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RESEARCH PAPER

E. coli biosensor based on modular GFP and *luxI/luxR* cyclic amplification circuit for sensitive detection of lysine

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Abstract

In this study, an *E. coli* biosensor based on modular green fluorescent protein and *luxI/luxR* cycle amplification circuit was constructed for sensitive detection of bioavailable lysine. The results indicated that the *luxI/luxR* positive feedback circuit based on quorum sensing can be used as a signal amplifier to improve the sensitivity to lysine detection with the detection limit of 256 nM. The presented method was more sensitive than the previously reported whole-cell fluorescent microbial biosensors. In addition, the developed *E. coli* biosensor was specific for lysine detection, and other amino acids and proteins did not cause any interference. The constructed genetic engineered biosensor was accurate for lysine detection, the lysine content of 6.87 ± 0.36% in tryptone was successfully measured, and after adding 10, 30, and 50 μM lysine in tryptone, the recoveries of 109.98 ± 10.44%, 103.88 ± 7.66%, and 105.89 ± 6.34% were obtained, respectively. Furthermore, as the design of the genetic engineered biosensor is modular, it can conceivably be utilized as a component in the design of more complex synthetic gene circuits without any changes to the amplifier and reporter system.

Keywords Lysine · Biosensor · *LuxI* · *LuxR* · Amplification

Introduction

L-Lysine ($C_6H_{14}N_2O_2$), as one of the essential amino acids, has an important physiological function in the body. It is an indispensable factor in the synthesis of body proteins, and plays an essential role in the formation of enzymes, germ cells, skeletal muscle, hemoglobin, etc. [1, 2]. L-Lysine is also a component of some peptide hormones, and can be broken down into glucose or ketone to supply energy when the body lacks carbohydrates for energy metabolism. In addition, lysine can promote human development, enhance immune function, and improve the function of central nervous tissue [3]. The addition of L-lysine to animal feeds can significantly improve the balance of amino acid absorption in feeds and promote the healthy and rapid growth of livestock [4].

However, L-lysine cannot be synthesized by the human body and mammals, but can only be obtained through the diet [5]. For humans, insufficient lysine may lead to loss of appetite, poor growth, anemia, or reproductive disorders [6], while excessive lysine intake may manifest as epilepsy, psychomotor impairment, spasticity, etc. [7, 8]. For animals, insufficient lysine supplementation can reduce feed efficiency, while too much lysine can increase the amount of nitrogen in animal excretion, thus causing environmental pollution [9, 10]. Thus, the accurate and sensitive determination of lysine has great significance in the field of food, clinical diagnosis, and feed industry.

To date, various methods have been developed for the quantification of lysine, including the titrimetric method [11], fluorometric method [12], chromatographic method [13], voltammetric method [14], nuclear magnetic resonance (NMR) imaging [15], enzymic spectrophotometric method [16], and biosensors such as electrochemical, enzyme nanoparticles, microbial, etc. [17]. However, it must be noted that some of these methods have inherent disadvantages. For example, the titrimetric method is non-specific and can be interfered by other amino acids. The chromatographic method requires expensive equipment and professional operators; moreover, the sample is prone to be hydrolyzed leading to amino acid decomposition. The enzymic colorimetric method is less sensitive and requires a long time to prepare the sample.

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The fluorometric method shows the problems of toxicity and suffers from less lifetime of the fluorescent compound [18]. Among the detecting methods, biosensors are becoming the mainstream analytical method for detecting lysine in biological samples due to their high sensitivity, simple procedure, and high reproducibility [5]. For example, Nohwal developed a modified amperometric L-lysine biosensor by immobilizing lysine oxidase nanoparticles on a gold electrode and was used to detect lysine in serum and milk. A linear correlation ranging from 10 to 100 μM and the limit of detection (LOD) of 10 μM were obtained [19]. Endo et al. developed an optical enzyme biosensor system for L-lysine detection in foods such as scallop hepatopancreas [20] with the linear range from 0.1 to 3.0 mM. In addition, microbial biosensors developed by using lysine as a specific substrate metabolizing various compounds can also realize the detection of lysine, such as the microbial biosensor based on lysine decarboxylase [21, 22] and lysine oxidase/*Saccharomyces cerevisiae* [6].

Moreover, compared with other biosensors, the microbial biosensor based on the response of the microbial growth to determine lysine can better determine the bioavailability of lysine, thus successfully simulate the biological response in animal models [23]. Payne et al. proposed a method for the determination of available lysine in protein foods using the *E. coli* Lys auxotroph [23]. However, the sensitivity of the current microbial biosensor for lysine detection needs to be further improved [4, 24].

