Dual Promoters Enhance Leucine Dehydrogenase Expression and Fermentation in Bacillus subtilis (Postprint)

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Abstract

To promote efficient expression of leucine dehydrogenase in Bacillus subtilis, a strategy was adopted wherein inducible and constitutive promoters were added downstream of the native promoter PHpaII on plasmid pMA5 to investigate the effects of dual promoters on enzyme expression. Two inducible promoters (Pgrac, Pgl-M1) and four constitutive promoters (P43, Plaps, PHpaII, PamyQ) were selected for construction and expression. Among these, the dual promoter comprising the constitutive promoter PamyQ and PHpaII exhibited the best performance, effectively increasing the extracellular activity to 31.24 $U \cdot mL^{-1}$, representing a 3.4-fold improvement over the single PHpaII promoter. Building upon this optimal dual promoter, four signal peptides from the Sec and Tat pathways were individually fused; however, the combination of signal peptides with the dual promoter did not result in higher enzyme activity. The mutant strain exhibiting the highest enzyme activity, Bacillus subtilis 168/pW6 (PHpaII-PamyQ), was selected for enzyme production through fed-batch fermentation in a 7.5 L fermenter, achieving a LeuDH activity of 217.96 $U \cdot mL^{-1}$, which corresponds to a 6.97-fold increase over shake-flask levels, thereby providing valuable reference for industrial production of leucine dehydrogenase.

Full Text

Preamble

Dual-Promoter Strategy to Enhance Leucine Dehydrogenase Expression in *Bacillus subtilis* and Fermentation Study

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Abstract

To promote efficient expression of leucine dehydrogenase (LeuDH) in *Bacillus subtilis*, inducible and constitutive promoters were inserted downstream of the native PHpaII promoter on plasmid pMA5 to investigate the effects of dual-promoter systems on enzyme expression. Two inducible promoters (Pgrac, Pglv-M1) and four constitutive promoters (P43, Plaps, PHpaII, PamyQ) were constructed for LeuDH expression. The dual-promoter combination of PHpaII-PamyQ exhibited the best performance, effectively increasing extracellular activity to 31.24 $U \cdot mL^{-1}$, which was 3.4-fold higher than that achieved with the single PHpaII promoter. Based on this optimal dual-promoter system, four signal peptides from both Sec and Tat pathways were fused individually, but co-expression with signal peptides did not yield higher enzyme activity. The engineered strain *Bacillus subtilis* 168/pW6 (PHpaII-PamyQ) with the highest enzyme activity was selected for fed-batch fermentation in a 7.5 L bioreactor, achieving a LeuDH activity of 217.96 $U \cdot mL^{-1}$, representing a 6.97-fold improvement over shake-flask levels. These results provide valuable insights for industrial-scale production of leucine dehydrogenase.

Keywords: leucine dehydrogenase; *Bacillus subtilis*; promoter; signal peptide; enzyme production; fermentation

Leucine dehydrogenase (EC 1.4.1.9, LeuDH) is an oxidoreductase that plays important roles in catalyzing the production of chiral amino acid pharmaceutical intermediates and in clinical biochemical diagnostics, including neonatal screening for maple syrup urine disease and determination of branched-chain fatty acids and their keto analogs. *Bacillus subtilis* offers several advantages for heterologous protein expression, including ease of cultivation, non-production of endotoxins, reduced inclusion body formation, and direct secretion of expressed products into the extracellular medium, which facilitates downstream purification. These characteristics make it particularly suitable for expressing homologous proteins.

Efficient secretory expression of heterologous proteins in *B. subtilis* typically relies on strong promoters and appropriate signal peptides. Promoters precisely initiate transcription and control the expression level of foreign genes, with constitutive and inducible promoters being the most commonly used types. Constitutive promoters offer strong initiation, require no inducers, enable direct production of target proteins, and reduce costs; examples include P43, PHpaII, Plaps, and PamyQ. Inducible promoters allow regulation of heterologous protein expression at specific stages, offering controllability, rapid response, and high expression levels; examples include Pgrac, Pspac, and Pxyl. Numerous studies have demonstrated that promoter engineering effectively enhances foreign protein expression. For instance, Guan et al. reported that the dual-promoter PHpaII-PgsiB increased aminopeptidase activity by 2.3-fold and 2.2-fold compared to single promoters PHpaII and PgsiB, respectively. Zhou achieved a 53.77% increase in total lipase activity to 60.97 $U \cdot L^{-1}$ using a hybrid tandem

promoter P43-Pgrac. Fusion of appropriate signal peptides downstream of promoters can further enable efficient secretory expression; Xia et al. identified the Bpr signal peptide achieving 86% secretion efficiency for lipase A.

