibilities on how the metabolism can be controlled. This can also lead to activation of cellular responses to starvation, such as stringent response, which decreases the metabolic activity of the cell (Boutte & Crosson, 2013). For some organisms, such as *Escherichia coli*, research has been done to create strains that lack or have a reduced stringent response towards nutrient limitation like nitrogen (Ziegler et al., 2021).

Consequently, instead of limiting what the cells require to grow, the addition of inhibitory compounds or expression of toxins has also been used as a way of limiting biomass formation. These toxins/inhibitors often target different cellular proteins involved in processes related to growth, while also maintaining a level of metabolic activity. A study by Li and co-workers looked into using different toxic chemical inhibitors, such as kanamycin, tetracycline and 5-fluorouracil, targeting different cellular and metabolic processes in *E. coli* that are required for growth, as a way to limit biomass formation and thereby improve the production of tyrosine and mevalonate (Li et al., 2016). Their findings suggest that for the two products tested in the study, sulfate limitation was the most efficient way of increasing yield through growth inhibition. Another study by Chen and co-workers used indole to achieve quiescent but metabolically active cells that maintain GFP expression after growth arrest (Chen et al., 2015). Growth inhibition can also be achieved by inducible expression of toxin proteins. Bokinsky and co-workers showed that HipA could be overexpressed to stop growth, while maintaining mevalonate productivity at a level equivalent to non-arrested cells (Bokinsky et al., 2013). These studies show that while it is possible to suppress growth using inhibitors and toxins while maintaining a level of metabolic activity, the benefits to product formation may be limited, possibly due to uncharacterized off-target effects.

1.2 | Synthetic biology tools to control cellular growth and metabolism

Metabolic engineering of cell factories has long been applied to increase production, where the metabolism can be changed more precisely (Nielsen & Keasling, 2016). However, some genes cannot be targeted for knockouts if they have a highly deleterious effect on cell growth. In recent years, tools have emerged that enable the inducible

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repression of targeted genes without the need for deleting genes from the organism's genome. In this way, it is possible to target genes that limit cell growth to facilitate a TSF process. The deletion/repression of a target gene should also not negatively impact overall metabolic activity, as inhibition of metabolic genes can lead to stringent response, and downregulation of the translational machinery can lead to lower levels of metabolic enzymes and thus reduce productivity (Irving et al., 2021) (Traxler et al., 2008).

Methods for inducible repression of gene expression can generally be divided into two overall elements: a synthetic biology tool that can repress gene expression and an inducible promoter. Important properties for the tool include stability, fold of repression, and host organism compatibility. Additionally, the ability to switch the repression on and off multiple times can be necessary if the process is run with cell recycling, such as shown in Figure 1b. In recent years, CRISPR interference (CRISPRi) has emerged as a method for dynamic modulation of gene expression across various production hosts (Cleto et al., 2016; Momen-Roknabadi et al., 2020; Qi et al., 2013; Y. Wu et al., 2020). Table 1 lists some studies that have used CRISPRi to dynamically switch metabolism to favor product formation. CRISPRi can repress gene expression very effectively, up to 300-fold in *E. coli* and >100-fold in *Bacillus subtilis* (Qi et al., 2013; Y. Wu et al., 2020). Other methods of controlling gene expression include the pLac + pTet system used in a study reported by Soma and co-workers, where the target gene is controlled by the pTet promoter, and the TetR repressor is controlled by the pLac promoter (Soma et al., 2014). Another tool that has been applied for gene silencing is RNA interference (RNAi). Studies have been for example been made in the industrial relevant host organisms *E. coli* and *S. cerevisiae*; in *E. coli*, RNAi has been shown to be able to repress expression to similar levels compared to a knock-out, and in *S. cerevisiae*, RNAi has been shown to repress expression at ~80%–95% efficiency (Magistro et al., 2018) (Y. Chen et al., 2020). RNAi has yet to be studied in the context of TSF's. Whereas both inducible promoters, CRISPRi and RNAi assert their regulation on a transcriptional/posttranscriptional level, a study by Durante-Rodríguez and co-workers demonstrated a method to control the protein levels through targeting to proteosomal degradation (Durante-Rodríguez et al., 2018). Here a degradation signal is attached to the target protein via a linker that includes a site for a specific protease. When the specific protease is induced, the degradation signal is removed and the protein is stable, and vice versa.

