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Review

Research Progress on the Effect of Autolysis to *Bacillus subtilis* Fermentation Bioprocess

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Abstract: *Bacillus subtilis* is a gram-positive bacterium, a promising microorganism due to its strong extracellular protein secretion ability, non-toxic, and relatively mature industrial fermentation technology. However, cell autolysis during fermentation restricts the industrial application of *B. subtilis*. With the fast advancement of molecular biology and genetic engineering technology, various advanced procedures and gene editing tools have been used to successfully construct autolysis-resistant *B. subtilis* chassis cells to manufacture various biological products. This paper first analyses the causes of autolysis in *B. subtilis* from a mechanistic perspective and outlines various strategies to address autolysis in *B. subtilis*. Finally, potential strategies for solving the autolysis problem of *B. subtilis* are foreseen.

Keywords: *Bacillus subtilis*; cell autolysis; gene editing; fermentation technology; chassis cell

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1. Introduction

B. subtilis is a generally recognized as safe (GRAS) microorganism [1], which is famous for its strong ability for heterologous expression, clear physiological and biochemical characteristics, and legacy relatively simple to operate. In addition, they have the advantages of being easy to cultivate and robust in industrial fermentations [2]. Using *B. subtilis* as the chassis cell, the exogenous introduction of the desired synthetic pathway and the systematic optimization of the strain's own global metabolism and the coordination of the balance between the strain's metabolic network and the exogenous pathway allow for the efficient production of a large number of products. As a crucial industrial biotechnology powerful chassis cell, *B. subtilis* produces valuable enzymes and biopolymers [3]. For example, xylanase can be fermented in a recombinant strain of *Bacillus subtilis* to obtain an enzymatic activity of 38 U/mL [4], and L-asparaginase (ASN) can reach a yield of 407.6 U/mL (2.5 g/L) by *Bacillus subtilis* fermenters [5]. Biopolymers are primarily represented by riboflavin, surface activator, ethylene coupling, phytase, xylanase, L-asparaginase, chondroitin, N-ethyleneglycolate phytase, xylanase, L-asparaginase, chondroitin, N-acetyl acyl glucosamine, etc. [6] (shown in Figure 1). The yield of riboflavin in *Bacillus subtilis* was ahead of other strains of the chassis. As early as 1999, Perkins et al. applied genetic engineering technology to construct a high-yielding strain of riboflavin using *Bacillus subtilis* as the substrate cells, and the yield of riboflavin could reach 15 g/L at 56 h of fermentation [7]. In the agriculture field, *B. subtilis* is not only used to treat plant and animal illnesses and to replace hazardous chemical fungicides to minimize the danger to other species and the environment [8]. Regarding probiotic foods, *B. subtilis* can enter animals in the form of spores [9]. After germination, it can produce many antibacterial substances to inhibit the growth of harmful intestinal microorganisms such as *Escherichia coli* to achieve the effect of gastrointestinal protection [10].

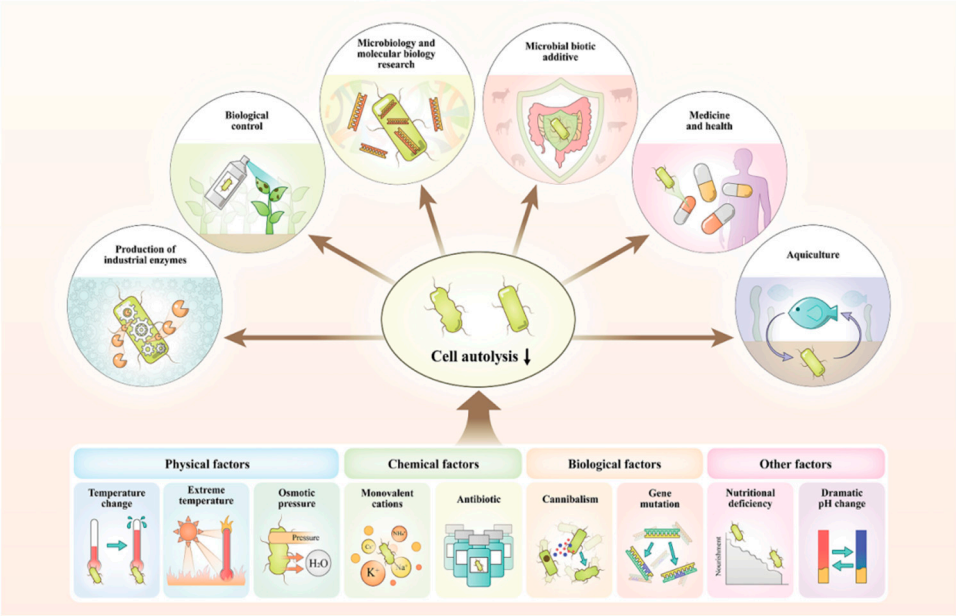

Figure 1. Application of *B. subtilis* as industrial production chassis and factors related to autolysis of *B. subtilis*.

However, there is a problem with cell autolysis in fermentation cultures of *B. subtilis*. Cell autolysis is widespread in microorganisms, such as bacteria, actinomycetes, and fungi. The researchers defined the self-structural degradation process that bacteria undergo at the end of their life cycle as "autolysis" and found that "autolysins" such as cytosolic hydrolases and peptidoglycan hydrolases, which catalyze the hydrolysis of the cell wall peptidoglycan layer, are closely related to the autolysis process. Reduced cell autolysis in *B. subtilis* leads to a significant increase in cell secretion of recombinant proteins [11] and other productions. In a later stage of fermentation or under fermentation conditions unsuitable for cell growth, the cells will begin to autolyze. This effect leads to a substantial reduction in cell biomass, which seriously affects the expression of products and fermentation efficiency [12]. In particular, high-yield strains modified by metabolic engineering are more likely to occur during cell autolysis. For example, WB800 [13] (*B. subtilis* with eight extracellular proteases knocked out), most often applied to express extracellular proteases, has more severe cell autolysis [14]. Therefore, it is essential to study the autolysis of *B. subtilis*. Studies have shown that the root cause of *B. subtilis* autolysis is that the activity of autolysins is controlled by various conditions to perform their respective functions and thus hydrolyze the peptidoglycan of the cells. These autolysis enzymes include those closely related to the growth and development of *B. subtilis* and those from prophages.

