

Multilevel balancing of laccase biosynthesis and secretion in *Bacillus subtilis* enables efficient tobacco straw delignification

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Abstract

Background Efficient extracellular production of laccase remains a major constraint in developing microbial platforms for biological delignification of lignocellulosic biomass. Here, we established a multilevel engineering strategy to coordinate laccase biosynthesis and secretion in *Bacillus subtilis*.

Results Screening of laccase-host combinations identified the *B. subtilis*-derived BsLac1 expressed in *B. subtilis* G600 as the most effective production system. Transcriptional output was enhanced using the maltose-inducible promoter P_{malA} , while extracellular export was improved by coupling BsLac1 with the signal peptide SP_{ykpC} . Translation initiation was subsequently fine-tuned using a ribosome-binding-site library to further balance protein synthesis with secretion capacity. The optimal variant, RBS10, achieved an extracellular laccase activity of 1764.82 U/L under shake-flask conditions. Upon fed-batch cultivation in a 3-L bioreactor, the engineered strain reached an OD600 of approximately 180 and a maximum extracellular laccase activity of 6339.61 U/L at 36 h. Importantly, the crude extracellular enzyme preparation was directly applicable to tobacco straw delignification, decreasing lignin content from 80.7 to 56.8 mg/g, corresponding to 29.6% lignin removal. SEM and FTIR analyses revealed disruption of the compact lignocellulosic matrix and reduction of lignin-associated structural features.

Conclusions Collectively, this study demonstrates that coordinated engineering of transcription, translation, and protein export can substantially enhance extracellular laccase production in *B. subtilis*, providing a scalable microbial platform for biological pretreatment of lignocellulosic residues.

Introduction

Lignocellulosic agricultural residues, including tobacco straw, are abundant renewable feedstocks for the production of biofuels and bio-based chemicals[1]. Their efficient conversion, however, is restricted by the recalcitrant architecture of lignocellulose, in which cellulose and hemicellulose are embedded within a highly cross-linked lignin matrix. Therefore, selective removal or disruption of lignin is an important prerequisite for the effective valorization of lignocellulosic residues[2, 3].

Laccase are multicopper oxidases that catalyze the single-electron oxidation of a broad range of phenolic and, in the presence of suitable mediators, non-phenolic compounds, while reducing molecular oxygen to water[4, 5]. Owing to their broad substrate range and mild reaction requirements, laccase have been investigated for lignin modification, wastewater remediation, removal of environmental contaminants, and synthesis of value-added chemicals[6, 7]. In lignocellulosic biomass processing, laccase-mediated delignification offers a potentially less energy-intensive and more environmentally compatible alternative to conventional thermochemical pretreatment methods[8].

The broader application of laccase nevertheless remains constrained by limited enzyme availability and the difficulty of obtaining high-activity preparations from native producers[9, 10]. Although fungi are the primary source of laccase, their long growth cycles and complex genetic manipulation significantly increase the difficulty of downstream purification[11, 12]. Heterologous expression in genetically

accessible microorganisms has therefore become an important strategy for laccase production. For instance, expression of a laccase from *Bacillus vallismortis* fmb-103 in *Escherichia coli* resulted in an activity of 1545.6 U/L[13]. Secretory expression of small laccase in *Streptomyces lividans* yielded a maximum activity of 1950 ± 99 U/L[14], whereas expression of a *Cerrena unicolor* laccase in *Pichia pastoris* produced an activity of 269.1 U/L[15]. Although *E. coli*, yeasts, and filamentous fungi are frequently used for recombinant laccase production[16], *B. subtilis* represents an attractive alternative because of its well-characterized genetic background, rapid growth, established cultivation processes, and capacity to secrete proteins directly into the culture medium[17, 18].

In this study, a multilevel engineering strategy was applied to construct a *B. subtilis* cell factory for extracellular laccase production. We comprehensively screened laccase candidates from diverse sources and identified the endogenous BsLac1 as exhibiting superior enzymatic activity. Further expression improvements were achieved by screening host strains to identify *B. subtilis* G600 as the optimal chassis. By rationally integrating the maltose-inducible promoter P_{malA} , the most effective signal peptide SP_{ykpC} , and a fine-tuned translation initiation variant, RBS10, the target protein yield was maximized. Scale-up fed-batch cultivation of the engineered strain G600-RBS10-BsLac1 was then implemented in a 3-L bioreactor. Finally, the practical application of the engineered laccase in the biological degradation of tobacco straw was systematically evaluated using compositional analysis, SEM, and FTIR. This study provides a valuable foundation for the high-yield microbial production of laccase and lays the groundwork for the future engineering of *B. subtilis* cell factories for industrial-scale biomass valorization.

Materials and methods

Strains and plasmids

E. coli DH5 α was employed for gene cloning and propagation. Laccase was expressed in *B. subtilis* 168 (BS168), *B. subtilis* G201 (G201), *B. subtilis* G401 (G401), *B. subtilis* G600 (G600), *B. subtilis* G601 (G601) and *B. subtilis* G800 (G800). Five laccase genes-originating from *Trametes maxima*, *Trametes versicolor*, *B. subtilis*, *Bacillus licheniformis*, and *B. vallismortis*-were cloned into the P_{43} vector for protein expression. All strains, plasmids and primers are listed in Table S1, S2 and S3.

Construction of laccase plasmid in *B. subtilis*

To construct the plasmids P_{43} -TmLac1, P_{43} -TvLac1, P_{43} -BsLac1, P_{43} -BILac1, and P_{43} -BvLac1, the initial vector P_{43} -His6 was first amplified using the primer pair P_{43} -LiF/R. Subsequently, the optimized laccase gene fragments (*Tmlac1*, *Tvlac1*, *Bslac1*, *Bllac1*, and *Bvlac1*) were amplified using the corresponding primers (TmLac1-F/R, TvLac1-F/R, BsLac1-F/R, BILac1-F/R, and BvLac1-F/R). The gene fragments and the amplified P_{43} -His6 were subsequently assembled using the Gibson Assembly system to generate the target plasmids[19]. The assembled plasmids were subsequently transformed into *E. coli* DH5 α . After 12 hours of incubation, individual colonies were selected for colony PCR analysis. Successful cloning was

validated using the primers P₄₃-VR, TmLac1-VF, TvLac1-VF, BsLac1-VF, BILac1-VF, and BvLac1-VF. Plasmids derived from colonies exhibiting the correct band size in agarose gel electrophoresis were subsequently subjected to sequencing.

Strain transformation

E. coli was transformed using chemical transformation, competent cells were thawed on ice, followed by the addition of the recombinant DNA to the cells. The mixture was gently mixed and incubated on ice for 30 minutes. Subsequently, the cells were subjected to a heat shock in a water bath at 42°C for 90 s, followed by immediate cooling on ice for 2 minutes. Antibiotic-free Luria-Bertani (LB) medium was then added, and the cells were cultured in a shaking incubator at 220 rpm and 37°C for 1 h. After incubation, the cells were spread on LB agar plates containing the appropriate antibiotics and incubated upside down at 37°C. After sufficient incubation, colonies were selected for further analysis.

