ARTICLE

Superior Performance Organic Light-Emitting Systems Employing an Organoterbium Emitter

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Abstract

From the luminescence performance of organic microorganisms, the establishment of a high-performance organic luminescence system can help to better realize the accurate detection of pathogenic bacteria. In the article, Staphylococcus aureus ATTC260 was used as the research object, and the enolase (Eno) gene in ATTC260 was screened by fusion targeting, and the Nluc, Teluc and Antares2 nucleotide sequences were combined with ATTC260/Eno through pUC57 vector by the knock-in method to prepare ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2 reporter strains. The growth of ATTC260/Eno was detected using ATP bioluminescence and Gompertz modeling, and ex vivo and in vivo luminescence assays were carried out for different types of strains. Finally, the organoluminescent system with the best luminescence intensity was tested with three different substrate solutions (FUR, DTZ and HFZ). The addition of Nluc, Teluc and Antares2 nucleotide sequences to the ATTC260/Eno strain did not affect its normal growth curve, and the error in the growth curve did not exceed 0.5% from that of the native ATTC260/Eno strain.The luminescence intensity of the ATTC260/Eno-Antares2 strain was maintained from 10 min to 50 min at more than $1*10^8$ photons/sec. Under three different catalytic substrate concentrations, ATTC260/Eno-Antares2/HFZ obtained the highest orange-red luminescence intensity, which was most suitable for the organoluminescence system, and HFZ was the most efficient catalytic substrate for ATTC260/Eno-Antares2. This paper provides a new theoretical basis for the study of bio-organic luminescence systems, and provides a new research feasibility for the detection and tracing of biological living cells.

Keywords: staphylococcus aureus, knock-in method, ATP technology, Gompertz model, organoluminescent system, microbial emitter

Citation

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1 Introduction

Organic light-emitting materials (OLMs) are a special class of materials that can emit visible light when they are energized or excited by external energy, and they are characterized by high luminous efficacy, high color purity, wide spectral range of light emission, good processability, and environmental friendliness [1, 2]. These characteristics make in the past decades, organic luminescent materials in the field of light-emitting devices, display technology and optoelectronics has made great development, and has been widely used in cell phone screens, television monitors, lighting and other aspects, and organic luminescent materials research and development is still a challenging and potential field [3–6].

The luminescence mechanism of organic luminescent materials is realized by means of excitation of electron leaps within the material. Its basic principle is to utilize the electron leaps or complexes will emit a specific photon, so as to realize the luminescence

[7, 8]. Specifically, in the organic compound molecule, the electron will be excited to a high energy state under the action of excitation energy, but due to the instability of the energy state, the electron will quickly return to a lower energy state, and in the process of releasing energy, the release of this energy can be converted into the form of light, that is, luminescence [9–12]. The color of the light emitted is related to the molecular structure, the parameters of the material, the excitation energy, etc. The molecular structure of organic light-emitting materials is very complex. The molecular structure of organic luminescent materials is very complex, usually consists of multiple luminescent groups and electron transport components. Factors such as the type, position, size, and distribution of the luminescent groups affect the luminescent properties of OLMs [13–16]. At the same time, the complex structure within the molecule and the diversity of constituent elements of organic luminescent materials also lead to their special photoelectric properties, which have shown good superiority in luminescence efficiency, color purity, lifetime, stability, etc. [17–20].

In the article, Staphylococcus aureus ATTC260 was used as the research object, and the ATTC260 strain was prepared by constant temperature shaker, and the enolase (Eno) gene was screened using the fusion target. In order to further enhance the luminescence performance of ATTC260/Eno microorganisms, three different types of reporter strains were prepared by combining the nucleotide sequences of Nluc, Teluc and Antares2 with ATTC260/Eno strains by knock-in method using the pUC57 vector. The growth data of ATTC260/Eno strains were detected by ATP technology and analyzed with Gompertz model. For the different types of reporter strains, the growth curves and ex vivo and in vivo luminescence characteristics were analyzed, and three different concentrations of catalytic substrates (FUR, DTZ and HFZ) were introduced to detect the luminescence intensities of the different types of strains, which provided a basis for the selection of optimal organic bioluminescence systems.

2 Materials and methods

In this study, the luminescence performance of Staphylococcus aureus was detected by ATP bioluminescence technology, and the emitter of Staphylococcus aureus strain was prepared by combining chromosomal recombination, and its in vivo and ex vivo luminescence performance was analyzed in depth, so as to provide organic microbial emitters for the preparation of high-performance organic luminescence system.

2.1 Staphylococcus aureus luminescence mechanism

2.1.1 ATP bioluminescence technology

1. Principle of ATP bioluminescence technology

   The principle of ATP bioluminescence is that under the action of luciferase E and Mg^{2+}, luciferin LH2 is activated by adenosine acylation with ATP, and the activated luciferin combines with luciferase to form a luciferin-AMP complex and emits pyrophosphate (PPi). The complex is oxidized by oxygen molecules to form the excited state complex P*-E-AMP, which emits CO_2 and emits light when the complex returns from the excited state to the ground state. And finally, oxidized worm fluorescein P and AMP are formed. The reaction process is as follows:

$$E+LH_2+ATP \overset{Mg^{2+}}{\longleftarrow} E\cdot LH_2-AMP+PPi \quad (1)$$

$$E\cdot LH_2-AMP+O_2 \rightarrow [P*-E\cdot AMP]+CO_2 \quad (2)$$

$$P*-E\cdot AMP \rightarrow E-P+hv+AMP \quad (3)$$

   After experiments, it was shown that luciferase is an atypical Michaelis-Menten's enzyme with an optimum pH of 7.5, and the enzyme kinetic curve for one of the substrates of its action, ATP, is S-shaped. In the appropriately low ATP concentration range, the speed of its reaction and ATP concentration into a first-order reaction relationship, that is, the intensity of the detected fluorescence value is proportional to the ATP concentration. For living microorganisms within a certain physiological period, all have a relatively constant level of ATP content, making a good linear relationship between ATP concentration and the content of living microorganisms. Moreover, after the death of microorganisms, their ATP will be degraded by intracellular enzymes, which will not affect the determination of living microorganisms.These characteristics of ATP make ATP bioluminescence method a better detection method for living microorganisms.