This review analyzes the relevant factors of *B. subtilis* autolysis in terms of mechanism, summarizes the functions of critical autolysins in its autolysis process, and outlines the various effective strategies that have been used in recent years to address autolysis in *B. subtilis* and the results they have achieved. Finally, future research directions for solving the autolysis problem of *B. subtilis* have been prospected.

2. Mechanism and Causative Factors of Autolysis Phenomenon

Microbial autolysis is mainly manifested in the action of enzymes on the cell wall. As shown in Figure 2, the cell wall of *B. subtilis* consists primarily of peptidoglycan and anionic polymers. The anionic polymers provide chemical bonds that can be hydrolyzed, thus generating a more significant number of potential active sites for bacterial autolysins action [15]. These actions eventually lead to disrupting the cross-linked structure of peptidoglycan and the peptidoglycan cleavage [16]. The following figure shows the structural units and autolysis action sites (four in total) of the *B. subtilis* metrocyte cell wall and spore cortex.

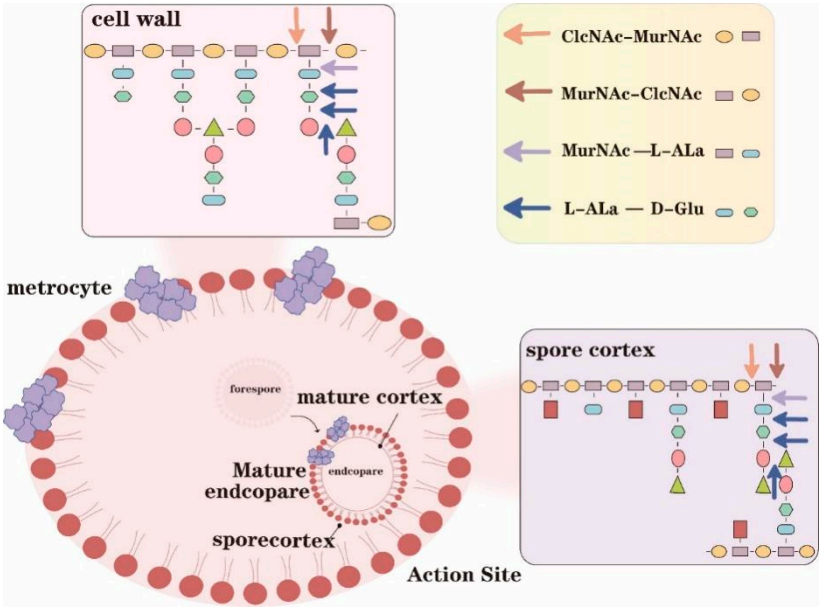

Figure 2. Mechanisms of autolysis in *Bacillus subtilis* site of action of autolysin. The peptidoglycan PG in the cell wall structure is the backbone component of the cell wall, and the side chains of the peptidoglycan are linked to anionic polymers, which provide the sites of action for autolysis enzymes.

In addition to the normal senescence and death of cells, any factors that are unsuitable for the growth and physiological metabolism of microorganisms and cause extreme disturbance in their physiological state can effectively induce autolysis expression and cause microbial autolysis. Comprehensive of the existing reports at home and abroad, we summarize the various factors that can cause cell autolysis in *B. subtilis*, as shown in Figure 1.

2.1. Physical Factors That Induce Autolysis

The main physical factors are temperature, osmotic pressure, and pH. Among them, temperature variation is an essential environmental parameter affecting microbial cell growth and reproduction. High-temperature fermentation has some advantages in production because it creates a harsh environment (preventing the growth of hybrid bacterium) that promotes the growth of the target strain. Nonetheless, raising the high temperature of the culture might have a detrimental impact on microorganism development and lead to autolysis of the cultured cells in certain situations.

B. subtilis can grow at 25~40 °C, and the optimum temperature for its growth is 35 °C. As mentioned in the article by Regamey and Karamat et al. [17], after *B. subtilis* was subjected to thermal excitation at 50 °C, prophage *spβ* was induced, and many cells underwent autolysis. Similarly, Nandy et al. [18] found that moving *B. subtilis* from 37 °C to 48 °C water bath conditions caused massive autolysis of *B. subtilis* cells. According to Antelmann et al. [19], high-temperature incubation caused the production of autolysis enzymes in some bacteria, leading to massive cell autolysis of these microorganisms. This phenomenon poses a severe threat to microbial culture. Therefore, using a higher temperature to culture microorganisms and avoid cell autolysis has become one of the main obstacles to high-temperature culture microorganisms. One of the most successful strategies to overcome this issue is to improve microbial strains to adapt to high-temperature fermentation settings [20]. In addition to this, autolysis of *B. subtilis* induced by supercooling was also found by Yamanaka et al. [21]. Following a rapid decrease in temperature, the physiology of *B. subtilis* cells changes profoundly; this phenomenon is called cold shock [22]. *B.subtilis* vegetative cells undergo autolysis when exposed to cold shock treatment.