Our research group previously improved LeuDH expression from *Bacillus cereus* ATCC14579 in *B. subtilis* through signal peptide screening. Building upon this work, the present study investigated the effects of dual-promoter systems by adding inducible promoters (Pgrac, Pglv-M1) and constitutive promoters (P43, Plaps, PHpaII, PamyQ) downstream of the native PHpaII promoter on plasmid pMA5. Subsequently, four signal peptides (AmyQ, SacB, PhoD, and YwbN) previously shown to be effective for LeuDH secretion were fused to the optimal dual-promoter PHpaII-PamyQ to identify the best promoter-signal peptide combination. Finally, scale-up fermentation was performed in a 7.5 L bioreactor with the best-performing recombinant strain to evaluate enzyme production levels, providing a reference for industrial-scale LeuDH production.

1.1.1 Strains and Plasmids

The cloning host *E. coli* JM109, expression host *B. subtilis* 168, plasmid pMA5, pMA5-leudh, and pHT43 were preserved in our laboratory. The recombinant plasmids constructed in this study included pW1 (PHpaII-Pgrac), pW2 (PHpaII-Pglv-M1), pW3 (PHpaII-P43), pW4 (PHpaII-Plaps), pW5 (PHpaII-PHpaII), pW6 (PHpaII-PamyQ), pW6I (PHpaII-PamyQ, SP), pW6II (PHpaII-PamyQ), pW6III (PHpaII-PamyQ), and pW6IV (PHpaII-PamyQ).

1.1.2 Reagents

Restriction enzymes, T4 DNA ligase, rTaq DNA polymerase, protein low molecular weight markers, and DNA markers were purchased from TaKaRa (Dalian). DNA gel extraction kits were from Axygen. Gram-positive bacterial DNA extraction kits, peptone, and yeast extract were from Sangon Biotech (Shanghai). All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd.

1.1.3 Culture Media

LB medium (g/L): peptone 10, yeast extract 5, NaCl 10. TB medium (g/L): peptone 12, yeast extract 24, glycerol 5, $K_2HPO_4 \cdot 3H_2O$ 16.43, KH_2PO_4 2.30. Fermentation medium (g/L): peptone 12, yeast extract 24, glucose 10, yeast extract 10, $K_2HPO_4 \cdot 3H_2O$ 16.43, KH_2PO_4 2.30, 1 mmol $\cdot$ L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mmol $\cdot$ L^{-1} $ZnSO_4$, kanamycin 100 μg/mL. SPI medium: SP salt solution supplemented with 1% (v/v) 50% glucose solution and 1% (v/v) 100×CAYE solution. SPII medium: SPI medium supplemented with 1% (v/v) 50 mmol/L $CaCl_2$ solution and 1% (v/v) 250 mmol/L $MgCl_2$ solution. SP salts: 0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% sodium citrate. CAYE (100×): 2% Casamino acid, 10% yeast extract. EGTA (100×): 10 mmol/L EGTA solution (pH adjusted to 8.0 with NaOH during dissolution).

1.2.1 Construction of Recombinant Plasmid pW1 (PHpaII-Pgrac)

Using plasmid pHT43 as template, primers Pgrac-F/R were designed to amplify the Pgrac promoter fragment via PCR. The amplified fragment and plasmid pMA5-leudh were double-digested with NdeI and BamHI, gel-purified, and ligated with T4 DNA ligase overnight at 4°C. The ligation mixture was transformed into *E. coli* JM109. Plasmids from clones were verified by restriction digestion and sequenced by Suzhou Hongxun Biotechnology Co., Ltd. The correctly sized bands and sequence-confirmed plasmid was designated pW1. Recombinant plasmids pW3 (PHpaII-P43), pW5 (PHpaII-PHpaII), and pW6 (PHpaII-PamyQ) were constructed similarly, except that pW3 and pW6 used *B. subtilis* 168 genomic DNA as template with primers P43-F/R and PamyQ-F/R, respectively, while pW5 used plasmid pMA5 as template with primers PHpaII-F/R.