2. ATP bioluminescence detection steps

   The detection steps of microbial luminescence technology mainly include image sampling, sample extraction, adding fluorescein, determining the amount of luminescence

of pollutants, and finding out the concentration of pollutants and the number of viable bacteria. Most of the time, the image sample can not be measured without processing. For measurement, the sample is fused with an extractant, which dissolves the cell membrane and cell wall, releasing unstable compounds. The unstable compound extractant is a reagent based on a surface active agent, and then the extracted unstable compound interacts with a fluorescein bioluminescent agent, and the amount of bioluminescence that results from the reaction between the unstable compound and the agent is measured using a luminescence detector. The total number of bacteria can be obtained from the amount of unstable compounds of live bacteria by using a standard curve of pre-measured unstable compounds.

2.1.2 Properties of Staphylococcus aureus

Staphylococcus aureus typically has a spherical morphology and can be observed as arranged bunches of grapes under the microscope, so it is named Staphylococcus aureus. The size of a single organism is about 0.8 μm in diameter. There are no germ cells and flagella, most of them do not have pods, and the Gram stain shows positive, which can be changed to negative after aging or death of the organism.

Most of the S. aureus has average requirements for nutrient environment, and can meet the growth requirements on normal nutrient agar medium, aerobic and parthenogenetic anaerobes. Resistant to desiccation, can survive for months in a dry environment, its growth temperature range 7-48.7 ℃, the optimal growth temperature is 37 ℃. Its growth temperature range is 7-48.7℃, optimal growth temperature is 37℃, and its growth pH range is 4.0-9.8, optimal growth environment is pH7.4. It can grow on medium containing 10-14% NaCl, and it has higher salinity tolerance than the general bacteria, and its growth water activity range is a_w0.86-0.97, a_w0.86 is the minimum water activity value required for its growth, and it has a weaker competitiveness with other bacteria.

The morphology of its colonies grown on the plate has the following characteristics:

Round with protrusions thicker, shiny, size of 1∼2mm colonies will form a transparent hemolytic ring around the blood plate.VP reaction shows weak positivity and methyl red reaction shows positivity. S. aureus has low sensitivity to sulfonamides, however, it has high sensitivity to penicillin and erythromycin.

2.2 Experimental materials and preparation methods

2.2.1 Experimental materials and reagents

1. Experimental strains

   Staphylococcus aureus (ATTC260) was purchased from HK Microbiology Ltd. 2 × Taq plus enzyme, rapid plasmid mini-extraction kit, endotoxin-free mini-extraction kit, agarose gel electrophoresis powder, agarose gel electrophoresis gel recovery kit, D2000 DNA Marker, genomic DNA extraction kit, methanol, FastKing one-step RT-PCR kit were purchased from BJ Tiangen Biotechnology Co. DMEM medium, Opti-MEM, fetal bovine serum were purchased from Gibco, ligation buffer was purchased from NEB, PBS buffer powder, SDS-PAGE gel configuration kit, color pre-stained molecular weight standard for medium molecular proteins (10-180 KDa), filter paper, PVDF membrane, ultrasensitive ECL. Chemiluminescence ready-to-use substrate, primary/secondary antibody dilution, GAPDH antibody, goat anti-mouse secondary antibody purchased from BSD Bioengineering Co. NP40, trypsin powder, Western cell lysate, BCA protein concentration assay kit, skimmed milk powder, 10 × TBSTw buffer, 5 × loading buffe purchased from BYT Biologicals, 100 × non-essential amino acids, glutamine, and penicillin-streptomycin double antibody were purchased from PB. pUC57-hTMPRSS2-Donor plasmid was synthesized by JWZ Biotechnology, and pX330 plasmid was purchased from ADD. Cre-logP mice, C57BL/6N mice (all animals used in this study were evaluated in a pathogen-free (SPF) animal facility in standard cages housed on a 12-hour daily cycle).

2. Experimental apparatus

   Vertical pressure steam sterilizer (YXQ-75SII) was purchased from Medical Equipment Factory of SH Industrial Co., Ltd, biochemical incubator (SHX150III) was purchased from SH IYUZHUO INSTRUMENT CO., LTD, intelligent constant temperature culture oscillator (HNY-2102C) was purchased from TJ OUNAO INSTRUMENT CO., LTD, and two-person double-side purification workbench (SW-CJ-2F) was purchased from SZ Purification Equipment Co. Ltd. High-speed tabletop centrifuge (5804R) was purchased from ABD Life Sciences, tabletop high-speed

centrifuge (TG16-WS) was purchased from XT Xiangyi Instruments, electronic balance (ME204) was purchased from METTLER TOLEDO INTERNATIONAL TRADING CO., LTD, gas chromatography and mass spectrometry (TSQ8000Evo) was purchased from SMF Shier Technology Co. Vertical refrigerator (YTR-62 single door) was purchased from BJ Weilan Electric Co. Ultrasonic cleaner (KQ5200E) was purchased from KS Ultrasonic Instrument Co., Ltd, electronic universal furnace was purchased from BJ Yongming Medical Instrument Co., Ltd, and electric thermostatic water bath (DK-S24) was purchased from SH Jinghong Experimental Equipment Co.