In the aspect of osmotic pressure, the suitable osmotic pressure for *Bacillus subtilis* is converted to a culture speed of approximately 200–250 rpm. Svarachorn et al. [23]

discovered that when *B. subtilis* cells lose their capacity to regulate osmotic pressure, the intracellular potassium ions of the bacterium will leak, and the cells will not take up glucose. If the bacterium is continuously incubated at 37 °C in high concentrations of near-monovalent cations, autolysis of cells will be observed. Sahoo et al. [24] and Yamanaka et al. [21] exposed *B. subtilis* to drastic pressure change conditions (rate of 1.482/s). After 10 h incubation, they found that the biomass of the bacterium decreased drastically. The effect of high pressure on the autolysis of *B. subtilis* can also be understood during the decontamination of HHP (high hydrostatic pressure) [25].

pH is a very important parameter for microbial growth, a comprehensive indicator of metabolic activity, and closely related to microbial autolysis. Different species of microorganisms have different pH requirements. The optimum pH for most bacteria is 6.5–7.5. The optimal culture pH for *B. subtilis* is about 7. Jolliffe et al. [26] found that *Bacillus subtilis* showed the highest autolysis of its cells when it was in alkaline medium (pH = 9).

2.2. Chemical Factors That Induce Autolysis

Chemical factors are mainly the effects of monovalent cations and antibiotics on cell autolysis. These substances affect the normal expression of autolytic enzymes and proteases in cells. For example, Svarachorn et al. [27] found that monovalent cations such as K^+, $NH_4{}^+$, Na^+, Cs^+, and Li^+ could cause intracell autolysis of *B. subtilis* 168 when the concentration of K^+, $NH_4{}^+$, Na^+, Cs^+, and Li^+ was 100 mM. The autolysis rate of cells in the above buffer was in the range of 2.8–4.0 k $\times$ 10^2/min. This shows that high concentrations of monovalent cations in buffers can promote autolysis in *B. subtilis* 168, mainly due to activating important, internal, and significant N-acetylmuramoyl-L-alanine amidase inside this bacterium, e.g., NH^+, Li^+, Rb^+, Cs^+, and Na^+ at 100 mM. Their enzyme activities were increased by 6.4, 3.9, 3.9, 3.6, and 3.4 times, respectively. Autolysis is more likely to occur when protein synthesis is active, but peptidoglycan synthesis is inadequate.

The role of proteases in antibiotic-induced cell lysis is also a more complex process [28]. Jolliffe et al. [29] investigated the impact of nafcillin on *B. subtilis* intracell autolysis and discovered that mutants that release many proteases are incredibly resistant to nafcillin-induced fatal and lytic effects. Under nafcillin induction, however, protease-deficient bacteria were more sensitive to mortality and lysis.

2.3. Biological Factors That Induce Autolysis

Biological factors mainly include external and internal factors, which are the microbial interactions and alterations in the autolysin gene expression level of *B. subtilis*. Among the external factors, *B. subtilis* in the same culture system compete with each other for nutrients. The interaction between microorganisms causes autolysis. As for internal factors, mutations in some genes that can affect the expression of autolysis enzymes can also have a consequence on cell autolysis.

2.3.1. Cell Autolysis Caused by Cannibalism

The phenomenon of "kill one's kind" in *B. subtilis* was reported by Pastor et al. [30]. They found that *B. subtilis* cells forming spores are prone to cannibalism, whereby the bacteria delay the formation of spores in other cells by cannibalism. The nature of cannibalism is that *B. subtilis* that have entered sporulation can produce and export a killing factor and a signaling protein. Synergistic action prevents other sister cells from forming spores and causes sister cell autolysis. Immediately after Allenby et al. [31] discovered the significance of the phenomenon of cannibalism, when *B. subtilis* was subjected to phosphate-deficient conditions, the harsh conditions significantly induced the lysis of non-spores, thus providing a good source of nutrients for other cells. To test this idea, the researchers transferred *B. subtilis* to a carbon-deficient medium and the bacteria underwent rapid cell autolysis [31,32]. Nandy et al. [33] revealed that this crucial regulatory protein of *B. subtilis*, Spo0A, which controls spore development, uses a certain antimicrobial factor to lyse *E. coli* cells under its regulation.

Gonzalez-Pastor et al. [34] analyzed the nature of *B. subtilis* "conspecific" and concluded that those cells entering the spore pathway produce and export a killing factor (SkfA) and a signal peptide protein (SdpC). The genes encoding these two proteins are located on the *skfA* operon and the *sdpC* operon, respectively, where the *skf* operon element is responsible for the output of SkfA and the product of immune function against SkfA [30]. Together, *skfA* and *sdpC* cause sister cell autolysis and prevent the formation of spores. The processing and secretion of the *sdpC* gene also produces a sporulation-delaying protein (SDP). SDP is a peptide toxin that kills cells outside the biofilm to support continued growth. SDP rapidly collapses the proton motive force (PMF) of *B. subtilis*, which induces dramatic autolysin-mediated lysis [35,36].

The *sdpC* operon is also expressed in Spo0A-active cells, producing a signaling protein (called delaying the formation of spore protein, SdpC) that causes transcription of the *yvbA* gene into the protein YvbA. The YvbA protein turns on the operon containing the ATP synthase gene (*atp*) and the lipid catabolism inhibitor gene (*yusLKJ*), thereby delaying the entry of Spo0A-activated cells to delay entry into the bud formation process. However, in Spo0A-inactivated cells, SdpC signaling protein also induces the production of YvbA protein, which promotes the death of Spo0A-inactivated cells by inhibiting a σ^W factor. The opened element of the metabolic inhibitor gene (*yusLKJ*) decomposes, thereby delaying the entry of Spo0A-inactivated cells into the formation process of spores. However, in Spo0A-inactivated cells, the SdpC signaling protein also induces the production of YvbA protein, which promotes the death of Spo0A-inactivated cells by inhibiting a σ^W factor. (This σ^W factor is associated with antibiotic resistance and detoxification and protects cells from lysis by killing aspects.) Thus, in Spo0A-inactivated cells, YvbA assists the killing factor in causing cell autolysis. The final result of the above process is that the nutrients released by the dead cells are used as food for the Spo0A-activated cells, keeping the Spo0A-activated cells growing instead of entering the formation process of spores [34].

