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Engineering of bacteria towards programmed autolysis: why, how, and when?

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Abstract
Programmed autolytic bacteria, also termed controlled self-disruptive or self-destructive bacteria, are bacterial systems that express certain lytic genes and undergo cell lysis at a predetermined time point to release the intracellular contents or to commit suicide. Such systems have wide applications in high-throughput screening of protein libraries, synthesis and recovery of bio-products, population control of heterogeneous cultures or synthetic co-cultures, drug delivery, and food fermentation. Recently, great achievements have been reported regarding on-demand control of cell autolysis for different purposes, highlighting the potential of autolytic strains in biomanufacturing and biomedicine. In this review article, we first introduce the various applications of such bacteria, followed by a summarization of the approaches used in the establishment of autolytic bacterial systems, including cell autolysis mediated by cell wall hydrolases with or without facilitating proteins and by membrane-disturbing proteins. Next, we describe in detail the methodologies adopted to control and initiate cell lysis, including induction by chemical inducers, stimulation by physical signals, auto-induction by metabolic status or nutrient limitation, and constitutive expression of the lytic genes. This article is ended with discussions on the remaining problems and possible future directions. This review provides comprehensive information on autolytic bacteria and insightful guidance to the development of highly efficient, robust, and smart autolytic bacterial platforms.

Keywords Autolysis, Endolysin, Release, Control, Bio-product

Background

Bacteria are commonly used in industries and laboratories for the synthesis of bio-polymers such as proteins, enzymes, and bioplastics. Purification of the intracellularly synthesized bio-polymers relies on efficient cell disruption using mechanical, chemical, and/or enzymatic methods, which are associated with problems such as partial denaturation of the protein/enzyme products, high processing costs, etc [1, 2]. Alternatively, programmed autolytic bacteria, also termed self-disruptive or self-destructive bacteria, can be utilized as production hosts, which undergo mild burst and lysis at a predetermined time point in response to endogenous or exogenous stimuli and allow the intracellular materials to migrate to the culture media (Fig. 1) [1, 3]. Cell lysates

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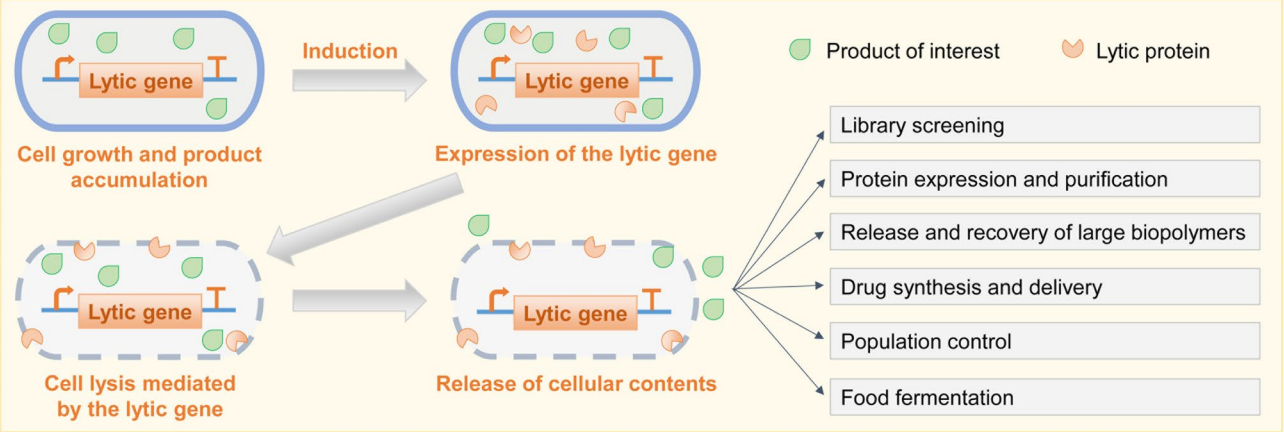

Fig. 1 Schematic representation of programmed autolytic bacterial systems and their applications. Programmed bacterial autolysis lies in the controlled expression of one or more lytic genes sometimes with facilitating proteins, aiming to disintegrate the cell wall and/or membrane structures. At a certain stage of bacterial growth, the lytic gene is induced in response to an external or internal stimulus or signal. This leads to lytic gene expression and cell disruption, thereby releasing intracellular contents for various purposes

obtained in such a way can be directly used for purification steps or for screening purposes, thereby eliminating the traditional cell disruption procedure and the corresponding equipment and cost.

Autolytic bacterial systems were first reported in 1990 [4] and have ever since been employed for the recovery of intracellularly synthesized polyhydroxyalkanoates (PHAs) aiming to reduce the downstream processing cost. PHAs are important degradable bioplastics naturally produced by certain bacteria as intracellular storage granules of carbon source and energy [5]. These granules occupy a large percentage of cellular space and cannot be easily secreted into the culture medium owing to their large size (0.2–0.5 μm) [5]. Traditionally, purification of these molecules involves incubation of the producing cells with enzymes followed by mechanical treatment and/or extraction by organic solvents [5]. The adoption of the autolytic strategy allows for facile recovery of PHA upon triggered cell lysis, which represents a great stride toward green manufacturing.

Autolytic bacteria have later been used in the expression of recombinant proteins and enzymes since 1998 [6]. This on the one hand simplifies protein purification without a traditional disruption method not only for large-scale processing but also for miniaturized culture sizes used in certain assays, and on the other hand greatly facilitates high-throughput screening of protein/enzyme mutant libraries for altered functions/activities or stability, bypassing the tedious procedure of in situ lysis for thousands of cells either in 96-well plates or on petri dishes [3, 7]. Recently, autolytic bacterial platforms have been extended to the microbial production of small-molecule chemicals, showing the versatility of such systems for the generation and release of bio-products regardless of their molecular weights and sizes [8].

In biomanufacturing, the metabolic status of the producing host strain is critically impactful on product yield and productivity. Nonetheless, dormant, metabolically inactive bacterial cells exist in all growth stages at distinct ratios owing to stochastic gene expression or uneven nutrient distribution, which take up resources yet contribute little to the bio-production. Elimination of such dormant cells constitutes one of the major contents of bacterial population control, in which autolysis can be a powerful tool via identification and killing of the non-dividing cells to free up growth space and to release intracellular metabolites as nutrients for the dividing cells [9]. In the past two decades, synthetic co-cultures are frequently employed for the biosynthesis of compounds, particularly plant natural products, with very complicated structures or extremely long synthetic pathways. The pathways are segregated into several modules and allocated into different microbial strains via a process called "division of labor" [10]. Despite its great potential in promoting the overall productivity, it is challenging to achieve a controlled ratio among various strains in the co-culture. Autolysis proves effective for such a scenario, in which a proper ratio among different strains in a consortium can be maintained via controlled suicide of the bacteria with a specific metabolic status [11].

Autolytic bacteria have also been explored, with albeit much less attention, in food processing and drug delivery. Programmed lysis of probiotic bacteria leads to release of intracellular enzymes that help with the development of food flavor and is beneficial for accelerated ripening of the fermented food [12, 13]. In drug delivery using bacterial therapy, the self-disruptive bacterial cells deliver the drug payload to a particular site of the patient upon lysis. These cells serve as both the drug-synthesizing factory

and the delivery vehicle, and are self-restrained in their population size [14].

The controlled autolytic system is composed of several key factors, i.e., (i) a lytic gene, whose protein product targets an essential structure or component of the bacteria, increases cell permeability, and reduces the cell viability; (ii) a promoter that controls the expression of the lytic gene in either a constitutive or an inducible manner; (iii) a stimulus in the case of inducible induction, either exogenous or intrinsic, that binds to the promoter and initiates the expression of the lytic gene, and (iv) a facilitator that is sometimes required either to initiate the autolysis or to accelerate the cell lytic process. This review article focuses on these factors and summarizes how the cells are lysed and how the lysis event is initiated. Briefly, this article first recapitulates the lytic systems that have been reported, including cell wall hydrolases, either with or without facilitating proteins, and membrane-disturbing proteins/enzymes. Then control over the expression of the lytic systems is reviewed, which includes inducible expression via chemical inducers or physical signals, auto-induction based on metabolic status or nutrient supply, and constitutive expression. At the end, the remaining problems are discussed with a brief guide to further directions to the development of highly efficient and stable autolytic bacterial strains.

Autolysis mediated by cell wall hydrolases

The bacterial cell wall is a critical structural component in the maintenance of cell integrity and tolerance to osmotic pressure. Bacteria lacking their cell wall are unstable under normal conditions and lyse rapidly. By targeting the cell wall using various cell wall hydrolases that are designed to express in the host cell, auto-disruption of the bacteria can be accomplished to liberate the cellular contents. However, most of the intracellularly produced cell wall hydrolases cannot automatically penetrate the cell membrane barrier, unless in the presence of some facilitating protein(s) or when the hydrolases are engineered in a certain way.

With facilitating protein(s)

Many bacteriophages utilize an intricate bio-device to release progeny after infecting their bacterial hosts, which mainly comprises a holin, an endolysin, and a spanin complex [15]. The holin is a pore-forming protein that accumulates in and destabilizes the cell membrane by dissipating the proton motive force and forming micron-scale holes [15], whereas the spanin complex contains proteins that destabilize the outer membrane in the case of a Gram-negative host bacterium [15]. The endolysin in the phage lytic device is a cell wall hydrolase with glycosylase, amidase, or peptidase activity. Among these, the glycosylase activity cleaves the glycan chain of the cell wall peptidoglycan and can be either a lytic transglycosylase or a muramidase depending on the glycosidic bond it acts on. In comparison, the amidase activity trims the peptide side chain from the glycan chain, whereas the peptidase activity digests peptidoglycan within the peptide side chain [16, 17].