2.2.2 Preparation of luminescent strains of S. aureus

1. Cultivation of Staphylococcus aureus ATTC260

   The freeze-dried S. aureus powder was inoculated into 120 mL of sterilized BHI liquid medium, and then incubated in a constant temperature shaker at 36℃ (160rpm) for about 9 hours to activate the strain. After that, a small amount of liquid medium with activated strain was dipped into the BHI solid medium with an inoculating needle and streaked, and incubated at 36℃ for 50 hours to obtain golden staphylococcal colonies, and the cultured strain was kept in the refrigerator at 5℃ for spare use. Each time you use it, use the inoculation needle to pick off the single colony saved for reserve, and inoculate it in 120mL of sterilized BHI liquid medium, and then put it into a constant temperature shaker at 35℃ (150rpm) for about 9 hours to make the concentration of the bacterial solution reach 105-109 CFU/mL (logarithmic phase) for reserve.

2. Screening of luciferase fusion targets in Aureobasidium aureofaciens ATTC260

   Based on the existing research, it is known that the enolase (Eno) gene in S. aureus ATTC2600 has strong fluorescence, in this paper, plasmid pUC57-Eno was constructed by linking the Nhe/Bsp118 cleavage site of pUC57 vector through the method of knock-in cloning. Plasmid pUC57-LGR was constructed through the method of fixed-point mutation and further constructed through the method of fixed-point mutation on the basis of LGR. The plasmid pUC57-LGR was constructed by targeted mutagenesis, and based on LGR, a luciferase mutant with improved activity and stability was further constructed by targeted mutagenesis to realize the fusion target screening of luciferase in S. aureus ATTC2600. The specific steps are as follows:

   Stellar competent cells were transformed with luciferase mutants, the organisms were coated on TSA plates containing 0.1% arabinose, incubated at 35°C for 20h, then left at room temperature for 20h, the organisms were collected, the organisms were lysed by ultrasonic crusher, and the centrifugation-collected supernatant was mixed with 5mL of HisPur Cobalt Resin at room temperature for 3h, and the protein was purified with a The protein was purified by gravity column, and washed with 5mL 60mmol/L HEPES (pH 7.6) buffer for 3 times. Then, it was washed 3 times with 5mL 60mmol/L HEPES as well as 10mmol/L imidazole (pH7.6), and then the luciferase mutant was eluted with 3mL 60mmol/L HEPES as well as 120mmol/L imidazole (pH7.6), and finally, it was desalted with a desalting column, and quantified with a protein quantification kit.

2.2.3 Construction of reporter strains

In order to be able to analyze the specific luminescence of different S. aureus, in this paper, we used plasmid pUC57-Eno as the basis, combined with the nucleotide sequences of SH gene chemically synthesized Nluc, Teluc, and Antares2, which were cloned into the pUC57-Eno plasmid using the knock-in method, and obtained pUC57-Nluc, pUC57-Teluc, and pUC57-Antares2. Taking the preparation of pUC57-Nluc as an example, the specific steps are as follows:

1. Plasmid transformation

   Step1 Remove the purchased Aureobasidium ATTC2600 receptor cells from -80℃ refrigerator, incubate on ice for 5 min, and leave to thaw.

   Step2 Take 1.8 mLEP tube, add 60 μL of complete recovery of the sensory cells and 60 ng of pUC57-Eno expression plasmid, 15 μL range pipette gun gently blowing, mix well and incubate on ice for 40 min.

   Step3 Set the thermostatic water bath to 40℃, heat-excite the above mixed products for 50s, and incubate on ice for 4min again.

   Step4 Add 600μL of antibiotic-free LB liquid medium to the 1.8mL EP tube, gently blow with a 120μL pipette gun, mix well, and then incubate for 2h in a thermostatic shaker, which was set at

36℃ and 250rpm/min.

Step5 Take 60μL of culture solution after incubation and spread it evenly on the LB agar plate containing ampicillin antibiotic.

Step6 Put the coated plate into a constant temperature incubator, and incubate for 20h after 12min of inversion.

2. Plasmid amplification

Step 1 Pick the single colony obtained from overnight culture with a lance tip, and put the lance tip with the attached single colony into a 20mL EP tube.

Step 2 Add 5mL of LB liquid medium containing ampicillin antibiotic to the 20mL EP tube that has completed the above operation, and incubate it in a constant temperature shaker for 4h, with the constant temperature shaker set at 35℃ and 200rpm/min.

Step 3 Take 60mL EP tube, add 3mL of the bacterial solution that completed the initial amplification and 50mL of LB liquid medium containing ampicillin antibiotic for the second amplification, and incubate it in a constant temperature shaker, which was set at 35℃ and 200rpm/min, and carry out the next operation after flocculent precipitation appeared in the 60mL EP tube.

3. Plasmid extraction

Step1 Expanded culture of bacterial broth. Select LB medium containing corresponding resistant antibiotics, add 30mL of medium into 60mL centrifuge tube, inoculate 120μL of bacterial solution, and place it in 35℃, 250rpm/min shaker for overnight expanded culture. The well-grown bacterial liquid should have visible sticky flocculent bacterial body.

Step2 Centrifuge the bacterial solution at RCF=6000g for 15min, at this time, the bacterial body should be sunk to the bottom of the tube, remove the supernatant, and pipette the residual small amount of medium.

Step3 Add 600μL Solution, resuspend the bacteria, and transfer to 3mL centrifuge tube.

Step4 Add 600μL of Solution and mix gently by inverting the solution, which should become clear and sticky. This process should be done gently, otherwise it may lead to genomic DNA contamination of S. aureus ATTC2600.

Step5 Add 300μL of pre-cooled Buffer N3 on ice and mix gently by inverting until a tight white protein mass is formed.

Step6 Centrifuge the completely lysed bacterial solution at 12,000g for 15min, and the protein should sink to the bottom of the tube.

Step7 Carefully take the supernatant and divide it into two 2mL centrifuge tubes. Add 12% volume of ETR Solution to each and incubate on ice for 12min, at which point the solution should become turbid.