Quorum sensing (QS) is a cellular communication mechanism that relies on the recognition of signal molecular density to regulate gene expression [25]. The *luxI/luxR* system is a typical QS system of Gram-negative bacteria, which is a common positive feedback mechanism used in the regulation of many gene circuits [26]. Wang et al. spliced *luxI/luxR* and suicide gene *ccdB* using the standard assembly method to construct a quorum sensing circuit, realizing the subtle control of population density in bacteria [27]. Many studies have described the mechanism of *luxI/luxR* and conducted in-depth exploration [28]. To our knowledge, no study has been conducted to develop microbial biosensors based on the *luxI/luxR* cyclic amplification circuit for the sensitive detection of lysine. In this study, the *E. coli* lysine auxotroph based on modular green fluorescence protein fused with the *luxI/luxR* system was constructed to realize the sensitive detection of bioavailable lysine, and the specificity and accuracy of the method were verified as well.

Materials and methods

Bacterial strain and plasmid

The *lysA* gene in the genome of *E. coli* K12 MG1655 (ATCC 700926) was knocked out by gene homologous recombination to obtain nutritional deficient *E. coli ΔlysA*. The *E. coli Trans 5α* competent cells were purchased from Sangon company (Shanghai, China). The components that construct the gene circuit and the plasmid pSB1C3, pSB1A2 were obtained from the plasmid plate provided by International Genetically Engineered Machine competition (iGEM), and the gene sequence can be found on the iGEM website by number (http://parts.igem.org/Main_Page). All the standardized gene element sequences used contain *Eco*RI, *Xba*I restriction sites at the 5′ end and *Spe*I, *Pst*I restriction sites at the 3′ end for gene splicing.

Construction of the *luxI/luxR* signal cyclic amplification gene circuit

The gene elements used to construct *luxI/luxR* signal cyclic amplification circuit strains are shown in Table S1, which were obtained from the plasmid plate of iGEM according to the number and transformed into *Trans 5α* competent cells for extended culture. The plasmid was obtained by plasmid extraction kit, and the gene elements were connected by enzyme digestion and enzyme ligation to obtain the plasmid containing signal cyclic amplification circuit, P_{j23100}-P_{lux}-*luxI*-*luxR*-GFP-T-T. Furthermore, in order to verify the effect of signal amplification, the P_{j23100}-GFP-T-T gene circuit was constructed as a control. The restriction enzyme *Eco*RI, *Xba*I, *Spe*I, *Pst*I, and T_4 DNA ligase were all purchased from NEW ENGLAND BioLabs (NEB, England).

Culture and assay conditions

E. coli ΔlysA was prepared as competent cells. The plasmids of the gene line with and without signal amplification elements were transformed into competent cells and were named as *E. coli ΔlysA gfp* and *E. coli ΔlysA luxI-luxR-gfp*; the positive clones were screened and cultured in Luria-Bertani (LB) medium. Then, the *E. coli ΔlysA*, *E. coli ΔlysA gfp*, and *E. coli ΔlysA luxI-luxR-gfp* were transferred to M9 minimal medium with chloramphenicol (34 μg/mL) for starvation treatment for 10 h to deplete lysine, respectively. One hundred microliters of each strain was added to M9 medium containing different concentrations of lysine (10, 20, 30, 40, and 50 μM). Two hundred microliters of each subculture was transferred to a 96-well microplate (Corning, USA), and the optical density at 600 nm (OD_{600}) and the fluorescence intensity with the excitation/emission wavelength at 488 nm/520 nm were measured by the fluorescence microplate reader (BioTek, USA) every 2 h.

Specificity of the *E. coli ΔlysA luxI-luxR-gfp* biosensor for lysine detection

One hundred microliters of the *E. coli ΔlysA luxI-luxR-gfp* strain was added to M9 medium containing 50 μM lysine,

50 μM glycine, 50 μM arginine, 50 μM tryptophan, 50 μM phenylalanine, non-essential amino acids (NEAA, 1%), and bovine serum albumin (BSA, 5 mg/mL), respectively, and was cultured for 10 h. And then 200 μL of each subculture was transferred to a 96-well microplate, and the optical density at 600 nm (OD_{600}) and the fluorescence intensity with the excitation/emission wavelength at 488 nm/520 nm were measured.

Accuracy of the *E. coli ΔlysA luxI-luxR-gfp* biosensor for lysine detection

Casein hydrolysate (tryptone, filter-sterilized) was used for lysine recovery experiments to verify the accuracy of the *E. coli ΔlysA luxI-luxR-gfp* biosensor. Tryptone with a final concentration of 30 μg/mL and lysine with different concentrations of 0, 10, 30, and 50 μM were added to the M9 medium, respectively. One hundred microliters of *E. coli ΔlysA luxI-luxR-gfp* was added and cultured for 10 h. The optical density at 600 nm (OD_{600}) and the fluorescence intensity with the excitation/emission wavelength at 488 nm/520 nm were measured. Lysine concentration in each sample was calculated according to the calibrated curve.

