Microbial Cell Factories

REVIEW

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Delaying production with prokaryotic inducible expression systems

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Abstract

Background Engineering bacteria with the purpose of optimizing the production of interesting molecules often leads to a decrease in growth due to metabolic burden or toxicity. By delaying the production in time, these negative effects on the growth can be avoided in a process called a two-stage fermentation.

Main text During this two-stage fermentation process, the production stage is only activated once sufficient cell mass is obtained. Besides the possibility of using external triggers, such as chemical molecules or changing fermentation parameters to induce the production stage, there is a renewed interest towards autoinducible systems. These systems, such as quorum sensing, do not require the extra interference with the fermentation broth to start the induction. In this review, we discuss the different possibilities of both external and autoinduction methods to obtain a two-stage fermentation. Additionally, an overview is given of the tuning methods that can be applied to optimize the induction process. Finally, future challenges and prospects of (auto)inducible expression systems are discussed.

Conclusion There are numerous methods to obtain a two-stage fermentation process each with their own advantages and disadvantages. Even though chemically inducible expression systems are well-established, an increasing interest is going towards autoinducible expression systems, such as quorum sensing. Although these newer techniques cannot rely on the decades of characterization and applications as is the case for chemically inducible promoters, their advantages might lead to a shift in future inducible expression systems.

Keywords Induction, Two-stage fermentation, Decoupling, Regulation, Recombinant protein, Pathway optimization

Introduction

Wild-type microorganisms have evolved for millions of years, thereby optimizing their internal metabolism to achieve maximal growth rates. This tightly regulated metabolism guarantees a maximal carbon flux towards growth. Within this complex network of regulated pathways, a vast array of molecules are produced that ensure growth and survival in all kinds of conditions. A lot of these molecules have proven to be interesting to humankind, such as lactic acid, penicillin, ethanol and 2,3-butanediol [1, 2]. Additionally, with the rise of targeted genetic engineering in the 1980s, the range of possibilities of these microorganisms for industrial biotechnology purposes increased rapidly. With this innovative technology, existing pathways can be tweaked and new pathways introduced. However, when scientists start engineering a microorganism, they interfere with its tightly regulated carbon flux, resulting in suboptimal growth (Fig. 1A). Additionally, the introduced or engineered pathways can cause stress or even toxicity for the host [3]. For example, the expression of the mevalonate-dependent pathway in *Escherichia coli*, responsible for the production of terpenoids, leads to ceased growth or spontaneous mutations due to the toxic accumulation of isoprenoid precursors [4]. When overexpressing recombinant proteins, this production drains energy and

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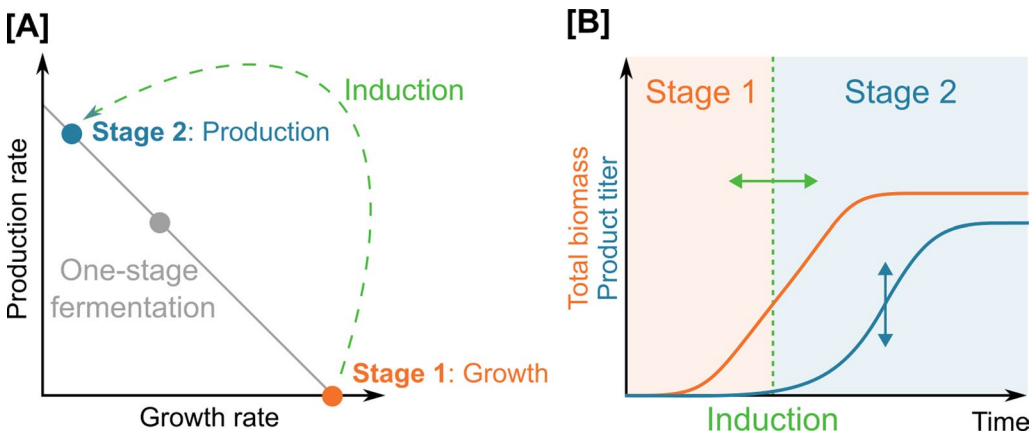

Fig. 1 **A** Illustration of the trade-off between the growth and production rate in a batch process. In a one-stage fermentation (grey dot) neither of the two are optimal. In a two-stage fermentation a maximal growth rate is obtained in the first stage (orange circle) and the production rate is maximized in the second stage (blue circle). **B** Illustration of a two-stage fermentation in which the switch is induced at a certain timepoint that can be tuned. Additionally, the expression level of the output can be adjusted to be higher or lower

protein synthesis machinery from the cell, thereby affecting its metabolism resulting in ceased growth or even "viable, but non-culturable" cells, which are no longer capable of dividing [5]. The reduced growth rates that are associated with this competition or toxicity cause lower volumetric productivity ($g.L^{-1}.h^{-1}$) and lower final product titers ($g.L^{-1}$). Consequently, to obtain the desired production levels, a longer fermentation run and larger fermenter are required, leading to higher fermentation costs. Alternatively, the production can be optimized by further metabolic engineering or optimization of the fermentation parameters, such as temperature [6–8]. Nevertheless, these strategies can become quite labor-intensive and do not guarantee an increase in volumetric productivity ($g.L^{-1}.h^{-1}$) and/or product titers ($g.L^{-1}$). Furthermore, for the production of toxic products, these strategies might not suffice for optimizing the production. Therefore, it is more compelling to separate growth and production in time, resulting in a two-stage fermentation process. The first stage is dedicated to reaching high cell density with minimal metabolic stress, followed by a phenotypic switch to a production stage (Fig. 1B). This production stage can be achieved by halting growth, resulting in an excess carbon flux flowing to production, or by strongly activating production, thereby pulling the carbon into this product flux, or a combination of both. The strategy of strongly activating production and the possibilities to achieve this, will be the focus of this review. Such a two-stage fermentation might result in increased volumetric productivity ($g.L^{-1}.h^{-1}$) and even higher yield and titer if the substrate uptake rate is retained [9]. Figure 1 illustrates how this separation in time can circumvent the trade-off between the growth and production rate, thereby optimizing both.

To achieve this phenotypic switch, the activation or repression of certain pathways needs to be obtained. By applying inducible promoters to regulate the expression of these pathways, an external trigger can be added to achieve the transition to the production stage (Fig. 2A). These inducible promoters allow to control the timing of expression and the expression level simultaneously, providing a broad range of optimization possibilities. Since the first description of an inducible promoter [10], many more inducers have been found and applied to induce the expression of a production pathway or recombinant protein. In this review, we first discuss how both chemical inducers and environmental triggers can be used to enable such a switch. Next, we review how the interference of adding a trigger on the growth process can be omitted by the use of autoinducible systems. In these systems, the cells will automatically switch from a growth to a production phenotype, triggered by the cell growth stage, medium composition or cell density (Fig. 2B). An overview of the different options for inducing the production stage is given in Fig. 2. Each application of one of the induction strategies needs individual optimization to reach the correct balance, which can become labor-intensive. Not only the metabolic pathways need to be optimized, but also the characteristics of these genetic switches, such as induction time and expression strength. Finally, this review addresses the tuning possibilities of the described inducible expression systems.

External induction systems

By responding to a certain input, inducible promoters form the starting point of numerous expression vectors [13]. Their broad use has led to a high interest in finding more and different inducible promoters to fulfill all

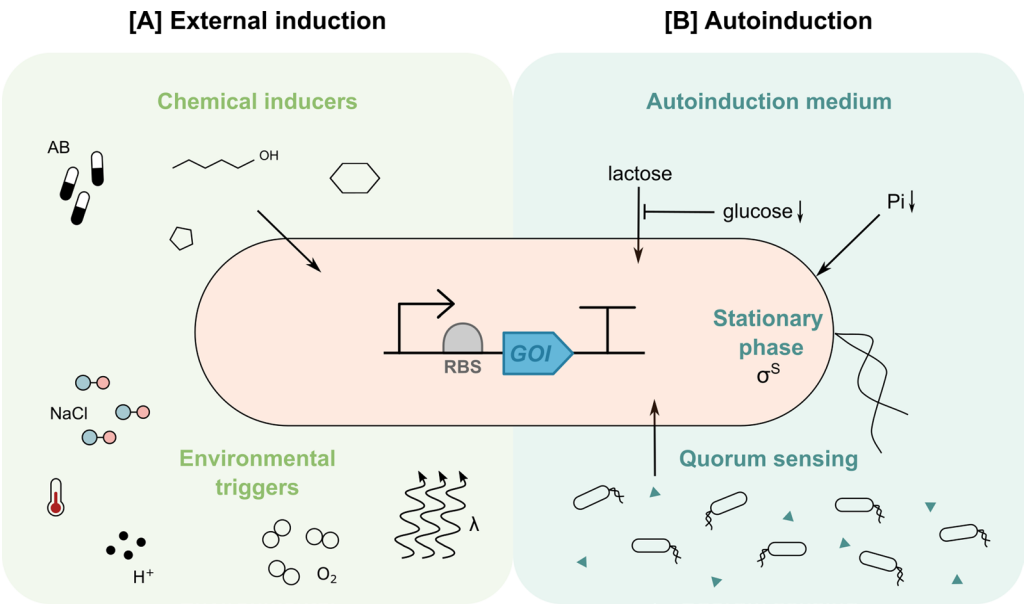

Fig. 2 Overview of the available options to obtain a two-stage fermentation. The systems can be divided into two groups: external induction (**A**), in which a trigger is externally added to the fermentation broth, and autoinduction (**B**), in which the medium or cells themselves cause the phenotypical switch to the production stage. Genetic parts are depicted according to SBOL conventions [11, 12]. *RBS* ribosome binding site, *GOI* gene of interest, *Pi* inorganic phosphate, λ light, *AB* antibiotics, σ^S stationary phase sigma factor

