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CRISPR/Cas9-Based *amyE* Regulatory Engineering for Enhanced α-Amylase Production to Support Sustainable Starch-Rich Industrial Crop Bioprocessing

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ABSTRACT

The sustainable processing of starch-rich industrial crops, such as potatoes, maize, and wheat, is central to the production of value-added products, including bioethanol and high-fructose corn syrup, from these feedstocks. Efficient conversion of crop-derived starch into fermentable sugars relies on robust α-amylase enzymes and their optimal concentrations. Here, we present a proof-of-concept CRISPR/Cas9-based strategy aimed at engineering the regulatory region of the *amyE* gene in *Bacillus subtilis*, a GRAS-certified bacterium widely used for industrial enzyme production. Our modular synthetic construct features combined elements, including a high-strength promoter, multiple ribosomal binding sites, and optimized secretion signal, enabling optimization of α-amylase expression and secretion. The modular plasmid was successfully assembled and validated in *Escherichia coli*, which is used for its rapid cloning capabilities and compatibility with plasmid construction, before transformation into the final *B. subtilis* production host. This integrated engineering approach in a single construct is expected to yield higher extracellular α-amylase titers than native systems, supporting more efficient industrial starch bioprocessing of starch-rich agricultural feedstocks and advancing green technologies for the industrial crop sector. Future work will focus on quantifying α-amylase activity in engineered strains and validating the approach on agricultural substrates to assess industrial scalability.

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1. Introduction

Starch-rich industrial crops such as potato, maize, wheat, and cassava are vital to the global bioeconomy, serving as essential raw materials for food, animal feed, and biorefineries. These crops significantly enhance food security by ensuring the availability of nutritious food and providing energy and protein for livestock (Ibba *et al.*, 2025). Moreover, they are crucial feedstocks for biorefineries that convert biomass into biofuels, biochemicals, and bioplastics, thereby promoting sustainability and reducing reliance on fossil fuels (Dammer *et al.*, 2023). Global starch production surpassed 100 million tonnes in 2023, with corn-derived starch still dominating at 75% of total output, while cassava, wheat and potato starches collectively account for over 20%. The industrial starch market is projected to grow at a compound annual growth rate of 6.3% through 2030, driven by demand for biodegradable plastics and biofuels, which now represent 12% of non-food starch applications (Vilpoux and Junior, 2023). This expansion intensifies pressure to innovate starch processing technologies for improved yield, cost-efficiency, and sustainability. The efficient conversion of starch reserves into fermentable sugars is essential for producing bioethanol and other value-added products such as sweeteners. However, this process is technically challenging due to the semi-crystalline structure of native starch granules (Yu *et al.*, 2019). While conventional acid hydrolysis achieves rapid starch depolymerization under high-temperature, acidic conditions, it often generates undesirable by-products and requires energy-intensive purification (Olawoye *et al.*, 2023). In contrast, enzymatic hydrolysis using microbial amylases (e.g., Bacillus α-

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amylases) enables selective and mild starch degradation with fewer inhibitors, offering a sustainable, environmentally friendly, and energy-efficient alternative to conventional methods. However, this approach typically incurs higher enzyme costs and slower reaction kinetics compared to acid hydrolysis (Singh *et al*., 2024).

Central to this process is α-amylase, an enzyme that catalyzes the hydrolysis of α-(1,4)-D-glucosidic linkages in starch, breaking it down into simpler sugars such as glucose, maltose, and dextrin (Abedi *et al*., 2024). This enzymatic liquefaction is essential for producing glucose syrups, maltodextrin, and other derivatives from starches sourced from crops like maize, cassava, potato, and wheat (Rana *et al*., 2013). The diversity and regulation of α-amylase genes in crops such as potato, exemplified by the StAMY family's role in starch degradation, tuber dormancy, and stress responses, highlight the natural complexity of plant-derived enzyme systems (Duan and Jin, 2024; Hou *et al*., 2019). While plant α-amylases are critical for in vivo starch metabolism, their industrial application faces inherent limitations, including low expression levels, susceptibility to environmental fluctuations, and challenges in large-scale extraction. For instance, bioengineered crops like Syngenta's amylase-expressing corn demonstrate the potential of in planta enzyme production (Urbanchuk *et al*., 2009), yet such approaches often suffer yield penalties or require extensive optimization to avoid deleterious effects on plant growth and likely can be used for specific starch modifications, particularly where post-harvest processing is not feasible (Hebelstrup *et al*., 2015). In contrast, microbial α-amylase production offers unparalleled advantages for industrial crop processing, including rapid scalability, cost-effective fermentation, and the ability to engineer enzymes for specific substrates or process conditions (e.g., high-temperature biorefineries).

Microbial systems circumvent the need for crop-specific tailoring, enabling consistent enzyme supplies for diverse feedstocks (e.g., corn, cassava, wheat) while minimizing energy and water use compared to plant-based extraction. This flexibility is critical for modern biorefineries, which require modular, biomass-dedicated solutions to achieve total valorization of agricultural waste (Ashok *et al*., 2024). Among microbial sources, Bacillus species stand out for their high enzyme yield, robustness under harsh industrial conditions, and amenability to genetic engineering. Engineering *B. subtilis* to overexpress α-amylase enzymes represents a promising strategy to enhance bioprocessing pipelines, enabling more efficient conversion of crop starch into valuable bioproducts (Jujjavarapu and Dhagat, 2019). Amylases derived from extremophiles-including thermophiles, halophiles, alkaliphiles, and others-exhibit exceptional stability under extreme conditions, making them highly suitable for diverse industrial processes. Notably, *Bacillus amyloliquefaciens*, *B. licheniformis*, and *B. stearothermophilus* are recognized producers of thermostable α-amylase, valuable for various industrial crop processing (Gangadharan *et al*., 2020). The incorporation of extremophile-derived α-amylase genes into *B. subtilis*, together with the engineering of regulatory elements controlling their expression, provides an opportunity to optimize both enzyme properties and production levels, resulting in more robust and efficient biocatalysts for industrial applications. CRISPR/Cas9 enables precise, marker-free, and stable chromosomal integration of genes in *B. subtilis*, overcoming the instability and variability often seen with plasmid-based systems. Its high efficiency and capacity for multiplex editing make it ideal for developing robust strains with consistently enhanced protein production (Yuzbashev *et al*., 2023).