2.3.2. Gene Mutations Cause Cell Autolysis

In *B. subtilis*, the expression of autolysis enzymes is regulated by a variety of genes. However, with the use of *B. subtilis* in industrial development, many of the mutants produced by genetic modification to improve fermentation yield are more prone to autolysis because they disrupt this regulatory balance. According to Kodama, the *B. subtilis* gene mutant is an excellent host for exogenous protease synthesis [37]. Stephenson [14] reported in the early years that mutants deficient in the extracellular proteases NprE and AprE were more likely to increase the intracellular hydrolysis of *B. subtilis* between the transition and stabilization phases of batch cultures of the bacterium. Afterward, the Wong group [38] constructed deletion mutants of eight major exogenous protease genes in *B. subtilis*. Although the production of cellulase was improved in this mutant, the cells were highly susceptible to autolysis during culture. In recent studies, a number of other genes have also been found to be associated with autolysis. Palomino et al. [39] found that high salt and *pbpE* mutations lead to cell wall defects. The resulting mutant peptidoglycan shows increased solubility and sensitivity to mutant autolysins. Perez [40] found that *spoVG* mutations decreased and *spoVS* mutations increased *sigma*(*D*)-directed gene expression, causing cell separation and autolysis.

2.4. Other Factors That Induce Autolysis

Autolysis of bacterial cells is readily caused by the severe circumstances to which bacteria are exposed, including nutritional deficits. Such harsh conditions act as an inducing factor, resulting in the cannibalism mentioned above. For example, Allenby et al. [30] reported that when *B. subtilis* was subjected to phosphate-deficient conditions, the harsh conditions significantly induced transcription of the *skfA* operon element in *B. subtilis*, which made it enter a state of cannibalism, leading to cell autolysis. It is worth adding that Jolliffe et al. [41] discovered that adding chemicals that distribute charge or pH gradients to *B. subtilis* cells might lyse the bacterium.

3. *B. subtilis* Key Autolysins Enzymes

From the above studies, it is clear that autolysis in *B. subtilis* is ultimately caused by autolysins. The final results of various evoked autolytic factors are stimulated autolysins expression. Therefore, the research conducted on autolysins is the key to solving the autolysis problem. *B. subtilis* produces several hydrolases during the trophic growth phase [42–44]. Smith et al. [45] nicely summarized the autolysins associated with *B. subtilis* growth and development. Based on the specificity of autolysins' hydrolysis of *B. subtilis* cell wall peptidoglycan and the specificity of the hydrolytic chemical bonds of the autolysins, autolysins and peptidoglycan hydrolases can be classified as muramidase, acetyl-glucosaminidase, acetylmuramoyl alanine amidase, and endopeptidase. The functions of several of the most critical enzymes and their mechanisms of action in autolysis are detailed in Figure 3.

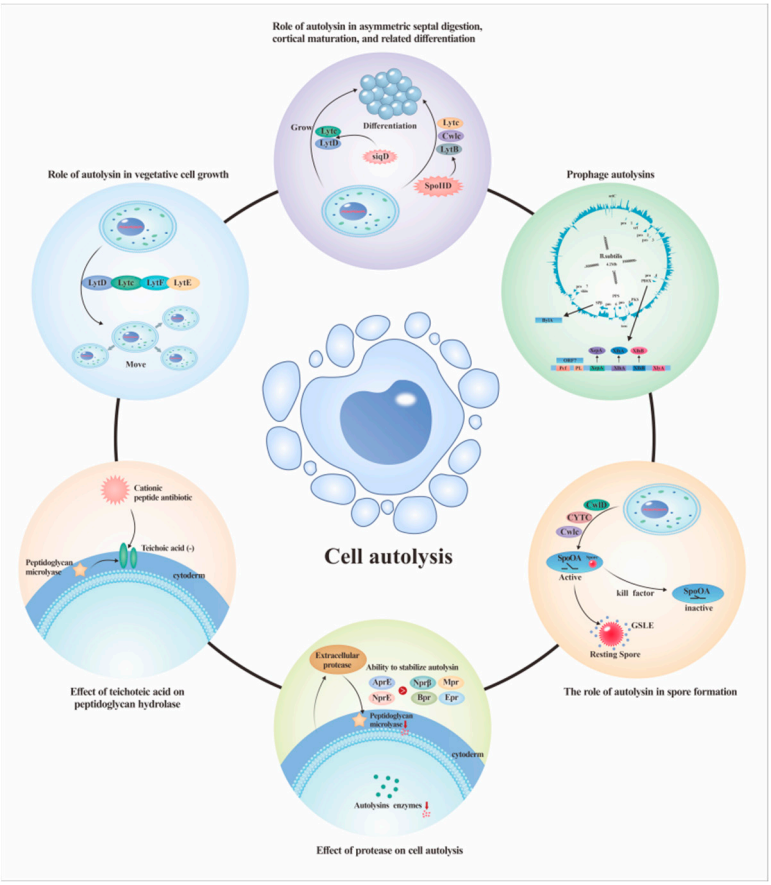

Figure 3. The role of key autolysis enzymes of *B. subtilis* in life processes and the factors affecting them.

3.1. Analysis of the Mechanism of Action of Related Enzymes

The respective functions of the life cycle of *B. subtilis* autolysis enzymes are demonstrated in Figure 3. Among these hydrolases, N-acetylmuramoyl-L-alanine amidase (amidase, LytC or CwlB) and N-acetyl-β-glucosaminidase (glucosaminidase, LytD or CwlG) are two of the significant autolysins [46–48]. During the nutritional development stage of bacteria, they carry the majority of the autolysis activity. Inactivation of LytC and LytD show that these two autolysins bear 95% of the autolytic activity of the cells [49,50]. In addition to the main autolysins described above, other less significant enzymes were discovered individually in nutritional cell surface extracts, including LytF (CwlE) and LytE (CwlF) [51–53]. Table 1 summarizes the peptidoglycan hydrolase-related genes. People are still researching the roles of various additional, less significant, but potentially vital autolysins. Different groups studying the functions of the above autolysins have come to different conclusions due to subtle differences in strain genetic background and experimen-

tal conditions. Still, they all have the same impression about these autolysins. There is a high degree of functional redundancy in these various multifunctional autolysins.

