Optimizing Luc Reporter Output: "Media and Temperature Effects on Bacterial Bioluminescence"

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The firefly luciferase (Luc) reporter assay is a powerful tool used to analyze promoter activities in living cells. Past studies have indicated that the short duration of luciferin-based chemical illumination poses a challenge for long-term observations. In this report, we successfully established and expressed the firefly Luc reporter gene detection system in *DC3000/PLH-luc*. The expression of the *hrpA* promoter was enhanced in the presence of 2% glucose. The system was researched under various conditions, including Mg^{2+}, Ca^{2+}, different pH ranges, and temperatures. It was found that the optimal reaction conditions for long-term tests were $24-37°C$, while for short-term tests, the bioluminescence was highest at 42°C. The optimal pH range for this system, while within the cell or in the crude extraction lysate, is 5 to 9. As the pH value drops to 3 or rises to 11, the enzymatic reaction rapidly decreases. The optimal activation concentration of Mg^{2+} is around 7.5 mM, while 1 mM Ca^{2+} is the optimal activation concentration. However, the activation effect of Ca^{2+} is only about 50% of that of Mg^{2+}. When the concentration of these metal ions is too high, it inhibits the enzyme's activity. Therefore, our research results indicate that by adjusting the reaction conditions, the reporter system can be made more sensitive and stable.

Keyword: Luciferase reporter gene system; Ions; Reaction system; Bioluminescence.

Introduction & Literature Review

Bioluminescence is the production of light through an enzymatic reaction involving the oxidation of a chemical substrate (luciferin) by an enzyme (luciferase)[1]. In 1947, McElroy isolated luciferase from fireflies. In the luminescent cells of fireflies, two chemical substances, luciferin and luciferase, are present. Luciferin undergoes an oxidation reaction catalyzed by luciferase, generating light[2]. In 1985, de Wet et al. successfully cloned and expressed the North American firefly luciferase gene using a prokaryotic expression system[3], and luciferase was subsequently applied as a reporter gene in detection systems. The luciferase reporter system utilizes the properties of luciferase to catalyze the oxidation of the substrate luciferin and emit a light signal, enabling quantitative analysis of the activity of target genes or regulatory elements by detecting the light signal. It is widely used in molecular biology and biotechnology to study gene expression, signaling pathways, protein interactions, and drug screening[3,4].

This system has been applied for analyzing promoter and enhancer activity. The promoter or regulatory sequence of the target gene is inserted into the reporter vector to drive the expression of the luciferase gene. When the reporter vector is transfected into cells, the luciferase expression level is proportional to the activity of the target promoter or regulatory sequence. After the addition of the luciferase substrate, the luciferase activity is quantitatively analyzed by measuring the luminous intensity (typically using a photometer or imaging system), thereby indirectly reflecting the activity of the target gene or regulatory element[5]. This system is also used for miRNA target gene verification and in vivo drug screening in animals.

The luciferase reporter system is a powerful and flexible tool, equipped with high sensitivity, a wide dynamic range, and low background interference, making it an important technique in molecular biology research. Current research has utilized firefly luciferase to assist in quantifying the degree of ultrasonic disruption in *Escherichia coli*[6]. This method not only accurately reflects the disruption level of target cells but also provides a reference for preserving protein activity during the disruption process.

The characteristics in their applications for measurement and research. In particular, this enzyme is highly prone to inactivation at high temperatures[7–9], and its bioluminescence spectrum exhibits a significant red shift under low pH values and unstable conditions[10,11]. Additionally, in studies on kiwifruit canker caused by *Pseudomonas syringae* pv. *actinidiae (Psa)*, a luciferase reporter strain of *Psa* was constructed and cultured in nutrient-rich KB medium and HDM (hrp-derepressing medium), which simulates the plant environment. It was found that HDM significantly induced luciferase expression[12]. In this system, the production of light usually requires auxiliary factors, supplementary enzymes, ATP, and intermediate steps. Regarding the