For *B. subtilis*, take a certain amount of plasmid and add it to the *B. subtilis* competent cells. Then, incubate them at 220 rpm for 37°C for 2 minutes. After incubation, the cells were spread on LB agar plates containing the appropriate antibiotics and incubated upside down at 37°C. After sufficient incubation, colonies were selected for further analysis.

Construction of plasmids expressing laccase with different promoters

To construct plasmids with different promoter-laccase combinations, the promoters P_{araA}, P_{glpD}, P_{malA}, P_{manA} and P₂₁ were amplified from plasmid templates preserved in our laboratory, using primers P_{araA}-F/R, P_{glpD}-F/R, P_{malA}-F/R, P_{manA}-F/R and P₂₁-F/R. Following this, PCR amplification was performed on the P₄₃-BsLac1 plasmid using primers P₄₃-Pro-LiF/R. The Gibson Assembly system was then employed to assemble the amplified fragments into the plasmids P₄₃-P_{araA}-BsLac1, P₄₃-P_{glpD}-BsLac1, P₄₃-P_{malA}-BsLac1, P₄₃-P_{manA}-BsLac1, and P₄₃-P₂₁-BsLac1. After 12 h of incubation, the transformants were subjected to colony PCR for verification. The positive transformants were cultured for an additional 12 h, and plasmids were then extracted and sequenced to confirm the presence of the recombinant constructs.

Construction of plasmids expressing laccase with different signal peptides

To construct plasmids containing various signal peptides, eight distinct signal peptides were amplified from laboratory-stored templates (Table S4). The amplified fragments were then integrated into the expression vector using the Gibson Assembly system. Following 12 h of incubation, the transformants were verified via colony PCR. Positive clones were further cultured for an additional 12 h, after which the

plasmids were extracted and sequenced to confirm the successful construction of the recombinant vectors.

Construction of plasmids expressing laccase with different RBS

To construct the RBS library, an initial RBS sequence was optimized using the online tools RBS Calculator and RBS Library Calculator (<https://www.denovodna.com/software/>). A mixed RBS library, containing 22 variants with different translation initiation rates (Table S5), was generated using the degenerate primers RBS-F and RBS-R. The original RBS sequence in the P₄₃-P_{malA}-BSLac1 plasmid was replaced with the library sequences via one-step cloning. The resulting constructs were transformed into *E. coli* DH5α for library amplification. After 12 h of incubation, the transformants were subjected to colony PCR for verification. The positive transformants were cultured for an additional 12 h, and plasmids were then extracted and sequenced to confirm the presence of the recombinant constructs.

Fed-batch cultivation in bioreactor

Scale-up cultivation was carried out using the engineered strain G600-RBS10-BsLac1 in a 3-L bioreactor loaded with 1.5 L of Terrific Broth (TB) medium supplemented with 50 µg/mL kanamycin. 4% (v/v) of the seed culture was inoculated into the initial fresh medium, which was composed of 3% tryptone, 5% yeast extract, and 0.6% K₂HPO₄. The cultivation was performed at 37°C, with an initial agitation speed of 600 rpm, an aeration rate of 1.8 vvm, and a total cultivation duration of 48 h. To maintain the dissolved oxygen (DO) concentration above 15%, the agitation speed was dynamically regulated within the range of 600–1000 rpm. When the culture entered the stationary growth phase, the feed medium containing 5% yeast extract was continuously added to the bioreactor at a constant flow rate of 20 mL/h.

Culture conditions

For *E. coli*, LB medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl) was used unless otherwise specified. *B. subtilis* was cultured in TB medium (24 g/L yeast extract, 12 g/L tryptone, 4 ml/L glycerin, 16.43 g/L K₂HPO₄·3H₂O and 2.31 g/L KH₂PO₄). The following antibiotics were used for *E. coli*: 100 µg/mL Ampicillin, 50 µg/mL kanamycin. For *B. subtilis*, the antibiotics used were 50 µg/mL kanamycin.

The single colony was inoculated into LB medium. The seed cultures were obtained after 12 h incubation at 37 °C, 220 rpm. Then, 1 mL seed cultures were added to 20 mL of TB medium, where they were continued to grow for an additional 48 h. To maintain plasmid replication, the appropriate antibiotics (50 µg/mL kanamycin) were added to the culture medium.

Protein analysis

One milliliter of culture sample was collected at 24 h of cultivation and centrifuged (12 000 ×g for 10 minutes at 4°C). The supernatant and cell precipitate were separately taken. The cell precipitate was suspended in ddH₂O, and then the cells were disrupted using an ultrasonic processor to obtain the cell lysate. The supernatant and the cell lysate were added to 4× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for treatment, and then boiled for 20 minutes to prepare protein samples. Protein separation was performed using SDS-PAGE on 12% Bis-Tris protein gels (Thermo Fisher Scientific), followed by staining with Coomassie Brilliant Blue R250. Images of the gels were captured using a Gel Doc imaging system (Bio-Rad). Western blot (WB) analysis was conducted using a commercial WB kit, and chemiluminescent signals were detected and recorded using a chemiluminescent gel imaging analyzer (Bio-Rad). To maintain the stability and integrity of laccase, all samples were kept at 4°C throughout the entire process.

Enzyme activity assay

The laccase activity was assessed at room temperature by monitoring the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as the substrate. The reaction mixture contained 10 mM ABTS, 0.1 M sodium acetate buffer (pH = 4.5), and culture supernatant. The method to detect enzyme activity used in this study was modified based on the previously reported research[20]. It was then incubated at room temperature for 5 minutes, after which the laccase activity was determined by the increase in absorbance at 420 nm ($\epsilon = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$). One unit of enzyme activity was defined as the amount of enzyme needed to oxidize 1 μmol of ABTS per minute. The assay was carried out in 96-well plates and read by a microplate reader.

Characterization of lignocellulosic samples

The tobacco straw was oven-dried at 80°C for 24 h and subsequently milled to a uniform powder. For the enzymatic treatment, 2.0 g of the prepared biomass was transferred into an Erlenmeyer flask supplemented with 40 mL of the crude laccase solution. The reaction mixture was then maintained in an orbital shaker at 37°C and 220 rpm for 48 h. After separation and drying, the solid residues were analyzed using specific assay kits to determine the contents of lignin, hemicellulose, and cellulose. Using the inactivated enzyme solution as the blank control, the degradation efficiency was calculated according to the following equation: $\text{Degradation efficiency}(\%) = (C_{\text{untreated}} - C_{\text{treated}}) / C_{\text{untreated}} \times 100\%$

The untreated and treated lignocellulosic samples were dehydrated using an air dry oven. Scanning electron microscopy was used to characterize the changes in surface morphologies of tobacco straw samples untreated and treated with laccase. All samples were sputter-coated with gold and photographed at a magnification of 1000×.