B. subtilis 168 , a Generally Recognized As Safe (GRAS) organism, is a preferred host for recombinant protein production, second only to *E. coli*. Its ability to secrete proteins extracellularly simplifies downstream processing. However, significant challenges remain that can limit overall yields. For example, while *B. subtilis* can achieve product yields exceeding 20 g L^{-1} for native enzymes, yields for heterologous proteins are often drastically lower or even undetectable, primarily due to challenges such as proteolytic degradation and the need to identify optimal signal peptides for each target protein, underscoring the necessity for systematic optimization of secretion pathways and host strains (Freudl, 2018; Fu *et al*., 2018). Notably, proteolytic degradation by extracellular proteases can result in the loss of 50–80% of heterologous proteins (Krishnappa *et al*., 2014). While recombinant DNA technology and optimized fermentation parameters (temperature, pH, cultivation duration) are foundational (Farooq *et al*., 2021; van Dijl and Hecker,

2013), advances in strain engineering now enable precise enhancements at the transcriptional, translational, and secretory levels. To enhance recombinant protein production, several key strategies can be employed. These include optimizing promoters, ribosome binding sites (RBS), and signal peptides, enhancing the secretory pathway and chaperones, minimizing the genome, and silencing proteases (Zhang *et al.*, 2020). Among these, promoter engineering is particularly noteworthy because transcription serves as the primary regulatory checkpoint in prokaryotes. Therefore, optimizing promoters is essential for maximizing protein expression (Jun *et al.*, 2023; Miao *et al.*, 2020; Nataraj and Sudhakaran, 2023). Modifications to promoter regions (e.g., -10/-35 sequences and upstream elements) have dramatically improved protein yields, exemplified by the engineered *B. subtilis* P3510 promoter, which boosted sfGFP fluorescence 195-fold and elevated β-galactosidase to 30–43% of total cellular protein as well as extracellular proteins like methyl parathion hydrolase and chlorothalonyl hydrolytic dehalogenase (Zhou *et al.*, 2019). Similarly, optimizing spacer lengths (e.g., 17-nucleotide between -35/-10 regions) and incorporating sequences like ATG at the -15 position enhanced promoter strength by over 100-fold (Phan *et al.*, 2012; Song *et al.*, 2016).

Engineering the RBS is another important strategy for boosting protein expression. Eight RBS sequences (20–24 bp) with the Shine-Dalgarno sequence GGAGG were tested in *B. subtilis*, yielding 50- to 600-fold higher GFP expression (Guiziou *et al.*, 2016). The optimal Shine-Dalgarno sequence TAAGGAGG, with a 7–9 nucleotide spacer to the start codon, further improved expression (Volkenborn *et al.*, 2020). Additionally, using multiple RBSs in *B. licheniformis* increased GFP expression to over 50% of total intracellular protein, with an optimal 8-nucleotide spacer and 15–21 nucleotides between adjacent Shine-Dalgarno sequences (Zhang *et al.*, 2022). Replacing the start codon GUG with AUG also enhanced expression efficiency (Zhang *et al.*, 2022). Signal peptide optimization also enhances protein secretion. Screening 173 *B. subtilis* signal peptides identified SPyojl as the most effective, which, combined with random mutagenesis of the α-amylase gene and chaperone overexpression, achieved a record-high α-amylase activity of 1.92 U ml^{-1} in *B. subtilis* after 92 hours (Yao *et al.*, 2019). These successes highlight the potential of integrating transcriptional enhancement with translational and secretory optimizations. While the simultaneous engineering of promoters, ribosome binding sites (RBS), and signal peptides has been shown to be a promising strategy for increasing recombinant protein production, only a limited number of studies have combined the optimization of all these regulatory elements in *B. subtilis*. For example, the combination of an engineered dual promoter and a highly efficient signal peptide in *B. subtilis* has been reported to enhance the production of aminopeptidase (Guan *et al.*, 2016) and alkaline serine protease (Liu *et al.*, 2019).

Additionally, the engineering of strong promoters together with other regulatory sequences, such as the Shine–Dalgarno sequence, has improved reporter protein production (Jun *et al.*, 2023). A systematic combinatorial approach was used to optimize RBSs in conjunction with signal peptides and engineered promoters, resulting in a 7.5-fold increase in the activity of Serratia marcescens nonspecific endonuclease compared to the control (Li *et al.*, 2022). These studies have often focused on specific proteins, and comprehensive approaches that simultaneously optimize promoters, RBSs, and signal peptides for broader applications in *B. subtilis* remain scarce. Therefore, simultaneous engineering of all key regulatory elements for enhanced recombinant protein production in *B. subtilis* remains an ongoing area of research.

We hypothesized that CRISPR/Cas9-mediated simultaneous engineering of the promoter, ribosome binding site (RBS), and signal peptide would synergistically enhance α-amylase production in *B. subtilis* 168, creating a scalable system for industrial biocatalyst development. Our objectives were to: (1) generate the synthetic promoter NBP351015 by introducing an ATG sequence at the -15 position of the non-inducible NBP3510 promoter; (2) optimize AmyE translation efficiency using five enhanced RBS variants; and (3) integrate the SPyojL signal peptide to improve secretion. To test this, we designed a synthetic construct combining the NBP351015 promoter, multiple high-efficiency RBSs, and SPyojL signal peptide, which was cloned and validated in *E. coli*. This study demonstrates the assembly and PCR validation of a CRISPR/Cas9 editing construct targeting the *amyE*

locus. While sequencing validation remains pending, the construct provides a foundation for future transformation into *B. subtilis* 168 to edit native regulatory elements. Subsequent work will analyze α-amylase expression and characterize production efficiency on both defined substrates and starch-rich crops like potato, maize, and wheat. These modifications, combined with the future replacement of the native *amyE* coding region with extremophile-derived thermostable variants, aim to yield robust α-amylase enzymes capable of high-performance catalysis under industrial conditions.