Promoter sequences Pglv-M1 and Plaps were synthesized by Suzhou Hongxun Biotechnology Co., Ltd. and cloned into pMA5-leudh to generate recombinant plasmids pW2 and pW4.

1.2.2 Construction of Recombinant Plasmids pW6I (PHpaII-PamyQ, AmyQ), pW6II (PHpaII-PamyQ, SacB), pW6III (PHpaII-PamyQ, PhoD), and pW6IV (PHpaII-PamyQ, YwbN)

Based on the optimal dual-promoter PHpaII-PamyQ, four signal peptides (AmyQ, SacB, PhoD, and YwbN) previously identified as effective for LeuDH secretion were fused to construct secretory expression plasmids. A SalI site was introduced via overlap PCR to facilitate construction of pW6I. Using *B. subtilis* 168 genomic DNA and synthesized leudh gene as templates, the AmyQ signal peptide and leudh gene were amplified with primer pairs SamyQ-F1/R1 and SamyQ-F2/R2, respectively. The purified fragments were mixed in equal proportions and used as template for fusion PCR with primers SamyQ-F1/R2. The resulting fusion fragment was cloned into plasmid pW6 via BamHI and MluI sites to construct secretory expression plasmid pW6I.

The SalI site introduced in pW6I facilitated subsequent signal peptide replacement. Signal peptides SacB, PhoD, and YwbN were amplified with BamHI and SalI sites at their termini and used to replace the AmyQ signal peptide in pW6I through double digestion and ligation, generating plasmids pW6II, pW6III, and pW6IV.

1.2.3 Preparation and Transformation of *B. subtilis* Competent Cells

A 100 µL aliquot of overnight seed culture was inoculated into 5 mL SPI medium and incubated at 37°C with shaking. When the culture reached late logarithmic phase (OD_{600} 3.0, approximately 3 h), 200 µL was quickly transferred to 2 mL SPII medium and incubated at 37°C, 100 rpm for 1.5 h. Then 20 µL of 100× EGTA solution was added and incubation continued at 37°C, 100 rpm for 10 min. The culture was aliquoted into 1.5 mL tubes (500 µL per tube). Appropriate amounts of plasmid DNA were added, mixed gently, and incubated at 37°C, 100

rpm for 30 min. Tubes were then transferred to a shaker at 250 rpm, 37°C for 1.5 h. Cells were collected by centrifugation at 4000 rpm, supernatant was partially removed, and the pellet was resuspended in the remaining 100 µL and plated on selective agar plates for overnight incubation at 37°C. The detailed protocol was adapted from the doctoral dissertation of Xia Yu at Jiangnan University.

1.2.4 Culture Conditions for Recombinant Strains

Single colonies were inoculated into LB medium (50 µg/mL kanamycin) and cultured overnight. The next day, 4% (v/v) inoculum was transferred to 50 mL TB medium (50 µg/mL kanamycin). For strains containing inducible promoters, IPTG was added to a final concentration of 0.8 mmol/L when OD_{600} reached 0.6–0.8. Strains with constitutive promoters were cultured continuously with periodic sampling.

1.2.5 Enzyme Activity Assay

The reaction mixture (3 mL) contained 20 mM leucine in 0.2 M glycine-KCl-KOH buffer (pH 10.5) and 12.5 mM NAD^+ solution. After equilibration at 37°C for 5 min, enzyme solution was added and mixed. A control without enzyme was prepared simultaneously. Absorbance changes at 340 nm were monitored for 3 min. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol NADH per minute under these conditions. Recombinant enzymes were analyzed by SDS-PAGE for apparent molecular weight estimation.

1.2.6 SDS-PAGE Analysis

Culture supernatants from 48 h fermentation were centrifuged at 4°C, 8000 rpm for 15 min. Thirty microliters of supernatant were mixed with 10 µL of 4× SDS loading buffer, boiled for 10 min, cooled, and centrifuged. The supernatant was loaded onto a 5% stacking gel and 12% separating gel. Electrophoresis was performed in Tris-Glycine buffer (pH 8.3), followed by Coomassie brilliant blue staining.