FTIR spectrometer and KBr pelleting were used to investigate the chemical structure changes of tobacco straw untreated and treated with laccase in the range of 400–4000 cm^{-1} .

Statistical analysis

All experiments were performed in at least triplicate, and data are presented as mean $\pm$ SD. Statistical significance was evaluated using Two-tailed Student's t-tests and one-way ANOVAs. Statistical significance was indicated as * for $p < 0.05$ and ** for $p < 0.01$, respectively.

Results and discussion

Screening of laccase candidates and *B. subtilis* host strains

To identify a suitable enzyme–host combination for laccase production, *B. subtilis* was selected as the initial expression platform because of its well-characterized genetic background and protein production capacity. Five laccase genes from diverse fungal and bacterial sources, including *T. maxima* (TmLac1), *T. versicolor* (TvLac1), *B. subtilis* (BsLac1), *B. licheniformis* (BILac1), and *B. vallismortis* (BvLac1), were evaluated. The laccase sequences are provided in Table S6, and their sources and principal characteristics are summarized in Fig. 1A. The corresponding genes were codon-optimized for expression in *B. subtilis*, synthesized, and individually cloned into the P₄₃-His6 expression vector (Fig. 1B). The resulting recombinant plasmids were introduced into BS168 to generate the respective expression strains. The protein expression was evaluated by SDS-PAGE and WB analysis (Fig. 1C, D). However, SDS-PAGE analysis did not reveal discernible bands corresponding to the target proteins, likely due to low expression levels and interference from abundant endogenous proteins (Fig. 1C). In contrast, WB analysis revealed specific immunoreactive bands for TvLac1 (~ 55.82 kDa), BsLac1 (~ 58.51 kDa), BILac1 (~ 59.10 kDa), and BvLac1 (~ 58.35 kDa), all of which migrated within the expected molecular-mass range of approximately 50–60 kDa. No corresponding band was detected for TmLac1 or in the BS168 control. The failure to detect TmLac1 may reflect limited compatibility of this fungal protein with the *B. subtilis* expression system, potentially involving inefficient folding, reduced stability, or rapid degradation within the host[17, 21].

Laccase activity and cell growth, represented by the optical density at 600 nm (OD₆₀₀), were subsequently monitored over time (Fig. 1E, F). The highest activities were generally observed at 24 h. Among the five candidates, the *B. subtilis*-derived BsLac1 showed the highest activity, reaching 49.04 U/L. No detectable laccase activity was observed for the BS168 control, despite the presence of native laccase-encoding genes in its genome. This result may be attributable to the low basal expression of chromosomal laccase and/or degradation of the corresponding proteins by endogenous proteases[22, 23]. Consistent with the absence of a detectable WB signal, no activity was observed for TmLac1. Based on the combined protein-expression and enzyme-activity results, BsLac1 was selected for subsequent host screening.

Because the genetic background of the production host can substantially influence recombinant protein expression and stability[24], the BsLac1 expression plasmid was further introduced into five additional *B. subtilis* strains, namely G201, G401, G600, G601, and G800. All five strains were derived from the parental strain BS168 and successfully engineered into protease-deficient mutants via the targeted deletion of various protease genes[25]. As observed in BS168, SDS-PAGE analysis did not reveal distinct BsLac1

bands in any of these strains, whereas WB analysis confirmed BsLac1 expression in all five hosts (Fig. 1G, H). Since the initial time-course analysis showed that laccase activity peaked at 24 h, enzyme activity in the host-screening experiment was compared at this time point. Among the tested strains, G600 exhibited the highest laccase activity, reaching 65.45 U/L, which was 1.33-fold higher than that obtained with BS168 (Fig. 1I).

The improved performance of G600 may be associated with its reduced extracellular protease background, which could enhance the stability and accumulation of the recombinant protein[25]. Differences in growth behavior and host physiology may also have contributed to the observed variation in laccase production. Taken together, these results identified BsLac1 and G600 as the preferred enzyme-host combination for subsequent optimization of transcription, translation, and extracellular secretion.

Enhancing laccase expression through optimizing promoter engineering

The optimization of promoters represents a key strategy for enhancing recombinant protein expression in *B. subtilis*[26]. To investigate the impact of promoter strength and regulation on BsLac1 expression, a constitutive promoter (P_{21}) and four inducible promoters (P_{araA} , P_{glpD} , P_{malA} , P_{manA}) were selected to construct recombinant strains. P_{21} promoter is endogenous to promoter of *B. subtilis*, whereas the P_{araA} , P_{glpD} , P_{malA} , and P_{manA} promoters are induced by arabinose, glycerol, maltose, and mannose, respectively[27]. The original P_{43} promoter was replaced using seamless cloning technology (Fig. 2A). Following cultivation, expression levels were analyzed by SDS-PAGE and WB analysis. As shown in Fig. 2B, no distinct protein bands were detected by SDS-PAGE analysis. WB analysis revealed that the expression levels driven by the P_{araA} and P_{21} promoters were relatively low, while the expression levels mediated by the P_{malA} , P_{glpD} and P_{manA} promoters were significantly higher (Fig. 2C). Enzymatic activity analysis demonstrated that the P_{malA} promoter mediated the highest expression of BsLac1, with an activity level of 813.37 U/L, which was 1.28-fold higher than that of the P_{glpD} promoter and 1.63-fold higher than that of the P_{manA} promoter (Fig. 2D). Compared with the activity obtained using the constitutive P_{43} promoter in G600 (65.45 U/L), the P_{malA} -driven system increased laccase activity 12.4-fold. This improvement may be attributable to the dual role of maltose as both an inducer of the P_{malA} promoter and a carbon source. Following cellular uptake, maltose enters central carbon metabolism and may help meet the energy and precursor demands associated with recombinant protein synthesis and folding[28, 29]. Therefore, the P_{malA} promoter was selected for subsequent BsLac1 expression studies.

Screening and optimization of signal peptides for enhanced extracellular secretion of BsLac1

The secretion and expression of proteins are dependent on the role of signal peptides, particularly within the *B. subtilis* expression system[30]. However, sequence analysis revealed that BsLac1 lacks a native signal peptide. To investigate the intracellular distribution of laccase, this study compared the enzymatic activities of P_{malA}-BsLac1 strain in the supernatant and cell lysate (Fig. 3A, B). As shown in Fig. 3B, laccase activity was also detected in the cell lysate and was higher than that in the supernatant. This indicates that while BsLac1 can be expressed, a significant portion of the protein remains trapped within the cytoplasm, potentially making it susceptible to degradation by endogenous intracellular proteases[17, 31].