needs. These promoters are used to minimize expression of recombinant proteins or pathways during the cloning process. Additionally, they can be applied to induce specific genes at a defined time point in the cultivation. For these applications, an ideal inducible promoter has strict control over gene expression, a fast response, low leaky expression under non-inducing conditions, fine-tuned expression levels and no limitations or variable response dependent on the use of media, for example its carbon source [14]. The inducers can be divided into two main groups: chemical inducers and environmental triggers (Fig. 2A). Chemical inducers are specific molecules that can be sensed by the host organism. The receptor is often a transcription factor that either activates or represses its cognate promoter. Environmental triggers are fermentation parameters that can be tweaked to lead to a more indirect way of activating certain promoters, usually through two-component systems. The induced promoter can then be used to control the expression of the gene of interest. Additionally, by combining promoters that get activated upon induction with inverters, competing pathways can be repressed when the inducer is present.

Chemical inducers

Chemically inducible promoters are often used for recombinant gene expression or pathway regulation. Throughout the years, many inducible promoters have been found for usage in different host organisms. An overview is given in Table 1. In general, the popularity of these inducible systems can be attributed to their ease of use and broad range of tuning possibilities. Despite the wide range of chemical inducers, in the context of two-stage fermentation processes, three major classes are of interest: sugars, antibiotics and organic acids.

Sugars are cheap inducers, making them appealing for industrial applications. However, in many cases these inducible systems suffer from carbon-catabolite repression (CCR), which can impair its functionality in three ways: inducer exclusion, inducer expulsion and promoter repression (Fig. 3) [15, 16]. CCR is an important regulation mechanism in bacteria to reassure the sequential uptake and metabolism of carbon sources. Since sugar-inducers can be seen as carbon sources by the host organism, the CCR can interfere with the envisioned induction. When a substrate of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS), such as glucose, is present, transport by permeases for non-PTS sugars, such as the inducers, is inhibited, resulting in inducer exclusion [17]. Additionally, inducer expulsion occurs in some gram positive bacteria, but its mechanism remains unclear [15]. Furthermore, many sugar- or alcohol-inducible promoters require CRP (cyclic adenosine monophosphate receptor protein) for the recruitment of RNA-polymerase to initiate transcription [16]. However, this protein acquires its active conformation by binding with cyclic adenosine monophosphate (cAMP), a

Table 1 Overview of often used chemically inducible promoters and their applications

Inducer	Transcription factor	Promoter	Organism of origin	Engineered	Additional mutation(s)	Application field	References
Sugar							
Lactose/IPTG/TMG	LacI	P_{lac}	*E. coli*	/	*ΔlacY*: lactose transport *ΔlacA*: acetylation of TMG/IPTG	Recombinant protein production	[168, 169]
		P_{lacUV5}	*E. coli*	2 bp mutations in -10 box of P_{lac}	/	Expression of T7-RNAP	[170]
		P_{Tac}	*E. coli*	Hybrid $P_{lacUV5} + P_{trp}$	/	Recombinant protein production	[171, 172]
		P_{T7lac}	*E. coli*	P_{T7} + *lac* operator	/	Recombinant protein production	[43, 173]
		P_{trc}	*E. coli*	$P_{trp} + P_{lacUV5}$: spacer is 1bp shorter than P_{Tac} to have optimal spacing	/	Recombinant protein production	[174, 175]
		P_{grac}	*B. subtilis*	Synthetic: lac operator in P_{groE}	/	Recombinant protein production	[176]
Maltose	MalR	P_{malA}	*B. subtilis*	Deletion of first 50 bp of the promoter and of the first 30bp of the spacer	*ΔmalL* and *ΔyvdK*: maltose utilization	Recombinant protein production	[177]
L-arabinose	AraC	P_{araBAD}	*E. coli*	/	*ΔaraBAD*: arabinose metabolism	Recombinant protein production, pathway optimization	[41, 178–180]
L-rhamnose	RhaR, RhaS[1]	P_{rhaBAD}	*E. coli*	/	*ΔrhaT*: L-rhamnose transport frameshift in *rhaB*: L-rhamnose catabolism	Recombinant protein production	[181]
		P_{rhaT}	*E. coli*	/		Recombinant protein production	[182]
D-xylose	XylR	P_{xylA}	*B. megaterium*	/	/	Recombinant protein production	[183, 184]
Organic acid							
Propionate	PrpR[2]	P_{prpB}	*C. glutamicum*	/	/	Recombinant protein production	[185]
Indoleacrylic acid[3]	TrpR	P_{trp}	*E. coli, S. enterica*	/	*ΔtnaA*: tryptophan degradation	Recombinant protein production	[186, 187]
Antibiotic							
(Anhydro)tetracycline	TetR	P_{TetA}	*E. coli*	/	*tetR* uncoupled from Tet-feedback	Recombinant protein production	[188]
Nisin	NisK/NisR[4]	P_{NisA}	*L. lactis*	/	/	Recombinant protein production	[189, 190]
Thiostrepton	TipAL	P_{TipA}	*S. lividans*	/	*tsr* resistance gene	Recombinant protein production	[191, 192]
Bacitracin[4]	LiaR/LiaS[5]	P_{liaI}	*B. subtilis*	/	/	Recombinant protein production	[193]

IPTG isopropyl-β-D-1-thiogalactopyranoside, TMG methyl-1-thio-β-d-galactopyranoside, *E. Coli Escherichia coli*, *B. Subtilis Bacillus subtilis*, *B. Megaterium Bacillus megaterium*, *C. Glutamicum Corynebacterium glutamicum*, *S. Enterica Salmonella enterica*, *L. Lactis Lactococcus lactis*, *S. Lividans Streptomyces lividans*, bp basepair(s), T7-RNAP T7-RNA Polymerase

[1] RhaR is a transcriptional activator regulating the expression of *rhaS*. RhaS is L-rhamnose dependent and activates the inducible P_{rhaBAD}

[2] PrpR is activated by 2-methylcitrate. Propionate, added to the medium, will be converted to 2-methylcitrate by the bacteria, which will then activate PrpR

[3] Tryptophan binds the TrprR regulator that represses this promoter, whereas indole acrylic acid competes with it for binding PrpR, resulting in a non-functional repressor [187]

[4] These are two-component systems instead of transcription factors

[5] The LiaR/LiaS system is triggered by cell envelope stress caused by antibiotic, such as bacitracin