At the end of the phage infection cycle, the holin turns on the lysis clock, triggers the deformation of the cell membrane, and presents the endolysin to its cell wall substrate for peptidoglycan degradation. Afterwards, the spanin complex is exposed to and disturbs the outer membrane, releasing the newly synthesized phage particles into the environment [15]. The spanin complex is dispensable when the bacterial cell culture is subjected to shear force, i.e., incubated in a rotary shaker in shake flasks [15]. For commonly used bacteria of industrial relevance, cells are cultivated in shake flasks or in fermenters with agitation, therefore it is unnecessary to express the spanin gene(s).

Based on the phage lytic cassette, it is possible to construct an autolytic bacterial strain using the holin-endolysin genes either cloned onto a plasmid or incorporated into the genome (Fig. 2A). Here, the endolysin is the major cell-disrupting agent while the holin acts as a facilitating protein. One of the most frequently adopted holin-endolysin combinations is the SR cassette from phage lambda, with S and R being the holin and the endolysin with transglycosylase activity, respectively. Such a cassette has been transferred to *Escherichia coli*, *Halomonas campaniens*, and *Synechocystis* sp. for the release of recombinant proteins and bio-polymers (Table 1) [18–24]. The *Rz* gene encoding the spanin is often adopted due to its presence in the same operon as *SR*. Other lysis cassettes have also been tested, such as the holin-endolysin pairs from *Bacillus amyloliquefaciens* phage [25] or *E. coli* phage T4 [26], EJh-EJl (holin and endolysin, respectively) from pneumococcal phage [27], and GP 13 15 19 (holin, auxiliary protein, endolysin, respectively) of *Salmonella* phage P22 [24] (Table 1). In certain cases, the autolytic strain does not carry a functional holin gene; as a result, expression of the endolysin, such as the R endolysin of phage lambda and T7 lysozyme of T7 phage, allows its accumulation without causing cell disruption, and cell lysis is induced when cells are resuspended in solutions containing membrane-destabilizing agents such as Tris, EDTA, and Triton X-100 [28–31].

In the holin-endolysin based autolytic strains, cell lysis can be enhanced by co-adopting lysis cassettes from various phages, as demonstrated in *Synechocystis* sp. [24]. The cell death rate induced by 20 μM $NiSO_4$ was 48.7% and 11.5% when using *Salmonella* phage P22 GP13 15 19 and lambda phage SRRz, respectively, which was elevated to 60.3% when both cassettes were expressed. Since GP19 of phage P22 is a lysozyme (muramidase) [32] and

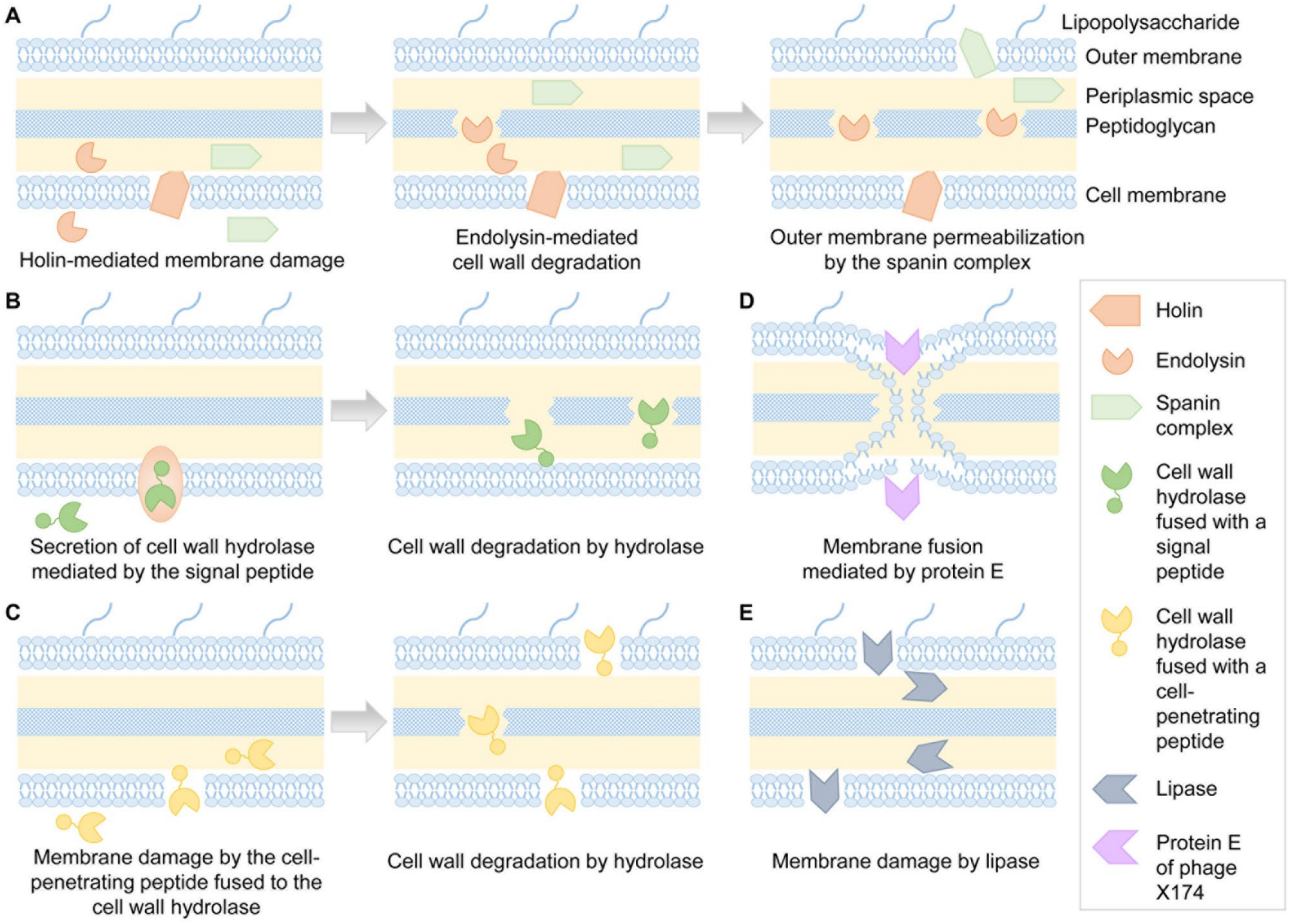

Fig. 2 Schematic representation of bacterial autolysis via different mechanisms. (**A**) Lysis of bacterial cells by the holin-endolysin-spanin cassette. (**B**) Cell lysis by a cell wall hydrolase fused with a signal peptide for secretory expression. (**C**) Cell wall hydrolase fused with a cell-penetrating peptide for simultaneous membrane destruction and cell wall degradation. (**D**) Membrane deformation and fusion caused by protein E of phage X174. (**E**) Membrane digestion by lipases

lambda R is a lytic transglycosylase [15], co-induction of these enzymes leads to simultaneous peptidoglycan digestion at two different sites of the glycan chain and hence higher lysis efficiency. However, cells carrying these lytic cassettes had long doubling time before induction, probably due to the leaky expression and partial growth inhibition.

It is interesting to note that the facilitator of the endolysin is not necessarily a holin. For instance, T7 lysozyme is an amidase encoded by T7 phage [33] and is unable to lyse *E. coli* cells from the inside on its own in the autolytic strain, whereas efficient cell lysis can be achieved when D-amino acid oxidase is co-expressed [34]. This enzyme consumes cellular pool of D-amino acids and sabotages cell wall crosslinking [35]. Although expression of D-amino acid oxidase in isolation resulted in significant release of the reporter protein GFP (green fluorescent protein), co-expression of T7 lysozyme increased product discharge by 40% [34].

Without facilitating proteins

The holin-endolysin autolytic system expresses both the holin and the endolysin, and sometimes other auxiliary proteins, thereby imposing metabolic burden to the host cell and leading to prolonged cell lysis and product release. To reduce such burden and to save cellular resources, researchers have devised self-disruptive systems using a single lytic protein that is either natural or engineered.

Some cell wall hydrolases are strong enough to disturb the cellular structure without the facilitation of any other protein, and can serve as good candidates in the engineering of autolytic bacteria. For example, VanX is a zinc-dependent D-Ala-D-Ala dipeptidase involved in resistance to vancomycin in Gram-positive bacteria [36]. When overexpressed in *E. coli* below 30 °C in soluble and folded form, this enzyme impairs cell wall biosynthesis via inhibition of the incorporation of D-Ala-D-Ala into elongating peptidoglycan, leading to weak cells that are susceptible to osmotic shock and shear force [37].

Table 1 List of lytic proteins/enzymes used for cell autolysis and release of various products