To guide the secretion of BsLac1 and increase extracellular yield, eight signal peptides derived from *B. subtilis*-SP_{appA}, SP_{wapA}, SP_{oxaA}, SP_{ykpC}, SP_{yxiP}, SP_{ytrF}, SP_{ydjN}, and SP_{yvgO}-were selected. Under the regulation of the P_{malA} promoter, these signal peptide sequences were integrated upstream of the BsLac1 (Fig. 3C). After cultivation, extracellular expression levels were detected by SDS-PAGE and WB analysis (Fig. 3D, E). WB analysis confirmed that all proteins were expressed following the introduction of signal peptides. Enzymatic activity analysis demonstrated that the strain carrying SP_{ykpC} exhibited the highest extracellular activity, reaching approximately 1212.91 U/L, representing a 1.49-fold increase compared to the P_{malA}-BsLac1 control without a signal peptide. The OD₆₀₀ values across all strains remained relatively stable, indicating that the enhanced secretion process did not impose a significant metabolic burden on the host cell (Fig. 3F). Therefore, SP_{ykpC} was identified as the optimal signal peptide for efficient BsLac1 secretion and was used for all subsequent expression optimization experiments.

Enhancing laccase expression through optimizing RBS

Translation efficiency is an important determinant of recombinant protein production and is jointly influenced by translation initiation and elongation[32]. Translation initiation is particularly sensitive to the sequence of RBS, the accessibility of the start codon, and the secondary structure and folding free energy of the translation initiation region in messenger RNA (mRNA)[33, 34]. An RBS library was constructed using the original RBS as a template. Utilizing seamless cloning technology, the original RBS sequence in the expression vector was precisely replaced by selected mutant sequences (Fig. 4A), resulting in a series of recombinant strains with varying translation strengths. As shown in Fig. 4B, no discernible protein bands were detected by SDS-PAGE due to interference from endogenous proteins. And WB analysis revealed that most RBS variants achieved successful expression, with the exception of clones RBS5, RBS8, and RBS9, which failed to show target protein signals (Fig. 4C). This might be due to the RBS sequence or the start codon being incorporated into a stable secondary structure, preventing the ribosome from accessing and binding to the mRNA, and the translation rates of RBS8 and RBS9 being too high, resulting in the obstruction of translation initiation[35, 36]. Enzymatic activity analysis further confirmed the efficacy of translation optimization; among all screened variants, RBS10 mediated the highest expression level of BsLac1, with extracellular activity reaching 1764.82 U/L, a 1.46-fold increase over the original RBS sequence (Fig. 4D). The OD₆₀₀ growth curves indicated that the biomass of all recombinant strains remained fundamentally stable.

Cultivation optimization for enhanced laccase production in a 3-L bioreactor

To further evaluate the production potential of the engineered strain and scale up laccase expression, fed-batch cultivation was conducted in a 3-L bioreactor utilizing the optimal construct G600-RBS10-BsLac1. The configuration of the cultivation system, incorporating real-time feedback sensors for DO, pH, temperature, and level regulation via automated peristaltic pumps, is illustrated in Fig. 5A. Key cultivation parameters, including biomass OD₆₀₀, laccase extracellular activity, residual maltose concentration, pH, and DO, were tracked systematically over 48 h (Fig. 5B). During the initial 24 h of batch cultivation, the strain exhibited rapid growth kinetics, entering an exponential phase after 6 h, which coincided with the efficient consumption of the primary carbon source, maltose. As the translation and accumulation of BsLac1 progressed rapidly during this phase, a simultaneous and sharp decline in the DO concentration was observed, dropping from an initial 100% to approximately 20% by 24 h due to the high respiratory demand of the dense cell culture. Upon entering the stationary growth phase at 24 h, a feeding medium containing 5% yeast extract was continuously supplied at a steady flow rate of 20 mL/h to alleviate potential nutrient limitations and sustain protein synthesis. Following the initiation of this fed-batch strategy, the extracellular laccase activity experienced a dramatic surge, rising sharply from about 5399.67 U/L at 24 h and reaching its maximum at approximately 36 h. The maximum extracellular enzyme activity reached a level of 6339.61 U/L, while the final biomass OD₆₀₀ reached approximately 180. Meanwhile, the DO levels stabilized safely around 15%-20% during the late cultivation stage through dynamic agitation tuning. To directly validate the expression and secretion of laccase during cultivation at the protein level, SDS-PAGE and WB analyses were performed on culture samples collected at various time points, as shown in Fig. 5C, D. As the cultivation progressed, the total protein concentration increased steadily, and the WB results further specifically confirmed the identity of the target protein as BsLac1.

Application of recombinant laccase in tobacco straw degradation

To evaluate the efficacy of the recombinant laccase in degrading tobacco straw, the compositional changes of the biomass were first quantitatively analyzed. After treatment with laccase enzyme solution, the contents of lignin, cellulose and hemicellulose all changed (Table 1). The substantial reduction in lignin content underscores the robust oxidative capacity of the recombinant laccase, whose primary function likely involves the oxidation of phenolic moieties within the complex lignin polymer. Although laccase inherently lacks the ability to directly hydrolyze glycosidic bonds in polysaccharides, the observed minor loss of cellulose and hemicellulose can likely be attributed to the extensive cleavage of lignin-carbohydrate complexes (LCCs) during the delignification process. Concomitant with the degradation of the lignin matrix, structurally associated polysaccharides are either released or rendered water-soluble[37]. Furthermore, highly reactive free radicals generated during the laccase-mediated oxidation of lignin may inflict non-specific, localized attacks on adjacent carbohydrate chains[38].

Notably, this targeted action efficiently dismantles the recalcitrant lignin barrier while largely preserving the bulk of fermentable sugars (cellulose and hemicellulose).

Table 1
Degradation efficiency of recombinant laccase on lignin, hemicellulose, and cellulose.

Lignin	Control (mg/g)	Treatment (mg/g)	Degradation (%)
	81.6 ± 3.3	57.5 ± 2.6	29.5 ± 4.3
Hemicellulose	470.7 ± 13.1	459.1 ± 11.6	2.5 ± 3.7
Cellulose	274.9 ± 4.4	248.3 ± 0.9	9.7 ± 1.5

To further elucidate the effects of recombinant laccase treatment on the physicochemical structure of tobacco straw, SEM and FTIR were employed to characterize the substrate before and after treatment. As shown in Fig. 6, the untreated tobacco straw exhibited a smooth, flat surface with a compact and highly ordered structure (Fig. 6A). In contrast, following the laccase treatment, the structural integrity of the biomass surface was severely disrupted, becoming notably rougher with localized fractures and a loose, disordered texture (Fig. 6B). These morphological alterations are consistent with previous findings[39]. The results indicate that the degradation by laccase effectively deconstructs the compact barrier of lignocellulose, thereby increasing the surface porosity and roughness. Such structural modifications significantly expand the specific surface area of the biomass, which consequently enhances substrate accessibility to subsequent hydrolytic enzymes, ultimately improving the overall bioconversion efficiency of lignocellulose[40].