Comparing the methods, CRISPRi is the most studied. It has been shown to function in many industrial chassis organisms, and some nonconventional host organisms, such as halophilic bacteria and obligate anaerobes like *Clostridium* species (Lin et al., 2021; Tao et al., 2017; Woolston et al., 2018). In terms of stability, a study by Li and co-workers deployed CRISPRi based growth decoupling for up to 48 h (Li, Jendresen, Grünberger, et al., 2016). Likewise, the two first studies listed in Table 1 also ran their fermentations for 48 h. CRISPRi has been deployed in fermentations up to 144 h, although it is not clear from the results if the repression remains effective throughout (Ni et al., 2019). It has also been demonstrated that it is possible to

TABLE 1 Examples on the use of CRISPRi as a metabolic switch to improve production of chemicals.

Organism	Product	Target(s)	Duration of fermentation	Titer	Rate	Yield	Ref.
E. coli	n-butanol	*pta, frdA, ldhA, and adhE*	48 h	—	3. 2-fold increase	5. 4-fold increase	Kim et al. (2017)
E. coli	Butanoic acid	*fabI*	48 h	—	6.1-fold increase	5.9-fold increase	Ji et al. (2020)
K. marxianus	Ethyl acetate	*ACO2b, SDH2, RIP1, and MSS51*	14 h	—	3. 8-fold increase	—	Löbs et al. (2018)
S. cerevisiae	Beta-amyrin	*ADH1, ADH4, ADH5, ADH6, CIT2, MLS2 and ERG7*	144 h	43% increase	—	—	Ni et al. (2019)
B. subtilis	GlcNAc	*zwf, pfkA, glmM*	90 h	13.2% increase	—	84.1% increase	Wu et al. (2018)

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deactivate the repression from CRISPRi with a pTet-promoter by washing the cells in fresh media without an inducer (Qi et al., 2013). For direct control by inducible promoters (Soma and coworkers), the fermentation was run for 72 h, and the protein degradation based approach by Durante-Rodriguez and coworkers was tested for 24 h (Soma et al., 2014) (Durante-Rodríguez et al., 2018). Although CRISPRi can potentially be stable enough for typical durations of industrial fermentations, growth decoupling can also be susceptible to mutations, for example, in the dCas9 gene in the case of CRISPRi, that enable the cells to escape the growth decoupling and take over the population. This is especially challenging if one wishes to utilize biomass recycling in the process. One aspect worth considering is the added cellular burden of expressing and then degrading the target gene when control is exerted at the protein level. In this regard, RNAi or controlling the target gene directly with an inducible promoter could potentially be the least burdensome, as CRISPRi also involves expressing dCas9, which is a large protein.

Inducible promoters are needed to control when in the fermentation the target gene(s) are repressed. The most important properties of inducible promoters are homogeneity of expression, "leakiness", that is, level of expression without the presence of the inducer, expression strength when induced, stability and host compatibility. Table 2 lists inducible promoters that have been used in conjunction with CRISPRi as well as a few other commonly used inducible promoters. Leaky expression can increase the likelihood of escapee mutations, as there would be a selective pressure favoring escapees already during the pre-culture stage. The inducer prices vary greatly, and can be prohibitory, especially with lower value specialty and commodity chemicals. Furthermore, promoters that get induced by sugars can limit what feedstocks can be used. A few studies have looked at using inducer-free promoters using optogenic (blue light), quorum sensing, (nonessential) nutrient depletion, or temperature control to induce expression (Wu et al., 2021) (Tian et al., 2020) (Landberg et al., 2020) (Restrepo-Pineda et al., 2021). Some of these have not been studied at large scale, which could pose problems for some inducer-free promoters; it might for example, be challenging to apply blue light to the entire reactor. Temperature gradients could affect temperature-based induction, depending on how sensitive the promoter is. Out of these mechanisms, nutrient depletion based promoters, such as the tryptophan system developed by Landberg and coworkers, has the most potential as an alternative to inducer dependent promoters (Landberg et al., 2020).

