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Rapid High-Throughput Screening of Curdlan-Producing Mutants via a Microscale Aniline Blue Colorimetric Assay

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Abstract

Curdlan is an industrially important β-1,3-glucan with applications in the food, pharmaceutical, and biomaterial industries. However, the identification of high-yielding curdlan-producing strains is hindered by the absence of rapid and efficient screening methods. To address this limitation, we developed a systematically optimized integrated microscale workflow combining 48-well plate fermentation with a quantitative aniline blue-based colorimetric assay in 96-well plates for high-throughput screening of curdlan-producing strains. Fermentation was miniaturized using 48-well plates, while curdlan quantification was performed in 96-well plates through formation of a curdlan–aniline blue complex. Key parameters were systematically optimized. Under optimal conditions, curdlan dissolved in 1.0 mol/L NaOH was reacted with 2.0 mg/mL aniline blue in 0.5 mol/L phosphate buffer (pH 7.0) for 90 min, and absorbance was measured at 550 nm. The assay demonstrated excellent linearity between curdlan concentration and absorbance ($y = 1.0008x + 0.1536$, $R^2 = 0.9957$, $p < 0.001$). To validate the method, curdlan yields from nine mutants derived from ATCC31749 were determined using both gravimetric and colorimetric approaches, revealing a strong correlation ($R^2 = 0.8683$, $p < 0.001$), that confirmed the assay's reliability for rapid screening. Application of this platform to 132 UV-mutagenized strains identified nine mutants with enhanced curdlan production. The best-performing strain, UV150824-02, produced 46.8 ± 0.08 g/L curdlan, an 11.4% increase over the wild-type strain ATCC31749 (41.6 ± 1.54 g/L), and maintained stable curdlan production over nine laboratory passages (coefficient of variation = 2.72%). This method significantly improves the efficiency of mutagenesis-based strain screening and provides a practical and efficient tool for accelerating strain improvement in industrial polysaccharide fermentation.

Keywords: curdlan; aniline blue; microscale analysis; high-throughput screening

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1. Introduction

Curdlan is a water-insoluble polysaccharide composed exclusively of β-1,3-D-glucose residues, primarily synthesized by *Agrobacterium* and *Rhizobium* species under nitrogen-limited conditions [1,2]. Its unique gel-forming properties initially led to its application as a food additive for improving food quality [3]. Despite its negligible water solubility, various modification strategies have been developed to enhance this property, yielding numerous derivatives that have expanded the functional repertoire of this biopolymer [4]. These derivatives exhibit diverse bioactivities, including antitumor, antiparasitic, antifungal,

antiviral, and immunomodulatory effects [5]. Additionally, curdlan's capacity for controlled drug release has positioned it as a promising carrier for drug delivery systems [6,7], while its use as a cement admixture to enhance fluidity highlights its broad industrial potential [8,9].

Among the various strategies to maximize curdlan production, optimization of fermentation parameters including temperature, pH, agitation, aeration, and nutrient composition has been extensively studied to maximize curdlan yields [10]. However, for these factors, strain performance is critical [11]. Despite its importance, mutagenesis-based strain improvement remains underexplored. Over the past two decades, only a few studies have investigated mutagenesis breeding of curdlan-producing strains. Early efforts by Kim et al. generated an *Agrobacterium* sp. mutant with enhanced curdlan production [12], while West et al. and Kalyanasundaram et al. isolated higher-yielding mutants through chemical mutagenesis [13,14]. However, the efficiency of mutant screening has been severely constrained by the limitations of traditional quantification methods, which hinders the application of mutagenesis breeding in curdlan production.

Conventional curdlan quantification relies on gravimetric analysis, which is time-consuming, labor-intensive, and incompatible with large-scale screening [15]. Flask-based fermentation, commonly used for mutant evaluation, further limits throughput due to its low processing capacity. Since the success of mutagenesis breeding depends heavily on the number of mutants screened, there is an urgent need for rapid, high-throughput quantification methods to accelerate strain improvement.

Aniline blue, an acid dye with a stable molecular structure, binds specifically to β-1,3-glucans such as curdlan, forming a fluorescent complex with a characteristic absorption peak near 550 nm [16,17]. Nakanishi et al. demonstrated that curdlan and aniline blue form complexes in dilute alkaline solutions, with absorbance correlating linearly to the curdlan concentration [18]. Compared with HPLC and enzymatic methods, aniline blue-based colorimetric assays offer advantages in terms of cost-effectiveness, simplicity, and compatibility with high-throughput formats, making them ideal for large-scale mutant screening. While this principle has been applied in plant histology and food analysis, its potential for high-throughput screening in microbial strain development has not been fully realized.

Recent advances have demonstrated the feasibility of integrated high-throughput platforms for microbial exopolysaccharide screening. Rühmann et al. developed a comprehensive platform capable of accomplishing strain cultivation, exopolysaccharide quantification, monosaccharide composition analysis, and carbohydrate fingerprinting within a single working day using microplate-based workflows [19]. While such platforms confirm the broad utility of miniaturized screening approaches for polysaccharide producers, a complete integrated workflow specifically combining miniaturized fermentation with a systematically optimized aniline blue-based colorimetric assay for mutagenesis-based curdlan strain improvement has not been described previously [20].

To address this limitation, we developed a systematically optimized integrated microscale high-throughput screening platform that couples 48-well plate fermentation with 96-well plate-based aniline blue colorimetric quantification for the rapid screening of curdlan-producing strains (Figure S1). This platform markedly improves screening efficiency compared with conventional approaches by reducing sample consumption, shortening analytical turnaround time, and enabling parallel evaluation of large mutant libraries. The developed workflow provides a practical and scalable strategy for accelerating microbial strain improvement and enhancing curdlan fermentation performance.

2. Results and Discussion

2.1. Determination of the Maximum Absorption Wavelength

To establish the optimal detection wavelength, absorption spectra of the curdlan–aniline blue complex were scanned from 400 to 700 nm at varying curdlan concentrations (Figure 1). Regardless of the curdlan concentration, all spectra exhibited a single, well-defined absorption peak at 550 nm with minimal background interference at other wavelengths, with absorbance increasing up to this wavelength and declining thereafter. Consequently, 550 nm was selected as the detection wavelength for all subsequent analyses. This finding aligns with previous reports indicating that aniline blue forms a strongly fluorescent complex with β-1,3-glucans such as curdlan, producing a characteristic absorption peak near this wavelength [21].

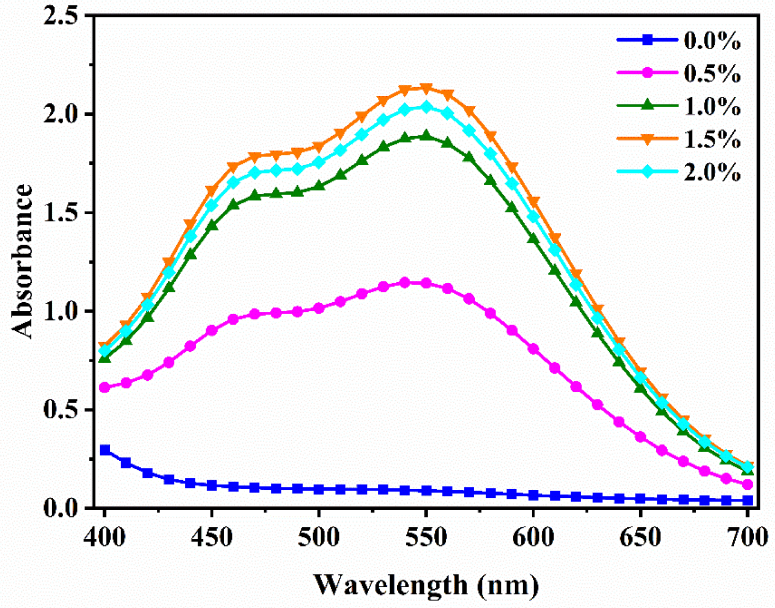


Figure 1. Absorption spectra of the curdlan–aniline blue complex from 400 to 700 nm at varying curdlan concentrations.

2.2. Effect of NaOH Concentration on Colorimetric Assay Performance

Curdlan is water-insoluble but readily dissolves in alkaline solutions such as NaOH [22]. However, NaOH concentration may influence both the solubility of curdlan and the formation of the curdlan–aniline blue complex. To investigate this effect, a range of NaOH concentrations (0.3–1.0 mol/L) were systematically evaluated.