To thoroughly evaluate the chemical structural modifications induced by the crude laccase solution expressed by *B. subtilis* on tobacco straw, FTIR spectroscopy was performed (Fig. 6C). FTIR spectroscopy serves as an effective tool for probing structural alterations in lignocellulosic matrices, allowing for the quantitative assessment of laccase treatment performance[41]. The spectra revealed a broad absorption band in the region of 3300–3500 cm⁻¹, which is attributed to the stretching vibrations of intra- and intermolecular hydroxyl groups; the attenuation of this peak intensity in the laccase-treated group indicated the disruption of the internal hydrogen-bonding network within the biomass matrix. Notably, the characteristic absorption bands spanning 1500–1650 cm⁻¹ correspond primarily to the C = C aromatic ring skeleton vibrations and C = O stretching vibrations in lignin. Compared to the untreated control, a significant decline in spectral absorbance within this region was observed post-treatment, confirming the partial degradation and delignification of the straw. Furthermore, a decreasing trend in signal intensity was detected at 1200–1250 cm⁻¹, assigned to the C-O vibrations of guaiacyl rings in lignin, as well as at 1000–1030 cm⁻¹, corresponding to the C-O-C stretching vibrations of cellulose and hemicellulose. This attenuation can likely be attributed to the exposure of polysaccharide structures following the removal of the protective lignin barrier, or to the concomitant degradation of glycans during enzymatic treatment[42–44]. Taken together, the FTIR results demonstrate that the crude laccase solution effectively targets and attacks the aromatic backbone of lignin, leading to the dissociation and

loosening of the recalcitrant lignocellulosic network, thereby achieving efficient degradation of tobacco straw.

Conclusion

In this study, a highly efficient *B. subtilis* cell factory was successfully engineered for the high-yield expression and extracellular secretion of laccase. Through a systematic optimization strategy, the endogenous laccase BsLac1 and the host chassis G600 were identified as the optimal combination for enzyme production. Subsequent genetic engineering utilizing the maltose-inducible promoter P_{malA} and the SP_{ykpC} significantly enhanced the expression levels and extracellular translocation of the target protein. By fine-tuning translation initiation via an RBS library, the RBS10 variant was selected to further maximize the protein yield. Scale-up fed-batch cultivation in a 3-L bioreactor, coupled with a targeted nutrient-feeding strategy at the stationary phase, achieved a final biomass OD_{600} near 180 and a remarkable peak extracellular enzyme activity of 6339.61 U/L at 36 h. Furthermore, the practical application of the crude recombinant laccase demonstrated exceptional efficacy in the biological degradation of tobacco straw, with compositional analysis, SEM, and FTIR characterizations confirming the successful disruption of the recalcitrant lignocellulosic matrix and significant delignification. Overall, this work establishes a robust foundation for the large-scale microbial production of laccase and highlights the vast potential of engineered *B. subtilis* platforms in industrial-scale biomass valorization and biocatalysis.

Declarations

Acknowledgments Not applicable.

Author contributions Hailin Zhang: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. Zhendong Li: Supervision, Visualization, Writing - review and editing. Xueqin Lv: Investigation, Methodology. Chang Liu: Conceptualization, Validation. Jian Mao: Supervision, Visualization, Writing - review and editing. Jingjing Liu: Supervision, Visualization, Writing - review and editing.

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Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Figures