3.1.1. The Role of Autolysins in the Formation of Spores

Autolysins aid cell differentiation. Regarding *B. subtilis* differentiation activities, including the formation of spores and germination, which need substantial peptidoglycan rearrangements and alterations, almost all of them are affected by autolysins [54,55].

Even though spores have considerable resistance and dormancy, they possess a stress-sensitive system that reacts to germination agents. Maternal cell autolysis aids in the dispersion of mature spores to a more favorable environment. Foster et al. [56] found the presence of a 30 kDa sized protein associated with maternal cell autolysis in the formation of spores that are well expressed before maternal cell autolysis occurs. It is presumed to be primarily responsible for maternal cell autolysis. Then, Kuroda et al. [57] cloned and identified the gene responsible for encoding this protein and named it "*cwlC*." They tested the function of the *cwlC* deletion mutant. They showed that the mutant did not undergo significant phenotypic changes in shoot cell formation, germination, and shoot resistance to heat and autolysins. Moreover, in the *cwlC* mutant, the mother cell still underwent cell autolysis. They also found that CwlC proteins hydrolyze both the nutrient cell wall peptidoglycan of *B. subtilis* and the peptidoglycan of spores. Mother cell autolysis required for the release of mature endosperms during the formation of spores is mainly dependent on two amidases of the LytC family, CwlC and LytC; CwlC and LytC play complementary roles in hydrolyzing the cell wall peptidoglycan of mother cells [44,56].

In addition, endospore formation in *B. subtilis* produces a germination-specific lyase (GSLE) that can only be isolated from germinating spores in its active form and can only hydrolyze the stress peptidoglycan of permeabilized, undamaged spores in vitro. GSLE is present in dormant spores as an inactive preprotein [58].

3.1.2. Role of Autolysins in the Digestion of the Asymmetric Septum, Cortical Maturation, and Related Differentiation Processes

The *B. subtilis* autolysins play an essential role in asymmetric septum digestion, cortical maturation, and related differentiation processes [15]. *spoIID* is involved in the digestion of the asymmetric septum, as strains carrying mutations in this gene are found when the septum is only partially digested and remains in an encircled state around the cell. *spoIID* encodes a modified protein similar to the LytB sequence. Studies claim that *spoIID* may control the movement of an autolysin to digest peptidoglycan from asymmetric septa [59].

3.1.3. The Role of Autolysins during the Vegetative Phase

The main autolysins during the vegetative phase of *B. subtilis* are LytC and LytD; the *lytC* gene encodes LytC, and *lytC* is located within the operon element *lytLABC* [50]. *lytC* is active at the end of the logarithmic growth phase of *B. subtilis*, and this autolytic activity is maintained until late in the stabilization phase. It remains active even during the formation of spores (referred to in Section 3.1.1 mentioned above). LytC and LytD are the main autolysins produced by *B. subtilis* during growth. Both of them are transcriptionally regulated by *sigD*. *sigma D* is expressed during vegetative growth and is associated with exponential growth and transcription of genes expressed during early stationary periods [60,61]. Furthermore, both proteins are required for bacterial motility. Knocking out the *lytC* gene in *B. subtilis* ATCC 6051 increased bacteriophage density and amylase activity by 1.6-fold. *lytC* and *lytD* deletion also inhibited cell lysis, but the inhibition was less than in the *lytC*-only mutant.

lytE and *lytF* encode peptide chain endopeptidases and are involved in bacterial segregation. Deleting *lytE* and *lytF* alone resulted in mutants with defective growth segregation (division or separation). Still, the inhibitory effect on cell autolysis was not significant. In addition, during cell growth, cells must expand covalently closed macromolecules of the cell membrane [62]. *B. subtilis* contains two functionally redundant D, L-endopeptidases

(CwlO and LytE) that cleave peptide crosslinks to allow expansion of PG (the cell wall peptidoglycan) [63]. Double deletion of *lytE* with the *cwlO* mutant, which affects cell length increase, leads to a bacteriophage lethal effect [64].

3.2. Prophage Autolysins

In addition to the numerous autolysins involved in the growth and development, there are also autolysins from *B. subtilis* prophage. When the expression of autolysin encoded by prophages (*PBSX*) is induced, pores with auxiliary or unknown functions are produced in the peptidoglycan cell wall. The cytoplasmic membrane material protrudes through these openings and is released as a membrane sac (MV) [65]. The induced cells eventually die due to loss of membrane integrity. The vesicle-producing cell leads to cell autolysis through the enzymatic action of releasing autolysin, which induces the formation of MV in neighboring cells [66]. The study of prophage sequences has been studied since as early as 1997; Presecan [67] identified many potentially non-essential chromosomal motifs in the whole genome nucleotide sequence of *B. subtilis*, particularly 10 relatively large AT-rich regions representing known prophages and prophage-like regions scattered across the genome. Analog sequences (*pro*1-7) are obtained by horizontal gene transfer [68–70]. Prophage *PBSX*, *spβ*, and *skin* remain lysogenic [17,71,72]. Lysogenic phages usually encode peptidoglycan hydrolases, called prophage autolysins. Under certain conditions, it can lyse host cell walls and release phage particles after replication.