Although higher NaOH concentrations enhanced curdlan solubility, the absorbance of the complex gradually declined with increasing NaOH concentration. This phenomenon may be attributed to conformational changes in curdlan molecules under alkaline conditions. In dilute alkali, curdlan typically adopts a stable triple-helix structure with a hydrophobic core and hydrophilic surface. As the NaOH concentration increases, this structure progressively transitions to a single-stranded helix and eventually unfolds into a more extended conformation [23]. Such structural alterations may disrupt the interaction between curdlan and aniline blue, thereby reducing absorbance intensity.

Although increasing the NaOH concentration resulted in a decrease in the absolute absorbance of the curdlan–aniline blue complex, the linear relationship between curdlan concentration and OD_{550} was improved (Figure 2a,b). This observation can be attributed to the reduced background contribution under higher alkaline conditions, which enhanced the relative signal-to-background ratio and improved the proportional response between curdlan concentration and absorbance. Therefore, the improved linearity does not reflect

an increase in absolute color intensity but rather a more reliable quantitative relationship between curdlan concentration and OD_{550}. The highest correlation coefficient (R^2 = 0.9939) was obtained at 1.0 mol/L NaOH, which was selected as the optimal condition for curdlan dissolution in subsequent experiments.

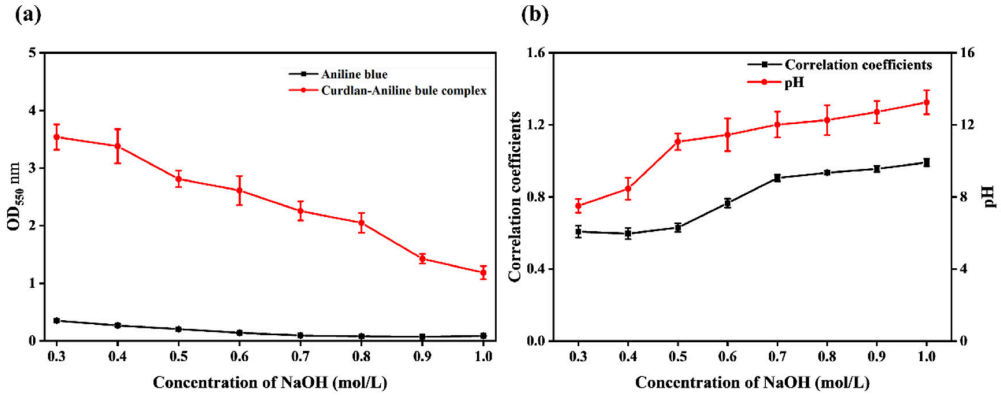


Figure 2. Optimization of NaOH concentration. (**a**) Effect of NaOH concentration on the absorbance of the curdlan–aniline blue complex. (**b**) Effect of NaOH concentration on correlation coefficient (R^2) and pH.

2.3. Effect of Aniline Blue Concentration on Colorimetric Assay Performance

Aniline blue concentration is critical for determining assay sensitivity and accuracy. As shown in Figure 3, when aniline blue concentrations ranged from 2.0 to 3.0 mg/mL, OD550 values increased linearly with curdlan concentration. Among the tested conditions, 2.0 mg/mL aniline blue yielded the strongest linear relationship between OD550 and curdlan concentration (R^2 = 0.9930). Higher dye concentrations likely increased the amount of unbound aniline blue in the reaction system, elevating background absorbance and reducing measurement accuracy. Accordingly, 2.0 mg/mL was selected as the optimal aniline blue concentration. This result is consistent with previous studies demonstrating that curdlan specifically binds aniline blue to form a blue complex, with color development dependent on the concentrations of both reactants [24].

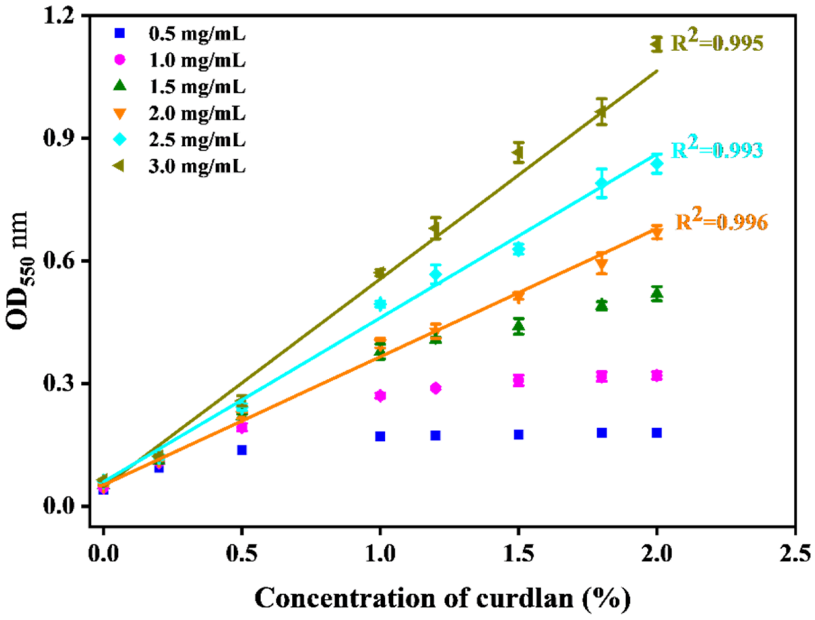


Figure 3. Effect of aniline blue concentration on the absorbance of the curdlan–aniline blue complex at different curdlan concentrations.

2.4. Effect of Phosphate Buffer Concentration and Reaction Time on Complex Formation

Phosphate buffer maintains stable pH during complex formation. As shown in Figure 4, when the buffer concentration was below 0.3 mol/L, the absorbance values of the curdlan–aniline blue complex and aniline blue alone were nearly identical, indicating insufficient differentiation. When the buffer concentration increased to 0.5–1.0 mol/L, the absorbance of the complex increased significantly while that of free dye remained relatively stable. Among the tested conditions, 0.5 mol/L phosphate buffer provided clear differentiation between the complex and free dye while maintaining stable absorbance values. Additionally, OD_{550} values stabilized when reaction time exceeded 90 min. Therefore, 0.5 mol/L phosphate buffer and a 90 min reaction time were selected as optimal conditions.