Bacterium	Lytic enzyme/protein	Product	Ref.
Pseudomonas putida	Ejh (holin) and Ejl (endolysin) from pneumococcal phage EJ-1	Polyhydroxyalkanoates (PHA)	[27]
Escherichia coli	Lytic cassette SRRz from *E. coli* phage lambda with an amber mutation of S	PHA	[28]
Escherichia coli	Lytic cassette SRRz from *E. coli* phage lambda	β-Glucosidase, β-galactosidase, amadoriase, GFP, lipase	[18–20, 22, 23]
Halomonas campaniensis	Lytic cassette SRRz from *E. coli* phage lambda	GFP	[20]
Synechocystis sp.	Lytic cassette GP13 15 19 from *Salmonella* phage P22 and lytic cassette SRRz from *E. coli* phage lambda	Membrane lipids	[24]
Bacillus megaterium	Holin-endolysin from *Bacillus amyloliquefaciens* phage	PHA	[25]
Escherichia coli	Holin-endolysin from *E. coli* phage T4	Red fluorescent protein	[26]
Escherichia coli	Holin of T4 phage, T7 lysozyme	β-Glucuronidase, β-galactosidase	[6, 39]
Escherichia coli	D-Amino acid oxidase, T7 lysozyme	GFP	[34]
Escherichia coli	Endolysin R from *E. coli* phage lambda	GFP	[29, 30]
Escherichia coli	VanX, a D-Ala-D-Ala dipeptidase	GFP, luciferase	[38]
Escherichia coli	T7 lysozyme from *E. coli* phage T7	PHA	[31]
Escherichia coli	T4 lysozyme with N-terminal fusion of two copies of an amphipathic cell-penetrating peptide	Lysostaphin, GFP, trehalose synthase	[2, 46]
Escherichia coli	T7 lysozyme with N-terminal fusion of FhuD signal peptide for secretion and C-terminal fusion of SsrA tag for recognition by ClpX protease	GFP, human growth hormone, human necrosis factor α, α amylase	[41]
Escherichia coli, *Pseudomonas putida*	C-Type lysozyme with N-terminal fusion of a signal peptide for secretion	PHA	[40]
Escherichia coli	Toxicity domain of colicin M with N-terminal fusion of PelB signal peptide and SsrA degradation tag	Poly(lactate-co-3-hydroxybutyrate), acetate	[8]
Escherichia coli	Chimeric enzyme ClyN targeting streptococci	Enzyme mutants	[47]
Salmonella enterica serovar *Typhimurium*	Protein E from *E. coli* phage X174	Haemolysin E, GFP, cyan fluorescent protein	[11, 14]
Escherichia coli	Protein E from *E. coli* phage X174	PHA, GFP, β-galactosidase	[9, 55, 56]
Synechocystis sp.	Lipase from *Fervidobacterium nodosum* Rt17-B1	Membrane lipids	[58]
Synechocystis sp.	Lipase from *Staphylococcus hyicus*, phospholipase from *Fusarium oxysporum*, or lipase from guinea pig	Membrane lipids	[59]

Induced expression of VanX and the reporter protein GFP at the same time in *E. coli* led to gradual release of GFP in 24 h; the addition of surfactants that destabilize the membrane structures at the end of protein expression triggered almost complete GFP release [38].

Most of the known cell wall hydrolases possess no membrane-disturbing activity but can be engineered via various means to become dual-function enzymes that can both traverse the cell membrane and degrade the cell wall. The easiest way to achieve this in an autolytic *E. coli* system is to fuse a signal peptide to the enzyme for secretory expression in the periplasmic space (Fig. 2B), as performed by fusion of the signal peptide of *E. coli* transmembrane protein PelB to the N-terminus of *E. coli* autolysin colicin M [8] or T4 lysozyme derived from T4 phage [6, 39], fusion of the first 21 amino acids of *Pseudomonas stutzeri* glucan 1,4-alpha-maltotetraohydrolase to the N-terminus of C-type lysozyme from *Gallus gallus* [40], and fusion of the FhuD signal peptide to the N-terminus of T7 lysozyme [41]. In the last case, the FhuD-T7 lysozyme fusion was further fused at the C-terminus to the SsrA degradation signal peptide that directs the un-secreted enzyme to the cytoplasmic protease ClpX to prevent cytotoxicity of the leaky expression [41].

An alternative approach to the engineering of cell wall hydrolases focuses on cell-penetrating peptides (CPPs), the membrane-destabilizing peptides generally used for drug delivery [42], for their fusion to the N-terminus of T4 lysozyme spaced by a flexible peptide linker (Fig. 2C) [2, 43]. Screening of CPP property identified that the highest efficiency of cell autolysis was obtained when an amphipathic CPP was used, which could be further improved by increasing the copy number of the CPP. By using such an autolytic *E. coli* strain carrying 2xCPP-T4 lysozyme fusion, the reporter enzyme lysostaphin, after 3 h of expression, was released at almost 100% upon 4 h of induced expression of the lytic enzyme; in addition, lysostaphin purified from the culture supernatant of the autolytic system had 1.63-fold higher activity than that purified from the cell lysate prepared by traditional freeze-thawing and sonication. This study highlights the potential of autolytic bacteria for bio-production and downstream processing particularly for enzymes that are susceptible to deactivation by mechanical force, and also

provides an advancement in the efficient production and purification of highly active lysostaphin without mechanical cell disruption, which is critical given the application potential of this particular enzyme. Lysostaphin is a very important antimicrobial enzyme used in research for the isolation of staphylococcal genomes or for anti-staphylococci studies, and can thus be applied in the preparation of animal feed or in the development of staphylolytic therapy [44]. Moreover, lysostaphin is a novel protease toward glycine-rich peptide linkers, thereby facilitating linker removal in protein manipulation [45]. This cell-autolysis-based strategy for protein production was recently verified in an *E. coli* probiotic strain Nissle 1917 for the release of GFP and trehalose synthase with promising results [46].

One more engineering method involves the construction of chimeric enzymes via fusion of modular domains from different enzymes. ClyN is a chimeolysin with the LysM domain fused to the C-terminus of a lysozyme intrinsic to *E. coli* [47]. The LysM domain is involved in cell wall binding and is also proposed to interfere with membrane integrity [47, 48]. Induced expression of ClyN caused rapid *E. coli* lysis from within and allowed the release of engineered enzyme mutants with excellent anti-streptococcal activities for high-throughput activity screening [47].

Autolysis mediated by membrane-disturbing proteins/enzymes

Gram-negative bacteria contain a thin layer of cell wall yet two layers of membranes, i.e., the cell membrane (also termed the inner membrane or the cytoplasmic membrane) and the outer membrane, which play essential roles in maintaining cell turgor [49]. These membranes are home to a large variety of proteins, and their expression levels are vital for cell susceptibility to osmotic pressure. The *Pseudomonas* sp. contain OprF and OprE in the outer membrane and the MscL protein in the inner membrane. OprF is a nonspecific porin and is responsible for cell permeability and integrity. OprE as a specific porin is activated under oxygen-limiting conditions. MscL is a mechanosensitive channel responsive to osmotic pressure. An autolytic *Pseudomonas putida* was constructed via deletion of *mscL* and overexpression of *oprF* and *oprE* genes [50]. By cultivating the cells for 30 h for PHA accumulation and 18 h for OprF/E expression, followed by NaCl treatment for 1 h and cell resuspension and incubation in water for 3 h, cells were lysed by 96%; moreover, the engineered strain produced 10% more PHA than the control strain without the autolytic module, and 93% of the total PHA could be recovered by chloroform extraction [50].

Large phages such as lambda and T4 require holin-endolysin-spanin for complete lysis of the host cell and release of progeny virions [15]. In comparison, the smaller phage X174 uses a single protein E to perform such a function [51]. E-mediated cell lysis does not involve peptidoglycan degradation as E lacks murein hydrolase activity [51]. Instead, E mediates the formation of transmembrane tunnels traversing both the cell membrane and the outer membrane of *E. coli* (Fig. 2D) [52]. In addition, E is an inhibitor of MraY, which is a translocase involved in the first step of cell wall biosynthesis [53, 54]. In consequence, gene *e* on its own is sufficient for the construction of autolytic *E. coli* or *Salmonella enterica* serovar *Typhimurium* for the production and release of PHA or recombinant proteins [14, 31, 55, 56] or for population control [9]. Induced expression of E launches rapid cell lysis in exponential phase cultures but is less efficient toward stationary phase cultures, when the bioproducts start to accumulate to high levels [31, 56]. To solve this problem, Resch et al. attempted to add Mg^{2+} prior to induction of gene *e* to stabilize membrane and to protect cells from lysis, so that the product biosynthesis continues [56]. At the end of the fermentation, cells were resuspended in water or a buffer with low ionic strength, and the osmotic pressure forced cells to form micron-scale holes and to release 90% of PHA immediately.

Photosynthetic cyanobacteria are intensively investigated for biofuel production because they harbor strong lipid synthesizing pathways and their cell membrane is mainly composed of diacylglycerol lipids [57]. Autolytic cyanobacteria based on membrane disruption are thus advantageous for lipid recovery from membranes. Toward this end, lipase genes from various microbes have been codon-optimized and introduced into *Synechocystis* sp. PCC6803 individually or in combination [58, 59]. Induced expression or activation of the lipases cause membrane damage and cell death (Fig. 2E), and the degraded membranes can be extracted with organic solvents for lipid recovery.

Control and initiation of cell autolysis

A critical factor of an autolytic system is how and when the cell lysis is launched. While it is very straightforward to induce the expression of the lytic cassette, the cost related to the inducer and the metabolic burden imposed to the cell when the lytic cassette contains multiple components should be carefully considered. In general, current studies have adopted chemical inducers, physical signals, metabolic status or nutrient supply to drive cell lysis. In certain cases, constitutive expression of the lytic gene also proves effective or even more powerful.

Inducer-dependent induction

The easiest way to control the timing of programmed cell lysis is to use a small-molecule inducer. To achieve this, the lytic cassette needs to be driven by a proper promoter

that can be activated or derepressed by the inducer. For the selection of the promoter-inducer combination, one should bear in mind that (i) the strength of the promoter has a direct impact on the speed of lytic element expression and cell lysis, and (ii) the promoter should be orthogonal to the promoter(s) used in the biosynthesis of the bio-product of interest.

In all the reported autolytic systems, the most frequently used promoters are P_{T7} and P_{araBAD}. This is due on the one hand to the commercial availability of the plasmid series carrying these promoters, and on the other hand to their universal applicability in various bacteria. P_{T7} is a strong promoter inducible by IPTG (isopropyl β-D-1-thiogalactopyranoside) or lactose. Lactose is utilized as a carbon source by many bacteria and is not a preferred inducer despite its low price. In contrast, IPTG as a structural analog of lactose cannot be metabolized by bacteria, thereby resulting in strong and steady induction of the target gene downstream of the T7 promoter even at a low dose, as showcased by the faster cell lysis induced by IPTG than by lactose in an autolytic *E. coli* strain [18]. Compared with P_{T7}, P_{araBAD} is a relatively weak promoter inducible by arabinose [60]. To construct an autolytic strain, the lytic cassette can be placed downstream of either P_{T7} or P_{araBAD}, depending on the genetic context. Generally, P_{T7} together with IPTG guarantees strong expression of the lytic cassette and fast cell lysis even at the late-log or stationary phase [2], while a delay is observed with the P_{araBAD}-arabinose combination particularly when cells transition into the late-log or stationary phase [41]. It is preferable to adopt the P_{T7} promoter and use IPTG as the inducer for the lytic gene when the gene(s) relevant to the target product biosynthesis is constitutively expressed or regulated by a different and irrelevant inducible promoter. However, for the product biosynthetic pathway and the lytic cassette, if one has to be controlled by P_{T7} whereas the other by P_{araBAD}, there is always a trade-off between the amount of product accumulation and the speed and extent of cell lysis.