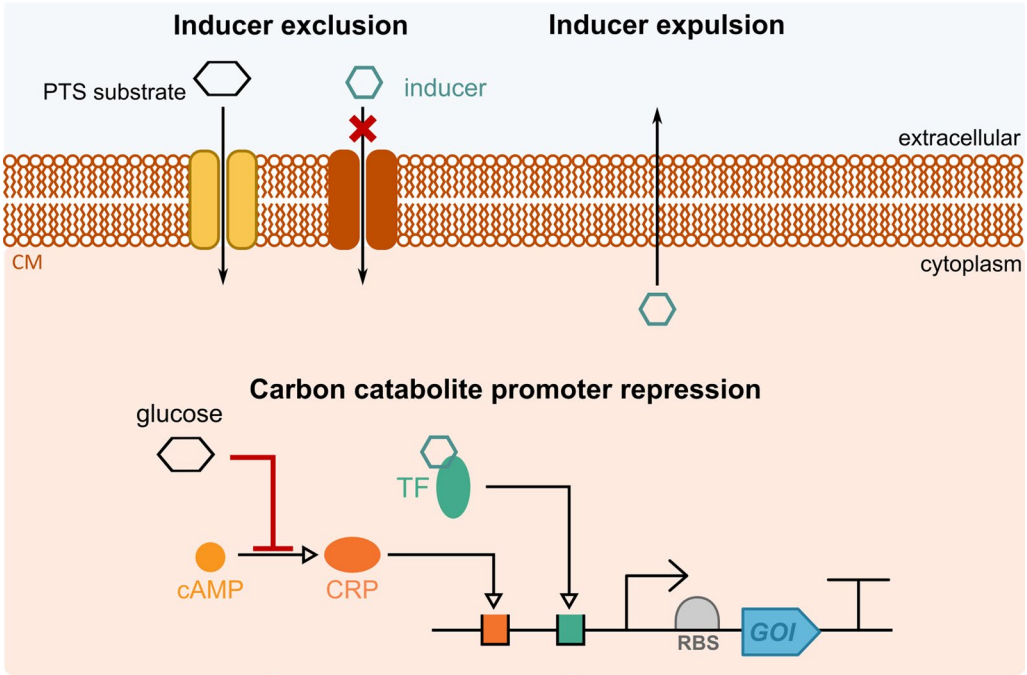

Fig. 3 Overview of the three ways in which carbon catabolite repression can cause undesired repression of the gene of interest (GOI). Inducer exclusion: the presence of a phosphotransferase system (PTS) sugar, such as glucose, inhibits the import of the sugar inducer. Inducer expulsion: occurs via an unknown mechanism. Promoter repression: the cyclic adenosine monophosphate (cAMP) concentration is inversely related with the glucose concentration. cAMP works as an activator of CRP (cAMP Responsive Protein), which in turn activates the inducible promoter together with the transcription factor (TF) responsible for detecting the inducer. In the presence of glucose, CRP is inactive and the inducible promoter cannot be activated (or derepressed) by the ligand-bound transcription factor. The orange and green boxes depict the operator sites in the inducible promoter region of CRP and the transcription factor, respectively. Genetic parts are depicted according to SBOL conventions [11, 12]. *CM* cell membrane, *TF* transcription factor, *RBS* ribosome binding site

global signal molecule in the cell, which concentration is inversely correlated with the glucose concentration. This issue has been tackled in some promoters, such as P_{lac}, by removing the CCR site in the promoter region, thereby eliminating the need for CRP for promoter activation. The use of non-sugar carbon sources can omit the CCR, but is associated with slow growth [18]. Another strategy to eliminate promoter repression by CCR is using CRP* strains. These contain a mutant of CRP (in an adenylate cyclase lacking background) that is less dependent on cAMP for activating gene expression and will hence also activate the promoter in the presence of glucose [19, 20]. For tackling inducer exclusion, two mutant strains were reported. Firstly, disruption of the gene coding for PtsG, a glucose phosphotransferase part of the PTS, leads to higher intake of mixed sugars, thereby reducing inducer exclusion [21]. Secondly, a specific mutation in the *mlc* promoter, indicated by *mlc**, leads to overexpression of Mlc, a transcriptional repressor of the *ptsG* gene [22]. Hence, the result of this mutation is similar to the *ΔptsG* mutation [21].

Besides possible issues with CCR, these sugars can often be metabolized by the host organism. This implies that the induction diminishes over time, leading to reduced and unpredictable product synthesis during the production stage. Therefore, deletions in the host are often required to abolish metabolism of this sugar, allowing more precise control of the promoter (Table 1). Alternatively, synthetic mimics of the inducers can be used. The most well-known example is isopropyl-β-D-1-thiogalactopyranoside (IPTG) [10], which resembles allolactose, a lactose metabolite that triggers LacI to derepress P_{lac}. Unlike (allo)lactose, this synthetic molecule cannot be metabolized by the host [10]. However, it comes at a higher economic cost and can lead to toxicity to the cell [23]. Additionally, the use of chemical inducers could raise concerns about the safety of the end products for the food and pharmaceutical industry.

Despite the possible issues with sugar inducers, they remain the biggest and mostly used group of chemical inducers. Alternatively, organic acids or antibiotic inducers have been applied. However, organics acids have the side effect of influencing the pH of the system, thereby possibly inducing other response pathways as well as the production pathway. Antibiotic inducers, on the other

hand, require resistance of the host organism to the specific antibiotic inducer.

For each specific production pathway activated by chemically inducible promoters, multiple design choices need to be made. First of all, an inducible promoter and ligand combination that fits the envisioned application needs to be chosen. This decision will mainly depend on the production pathway itself and on the growth medium that will be used. Multiple methods have been created to ease the search for new inducible promoters, such as screening of promoter libraries [24, 25], transcriptome analysis [26], a genome-wide approach [27], SELEX [28], protein binding microarrays [29]. However, this research is more relevant in the field of biosensors where researchers aim to find inducible systems for specific ligands to facilitate high-throughput screening or dynamic pathway regulation. Specifically for the application of temporal regulation, the available range of inducible promoters is usually sufficient. Nevertheless, a better inducible system might exist for the specific application. As such, the ligand can also be bifunctional as both inducer and substrate. For example, the substrate hydroxycinnamic acid was used as an inducer to activate the conversion of lignin constituents to vanillin to avoid early expression of unnecessary proteins [30].

After choosing the appropriate inducible promoter, the next important parameter is the timing of induction (Fig. 4). Early induction leads to metabolic burden and early downregulation of competitive metabolic pathways might impair cell growth. Conversely, late induction leads to suboptimal product yield [31, 32]. In general, production is induced in the mid- or late-exponential phase [33, 34]. As cell growth is extremely sensitive to changes in its environment, the optimal timing of induction will differ between batches. For example, it is very well known that temperature influences the growth rate. However, temperature also influences processes on a single cell level. The dynamics of transcription of the inducible $P_{lac/ara-1}$ promoter were investigated with changing temperatures and clear influences could be observed [35]. This implies that changing fermentation parameters, such as the temperature, would require new characterization and optimization of the induction characteristics. Hence, due to batch-to-batch differences, cell growth will have to be monitored for every application to decide the optimal induction.

Besides the optimal promoter and induction timing, the inducer concentration is the next crucial parameter for optimizing an inducible expression system. This concentration depends on the used inducible system and application and, therefore, often requires intensive characterization and optimization. In general, 1 mM IPTG is considered a good starting concentration for inducible systems based on P_{lac} or its variants [36–38]. However, even though this concentration should allow maximal promoter activation, this does not necessarily mean that the highest possible yield is achieved due to the accumulation of non-soluble proteins [39]. Hence, case-by-case optimization is the most reliable way to get optimal expression of the pathway or protein of interest. Together

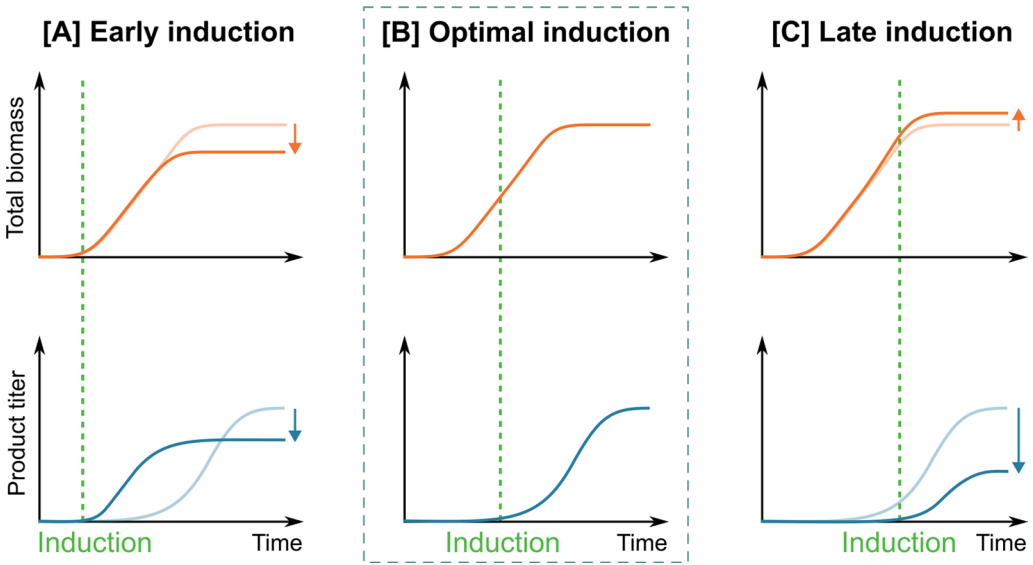

Fig. 4 Schematic representation to indicate the effect of early (**A**) and late (**C**) induction compared to the optimal induction timing (**B**) on total biomass (orange) and production (blue) in a batch process. The dark green dotted line indicates the induction timepoint. The orange and blue arrow depict a change in growth and product titer, respectively, compared to the optimal induction timepoint

with the timing of induction, Mühlmann et al. [40] optimized these parameters in a high-throughput, robotic system [40]. In this research, 36 combinations of induction timing and IPTG concentration were analyzed for four different temperatures, to create an induction profile of P_{lacUV5} using an automated robotic system and a fluorescent reporter protein. Doing this, they found that the optimal inducer concentration is highly dependent on the timing and cultivation temperature, but clear trends could be seen to find the optimal parameters. In general, this optimal inducer concentration was much lower than the often recommended 1 mM IPTG. This finding is also supported by the work of Huber et al. [32] who conducted 'induction profiling' to analyze the effect of induction timing and strength on the expression [32].