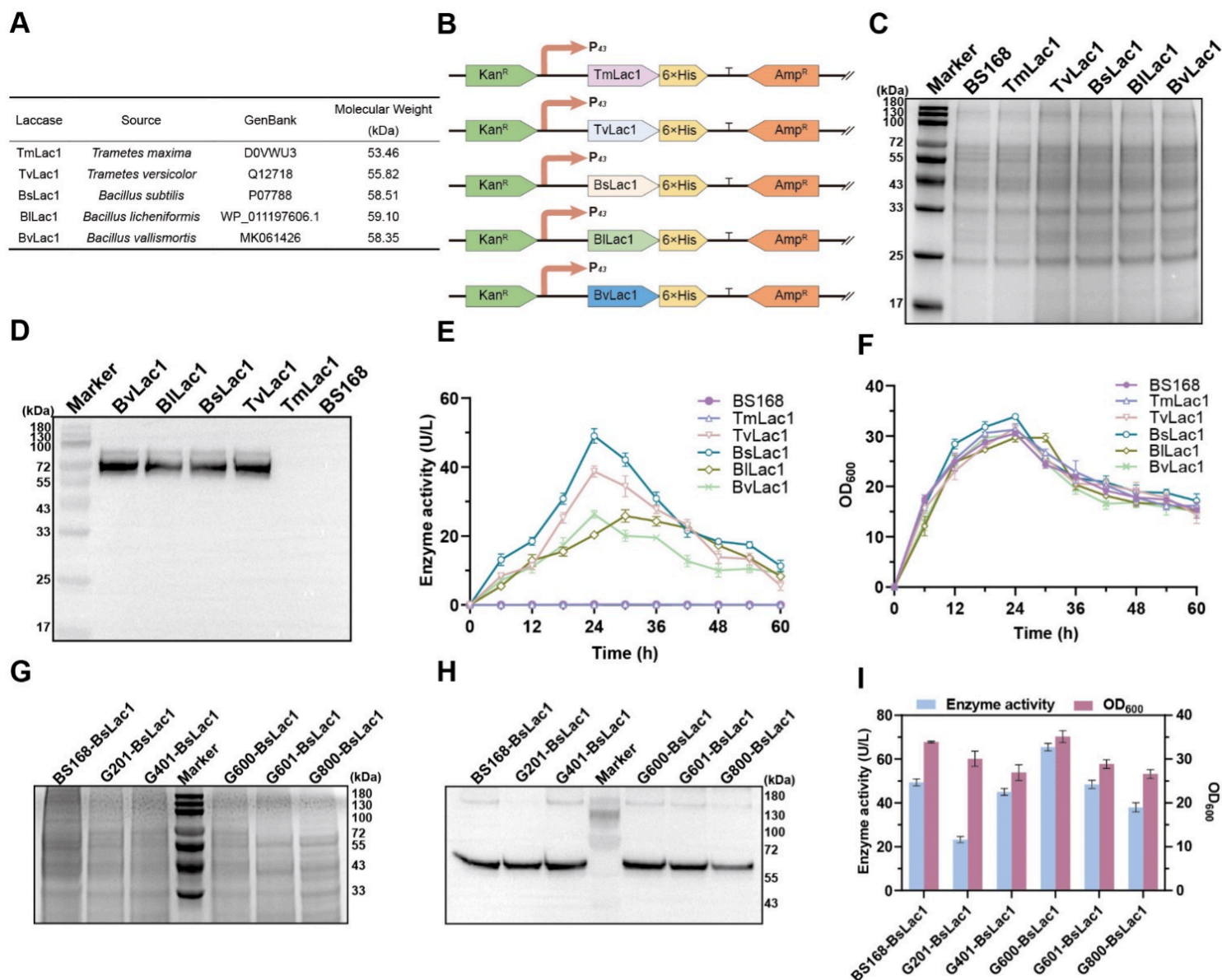


Figure 1

Expression of different laccases in *B. subtilis*. (A) Laccase candidates used in this study. (B) Schematic representation of the plasmid construction strategy. (C) Detection of the expression of different laccase by SDS-PAGE. (D) Expression of different laccase detected by WB. (E) Enzyme activity of different laccase in *B. subtilis*. (F) Reconstruction of the growth curve of *B. subtilis*. (G) Effects of different *B. subtilis* hosts on laccase production detected by SDS-PAGE. (H) Effects of different *B. subtilis* hosts on laccase production detected by WB. (I) OD₆₀₀ and enzyme activity of laccase strains with different *B. subtilis* hosts.

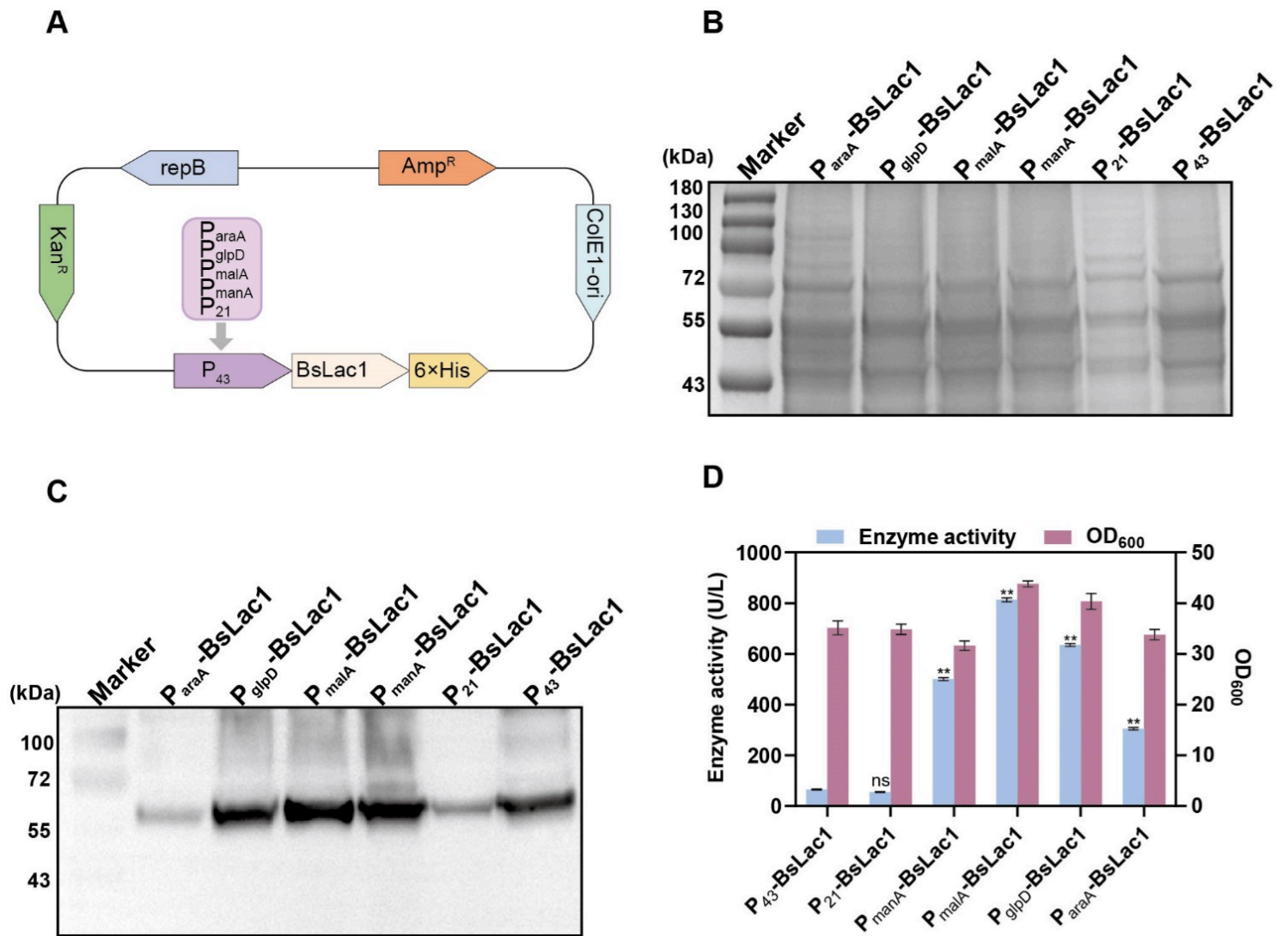


Figure 2

Effects of different promoters on laccase expression. (A) Schematic illustration of plasmid construction for promoter optimization. (B) Effects of different promoters on laccase production detected by SDS-PAGE. (C) Effects of different promoters on laccase production detected by WB. (D) OD₆₀₀ and enzyme activity of laccase strains with different promoters.