1.2.7 Determination of Biomass

Culture samples were diluted appropriately and OD_{600} was measured spectrophotometrically. At regular intervals, 15 mL culture was centrifuged at 4°C, 8000 rpm for 10 min. The pellet was washed twice with PB buffer and dried at 105°C to constant weight. A linear correlation between OD_{600} and dry cell weight was established: 1 OD_{600} unit corresponded to approximately 0.41 $g \cdot L^{-1}$ dry cell weight.

1.2.8 Glucose Concentration Measurement

One milliliter of fermentation broth was centrifuged and the supernatant was diluted to bring glucose concentration within the 0–100 $mg \cdot (100\ mL)^{-1}$ range.

Glucose content was measured using a SBA-40C biosensor analyzer. The instrument was calibrated with 25 µL glucose standard solution after automatic washing. When the sampling indicator was steady (not flashing), 25 µL of diluted sample was injected and the displayed value was recorded.

2.1.1 Construction of Recombinant Strains with Inducible Promoters

The inducible promoters Pgrac and Pglv-M1 were inserted downstream of the native PHpaII promoter on plasmid pMA5 to construct recombinant plasmids pW1 (PHpaII-Pgrac) and pW2 (PHpaII-Pglv-M1), respectively, to evaluate their effects on LeuDH expression. The construction strategy is illustrated in [Figure 1: see original paper].

The Pgrac promoter was amplified from plasmid pHT43 using primers Pgrac-F/R. The amplified fragment was digested with NdeI and BamHI and ligated into similarly digested pMA5-leudh. Recombinant plasmids were verified by double digestion and sequencing. The digestion yielded fragments of approximately 180 bp (Pgrac) and 8.6 kb (plasmid containing leudh), as shown in Figure 2: see original paper. Promoter Pglv-M1 was synthesized by Suzhou Hongxun Biotechnology Co., Ltd. and cloned into pMA5-leudh. The verified plasmids were transformed into *B. subtilis* 168 to generate strains *B. subtilis* 168/pW1 (PHpaII-Pgrac) and *B. subtilis* 168/pW2 (PHpaII-Pglv-M1).

2.1.2 Expression of Recombinant Strains with Inducible Promoters

Growth and enzyme activity profiles were determined for strains containing inducible promoters. Growth curves were recorded from the time of inducer addition at logarithmic phase. *B. subtilis* 168/pW1 (PHpaII-Pgrac) reached maximum OD_{600} of 8.06 at 24 h, while *B. subtilis* 168/pW2 (PHpaII-Pglv-M1) reached maximum OD_{600} of 9.12 at 12 h. The shortened lag phase for pW2 may be attributed to maltose serving as a carbon source that accelerated cell growth, as shown in Figure 3: see original paper.

Enzyme activity curves for strains with inducible promoters Pgrac and Pglv-M1 are presented in Figure 3: see original paper. Although Pgrac showed better performance than Pglv-M1, neither achieved the desired regulatory effect. Induction of *B. subtilis* 168/pW1 with 0.8 $mmol \cdot L^{-1}$ IPTG yielded total enzyme activity of 20.58 $U \cdot mL^{-1}$ at 24 h, but extracellular activity remained low at 1.87 $U \cdot mL^{-1}$. Induction of *B. subtilis* 168/pW2 with 2% maltose produced maximum activity at 32 h, yet total activity was only 1.36 $U \cdot mL^{-1}$. Detailed enzyme production data and SDS-PAGE analysis are provided in and [Figure 4: see original paper].

2.2.1 Construction of Recombinant Plasmids with Constitutive Promoters

Constitutive promoters PHpaII, P43, and PamyQ were amplified from *B. subtilis* 168 genomic DNA. PCR products of approximately 302 bp, 312 bp, and 367 bp were obtained, matching theoretical sizes Figure 5: see original paper. After double digestion with NdeI and BamHI, these fragments were ligated into pMA5-leudh to generate dual-promoter plasmids. Restriction digestion verified the correct fragment sizes Figure 5: see original paper, and sequencing confirmed the sequences.