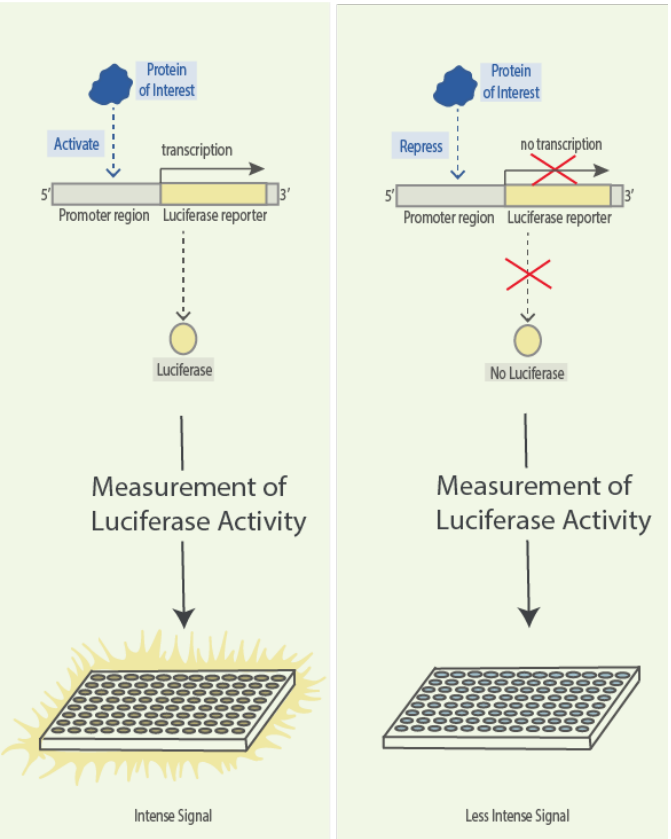

Fig. 1 A schematic of the luciferase reporting system to study whether a transcription factor can interact with a target promoter fragment[4]

timing of the emission, luciferase exhibits a unique mechanism: it binds to oxygen-containing luciferin and becomes stable, emitting light only when Mg^{2+} or Ca^{2+} cations are present[13]. The application of luciferase reporter genes provides a quantitative output for promoter activation.

The luciferase activity within cells is usually influenced by various external factors, such as the environment, which results in a decrease in light emission intensity over time and limits the duration of observation[14]. A common issue in existing experiments is the short duration of luciferin-based chemiluminescence, which makes repeated experiments time-consuming. For example, in some studies, after intraperitoneal injection of luciferin, cells expressing luciferase began to emit light within about 1 minute, reached a stable peak intensity after 10 minutes, and started to decay after 20-30 minutes. Approximately 3 hours later, the luciferin was excreted, and the luminescence completely disappeared[15,16]. Based on these experiments, the duration of luciferin luminescence is insufficient to support longer observations.

This study aims to reveal the effects of medium and temperature on the kinetic dynamics of the luciferase reaction in fireflies, as well as the role of external factors in enhancing enzyme activity. We constructed an expression plasmid capable of expressing luciferase in bacteria and selected glucose as the inducer of the promoter because, compared with glycerol and sucrose solutions, glucose has been proven to be a signaling molecule that can induce promoter expression[17]. Our data indicate that the luciferase expressed within the bacteria remains active in the presence of Mg^{2+} and Ca^{2+}, but its activity is inhibited at concentrations of 7.5 mM Mg^{2+} and 1 mM Ca^{2+}. Conversely, when the enzyme was purified through rough separation, the optimal ion concentrations for luciferase were 7.5 mM Mg^{2+} and 1 mM Ca^{2+}. We also found that the addition of glucose significantly enhances the enzyme's activity. Analysis of the optimal reaction temperature for luciferase revealed that the bioluminescence can be measured for a long time within the temperature range of $24-37°C$, while the maximum bioluminescence occurs at 42°C within 0-5 minutes. The study showed that enzyme activity decreases over time. Therefore, we enhanced the expression of luciferase in bacteria through an inducible enhanced promoter and conducted a detailed comparative study on the effects of different media and temperatures. This has never been done before.

Materials & Methods

Materials

The following bacterial strains were used in this study: *Escherichia coli* DH5α and *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000). *E. coli* DH5α was grown at 37°C and DC3000 was grown at 28°C. The following concentrations of antibiotics were used: ampicillin 100 μg/mL, kanamycin 50 μg/mL, and rifampicin 50 μg/mL with LB medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract).

Methods

Construction of Plasmids and Luciferase Reporter Strains

Luciferase-based assays have become an invaluable tool for analyzing cloned promoter DNA fragments, both for verifying the ability of a potential promoter fragment to drive the expression of a luciferase reporter gene in various cellular contexts and for dissecting binding elements in the promoter[18]. Here, the *hrpA* promoter of DC3000 were cloned by polymerase chain reaction (PCR) with TransStart FastPfu Fly DNA Polymerase. Using DC3000 genomic DNA as a template, *hrpA* fragments were amplified by PCR and linked with *luc* fragments, which were fused with the pLAFR6 vector. The constructs were transformed into *E. coli* strain, resulting in *pLAFR6/hrpA*, used for strain DC3000.