3.2.1. Prophage Autolysins PBSX

PBSX, a phage-like phibacin of *B. subtilis* 168, carried by bacteria can be induced to lyse DNA-damaging agents and produce *PBSX* particles; however, these particles cannot transmit the *PBSX* genome [73]. This suicide reaction produces particles that kill non-lysogenic strains of *PBSX*. The prophage *PBSX* includes *xlyA*, which encodes the peptidoglycan hydrolase XlyA mentioned above, *xepA* encodes an export protein XepA, *xhlA* encodes a membrane-associated protein XhlA, and *xhlB* encodes a perforin protein XhlB. The proteins encoded by the latter three genes play an assisting role in cell autolysis. In subsequent studies conducted by Krogh et al. [74], it was found that a late operon located within *B. subtilis PBSX* was used to express autolysins associated with the prophage *PBSX*, as shown in Figure 3. Four different genes on late operon encode these autolysins proteins [73].

Table 1. Summary of peptidoglycan hydrolase-related genes.

	Gene	Comments	Function	Ref
1	*cwlC*	Amidase, mother cell-specific	Cell separation, spore	[43,55]
2	*lytC*	Amidase, vegetative/sporulation expressed only during vegetative growth	Cell separation, motility, cell lysis, mother cell lysis	[46–48]
3	*lytD*	Glucosaminidase, vegetative	Motility	[48,49]
4	*lytE lytF*	Encoding peptide chain endopeptidase	Participation in bacteriophage isolationCell separation, motility, cell lysis, mother cell lysis	[46–48,50–52]
5	*gslEgslE*	GSLE-related proteinsGSLE-related proteins	Cortex hydrolysisCortex hydrolysis	[57]
6	*spoIIDlytE lytF*	Control of the activity of an autolysin to digest peptidoglycan from asymmetric septaEncoding peptide chain endopeptidase	digestion of the asymmetric septumParticipation in bacteriophage isolation	[50–52,59]
7	*xlyAspoIID*	Amidase, mitomycin C-inducibleControl of the activity of an autolysin to digest peptidoglycan from asymmetric septa	PBSX lysisdigestion of the asymmetric septum	[59,75]
8	*cwlAcwlC*	Amidase, silent gene in the skin elementAmidase, mother cell-specific	Cryptic prophageCell separation, spore	[43,55,76,77]

3.2.2. XlyA Amidase Family

As further research into it, Longchamp et al. [72] divided and identified two peptidoglycan hydrolases of the XlyA amidase family, XlyA and YomC, and found YomC is within the *spβ*, respectively, and belongs to the *B. subtilis* prophage autolysins. Another enzyme from the XlyA family, N-acetylmuramoyl-L-alanine amidase, is an active peptidoglycan hydrolase [75]. The protein N-acetylmuramoyl-L-alanine amidase (CwlA) is related to cell autolysis. Later studies showed that CwlA is an autolysin gene located within the *skin* component of the prophage [76]. Kunst et al. [77] also found that the two open reading frames *yqxH* and *yqxG* immediately adjacent to *cwlA* encode two other proteins, respectively, which they hypothesized to be related to the autolytic activity of CwlA and named them "Yqxh" (similar to holin) and "Yqxg" (similar to phage-related lytic exoenzyme), respectively.

3.2.3. Prophage Autolysins BlyA

Regamey and Karamata [17] identified and characterized a DNA fragment in *B. subtilis* and named it "*blyA*" gene, which encodes a protein with a molecular weight of 39.6 kDa and contains 367 amino acids (BlyA). BlyA exhibited N-acetylcytidyl-L-alanine amidase activity associated with *spβ* phage-mediated cell autolysis. They conclusively demonstrated that BlyA belongs to the prophage *spβ* autolysins and that heat stimulation of *B. subtilis* CU1147 (CU1147 is an *Spβ*c2 lysogenic strain, and *Spβ*c2 is a temperature-sensitive phage) would induce this *Spβ*c2 to express BlyA and lead to cell autolysis.

3.3. The Effect of Phosphopiridic Acid on Peptidoglycan Hydrolase

It has been shown that the peptidoglycan hydrolase associated with *B. subtilis* autolysis is regulated by lipoteichoic acid in the cell wall and the action of extracellular proteases. Lipoteichoic acid (LTA) can absorb Mg2+ to support the activity of several synthetic enzymes in the cell membrane. It also functions as a storage element and regulates the activity of intracell autolysis enzymes [78]. In *B. subtilis*, LTA modification is controlled by the *dltA-E* operon. Previous studies have shown that deletion of the *dltA-D* gene disrupts LTA modification, which further alters the cell surface microenvironment and enhances cell autolysis [79–81].

3.4. Effect of Proteases on Cell Autolysis

B. subtilis produces at least eight extracellular proteases [82]. Some examples suggest that these extracellular proteases are associated with cell autolysis. Jolliffe et al. reported that *B. subtilis* protease mutants have autolysin levels in the cell wall equal to or higher than wild-type strains [35]. Extracellular proteases have been identified as involved in degrading peptidoglycan hydrolases in the cell walls. Extracellular protease deficient strains showed increased lysis, where the extracellular proteases AprE and NprE had a greater ability to stabilize autolysins than NprB, Bpr, Mpr, and Epr [83]. Coxon et al. [84] found that the *B. subtilis* protease deletion mutant had a higher peptidoglycan folding rate while increasing susceptibility to intracell autolysis. In his research, Stephenson found that *B. subtilis* strains with inactivated protease genes exhibited cell autolysis, and mutants lacking multiple extracellular proteases became more susceptible to autolysis [14].