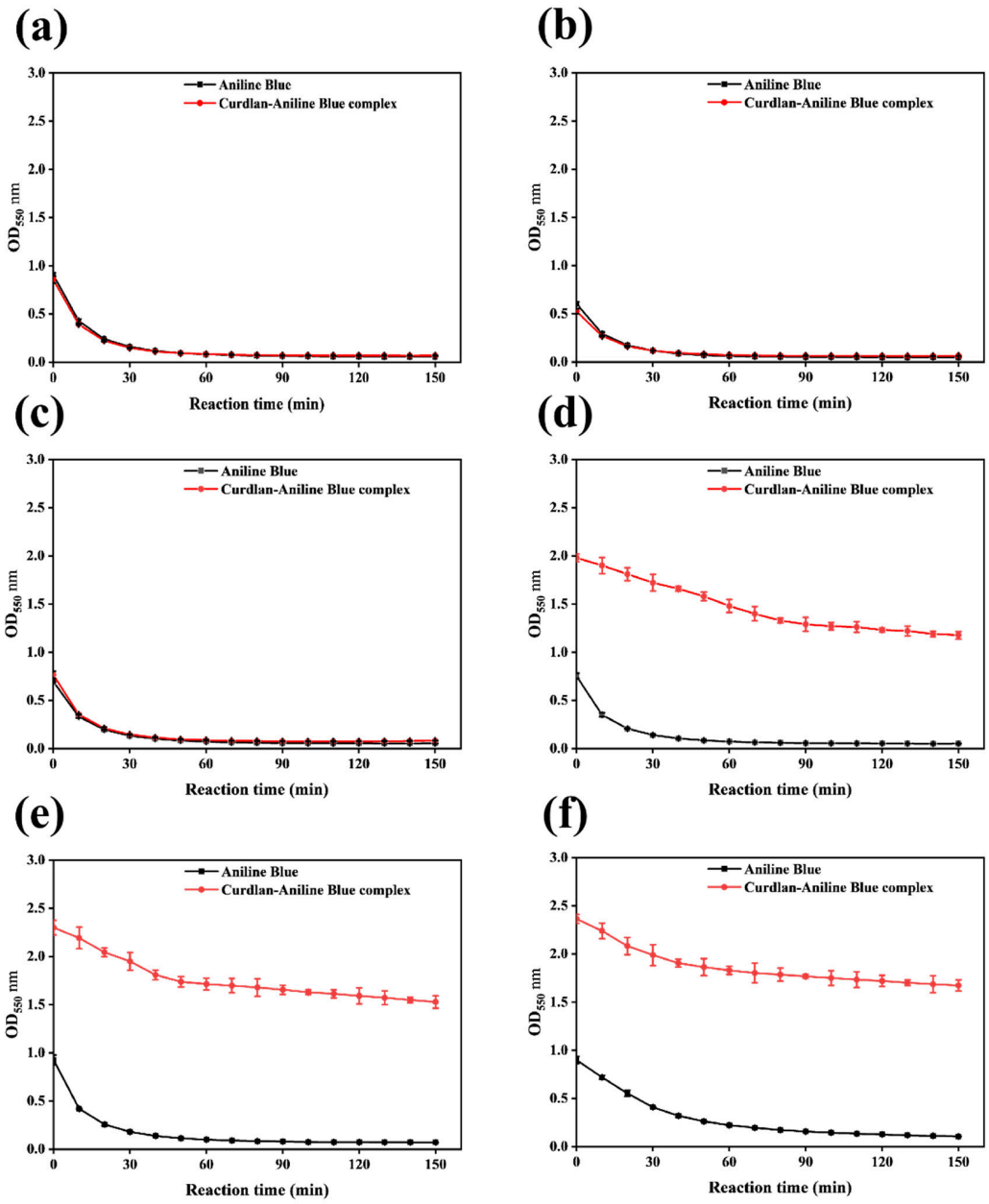


Figure 4. Effect of phosphate buffer concentration and reaction time on the absorbance of the curdlan–aniline blue complex. Panels (**a**–**f**) represent phosphate buffer concentrations of 0, 0.1, 0.3, 0.5, 0.7, and 1.0 mol/L, respectively.

2.5. Establishment of a Curdlan Micro-Assay Method Using 96-Well Plates

Based on the optimized parameters, a standard curve for curdlan quantification was established using the 96-well plate colorimetric method. As shown in Figure 5, OD_{550} values increased linearly with curdlan concentration. Linear regression analysis yielded the following equation:

$$y = 1.0008x + 0.1536 \ (R^2 = 0.9957, p < 0.001)$$

The high correlation coefficient and statistical significance confirm excellent linearity within the tested range (0.5–2.0% curdlan), validating the reliability of the assay for curdlan quantification. Beyond its accuracy, the microplate-based colorimetric method enables the simultaneous analysis of numerous samples, making it particularly suitable for high-throughput screening applications [25].

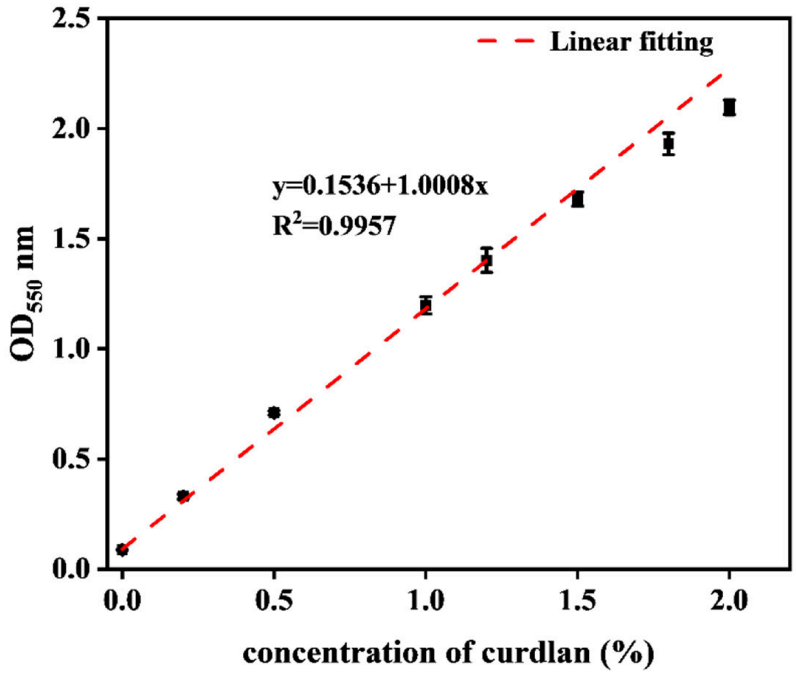


Figure 5. Standard calibration curve for curdlan quantification using the aniline blue colorimetric assay. Absorbance at 550 nm increased linearly with curdlan concentration under optimized reaction conditions. Data points represent the mean OD_{550} values of three independent replicates, and the dashed line indicates the fitted linear regression.

A visual demonstration of the 96-well plate-based aniline blue assay for high-throughput curdlan detection is shown in Figure S2. The gradual increase in blue color intensity with increasing curdlan concentrations corresponded well with the quantitative OD_{550} measurements, further supporting the reliability and applicability of the assay for high-throughput screening.

To evaluate the selectivity of the aniline blue assay within the *Agrobacterium* fermentation matrix, three types of samples were evaluated under the optimized conditions: aniline blue reagent alone (reagent blank), fermentation broth supernatant, and succinoglycan, an acidic exopolysaccharide containing β-1,3/1,6-glycosidic linkages that can be co-produced by *Agrobacterium* sp. ATCC 31749 [26]. As shown in Figure S3, none of these samples exhibited visible blue color development after 90 min of incubation, and their OD_{550} values showed no significant differences compared with the reagent blank ($p > 0.05$). These results indicate that components present in the fermentation matrix and the co-produced succinoglycan did not generate detectable interference in the aniline blue response, supporting the

selective determination of curdlan (β-1,3-glucan) in *Agrobacterium* fermentation samples. Therefore, the developed method is applicable for the direct determination of curdlan concentration without additional purification steps.

2.6. Correlation Between Gravimetric and Colorimetric Methods

To validate the newly developed method, nine UV-mutagenized strains were randomly selected and cultured in shake flasks. Curdlan yields were measured using both the traditional gravimetric method and the colorimetric assay. Regression analysis revealed a strong correlation between the two methods (Figure 6):

$$y = 0.00864x + 0.0351 \ (R^2 = 0.8683, p < 0.001).$$

These results demonstrate that the colorimetric assay provides comparable accuracy to the gravimetric method while offering significantly higher throughput. Consequently, the colorimetric assay represents an effective alternative for the rapid screening of large mutant libraries [27].

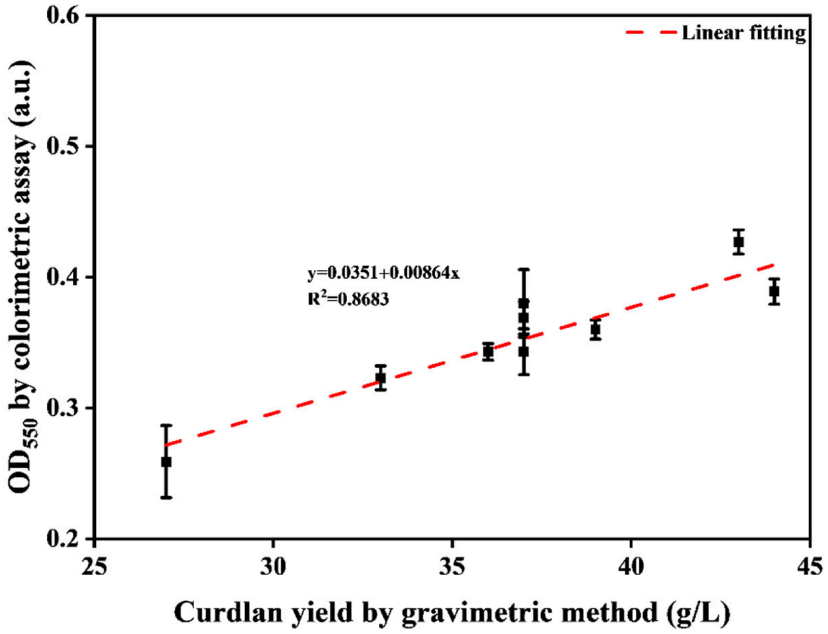


Figure 6. Correlation between curdlan concentration determined by the colorimetric assay and curdlan yield measured by the gravimetric method for nine UV-mutagenized strains.