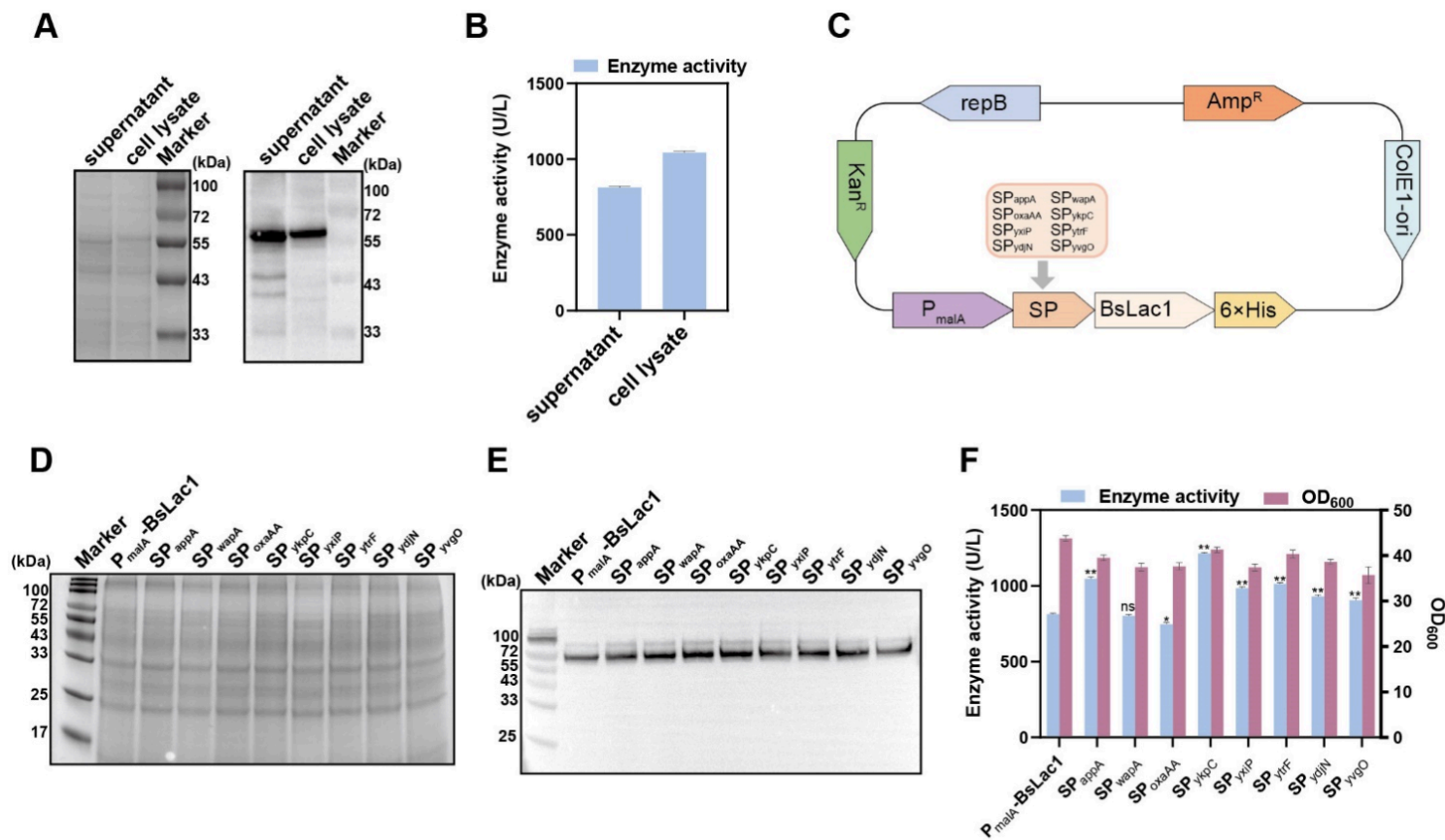


Figure 3

Effects of different signal peptides on laccase expression. (A) The supernatant and cell lysate of the P_{malA}-BsLac1 were analyzed by SDS-PAGE and WB. (B) Laccase activity was measured on the supernatant and cell lysate of P_{malA}-BsLac1. (C) Schematic illustration of plasmid construction for signal peptides optimization. (D) Effects of different signal peptides on laccase production detected by SDS-PAGE. (E) Effects of different signal peptides on laccase production detected by WB. (F) OD₆₀₀ and enzyme activity of laccase strains with different signal peptides.

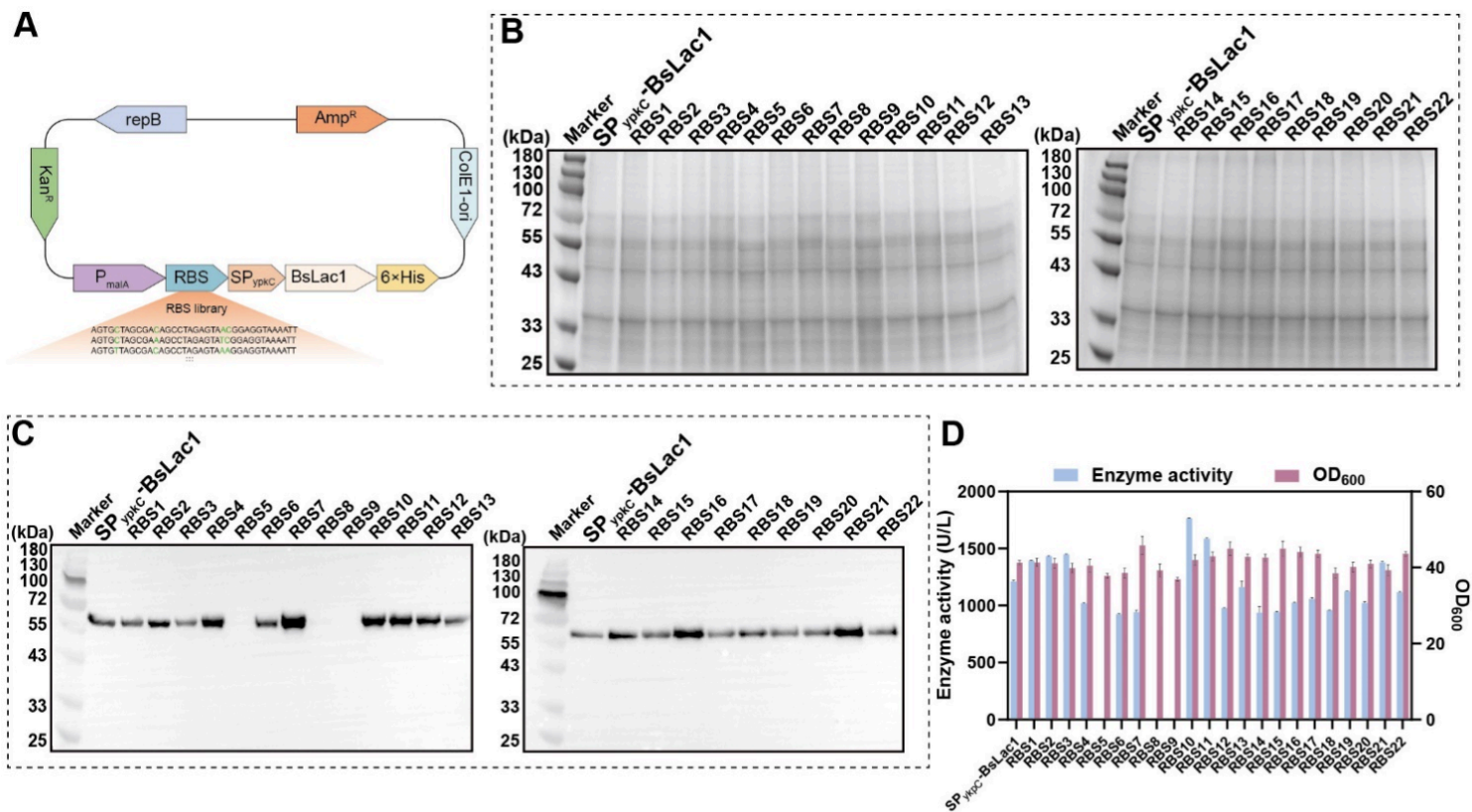


Figure 4

Effects of different RBS on laccase expression. (A) Schematic illustration of the strategy for RBS library construction and plasmid assembly. (B) Effects of different RBS on laccase production detected by SDS-PAGE. (C) Effects of different RBS on laccase production detected by WB. (D) OD₆₀₀ and enzyme activity of laccase strains with different RBS.