2.2.2 Expression of Recombinant Strains with Constitutive Promoters

The addition of different promoter elements affected both cell growth and enzyme production characteristics. Fermentation profiles revealed that recombinant strains containing P43, Plaps, PHpaII, and PamyQ reached maximum OD_{600} values of 7.32, 7.5, 8.75, and 9.69, respectively, at 24 h. Compared to the control strain *B. subtilis* 168/pMA5-leudh (OD_{600} = 11.47 at 24 h), growth was inhibited to varying degrees, particularly for strains with P43 and Plaps promoters. This inhibition may be due to the composite nature of these promoters, which could impose metabolic burden on the host cells.

Extracellular enzyme activities varied significantly among the four promoters Figure 6: see original paper. PamyQ demonstrated the best performance, achieving 31.24 $U \cdot mL^{-1}$ extracellular activity at 48 h—3.45-fold higher than the original strain. In contrast, P43, Plaps, and PHpaII showed poor regulatory effects, with extracellular activities of only 0.99 $U \cdot mL^{-1}$, 0.32 $U \cdot mL^{-1}$, and 0.80 $U \cdot mL^{-1}$, respectively. While Yang et al. reported that Plaps increased enzyme expression 14-fold compared to P43, this promoter showed minimal effect on LeuDH expression in our study. Detailed enzyme production data are summarized in .

SDS-PAGE analysis of fermentation supernatants [Figure 7: see original paper] revealed that the band corresponding to strain *B. subtilis* 168/pW6 (PHpaII-PamyQ) was significantly more intense than other lanes, confirming that PamyQ provided the most effective enhancement of LeuDH expression. The poor performance of PHpaII, Plaps, and P43 may be attributed to inappropriate spacing between the Shine-Dalgarno sequence and start codon, interference with cellular metabolism, or improper protein folding. The superior performance of PamyQ likely resulted from enhanced transcriptional strength and increased mRNA levels.

2.3.1 Construction and Verification of Recombinant Plasmids pW6I, pW6II, pW6III, and pW6IV

Although the PHpaII-PamyQ dual-promoter system significantly improved LeuDH activity, substantial intracellular protein accumulation was observed. To better investigate the combined effects of promoters and signal peptides on secretory expression, four signal peptides (AmyQ, SacB, PhoD, and YwbN) were fused to the optimal PHpaII-PamyQ dual-promoter, aiming to direct intracellular protein to the extracellular space and increase secreted yields. The construction of secretory expression plasmid pW6I (PHpaII-PamyQ, AmyQ) is illustrated in [Figure 8: see original paper].

The AmyQ signal peptide and leudh gene were amplified as fragments of approximately 96 bp and 1119 bp, respectively. The fused fragment of about 1215 bp matched the theoretical size Figure 9: see original paper. This fragment was cloned into plasmid pW6 via BamHI and MluI sites to construct pW6I. Due to the small size of the AmyQ fragment, double digestion with BamHI and SalI did not produce visible bands, so the signal peptide and target gene were treated as a single unit for verification Figure 9: see original paper.

Plasmids pW6II (PHpaII-PamyQ, SacB), pW6III (PHpaII-PamyQ, PhoD), and pW6IV (PHpaII-PamyQ, YwbN) were constructed by replacing the AmyQ signal peptide in pW6I with SacB, PhoD, and YwbN signal peptides, respectively, using BamHI and SalI sites introduced at both ends of each signal peptide. Double digestion verification of all recombinant plasmids is shown in [Figure 10: see original paper].

2.3.2 Co-expression with Optimal Dual-Promoter and Signal Peptides

Growth and enzyme production profiles were determined for recombinant strains containing both dual-promoters and signal peptides. Strains *B. subtilis* 168/pW6I (PHpaII-PamyQ, AmyQ), *B. subtilis* 168/pW6II (PHpaII-PamyQ, SacB), and *B. subtilis* 168/pW6IV (PHpaII-PamyQ, YwbN) reached maximum OD_{600} values of 7.54, 7.83, and 11.07, respectively, at 24 h. *B. subtilis* 168/pW6III (PHpaII-PamyQ, PhoD) reached maximum OD_{600} of 8.87 at 36 h, with delayed growth likely due to the larger size of the PhoD signal peptide (approximately 153 bp) imposing greater metabolic stress.