To more rigorously assess the agreement between the two methods, a Bland–Altman analysis was performed (Figure S4). The mean bias was +0.24 g/L (gravimetric minus colorimetric-estimated yield), with 95% limits of agreement ranging from −3.86 to +4.34 g/L. All data points fell within the limits of agreement, and no proportional bias was observed across the measurement range. The moderate R^2 = 0.8683 is attributable to inherent differences in the measurand between the two methods: the gravimetric method quantifies all precipitable curdlan, while the colorimetric assay measures only the NaOH-soluble fraction in the supernatant. Given that the mean bias of 0.24 g/L is negligible relative to the yield difference between high-producing mutants and the wild-type strain (~5 g/L), the colorimetric assay is considered fit for purpose as a primary screening tool for relative mutant ranking.

2.7. Optimization of Culture Time in 48-Well Plates

Culture duration is a key determinant of curdlan production during fermentation [28]. To establish the optimal screening time in 48-well plates, the correlation between curdlan content measured in microplate cultures and final yield obtained in 96 h shake-flask fermentation was analyzed.

As shown in Figure 7, good correlations were observed for all tested culture times (24, 48, 72, and 96 h), with correlation coefficients (R^2) of 0.7309, 0.8337, 0.8502, and 0.8540, respectively. Notably, the correlation coefficient at 48 h (R^2 = 0.8337) was comparable to those at 72 h (R^2 = 0.8502) and 96 h (R^2 = 0.8540). Considering both screening efficiency and experimental time, a 48 h cultivation period was selected for subsequent high-throughput screening experiments.

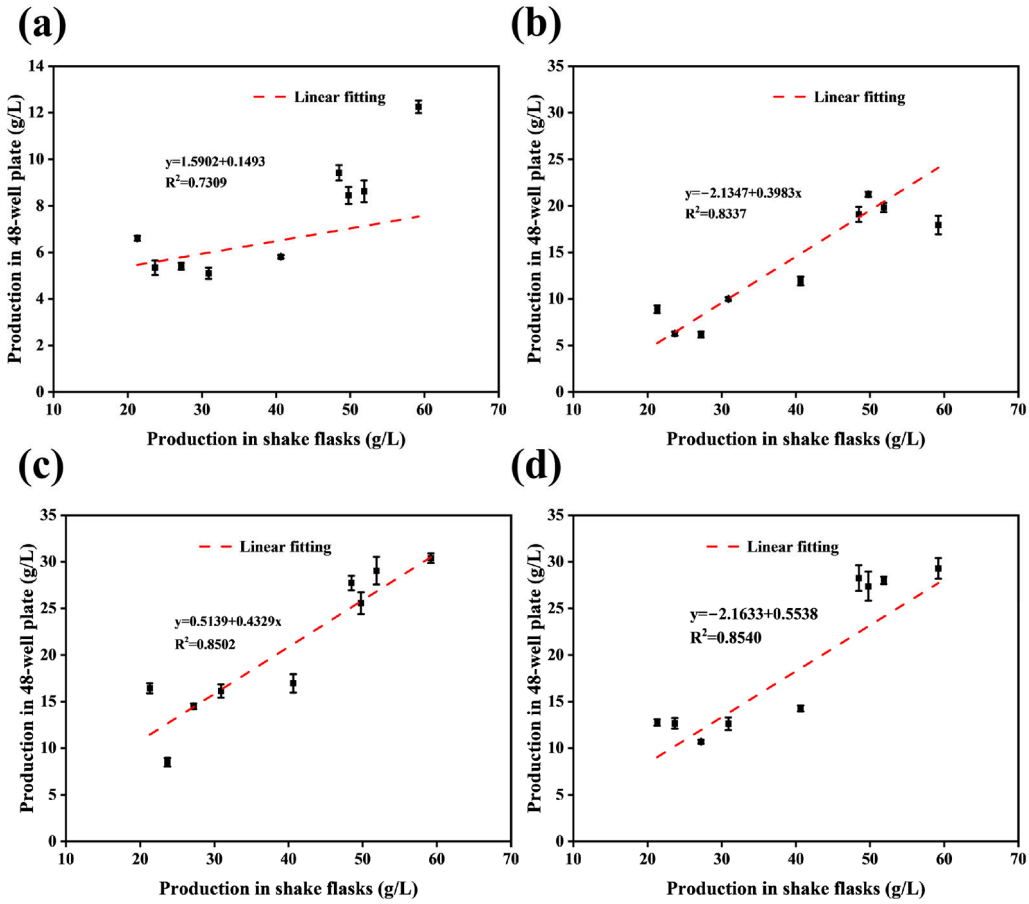


Figure 7. Correlation between curdlan production measured in 48-well plate cultures and yields obtained from 96 h shake-flask fermentation. Panels (**a**–**d**) represent microplate cultivation times of 24, 48, 72, and 96 h, respectively. The squares represent individual data points for each mutant strain, and the dashed line indicates the fitted linear regression.

2.8. Evaluation of Curdlan Production in 48-Well Plate Cultures

To assess the reproducibility of the microplate screening system, 48 independent clones of the parental strain ATCC31749 were cultured in a 48-well plate and analyzed using the colorimetric assay. As shown in Figure 8, the average curdlan concentration was 14.88 ± 1.43 g/L, with a coefficient of variation of 9.62%. These results indicate good reproducibility and stability of the microplate-based cultivation and detection system, confirming its suitability for high-throughput screening of curdlan-producing mutants.

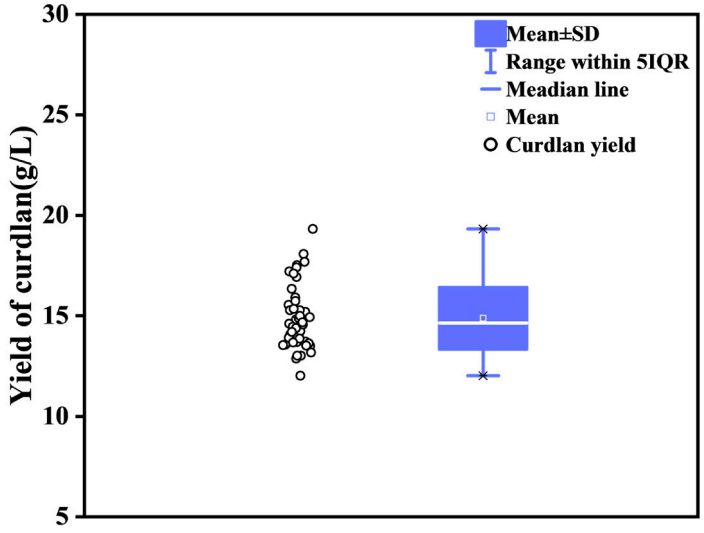


Figure 8. Reproducibility of curdlan quantification in 48-well plate cultures using the colorimetric assay. Data represent 48 independent clones of the parental strain ATCC31749.

2.9. High-Throughput Screening of UV-Mutagenized Strains

UV irradiation is widely used to induce random mutations in microbial genomes, generating strains with altered phenotypes [29]. Using the integrated aniline blue colorimetric assay and 48-well plate screening platform, 132 surviving colonies obtained after UV mutagenesis were subjected to initial screening in 48-well plates, followed by rescreening in shake flasks.

Nine strains exhibited higher curdlan yields than the wild-type strain ATCC31749 (41.6 ± 1.54 g/L) (Figure 9). The highest-producing mutant, designated UV150824-02, produced 46.8 ± 0.08 g/L of curdlan, representing an 11.4% increase over the wild type (Figure 10). Compared with previously reported mutants, UV150824-02 produced 46.8 g/L, which is comparable to the 45.2 g/L reported by Kim et al., but was obtained with significantly higher screening throughput (132 mutants screened in 2 days vs. approximately 50 mutants in 2 weeks using conventional flask-based methods) [12,13].