These inducible systems have been successfully applied to increase the production of a range of products (Table 1). Even though most applications focus on the overexpression of recombinant proteins for purification purposes, these inducible systems have also been applied to pathway optimization. For example, Kim and Keasling [41] used an arabinose-inducible expression system to control the expression of the DXP synthase gene, part of the mevalonate-independent pathway, for the production of lycopene [41]. By inducing with 13 mM arabinose at an OD of 0.8, corresponding to the exponential growth phase, the researchers reached a four times higher lycopene production than their controls [41].

To further reduce the burden of the introduced pathway, delaying production can be combined with minimizing competition for cellular resources. As such, the inducible promoter, mostly P_{lacUV5}, can be used to control the expression of T7-RNA polymerase (T7-RNAP) [42]. When induced, this RNAP is produced and can start transcription of the recombinant gene controlled by the T7 promoter. This creates minimal leakage and less competition between the host metabolism and the introduced gene for the same RNAP. It also allows the use of weak inducible promoters to still obtain high expression due to the strength of the T7 promoter. Additionally, a *lac*-operator can be added to the T7 promoter to eliminate leaky expression [43]. This lies at the basis of the well-known pET expression vectors, mostly used for recombinant protein expression [36].

The use of T7-RNAP variants, combined with a double inducible system was used to regulate the orthogonal production of both lycopene and deoxychromoviridans [44]. The first system is induced by IPTG and the second promoter is only activated in the presence of both IPTG and anhydrotetracycline (aTc). These promoters are then used to regulate two different orthogonal T7-RNAP variants. Consecutively, the RNAPs will transcribe mRNA of their respective promoters that regulate the operon for lycopene and deoxychromoviridans, respectively. A double inducible system was also designed by Venayak et al. (2018) to create a bistable switch in which the growth and production stage are each triggered by a different inducer. This switch was applied for lactate production in a two-stage anaerobic fermentation, scaled up to a 500 mL bioreactor scale. A knock-out of two enzymes, competing with lactate production, was combined with induced expression of these two-enzymes during the growth stage [45]. Despite the successful implementation in these two research papers, the use of more complex genetic circuitry regulated by multiple inducers to obtain separation of growth and production is limited.

Environmental triggers

As simple single-celled organisms in a complex environment, bacteria have evolved numerous mechanisms to sense and respond to their surroundings. Bacteria are able to respond to a range of stress sources, going from carbon starvation to radiation. The range of pathways that respond to these environmental triggers are a major source of interesting inducible systems to obtain a two-stage fermentation (Fig. 2A). Many of these responses are based on two-component systems that consist of a membrane-bound histidine kinase that senses the environmental trigger. Upon induction, this kinase will autophosphorylate and transfer its phosphoryl group to the response regulator. This activated regulator is then responsible for up- or downregulating pathways involved in the cellular response to this trigger, such as temperature, light, oxygen or pH [46]. It is important to note that there is no induction system that can be applied to every production process, especially in the case of environmental triggers. The choice for such an inducible expression system can be influenced by the characteristics of the product or protein of interest, the expression host and fermentation conditions.

Thermosensitive promoters

The use of thermosensitive promoters is well-established with the $\lambda P_L/P_R$-cI857 thermo-inducible system as the most well-known example. In 1966, thermosensitive variants of the lambda phage repressor cI were found [47]. The most used mutant, cI857, becomes unstable at higher temperatures. Therefore, derepression of P_L/P_R by this variant occurs upon heat shock at 42 °C. Thermo-inducible systems are popular on an industrial scale due to their reduced interference with the medium compared to chemical inducers, resulting in lower contamination risk [48]. However, as is the case for many environmental triggers, not only is the desired pathway activated, but also the metabolism of the host organism is influenced. Heat induction can

be an extra source of stress on cells that might already be experiencing stress due to the activated production pathways [3]. Transcriptomics and metabolomics studies have shown that 163 genes are differently regulated in cells experiencing stress caused by the combination of heat-induction and protein overexpression, than in cells experiencing only one of the two stress sources [49]. For example, genes encoding RNA polymerase are downregulated, resulting in lower expression levels of the introduced genes. Other genes are involved in pyruvate metabolism and glutamine biosynthesis, indicating important changes in the cell metabolism that can interfere with the synthesis of the product or protein of interest [49]. Additionally, the activation of the heat-shock response leads to elevated levels of heat shock proteins, such as proteases [49, 50]. These increased expression levels require resources that will compete with the desired production pathway or recombinant protein expression [51]. Additionally, they can directly influence the production, folding and degradation of the proteins of interest. Nevertheless, the dual stress also results in higher amino acid-tRNA and chaperone gene transcriptome levels [49]. This upregulation can exert a positive effect possibly outbalancing the stress caused by recombinant protein expression. This can be a possible explanation for the often higher productivity observed for the recombinant protein production at higher temperatures [40, 52, 53]. To circumvent activation of the heat-shock response, Caspeta et al. [51] demonstrated that a lower heating rate will give the cells enough time to adapt without having to activate the stress response, leading to higher protein production [51]. Furthermore, these lower heating rates are more feasible in larger scale fermenters, where heat transfer becomes limited. Some examples of successful thermo-inducible production systems can be found in Table 2.

Alternatively to the heat-inducible systems, some promoters exist that get activated upon cold shock. However, their application appears limited. This might be due to the practical complications of cooling down fermenters at larger scales and the cost related to it. Nevertheless,

Table 2 Overview of inducible expression systems triggered by an environmental parameter and their application field

Trigger	Receptor	Promoter	Organism of origin	Application field	References
Temperature					
< 20°C	CspA	P_{cspA}	*E. coli*	Recombinant protein production: psychrophilic enzymes, toxic mesophilic and thermophilic proteins, chitinase	[194, 195]
< 25°C	DesK/DesR	P_{des}	*B. subtilis*	Recombinant protein production	[54]
> 37°C	λ cI857[1]	P_R	Phage lambda	Recombinant protein production	[196]
> 37°C	λ cI857[1]	P_L	Phage lambda	Recombinant protein production	[51]
> 37°C	LacIts[2]	P_{A1}	*E. coli*	Flavor esters	[197, 198]
> 42°C	Heat shock	P_{dnaK}	*E. coli*	Curcumin, p-coumaric acid, caffeic acid	[199]
> 42°C	Heat shock	P_{ibpA}	*E. coli*	Caffeic acid	[199]
Light					
UV	EL222	P_{C120}	*E. litoralis*	Lactic acid, isobutanol	[200]
Green light (520 nm)	CcaS/CcaR	P_{cpcG2}	*Synechocystis* sp. PCC 6803	Isobutanol, 3-methyl-1-butanol	[201]
Blue light	EL222	P_{C120}	*E. litoralis*	Muconic acid	[63]
Oxygen					
Anaerobic conditions[3]	FNR, NarL	P_{nar}	*E. coli*	D-lactate, 2,3-butanediol, 1,3-propanediol	[65]
Increased oxygen	SoxR	P_{soxS}	*E. coli*	Recombinant protein production	[70, 202]
Osmolarity					
NaCl	/[4]	P_{proU}	*E. coli*	Carboxytransferase	[74, 75]
		P_{opuAA}	*L. plantarum*	Recombinant protein: halophilic proteases	[76, 77]
pH					
pH 6	CadR	P_{CadA}	*E. coli*	Beta-galactosidase	[80]

E. coli Escherichia coli, *B. subtilis Bacillus subtilis*, *E. litoralis Erythrobacter litoralis*, *L. plantarum Lactobacillus plantarum*

[1] A temperature sensitive variant of the phage lambda repressor cI was used

[2] A temperature sensitive variant of the LacI repressor has been applied

[3] The system is also sensitive to nitrate. A higher induction is obtained in the absence of nitrate [65]

[4] The mechanism behind the regulation of these osmolarity induced promoters remains unclear. Two mechanisms have been proposed for the regulation of P_{proU} [203]

a colder temperature during the production stage could allow better protein folding of otherwise complex aggregating proteins [54, 55].