By enabling precise, marker-free chromosomal edits, this CRISPR/Cas9-based approach avoids plasmid-associated instability and ensures consistent enzyme expression, critical for scaling industrial processes. Ultimately, this work advances a sustainable framework for microbial production of high-performance α-amylase, directly applicable to biorefinery workflows for converting agricultural feedstocks into biofuels and value-added chemicals.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. subtilis 168 provided genomic DNA for *amyE* flanking regions. *E. coli* TOP10 strain (Thermo Scientific) was used (due to its higher transformation efficiency) for plasmid propagation. Luria-Bertani (LB) medium (25 g L^{-1} liquid, 35 g L^{-1} solid) was used, supplemented with kanamycin (50 μg mL^{-1}) or ampicillin (100 mg L^{-1}) for *E. coli* selection, and X-Gal (40 μg mL^{-1}) for blue-white screening. Strains were propagated in LB liquid medium (with 220 rpm agitation) and on LB agar plates at 37°C. The CaCl2 heat-shock method was employed for *E. coli* TOP10 strain transformations (Casali and Preston, 2003).

2.2. Plasmids and primers

The CRISPR plasmid pJOE8999 (Altenbuchner, 2016), featuring kanamycin resistance and a temperature-sensitive pE194ts origin for *B. subtilis* replication, was selected for its ability to enable plasmid curing at 42°C and single-vector integration of Cas9 (controlled by a mannose-inducible promoter), sgRNA (via modular *Bsa*I sites for spacer insertion), and homology templates (via *Sfi*I sites for ordered assembly), streamlining CRISPR-Cas9 editing in *B. subtilis*. The pJET1.2/blunt vector (CloneJET PCR Cloning Kit, Thermo Scientific) was assembled with the editing template. The primers (Table 1) were designed using Gene Runner and Primer 3 (Kõressaar *et al.*, 2018), with sequences from NCBI and SubtiWiki, verified by Primer BLAST and OligoAnalyzer.

Table 1. sgRNA and primer sequences used in this study

Primer name	Sequence (5' to 3')	Tm (°C)
Vector CRISPR F	TTTCCTTTTTGCGTGTGATG	53
Vector CRISPR R	ACGCATTGATTTGAGTCAGC	54
amyE_sgRNAF	TACGAAATTCTCCAGTCTTCACAT	54
amyE_sgRNAR	AAACATGTGAAGACTGGAGAATTT	53
amyE-LF	AAGGCCAACGAGGCCTGATCGG	66
amyE-LR	AAGAAGACGAGAAGTCAAAACAAC TTGGCAGAGTG	64
amyE-ProF	AAGAAGACGACTTCTCAAAGATCCC	57
amyE-ProR	AAGAAGACCCGCGGGTGCTG	62
amyE-RF	AAGAAGACACCCGCGGAAGCAGAA ACGGCGAAC	70
amyE-RR	AAGGCCTTATTGGCCAGTTAGAC	61
pJET-F	CGACTCACTATAGGGAGAGCGGC	66
pJET-R	AAGAACATCGATTTTCCATGGCAG	60

2.3. The amyE targeting _sgRNA design and cloning

A 20-nucleotide gRNA targeting the *ycgB-amyE* intergenic region was designed using CHOPCHOP (https://chopchop.cbu.uib.no/) (based on default criteria), synthesized, and annealed. The pJOE8999 plasmid was digested with *BsaI* (NEB) at 37°C for 10 h, heat-inactivated at 65°C for 20 min, and ligated with *amyE* targeting _gRNA using T4 DNA Ligase (Bio Basic) at 16°C overnight. Ligation products were transformed into *E. coli* Top10 via heat shock method, screened on LB-kanamycin-X-Gal plates, and verified by colony PCR using Vector CRISPR F/R primers.

2.4. Synthetic fragment design and assembly

The NBP351015 promoter was derived from the NBP3510 promoter (Zhou *et al.*, 2019) by introducing an ATG sequence at the -15 position. A synthetic fragment comprising the NBP351015 promoter, the SPyojL signal peptide (Yao *et al.*, 2019), and five ribosomal binding sites (RBSs; TAAGGAGG Shine-Dalgarno, 8-nucleotide spacer to GTG start codon, 15–21 nucleotides between adjacent RBSs (Zhang *et al.*, 2022)) was synthesized by GenScript. Flanking regions of the *amyE* gene (left: 782 bp; right: 751 bp) were amplified from *B. subtilis* 168 genomic DNA using primers amyE-LF/LR and amyE-RF/RR using Q5 High-Fidelity DNA Polymerase (New England Biolabs). Fragments were digested with *Bbs*I (New

England Biolabs), ligated via Golden Gate assembly (T4 DNA Ligase, 25 cycles: 37°C for 5 min, 16°C for 10 min), and cloned into pJET1.2/blunt (Thermo Fisher Scientific). The resulting constructs were transformed into *E. coli* TOP10, selected on LB agar with ampicillin (100 µg mL^{-1}), and verified by colony PCR using primers pJET-F/R.

2.5. CRISPR construct assembly

The pJOE8999-gRNA and pJET-editting template were each digested with *Sfi*I (New England Biolabs) at 65 °C for 16 hours. Following digestion, the vector and editing template were purified using phenol-chloroform extraction. The purified DNA fragments were then ligated using T4 DNA ligase (Biobasic) at 22 °C for 3 hours. The ligation mixture was transformed into *E. coli* TOP10 competent cells. Correct assembly of the CRISPR construct was confirmed by Colony PCR.

2.6. Rationale and application context

The CRISPR-Cas9-based genetic modification described in this study is specifically designed for application in processing starch-rich industrial crop residues, such as those derived from potato, maize, wheat, and cassava. This focus aligns with the growing demand for efficient enzymatic conversion of agricultural biomass into fermentable sugars for biofuels, sweeteners, and bioplastics. While current work focuses on vector construction, future work will evaluate the activity and efficiency of the enhanced α-amylase in edited *B. subtilis* on real crop-derived substrates, including potato and corn starch, to validate its industrial applicability.

3. Results and discussion

3.1. In silico promoter optimization for enhanced α-amylase expression in B. subtilis *168*

Strong promoters are crucial for biotechnological and industrial crop processing. Constitutive promoters such as P43 promoter are widely utilized in *B. subtilis* and are known for their ability to drive high levels of protein expression, making them advantageous for large-scale protein production (Yang *et al.*, 2013; Zhou *et al.*, 2019). However, its application can lead to significant challenges, including altered expression patterns that may result in protein aggregation and misfolding. The *Pylb* promoter in *B. subtilis* has demonstrated remarkable efficiency in inducing high levels of protein expression during the transition from the late log phase to the stationary phase. This promoter has been shown to outperform the widely utilized P43 promoter, particularly in the overexpression of proteins such as active β-galactosidase, EGFP, RFP, pullulanase, and organophosphorus hydrolase (Xu *et al.*, 2020; Yu *et al.*, 2015; Zhou *et al.*, 2019).