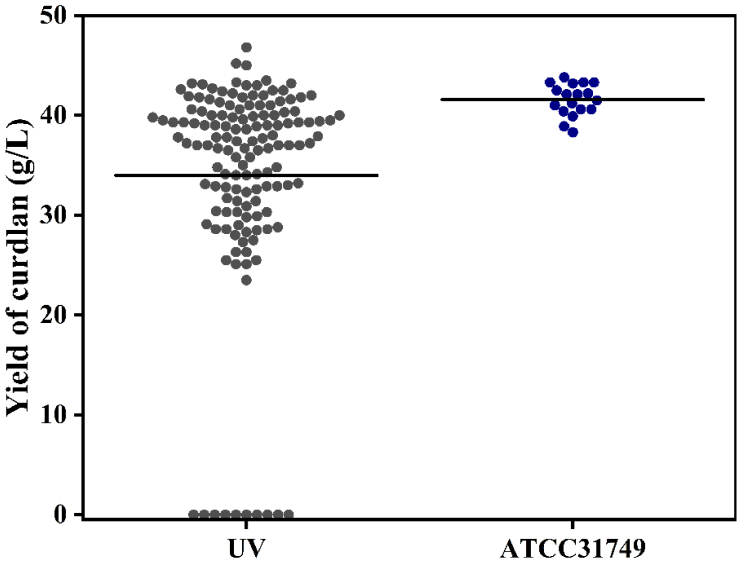


Figure 9. Curdlan yields of UV-mutagenized strains following rescreening in shake flasks. Horizontal lines represent the mean curdlan yields of the wild-type strain ATCC31749 (right) and selected UV-mutagenized strains (left).

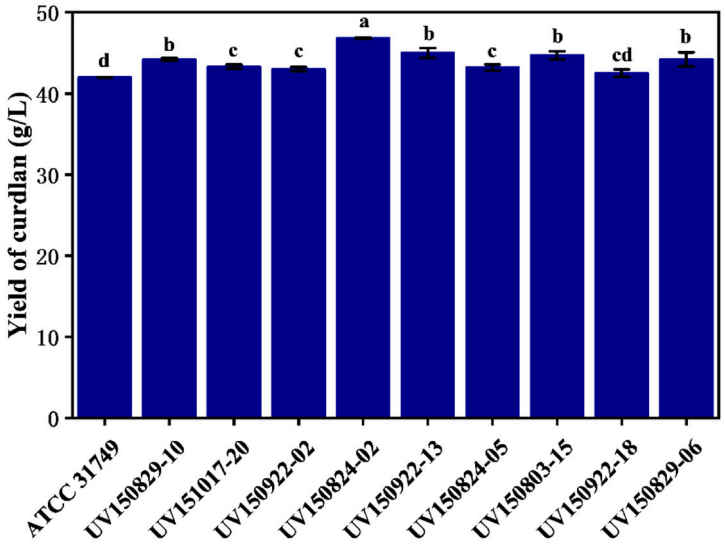


Figure 10. Curdlan yields of nine mutant strains exhibiting higher production levels than the wild-type strain *Agrobacterium* sp. ATCC 31749. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Different letters indicate significant differences among strains ($p < 0.05$).

To evaluate genetic stability, mutant UV150824-02 was serially passaged nine times on LB agar. The coefficient of variation in curdlan yield across these passages was only 2.72%, with an average production of 45.0 ± 1.23 g/L (Figure 11). These results indicate that UV150824-02 maintained consistent curdlan production over nine successive passages (CV = 2.72%), with long-term stability under production conditions requiring further validation.

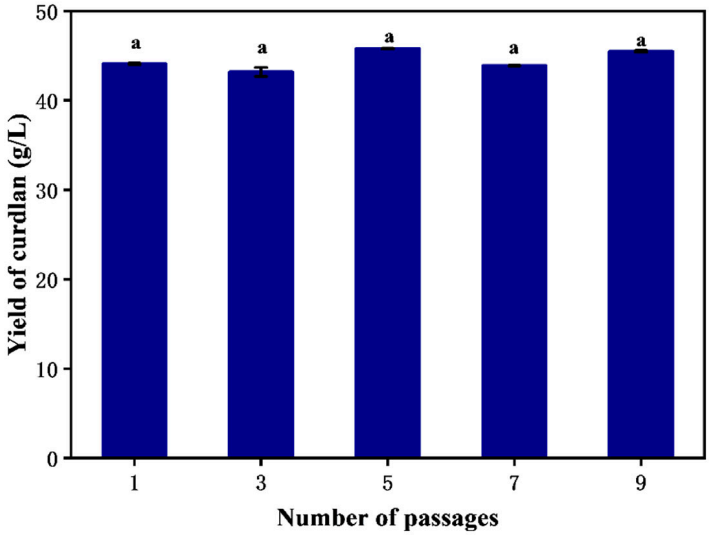


Figure 11. Genetic stability of strain UV150824-02. Curdlan yields over nine consecutive passages demonstrate stable production capacity. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. The same letter (a) above the bars indicates no significant differences among passages ($p \geq 0.05$).

The molecular and metabolic mechanisms underlying the enhanced curdlan production of strain UV150824-02 remain to be elucidated. Future studies involving whole-genome sequencing and comparative physiological and metabolic analyses will provide further insights into the genetic and metabolic basis of this improved phenotype.

2.10. Method Performance and Limitations

To further contextualize the performance of the developed assay, a systematic comparison with existing curdlan quantification approaches was conducted (Table 1). The gravimetric method remains the most widely used reference approach for curdlan determination; however, it is inherently low-throughput, requiring 24–48 h per batch due to drying procedures and typically supporting only 20–40 samples per day [15]. HPLC-based methods provide high analytical sensitivity (minimum detectable concentration approximately 0.084 mg/L) but require specialized instrumentation, extensive sample preparation, and relatively low sample throughput (2–4 samples/day) [30]. Enzymatic approaches based on endo-β-1,3-glucanase offer rapid and selective detection (~25 min), but their application is limited by reagent cost and throughput constraints (3–8 samples/day) [31]. The Congo red assay provides a simple colorimetric alternative (1–3 min) but was primarily developed for β-1,3/1,6-glucans from edible mushrooms and exhibits lower sensitivity (~5 μg/mL) compared with other analytical methods [32]. In contrast, the present aniline blue-based microplate assay enables rapid analysis of 200–400 samples per day with a low reagent cost (<$0.10 per sample), providing a practical solution for large-scale screening of curdlan-producing mutants.

Table 1. Comparison of analytical methods for curdlan quantification.

Parameter	This Work (Aniline Blue, 96-Well)	Gravimetric [15]	HPLC [30]	Enzymatic [31]	Congo Red [32]
Minimum detectable concentration	0.5% (*w*/*v*)	/	0.084 mg/L	/	5 μg/mL
Linear range	0.5–2.0% (*w*/*v*)	Broad (weight-based)	0.5–400 μg/mL	/	50–150 μg/mL
Analysis time per sample	1.5–2.0 h (including dissolution)	24–48 h (drying required)	4–5 h	25 min	1–3 min
Throughput (samples/day)	200–400	20–40	2–4	3–8	/
Cost per sample	Low (<$0.10)	Very low	High	High	Low
Main equipment required	Microplate reader	Balance, oven	HPLC	HPAEC-PAD	UV-Vis spectrophotometer
Structural information	No	No	No	Partial	Partial
Suitable for HTS	Yes	No	No	No	No

Notes: Abbreviations: LOD, limit of detection; HTS, high-throughput screening; HPLC, high-performance liquid chromatography; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection. LOD values are approximate and based on the published literature for each method class.

The efficiency gains demonstrated by the present platform are consistent with recent advances in high-throughput exopolysaccharide screening. Rühmann et al. reported an integrated microplate-based workflow capable of completing strain cultivation, polysaccharide quantification, monosaccharide composition, and carbohydrate fingerprinting within a single working day [19], highlighting the growing importance of miniaturized, rapid analytical platforms in microbial polysaccharide research. The present study extends this concept by providing a complete mutagenesis-oriented screening workflow specifically optimized for curdlan production, from UV irradiation through 48-well plate fermentation and aniline blue colorimetric quantification to shake-flask rescreening and genetic stability validation.

The developed method offers significant advantages in terms of throughput and speed but also has inherent limitations. The specificity of the aniline blue assay for curdlan was experimentally validated by testing representative fermentation matrix compo-

nents under the optimized assay conditions, including fermentation medium blank and succinoglycan—the primary co-produced extracellular polysaccharide of *Agrobacterium* sp. ATCC 31749. Neither the medium components nor succinoglycan produced absorbance values significantly different from the buffer blank ($p > 0.05$), confirming that the colorimetric signal is attributable specifically to the linear (1 → 3)-β-D-glucan backbone of curdlan (Figures 8 and 9). This specificity is consistent with the established structural requirements for aniline blue binding, which necessitates the ordered helical conformation of linear β-1,3-glucan chains—a feature absent in the branched, charged architecture of succinoglycan.