A less commonly used promoter is responsive to nickel in the cyanobacterium *Synechocystis* sp. PCC 6803, which naturally carries a two-component signal transduction system *nrsRS* and a downstream *nrsBACD* operon involved in nickel resistance [61]. Ni^{2+} is sensed by NrsS (sensor kinase) to phosphorylate NrsR (response regulator), which then binds to the *nrsRS-nrsBACD* intergenic region and activates the transcription of *nrsBACD* [62]. When *Salmonella* phage holin/endolysin cassette was inserted downstream of the natural promoter of *nrsB*, induction with 7 μM $NiSO_4$ for 12 h led to more than 99% cell lysis, and 24 h of induction turned the cells into ghosts, indicating complete cell wall degradation [24]. Other promoters have also been reported, including the P_{CAT} inducible by chloramphenicol [20], *xylS*-P_m inducible by toluic acid or 3-methylbenzoate [27, 40], P_{lux} inducible by 3-oxohexanoyl-L-homoserine lactone [26], and P_{nisA} inducible by nisin [12].

Independent control of product synthesis and cell lysis offers much flexibility and high efficiency to the overall regulation of the autolytic system. Nonetheless, the induction point is essential for product accumulation and release. Ideally, cells should be cultivated and induced for a long enough period to maximize the biosynthesis of the target product, followed by the induction of the lytic cassette for product liberation. The real case is that at the end of product synthesis, especially for biopolymers that require more than 10 h for accumulation such as PHA, cells are already in the stationary phase and are inefficient in expressing the lytic cassette. To solve this problem, the lytic cassette can be controlled by a weak promoter and induced at an earlier time point, before the product accumulates to the maximum level. The slow course of cell lysis still allows continuous product formation in the unlysed cells, as proved by GFP production in autolytic *E. coli*, in which the extent of cell lysis and the amount of protein release were inversely correlated with the time delay between the induction of the lytic gene and the induction of the product [38].

Occasionally, the target gene and the lytic cassette, although placed at different genetic locations, can employ the same promoter and share the inducer. In a GFP-producing *E. coli* strain that expressed VanX peptidase for autolysis, both genes were cloned on the same plasmid using the T7 promoter but in different open reading frames. Induction by 1 mM IPTG for 24 h allowed the release of 30% of the expressed GFP into the culture (Table 2). When the two genes were cloned into different plasmids both downstream of the T7 promoter, the same amount of GFP was released after 12 h of induction by 1 mM IPTG [38]. Despite the simple procedure, the inducer has to be allocated between two genes with a lowered induction intensity, leading to a low level of product accumulation and slow product discharge even when a strong promoter is used. In comparison, when the two genes were controlled by independent promoters but induced together, a five-fold higher product concentration could be detected in the culture supernatant [38].

Induction responsive to physical signals

To avoid the use of expensive chemical inducers such as IPTG, physical induction has been designed using temperature-sensitive promoters. P_R is a heat-inducible strong promoter originated from phage lambda. This promoter is repressed by its natural repressor CI587 through direct binding below 35 °C. When the temperature rises to 37–42 °C, the repressor is inactivated and the promoter is derepressed, thereby initiating the expression of the downstream genes [63]. When both P_R and *cI587*

Table 2 List of efficiencies of various autolytic bacterial systems

Bacterium	Gene expression	Efficiency of cell lysis and product release	Ref.
Escherichia coli	Arabinose induction of the product gene at OD_{600} = 0.6 for 3 h, followed by IPTG induction of the lytic gene	98.97% cell death and 100% lysostaphin release in 4 h of IPTG induction; higher lysostaphin activity than when cells were mechanically disrupted	[2]
Escherichia coli	Constitutive expression of the product gene, followed by IPTG induction of the lytic gene at OD = 0.8 or 1.3	90% release of β-galactosidase after 20 min of induction at OD = 0.8; 50% release after 1 h of induction at OD = 1.3; 100% release when cells were further resuspended in water	[6]
Escherichia coli	Auto-induction of the lytic gene using a stationary phase promoter and a protease switch	71% release of poly(lactate-co-3-hydroxybutyrate) after 56 h of cultivation	[8]
Escherichia coli	Light induction of product gene and lactose induction of the lytic gene both at OD = 0.6 in shake flasks or OD = 10 in a 3 L fermenter	84.5% and 77.2% release of β-glucosidase after 12 h of induction in shake flasks and a 3 L fermenter, respectively; 91.8% release was achieved when induced with IPTG	[18]
Escherichia coli	IPTG induction of the product at OD = 0.5 for 1.5 h, followed by thermal induction of the lytic gene	96% release of amadoriase after 30 min of induction	[19]
Escherichia coli, *Halomonas campaniensis*	Constitutive expression of the lytic gene till the stationary phase, followed by induction with chloroform treatment	85% GFP release from *E. coli* after 5 min chloroform treatment with shaking; 90% GFP release from *H. campaniensis* after 6 h chloroform treatment with shaking	[20]
Escherichia coli	IPTG induction of the product gene at early exponential phase for 1 h, followed by UV induction of the lytic gene	63.4% cell lysis after 40 s UV induction and 2 h incubation	[22]
Escherichia coli	IPTG induction of the product gene at OD = 0.4 for 1.5 h followed by thermal induction of the lytic gene at OD = 0.8-1.0	90.4% release of β-galactosidase after 2 h of induction and 2 h of incubation	[23]
Escherichia coli	Constitutive expression of the product gene, followed by induction of the lytic gene at OD = 0.6–0.7 by homoserine lactone	96% RFP release after 125 min of induction	[26]
Escherichia coli	Constitutive expression of the lytic genes and the product genes	Release of almost all PHA after 24 h of cultivation and resuspension in Tris-EDTA	[28]
Escherichia coli	Induction of both product gene and lytic gene upon phosphate limitation	55% cell lysis after 24 h of induction and 1 h of Triton X-100 treatment; 90% lysis if further freeze-thawed for 30 min	[30]
Escherichia coli	Constitutive expression of the lytic gene	99% cell lysis and good release of poly-β-hydroxybutyrate (data not available) after 50 h of cultivation and treatment with Tris, EDTA, and Triton X-100.	[31]
Escherichia coli	Constitutive expression of the product gene, followed by IPTG induction of the lytic gene at OD = 1.0	60% GFP release after 12 h of induction; 92% after a freeze-thawing cycle	[34]
Escherichia coli	IPTG induction of both VanX and GFP at OD = 0.5	30% GFP release in 24 h	[38]
Escherichia coli	Constitutive expression of the product gene, followed by IPTG induction of the lytic gene at OD = 0.5 or 1.5	100% release of β-glucuronidase after 2–6 h of induction at OD = 0.5 or 1.5, respectively	[39]
Escherichia coli	Induction after 8 h of cell growth with tuluic acid	15% and 99% decrease in cell viability after 15 and 25 h of induction, respectively	[40]
Escherichia coli	IPTG induction of the product gene at OD = 0.6–0.8 for 10 h at 18 °C, followed by arabinose induction of the lytic gene	58% GFP release in 10 h of induction; 82% release in 10 h when cells were further treated with Triton X-100 and NaOH; 90% in 8 h of induction and Triton X-100/NaOH treatment in a 5 L fermenter	[41]
Escherichia coli	IPTG induction of the lytic gene at OD = 0.7	99.87% decrease in cell viability after 30 min induction	[47]
Escherichia coli	Auto-induction of the lytic gene using quorum sensing	60% release of superoxide dismutase after 14 h of lysis; 58% release of GFP after 16 h of lysis	[55]
Escherichia coli	Thermal induction in the stationary phase	Almost 100% cell lysis and 90% PHA release after 30 min of Mg^{2+} protection, 30 min of induction, 30 min of incubation, and resuspension in water	[56]
Pseudomonas putida	14 h of cell growth followed by co-induction of product synthesis and cell lysis in a Δ*tolB* background	100% cell lysis and 81.6% PHA release after 24 h of induction and 8 h of treatment with EDTA and SDS	[27]
Pseudomonas putida	Induction with toluic acid after 20 h of cell growth in shake flasks or after 72 h of growth in fermenter	99% decrease in cell viability after 20 h of induction in shake flasks; 98% decrease in cell viability after 16 h of induction in fermenter; 75% PHA release after 24 h of induction and 3 h of chloroform/EDTA treatment in fermenter	[40]
Synechocystis sp.	$NiSO_4$ induction of the lytic gene; induction time point not mentioned	62% cell death per hour after induction	[24]
Bacillus megaterium	20 h of product synthesis followed by induction of the lytic gene by xylose	50% reduction in cell dry weight and 70% PHA release in 30 h of induction	[25]

are introduced into bacteria to control the expression of the lysis elements, normal cell growth can be observed at a lower temperature whereas fast cell lysis is achieved when the culture is shifted to 42 °C (Table 2) [19, 23, 26, 31, 56]. Better cell lysis and release of recombinant amadoriase could be accomplished when the induction temperature was elevated to 45 °C [19].

An alternative physical signal to stimulate cell lysis is ultraviolet (UV) irradiation. UV-inducible gene expression is part of the SOS response in bacteria. Exposure to UV causes reversible activation of the protease activity of RecA, which then cleaves and inactivates LexA, a repressor of many genes, leading to high-level expression of the LexA-controlled genes [64]. For *E. coli* BL21 cells carrying the lambda phage *SRRz* lysis cassette under the control of the *recA* promoter, 63% of exponentially growing cells were lysed in 2 h post UV treatment for 40 s at 254 nm (3840 J cm^{-2} s^{-1}) in a petri dish with a culture height of 0.94 mm (Table 2) [22]. Despite the fast response and ease of operation, this method is associated with uneven induction of the culture particularly when a large volume has to be processed.