Luciferase Activity Measurement

Luciferase activity was measured using a TransDetect® Single-Luciferase (Firefly) Reporter Assay Kit (TRAN). For DC3000, the culture was collected and diluted with PBS buffer (10 mM PO_4^{3-}, 0.8% NaCl) to a cell suspension (OD_{600}=1.0). First, 1.5 mL of the suspension was harvested by centrifugation at 12,000× g for 1 min at room temperature and washed three times with PBS buffer. Next, 100 μL of supernatant was collected and transferred into a 96-well multifunctional enzyme-linked microplate, and 10 μL of D-luciferin (15 mg/mL) was added. The bioluminescence using a multifunctional microplate reader (TECAN Infinite 200 PRO).

Efficiency of Glucose, Mg^{2+} and Ca^{2+} on Luciferase Bioluminescence

This experiment was adapted from that described in Section above. For live strain cells, the cells were diluted with PBS buffer to an OD_{600} of 1.0, and then 1.0 mL of the cell suspension was harvested by centrifugation at 12,000×g for 1 minute at room temperature and washed twice with PBS buffer. Next, the collected cells were separately incubated with 2% glucose, Mg^{2+}, or Ca^{2+} for 10 minutes at room temperature. For cell lysate, the cells were diluted with PBS buffer containing Mg^{2+} or Ca^{2+} to an OD_{600} of 1.0, and then 1.0 mL of the cell suspension was harvested by centrifugation at 12,000×g for 1 minute at 4°C and washed twice with PBS buffer. The collected cells were lysed by adding 200 μL of cell lysis buffer (1×) and separately incubated with different concentrations of Mg^{2+} or Ca^{2+} for 20 minutes at 4°C. Finally, 100 μL of the supernatant was collected and transferred into a 96-well multifunctional enzyme-linked microplate, and 10 μL of D-luciferin (15 mg/mL) was added. The bioluminescence was measured using a multifunctional microplate reader (TECAN Infinite 200 PRO). Samples without the substrate (D-luciferin) were used as controls to measure background bioluminescence simultaneously.

Efficiency of pH on Luciferase Bioluminescence

This experiment was adapted from that described in Section "Luciferase Activity Measurement". After washing twice with PBS buffer, 1 mL of the cell suspension was incubated with PBS at pH 3, 5, 7, 9, or 11 for 10 minutes at room temperature. Then, 100 μL of the supernatant was collected and transferred into a 96-well multifunctional enzyme-linked microplate, and 10 μL of D-luciferin (15 mg/mL) was added. The bioluminescence was measured using a multifunctional microplate reader (TECAN Infinite 200 PRO). Samples without the substrate (D-luciferin) were used as controls to measure background bioluminescence simultaneously.

Efficiency of Temperature on Luciferase Bioluminescence

This experiment was adapted from that described in Section "Luciferase Activity Measurement". A total of 100 μL of the supernatant was collected and transferred into a 96-well multifunctional enzyme-linked microplate, and 10 μL of D-luciferin (15 mg/mL) was added. Next, the bioluminescence was measured at 1-minute intervals for a total of 21 time points under four different temperature conditions (24°C, 28°C, 37°C, and 42°C) by automatic detection. The bioluminescence was measured using a multifunctional microplate reader (TECAN Infinite 200 PRO). The same samples without the substrate (D-luciferin) were used as controls to measure background bioluminescence simultaneously.

Statistical analysis

All measurements were performed in triplicate, and experiments were repeated at least three times. Statistical analyses were performed using GraphPad Prism 9.0. The error bars represent the standard deviation (SD). Significance was assessed by calculating an independent-samples t-test. For ns, not significantly different; ***$P < 0.001$, extremely significant. The bioluminescence values of the experimental group were calculated using the formula: bioluminescence value (with D-luciferin) - bioluminescence value (without D-luciferin).

Results & Discussion

A Luciferase Reporter Assay System Was Successfully Expressed in DC3000/PLH-luc

To examine the utility of the Luc reporter system in DC3000, the *hrpA* promoter region linked with the *luc* gene was connected to the pLAFR6 plasmid and expressed in DC3000. PCR detection and sequencing were used to confirm the correct positive clone (Figure 2). The clones were further tested through luciferase activity experiments. As showed in Figure 3, in *DC3000/PLH-luc-1*, higher luminescence was detected after adding the reaction substrate D-luciferin. This further verified that the luciferase reporter assay system was successfully expressed in *DC3000/PLH-luc-1*, and it was used to the following experiments in Figure 3.