Step8 At the end of the ice bath place in a 40°C water bath and keep for 6min, at this point the solution should become clear.

Step9 Centrifuge at 11000g for 2min, ETR Solution sinks to the bottom of the tube. Carefully remove the supernatant into a 1.5mL centrifuge tube and combine the two tubes into one. Be careful to avoid the lower layer of ETR Solution, because endotoxin exists here.

Step10 Add 50% volume of anhydrous ethanol, batch the solution over the adsorption column, 750μL each time, centrifuge at 12000g for 2min, discard the waste solution.

Step11 Add 600μL HBC Buffer, centrifuge at 12000g for 2min, discard the waste solution.

Step12 Add 600μL of DNA Wash Buffer, centrifuge at 12000g for 2min, discard the waste liquid, and repeat the process three times.

Step13 Spin in vacuo. Centrifuge at 12000g for 1min with the purpose of removing reagent residue.

Step14 The adsorption column was placed on a 2mL centrifuge tube, 120μL Elution Buffer was added dropwise to the center of the adsorption column and left for 2min.

Step15 Centrifuge at 12000g for 2min to elute the plasmid. Measure the concentration, label and store at -22°C.

4. Primer design and PCR amplification

Primers for amplification of specific fragments of the genome were designed using the NCBI online primer design website and synthesized

by WH Aoke Dingsheng Biotechnology Co. Primers need to be mapped for annealing temperature to reduce non-specific amplification, and generally five temperature gradients of 55℃, 57℃, 59℃, 61℃ and 63℃ were designed for optimization of annealing temperature, and the appropriate annealing temperature was selected for subsequent amplification. The primers for amplification of plasmid fragments do not need to be designed, due to the plasmid as a PCR template, non-specific interfering factors are less, directly select the target position of 20 bases as primers, and its annealing temperature is generally 60 ℃ can be taken.

The principle of polymerase chain reaction (PCR) is the use of primers, dNTPs and other raw materials, DNA polymerase at the appropriate temperature for the process of DNA strand amplification, after the cycle of denaturation-annealing-extension process, a large number of amplification of the target fragments.PCR reaction on the concentration of each reagent, the temperature of a certain requirement, in general, these requirements according to the different DNA polymerase.

After the above steps, the Nluc gene on the pUC57-Nluc plasmid can be successfully knocked into Aureobacterium aureus ATTC260/Eno, and subsequently the reporter strain ATTC260/Eno-Nluc can be obtained.ATTC260/Eno-Teluc as well as ATTC260/Eno-Antares2 can be produced by the same steps.

2.3 Luminescence properties of S. aureus luminescent strains

2.3.1 Microbial growth modeling

For the growth of S. aureus ATTC260, the data obtained from the experiments were entered into Origin software for analysis and statistics, and the modified Gompertz function was used to fit its growth model to determine the longest realization of the suitable preservation of S. aureus ATTC260 at different temperatures with initial contamination amount of 103CFU/g. The maximum growth rate and retardation period of Aureobasidium in the nutrient base were also calculated.The Gompertz equation can be expressed as:

$$Log(N_t/N_0) = a \times \exp\{-\exp[-k \times (t - x_c)]\} \quad (4)$$

The maximum growth rate and the retardation period can be expressed as, respectively:

$$\mu_{\max} = a \times k/e \quad (5)$$

$$\lambda = x_c - 1/k \quad (6)$$

where t is the time, N_t, N_0 is the number of microorganisms CFU/g corresponding to time t and the initial time, respectively, x_c is the time required when the bacteria are at the relative maximum growth rate, k is the relative growth rate at time x_c, a is the difference between the number of bacteria at the time of stabilization and the number of bacteria at the time of inoculation, $\mu_{\max}$ is the maximum specific growth rate, and λ is the lag period.

2.3.2 Bioluminescence detection of Aureobasidium strains

The strains of ATTC260/Eno, the reporter strains ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2, which were produced in the previous paper, were configured into a solution with a concentration of 1.5 mM, and then 12 μL of the solution was taken and diluted to 12 μM in 2 mL EP tubes. They were applied to a warm bed and incubated at 36°C for 3 h. The emission spectra of the different strains were determined using ATP bioluminescence with an excitation wavelength of 320 nm.

1. In vitro luminescence detection of Aureobasidium strains

   The ATTC260/Eno strain, the reporter strains ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2 strains obtained from previous preparations were used as examples, and the mutant proteins were purified and stored in the refrigerator at -90°C for backup. The activity of the Aureobasidium strains was determined by monitoring luminescence at 480 nm. A typical experimental sample contains reaction mixture A and B. To facilitate the measurement, 60 mL of reaction mixture A (containing 20 mM HEPES pH 7.6, 160 mM NaCl, 6% glycerol, 1.5 mM MgCl2, 3 mM DTT, 30 μM C14:0, 30 μM FMN, 2 mM ATP, 12 μM LuxAB with 3 μM Fre) were dispensed in 2 mL/tube and frozen. For each experiment, the reaction was initiated by adding Reaction Mix B (5 mM NADPH, 25 μM ATTC260/Eno) to Reaction Mix A. The reaction was then run on a Flex Station Enzyme Marker. All assays were performed on a 120 μL scale in 96-well microtiter plates at 26°C using a Flex Station enzyme labeler. The Flex Station zymograph was set to continuous

detection mode with Luminescence at 480 nm for 45 min. Each set of experiments was repeated three times, and statistical analysis was performed using GraphPad Prism.