Osmotic pressure has also been harnessed to control cell lysis. In a *Pseudomonas putida* strain engineered to overexpress the outer membrane porins OprF and OprE together with the loss-of-function mutation of the mechanosensitive channel MscL, cells became hypersensitive to hypotonic conditions, and 96% loss in cell viability was achieved in 3 h upon cell resuspension in water after 30 h of PHA production, 18 h of OprF/E overexpression, and 1 h of NaCl treatment (10 g/L) [50]. Actually, for those autolytic cells with unsatisfactory lysis efficiency due to insufficient cell wall degradation, resuspension in a low-salt solution can always improve cell lysis due to the difference in osmotic pressure between the intracellular and the extracellular environment (Table 2) [6, 39, 56].

Carbon dioxide is an important nutrient for photosynthetic cyanobacteria, the cell membrane of which is a good source of free fatty acids as biofuels. Among these, *Synechocystis* sp. PCC6803 was engineered to express a lipase from *Staphylococcus hyicus* (Shl) with a broad substrate spectrum and a phospholipase from *Fusarium oxysporum* (Fol) that also exhibits galactolipase activity, and the relevant genes were placed under the control of CO_2-limitation-inducible promoter P_{cmp} [59]. Thirty minutes after CO_2 limitation (stop of aeration), almost complete cell death was observed due to membrane damage, and the yield of free fatty acids released from the membrane increased by 4.5-fold compared to the unengineered control cells. However, this system must be exposed to sufficient light for cells to accumulate enough energy to drive the expression of Fol and Shl upon CO_2 limitation. To save energy, *Synechocystis* sp. PCC6803 was optimized by introducing a thermostable lipase from *Fervidobacterium nodosum* Rt17-B1 (Fnl) under the control of P_{trc}, which is further controlled by P_{cmp} [58]. CO_2 limitation induced Fnl expression, which is then activated at 47 °C to digest the cell membrane and lyse the cells, releasing membrane fatty acids.

Auto-induction dependent on metabolic status or nutrient supply

To further reduce the processing cost and to simplify the induction procedure, auto-inducible self-disruptive bacteria have been designed based on the metabolic status of the bacteria or the consumption of certain nutrients, which relies on specific promoters that are responsive to such conditions.

Quorum sensing is a cell-density-based inter-cellular communication system and is frequently used in the design of density-dependent regulatory networks and genetic circuits. Some of the well-defined quorum sensing systems include LuxI/LuxR in *E. coli*, YasI/YasR in *Pseudoalteromonas* sp. R3, and RpaI/RpaR in *Rhodopseudomonas palustris*. The former system relies on acyl homoserine lactones (AHLs), with LuxI being an AHL synthase and LuxR being an AHL receptor. Binding of AHL to LuxR activates the promoter P_{luxI} [65]. The RpaI/RpaR pair works in a similar way except that the signal molecule is *p*-coumaroyl homoserine lactone [66]. Such systems combined with cell-killing enzymes have been proposed in the design of self-inducible autolytic bacteria. One study used LuxI/LuxR and RpaI/LuxR to orthogonally control the lysis of two strains of *Salmonella enterica* serovar *Typhimurium* by inserting the phage X174 lytic gene *e* downstream of P_{luxI} or P_{rpaI} [11]. The signal molecules were synthesized and accumulated based on the cell density till a certain concentration threshold, which then triggered gene *e* expression and cell lysis. However, this method does not have a high specificity toward the growth phase of the cells. As an improvement, gene *e* with a 3' degradation tag s*srA* for tight control of the protein leaky expression was placed under the control of P_{luxI}, whereas *luxR* was removed from the *luxI/R* cassette and placed downstream of the stationary phase promoter P_{osmY} [9]. When *E. coli* MG1655 cells entered the stationary phase, LuxR expression was activated, which then interacted with a low concentration of *N*-(3-oxohexanoyl)-L-homoserine lactone present in the medium to activate the expression of protein E. This led to rapid cell lysis and elimination of stationary-phase or non-dividing cells. This method is powerful in population control in synthetic cell consortia that are nowadays frequently utilized for the biosynthesis of compounds with complicated structures or long pathways. Another study used a similar approach yet for a more advanced application, i.e., on-site drug synthesis and delivery [14]. *Salmonella enterica* serovar *Typhimurium* was engineered to

express protein E under the control of P_{luxI}; meanwhile, the bacterium also expressed the anti-tumor toxin haemolysin E from *E. coli* or other tumor-inhibiting proteins under the control of P_{tac}. Cells grown to a certain point with sufficient AHL accumulation turned on the expression of protein E to lyse cells, release haemolysin E, and kill tumor cells within 2 h of cell lysis. Interestingly, the unlysed cells could resume growth and multiply to increase AHL concentration, thus forming an oscillatory fashion of drug release. By adding an SsrA degradation tag to LuxI, the timing of cell lysis and the cycle of lysis-growth could be tuned. This system was further tested in animal models using various injection methods or via oral delivery, all leading to exciting protection. This technology greatly advances bacterial therapy in that repetitive treatment can be achieved with a single dosage, and that the bacterial population used for drug synthesis and delivery can be restrained to a small size without significantly harming the microenvironment or causing severe microbiota imbalance.

In the YasI/YasR system, YasI is a C8-HSL (C8-acyl homoserine lactone) synthase while YasR is a C8-AHL receptor. YasR binds to C8-HSL and forms a dimer that actives the expression of genes under the control of the *yasI* promoter. By transferring this system to *E. coli* in the form of a plasmid and by incorporating the phage X174 lytic gene *e* together with P_{yasI} onto a separate plasmid, cells started to lyse at an optical density of 0.4 at 600 nm and released 60% of intracellularly expressed GFP reporter protein in 14 h [55]. To further improve the flexibility of the system hoping to achieve cell lysis at any optical density when desired, the sequence of P_{yasI} and the C8-HSL-binding site of YasR were subjected to mutagenesis and engineering, and the combination of different mutants could allow for cell autolysis initiated at different optical densities ranging from 0.2 to 0.9 either on a small scale in shake flasks or in high-density fermentation in a bench-top fermenter [55]. Compared with other autolytic platforms, this system is mild and slowly acting in product release with gentle increase in culture turbidity, which ensures continuous product synthesis despite cell lysis at a relatively low optical density before reaching the stationary phase.

Bacteria engineered for the production of secondary metabolites accumulate the products usually at the stationary phase. Therefore, it is beneficial to achieve cell lysis at a desired time point of the stationary phase to maximize product biosynthesis. The adoption of a stationary phase promoter alone to control the expression of the lysis module is not sufficient because cells start to synthesize the lytic protein(s) as soon as they enter the stationary phase, before the product accumulates to the highest level. This can be resolved by the concomitant use of a protease switch involving two inter-regulating proteases to regulate the expression of the lytic gene [8]. The protease TEVp (tobacco etch virus protease) was fused at the N-terminus with F-degron and the recognition sequence of TVMVp (tobacco vein motting virus protease), and the gene was placed downstream of the stringent stationary phase promoter P_{fic}. TVMVp was fused at the N-terminus to F-degron and the recognition sequence of TEVp, and the gene was controlled by the stationary phase promoter P_{rpsM}. The TEVp and TVMVp modules were assembled as the repression arm and action arm of the switch, respectively, in an *E. coli* strain that expressed the cell-wall-degrading toxicity domain of colicin M with N-terminal fusion of the PelB signal peptide, the F-degron, and the TVMVp recognition site under the control of P_{fic}. When cell growth entered the stationary phase, the difference in gene expression speed and extent could affect the abundance of TVMVp and the engineered colicin M, thereby determining when cell lysis was launched and how fast it proceeded. By modifying the sequences of the ribosome-binding sites, the degron, and the start codon, cell lysis could be initiated at different stages of the stationary phase. This strategy was successfully applied to the biosynthesis of poly(lactate-co-3-hydroxybutyrate) in a monoculture in a 5-L bioreactor and the product was released from 60 h to72 h of cultivation. This autolytic platform was also verified in butyrate biosynthesis in a co-culture for the upstream strain that utilized fatty acids to produce acetate.

E. coli contains the PhoRB two-component regulatory system that senses and responds to environmental phosphate concentrations. PhoR (sensor kinase) senses low concentrations of phosphate (usually below 4 μM) to phosphorylate PhoB, which is then activated and binds to the Pho box in the promoter region of many genes to start their transcription [67]. One of these genes is *yibD* (metal ion stress response gene) [67, 68], whose promoter can be used for high-level, tight-control and scalable expression of recombinant proteins inducible by phosphate limitation [69]. This strategy was adopted in an autolytic *E. coli* strain to control the expression of the lambda phage endolysin R upon phosphate starvation in the stationary phase [30]. Cells were cultivated in auto-induction media for 24 h to express GFP (the model protein product) and endolysin R, and treated with Triton X-100 to destabilize the cell membrane and the outer membrane, which caused release of 55% of total GFP in 1 h due to R-mediated cell wall degradation and cell lysis.