To maximize α-amylase production for industrial crop starch processing, we aimed to select an optimal promoter to enhance amyE expression in *B. subtilis* 168. For this purpose, we analyzed a *B. subtilis* microarray dataset (NCBI Accession No. GSE19831), which contains expression data for 4,169 genes across 40 time points representing distinct growth phases, building on prior studies (Blom *et al.*, 2011; Yu *et al.*, 2015). The *Pylb* expression profile was compared to that of the *amyE* gene to inform promoter selection (Fig. 1). Similar expression pattern of *amyE* and *Pylb* during specific growth phases supports the potential of *Pylb*-derived promoters for effective *amyE* expression.

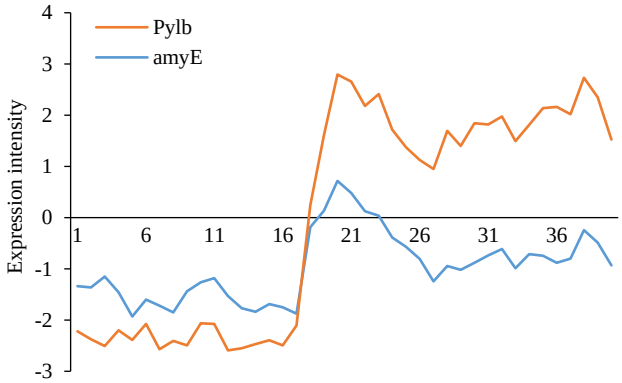

Figure 1. Similar expression pattern of *Pylb* and *amyE* in *B. subtilis* 168. Comparison of transcript levels showing co-regulation of the *Pylb* and the *amyE* gene. The y-axis represents expression intensity across multiple time points (x-axis). Data were sourced from Yu et al. (2015).

3.2. Synthetic regulatory module assembly

The synthetic regulatory module, comprising the NBP351015 promoter, SPyojL secretion signal, and five optimised ribosomal binding sites, was successfully assembled and cloned. The construct was validated in *E. coli*, confirming the feasibility of this modular engineering approach.

3.3. Optimized NBP351015 promoter designed for superior α-amylase production in B. subtilis *168*

To enhance α-amylase (*amyE*) production in *B. subtilis* 168, we optimized the high-performance Pylb

promoter, selected for its superior activity (Zhou *et al*., 2019). The Pylb promoter's -10 and -35 regions had been modified to their consensus sequences, and its upstream region was engineered to create the strong, non-inducible NBP3510 promoter, which drove intracellular expression of sfGFP (195-fold higher fluorescence than the wild-type promoter) and β-galactosidase (BgaB; 30–43% of total cellular protein) and enhanced extracellular expression of methyl parathion hydrolase (MPH) and chlorothalonil hydrolytic dehalogenase (Chd) (Zhou *et al*., 2019). Building on findings that an ATG sequence at the -15 position, particularly when combined with -10 and -35 consensus sequences, increases promoter strength and β-galactosidase activity over 100-fold (Phan *et al*., 2012), we replaced the CGT sequence at the -15 position of NBP3510 with ATG, generating the synthetic NBP351015 promoter. This promoter was combined with five ribosomal binding sites (RBSs; TAAGGAGG Shine-Dalgarno, 8-nucleotide spacer to GTG start codon, 15–21 nucleotides between adjacent RBSs (Zhang *et al*., 2022) and the SPyojL signal peptide, selected from 173 screened signal peptides in *B. subtilis* (Yao *et al*., 2019), in a GenScript-synthesized fragment (Fig. 2), designed to significantly enhance *amyE* expression.

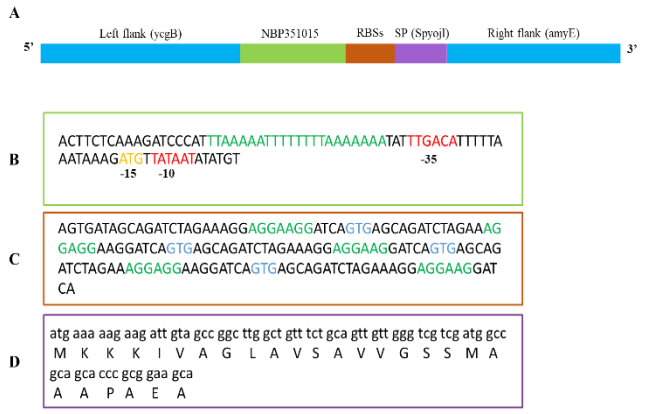

Figure 2. Components of the synthetic regulatory regions for enhancement of *amyE* expression in *B. subtilis* 168. (A) Schematic structure of editing template. (B) Sequence of the new NBP351015 synthetic promoter, including the upstream region (green) and optimized -15 (ATG, orange) and -10 and -35 regions (red). (C) Sequence of multiple ribosomal binding sites (5xRBS, in green) with the optimized GTG start codon (blue). (D) Sequence of the SPyojL signal peptide, selected for optimal secretion.

3.4. gRNA design and CRISPR/Cas9 construction and validation

To enhance *amyE* expression in *B. subtilis* 168 via CRISPR-based editing of its regulatory regions, we designed a 20-nt specific spacer targeting *amyE* using the CHOPCHOP tool. The gRNA sequence was completed by adding complementary overhangs compatible with *BsaI*-digested CRISPR plasmid ends (Fig. 3). Two single-stranded oligonucleotides were annealed through temperature cycling to form a double-stranded fragment, which was ligated into the *BsaI*-digested pJOE8999 plasmid (375 bp and 7423 bp bands; Fig. 4A) and transformed into *E. coli* TOP10.

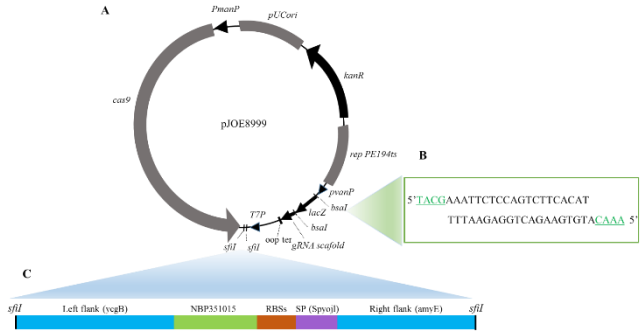

Figure 3. Physical map of CRISPR-Cas9 vector and editing template. (A) Map of the original pJOE8999 CRISPR/Cas9 vector. (B) *amyE*-targeting gRNA spacer sequence designed for this study. (C) schematic structure of the assembled editing template, flanked by *SfiI* sites.