Light-inducible promoters

Light-inducible promoters, also known as optogenetic tools, are increasingly popular and researched systems to regulate gene expression. Different mechanisms and types of photoreceptors have been discovered and created and are applied in a broad range of research domains. In a review by Chia et al. [56], a nice overview of the advancements for synthetic biology is given [56]. These advancements are driven by the advantages that light as a trigger offers, such as tunability by adjusting the frequency and intensity, and orthogonality to biological systems [57]. Additionally, this type of trigger does not require interference with the broth culture, thereby reducing the contamination risk [58]. However, issues with light penetration hamper upscaling processes. In general, the bioreactor needs to allow illumination, posing an infrastructural challenge for the most common steel wall bioreactors. Stirred tank photobioreactors for the cultivation of cyanobacteria or microalgae exist, but are limited in size up to 100 L. To allow larger scale cultivation of microalgae, different types of photobioreactors are being used, such as flat panel, tubular or bubble column photobioreactors [59]. Despite their practical value for the cultivation of these phototrophic organisms, a stirred tank reactor remains the most optimal reactor for non-phototrophic bacteria. Alternatively, existing bioreactors can be tuned with internal illumination, but this requires a lot of optimization with additional cost [60]. These practical implications can be minimized by applying a light-repressible system. In this way, light will repress the production stage in the beginning of the fermentation when cell density, and hence the turbidity, is still low. When shifting to the production stage, the cells will be grown in the dark. This system can be implemented by adding inverters to light-inducible systems, as was done in the OptoLAC system that will be discussed later [61].

Many of the existing light-inducible systems in bacteria are making use of LOV domains. These are protein photosensors that detect blue light [62]. For example, the LOV photosensory domain of YtvA from *Bacillus subtilis* was combined with the FixL histidine kinase from *Bradyrhizobium japonicum* to create a blue light-repressible histidine kinase, called YF1. In the absence of blue light, the phosphorylated kinase YF1 will activate the response regulator FixJ which then binds and activates P_{FixK2}. This promoter is then used to regulate expression of the gene of interest in the pDusk expression plasmids [58]. To create a light-inducible system, the previously discussed P_{FixK2} promoter was used to express the lambda phage repressor cI. P_R, repressed by cI, is then used to control the expression of the gene of interest. These expression plasmids are referred to as pDawn [58]. An extra layer of regulation was added by Lalwani et al. (2022), to allow the use of light-regulated plasmids for genes that were originally induced by IPTG. To obtain this, pDawn was adapted to express LacI from P_R. The gene of interest was then regulated by $P_{T5\text{-}lacO}$ resulting in a light-repressible expression system called OptoLAC. This principle was applied and upscaled for the production of mevalonate and isobutanol [61]. Similarly, light-regulated metabolic flux regulation was done by the blue light-sensitive EL222 protein. As such, muconic acid synthesis in *E. coli* was significantly improved by turning off competitive pathways with the dCpf1-mediated CRISPRi system, regulated by EL222 [63].

Oxygen-sensitive promoters

During anaerobic growth, cells are forced to halt respiration due to the lack of electron acceptors. Therefore, the cells will move to anaerobic fermentation pathways to recycle NADH by, e.g., converting pyruvate to fermentation products such as organic acids and alcohols [64]. When these products, or their derivatives, are of interest, it can be beneficial to conduct the growth stage in the absence of oxygen. Additionally, the production pathways can be controlled by a promoter that is active in anaerobic circumstances. This was applied for the production of D-lactate, 1,3-propanediol and 2,3-butanediol using the *nar* promoter [65]. After switching to microaerobic conditions on bioreactor scale, the induction time was about an hour, which is comparable to chemically inducible promoters such as P_{lac} and P_{araBAD}. The obtained yield and productivity of D-lactate were comparable to strains regulating the expression with temperature sensitive promoters [66]. Researchers created synthetic variants of the *nar* promoter resulting in weak, medium and strong expression, allowing individual and optimized expression of each pathway enzyme. By applying these for the expression of 2,3-butanediol, an increase of 72% in titer was obtained compared to the wild-type promoter [67]. Similarly, Wichmann et al. [68] characterized 15 oxygen depletion-induced promoters and applied one for the production of D-lactate in the anaerobic production stage. By controlling the expression of an ATPase by this promoter, ATP wasting was enforced during the production stage, thereby boosting the D-lactate productivity [68].

Besides promoters activated in anaerobic conditions, the promoters of the *soxS* and *sodA* genes are found to be upregulated at increased dissolved oxygen levels, up to 300% air saturation [69]. The potential of P_{soxS} as an

inducible promoter was demonstrated by regulating the expression of a fluorescent reporter protein induced at different dissolved oxygen levels. Additionally, the authors demonstrated no effect on cell growth of this increased oxygen and even proved its functionality in a fed-batch system. Despite the advantages of precise control over the induction timing and easy implementation, this is the only report of such an oxygen-inducible system [70].

Osmoresponsive promoters

Despite the research and interest in osmoregulated induction [71–73], the number of applications remains limited. Nevertheless, the tools and expression vectors for osmoresponsive systems are available. Both Herbst et al. [74] and Bhandari and Gowrishankar [75] have created *proU*-based expression systems for *E. coli*, with the main difference that the expression system by Bhandari and Gowrishankar [75] uses the osmoresponsive *proU* promoter to regulate the expression of the T7 RNAP on the genome [74, 75]. Therefore, the strain can be combined with expression vectors regulating the gene of interest by the T7-promoter [75]. For osmoregulation in *B. subtilis*, a salt-inducible expression vector using the *opuAA* promoter from *Lactobacillus plantarum* BCC9546 was created. Additionally, the expression vector includes a signal peptide of subtilisin E leading to the secretion of the product. The expression vector was applied for the expression of multiple proteases [76, 77]. The limited use of these osmoresponsive promoter might be attributed to the effect of increased osmolarity on bacterial growth. Cells can recover osmotic shock, but will remain to grow more slowly [78, 79]. Furthermore, stress responses are known to be intertwined and change the resource management of the bacterial cell towards recovery [3]. Therefore, the metabolic capacity of the bacteria to produce might be impacted negatively.

pH-controlled promoters

The use of pH as trigger offers quite some advantages, such as the short response time, its low cost and minimal effect on cell physiology and growth by this trigger. Additionally, in the system found by Chou et al. [80], the promoter is strong with a wide range of inducibility, and the expression is relative to the pH [80]. This promoter regulates the *cadA* gene in *E. coli* and by including the expression of the transcription factor CadC, the induction can be improved. Nevertheless, to our knowledge, this is the only application of a pH-inducible promoter. The limited interest in these promoters could be explained by the difficulties with scaling-up the production process. While in bioreactors with pH control it is very straightforward to keep the pH constant up until the moment of induction, this is more complicated on a smaller scale without such automated pH control. In this case the pH will shift throughout the growth [81] and might trigger the inducible promoter at an undesired timepoint. Furthermore, a change in pH is known to influence the cell metabolic activity as it needs to acclimate to this new pH level [82]. Hence, productivity might not be ensured. Additionally, the produced proteins also need to be resistant to the harsh environment without losing activity or stability.

Autoinducible systems

Besides the earlier mentioned disadvantages of external inducers, such as the cost, need for human intervention and diffusion limitations, batch-to-batch variations of biomass might also influence the optimal induction time. This could be caused by small variances in inoculation volume or slight differences in medium composition. Since biomass formation is closely intertwined with the optimal induction time, these variations might lead to the application of suboptimal induction times in practice. Process analytical technologies can aid in automated induction by monitoring, e.g. cell density or pH [83]. However, in this section the focus lies on biological approaches to achieve autoinduction. In an autoinducible system, the variables of the culture itself will determine the induction time, thereby offering a solution for the beforementioned problems. This variable is either the cell density itself or linked to it, e.g., nutrient concentrations. Additionally, the use of autoinducible systems abolishes the need for interference with the fermentation process and diffusion limitations of external inducers. Multiple options exist to obtain this autoinduction (Fig. 2B). Cells naturally possess promoters that are upregulated during the stationary phase as well as promoters that react to nutrient deprivation. Furthermore, cell density can be indirectly measured by the cell with quorum sensing: a natural cell–cell communication mechanism. These options will be discussed in the following sections and an overview is given in Table 3.