2. In vivo luminescence detection of Aureobasidium strains

   The ATTC260/Eno strain, the reporter strains ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2 strains obtained from the previous preparation were co-transformed into Cre-logP mice in order to obtain the luminescent Aureobacter strains. The transformants were activated overnight in 6 mL of antibiotic-containing LB medium, then inoculated into 50 mL of LB and incubated to mid-logarithmic stage at 32°C. The incubation was continued by adding 0.6 mM IPTG. Luminescence was detected by taking 300 μL of the bacterial solution every 1 h using a Flex Station enzyme labeler at 480 nm. Successive samples were taken several times to determine the maximum luminescence intensity of the different Aureobasidium strains. Each set of experiments was repeated three times and statistically analyzed using GraphPad Prism.

3 Results and analysis

The fluorescence emission intensity of organic microorganisms is used as the basis for the preparation of organic luminescence systems, which require microorganisms with high fluorescence intensity to be realized. Based on the previously prepared Aureobasidium ATTC260/Eno and three different types of reporter strains, this chapter provides a comparative validation of their luminescence intensities, which provides the basis of excellent microbial fluorescence emitters for the preparation of high-performance organic bioluminescence systems.

3.1 Growth curves of Aureobasidium strains

3.1.1 Growth pattern of Aureobasidium strains

In this article, the growth model of Aureobasidium strain ATTC260 was established with modified Gompertz function, in order to analyze its growth pattern in nutrient substrate, this article fitted its growth at different temperatures by Gompertz model and Logistic model, and in this way, we selected the model with more accurate growth pattern prediction to analyze the growth pattern of Aureobasidium strain ATTC260. Using the coefficient of determination (R^2) and root-mean-square error (RMSE) as evaluation indexes, the results of the comparison of the two models' fitting are shown in Table 1.

As can be seen from the table, the fitting coefficients of the 2 models, R^2, were greater than 0.95, which well described the growth of Aureobasidium in nutrient bases at different temperatures and different initial concentrations. Compared to the Logistic model, the Gompertz model fit yielded an R^2 closer to 1, with a mean value of 0.9784, and a smaller root mean square error mean (0.0957). It indicates that among the selected models, the Gompertz model fitting is relatively better, so the Gompertz model can be used to fit the growth pattern of Aureobasidium in nutrient substrate at 20∼35℃.

Figure 1 shows the Gompertz model fitted to the growth curves of S. aureus at different initial inoculum concentrations, in which Figures 1(a) to (c) show the changes in the growth curves at initial inoculum concentrations of 1*10^{3}CFU/g, 1*10^{4}CFU/g and 1*10^{5}CFU/g, respectively. The results showed that the growth of S. aureus existed in three phases of hysteresis, logarithmic growth and stabilization, showing an "S" shape. The lower the temperature, the smoother the curve was, and the higher the temperature, the steeper the curve was, indicating that the higher the temperature, the shorter the time for S. aureus to enter the logarithmic phase of growth, which may be due to the fact that the proliferation rate of the bacteria increases significantly under high temperature conditions. This shows that temperature is a significant factor affecting the growth of S. aureus in the nutrient base. In carrying out the culture of Aureobasidium ATTC260/Eno, the temperature of 35°C was used as a condition to obtain more Aureobasidium ATTC260/Eno quickly, which provided a stable source of native strains for the preparation of different types of Aureobasidium strains later.

3.1.2 Growth curves of the reported strains

In order to investigate whether the introduced exogenous plasmids pUC57-Nluc, pUC57-Teluc, and pUC57-Antares2 had any effect on the growth of Aureobasidium strain ATTC260/Eno, the growth curves of the three ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-antares2 strains and their corresponding Aureobasidium strains ATTC260/Eno were first compared. Antares2 strains with their corresponding Aureobasidium strain ATTC260/Eno growth curves, followed by evaluating the correlation between the luminescence intensity

Temperature	Initial concentration (lg CFU/g)	Gompertz		Logistic	
		R^2	RMSE	R^2	RMSE
20℃	$1*10^3$	0.9842	0.0503	0.9824	0.0524
	$1*10^4$	0.9769	0.0615	0.9751	0.0638
	$1*10^5$	0.9803	0.0683	0.9231	0.0915
25℃	$1*10^3$	0.9853	0.0791	0.9724	0.0943
	$1*10^4$	0.9832	0.0854	0.9806	0.0916
	$1*10^5$	0.9706	0.1103	0.9563	0.1243
30℃	$1*10^3$	0.9743	0.1024	0.9676	0.0998
	$1*10^4$	0.9859	0.0768	0.9852	0.0894
	$1*10^5$	0.9892	0.0735	0.9313	0.0835
35℃	$1*10^3$	0.9637	0.1842	0.9734	0.1674
	$1*10^4$	0.9825	0.1153	0.9696	0.1195
	$1*10^5$	0.9641	0.1416	0.9703	0.1235
Means	-	0.9784	0.0957	0.9656	0.1001

Table 1. The matching results of the two models are compared

of the luminescent strains and OD_{600}nm. Figure 2 shows the results of the plasmid transfer-based determination of the growth curve and luminescence curve of the Aureobasidium strain ATTC260/Eno, in which Figures 2(a)∼(b) show the growth curve and luminescence curve, respectively.