Blue-white screening identified white colonies lacking *lacZ* expression, amplifying a 378-bp fragment in positive colonies, confirming successful *amyE*-targeting sgRNA insertion (Fig. 4B). To confirm the presence of the *amyE* sgRNA spacer in colonies from the ligation (Fig. 4B), colony PCR was performed using Vector CRISPR F/R primers (Fig. 4C). Undigested pJOE8999 plasmid, serving as a negative control, yielded a 725-bp fragment (Fig. 4C).

To assemble the editing template sequence, the synthesized fragment was amplified via PCR using primers amyE-ProF/amyE-ProR. The *amyE* gene's left (782 bp) and right (751 bp) flanking regions were amplified from *B. subtilis* 168 genomic DNA using primers amyE-LF/amyE-LR and amyE-RF/amyE-RR, respectively (Fig. 5A and 5B). After purification, the three fragments (left flank, synthetic fragment, and right flank) were digested with *BbsI*. The digested fragments were ligated using the Golden Gate assembly method using T4 DNA ligase. The resulting construct was cloned into the pJET1.2/blunt plasmid and transformed into *E. coli* TOP10. The transformants carrying the pJET1.2 plasmid were selected on LB agar containing 50 μg mL^{-1} ampicillin (Fig. 5C). Cloning was verified by PCR using primers pJET-F and pJET-R (Fig. 5D). The final 1806 bp amplicon (Fig. 3C and

5D) was confirmed by sequencing and digested with *SfiI*. The digested editing template ligated to the *SfiI*-digested pJOE8999-gRNA vector to generate the final *amyE* pro_CRISPR/Cas9 (Fig. 5E). In summary, we successfully assembled an editing template comprising homology flanking sequences and a novel synthetic regulatory region, which includes an engineered promoter, multiple efficient ribosome binding sites, and an optimised signal peptide. We also demonstrated the successful establishment of a CRISPR vector that, after transformation into *B. subtilis*, will edit its *amyE* regulatory region.

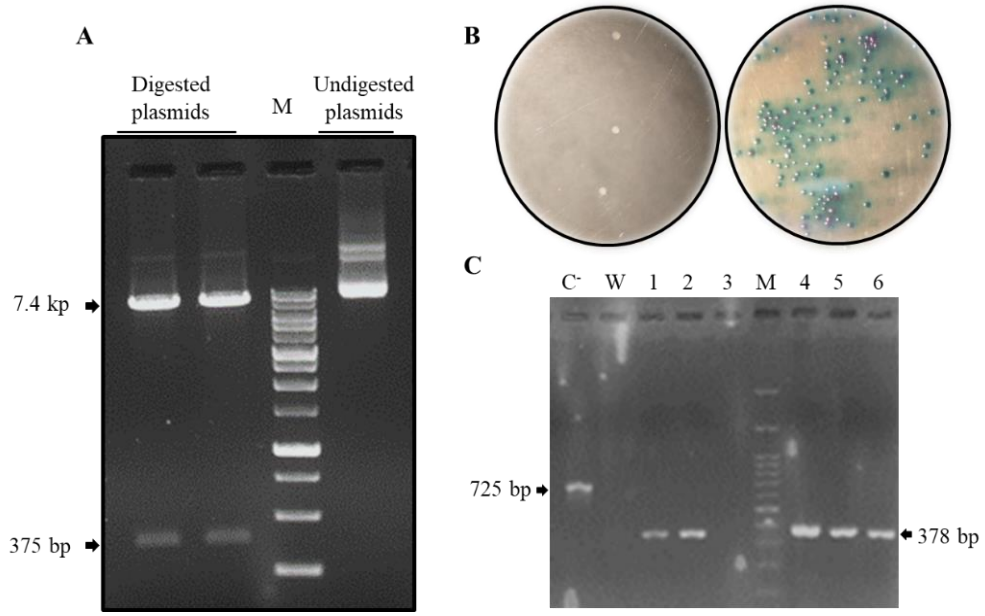

Figure 4. Validation of *amyE*-targeting sgRNA insertion in the pJOE8999 plasmid (A) *SfiI* digestion of the pJOE8999 plasmid, yielding fragments of 7423 bp and 375 bp; M denotes the DM3100 DNA ladder. (B) Blue-white screening showing white colonies lacking *lacZ* expression (left) and blue colonies as a negative control (right). (C) Colony PCR with Vector CRISPR-F/R primers, amplifying a 378-bp fragment in positive white colonies, confirming successful *amyE* sgRNA insertion, and a 725-bp fragment from the undigested pJOE8999 plasmid as a negative control. Sterile water was used as a no-template control (W); M denotes the DM2300 DNA ladder.