Extracellular enzyme activity profiles [Figure 11: see original paper] showed that peak enzyme production did not coincide with maximum cell density, with significant activity increases observed during late fermentation stages. Strain *B. subtilis* 168/pW6IV (PHpaII-PamyQ, YwbN) exhibited the highest activity, reaching 21.76 $U \cdot mL^{-1}$ at 48 h. In contrast, strains pW6I and pW6II showed poor production (0.44 $U \cdot mL^{-1}$ and 1.69 $U \cdot mL^{-1}$, respectively), while pW6III produced 6.49 $U \cdot mL^{-1}$. Detailed activity data are presented in .

Although the YwbN signal peptide with PHpaII-PamyQ dual-promoter showed

the best performance (21.76 $U \cdot mL^{-1}$), this represented a decrease compared to the strain without signal peptide. This reduction may be attributed to altered spacing between the Shine-Dalgarno sequence and the target gene, diminishing promoter strength. However, compared to our previous study where YwbN was fused directly to the native PHpaII promoter, both extracellular activity and secretion efficiency were significantly improved, likely due to enhanced transcription initiation by PamyQ and better compatibility between the PHpaII-PamyQ dual-promoter and YwbN signal peptide.

7.5 L Fermenter Production of LeuDH

The best-performing strain *B. subtilis* 168/pW6 was evaluated in a 7.5 L fermenter to assess growth and enzyme production at pilot scale. Activated seed culture was inoculated at 4% (v/v) into 3 L fermentation medium. Aeration was maintained at 3 $L \cdot min^{-1}$, dissolved oxygen was controlled at approximately 30% by adjusting agitation speed (400–600 rpm), pH was maintained at 7.0 using 50% H_3PO_4 and 30% $NH_4 \cdot H_2O$, and temperature was set at 37°C. Both non-fed and continuous fed-batch strategies were investigated (feeding began at approximately 12 h when glucose was nearly exhausted, at a rate of ~2 $g \cdot L^{-1} \cdot h^{-1}$ glucose) to evaluate their effects on LeuDH production.

Without feeding Figure 12: see original paper, glucose was depleted by 16 h, biomass reached 4.58 g (dry weight)$\cdot L^{-1}$, and extracellular activity was 40.90 $U \cdot mL^{-1}$ at 60 h, comparable to but slightly lower than shake-flask levels. Nutrient limitation during late fermentation negatively impacted cell growth and enzyme production.

With continuous glucose feeding Figure 12: see original paper, both biomass and LeuDH activity increased significantly. Dry cell weight reached 33.29 $g \cdot L^{-1}$ (7.27-fold higher than non-fed culture), and total LeuDH activity peaked at 664.76 $U \cdot mL^{-1}$ at 60 h, with intracellular activity of 446.80 $U \cdot mL^{-1}$ and extracellular activity of 217.96 $U \cdot mL^{-1}$—6.97-fold higher than shake-flask levels.

Summary

The *B. cereus* leucine dehydrogenase gene has been successfully expressed in *B. subtilis* 168, with enzyme activity improved through signal peptide screening in our previous work. This study further enhanced LeuDH expression through dual-promoter and signal peptide strategies, and conducted preliminary pilot-scale fermentation in a 7.5 L bioreactor.

Six different promoters (inducible Pgrac and Pglv-M1; constitutive P43, Plaps, PHpaII, and PamyQ) were inserted downstream of PHpaII. The PHpaII-PamyQ dual-promoter combination provided the most significant enhancement, achieving 31.24 $U \cdot mL^{-1}$ extracellular activity (3.4-fold improvement). Inducible promoters Pgrac and Pglv-M1, induced with 0.8 $mmol \cdot L^{-1}$ IPTG and 2% maltose for 24 h and 32 h, respectively, produced only 1.87 $U \cdot mL^{-1}$ and 0.82 $U \cdot$

mL^{-1} activity, proving unsuitable for LeuDH expression. Four signal peptides (AmyQ, SacB, PhoD, and YwbN) were fused to the optimal PHpaII-PamyQ dual-promoter, with YwbN showing modest positive effects, though none of the combinations further increased LeuDH activity beyond the dual-promoter alone.

Fed-batch fermentation of the best strain *B. subtilis* 168/pW6 in a 7.5 L bioreactor achieved enzyme production of 217.96 $U \cdot mL^{-1}$ (6.97-fold higher than shake-flask culture), providing a valuable reference for industrial-scale LeuDH production.

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Note: Figure translations are in progress. See original paper for figures.