The detection range (0.5–2.0% curdlan) is suitable for screening high-yielding mutants but may require sample dilution at extremely high concentrations. Compared with enzymatic or HPLC-based methods, the aniline blue assay is faster and more cost-effective but provides less structural information. Future work should focus on (1) validating the method with chemical and transposon mutagenesis, (2) extending its application to other β-1,3-glucan producers such as *Alcaligenes* and *Rhizobium*, and (3) integrating automated liquid handling systems for ultra-high-throughput screening.

Although the developed platform showed good agreement between the aniline blue-based colorimetric assay and the conventional gravimetric method, a systematic evaluation of false-positive and false-negative rates was not performed in this study. Such analysis requires larger mutant libraries, predefined screening criteria, and extensive independent validation. Future studies will focus on evaluating the classification accuracy of the platform using expanded screening datasets and further improving its reliability for large-scale strain development.

3. Materials and Methods

3.1. Materials

Chemical reagents, including sucrose, $(NH_4)_2HPO_4$, KH_2PO_4, $MgSO_4 \bullet 7H_2O$, $CaCO_3$, NaOH, KH_2PO_4-$K_2HPO_4 \bullet 3H_2O$, and aniline blue, were purchased from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China). Curdlan standard was obtained from Kirin Kyowa Foods Company, Ltd. (Shinagawa-ku, Tokyo, Japan).

3.2. Strains

The curdlan-producing strain *Agrobacterium* sp. ATCC31749 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). UV-mutagenized derivatives of ATCC31749 were generated, isolated, and preserved by the School of Life Sciences, East China Normal University. Mutant strains were assigned strain numbers UV150824-01 to UV150824-132.

3.3. Media

LB agar slant medium contained (*w*/*v*) 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar, with initial pH adjusted to 7.2 [33]. Seed medium contained (*w*/*v*) 2% sucrose, 0.5% $(NH_4)_2HPO_4$, 0.15% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.2% $CaCO_3$, with initial pH adjusted to 7.0 [34]. Fermentation medium contained (*w*/*v*) 9% sucrose, 0.2% $(NH_4)_2HPO_4$, 0.2% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCO_3$, 0.01% $FeSO_4 \cdot 7H_2O$, 0.004% $MnSO_4$, and 0.002% $ZnCl_2$, with initial pH adjusted to 7.0 [15]. All media were sterilized at 121 °C for 20 min.

3.4. Culture Conditions

For microplate culture, single colonies of *Agrobacterium* sp. from LB agar plates were inoculated into 48-well plates containing 1 mL of fermentation medium per well

to achieve an initial OD_{600} of approximately 0.1. Cultures were incubated at 30 °C with shaking at 250 rpm for 24, 48, 72, or 96 h. For flask culture, strains were first grown in 250 mL Erlenmeyer flasks containing 50 mL of seed medium at 30 °C and 250 rpm for 18 h. Subsequently, 5 mL (10%, *v*/*v*) of seed culture was transferred to 50 mL of fermentation medium and incubated at 30 °C and 250 rpm for 24, 48, 72, or 96 h [35].

3.5. Determination of Maximum Absorption Wavelength

Standard curdlan solutions at concentrations of 0, 0.5, 1.5, and 2.0% (*w*/*v*) were prepared in 1.0 mol/L NaOH. Aniline blue solution (2.0 mg/mL) was prepared in 0.5 mol/L phosphate buffer (pH 7.0). In 96-well plates, 50 µL of each curdlan solution was mixed with 50 µL of aniline blue solution and incubated in the dark at 25 °C with shaking at 200 rpm for 120 min (initial conditions prior to optimization). Blank controls (NaOH only) and reagent controls (aniline blue only) were included in each assay to correct for background absorbance. Absorbance spectra were recorded from 400 to 700 nm using a Synergy HT microplate reader from BioTek Instruments, Inc. (Winooski, VT, USA) with a path length correction applied for 96-well plates.

3.6. Effect of NaOH Concentration on Curdlan Determination

Aniline blue (2.0 mg/mL) was dissolved in NaOH solutions of varying concentrations (0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mol/L). Separately, curdlan (2.0 g) was dissolved in 100 mL of the corresponding NaOH solutions to prepare curdlan–aniline blue complex solutions. Absorbance at 550 nm was measured using a microplate reader. For each NaOH concentration tested, a matched blank was prepared containing aniline blue (2.0 mg/mL) in the same NaOH solution without curdlan.

3.7. Optimization of Aniline Blue Concentration

Aniline blue solutions at concentrations of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/mL were prepared in 0.5 mol/L phosphate buffer (pH 7.0). In 96-well plates, 50 µL of each aniline blue solution was mixed with 50 µL of curdlan solution (0.5%, 1.0%, 1.5%, or 2.0% in 1.0 mol/L NaOH). The plates were incubated in the dark at 25 °C with shaking at 200 rpm for 2 h, after which absorbance at 550 nm was measured using a microplate reader. A blank was prepared at each aniline blue concentration by substituting the curdlan–NaOH solution with an equal volume of 1.0 mol/L NaOH alone.

3.8. Optimization of Phosphate Buffer Concentration

Aniline blue solutions (2.0 mg/mL) were prepared in phosphate buffers at concentrations of 0, 0.1, 0.3, 0.5, 0.7, and 1.0 mol/L. A 2.0% curdlan standard solution was prepared in 1.0 mol/L NaOH. In 96-well plates, 50 µL of aniline blue solution was mixed with 50 µL of curdlan solution and incubated in the dark at 25 °C with shaking at 200 rpm for varying durations (30, 60, 90, 120, and 150 min). It should be noted that the phosphate buffer was not intended to neutralize the NaOH solution; rather, upon 1:1 mixing, the resulting reaction mixture reached a measured pH of 9.4 ± 0.2, which is sufficient to maintain curdlan in solution and to support stable curdlan–aniline blue complex formation. Absorbance at 550 nm was measured using a microplate reader. For each buffer concentration and time point, a matched blank was prepared by replacing the curdlan–NaOH solution with an equal volume of 1.0 mol/L NaOH.

3.9. Determination of Curdlan Concentration Using 96-Well Plates

Standard curdlan solutions at concentrations of 0.5, 1.0, 1.5, and 2.0% were prepared in 1.0 mol/L NaOH. Aniline blue solution (2.0 mg/mL) was prepared in 0.5 mol/L phosphate buffer (pH 7.0). In 96-well plates, 50 µL of aniline blue solution was mixed with 50 µL of

curdlan solution and incubated in the dark at 25 °C with shaking at 200 rpm for 90 min. Absorbance at 550 nm was measured using a microplate reader.

For sample analysis, culture broth from 48-well plates was mixed with 2 mL of 1 mol/L NaOH and shaken at 200 rpm at 25 °C for 60 min, followed by centrifugation at 3000× *g* for 10 min. The supernatant (50 µL) was transferred to a 96-well plate, mixed with 50 µL of 2 mg/mL aniline blue, and incubated in the dark at 25 °C for 90 min. Absorbance at 550 nm was measured using a microplate reader. Blank controls (culture medium without cells) were included in each assay.

To assess assay specificity, three controls were prepared and tested in parallel with curdlan standards: (1) aniline blue reagent alone (2.0 mg/mL in 0.5 mol/L phosphate buffer, pH 7.0) combined with 1.0 mol/L NaOH in the absence of any polysaccharide; (2) fermentation broth medium supernatant obtained by centrifugation at 3000× *g* for 10 min and dissolved in 1.0 mol/L NaOH at the same volume ratio used for curdlan samples; and (3) succinoglycan dissolved in 1.0 mol/L NaOH at concentrations of 0.5–2.0% (*w*/*v*). Each control (50 µL) was mixed with 50 µL of aniline blue solution in 96-well plates and incubated under the optimized conditions (25 °C, 200 rpm, 90 min, dark). Absorbance at 550 nm was measured using a microplate reader, and photographs were taken before and after incubation to document color development.

3.10. Determination of Curdlan Production by Gravimetric Method

Curdlan production was determined gravimetrically as previously described [36]. Briefly, 10 g of fermentation broth was mixed with 100 mL of 1 mol/L NaOH and stirred for 3 h to dissolve curdlan. The mixture was centrifuged at 9000× *g* for 10 min, and curdlan in the supernatant was precipitated by adding 100 mL of 1 mol/L HCl. The precipitate was collected by centrifugation at 9000× *g* for 10 min, washed three times with deionized water to remove salts, and dried to a constant weight at 80 °C.