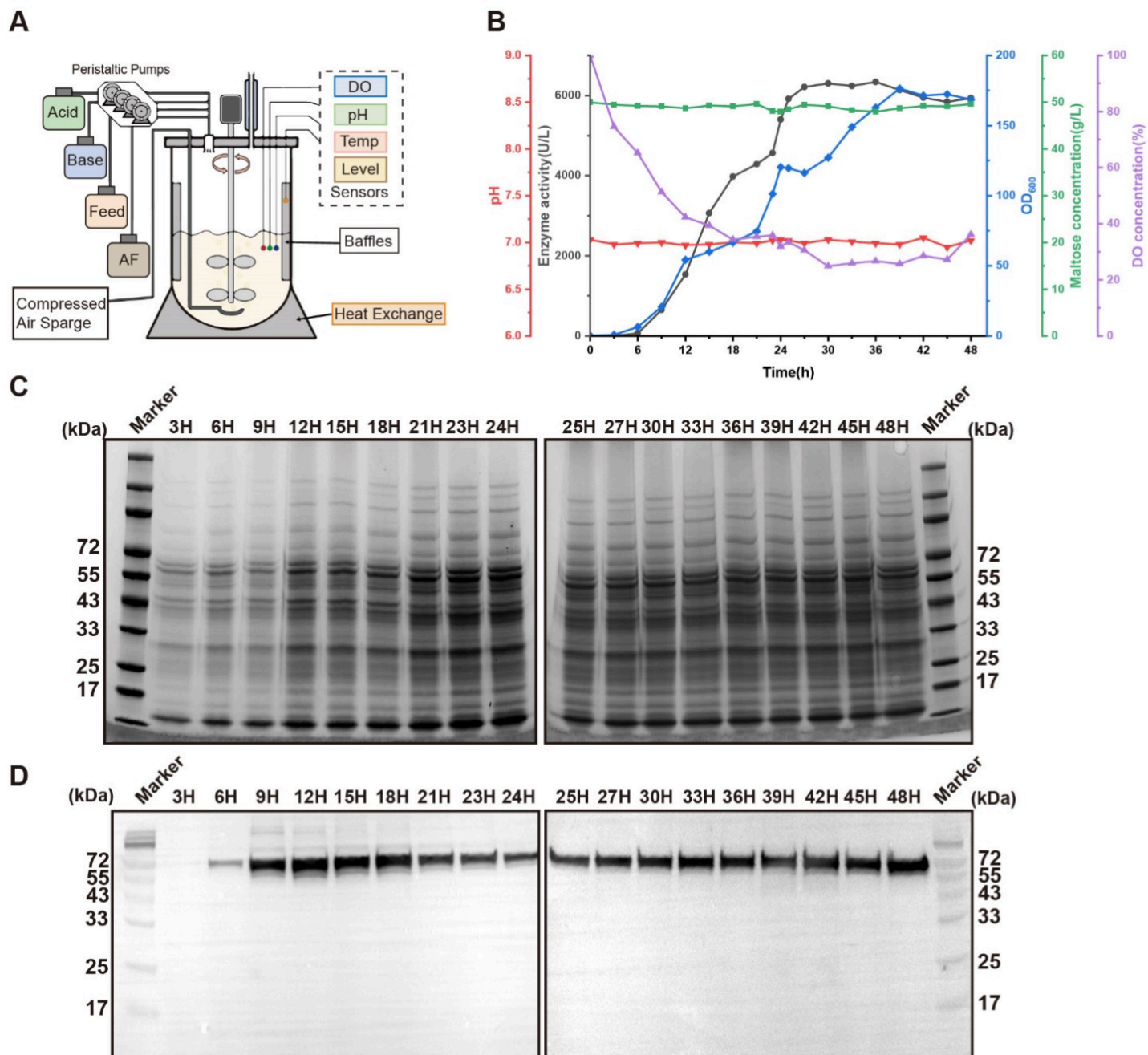


Figure 5

(A) 3-L cultivation tank diagram. (B) High-density cultivation results of the strain G600-RBS10-BsLac1 in a 3-L fermenter at 37 °C. Culture samples were taken every 3 h between 0 and 48 h to assess the enzyme activity (black), pH (red), biomass (blue), maltose concentration (green), and DO (purple) during cultivation. The feeding medium (5 % yeast extract) was fed into the fermenter at a steady flow rate of 20 mL/h at 24 h. (C) Effects of different times on laccase production detected by SDS-PAGE. (D) Effects of different times on laccase production detected by WB.

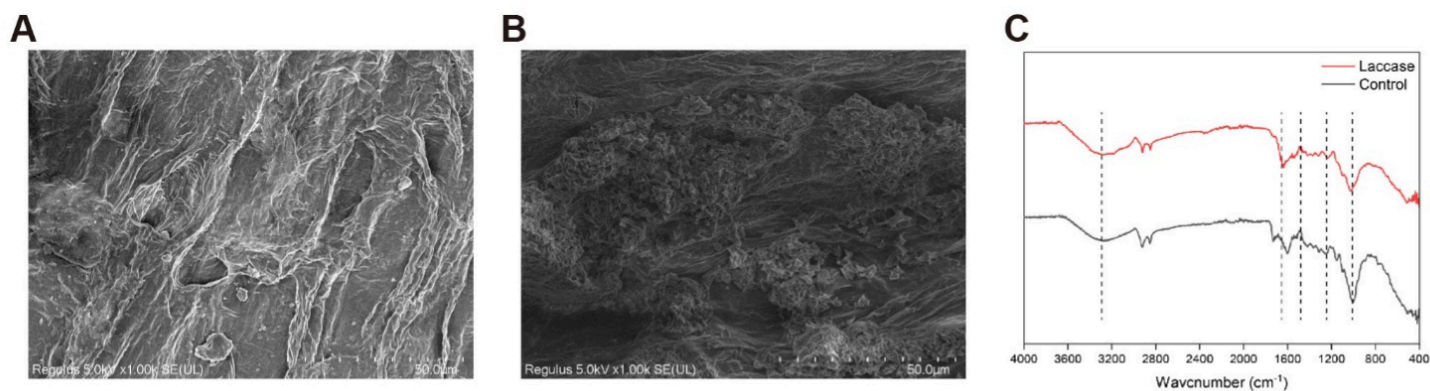


Figure 6

SEM microscopic images and FTIR of untreated and enzyme-treated tobacco straw. (A) SEM images of untreated tobacco straw. (B) SEM images of enzyme-treated tobacco straw. (C) FTIR analysis of untreated and treated tobacco straw with laccase enzyme solution.

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