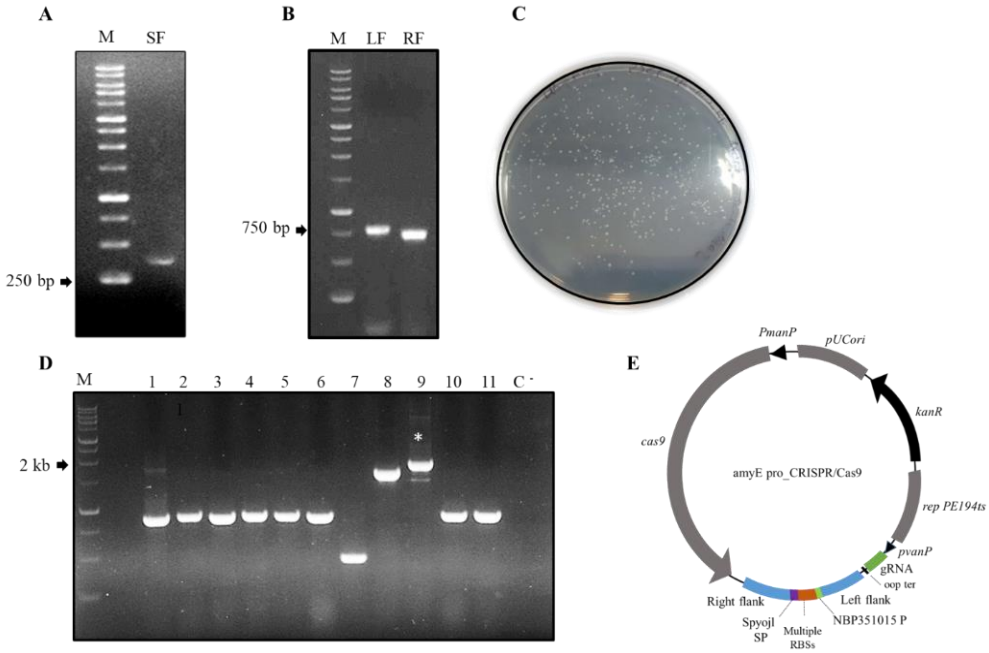

Figure 5. Validation of the assembled editing template in the pJOE8999 plasmid harboring the *amyE*-targeting gRNA (A) PCR amplification of the synthetic fragment (SF, 321 bp). (B) PCR amplification of the left and right flanking regions (LF, 782 bp and RF, 751 bp). (C) LB-ampicillin plates displaying colonies transformed with the pJET1.2 plasmid containing the Golden Gate-ligated construct. (D) Colony PCR validation of the assembled editing template (1806 bp) in selected colonies (The correct band is labeled with an asterisk); M denotes the DM3100 DNA ladder. (E) The schematic map of the final *amyE* pro_CRISPR/Cas9 for engineering of *amyE* regulatory regions.

The development of a CRISPR/Cas9-based strategy for optimizing α-amylase production in *B. subtilis* represents a significant advancement for the bioprocessing of starch-rich industrial crops. By integrating a high-strength promoter, optimized secretion signal, and multiple ribosomal binding sites, our approach addresses key bottlenecks in microbial enzyme production.

Enhanced α-amylase yields will directly benefit the processing of major industrial crops, enabling more efficient conversion of crop-derived starch into sugars for biofuel, sweetener, and biopolymer production. This microbial platform also enhances the use of agricultural residues, supporting circular bioeconomy initiatives and decreasing waste.

The escalating global demand for α-amylase, projected to reach a market value of $2,692.5 million by 2030 (Persistence Market Research, 2023), underscores the need for efficient, scalable microbial production systems. *B. subtilis* 168, with its GRAS status and robust secretion capacity, is a prime candidate for recombinant protein production, yet its native *amyE* expression levels often fall short of industrial benchmarks, such as 1,920 U mL^{-1} reported for optimized systems (Yao *et al.*, 2019). This proof-of-concept study developed a CRISPR/Cas9 construct integrating a synthetic NBP351015 promoter, SPyojL signal peptide, and five optimized ribosomal binding sites to enhance *amyE* expression. Assembly was validated in *E. coli* TOP10, with transformation into *B. subtilis* 168 and α-amylase activity quantification pending.

Our multi-element approach builds on prior single-approach studies. For instance, Zhou et al. (2019) demonstrated that the NBP3510 promoter, a precursor to NBP351015, achieved 195-fold higher sfGFP fluorescence and 30–43% of total cellular protein as β-galactosidase, highlighting its strength. By introducing an ATG sequence at the -15 position (Phan *et al.*, 2012), we further optimized NBP351015, potentially amplifying *amyE* transcription beyond NBP3510's capabilities. Similarly, Yao et al. (2019) identified SPyojL as an optimal signal peptide among 173 candidates, enhancing extracellular protein secretion in *B. subtilis*. The inclusion of five RBSs, with a TAAGGAGG Shine-Dalgarno sequence and optimized GTG start codon (Zhang *et al.*, 2022), likely boosts translation efficiency, as multi-RBS systems have increased protein yields by up to 2.5-fold in related bacilli (Zhang *et al.*, 2022). Integrating these elements via CRISPR/Cas9 to target the *ycgB-amyE* intergenic region is novel and may yield synergistic effects, potentially surpassing single-element optimizations (e.g., 1,500 U mL^{-1} in the study of Xu et al. (2020)).

Despite these advances, limitations remain. The lack of functional *amyE* expression data (e.g., enzyme activity in U mL^{-1}) restricts our ability to quantify improvements over native *B. subtilis* 168 or benchmarks. Preliminary *B. subtilis* transformation indicates integration, but off-target effects or recombination efficiency, common challenges in CRISPR/Cas9 systems (Altenbuchner, 2016), require validation through sequencing and phenotypic assays. Additionally, *B. subtilis*'s proteolytic activity may degrade secreted α-amylase, necessitating protease-deficient strains or further signal peptide optimization (Farooq *et al.*, 2021). This study's multi-approach strategy—combining promoter engineering, signal peptide optimization, and RBS tuning via CRISPR/Cas9—lays a robust foundation for enhancing *B. subtilis* as a microbial cell factory, with broader implications for recombinant protein production in synthetic biology. The engineered regulatory module is designed for targeted integration into the *B. subtilis* genome, enabling high-level secretion of α-amylase. This system will facilitate efficient hydrolysis of starch from industrial crop residues, supporting sustainable conversion of agricultural biomass into fermentable sugars for bioethanol and bioproduct manufacturing.

Future work will focus on deploying the engineered *B. subtilis* strains in bioreactor systems, using real crop-derived substrates to validate enzyme performance and scalability. The modular nature of our construct allows for further adaptation to other industrially relevant enzymes, broadening its impact across the agricultural biotechnology sector.

4. Conclusion

This proof-of-concept study demonstrates the successful assembly of a CRISPR/Cas9 construct integrating NBP351015, SPyojL, and multiple RBSs to enhance *amyE* expression in *B. subtilis* 168. Pending functional validation, this multi-element CRISPR/Cas9-based engineering approach in *B. subtilis* provides a promising microbial cell factory for the sustainable and efficient bioprocessing of starch-

rich industrial crops, supporting the advancement of green technologies in agriculture and industry. Future work will address these gaps by: (1) sequencing the *amyE* locus to confirm precise CRISPR-mediated integration, (2) quantifying α-amylase activity (U mL^{-1}) in shake-flask and bioreactor cultures to assess yield improvements, (3) evaluating protein stability against proteases, and (4) testing scalability under industrial fermentation conditions. While our modular design (simultaneously optimizing promoter, signal peptide, and RBS) enables adaptation to other enzymes or crop feedstocks, scaling this approach may face challenges such as metabolic burden in *B. subtilis* during large-scale fermentation or regulatory constraints for genetically modified organisms in agricultural settings.