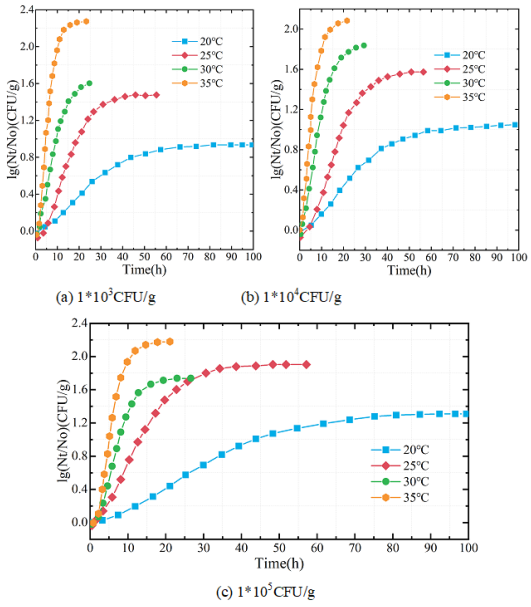
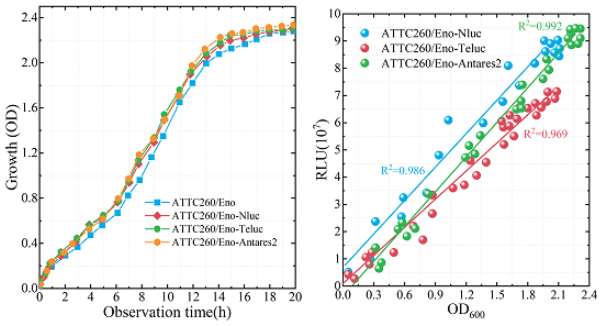


Figure 1. The growth curve of the gold gluteum

The results showed that the growth trend of the reporter strains with the addition of exogenous plasmids pUC57-Nluc, pUC57-Teluc, and pUC57-Antares2 was closer to that of the native Aureobasidium strain ATTC260/Eno as the observation time continued to increase, and the overall error did not exceed 0.5%. This indicates that Aureobasidium strain ATTC260/Eno still grows well with the addition of exogenous plasmids pUC57-Nluc, pUC57-Teluc, and pUC57-Antares2, and the introduction of the exogenous plasmids does not affect its growth. From the linear fit, the luminescence intensity of TTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2 strains showed a significant positive correlation with OD_{600}, with coefficients of determination of 0.986, 0.969, and 0.992, respectively. The positive correlation between the luminescence intensity and OD_{600} was stronger for the ATTC260/Eno-Antares2 strain, which also indicates that the survival status of S. Typhimurium can be represented in real time by the luminescence intensity of the organic microorganisms, providing strain markers for luminescence tracing of S. Typhimurium.



(a) Growth curve (b) Luminescence curve

Figure 2. The growth curve of different strains and the luminescence curve

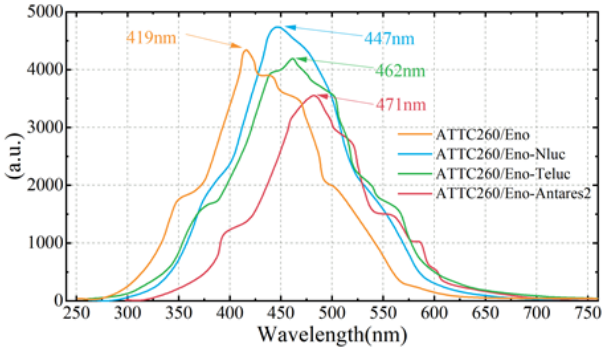


Figure 3. Spectral distribution of luminescent characteristics

3.2 Luminescent properties of Aureobasidium strains

3.2.1 Luminescence characteristics of each strain

Based on the ATTC260/Eno strain prepared in the previous section and three reporter strains with different exogenous plasmids as an example, the luminescence characteristic spectra of each strain were determined using ATP luminescence technology with a fluorescence spectrophotometer. Figure 3 shows the distribution of luminescence characteristic spectra of the four Aureobacter strains.

As can be seen from the figure, the maximum emission wavelengths of ATTC260/Eno, ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2 strains were 419 nm, 447 nm, 462 nm, and 471 nm, respectively. Antares 2 strain had the highest maximum emission wavelength. This also indicates to a certain extent that the ATTC260/Eno-Antares2 strain has a high luminescence intensity, and its application in the preparation of organic luminescent systems has a certain degree of feasibility.

The luminescence of S. aureus is density-dependent, the growth and luminescence are not synchronized, the bacterial concentration needs to reach a certain amount before luminescence, and the luminescence intensity varies greatly among different strains. The effects of incubation time on the luminescence and growth of different luminescent strains were investigated under the same medium composition and the same culture conditions. The experimental results are shown in Figure 4, where 4(a)∼(d) are the experimental results of ATTC260/Eno, ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2, respectively.

Compared with the other strains, the luminescence intensity of ATTC260/Eno-Antares2 strain was high, and it started to enter the logarithmic growth period at 10 h, and entered the growth stabilization period at 20 h, with a continuous stable luminescence time of up to 12 h. It was much longer than the stable continuous luminescence time of the exogenous plasmid strains, ATTC260/Eno-Nluc and ATTC260/Eno-Teluc, which had a longer luminescence time. And ATTC260/Eno-Nluc and ATTC260/Eno-Teluc. In addition, the luminescence of ATTC260/Eno-Nluc and ATTC260/Eno-Teluc was unstable, with intermittent luminescence, and the intensity of each luminescence was decreasing in a wave shape. It was suggested that this might be due to the fact that the substrates required for luminescence needed to be accumulated to a certain extent, and each luminescence peak was a short-lived release of the accumulated substrates, which needed to be accumulated again after the release was completed. This also confirms that the luminescence of luminescent bacteria requires the accumulation of substrate, and the ATTC260/Eno-Antares2 strain has sufficient substrate, which does not need to be accumulated for a long period of time, and the utilization and production of substrate can be equal within a certain period of time.

In the actual detection, strains with short onset luminescence time and long sustained stable luminescence time are easy to operate and control. From this point of consideration, the conventional ATTC260/Eno as well as the reporter strains ATTC260/Eno-Nluc and ATTC260/Eno-Teluc are not suitable for use as standard detection strains for organic luminescence systems. This will be further developed in the validation analysis later.