Stationary phase promoters

Due to the deprivation of nutrients, bacteria will enter the stationary phase after their exponential growth phase. This transition entails a complete adaptation of the cells to these more limiting conditions, with around 20% of genes being expressed at higher levels in *E. coli* [84]. These genes are usually involved in the survival of the bacteria during this new growth phase. The promoters of these genes are often recognized by σ^S, the stationary phase sigma factor, rather than σ^{70}, the housekeeping sigma factor [85, 86]. The autoinduced property of these promoters, together with the ideal

Table 3 Overview of autoinducible expression systems and their application field

Inducer	Effector	Promoter	Organism of origin	Application field	References
Stationary phase					
Stationary phase	σ^B	P_{ylb}	*B. subtilis*	Pullulanase, hydrolase, dehalogenase	[204, 205]
		P_{ohrB}	*B. subtilis*	Xylanase	[91]
	σ^S	P_{rpoS}	*E. coli*	1,3-propanediol, plyhydroxybutyrate	[206, 207]
		P_{fic}	*E. coli*	Phloroglucinol	[94]
		P_{yliH}	*E. coli*	P(LA-*co*-3HB)	[208]
		P_{dps}	*E. coli*	P(LA-*co*-3HB)	[208]
Autoinduction medium					
Lactose	LacI	P_{tac}	*E. coli*	Recombinant protein production	[96, 209]
Phosphate depletion	PhoR/PhoB	P_{phoA}	*E. coli*	Recombinant protein production	[105, 106]
		P_{yibD}	*E. coli*	Recombinant protein production	[210]
Quorum sensing					
ComX peptide	ComP/ComA	P_{srfA}	*B. subtilis*	Aminopeptidase	[211]
N-butanoyl-L-homoserine lactone	RhlR/RhlI	P_{rhlI}	*P. aeruginosa*	Recombinant protein production	[212]
N-(3-oxohexanoyl)-L homoserine lactone	EsaR/EsaI	P_{esaR}/P_{esaS}	*P. stewartii*	*Myo*-inositol, glucaric acid	[119]
	LuxR/LuxI	P_{luxI}	*V. fisheri*	Bisabolene, salicylic acid, 4-hydroxycoumarin	[117, 213]
Phr60	Phr60/Rap60/Spo0A	P_{spo0}/P_{abrB}	*B.subtilis*	Menaquinone-7	[124]

B. subtilis Bacillus subtilis, E. Coli Escherichia coli, P. Aeruginosa Pseudomonas aeruginosa, P. stewartii Pantoea stewartii. V. Fisherii Vibrio fisherii

timing, render them alluring regulatory elements to induce gene expression in a separate production stage.

Despite their easy implementation, applications are often still limited to cases where a low expression strength of the gene or pathway of interest is desired. Promoters with high specificity for the stationary phase usually possess a weak -35 box or even lack one [87]. Increasing the strength of this box will lead to reduced specificity due to the affinity of the housekeeping sigma factor for this sequence. Notwithstanding these difficulties, a synthetic strong variant of an existing stationary phase promoter was observed by repeated random mutagenesis [88]. Furthermore, a library of stationary phase promoters was created with varying strength and induction time [89]. This provides tuning possibilities for different applications. A similar strategy was used to optimize a stationary phase promoter in *Corynebacterium glutamicum*. From a promoter library, the most promising variant was selected and applied for the large-scale production of the recombinant protein glutathione *S*-transferase as proof-of-concept [90]. Despite the use of stationary phase promoters in different bacteria, their portability is limited due to differences in sigma factors between different species. In *B. subtilis*, the *ohrB* promoter was used to regulate the production of xylanase. This promoter is controlled by σ^B, the stress-related sigma factor of *B. subtilis* that is active during the stationary phase [91].

The reported 80% reduction in protein synthesis during the stationary phase raises concerns about the potential of overexpressing proteins or pathways in this growth phase [92]. Despite this reduction in protein synthesis, Gefen et al. [93] obtained constant production of a specific protein up to 60 h after entry into the stationary phase. This phenomenon is also referred to as the Constant Activity Stationary Phase [93]. Hence, the measured 80% reduction in protein synthesis is not necessarily linked to a resource deficit but might be the result of the lower activity of the housekeeping sigma factor, regulating many growth-related genes. To demonstrate this, researchers checked if the depletion of resources (e.g., amino acids, nucleotides) or competition for machinery (e.g., ribosomes, RNA-polymerases) are limiting factors for protein production. No depletion of resources was observed, and the protein production was competing for only 20% of the total protein machinery, with no measurable influence on the population fitness [93].

Despite the abovementioned concerns, stationary phase promoters were already successfully applied in many cases. The production of phloroglucinol in *E. coli*, for example, was separated from the growth stage by using the stationary phase promoter P_{fic}. Phloroglucinol is a precursor of many antibiotics and due to its toxicity requires this delayed induction. The final titer was 22% higher than when the IPTG-inducible system was used [94]. This *fic*-promoter has also been applied

in different closely related gram negative bacteria [95]. Since these promoters are only active during the stationary phase, their application is generally limited to batch fermentations.

Autoinduction media

The phenomenon of autoinduction in medium was first described by Studier [96] when he observed that P_{lac} was induced by low lactose concentrations once a high cell density was obtained [96]. This inducer is not imported in the cell in the presence of glucose due to inducer exclusion. However, once the medium is depleted of glucose, this mechanism is aborted, lactose imported, converted to allolactose and P_{lac} activated. Therefore, this promoter can be applied in combination with a specific autoinduction medium to obtain an automated delay of expression. After the first description of this autoinduction medium, the range of possible medium compositions has expanded and has been widely applied for the expression of recombinant proteins [97–103].

In a similar manner, an autoinduction medium based on phosphate depletion has been described. In this type of medium, the phosphate concentration is set to be depleted once high cell densities are obtained. In this case, the expression of the genes of interest is regulated by a promoter based on the PHO Pi-starvation system that is induced under inorganic phosphorus (Pi) depletion. The PHO Pi-starvation system is a two-component system, with PhoR as a sensor kinase and PhoB the response regulator [104]. A large set of genes in *E. coli* is known to be activated by PhoB. The best characterized and most used phosphate-activated promoter is P_{phoA}, which has been applied for the expression of many recombinant proteins [105, 106]. To enlarge the set of available promoters and desired characteristics, Torres-Bacete et al. [107] created a library of phosphate depletion-regulated promoters with higher expression strengths [107]. To this end, they introduced a library of PHO-boxes, the DNA-binding sequences recognized by PhoB, into a strong, constitutive promoter. Additionally, this library was analyzed in multiple industrially relevant microorganisms. Since the PHO Pi-starvation system is widely distributed among bacteria, these promoters are highly portable [108].

Quorum sensing-regulated inducible systems

All the strategies described previously are based on static regulation systems. In these static systems, the expression level and timing of the protein expression are constant and predetermined [109]. However, small fluctuations in the fermentation process can result in suboptimal induction conditions. Therefore, dynamic systems are often considered for pathway regulation. These can involve inducible systems that respond to intermediates of the production pathway and then activate the next enzymes in the production pathway [110]. However, these systems are context dependent and not applicable for all production pathways, because of the lack of biosensors for all molecules. Additionally, these regulation systems, although proven useful in many cases, do not usually decouple growth and production. Another dynamic regulation system that is context-independent and aims to decouple growth and production is quorum sensing (QS), a bacterial cell–cell communication mechanism. It regulates gene expression in response to cell density by sensing an autoinducer (AI) molecule.

Three main types of quorum sensing in the prokaryotic world can be described. The first type is based on the small, diffusible acyl-homoserine lactone (AHL) molecules as autoinducers [111, 112]. These molecules are produced by some gram negative bacteria of which *Vibrio fisheri* is the most well-known example, as the phenomenon of quorum sensing was first described in this species [113]. The AHL-molecules are recognized by LuxR-type transcription factors that consequently activate a range of promoters regulating cellular processes, going from luminescence to biofilm formation and virulence factors (Fig. 5). In the second quorum sensing type, the autoinducer is a peptide, secreted by specific ATP-binding cassette transporters [114]. The recognition goes through a signal cascade with a two-component system. This form of autoinducer is produced by some gram positive bacteria. Third, autoinducers with an unknown structure called autoinducer 2 (AI-2) are also recognized by a two-component system or by an ABC transporter [112, 115]. Homologues of its synthase, LuxS, can be found in both gram positive and negative bacteria [116].

Mainly the AHL-based quorum sensing systems already have numerous applications due to their easy implementation. The system solely relies on a synthase, responsible for the autoinducer production, a specific transcription factor and the promoter containing the corresponding transcription factor binding site (Fig. 5). This promoter can then be used to express the gene(s) of interest. With this principle, a 44% improvement in bisabolene titers was obtained by using the LuxI/LuxR controlled dynamic system compared to the IPTG-inducible P_{Trc} promoter [117].