Glucose Enhanced Luciferase Activity by Binding the *hrpA* Promoter

It has been reported that HDM (*hrp*-derepressing medium) can affect luciferase expression [12]. Studies have shown that *hrpA* can enhance the expression of downstream genes by responding to glucose [12,19,20]. The overnight-cultured strain was resuspended in LB medium with 2% glucose, and the bioluminescence was measured immediately. The results show that, compared with

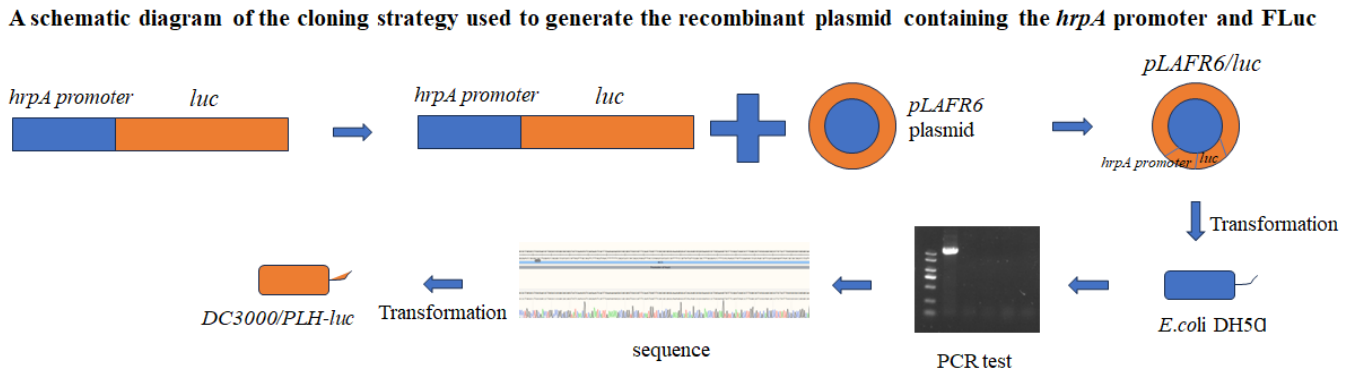

Fig. 2 The principle of constructing the expressing strain *DC3000/PLH-luc*.

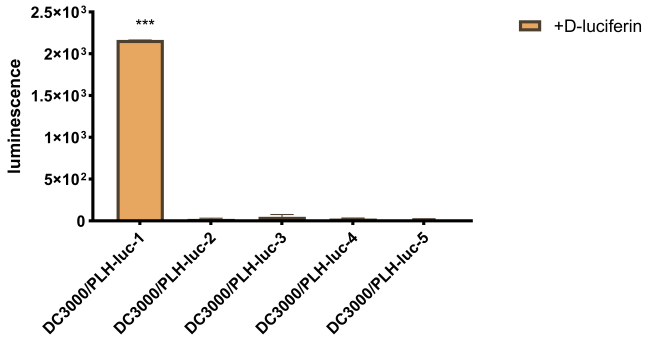

Fig. 3 The relative bioluminescence of *DC3000/PLH-luc* strains

the culture medium without glucose, the bioluminescence of *DC3000/PLH-Luc* significantly increased when glucose was present. This indicates that glucose can enhance the expression of the *hrpA* promoter (Figure 4).

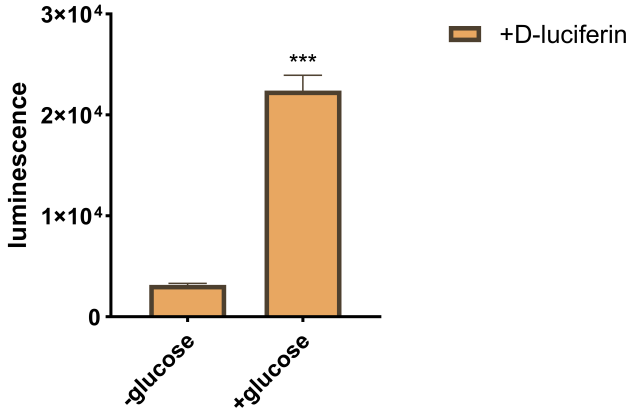

Fig. 4 The relative bioluminescence of *DC3000/PLH-luc* in LB medium with glucose