4. Strategies to Inhibit Cell Autolysis in *B. subtilis*

4.1. Inactivation-Associated Autolysins Genes

Inactivating single or few autolysin genes in *B. subtilis* cells becomes the first choice on the road to solving the autolysis problem. The strategies and results for modifying the autolysis aspects are presented in Table 2. Starting from the main control of autolysis gene *lytC*, Kuroda and Sekiguchi et al. [49] reported that *lytC* mutants are resistant to cell autolysis even after 6 days of culture at 37 °C. Similarly, Yamamoto et al. [64] found that inactivation of the *lytC* gene effectively inhibited the cell autolysis in wild-type *B. subtilis* cells and found that it can reduce the cell wall hydrolysis activity in stable cells by approximately 90%. On this basis, Smith and Foster et al. [44] found that dual mutants

of the *lytC* and *cwlC* genes were effective against autolysis of mother cells during late *B. subtilis* bacteriophage formation. Nugroho et al. [85] reported that a double mutant of the *cwlC* and *cwlH* genes inhibited autolysis in *B. subtilis* cells. In addition, other knockout strategies for spore-associated autolysin genes are gradually emerging. Gonzalez-Pastor et al. [34] found that a mutant of a killing factor (SkfA) in *B. subtilis* protects non-spores from lysis by cells forming spores. Kodama et al. [37] found that introducing a significant autolysin gene mutation (*lytC* mutation) into *B. subtilis* mutant strain with a deletion of *spo0A* effectively suppressed cell autolysis caused by the *spo0A* mutation. Chen R. et al. [60] demonstrated that appropriate deletion of autolysin genes could improve recombinant protein yield in extracellular protease-deficient strains, and knockdown of autolysin-related genes *lytC* and *sigD* in tandem with eight extracellular proteases could effectively increase exogenous protein yield and delay autolysis.

4.2. Inactivation Operons and Regulatory Genes

Inhibition of autolysis can also be achieved by regulating the operon of autolysin, phibacin, or other regulatory genes. The *dlt* operon mediates lysophosphatidic acid-D-alanylation, the primary net adverse charge modification process on the cell surface. *dlt* operon deficiency can improve the net negative charge of the cell wall. *dlt*ABCD deletion studied by Chen et al. [78] increased nattokinase, α-amylase, and β-mannanase of *B. subtilis* by 37.13%, 44.53%, and 53.06%.

Genevieve S Dobihal et al. [86] enabled artificial control of *B. subtilis* cell wall hydrolysis by regulating the WalRK two-component signaling pathway, homeostasis of cell membrane cleavage, and elongation. They also found that the cleavage products of PG hydrolases (LytE and CwlO) in the WalR regulon could effectively inhibit WalK signaling, thus offering the potential for therapeutic development.

Zhou et al. [87] knocked down *spo0A*, a regulatory gene regulating *skf* and *sdp*. They found that the knockdown *spo0A* strains showed significantly faster autolysis rate and lower production of exogenous alkaline protease. The results also confirmed the complexity of the regulatory gene *spo0A* in the whole metabolic network of the bacterium, and further exploration of the critical regulators and signaling of the genes related to similar feeding is needed.

In the operon of prophage autolysins, it was reported that inactivation of *Pcf* factor would inhibit the expression of the prophage *PBSX* autolysin genes *xlyA*, *xhlA, xhIB*, and *xepA,* thereby inhibiting the cell autolysis of the bacterium [88].

4.3. Deletion of the Prophage Sequence

As previously stated, prophage sequences include many hydrolytic enzymes related to cell autolysis. Therefore, the elimination of these sequences improves autolysis significantly. Analysis of the genome of *B. subtilis* revealed that 4.2 Mb *B. subtilis* genome contains 10 horizontally obtained prophages (*PBSX* and *spβ*) and prophage-like sequences (*pro*1-7 and *skin)* [89]. In addition, 2.8% of the genome contains two large operons that produce secondary metabolites (*pks* and *pps*). Westes reported a 7.7% (0.53 Mb) reduction in the genome of *B. subtilis* Δ6 mutant strains produced by deleting two prophages (*spβ* and *PBSX*), three primary phage-like sequences (*pro*1, *pro*6, and *skin*), and the *pks* operon [12]. Takuya Morimoto et al. [90] generated a strain MGB469 in which all prophage and prophage-like sequences except *pro*7 and *pks* and *pps* operons were deleted. In this case, cell growth was normal. Li et al. [91] deleted all known prophages (prophage1-7, *spβ*, *skin*, and *PBSX*) on the *B. subtilis* 168 genomes, and the autolysis rate of the deleted strain *B. subtilis* BSK756 was significantly reduced compared to that of the parents, which further confirms that the deletion of the prophage sequence containing autolysin has an ameliorating effect on cell autolysis.

4.4. Tool-Mediated Genome-Wide Editing by CRISPR and Others

As the research of the cell hydrolytic enzymes of *B. subtilis* continues, key genes are being explored and genetic editing is being carried out in large quantities to improve

autolysis and produce a variety of products efficiently. In this process, CRISPR, a tool more suitable for large-scale gene editing, has played an important role. Gerald E. M et al. [92] found that sequential deletion of *skfA*, *sdpC*, and *xpf* genes reduced cell lysis and increased biomass. Zhao et al. [93] inactivated peptidoglycan hydrolase-related genes alone or in different combinations, including *sigD*, *lytE*, *lytF*, *lytC*, *lytD*, and lytG. Compared to *B. subtilis* 168, mutants with multiple gene inactivations (e.g., Δ*sigD* Δ*lytE* Δ*lytD*) exhibited easier sedimentation, significantly increased growth rate, improved sensitivity to antibiotics, and improved α-amylase production. Mutants Δ*sigD* Δ*lytE* Δ*lytD* and Δ*sigD* Δ*lytE* Δ*lytC* Δ*lytD* also showed increased tolerance to the high osmotic pressure of sodium chloride. They allowed unsterilized fermentation, all of which contributed to lower processing costs.

Table 2. Strategies and results of the modification of autolysis aspects.