Salmonella enterica serovar *Typhimurium* contains a Mg^{2+}-responsive two-component system PhoQP and two Mg^{2+}-inducible transporters MgtA and MgtB. When the Mg^{2+} concentration is low as sensed by PhoQ, the PhoP protein is phosphorylated and derepresses the expression of MgtA and MgtB even in the absence of cell growth [70, 71]. By introducing the promoter of

mgtB to *E. coli* to control the lambda phage *SRRz* genes, cells could grow normally in the presence of abundant $MgCl_2$ (10 mM) but lost 80% viability and released 85% of intracellularly expressed GFP after 2 h cultivation in a low Mg^{2+} medium (<1 μM) [29]. However, cell lysis in this strain was not stringently controlled and could still be activated even at 50 μM $MgCl_2$. As an improvement, the 5'-untranslated region (UTR) of *mgtA* was inserted downstream of its promoter, forming the promoter *mgtA-UTR* [21]. This 5'-UTR acts as a riboswitch with a changing secondary structure according to the Mg^{2+} concentration and decides whether *mgtA* is transcribed [70]. Employment of $P_{mgtA-UTR}$ to control the lambda phage *SRRz* genes in a PHA-producing *E. coli* strain allowed for normal cell growth and PHA accumulation for 48 h in a medium containing 10 mM $MgSO_4$ at the beginning; cell lysis and PHA release was initiated when cells were resuspended to a medium without $MgSO_4$ [21].

Bacillus megaterium naturally harbors a xylose utilization pathway involving the *xylAB* operon, which encodes xylose isomerase and xylulokinase, respectively. The upstream region encodes the XylR repressor, which binds to the operator region to repress *xylAB* expression. Xylose as an inducer directly interacts with XylR and strongly initiates gene expression. However, glucose competes with xylose for binding to XylR and acts as an anti-inducer to prevent gene transcription [72]. As glucose is used preferably as a carbon source over xylose by bacteria, a combination of glucose and xylose can be fed to the cells for controlled gene expression upon glucose starvation, as reported in a xylose-inducible autolytic *B. megaterium* for PHA production [25]. Genes encoding the endolysin and holin from *Bacillus amyloliquefaciens* phage were inserted into the genome of *B. megaterium* downstream of *xylA*. Cells cultivated in the presence of 20 g/L of glucose and 8 g/L of xylose accumulated 2 g/L of PHA in 20 h, which gradually leaked into the medium in 15 h after glucose depletion with complete cell lysis.

In all these auto-induction systems responsive to nutrient limitation, the culture media have to be designed to contain proper amounts of the nutrients at the beginning, which could be adjusted to finely tune the timing of cell lysis.

Constitutive expression of the endolysin gene

Taking advantage of the collaborative nature of an endolysin and a corresponding holin, it is possible to separate their expression while still achieving efficient cell lysis. Since the endolysin alone is unable to cross the cell membrane barrier, the cell stays intact and continues to generate the bio-product of interest in the presence of the endolysin. Therefore, the endolysin can be expressed constitutively, enabling its accumulation to a sufficient concentration in the cytoplasm when the cell is metabolically active. The holin can then be expressed upon induction when the synthesis of the bio-product is close to an end, and cooperates with the endolysin to initiate cell lysis. By adopting such a strategy, only one component of the lytic cassette, i.e., the holin, needs to be expressed at the late-log or stationary phase when the cell is metabolically less active. This partially relieves the metabolic burden of the cell and ensures fast onset of cell lysis and product release even when the holin is generated at a moderate concentration. For example, in the biofuel-producing cyanobacterium *Synechocystis* sp. PCC 6803 carrying the *Salmonella* phage P22 lysis cassette GP13 15 19, faster cell lysis was achieved when the endolysin gene was expressed constitutively while the holin gene was expressed upon induction than in the case when both were induced for expression [24]. This is the first demonstration showing that constitutive expression of an endolysin combined with inducible expression of a holin makes up an efficient autolytic system.

It is worth mentioning that *E. coli* strains harboring the pLysS plasmid, such as BL21 (DE3) pLysS, constitutively express T7 lysozyme, which is also an endolysin cleaving the peptide stems from peptidoglycan with an extra function as a natural inhibitor of T7 RNA polymerase and is used to suppress leaky expression of genes. When these strains are engineered via implementation of the lytic cassette, the observed cell lysis reflects the joint effect of the designed lytic module and the passive degradation of cell wall by T7 lysozyme when the cell membrane is compromised. For example, in an autolytic *E. coli* strain with inducible expression of D-amino acid oxidase, which consumes the cellular pool of D-alanine and inhibits crosslinking of the cell wall peptidoglycan, introduction of the pLysS plasmid enhanced cell lysis and GFP release by 40% [34].

Given that T7 lysozyme is an endolysin derived from T7 phage, researchers have developed autolytic systems by merely introducing a holin gene into *E. coli* cells that carry the pLysS plasmid, such as the *E. coli* strain BL21 (DE3) pLysS that expresses the holin gene *t* from T4 phage [6]. Induction of gene *t* expression with IPTG at logarithmic phase resulted in immediate cell lysis and release of 90% of intracellular β-galactosidase within 20 min of induction. Similarly, in the β-glucuronidase-producing *E. coli* strain JM109 (DE3) pLysS, induction of gene *t* at the late-log phase caused 100% release of β-glucuronidase in 6 h (Table 2) [39].

As an extreme condition of the two-stage expression of the endolysin-holin system, both genes can be expressed constitutively when the host cell is naturally robust enough to withstand the lytic effect. In a GFP-producing *Halomonas campaniensis*, constitutive expression of the phage lambda SRRz lysis cassette integrated into the genome allowed normal cell growth and GFP production

in high-density fermentation; interestingly, spontaneous cell lysis and complete GFP release occurred when cells were centrifuged for 1 h, shock for 15 min at 2000 rpm in the recovered supernatant, and incubated overnight without shaking (Table 2) [20].

Bacterial cells naturally undergo autolysis at the stationary phase due to nutrient limitation, which can be exploited for a controlled and complete cell lysis. *Lactococcus lactis* that is widely used in cheese manufacturing has been engineered to constitutively express a ΦvML3 phage lysin [13]. When cell growth reaches the stationary phase, the natural level of autolysis results in the release of the lysin to the culture, which then acts from the outside to efficiently lyse almost the entire population of cells. This facilitates the liberation of the intracellular enzymes and accelerates milk clotting and cheese ripening. The extent of cell lysis can be adjusted with the addition of various concentrations of sucrose for protection against osmotic challenge.

Limitations of the current autolytic bacterial systems

Autolytic bacteria have shown great promise for applications not only in library screening and product recovery but also in population control and drug delivery. Moreover, the advancement of synthetic biology has dramatically potentiated on-demand design of novel autolytic strains that can lyse automatically without any external stimulus at a desired time point. However, problems remain with respect to the efficiency, cost, stability, product retention, etc., which definitely needs extra investigation, engineering, and optimization.

In the evaluation of the autolysis efficiency, the easiest and most commonly used method is measurement of the culture turbidity over time, and a drop in turbidity is always regarded as an indication of cell lysis, which is, nonetheless, not the universal case. A culture with a decreasing turbidity can experience increasing cell viability, whereas an unchanging or rising turbidity can be associated with lowering cell viability. Such discrepancy may be a combined result of several factors. Firstly, the released cellular proteins may aggregate or precipitate based on the composition of the growth medium, and the nucleic acids, especially the genomic DNA, may form sticky and viscous aggregates, thereby increasing the culture turbidity. Secondly, cells undergoing lysis can have an altered morphology, such as the formation of spheroplasts or ghosts or expansion in cell size. In certain cases, cells do not fracture into pieces but instead are punctured, leaving holes of various sizes on the cell surface. These ultimately have an impact on the optical density. Thirdly, lysing cells on their own may be prone to agglomeration attributed to the exposure of distinctly charged molecules on the cell surface, leading to a higher apparent optical density. In consequence, turbidity should never be used as the only criterion in judging the rate and extent of cell lysis; it should be presented together with other data such as cell viability, cell staining results, microscopic images, etc., as solid evidence supporting cell lysis.

Cell lysis initiated in the stationary phase is necessary for the high-level accumulation of bio-products such as proteins and bioplastics. However, the lysis is usually much slower under such conditions than lysis induced in the exponential phase unless the cells are treated with certain chemical reagents or induced with strong promoters (Table 2). Even when cells are designed to lyse at the stationary phase, lysis occurs in a prolonged period. On the one hand, cells at the stationary phase are metabolically less active under nutrient starvation, so expression of the cell-disruption proteins/enzymes is a sluggish process. On the other hand, cells at the stationary phase may alter the structure and compactness of the cell wall and cell membrane, leading to lower susceptibility to cell wall hydrolases or membrane-disturbing enzymes. The former issue may be addressed via nutrient feeding at the time of lysis induction, while the latter issue can probably be solved by using multiple lytic genes with different targets on the cell surface at the expense of higher metabolic burden.

Some autolytic systems experience resumption of cell growth after fast and efficient lysis. While stochastically low-level expression of the lytic gene may play a role [11, 14], cell mutation can also be a culprit [24]. A study collected the regrown cells and found that they could not lyse upon induction [26]. Isolation of single colonies followed by sequencing of the plasmid carrying the lytic gene identified single point insertion or large deletions either in the lytic gene or in the promoter region. In addition, plasmid stability showed large variations among different strains [26]. Phenotype stability and strain mutation were also reported for autolytic *Synechocystis* sp. PCC 6803 [24]. These explorations indicate the necessity to evaluate strain stability for industrial applications.

Cell lysis occurring at high efficiency does not always translate into equally efficient product liberation either for bioplastics or for proteins even with the aid of surfactants or membrane-destabilizing reagents to completely fractionate cells. This is probably due to the measure of cell lysis being cell viability instead of morphological change. A dead cell is not necessarily a lysed ghost or cell debris. Moreover, the product may interact with certain cellular debris such as membrane fragments or nucleic acids due to electrostatic or hydrophobic interactions, preventing their recovery in downstream processing [25]. In addition, the lesion developed on the cell surface during lysis may not be large enough for the bio-products to diffuse out. Currently, there lacks information regarding

the correlation between the size of the holes/tunnels formed on the cell surface and the size of the products. Although reports suggest that holes on *E. coli* surface are stretchable to allow the passage of the large PHA granules [31, 56], it is not clear how elastic these PHA granules are. Therefore, these results are unconvincing and need in-depth investigation.