Most of the time the quorum sensing promoter is activated by the ligand-bound transcription factor. However, in the case of the EsaI/EsaR quorum sensing system of *Erwinia stewartii*, the transcription factor EsaR binds the promoter region in the absence of the autoinducer produced by EsaI. Additionally, this promoter is bidirectional; in the absence of the autoinducer P_{esaS} is activated, while P_{esaR} is repressed [118]. This provides the option to

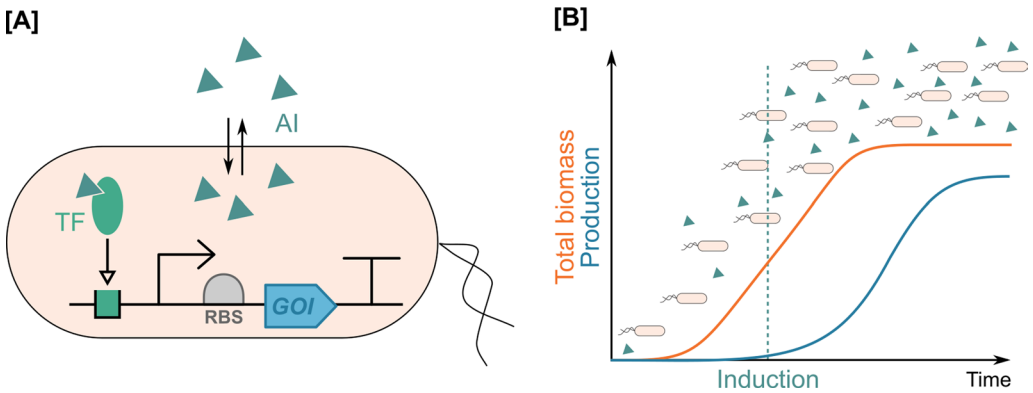

Fig. 5 The general principle of quorum sensing. **A** Depicts quorum sensing on an intracellular level. The bacteria produce an autoinducer (AI) that either diffuses or gets transported to the extracellular environment. When the AI concentration is high enough, a transcription factor (TF) will bind it and the complex activates the promoter regulating the gene of interest (GOI). **B** Overview of the quorum sensing principle related to growth and production in a batch process. The AI concentration (green triangle) will increase as the cell density increases. Once a threshold concentration is reached, the production stage will be activated. Genetic parts are depicted according to SBOL conventions [11, 12]. *RBS* ribosome binding site

create a bifunctional switch that can simultaneously up- and downregulate genes. Downregulating genes can be applied to redirect the flux towards the product of interest. As such, the EsaI/EsaR QS system was used to channel the flux from the endogenous pathways to the desired products by downregulating the glycolytic flux [119]. Upscaling to 3 L-scale benchtop bioreactors resulted in ten- and fivefold increases in titers of *myo*-inositol and glucaric acid, respectively, compared to strains without any downregulation of the glycolytic flux. Additionally, the regulation system was also applied for the production of shikimate [119]. Similarly, a bifunctional switch was created with the same quorum sensing system and applied to the production of 5-aminolevulinic acid (ALA) and poly-beta-hydroxybutyrate (PHB). The bifunctional switch simultaneously knocks down competing pathways and activates the production pathway [120]. In general, most AHL-based quorum sensing systems do not have this bidirectional promoter to allow for downregulation of competing pathways. Therefore, quorum sensing circuits can be combined with inverters to obtain the desired output. For example, for the optimization of naringenin and salicylic acid production, the P_{lux} promoter drives the expression of *dCas9* and a single guide RNA. This guide RNA then targets competing acetyl-CoA-consuming and fatty acid synthesis reactions [121]. Similarly, the upregulated promoter can be used for the expression of a sRNA, targeted at the competing pathway [122].

In the popular gram positive host organism *B. subtilis*, the PhrQ-RapQ-DegU-based QS system was used for the automatic regulation of poly-γ-glutamic acid production [123]. With a similar quorum sensing system, a bifunctional switch was optimized for the production of the nutraceutical menaquinone-7 (MK-7). Synthesis of the toxic intermediates was delayed by expressing them from an upregulated quorum sensing promoter. Simultaneously, *pyk* and *uppS* were inhibited because of their adverse effect on the cell growth [124].

The easy implementation of quorum sensing systems allows straightforward integration in more complex genetic circuitry. A 28.3% increase in serine productivity was obtained by combining quorum sensing and the well-known bistable toggle switch to repress competing pathways [125]. In a recent research paper, the temporal control that QS offers was combined with spatial control by controlling the export of L-cysteine, the end product, by an L-cysteine-responsive promoter [126]. Similarly, quorum sensing regulation was combined with a biosensor for D-glucaric acid production. The biosensor, the transcriptional regulator IpsA, detects the intermediate *myo*-inositol and regulates the expression of a downstream enzyme. Additionally, the quorum sensing system downregulates the glycolytic flux [127].

Tuning

An inducible system present in nature, whether it is an external or autoinduction system, is not necessarily optimal for all production purposes. Furthermore, each application has its own optimal parameters, varying in induction time, strength and possible leaky expression. Therefore, the existing natural inducible systems cannot always be used as such, but might require some tuning (Table 4). Increasing the expression level of the output of interest can easily be obtained by combining the inducible promoter with a stronger ribosome binding site [128, 129]. However, since this might also influence the leaky expression, other strategies to directly tune the promoter strength and output characteristics might be

Table 4 Overview of the possible tuning strategies for the different induction systems

Tuning strategy	Chemically inducible	Environmental triggers	Stationary phase promoters	Autoinduction medium	Quorum sensing
GOI expression level					
Promoter engineering	X	X	X	X[1]	X
Transcription factor tuning	X	X			X
Synthase level					X
Inducer concentration	X				
Intensity environmental trigger		X			
Other		X			
Induction timing					
Addition inducer	X				
Environmental trigger activation		X			
Synthase level					X
Transcription factor level					X
Medium composition				X	

This table is based on the references provided in the section about the tuning of induction systems

GOI Gene of interest

[1] It should be theoretically possible to perform promoter engineering as a tuning strategy for autoinduction broth, but no actual application has been reported to our knowledge

of interest. These strategies could reduce the leakiness of the promoter in the uninduced state, leading to lower unwanted expression during the growth stage. Additionally, the expression level of the gene(s) of interest could be adapted. Lastly, increasing the sensitivity of the system might lower the amount of inducer required to activate the production stage and, therefore, lead to a cost reduction. Most of the discussed tuning strategies focus on the molecular level, by changing the dynamics of the system. Nevertheless, additional tuning can be done on a bioreactor level by optimizing fermentation parameters, such as medium composition, feeding rate in fed-batch and dilution rate in continuous processes.

Promoter engineering

The strength and characteristics of an inducible promoter are determined by the combination of multiple factors: (1) the affinity of the transcription factor (TF) for its binding site, also called the operator; (2) the number of operator sites; (3) the spacing between the operator sites; (4) the position of the operator sites and (5) the strength of the core promoter [130].

A different affinity of a TF for its binding site would influence the equilibrium between bound and unbound TF. Consequently, the promoter activity can be varied, since the occupancy of the promoter by RNA-polymerase is dependent on the binding of the TF to the promoter region. By placing a library of 10^5 different operator sites in the spacer region of a constitutive promoter, a distribution of dynamic ranges, being the ratio of the maximum and minimum output, ligand sensitivities and response cooperativity was obtained by Liu et al. [25]. This was done for three different repressors from the TetR-family. Remarkably, TF binding relied on a core sequence motif within the operator site. Furthermore, the relative position of this core within the operator and the bases surrounding it impact the actual response. Hence, these can be used as tuning parameters if this core sequence is known [25]. By creating a full random library of the operator, information can also be obtained about the binding energy of the TF to the operator. This allows to fully characterize the operator sequence and identify interesting mutations to obtain the desired dynamic range [131, 132]. Nevertheless, it was observed by Mannan et al. [133] that the increased dynamic range associated with mutated LacI operator sites simultaneously led to a decrease in sensitivity of the system for IPTG. Therefore, higher inducer concentrations were required to activate the inducible system [133].

Alternatively, the affinity of a TF for the promoter can be increased by adding extra operator sequences to the promoter region. An additional *lac* or *tet* operator leads to a tenfold increase in dynamic range [134]. One of the reasons is the formation of loops in the DNA sequence, thereby blocking access of the RNA-polymerase for the promoter region [135, 136]. However, adding a second EsaR operator site in P_{esaR} did not lead to an increase in the dynamic range [137]. Hence, the mechanism of action of the TF, combined with the promoter architecture will still have a key role and, therefore, the outcome of certain tuning methods seems unpredictable. It is of importance to note that the distance between the operator sites

influences the effect of additional operators as well [138]. This could be explained by the importance of the DNA structure and the location of an operator sequence on the helical DNA. Furthermore, the position of an operator can even influence the functionality of the TF [137, 139].

Finally, the strength of the core promoter can be influenced by mutating the -10 and -35 boxes. Accordingly, a collection of dynamic ranges was obtained for an AraC- and LasR-regulated promoter [140]. Similarly, as discussed earlier, these strategies can also be applied for creating variants of existing stationary phase promoters [88–90].