2-10 mM Mg^{2+} Enhance Luciferase Activity

According to previous studies, Mg^{2+} can catalyze the oxidation of luciferin into oxyluciferin. Due to the relatively small physiological changes in Mg^{2+} concentration, it can respond most effectively to the common intracellular Mg^{2+} concentration, which typically ranges from approximately 0.1-6 mM[21]. Four concentrations (0, 2.5, 5, 7.5 mM) of Mg^{2+} were used to stimulate luciferase activity in live cells. Adding 2.5 mM Mg^{2+} did not affect the enzymatic activity within the cells, but when the concentration was increased to 5 and 7.5 mM, the luciferase activity was inhibited (Figure 5A). Moreover, overnight-cultured *DC3000/PLH-luc* was washed with PBS twice and then stimulated with different concentrations (0, 2.5, 5, 7.5, 10 mM) of Mg^{2+}. The luciferase activities were detected 10 minutes after the Mg^{2+} stimulation. The bioluminescence increased with the increase in Mg^{2+} concentration, but the luciferase activity dropped at 10 mM Mg^{2+} compared to that at 7.5 mM Mg^{2+} (Figure 5B). The results suggested that Mg^{2+} acts as a catalyst with feedback inhibition. Excessively high Mg^{2+} concentrations can inhibit enzyme activity. In cells, due to their ability to respond to the influence of Mg^{2+} on metabolism or to maintain magnesium ion homeostasis, excessive ion concentrations are prevented from entering the cells.

Ca^{2+} Inhibit Luciferase Activity in Enzyme Crude Extract

The presence of Ca^{2+} pumps ensures that the intracellular Ca^{2+} concentration remains low. To test the effect of Ca^{2+} on enzyme activity, four concentrations (0, 1, 3, 5 mM) of Ca^{2+} were added to PBS. As shown in Figure 6A, inside the cells, Ca^{2+} does not affect the activity of luciferase. However, the enzyme activity decreases as the Ca^{2+} concentration increases in the cell lysis buffer, and 1 mM Ca^{2+} is the optimal activation concentration. When the Ca^{2+} concentration is too high, it inhibits the enzyme's activity (Figure 6B). This indicates that the presence of Ca^{2+} inhibits enzyme activity.

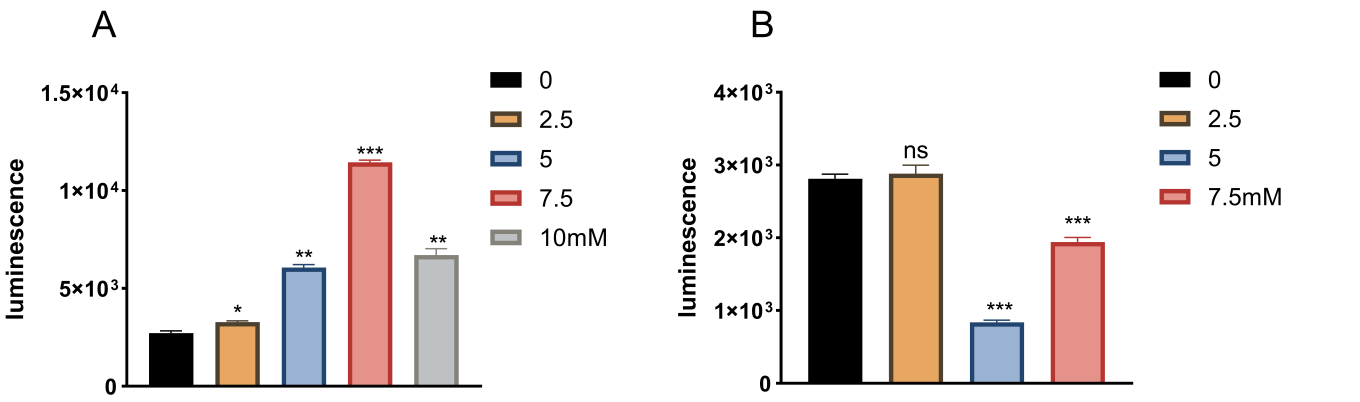

Fig. 5 The effect of different concentrations of Mg^{2+} on luciferase activity

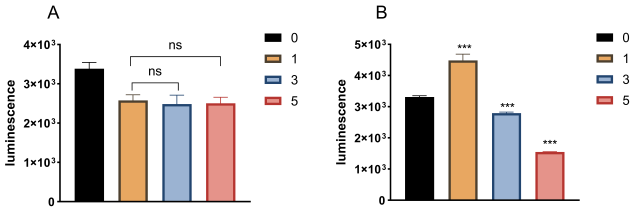

Fig. 6 The effect of different concentrations of Ca^{2+} on luciferase activity

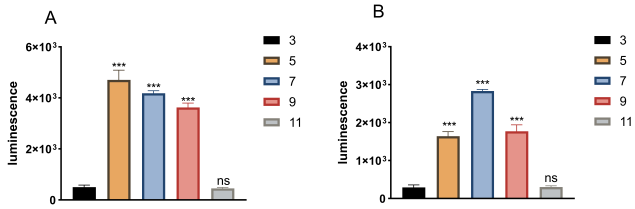

Fig. 7 The effect of different pH levels on bioluminescence in cells and cell crude extracts