	Gene	Remodeling Method	Remodeling Results	Ref
1	*skfA*	Deactivation	Protects cells that do not form budding spores from lysis	[29]
2	*lytC*, *cwlC*	Deactivation	The cells were still resistant to autolysis after six days of incubation at 37 °C.	[56]
3	*lytC* + *sigD*	Knockout	Slowed autolysis, improved exogenous protein production	[59]
4	*dlt* operon	Knockout	Nattokinase, α-amylase, and β-mannanase increased by 37.13%, 44.53%, and 53.06%, respectively.	[77]
5	*bylA*, *cwlH*	Deactivation	Reduction of heat stress-induced autolysis	[84]
6	*Pcf*	Deactivation	Inhibition of autolysis genes and thus cell autolysis.	[87]
7	prophage1-7 + *spβ* + *skin* + *PBSX*	Delet	Significantly lower autolysis rate	[88]
8	*sigD* + *lytE* + *lytD* + *lytC*	Knockout	Easier sedimentation, significantly increased growth rate, improved sensitivity to antibiotics, and increased alpha-amylase production. Tolerance to the high osmotic pressure of sodium chloride was improved.	[93]

Liu et al. [94] used *B. subtilis* ATCC6051 as the expression host and deleted eight extracellular proteases (*aprE, nprE, nprB, epr, mpr, bpr, vpr*, and *wprA*), *spoIIAC*, and *srfAC* to produce mutant *B. subtilis* ATCC6051Δ10. The final maximum extracellular PUL activity (625.5 U/mL) showed the highest expression level (stem cell weight 18.7 g/L).

Zhang et al. [95] used *B. subtilis* strain WS5 (already deficient in the protease-encoding genes *nprE* and *aprE*) as a starting strain and disrupted the six protease-encoding genes (*nprB, bpr, mpr, epr, vpr*, and *wprA*) in the genome in sequence using the CRISPR/Cas9 system to finally obtain a recombinant strain WS9PUL that achieved 5951.8 U/mL of branched-chain amylase activity.

Wang et al. [11] reduced cell lysis and enhanced biomass by deleting *skfA*, *sdpC*, *xpf*, and *lytC* alone. A multiple deletion mutant LM2531 (*skfA sdpC lytC xpf*) was constructed. After 4 h of incubation, its biomass production was significantly increased compared to the prototype *B. subtilis* 168 (wild type) strain, lysing 15% and 92% of cells in LM2531 and wild-type cultures, respectively. In addition, two expression vectors were constructed under the control of the P43 promoter to produce recombinant proteins (β-galactosidase and nattokinase). Cultures of LM2531 and wild-type transformants had 13,741 U/mL and 7991 U/mL of intracellular β-galactosidase, respectively (1.72-fold increase). In addition, strain LM2531 produced a 2.6-fold increase in secreted nattokinase compared to the wild type (5226 IU/mL and 2028 IU/mL, respectively).

4.5. Minimal Genome of B. subtilis

As more and more autolysis-related genes are deleted, it has been found that "minimal genome" may also be an effective approach for addressing autolysis. Since completing the whole genome sequencing of *B. subtilis*, Kunst et al. [77] found that the construction of the minimal genome of *B. subtilis* has also started to develop rapidly. The *B. subtilis* genome has been deleted from 7.7% in the beginning to 36.5% in the present. The corresponding non-essential genes (e.g., spore, motility, antibiotic synthesis, secondary carbon sources

metabolism, and unknown function genes) have been heavily depleted [96]. The strains obtained by deleting different fragment lengths have various performances. Specialized *B. subtilis* genomic databases have been established, such as DBTBS, SubiWiki, MetaCyc, SubtiList, SubtiPathways, and SubtInteract databases. Researchers can extract the required information from these databases and analyze data, including DNA sequences, metabolic pathways, protein interactions, and gene transcriptional regulation, to enable genomic modification of *B. subtilis* as a chassis host.

5. Conclusions

This review is devoted to the summary of autolysis in *B. subtilis*. We proceed from the mechanism of autolysis in *B. subtilis* (effect of autolysin on the cell wall) to (1) analyze the external and internal causes of autolysis, (2) introduce the currently known autolysis function, and (3) propose the solution measures to resist autolysis in recent decades. Researchers have been working on the autolysis problem of *B. subtilis* because the ability to produce recombinant proteins is a major advantage of using it, and the yield of these heterologous proteins is often tied to cellular biomass. In the case of the β-galactosidase described in the article by Wang et al. [11], wild bacteria, for example, and enzyme production began to decrease along with biomass after 7 h of incubation, while the biomass of the modified autolysis-resistant strain was still increasing and accompanied by enzyme production. Therefore, it can also be further understood that reducing the autolysis problem of *B. subtilis* can also extend the fermentation cycle and meet the needs of energy saving and efficient production. Although the external factors that induce autolysis in *B. subtilis* are largely understood, controlling these factors also provides researchers with a challenge. Moreover, multiple genes control autolysis in *B. subtilis*; therefore, it is currently difficult to accurately assess the specific contribution of each autolysis enzyme in the cell autolysis process. Researchers have been working to inactivate multiple autolysis-related genes simultaneously. With the in-depth study of autolysis enzyme function in *B. subtilis*, more and more regulatory factors controlling autolysis have been discovered and applied, and the study of prophages has led to the gradual implementation of minimal genome construction. In the future, more precise metabolic regulation techniques and holographic analysis will help to overcome the bottleneck of cell autolysis, and by combining techniques and strategies from the histological investigation, laboratory evolution, metabolic engineering, and synthetic biology, *B. subtilis* will be able to maintain stable biomass under high temperature and other environments, especially in industrial large-scale high-density fermentation production. New metabolic engineering tools such as the CRISPR-Cas system will be the mainstay to achieve this [97]. As research advances and bioinformatics, structural biology, and other fields advance, adopting innovative methods will be even more effective in improving the *B. subtilis* autolysis issue. In the future, with the deepening of the autolysis problems investigation and research, the precise autolyzed-related gene research and control, to further improve the fermentation production of *B. subtilis* such as chassis cell production capacity and efficiency, make the training cost reduced in the process of its application which is expected to achieve even closely integrated biomass and yield of high-efficiency production.

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