Bacterial cells, despite the lack of many organelles, are compartmentalized in the sense that the cytoplasm is not homogeneous and that different types of metabolites are present in distinct sub-cellular locations [73]. When the autolytic system is to be used for the recovery of an intracellularly expressed recombinant protein/enzyme, one should always bear in mind that release of the target product is accompanied with concomitant liberation of endogenous proteases, which may accumulate in different regions in the cytoplasm before cell lysis. This can cause product degradation and further lead to protein dysfunction or enzyme inactivation. This issue, although never reported, should not be neglected as it is directly related to quality control of the protein/enzyme product, especially when it takes a long time to achieve complete cell lysis and product release. It is advisable to always check the molecular weight and abundance of the protein/enzyme product by gel electrophoresis analysis, and to further verify the protein function or the enzyme activity over the entire course of cell lysis.

Conclusions and future perspectives

Autolytic bacteria are an excellent platform for the recovery of intracellular bio-products without an additional cell disruption procedure. Another way of achieving such a goal is to use cell-free systems, which utilize crude cell extracts or purified enzymes to carry out a series of enzymatic reactions for the synthesis of biopolymers, biomacromolecules, and small-molecule products [74]. This method completely eliminates the procedures and costs associated with cell disruption at the time of product recovery, greatly facilitates product purification, and allows for real-time detection of the concentrations of the products and other metabolites. However, cell-free systems are dependent on sufficient external supply of cofactors and are confronted with limited stability and activity of the enzymes. In comparison, bacterial autolytic systems can generate the required enzymes and cofactors in a continuous manner, thereby replenishing the enzyme/cofactor pool endogenously.

Besides the applications in the generation and recovery of intracellular bio-products, autolytic systems may be extended to the secretory synthesis of bio-products, in which the products are autonomously secreted or transported to the culture media. By eliciting partial cell lysis or by inducing the formation of small lesions on the cell surface near the end of product accumulation, the extent of product secretion may be elevated, thereby increasing the extracellular level of the product. Indeed, it has been shown that overexpression or deletion of certain outer membrane proteins increase outer membrane permeability in *E. coli* and contributes to a higher level of secretory expression of recombinant proteins [49]. For such applications, although the non-lytic bacterial systems require less strain engineering with better control on product synthesis, the autolytic systems lead to much less leakage of genetically modified bacteria and their genes into the environment and are hence environmentally much safer.

In the future, with the integration of synthetic biology and artificial intelligence, smart autolytic systems may be devised that can lyse on demand when sensing a change in temperature, in the intensity of light or a magnetic/electric field, in the concentrations of particular metabolites, pathogen toxins, or even disease markers, etc., for applications in basic lab research, food processing, biomanufacturing, industrial fermentation, or the biomedical industry. In the long march toward the ultimate goal of low cost, high efficiency, and high stability, it should never be ignored that process optimization can sometimes have tremendous impact on the overall performance of the autolytic cells and should be performed depending on the particular product, the application scenario, and the utilized process technology.

Abbreviations

PHAs	Polyhydroxyalkanoates
GFP	Green fluorescent protein
CPPs	Cell-penetrating peptides
IPTG	Isopropyl β-D-1-thiogalactopyranoside
UV	Ultraviolet
AHL	Acyl homoserine lactone
C8-HSL	C8-acyl homoserine lactone
TEVp	Tobacco etch virus protease
TVMVp	Tobacco vein motting virus protease
OD	Optical density
UTR	Untranslated region

Author contributions

X.W. and J.Z. conceived the idea and wrote the manuscript; C.D. and G.G. revised the manuscript; X.W., J.Z., C.D. and S.C. prepared the graphics and tables; J.R. collected literature.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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References

1. Wang Y, Ling C, Chen Y, Jiang X, Chen G-Q. Microbial engineering for easy downstream processing. Biotechnol Adv. 2019;37:107365.
2. Zha J, Liu Z, Sun R, Gong G, Dordick JS, Wu X. Endolysin-based autolytic *E. Coli* system for facile recovery of recombinant proteins. J Agric Food Chem. 2021;69:3134–43.
3. Gao Y, Feng X, Xian M, Wang Q, Zhao G. Inducible cell lysis systems in microbial production of bio-based chemicals. Appl Microbiol Biotechnol. 2013;97:7121–9.
4. Kalousek S, Dennis DE, Lubitz W. Release of poly-β-hydroxybutyrate granules from *Escherichia coli* by protein E-mediated lysis. International Symposium on Biodegradable Polymers. *Tokyo*. 1990.
5. Bedade DK, Edson CB, Gross RA. Emergent approaches to efficient and sustainable polyhydroxyalkanoate production. Molecules. 2021;26:3463.
6. Tanji Y, Asami K, Xing X-H, Unno H. Controlled expression of lysis genes encoded in T4 phage for the gentle disruption of *Escherichia coli* cells. J Ferment Bioeng. 1998;85:74–8.
7. Lin Z, Cai ZR. Cell lysis methods for high-throughput screening or miniaturized assays. Biotechnol J. 2009;4:210–5.
8. Diao W, Guo L, Ding Q, Gao C, Hu G, Chen X, Li Y, Zhang L, Chen W, Chen J, Liu L. Reprogramming microbial populations using a programmed lysis system to improve chemical production. Nat Commun. 2021;12:6886.
9. Bittihn P, Didovyk A, Tsimring LS, Hasty J. Genetically engineered control of phenotypic structure in microbial colonies. Nat Microbiol. 2020;5:697–705.
10. Roell GW, Zha J, Carr RR, Koffas MA, Fong SS, Tang YJ. Engineering microbial consortia by division of labor. Microb Cell Fact. 2019;18:35.
11. Scott SR, Din MO, Bittihn P, Xiong L, Tsimring LS, Hasty J. A stabilized microbial ecosystem of self-limiting bacteria using synthetic quorum-regulated lysis. Nat Microbiol. 2017;2:17083.
12. de Ruyter PG, Kuipers OP, Meijer WC, de Vos WM. Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening. Nat Biotechnol. 1997;15:976–9.
13. Shearman CA, Jury K, Gasson MJ. Autolytic *Lactococcus lactis* expressing a lactococcal bacteriophage lysin gene. Bio/Technology. 1992;10:196–9.
14. Din MO, Danino T, Prindle A, Skalak M, Selimkhanov J, Allen K, Julio E, Atolia E, Tsimring LS, Bhatia SN, Hasty J. Synchronized cycles of bacterial lysis for in vivo delivery. Nature. 2016;536:81–5.
15. Young R. Phage lysis: three steps, three choices, one outcome. J Microbiol. 2014;52:243–58.
16. Wu X, Kwon S-J, Kim J, Kane RS, Dordick JS. Biocatalytic nanocomposites for combating bacterial pathogens. Annu Rev Chem Biomol Eng. 2017;8:87–113.
17. Gerstmans H, Criel B, Briers Y. Synthetic biology of modular endolysins. Biotechnol Adv. 2018;36:624–40.
18. Chang F, Zhang X, Pan Y, Lu Y, Fang W, Fang Z, Xiao Y. Light induced expression of β-glucosidase in *Escherichia coli* with autolysis of cell. BMC Biotechnol. 2017;17:74.
19. Cai Z, Xu W, Xue R, Lin Z. Facile, reagentless and in situ release of *Escherichia coli* intracellular enzymes by heat-inducible autolytic vector for high-throughput screening. Protein Eng Des Sel. 2008;21:681–7.
20. Hajnal I, Chen X, Chen G-Q. A novel cell autolysis system for cost-competitive downstream processing. Appl Microbiol Biotechnol. 2016;100:9103–10.
21. Tamekou Lacmata S, Yao L, Xian M, Liu H, Kuiate J-R, Liu H, Feng X, Zhao G. A novel autolysis system controlled by magnesium and its application to poly (3-hydroxypropionate) production in engineered *Escherichia coli*. Bioengineered. 2017;8:594–9.
22. Li S-S, Xu L-H, Hua H, Ren C-P, Lin Z. A set of UV-inducible autolytic vectors for high throughput screening. J Biotechnol. 2007;127(4):647–52.
23. Xu L-H, Li S-S, Ren C-P, Cai ZR, Lin Z. Heat-inducible autolytic vector for high-throughput screening. Biotechniques. 2006;41:319–23.
24. Liu X, Curtiss R. 3rd. Nickel-inducible lysis system in *Synechocystis* sp. PCC 6803. Proc Natl Acad Sci. 2009;106:21550–4.
25. Hori K, Kaneko M, Tanji Y, Xing XH, Unno H. Construction of self-disruptive *Bacillus megaterium* in response to substrate exhaustion for polyhydroxybutyrate production. Appl Microbiol Biotechnol. 2002;59:211–6.
26. Pasotti L, Zucca S, Lupotto M, Cusella De Angelis MG, Magni P. Characterization of a synthetic bacterial self-destruction device for programmed cell death and for recombinant proteins release. J Biol Eng. 2011;5:8.
27. Martínez V, García P, García JL, Prieto MA. Controlled autolysis facilitates the polyhydroxyalkanoate recovery in *Pseudomonas putida* KT2440. Microb Biotechnol. 2011;4:533–47.
28. Yu H, Yin J, Li H, Yang S, Shen Z. Construction and selection of the novel recombinant *Escherichia coli* strain for poly(β-hydroxybutyrate) production. J Biosci Bioeng. 2000;89:307–11.
29. Zhang X, Pan Z, Fang Q, Zheng J, Hu M, Jiao X. An auto-inducible *Escherichia coli* lysis system controlled by magnesium. J Microbiol Methods. 2009;79:199–204.
30. Menacho-Melgar R, Moreb EA, Efromson J, Yang T, Hennigan JN, Wang R, Lynch MD. Improved, two-stage protein expression and purification via autoinduction of both autolysis and auto DNA/RNA hydrolysis conferred by phage lysozyme and DNA/RNA endonuclease. bioRxiv. 2020.
31. Fidler S, Dennis D. Polyhydroxyalkanoate production in recombinant *Escherichia coli*. FEMS Microbiol Rev. 1992;103:231–5.
32. Switt AIM, Sulakvelidze A, Wiedmann M, Kropinski AM, Wishart DS, Poppe C, Liang Y. Salmonella phages and prophages: genomics, taxonomy, and applied aspects, In: Schatten H, Eisenstark A. Salmonella: Methods and Protocols. Springer New York New York, NY. 2015, pp. 237 – 87.
33. Cheng X, Zhang X, Pflugrath JW, Studier FW. The structure of bacteriophage T7 lysozyme, a zinc amidase and an inhibitor of T7 RNA polymerase. Proc Natl Acad Sci. 1994;91:4034–8.
34. Chien LJ, Lee C-K. Synergistic effect of co-expressing D-amino acid oxidase with T7 lysozyme on self-disruption of *Escherichia coli* cell. Biochem Eng J. 2006;28:17–22.
35. Lin L-L, Chien HR, Wang W-C, Hwang T-S, Fu H-M, Hsu W-H. Expression of *Trigonopsis variabilis* D-amino acid oxidase gene in *Escherichia coli* and characterization of its inactive mutants. Enzyme Microb Technol. 2000;27:482–91.
36. Lessard IAD, Walsh CT. VanX, a bacterial D-alanyl-D-alanine dipeptidase: resistance, immunity, or survival function? Proc Natl Acad Sci. 1999;96:11028–32.
37. Sohya S, Kamioka T, Fujita C, Maki T, Ohta Y, Kuroda Y. Biochemical and biophysical characterization of an unexpected bacteriolytic activity of VanX, a member of the Vancomycin-resistance *vanA* gene cluster. J Biol Chem. 2014;289:35686–94.
38. Kamioka T, Sohya S, Wu N, Maki T, Matsuda T, Ikegami T, Nakamura H, Kuroda Y. Extraction of recombinant protein from *Escherichia coli* by using a novel cell autolysis activity of VanX. Anal Biochem. 2013;439:212–7.
39. Morita M, Asami K, Tanji Y, Unno H. Programmed *Escherichia coli* cell lysis by expression of cloned T4 phage lysis genes. Biotechnol Prog. 2001;17:573–6.
40. Borrero-de Acuña JM, Hidalgo-Dumont C, Pacheco N, Cabrera A, Poblete-Castro I. A novel programmable lysozyme-based lysis system in *Pseudomonas putida* for biopolymer production. Sci Rep. 2017;7:4373.
41. Zhang F, Fan X, Xu K, Wang S, Shi S, Yi L, Zhang G. Development of a bacterial FhuD-lysozyme-SsrA mediated autolytic (FLSA) system for effective release of intracellular products. ACS Synth Biol. 2023;12:196–202.
42. Raucher D, Ryu JS. Cell-penetrating peptides: strategies for anticancer treatment. Trends Mol Med. 2015;21:560–70.
43. Wu X, Fraser K, Zha J, Dordick JS. Flexible peptide linkers enhance the antimicrobial activity of surface-immobilized bacteriolytic enzymes. ACS Appl Mater Interfaces. 2018;10:36746–56.
44. Zha J, Li J, Su Z, Akimbekov N, Wu X. Lysostaphin: engineering and potentiation toward better applications. J Agric Food Chem. 2022;70:11441–57.
45. Liu Z, Gong G, Li Y, Xu Q, Akimbekov N, Zha J, Wu X. Peptidoglycan-targeting staphylolytic enzyme lysostaphin as a novel and efficient protease toward glycine-rich flexible peptide linkers. J Agric Food Chem. 2023;71:5293–301.
46. Ge C-E, Cai M-Z, Chen P-T. Construction of an autolytic system in *Escherichia coli* Nissle 1917 for efficient release of intracellular recombinant proteins. J Taiwan Inst Chem Eng. 2023;12:105271.
47. Yang H, Linden SB, Wang J, Yu J, Nelson DC, Wei H. A chimeolysin with extended-spectrum streptococcal host range found by an induced lysis-based rapid screening method. Sci Rep. 2015;5:17257.
48. Morita M, Tanji Y, Orito Y, Mizoguchi K, Soejima A, Unno H. Functional analysis of antibacterial activity of *Bacillus amyloliquefaciens* phage endolysin against Gram-negative bacteria. FEBS Lett. 2001;500:56–9.
49. Wu X, Liu J, Liu Z, Gong G, Zha J. Microbial cell surface engineering for high-level synthesis of bio-products. Biotechnol Adv. 2022;55:107912.
50. Poblete-Castro I, Aravena-Carrasco C, Orellana-Saez M, Pacheco N, Cabrera A, Borrero-de Acuña JM. Engineering the osmotic state of *Pseudomonas putida*