Luciferase Remains Activity at pH 3-11.

The stability of luciferase was evaluated under physiological conditions in cells and cell crude extracts. The activity of luciferase was measured over the pH range of 3-11. It was observed that the luciferase activity of cells showed a significantly higher level in the acidic and neutral ranges in vitro. Luciferase activity remained stable between pH 5-9, but the bioluminescence was relatively low at pH 3 or 11, both in cells and cell crude extracts. Furthermore, the highest activity and a slight but significant decrease in luciferase activity were observed at pH 7 in cell crude extracts and pH 5 in cells (Figures 7A and 7B).

Bioluminescence Decreases Over Time, and Thermal Inactivation of Luciferase Occurs at Different Temperatures

We measured the remaining luciferase activity at corresponding temperatures after incubating the enzymes for different time intervals at various temperatures. The kinetics of light emission in reactions catalyzed by luciferase was studied in PBS buffer at temperatures ranging from $24-42°C$. After mixing with substrates, the reaction kinetics is flash-like, with a rapid increase followed by a relatively slow decay of light intensity (Figure 8). Comparing luciferase activity at different temperatures, it can be seen that the reaction curve demonstrates its highest level of activity over a wider temperature range within 0-5 minutes (Figures 8A1-D1). However, the bioluminescence at the beginning of the reaction and during the first five minutes was higher at 42° compared to the other three temperatures. Meanwhile, after 20 minutes, the bioluminescence at 42° continued to decline, while that at the other three temperatures remained relatively stable. Longer heat treatment resulted in a consistent decrease in the remaining activity, which was well described by a single exponential function. The corresponding rate constants of thermal inactivation of bacterial luciferase are shown in Table 1. Comparing the thermal inactivation of luciferase, we found that at 42°, it is most sensitive, as its reaction rate was always higher than at other temperatures under the same conditions. The observed temperature effects on the activity of the enzymes indicate that (i) luciferase is more sensitive at 42° than at other temperatures, and (ii) the bioluminescence is highest when the enzyme interacts with the substrate at all temperatures.

Conclusion

In recent years, the luciferase reporter gene system has been widely used in prokaryotic, eukaryotic, and mammalian cells[22]. Tm-luc has similar morphological and functional characteristics to TM standard strains and can be used as a tool in cell infection