Data analysis

The three-dimensional coordinate plots of time, lysine concentration, and OD_{600}/fluorescence intensity of the engineered bacteria were established, respectively. Lysine recovery (%) in each artificial sample was calculated as a ratio of the experimentally obtained lysine content minus the obtained lysine content in tryptone and the added theoretical lysine content (10/30/50 μM). The data shown were the mean values (± standard deviation) of five independent experiments ($n = 5$).

Results and discussion

Construction and characterization of engineering bacteria with and without the *luxI/luxR* signal cyclic amplification gene circuit

The detection of lysine is of great significance in the fields of the feed industry, production metabolism, clinical, etc., and many detection methods have been developed [5]. With the development of synthetic biology, the low-cost, modular, and highly sensitive microbial biosensor has become one of the new methods for lysine detection [6, 22]. In this study, the *luxI/luxR* positive feedback line based on quorum sensing with green fluorescent protein was constructed for the detection of lysine in nutrient-deficient bacteria.

The gene line for the fluorescence signal amplification circuit based on the *luxR* and *luxI* system was constructed (Fig. S1a), and the gene line without signal amplification was chosen as a control (Fig. S1b) to evaluate the effect of signal amplification. The length of the two lines was characterized by agarose gel electrophoresis, and the results are shown in Fig. S1c and Fig. S1d, respectively. The electrophoresis results were consistent with the sequence length of the target fragment, and the correctness of the sequence was validated by sequencing.

For the signal amplification circuit, a strong constitutive promoter, P_{j23100}, was used to enhance the expression efficiency of downstream genes. The expression of downstream *luxR*, *luxI*, and GFP genes was regulated by the P_{lux} promoter [26]. The LuxI enzyme catalyzes the synthesis of acyl-serine lactone (AHL) molecules. The combination of AHL and its receptor protein LuxR leads to the efficient expression of the promoter P_{lux}, which can activate the transcription of bacterial quorum sensing, thereby forming a feedforward loop. When the bacterial population density and the expression level of LuxR are low, the synthetic AHL molecules will not accumulate in the cell, but quickly spread out of the cell. As the density of bacteria increases, the concentration gradient of extracellular AHL leads to the accumulation of AHL in the bacteria. Once the concentration of intracellular AHL reaches a certain threshold, AHL molecules bind to LuxR protein to form the AHL-*luxR* complex. Positive feedback regulates the activity of upstream promoters, promotes the expression of downstream *luxI/luxR* proteins, further promotes the synthesis of self-inducers, and forms a positive feedback regulatory mechanism to continuously activate the expression of the downstream GFP gene.

Response of *E. coli ΔlysA* strains to lysine

The OD_{600} of *E. coli ΔlysA* strains after adding different concentrations of lysine and culturing for different times is shown in Fig. 1. It can be found that the OD_{600} of the *E. coli ΔlysA* strains was enhanced continuously with the increase of culture time from 0 to 10 h and lysine concentration from 10 to 50 μM (Fig. 1a). The result indicated the successful knockout of the *lysA* gene in the genome of *E. coli* K12 MG1655. It can be also found that the correlation between lysine concentration and the OD_{600} values was not good within 4 h, but a good linear relationship can be obtained after 6 h of culture. Taking the results of 8 h as an example, the fitting curve of $y = 0.00189x + 0.0653$ ($R^2 = 0.989$) was obtained between lysine concentration and the OD_{600} values (Fig. 1b). Using the principle of the 3σ rule, the LOD was calculated to be 0.984 μM. The obtained result was consistent with that reported in the literature for the quantification of lysine using microbial growth [4, 23]. Of