3.11. UV Mutagenesis

Wild-type strain ATCC31749 was cultured in 5 mL of seed medium with shaking for 18 h. One milliliter of seed culture was diluted with 9 mL of sterile water to achieve an OD_{600} of approximately 0.4. The cell suspension was dispersed using a magnetic stirrer and subjected to UV irradiation (15 W, 254 nm) at a distance of 30 cm for 30 s. UV irradiation resulted in approximately 90% lethality, which is within the optimal range for mutagenesis. The irradiated suspension was diluted 10^5-fold with sterile water, and 100 µL was plated on LB agar. After overnight incubation at 30 °C, single colonies were selected for initial screening in 48-well plates, followed by rescreening in shake flasks.

3.12. Determination of Genetic Stability of High-Yielding Strains

The high-yielding strain UV150824-02 was serially passaged nine times on LB agar. Each generation was inoculated into seed medium, transferred to fermentation medium, and cultivated for 96 h. Curdlan yield was determined by the gravimetric method.

3.13. Statistical Analysis

All fermentation experiments were performed with at least three independent biological replicates ($n \geq 3$). Results are expressed as mean ± standard deviation (SD). Correlation coefficients (R^2) and linear regression analyses were calculated using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered significant. The coefficient of variation (CV) was calculated as (SD/mean) × 100%.

4. Conclusions

This study addresses a critical bottleneck in curdlan production by developing and validating a systematically optimized integrated microscale workflow combining 48-well plate fermentation with a quantitative aniline blue-based colorimetric assay. While aniline blue binding to β-1,3-glucans and microplate cultivation are individually established approaches, their systematic integration with full parameter optimization, method validation against the gravimetric reference method, and end-to-end application to mutagenesis-based strain improvement represent meaningful methodological contributions to industrial polysaccharide fermentation. Using optimized reaction conditions, we established a rapid colorimetric assay for curdlan quantification that demonstrated excellent linearity (R^2 = 0.9957) and strong correlation with the traditional gravimetric method (R^2 = 0.8683). Application of this platform to 132 UV-mutagenized strains successfully identified UV150824-02, a high-yielding mutant exhibiting an 11.4% increase in curdlan production (46.8 g/L) and consistent production over nine laboratory passages (CV = 2.72%), compared with the wild-type strain ATCC31749 (41.6 g/L). Compared with conventional flask-based screening, this method reduced screening time from approximately 2 weeks to 2 days while maintaining comparable accuracy.

This platform is readily adaptable to other β-1,3-glucan-producing organisms and may be coupled with alternative mutagenesis strategies, including chemical or transposon-based approaches, to broaden its utility in industrial strain engineering. Future studies should focus on (1) validating the method with chemical and transposon mutagenesis, (2) extending its application to other β-1,3-glucan producers such as *Alcaligenes* and *Rhizobium*, and (3) integrating automated liquid handling systems for ultra-high-throughput screening. While this platform significantly improves screening efficiency, further validation with diverse microbial systems and mutagenesis strategies is needed to fully establish its versatility.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors14080178/s1. Figure S1: Schematic illustration of the integrated microscale high-throughput screening platform for curdlan-producing strains. (A) Principle of curdlan detection: alkaline treatment with NaOH disrupts the higher-order structure of curdlan and makes the β-1,3-glucan chains accessible for interaction with aniline blue, generating a blue-colored complex with a characteristic absorbance at 550 nm. (B) Workflow of the high-throughput screening strategy for UV-mutagenized strains, including colony isolation, microscale fermentation in 96-well plates, colorimetric quantification, and secondary validation by shake-flask fermentation; Figure S2: Visual demonstration of the 96-well plate-based aniline blue assay for high-throughput curdlan detection. Representative wells containing increasing concentrations of curdlan (from left to right: 0, 0.5, 1.0, 1.5, and 2.0% (*w*/*v*)) were analyzed after 90 min of incubation at 25 °C under optimized assay conditions (1.0 mol/L NaOH, 2.0 mg/mL aniline blue, and 0.5 mol/L phosphate buff-er, pH 7.0); Figure S3: Interference assessment of the aniline blue colorimetric assay for curdlan quantification. Absorbance at 550 nm was measured for aniline blue reagent alone (reagent blank) (B), curdlan fermentation broth supernatant (T1), and succinoglycan solutions (2.0%, *w*/*v*) (T2) under the optimized assay conditions after 90 min of incubation. Data are presented as mean ± SD (n = 3). The OD_{550} values of the curdlan fermentation broth supernatant and succinoglycan solutions showed no significant differences compared with the buffer blank ($p > 0.05$, one-way ANOVA followed by Tukey's post hoc test); Figure S4: Bland–Altman plot comparing gravimetric and colorimetric methods for curdlan yield determination. The y-axis represents the difference between gravimetric and colorimetric yield measurements; the x-axis represents the mean of the two methods. The solid red line indicates the mean bias (+0.24 g/L); dashed green lines indicate the 95% limits of agreement (−3.86 to +4.34 g/L). All nine data points (individual strains)

fall within the limits of agreement, with no proportional bias observed across the measurement range (n = 9, SD = 2.09 g/L).

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References

1. Harada, T. Special Bacterial Polysaccharides and Polysaccharases. *Biochem. Soc. Symp.* **1983**, *48*, 97–116.
2. Rui, K.; Tian, J.; Tang, X.; Ma, J.; Xu, P.; Tian, X.; Wang, Y.; Xu, H.; Lu, L.; Wang, S. Curdlan Blocks the Immune Suppression by Myeloid-Derived Suppressor Cells and Reduces Tumor Burden. *Immunol. Res.* **2016**, *64*, 931–939. [CrossRef] [PubMed]
3. Chen, Y.; Wang, F. Review on the Preparation, Biological Activities and Applications of Curdlan and Its Derivatives. *Eur. Polym. J.* **2020**, *141*, 110096. [CrossRef]
4. Wang, Q.; Sheng, X.; Shi, A.; Hu, H.; Yang, Y.; Liu, L.; Fei, L.; Liu, H. β-Glucans: Relationships between Modification, Conformation and Functional Activities. *Molecules* **2017**, *22*, 257. [CrossRef] [PubMed]
5. Laroche, C.; Michaud, P. New Developments and Prospective Applications for β (1, 3) Glucans. *Recent. Pat. Biotechnol.* **2007**, *1*, 59–73. [CrossRef] [PubMed]
6. Fujimoto, K.; Kawaguchi, Y.; Shimohigoshi, M.; Gotoh, Y.; Nakano, Y.; Usui, Y.; Hayashi, T.; Kimura, Y.; Uematsu, M.; Yamamoto, T.; et al. Antigen-Specific Mucosal Immunity Regulates Development of Intestinal Bacteria-Mediated Diseases. *Gastroenterology* **2019**, *157*, 1530–1543.e4. [CrossRef] [PubMed]
7. Aquinas, N.; Bhat, M.R.; Selvaraj, S. A Review Presenting Production, Characterization, and Applications of Biopolymer Curdlan in Food and Pharmaceutical Sectors. *Polym. Bull.* **2022**, *79*, 6905–6927. [CrossRef]
8. Kahng, G.-G.; Lim, S.-H.; Yun, H.-D.; Seo, W.-T. Production of Extracellular Polysaccharide, EPS WN9, from *Paenibacillus* sp. WN9 KCTC 8951P and Its Usefulness as a Cement Mortar Admixture. *Biotechnol. Bioprocess. Eng.* **2001**, *6*, 112–116. [CrossRef]
9. Chang, I.; Cho, G.-C. Strengthening of Korean Residual Soil with β-1,3/1,6-Glucan Biopolymer. *Constr. Build. Mater.* **2012**, *30*, 30–35. [CrossRef]
10. Freitas, F.; Torres, C.A.V.; Reis, M.A.M. Engineering Aspects of Microbial Exopolysaccharide Production. *Bioresour. Technol.* **2017**, *245*, 1674–1683. [CrossRef] [PubMed]
11. Prakash, S.; Rajeswari, K.; Divya, P.; Ferlin, M.; Rajeshwari, C.T.; Vanavil, B. Optimization and Production of Curdlan Gum Using *Bacillus cereus* PR3 Isolated from Rhizosphere of Leguminous Plant. *Prep. Biochem. Biotechnol.* **2018**, *48*, 408–418. [CrossRef] [PubMed]
12. Kim, M.-K.; Ryu, K.-E.; Choi, W.-A.; Rhee, Y.-H.; Lee, I.-Y. Enhanced Production of (1→3)-β-d-Glucan by a Mutant Strain of *Agrobacterium* Species. *Biochem. Eng. J.* **2003**, *16*, 163–168. [CrossRef]
13. West, T.P. Elevated Curdlan Production by a Mutant of *Agrobacterium* sp. ATCC 31749. *J. Basic Microbiol.* **2009**, *49*, 589–592. [CrossRef] [PubMed]
14. Kalyanasundaram, G.T.; Doble, M.; Gummadi, S.N. Production and Downstream Processing of (1→3)-β-D-Glucan from Mutant Strain of *Agrobacterium* sp. ATCC 31750. *Amb. Express* **2012**, *2*, 31. [CrossRef] [PubMed]
15. Zhang, H.-T.; Zhan, X.-B.; Zheng, Z.-Y.; Wu, J.-R.; English, N.; Yu, X.-B.; Lin, C.-C. Improved Curdlan Fermentation Process Based on Optimization of Dissolved Oxygen Combined with pH Control and Metabolic Characterization of *Agrobacterium* sp. ATCC 31749. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 367–379. [CrossRef] [PubMed]
16. Young, S.-H.; Jacobs, R.R. Sodium Hydroxide-Induced Conformational Change in Schizophyllan Detected by the Fluorescence Dye, Aniline Blue. *Carbohydr. Res.* **1998**, *310*, 91–99. [CrossRef]