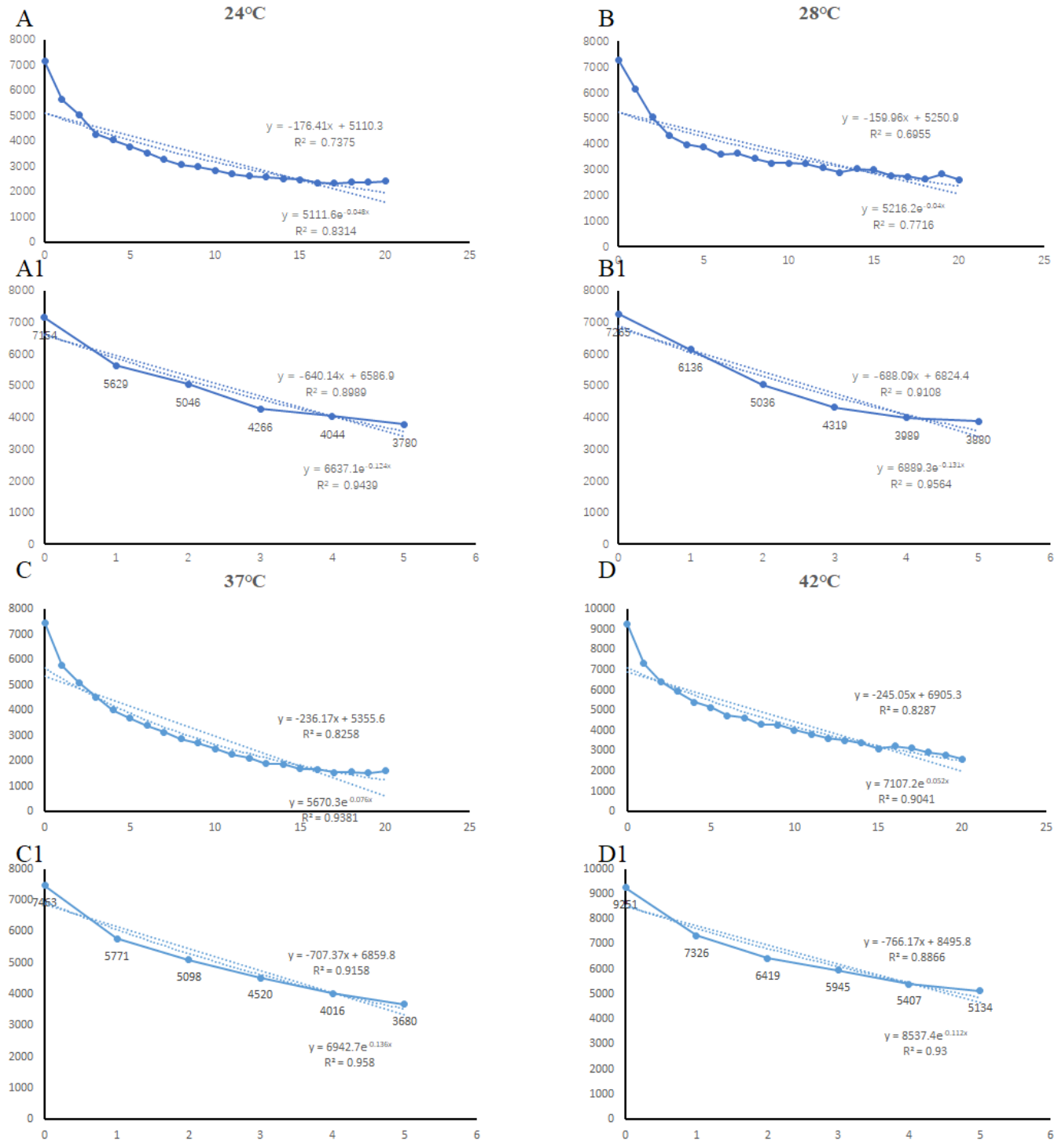

Fig. 8 The luciferase reaction rate constants at different temperatures

and animal infection models to more intuitively evaluate the interaction between TM and the host[23]. The establishment of the corresponding reporter strains provided a method for detecting the expression and activity of the enzyme[24]. However, problems such as low enzyme activity expression and no significant difference in bioluminescence during the detection process still

Table 1 The luciferase reaction rate constants at different temperatures

T,°C	K, min^{-1}	K, min^{-1} (0-5 min)
24	176.41	640.14
28	159.96	688.09
37	236.17	707.37
42	245.05	766.17

exist. This study explored and optimized the reaction conditions of the luciferin reporter system in bacteria by constructing a bacterial strain that successfully expressed the reporter system and verifying a promoter activated by glucose (Figure 4). The experiment proved that adding 2% glucose would increase the enzyme activity of the bacteria by five times (Figure 4). However, since glucose is a nutrient for bacteria, the impact of its external addition on bacterial metabolism, consumption, and activated signaling pathways remains unknown. Furthermore, it is unknown whether other sugars affect the promoter. It is necessary to further verify the induction effect of glucose on the promoter through MST experiments.