Besides the promoter and tool applied for inducible repression of a gene, equally important is determining the target gene(s). Earlier approaches to identify target genes have often been based on rational engineering (Soma et al., 2014) (Li et al., 2016). In the study by Soma and co-workers, the gene citrate synthase *gltA* was expressed with a tetR repressible promoter. Repressing the expression of the gene prevents the pre-cursor acetyl-CoA from going to the TCA cycle instead of the product pathway, while inhibiting the TCA cycle also reduces biomass formation (Soma et al., 2014). This resulted in an increase in titer and yield up to 3.7 and 3.1-fold respectively compared to a wild-type strain. However, this TSF process would only work for products derived from acetyl-CoA. Similarly, the studies listed in Table 1 also use target genes often derived from rational design, and aimed at improving the production of a specific compound. A study by Li and co-workers attempted to find more broadly applicable target genes. They reasoned based on existing literature that DNA origin of replication and pyrimidine biosynthesis would limit growth upon inhibition while not being associated with stringent response (Li et al., 2016). They found that using an inducible CRISPRi system to repress the pyrimidine biosynthesis gene *pyrF* gave an attenuation of growth, and a concomitant 2.16-fold increase in GFP titer, as well as a 2.9-fold increase in specific productivity and a 41% increase in yield when producing mevalonate as a proof of concept (Li et al., 2016). The group later published another study on a more systematic method of identifying potential targets for growth decoupling using CRISPRi, by screening a library of 12238 sgRNA's targeting all coding and also noncoding genomic locations in *E. coli* (Li et al., 2020).

1.3 | Future perspectives

While some TSF processes are used in industrial production, using switches such as nutrient limitation or aerobic growth phase/

TABLE 2 Examples of conventional inducible expression systems.

Organism	Promoter	Inducer	Price[†]	Stability of inducer	Leakiness
E. coli	pRha[‡] (Kim et al., 2017)	Rhamnose	0.14 USD/L	Depends on consumption	Low (Haldimann et al., 1998)
E. coli	pNEW[‡] (Ji et al., 2020)	Cumate	1.21 USD/L	–	Low (Choi et al., 2010)
Various	pTet[‡] (Qi et al., 2013) (Li et al., 2016)	aTc	3.06 USD/L	Half-life ~20 h [49]	Low (Lausberg et al., 2012)
B. subtilis	pXyl[‡] (Wu et al., 2018)	Xylose	0.38 USD/L	Depends on consumption	Low (Bhavsar et al., 2001)
Various	pLac	IPTG[§]	1.81 USD/L	No degradation at 32 h [49]	Medium (Kato, 2020)
E. coli, C. glutamicum	pAra	Arabinose	0.11 USD/L	Depends on consumption	Low (Guzman et al., 1995)

[†]Estimated based on the lowest price from Sigma-Aldrich. Prices may vary with bulk orders and other suppliers.

[‡]Used in studies to control CRISPRi expression.

[§]Lactose can be used as cheaper alternative.

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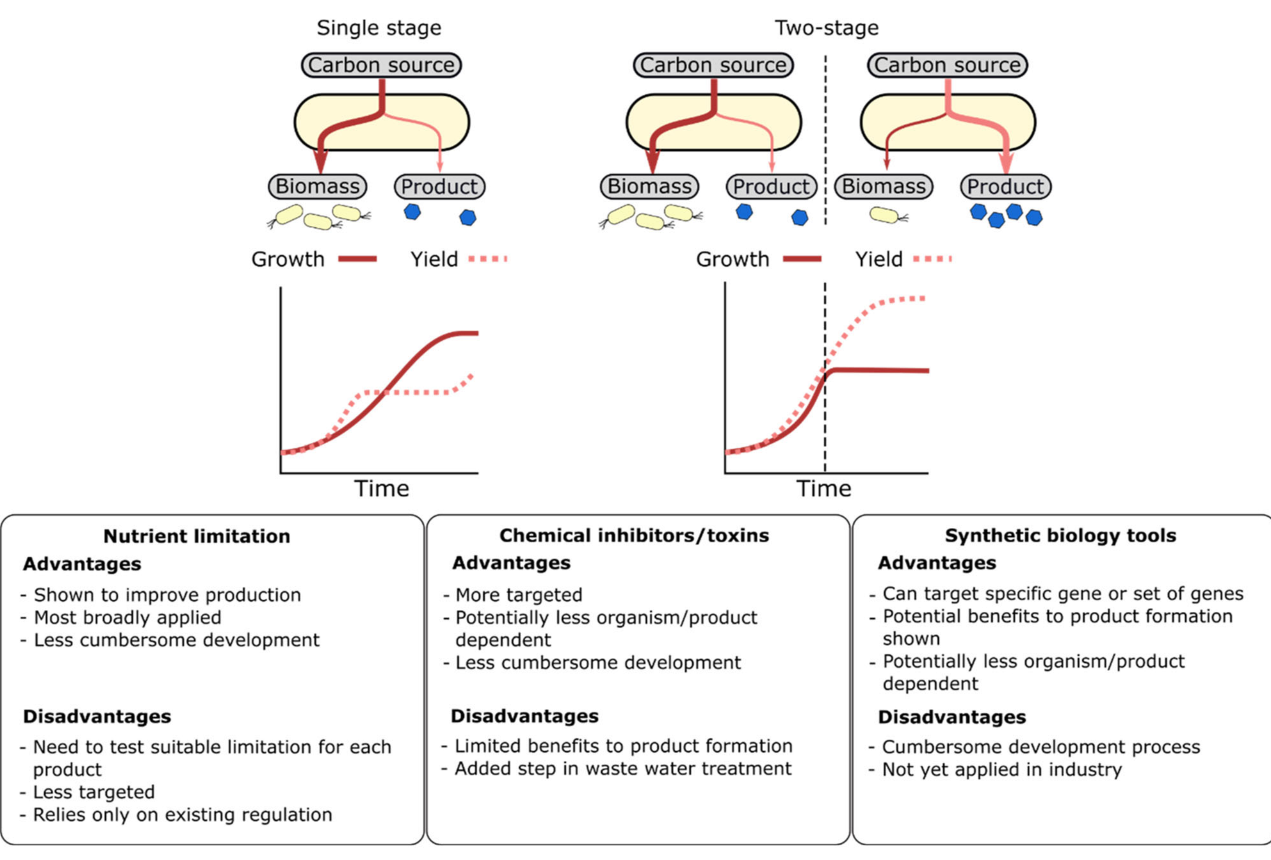