17. Young, E.S.; Kenyon, W.J. Interaction of the Dye Aniline Blue with the Curdlan-Type Exopolysaccharide from *Cellulomonas Flavigena* KU (ATCC 53703). *Ind. Biotechnol.* **2023**, *19*, 204–207. [CrossRef]
18. Nakanishi, I.; Kimura, K.; Kusui, S.; Yamazaki, E. Complex Formation of Gel-Forming Bacterial (1→3)-β-D-Glucans (Curdlan-Type Polysaccharides) with Dyes in Aqueous Solution. *Carbohydr. Res.* **1974**, *32*, 47–52. [CrossRef]
19. Rühmann, B.; Schmid, J.; Sieber, V. High Throughput Exopolysaccharide Screening Platform: From Strain Cultivation to Monosaccharide Composition and Carbohydrate Fingerprinting in One Day. *Carbohydr. Polym.* **2015**, *122*, 212–220. [CrossRef] [PubMed]
20. Li, X.; Zheng, T.; Xiao, Y.; Zhao, Y.; Wu, P. Field-Deployable Colorimetric Array for on-Site Diagnosis of Urinary Tract Infection and Identification of Causative Pathogens. *Anal. Chem.* **2024**, *96*, 14679–14687. [CrossRef] [PubMed]
21. Wood, P.J.; Fulcher, R.G. Specific Interaction of Aniline Blue with (1→3)-β-d-Glucan. *Carbohydr. Polym.* **1984**, *4*, 49–72. [CrossRef]
22. Ikeda, S.; Shishido, Y. Atomic Force Microscopy Studies on Heat-Induced Gelation of Curdlan. *J. Agric. Food Chem.* **2005**, *53*, 786–791. [CrossRef] [PubMed]
23. Tada, T.; Matsumoto, T.; Masuda, T. Influence of Alkaline Concentration on Molecular Association Structure and Viscoelastic Properties of Curdlan Aqueous Systems. *Biopolymers* **1997**, *42*, 479–487. [CrossRef]
24. El-Sayed, M.H.; Arafat, H.H.; Elsehemy, I.A.; Basha, M. Optimization, Purification and Physicochemical Characterization of Curdlan Produced by *Paenibacillus* sp. Strain NBR-10. *Biosci. Biotechnol. Res. Asia* **2016**, *13*, 901–909. [CrossRef]
25. Liu, Y.; Li, F.; Zhang, X.; Cao, G.; Jiang, W.; Sun, Y.; Zheng, P.; Zhang, D. A Fast and Sensitive Coupled Enzyme Assay for the Measurement of L-Threonine and Application to High-Throughput Screening of Threonine-Overproducing Strains. *Enzym. Microb. Technol.* **2014**, *67*, 1–7. [CrossRef] [PubMed]
26. Gao, M.; Liu, Z.; Zhao, Z.; Wang, Z.; Hu, X.; Jiang, Y.; Yan, J.; Li, Z.; Zheng, Z.; Zhan, X. Exopolysaccharide Synthesis Repressor Genes (exoR and exoX) Related to Curdlan Biosynthesis by *Agrobacterium* sp. *Int. J. Biol. Macromol.* **2022**, *205*, 193–202. [CrossRef] [PubMed]
27. Kwon, K.K.; Lee, J.; Kim, H.; Lee, D.-H.; Lee, S.-G. Advancing High-Throughput Screening Systems for Synthetic Biology and Bio-Foundry. *Curr. Opin. Syst. Biol.* **2024**, *37*, 100487. [CrossRef]
28. Aquinas, N.; Bhat, R.M.; Selvaraj, S. Submerged Fermentation and Kinetics of Newly Isolated *Priestia megaterium* for the Production of Biopolymer Curdlan. *J. Polym. Environ.* **2024**, *32*, 4683–4698. [CrossRef]
29. Laughery, M.F.; Wilson, H.E.; Sewell, A.; Stevison, S.; Wyrick, J.J. The Surprising Diversity of UV-induced Mutations. *Adv. Genet.* **2024**, *5*, 2300205. [CrossRef] [PubMed]
30. Zhang, X.; Xu, Z.; Yun, Z.; Bai, L.; Xu, X.; Luo, Q.; Purevtsogt, D.; Yang, L.; Liu, D. Determination of Yeast β-Glucan Content in Composite Yeast Culture by Enzymatic Hydrolysis HPLC Method. *Food Chem.* **2025**, *497*, 147058. [CrossRef] [PubMed]
31. Schiavone, M.; Vax, A.; Formosa, C.; Martin-Yken, H.; Dague, E.; François, J.M. A Combined Chemical and Enzymatic Method to Determine Quantitatively the Polysaccharide Components in the Cell Wall of Yeasts. *FEMS Yeast Res.* **2014**, *14*, 933–947. [CrossRef] [PubMed]
32. Nitschke, J.; Modick, H.; Busch, E.; Von Rekowski, R.W.; Altenbach, H.-J.; Mölleken, H. A New Colorimetric Method to Quantify β-1,3-1,6-Glucans in Comparison with Total β-1,3-Glucans in Edible Mushrooms. *Food Chem.* **2011**, *127*, 791–796. [CrossRef] [PubMed]
33. Gao, H.; Zhang, W.; Zhang, J.; Huang, Y.; Zhang, J.; Tian, J.; Niu, Y.; Zou, C.; Jia, C.; Chang, Z.; et al. Methionine Biosynthesis Pathway Genes Affect Curdlan Biosynthesis of *Agrobacterium* sp. CGMCC 11546 via Energy Regeneration. *Int. J. Biol. Macromol.* **2021**, *185*, 821–831. [CrossRef] [PubMed]
34. Gao, H.; Xie, F.; Zhang, W.; Tian, J.; Zou, C.; Jia, C.; Jin, M.; Huang, J.; Chang, Z.; Yang, X.; et al. Characterization and Improvement of Curdlan Produced by a High-Yield Mutant of *Agrobacterium* sp. ATCC 31749 Based on Whole-Genome Analysis. *Carbohydr. Polym.* **2020**, *245*, 116486. [CrossRef] [PubMed]
35. Yu, L.; Wu, J.; Liu, J.; Zhan, X.; Zheng, Z.; Lin, C.C. Enhanced Curdlan Production in *Agrobacterium* sp. ATCC 31749 by Addition of Low-Polyphosphates. *Biotechnol. Bioprocess. Eng.* **2011**, *16*, 34–41. [CrossRef]
36. Mohsin, A.; Sun, J.; Khan, I.M.; Hang, H.; Tariq, M.; Tian, X.; Ahmed, W.; Niazi, S.; Zhuang, Y.; Chu, J.; et al. Sustainable Biosynthesis of Curdlan from Orange Waste by Using *Alcaligenes faecalis*: A Systematically Modeled Approach. *Carbohydr. Polym.* **2019**, *205*, 626–635. [CrossRef] [PubMed]

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