3.2.2 Luminescence detection in vivo and in vitro

1. In vivo release imaging luminescence detection of reporter strains

   There are few examples of Staphylococcus aureus and its derivatives being used for in vivo imaging

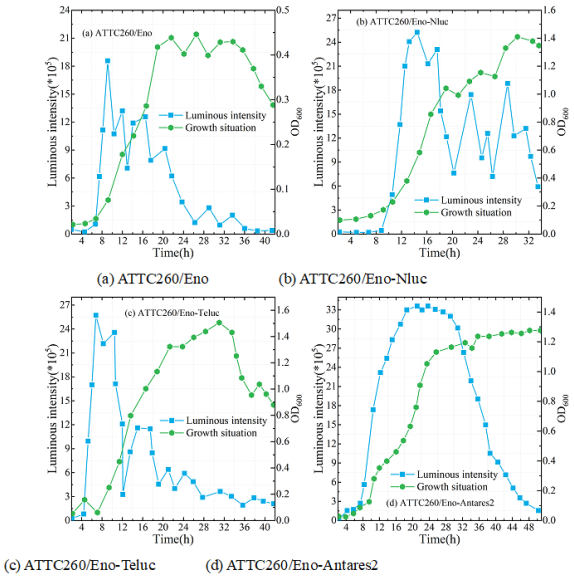


Figure 4. Culture time affects luminescence and growth

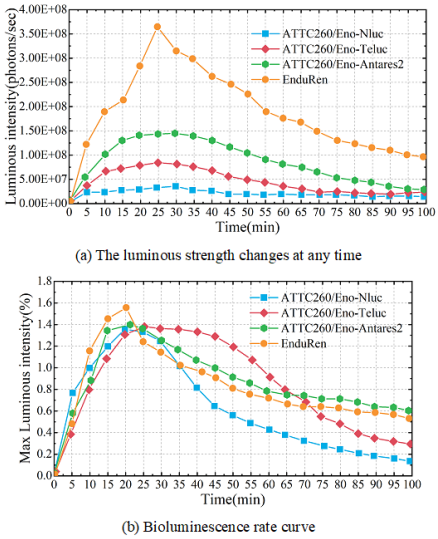


Figure 5. Luminescence detection of internal imaging

because the substrates are oxidized in vivo by substances such as serum proteins, which produce a large background signal. In addition, Staphylococcus aureus substrates are rapidly and irreversibly oxidized by Eno catalysis, so the signal generated does not last long. In order to prove that Staphylococcus aureus derivatives can continue to emit light in vivo, a model of axillary xenograft in nude mice was established, and then the activities of ATTC260/Eno-Nluc, ATTC260/Eno-Teluc and ATTC260/Eno-Antares2 strains in mice were compared, and the luminescence intensity of the marketed EnduRen was compared. Figure 5 shows the luminescence detection results of the in vivo release imaging of the reporter strains, where Figures 5(a)∼(b) show the luminescence intensity over time and the bioluminescence rate curves, respectively.

When four different types of strains were injected into the axillary tumor site of mice, the luminescence intensity of the ATTC260/Eno-Antares2 strain reached a maximum value of $1.45*10^8$ photons/second in 30 min, and then decreased at a faster rate, with its luminescence intensity being only $0.29*10^8$ photons/second after 100 min. the luminescence intensity of the ATTC260/Eno-Nluc vs. ATTC260/Eno-Teluc reporter strains also had longer sustained luminescence times, and although the ATTC260/Eno-Nluc strain would enter a plateau period ($0.18*10^8$ photons/sec) after 40 min, its overall change in light intensity was smaller. On the other hand, the luminescence intensity of ATTC260/Eno-Antares2 strain was maintained above $1*10^8$ photons/sec from 10min to 50min, and its bioluminescence could still be detected after 20h, which suggests that ATTC260/Eno-Antares2 strain is a promising luminescence emitter of organic microorganisms. The weaker luminescence intensity of ATTC260/Eno-Antares2 strain compared to EnduRen was due to the fact that EnduRen hydrolyzed more Eno enzymes than ATTC260/Eno-Antares2 strain hydrolyzed, which resulted in its better luminescence intensity. However, from the rate of release curve, after reaching the maximum value, the curve of ATTC260/Eno-Antares2 strain was always above EnduRen. This indicates that the degradation efficiency of ATTC260/Eno-Antares2 strain is slower, which is more suitable for prolonged and stable in vivo luminescence monitoring and imaging. In summary, the ATTC260/Eno-Antares2 strain prepared by introducing plasmid pUC57-Antares2 into ATTC260/Eno can be a promising long-lasting organic microbial fluorescence emitter, which can be used as the basis of an organic luminescence system to provide reliable fluorescence monitoring support for cell tracing.

2. Bioluminescence detection of different substrates catalyzed by the reporter strains

On the basis of analyzing the bioluminescence imaging of each strain, in order to further verify the feasibility of applying the ATTC260/Eno-Antares2 strain prepared and

obtained in this paper in the organoluminescence system, this paper chose to configure three different substrate solutions, namely furamidazin (FUR), luminaldehyde analog (DTZ), and hydroxylated furamidazin (HFZ). The reporter strains were separately incubated in BHI medium at 36°C until the OD_{600} was about 2.5, centrifuged at 12,000g 5°C for 15 min, and the bacteria were collected and adjusted to a concentration of 1×10^9 CFU/mL with PBS. 120 μL of Aureobasidium sp. ATTC260/Eno-Nluc, ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2 bacterial suspensions were taken and mixed with 12 μL of individual substrates (FUR, DTZ, and HFZ, at an initial concentration of 5 mM) in black 96-well enzyme labeling plates, and the reaction was carried out at room temperature for 2 min, using the SpectraMax full-wavelength zymography was used to measure the bioluminescence spectra, and the luminescence intensities at 2 nm intervals between 420 nm and 720 nm were collected, and the luminescence intensities of ATTC260/Eno-Teluc and ATTC260/Eno-antares2 were normalized and compared with the peaks of the reporter strains ATTC260/Eno-Nluc at 450 nm. Were normalized and compared to select the optimal Aureobasidium bioluminescence reporter strain/substrate system. Figure 6 shows the results of bioluminescence detection in vitro for different substrates catalyzed by the reporter strains, where Figures 6(a)∼(c) show the luminescence spectra, the change in bioluminescence intensity of catalyzed HFZ, and the total bioluminescence, respectively.