Abbreviation

amyE: Alpha-amylase gene; CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR- associated protein 9; GRAS: Generally Recognized As Safe; LB: Luria-Bertani; NBP351015: Synthetic promoter derived from NBP3510; RBS: Ribosomal Binding Site; sgRNA: Single-guide RNA; SPyojL: Signal peptide YojL; X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

Amir Mohammad Ghoorchibeygi performed molecular cloning and drafted the manuscript. Ali Mohammad Banaei-Moghaddam conducted CRISPR/Cas9 construct assembly, data analysis and revised the manuscript. Raheleh Karimi-Ashtiyani supervised the project, designed experiments, and revised the manuscript. All authors approved the final manuscript.

Informed consent

The authors declare not to use any patients in this research.

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References

Abedi E., Kaveh S., Hashemi S.M. 2024. Structure-based modification of a-amylase by conventional and emerging technologies: Comparative study on the secondary structure, activity, thermal stability and amylolysis efficiency. Food Chemistry 437: 137903. https://doi.org/10.1016/j.foodchem.2023.137903

Altenbuchner J. 2016. Editing of the *Bacillus subtilis* genome by the CRISPR-Cas9 system. Applied and Environmental Microbiology 82(17): 5421-5427. https://doi.org/10.1128/AEM.01453-16

Ashok P.P., Dasgupta D., Ray A., Suman S.K. 2024. Challenges and prospects of microbial α-amylases for industrial application: a review. World Journal of Microbiology and Biotechnology 40(2): 44. https://doi.org/10.1007/s11274-023-03821-y

Blom E.J., Ridder A.N., Lulko A.T., Roerdink J.B., Kuipers O.P. 2011. Time-resolved transcriptomics and bioinformatic analyses reveal intrinsic stress responses during batch culture of *Bacillus subtilis*. PLoS One 6(11): e27160. https://doi.org/10.1371/journal.pone.0027160

Casali N., Preston A. 2003. *E. coli* plasmid vectors : Methods and applications (1st ed. 2003. ed.). Totowa N.J.: Humana Press. https://doi.org/10.1385/1592594093

Dammer L., Carus M., Porc O. 2023. The use of food and feed crops for bio-based materials and the related effects on food security: Promoting evidence-based debates and recognizing potential benefits. Industrial Biotechnology 19(5): 257-271. https://doi.org/10.1089/ind.2023.29324.lda

Duan Y., Jin L. 2024. Genome-wide identification and expression profiling of the α-amylase (AMY) gene family in potato. Genes 15(6): 793. https://doi.org/10.3390/genes15060793

Farooq M.A., Ali S., Hassan A., Tahir H.M., Mumtaz S., Mumtaz S. 2021. Biosynthesis and industrial applications of α-amylase:

a review. Archives of Microbiology 203(4): 1281-1292. https://doi.org/10.1007/s00203-020-02128-y

Freudl R. 2018. Signal peptides for recombinant protein secretion in bacterial expression systems. Microbial Cell Factories 17(1): 52. https://doi.org/10.1186/s12934-018-0901-3

Fu G., Liu J., Li J., Zhu B., Zhang D. 2018. Systematic screening of optimal signal peptides for secretory production of heterologous proteins in *Bacillus subtilis*. Journal of Agricultural and Food Chemistry 66(50): 13141-13151. https://doi.org/10.1021/acs.jafc.8b04183

Gangadharan D., Jose A., Nampoothiri K.M. 2020. Recapitulation of stability diversity of microbial α-amylases. Amylase 4(1): 11-23. https://doi.org/10.1515/amylase-2020-0002

Guan C., Cui W., Cheng J., Liu R., Liu Z., Zhou L., Zhou Z. 2016. Construction of a highly active secretory expression system via an engineered dual promoter and a highly efficient signal peptide in *Bacillus subtilis*. New Biotechnology 33(3): 372-379. https://doi.org/10.1016/j.nbt.2016.01.005

Guiziou S., Sauveplane V., Chang H.J., Clerté C., Declerck N., Jules M., Bonnet J. 2016. A part toolbox to tune genetic expression in *Bacillus subtilis*. Nucleic Acids Research 44(15): 7495-7508. https://doi.org/10.1093/nar/gkw624

Hebelstrup K.H., Sagnelli D., Blennow A. 2015. The future of starch bioengineering: GM microorganisms or GM plants?. Frontiers in Plant Science 6: 247. https://doi.org/10.3389/fpls.2015.00247

Hou J., Liu T., Reid S., Zhang H., Peng X., Sun K., Du J., Sonnewald U., Song B. 2019. Silencing of α-amylase StAmy23 in potato tuber leads to delayed sprouting. Plant Physiology and Biochemistry 139: 411-418. https://doi.org/10.1016/j.plaphy.2019.03.044

Ibba M.I., Timsina J., Odjo S., Palacios N., Singh P.K., He X., Alakonya A., Krupnik T.J., Sonder K. 2025. Challenges in the global cereal supply chain. InFood Safety (pp. 245-270). Elsevier. https://doi.org/10.1016/B978-0-12-819340-2.00006-7

Jujjavarapu S.E., Dhagat S. 2019. Evolutionary trends in industrial production of α-amylase. Recent Patents on Biotechnology 13(1): 4-18. https://doi.org/10.2174/2211550107666180816093436

Jun J.S., Jeong H.E., Hong K.W. 2023. Exploring and engineering novel strong promoters for high-level protein expression in *Bacillus subtilis* DB104 through transcriptome analysis. Microorganisms 11(12): 2929. https://doi.org/10.3390/microorganisms11122929

Kõressaar T., Lepamets M., Kaplinski L., Raime K., Andreson R., Remm M. 2018. Primer3_masker: integrating masking of template sequence with primer design software. Bioinformatics 34(11): 1937-1938. https://doi.org/10.1093/bioinformatics/bty036

Krishnappa L., Monteferrante C.G., Neef J., Dreisbach A., van Dijl J.M. 2014. Degradation of extracytoplasmic catalysts for protein folding in *Bacillus subtilis*. Applied and Environmental Microbiology 80(4): 1463-1468. https://doi.org/10.1128/AEM.02799-13