Environmental conditions such as temperature, pH value, and solvent polarity affect the lifetime of excited molecules. For example, low temperatures generally increase the luminescence lifetime, while high temperatures may accelerate the deactivation of excited molecules through non-radiative pathways[25]. Inorganic ions and various other chemical compounds have also been studied as possible inhibitors. Some studies were conducted with different metal ions, particularly divalent ions, due to the fact that divalent ions can replace Mg^{2+}, and different anions (different salts) can result in variations in total ionic strength that could affect the bioluminescent reaction. All studies were performed by adding divalent ions and anions to the reaction mixture[26]. In this study, we measured the bioluminescence at different ion concentrations. The experimental results showed that two metal ions can enhance the activity of luciferase within a certain concentration range, acting as activators for this enzyme. Among them, the optimal activation concentration of Mg^{2+} is around 7.5 mM, while 1 mM Ca^{2+} is the optimal activation concentration (Figures 5 and 6). However, the activation effect of Ca^{2+} is only about 50% of that of Mg^{2+}. When the concentration of these metal ions is too high, it inhibits the enzyme's activity. Studies have shown that Mg^{2+} can form a compound with ATP more easily, which can combine with the active center of luciferase and facilitate the release of inorganic pyrophosphate (PPi) acid, thereby improving the efficiency of the enzymatic reaction[27,28]. Previous studies have also reported that acetate accumulation in *E. coli* can reach as high as 230 mM inside the cell when the extracellular concentration is only 8 mM. This turgor stress is thought to be partially relieved by reducing intracellular glutamate concentration[29]. Therefore, when storing and using luciferase, it is appropriate to add these two ions in moderation. The luciferase activity within the cells was measured at pH 3-11. The results showed that the bioluminescence was similar within the pH range of 5-9, while it was lower under overly acidic or alkaline conditions. The luciferase activity of the cells was measured in cell crude extracts at pH 3-11 (Figure 7). The results showed that the optimal pH for the enzyme reaction was around pH 7, which is consistent with the results of most luciferases (with an optimal pH range of 7.6-7.8)[30]. Acidity is one of the principal physicochemical factors that influence the behavior of microorganisms in any environment, and their response to it often determines their ability to grow and survive. At low pH values, the undissociated (protonated and uncharged) forms of weak acids diffuse through the cell membrane and dissociate within the cell, acidifying the intracellular pH. For example, when cells grow in a minimal culture medium with a pH of 5.5, genes such as *gadA, gadB, adiA, adiC, cadA, cadB, cadC, speF* and *potE* are induced[31,32]. However, the experimental results show that the bioluminescence is close to the blank control value at pH 3 and pH 11, which might be caused by the failure of the substrate. This result also indicates that it is necessary to avoid using ions that inhibit enzyme activity in the buffer solution for the enzyme dissolving agent, and the fluorescent substrate of fireflies is pH-sensitive. It should be prepared freshly and used immediately within the appropriate pH range. This acid tolerance behavior provides new and broad prospects for different industrial applications and the bioremediation of environmental pollutants. The acid tolerance of luciferase may improve the efficiency of transgenic bacteria in treating acidic industrial effluents.

The study on enzyme thermal inactivation at $24-42°C$ revealed the different sensitivities of luciferase to temperature (Figure 8). Specifically, the activity of luciferase showed significant changes in response to temperature variations within 0-5 minutes, while it provided approximately the same bioluminescence after 20 minutes. It was observed that the thermal property of luciferase remained consistent at $24-42°C$. In fact, although the activity of luciferase at $24-42°C$ was not the maximum, its long-term stability was significantly higher than at 42°C (Figure 8). Generally, the structure of luciferase is more stable at $24-37°C$, enabling it to function at a more suitable temperature rather than at lower or higher temperatures. It is worth noting that most known bacteria can survive in extreme environments. Therefore, the activity data of the studied luciferase at different temperatures should be carefully designed. In addition, there are still some experimental limitations in this study, such as the absence of a dual luciferase reporter system for internal reference normalization[33]. In future research, the author could introduce microfluidic technology or metabolomics analysis.

The lack of knowledge about thermostability and the activity of bacterial luciferase at different temperatures is one of the issues when it is implemented as a reporter for real-time monitoring of various processes in microbial or eukaryotic cells[8,9,34].

Previous studies demonstrated that in order to obtain a reproducible luminescent response and to understand this response, a detailed analysis of the characteristics of the environment on luciferase is necessary[34,35]. Therefore, our data has significant implications when using distinct media and temperatures in terms of both luciferase activity and stability as reporters.

Results

In the DC3000 strain, the luciferase gene driven by *hrpA* was successfully expressed. Under long-term detection, the bioluminescence did not disappear within the temperature range of $24-37°C$. Under short-term detection, the bioluminescence was strongest at 42°C, and its optimal pH range was 5-9. Like other luciferases, the enzyme activity expressed in this system is relatively sensitive to metal ions. Mg^{2+} shows the strongest effect on the enzymatic reaction at around 7.5 mM, while Ca^{2+} has only half the effect of Mg^{2+} at the optimal concentration of 1 mM. In the higher concentration range, these ions actually inhibit enzymatic activity.

The reaction conditions provided in this study are applicable for the application of bioluminescence detection methods in bacteria or mammals, and many aspects of product application design are included. The discovery of enhanced fluorescence by optimizing conditions could be a good option for researchers who have halted their research due to low bioluminescence signals. This study further provides an experimental foundation for the future application of luciferase in various fields.

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