KT2440 for efficient cell disruption and downstream processing of poly(3-hydroxyalkanoates). Front Bioeng Biotechnol. 2020;8:161.
51. Roof WD, Young R. Phi X174 E complements lambda S and R dysfunction for host cell lysis. J Bacteriol. 1993;175:3909–12.
52. Witte A, Bläsi U, Halfmann G, Szostak M, Wanner G, Lubitz W. Phi X174 protein E-mediated lysis of *Escherichia coli*. Biochimie. 1990;72:191–200.
53. Bernhardt TG, Struck DK, Young R. The lysis protein E of φX174 is a specific inhibitor of the MraY-catalyzed step in peptidoglycan synthesis. J Biol Chem. 2001;276:6093–7.
54. Bernhardt TG, Roof WD, Young R. Genetic evidence that the bacteriophage φX174 lysis protein inhibits cell wall synthesis. Proc Natl Acad Sci. 2000;97:4297–302.
55. Song X, Zhao Y, Ren Y, Liu R, Zhang M, Zhang Z, Meng Q, Zhu T, Yin J, Yu Z. Development of a quorum sensing-mediated bacterial autolytic system in *Escherichia coli* for automatic release of intracellular products. ACS Synth Biol. 2024;13:1956–62.
56. Resch S, Gruber K, Wanner G, Slater S, Dennis D, Lubitz W. Aqueous release and purification of poly(β-hydroxybutyrate) from *Escherichia coli*. J Biotechnol. 1998;65:173–82.
57. Liu X, Sheng J, Curtiss R III. Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci. 2011;108:6899–904.
58. Liu X, Curtiss R. Thermorecovery of cyanobacterial fatty acids at elevated temperatures. J Biotechnol. 2012;161:445–9.
59. Liu X, Fallon S, Sheng J, Curtiss R. CO_2-limitation-inducible green recovery of fatty acids from cyanobacterial biomass. Proc Natl Acad Sci. 2011;108:6905–8.
60. Greenfield L, Boone T, Wilcox G. DNA sequence of the *araBAD* promoter in *Escherichia coli* B/r. Proc Natl Acad Sci. 1978;75:4724–8.
61. García-Domínguez M, Lopez-Maury L, Florencio FJ, Reyes JC. A gene cluster involved in metal homeostasis in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol. 2000;182:1507–14.
62. López-Maury L, García-Domínguez M, Florencio FJ, Reyes JC. A two-component signal transduction system involved in nickel sensing in the cyanobacterium *Synechocystis* sp. PCC 6803. Mol Microbiol. 2002;43:247–56.
63. Restrepo-Pineda S, Valdez-Cruz NOP, Trujillo-Roldán NA. Thermoinducible expression system for producing recombinant proteins in *Escherichia coli*: advances and insights. FEMS Microbiol Rev. 2021;45:fuab023.
64. Little JW, Mount DW. The SOS regulatory system of *Escherichia coli*. Cell. 1982;29:11–22.
65. Tang R, Zhu J, Feng L, Li J, Liu X. Characterization of LuxI/LuxR and their regulation involved in biofilm formation and stress resistance in fish spoilers *Pseudomonas fluorescens*. Int J Food Microbiol. 2019;297:60–71.
66. Hirakawa H, Harwood CS, Pechter KB, Schaefer AL, Greenberg EP. Antisense RNA that affects *Rhodopseudomonas palustris* quorum-sensing signal receptor expression. Proc Natl Acad Sci. 2012;109:12141–6.
67. Baek JH, Lee SY. Novel gene members in the pho regulon of *Escherichia coli*. FEMS Microbiol Lett. 2006;264:104–9.
68. Yoshida Y, Sugiyama S, Oyamada T, Yokoyama K, Kim SK, Makino K. Identification of PhoB binding sites of the *yibD* and *ytfK* promoter regions in *Escherichia coli*. J Microbiol. 2011;49:285–9.
69. Menacho-Melgar R, Ye Z, Moreb EA, Yang T, Efromson JP, Decker JS, Lynch MD. Improved, scalable, two-stage, autoinduction of recombinant protein expression in *E. Coli* utilizing phosphate depletion. bioRxiv. 2020:820787.
70. Gall AR, Datsenko KA, Figueroa-Bossi N, Bossi L, Masuda I, Hou Y-M, Csonka LN. Mg^{2+} regulates transcription of *mgtA* in *Salmonella Typhimurium* via translation of proline codons during synthesis of the MgtL peptide. Proc Natl Acad Sci. 2016;113:15096–101.
71. Tao T, Grulich PF, Kucharski LM, Smith RL, Maguire ME. Magnesium transport in *Salmonella typhimurium*: biphasic magnesium and time dependence of the transcription of the *mgtA* and *mgtCB* loci. Microbiology. 1998;144(Pt 3):655–64.
72. Dahl MK, Schmiedel D, Hillen W. Glucose and glucose-6-phosphate interaction with xyl repressor proteins from *Bacillus* spp. may contribute to regulation of xylose utilization. J Bacteriol. 1995;177:5467–72.
73. Cornejo E, Abreu N, Komeili A. Compartmentalization and organelle formation in bacteria. Curr Opin Cell Biol. 2014;26:132–8.
74. Dong H, Yang X, Shi J, Xiao C, Zhang Y. Exploring the feasibility of cell-free synthesis as a platform for polyhydroxyalkanoate (PHA) production: opportunities and challenges. Polymers. 2023;15:2333.

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