Li Y., Wu Y., Liu Y., Li J., Du G., Lv X., Liu L. 2022. A genetic toolkit for efficient production of secretory protein in *Bacillus subtilis*. Bioresource Technology 363: 127885. https://doi.org/10.1016/j.biortech.2022.127885

Liu Y., Shi C., Li D., Chen X., Li J., Zhang Y., Yuan H., Li Y., Lu F. 2019. Engineering a highly efficient expression system to produce BcaPRO protease in *Bacillus subtilis* by an optimized promoter and signal peptide. International Journal of Biological Macromolecules 138: 903-911. https://doi.org/10.1016/j.ijbiomac.2019.07.175

Miao C.C., Han L.L., Lu Y.B., Feng H. 2020. Construction of a high-expression system in *Bacillus* through transcriptomic profiling and promoter engineering. Microorganisms 8(7): 1030. https://doi.org/10.3390/microorganisms8071030

Nataraj N.B., Sudhakaran R. 2023. Screening of various promoters for increased protein expression: Testing promoters for expression of proinsulin. Journal of Microbiology, Biotechnology and Food Sciences 13(3): e9863. https://doi.org/10.55251/jmbfs.9863

Olawoye B., Jolayemi O.S., Origbemisoye B.A., Oluwajuyitan T.D., Popoola-Akinola O. 2023. Hydrolysis of starch. InStarch: Advances in modifications, technologies and applications (pp. 83-101). Springer, Cham. https://doi.org/10.1007/978-3-031-35843-2_4

Persistence Market Research. 2023. Alpha amylase market. https://www.persistencemarketresearch.com/market-research/alpha-amylase-market.asp

Phan T.T., Nguyen H.D., Schumann W. 2012. Development of a strong intracellular expression system for *Bacillus subtilis* by optimizing promoter elements. Journal of Biotechnology 157(1): 167-172. https://doi.org/10.1016/j.jbiotec.2011.10.006

Rana N., Walia A., Gaur A. 2013. α-Amylases from microbial sources and its potential applications in various industries. National Academy Science Letters 36(1): 9-17. https://doi.org/10.1007/s40009-012-0104-0

Singh R., Jain R., Soni P., de Los Santos-Villalobos S., Chattaraj S., Roy D., Mitra D., Gaur A. 2024. Graphing the green route: enzymatic hydrolysis in sustainable decomposition. Current Research in Microbial Sciences 7: 100281. https://doi.org/10.1016/j.crmicr.2024.100281

Song Y., Nikoloff J.M., Fu G., Chen J., Li Q., Xie N., Zheng P., Sun J., Zhang D. 2016. Promoter screening from *Bacillus subtilis* in various conditions hunting for synthetic biology and industrial applications. PLoS One 11(7): e0158447. https://doi.org/10.1371/journal.pone.0158447

Urbanchuk J.M., Kowalski D.J., Dale B., Kim S. 2009. Corn amylase: Improving the efficiency and environmental footprint of corn to ethanol through plant biotechnology. AgBioForum 12(2): 149-154. https://agbioforum.org/389-2/

van Dijl J., Hecker M. 2013. *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. Microbial Cell Factories 12(1): 3. https://doi.org/10.1186/1475-2859-12-3

Vilpoux O.F., Junior J.F. 2023. Global production and use of starch. InStarchy crops morphology, extraction, properties and applications (pp. 43-66). Academic Press. https://doi.org/10.1016/B978-0-323-90058-4.00014-1

Volkenborn K., Kuschmierz L., Benz N., Lenz P., Knapp A., Jaeger K.E. 2020. The length of ribosomal binding site spacer sequence controls the production yield for intracellular and secreted proteins by *Bacillus subtilis*. Microbial Cell Factories 19(1): 154. https://doi.org/10.1186/s12934-020-01404-2

Xu J., Liu X., Yu X., Chu X., Tian J., Wu N. 2020. Identification and characterization of sequence signatures in the *Bacillus subtilis* promoter P ylb for tuning promoter strength. Biotechnology Letters 42(1): 115-124. https://doi.org/10.1007/s10529-019-02749-4

Yang M., Zhang W., Ji S., Cao P., Chen Y., Zhao X. 2013. Generation of an artificial double promoter for protein expression in *Bacillus subtilis* through a promoter trap system. PLoS One 8(2): e56321. https://doi.org/10.1371/journal.pone.0056321

Yao D., Su L., Li N., Wu J. 2019. Enhanced extracellular expression of *Bacillus stearothermophilus* α-amylase in *Bacillus subtilis* through signal peptide optimization, chaperone overexpression and α-amylase mutant selection. Microbial Cell Factories 18(1): 69. https://doi.org/10.1186/s12934-019-1119-8

Yu X., Xu J., Liu X., Chu X., Wang P., Tian J., Wu N., Fan Y. 2015. Identification of a highly efficient stationary phase promoter in *Bacillus subtilis*. Scientific Reports 5(1): 18405. https://doi.org/10.1038/srep18405

Yu Z., Huang C., Li Y., Du Y., Zhou J. 2019. Influence of the pretreatment method on the hydrolysis of cassava starch factory residue. Environmental Progress & Sustainable Energy 38(2): 624-629. https://doi.org/10.1002/ep.12963

Yuzbashev T.V., Yuzbasheva E.Y., Melkina O.E., Patel D., Bubnov D., Dietz H., Ledesma-Amaro R. 2023. A DNA assembly toolkit to unlock the CRISPR/Cas9 potential for metabolic engineering. Communications Biology 6(1): 858. https://doi.org/10.1038/s42003-023-05202-5

Zhang K., Su L., Wu J. 2020. Recent advances in recombinant protein production by *Bacillus subtilis*. Annual Review of Food Science and Technology 11(1): 295-318. https://doi.org/10.1146/annurev-food-032519-051750

Zhang M., Song J., Xiao J., Jin J., Nomura C.T., Chen S., Wang Q. 2022. Engineered multiple translation initiation sites: a novel tool to enhance protein production in *Bacillus licheniformis* and other industrially relevant bacteria. Nucleic Acids Research 50(20): 11979-11990. https://doi.org/10.1093/nar/gkac1039

Zhou C., Ye B., Cheng S., Zhao L., Liu Y., Jiang J., Yan X. 2019. Promoter engineering enables overproduction of foreign proteins from a single copy expression cassette in *Bacillus subtilis*. Microbial Cell Factories 18(1): 111. https://doi.org/10.1186/s12934-019-1159-0

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