FIGURE 2 Summary of advantages and disadvantages of different ways of achieving a two-stage fermentation.

anaerobic production phase, synthetic biology tool based methods have yet to be adopted (Gao et al., 2022) (Schmid et al., 2021) (Cao et al., 2013). Figure 2 lists some of the advantages and disadvantages of the different methods currently available.

Reasons for the limited uptake of synthetic biology based switches could be due to both technological barriers and incomplete knowledge. The main technological challenge is to improve the stability and robustness of these methods, which is crucial in industrial settings. Most studies are done in small lab-scale experiments without sufficient information to evaluate the feasibility of scale-up, which can be cumbersome and expensive (Schultz, 2023) (Delvigne et al., 2017). Furthermore, it can be resource heavy to develop systems for novel host organisms, even when following previously described methods. Lastly, it can be difficult to make a comprehensive evaluation of the potential benefits of TSF for a particular process. Limiting biomass concentration can affect the volumetric productivity, if an increase in specific productivity is not enough to compensate; the potential trade off in productivity in favor of increased yield should be considered, and the optimal balance of rate and yield likely depends on product value.

Other questions affecting the potential cost benefit at the end include: Does the organism grow fast and to a high enough cell concentration so it is relevant to limit growth? What is the value of the product compared to the cost of the substrate? Is aeration a crucial factor? In summary, synthetic biology tools are diverse and can enable finer control of cellular metabolism during a two-stage fermentation process, but further studies into industrial application and cost–benefit analyses, such as scale-down of industrial fermentations and detailed techno-economic analyses, are likely needed for broader adaptation and application in industrial settings.

ACKNOWLEDGMENTS

YR, JW and ATN conceptualized the article. YR drafted the outline with feedback from JW, ATN and SIJ. YR wrote the manuscript. YR, JW, ATN and SIJ revised the manuscript. This work was funded by the Novo Nordisk Foundation within the framework of the Fermentation-based Biomanufacturing Initiative (FBM), grant number: NNF17SA0031362. We further acknowledge funding from the Novo Nordisk Foundation, grant no. NNF20CC0035580.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Yixin Rong http://orcid.org/0000-0003-3325-2928
John M. Woodley http://orcid.org/0000-0002-7976-2483
Alex Toftgaard Nielsen http://orcid.org/0000-0001-6616-0187

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How to cite this article: Rong, Y., Jensen, S. I., Woodley, J. M., & Nielsen, A. T. (2024). Modulating metabolism through synthetic biology: Opportunities for two-stage fermentation. *Biotechnology and Bioengineering*, *121*, 3001–3008. https://doi.org/10.1002/bit.28791

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