The research of Yu et al. [138] tested the tuning strategies discussed above both separately and combined in the *lacZYA* promoter. Furthermore, a statistical mechanics model was created to predict the effect of operator sequences and core promoter strength on the promoter response [138]. Both the experimental results and the computational model demonstrate that the highest dynamic range is obtained by using (near) consensus -10 and -35 boxes, combined with a strong operator site.

Lastly, by changing the origin of replication of the plasmid on which the promoter is located and thereby its copy number, a change in dynamic range is possible [137].

Transcription factor tuning

On the level of the transcription factor (TF), there are two straightforward tuning methods: changing its expression level and mutating the TF itself.

By varying the expression level of the TF, its intracellular concentration will vary accordingly. It is known that decreasing the concentration of a repressor or increasing the concentration of an activator leads to increased sensitivity and dynamic range [141, 142]. The thermodynamic model developed by Bintu et al. [143] supports these observations by predicting an increase in dynamic range with increasing activator concentration. The opposite is true for repressors. This highlights the possibility of easy tuning by varying the TF expression level [143]. For quorum sensing-regulated transcription factors, such as LuxR, increased expression will result in earlier activation of the production stage [121, 141]. Besides varying the expression level, it could be considered to include negative autoregulation. In this case the TF represses its own production which has been shown to shorten response time, decrease noise and linearize the response curve [144].

Specific mutations in a TF can be sought to increase its sensitivity. A 100 times reduction in IPTG concentration was required to activate mutant LacIs. Furthermore, higher levels of induced gene expression could be observed by these mutants [142]. Similarly, by using directed evolution, an EsaR variant with more than 70-fold increase in signal sensitivity was found [145]. The variants of this QS-regulated transcription factor influenced the final gene expression and induction timing when combined with their synthase [137].

Inducer concentration and timing

Each inducible promoter has a specific response curve, meaning that each input inducer concentration corresponds to a certain promoter strength, within the operational range of the promoter. Therefore, varying the inducer concentration offers an easy and straightforward way of influencing the promoter strength. Depending on the gene(s) of interest, the highest promoter strength does not necessarily correspond to the highest yield. A trade-off exists between the high expression of the gene(s) of interest and the burden caused by its gene product. Furthermore, the timing of induction can be optimized for each application.

In the case of QS-regulated inducible systems, similar tuning can be obtained by differing the expression level of the synthase. The threshold QS-inducer concentration will be reached earlier with increasing synthase levels. This was successfully applied with changing the expression levels of the synthases EsaI and LuxI [119–121]. Furthermore, it was observed that the final activation of the promoter was increased accordingly [119, 120].

Other tuning strategies

Some tuning strategies are only applicable to a certain type of inducible systems. The induction timing in an autoinduction medium, for example, can be influenced by varying its composition [146]. Similarly, the response of pH-inducible promoters is related to the pH in the medium [147]. In light-inducible systems, the light intensity and illumination duration offer different tuning strategies that were discussed earlier [58]. Finally, in two-component systems, the dynamic range can be altered by changing the phosphatase activity of the sensor kinase. A range of residues have already been identified that can influence this activity [148].

Discussion

The expression of recombinant proteins or heterologous pathways might severely disturb the well balanced metabolism of the bacterial host. To minimize these effects, a two-stage fermentation process is suggested in literature in which a growth stage is followed by the activation of the production pathway. Different strategies are available to create this temporal separation of growth and production during a fermentation process. External triggers, such as chemical inducers or environmental conditions, can be manually introduced to activate the transition to

the production stage. There are numerous possibilities and successful applications known with these types of inducers. However, on an industrial scale there are still some limitations regarding the necessary optimization, lack of automation and limitations with upscaling. In the recent years, a switch towards autoinducible expression systems occurred. These systems do not rely on external triggers and their induction timing depends on the fermentation process itself. Quorum sensing systems, as autoinducible expression systems, offer the great advantage that they allow more tuning possibilities. Therefore, these systems have drawn a lot of attention.

Besides the numerous advantages that two-stage fermentation processes offer, there are some hurdles to be aware of. By delaying the activation of production, this stage mainly overlaps with the stationary phase in batch processes. This growth phase is known to have a major impact on the cellular metabolism and growth, since regulation is now shifted from the housekeeping to the stationary phase sigma factor. Hence, different pathways will be up- or downregulated. In general, this can lead to a reduced growth rate and substrate uptake rate, thereby limiting the carbon flux that can go into the product synthesis pathways [149–151]. Therefore, a complete halt of the growth in the production stage negatively impacts the flux towards product synthesis. In the optimal case, an intermediate growth rate is retained during the production stage. This was predicted by the computational tool developed by Raj et al. [9]. This tool can assess whether or not a two-stage fermentation process will outperform a one-stage fermentation process. Additionally, it provides information about the optimal fermentation parameters. This tool predicted that two-stage fermentations can outperform a growth-coupled production for multiple relevant objectives, such as increased productivity and yield if intermediate growth is retained [9]. To further boost the substrate uptake during the stationary phase, a high demand for energy can be created by enforced ATP-wasting [152]. This was shown to lead to an increased productivity, yield and titer of general fermentation products, such as ethanol, formate, acetate, lactate and succinate [152].

The second main concern regarding the use of genetic control elements and, by extension, genetic circuits is the robustness. As was shown by Moser et al. [153], the output of an AND-gate inducible system was not preserved upon upscaling [153]. However, this is mainly the case in more complex genetic circuits. Furthermore, it was shown that a two-stage fermentation can even improve robustness and scalability due to the lower selection pressure and burden during the growth stage [154, 155].

The induction strategies and references given in this review are mainly based on batch fermentation. However, two-stage fermentation can also be introduced in fed-batch or continuous fermentation. Nevertheless, not all strategies can be applied for each type of fermentation. Promoters, induced by chemical inducers, are often used in fed-batch fermentations [156, 157]. The inducer can be added as a pulse or continuously in the post-induction feeding [158]. Even in continuous fermentations, inducible promoters have been applied in combination with spatial separation of growth and production. A such, a cascade of bioreactors is created, where the first reactor allows growth and the second reactor supplies the inducer for the production stage [159–162]. Furthermore, some of the autoinduction methods have also been demonstrated in fed-batch fermentation. Both autoinduction medium and quorum sensing regulated induction have successfully been used in these reactors [163–165]. For strategies using an autoinduction medium, different feeding strategies can be used as an additional tuning method [163]. However, due to the fundamental difference in bacterial growth between batch and continuous fermentation systems, autoinduction is more complex this set-up. Nevertheless, the work by Dubern et al. (2023) indicates that the quorum sensing system of *Pseudomonas aeruginosa* is functional in a continuous fermentation process [166]. This highlights that there is still undiscovered potential for applying these systems for a two-stage continuous fermentation.

Conclusion

There are numerous possibilities at the moment to obtain a two-stage fermentation process, making it a very difficult choice to pick the optimal one for a specific application. Furthermore, the consequent tuning can be tedious and unpredictable. Nevertheless, the increased interest in modeling, machine learning and artificial intelligence causes promising trends in this field. We believe that these computational tools will highly contribute to speed up the whole optimization process: from choosing the optimal induction system [9] to optimal induction timing [167] and level. These promising models combined with the current state-of-the-art of inducible expression systems create a positive view on the future of two-stage fermentation processes. However, the application of inducible expression systems and the developed models could benefit from an increase in standardized characterization and documentation. General overviews of these inducible promoters with their dynamic and operational range together with their limitations are not available even though a lot of the data is present, spread over multiple research labs and papers. Additionally, standardized use of these inducible promoters is hampered by the influence of many parameters, such as plasmid copy number, genomic background, medium, etc., on the

inducible promoters. Hence, the system needs to be characterized and optimized for each individual application.

Even though two stage fermentation processes already have a long list of applications, the possibility of applying quorum sensing systems could even further increase the success. Additionally, the increased interest in computational modeling and standardization of parts will have a major contribution to the ease of use of these (auto) inducible expression systems.

Abbreviations

AHL	Acyl-homoserine lactone
AI	Autoinducer
CCR	Carbon Catabolite Repression
PTS	Phosphoenolpyruvate-dependent carbohydrate phosphotransferase system
RNAP	RNA-polymerase
TF	Transcription factor
QS	Quorum sensing

Acknowledgements
We would like to thank the UGent Core Facility 'HTS for Synthetic Biology'.

Author contributions
All authors were involved in the conceptualization of the manuscript. J. D.B. wrote the manuscript that was critically reviewed by all authors.

Funding
This work was supported by the Research Foundation—Flanders (FWO) [grant numbers 1S29521N, 1246323N] and the Interuniversity BOF Project (iBOF) [grant number BOF20/IBF/131]. We would also like to thank the UGent Core Facility 'HTS for Synthetic Biology'.

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 20 June 2024 Accepted: 4 September 2024
Published online: 13 September 2024

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