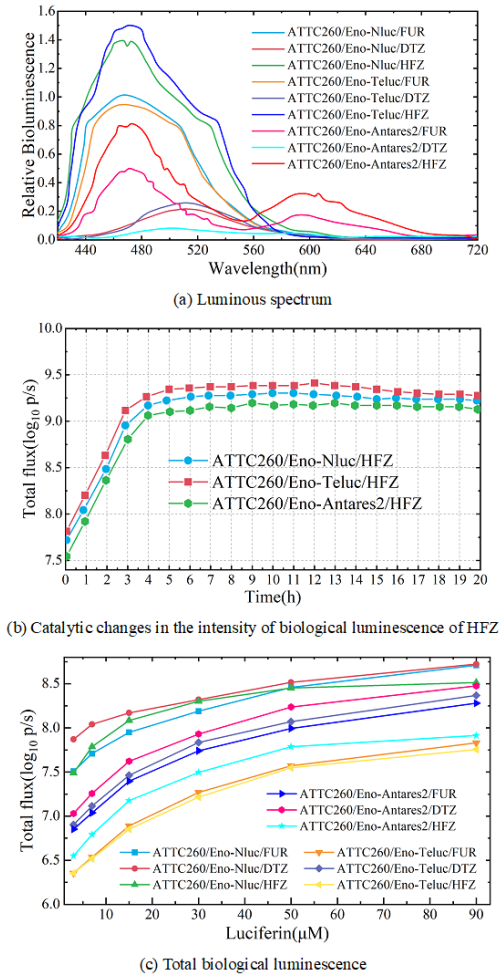


Figure 6. Biological external light detection results

In Figure 6(a), the Aureobasidium reporter strain ATTC260/Eno-Antares2 catalyzed the substrate HFZ to emit orange-red light at ∼594 nm in addition to cyan-blue light at ∼472 nm, where the production of >600 nm photons was also the highest, suggesting that ATTC260/Eno-Antares2/HFZ may have a better in vivo tracer of Aureobasidium application value. Based on the results of the analysis of ATTC260/Eno-Antares2/HFZ in Figure 6(a), the differences in the intensity of substrate luminescence catalyzed by the reporter strains with different incubation times were observed using HFZ as a substrate, and it was seen that with the extension of incubation time, the early ATTC260/Eno-Nluc, ATTC260/Eno-Teluc and ATTC260/ Eno-Antares2 catalyzed HFZ bioluminescence intensity gradually increased, and the luminescence signal stabilized after 7 h of incubation.

From the total bioluminescence of different substrates at different concentrations in Figure 6(c), the S. aureus reporter strain catalyzed the same concentration of HFZ to produce the highest signal brightness, followed by FUR, and DTZ was the weakest. Orange-red light signal acquisition using 590±10 nm filter revealed that although ATTC260/Eno-Teluc catalyzed HFZ produced the highest total luminescence intensity, it emitted orange-red light at a weaker intensity. In comparison, ATTC260/Eno-Antares2-catalyzed HFZ emitted the strongest intensity of orange-red light, followed by ATTC260/Eno-Antares2/FUR, suggesting that ATTC260/Eno-Antares2/HFZ has a better sensitivity for in vivo tracer imaging of Aureobasidium.

In summary, among the ATTC260/Eno-Nluc,

ATTC260/Eno-Teluc, and ATTC260/Eno-Antares2 reporter strains prepared in this paper, the orange-red luminescence signal produced by ATTC260/Eno-Antares2-catalyzed HFZ was the strongest in vitro. The results of this study revealed that the Aureobasidium ATTC260/Eno-Teluc/HFZ luminescence system is more suitable for in vitro tracer detection, while the ATTC260/Eno-Antares2/HFZ bioluminescence system is more preferable for in vivo tracing, which creates a prerequisite for the in-depth application of the Aureobasidium bioluminescence system.

4 Conclusion

In the article, three types of reporter strains based on Staphylococcus aureus were prepared by pUC57-Nluc, pUC57-Teluc, and pUC57-Antares2 and validated against their luminescent properties. Three different substrate solutions (FUR, DTZ and HFZ) were configured to explore the optimal organoluminescence system. The growth trend of the strains with the addition of exogenous plasmids pUC57-Nluc, pUC57-Teluc, and pUC57-Antares2 was closer to that of the native Aureobasidium strain ATTC260/Eno, and the error did not exceed 0.5%. And the luminescence intensity of the three reporter strains showed a significant positive correlation with OD_{600}. In the in vivo luminescence assay in mice, the luminescence intensity of ATTC260/Eno-Antares2 strains was maintained at more than $1*10^8$ photons/s from 10 min to 50 min, and the bioluminescence could still be detected after 20 h. In addition to emitting greenish-blue light at about 472 nm, the catalytic substrate HFZ of ATTC260/Eno-Antares2 also emitted orange light at about 594 nm. HFZ catalyzed by ATTC260/Eno-Antares2 emits orange-red light at about 594 nm in addition to cyan blue light at about 472 nm. In comparison, the intensity of the orange-red light emitted by the ATTC260/Eno-Antares2-catalyzed HFZ was the strongest, followed by ATTC260/Eno-Antares2/FUR. Therefore, HFZ catalyzed by S. aureus-conjugated plasmid pUC57-Antares2 yielded ATTC260/Eno-Antares2/HFZ organic microbial emitters with the highest intensity of orange-red light emission, which can be applied in organic luminescence systems for effective monitoring and tracing of cells in vivo.

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