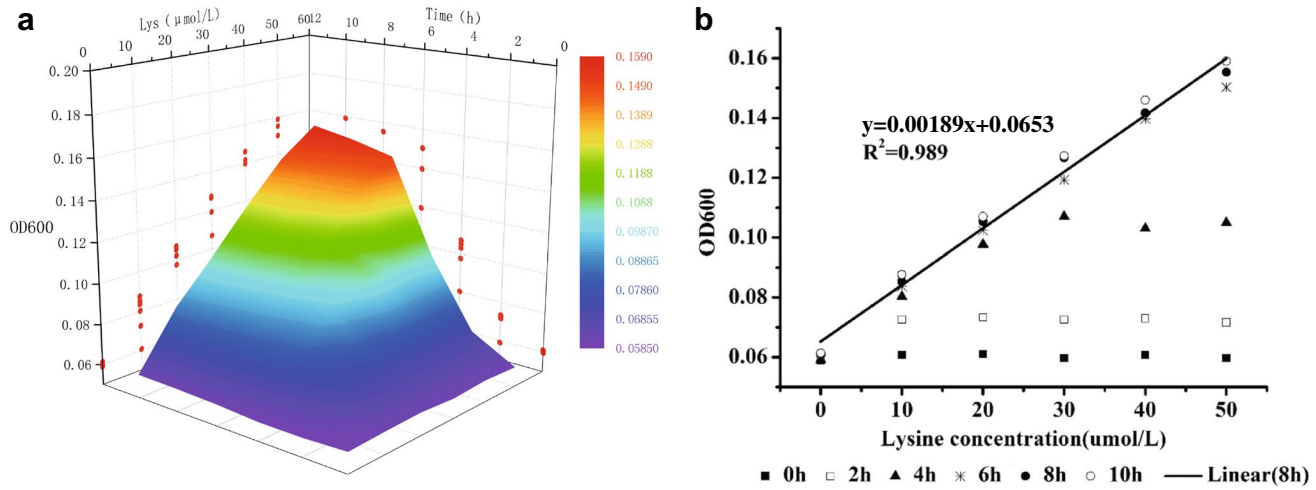

Fig. 1 Response of *E. coli ΔlysA* strains to gradient concentration of lysine at different culture time. **a** 3D graph of the relationship between culture time, lysine concentration, and OD_{600}. **b** Fitting curve of lysine concentration and OD_{600} after culture of *E. coli ΔlysA* strains for 8 h

course, the chosen strain and the integrity of the knockout gene sequence might affect the LOD to some extent.

Response of *E. coli ΔlysA gfp* strains to lysine

E. coli ΔlysA gfp strains were constructed by introducing plasmid containing P_{j23100}-GFP gene circuit into *E. coli ΔlysA*; OD_{600} values and the fluorescence intensity of *E. coli ΔlysA gfp* strains to gradient concentrations of lysine at different culture times are shown in Fig. 2. Figure 2a shows the three-dimensional relationship between lysine concentration, culture time, and OD_{600}. Similar to the responses of *E. coli ΔlysA* strains to lysine, *E. coli ΔlysA gfp* strains showed time- and dose-response; that is, with the increase of culture time and lysine concentration, the OD_{600} of the *E. coli ΔlysA gfp* strains was enhanced. The fitted curves between lysine concentration and OD_{600} are shown in Fig. 2b. A good linear relationship can be obtained ($y = 0.00218x + 0.0673$, $R^2 = 0.999$) taking the results of 8 h as an example. The LOD of 1.979 μM can be obtained according to the 3σ rule, which was comparable with that of *E. coli ΔlysA* strains (0.984 μM).

Similar to the results of OD_{600}, the fluorescence intensity of *E. coli ΔlysA gfp* strains increased with the increase of culture time and lysine concentration (Fig. 2c). The fitted curve between lysine concentration and fluorescence intensity is shown in Fig. 2d. It can be found that after culture for 8 h, a good linear correlation between lysine concentration and fluorescence intensity can be obtained ($y = 26.97x + 778.920$, $R^2 = 0.995$). The LOD of lysine was calculated to be 0.935 μM according to the principle of the 3σ rule, similar to that of OD_{600} (1.979 μM), which was not significantly changed compared to *E. coli ΔlysA* strains.

Response of *E. coli ΔlysA luxI-luxR*-gfp strains to lysine

P_{lux}-*luxI-luxR* was cloned between the P_{j23100} promoter and the *gfp* gene to construct the signal amplification circuit. In this gene line, *luxI-luxR* functions in a positive feedback loop as it can bind to the P_{lux} promoter and activate its own transcription. The responses of *E. coli ΔlysA luxI-luxR-gfp* strains to different concentrations of lysine for different culture times are shown in Fig. 3. It can be found that the OD_{600} of *E. coli ΔlysA luxI-luxR-gfp* strains showed an increasing tendency with the increase of lysine concentration and culture time (Fig. 3a), which was similar to that of *E. coli ΔlysA* strains and *E. coli ΔlysA gfp* strains. At the same time, a good linear relationship between lysine concentration and OD_{600} was be obtained ($y = 0.00214x + 0.0611$, $R^2 = 0.999$) (Fig. 3b), and the LOD of 1.266 μM can be calculated according to the 3σ rule.

Compared with the results of *E. coli ΔlysA gfp* strains, the fluorescence intensity of *E. coli ΔlysA luxI-luxR-gfp* strains was significantly amplified with the presence of the same concentration of lysine at the same culture time point (Fig. 3c). After adding lysine and culture for 8 h, a good linear relationship can be obtained between lysine concentration and fluorescence intensity ($y = 265.34x + 2296.050$, $R^2 = 0.998$) (Fig. 3d). The LOD of 256 nM was obtained according to the 3σ rule, which was reduced by 2.65 times compared with the results of *E. coli ΔlysA gfp* strains.

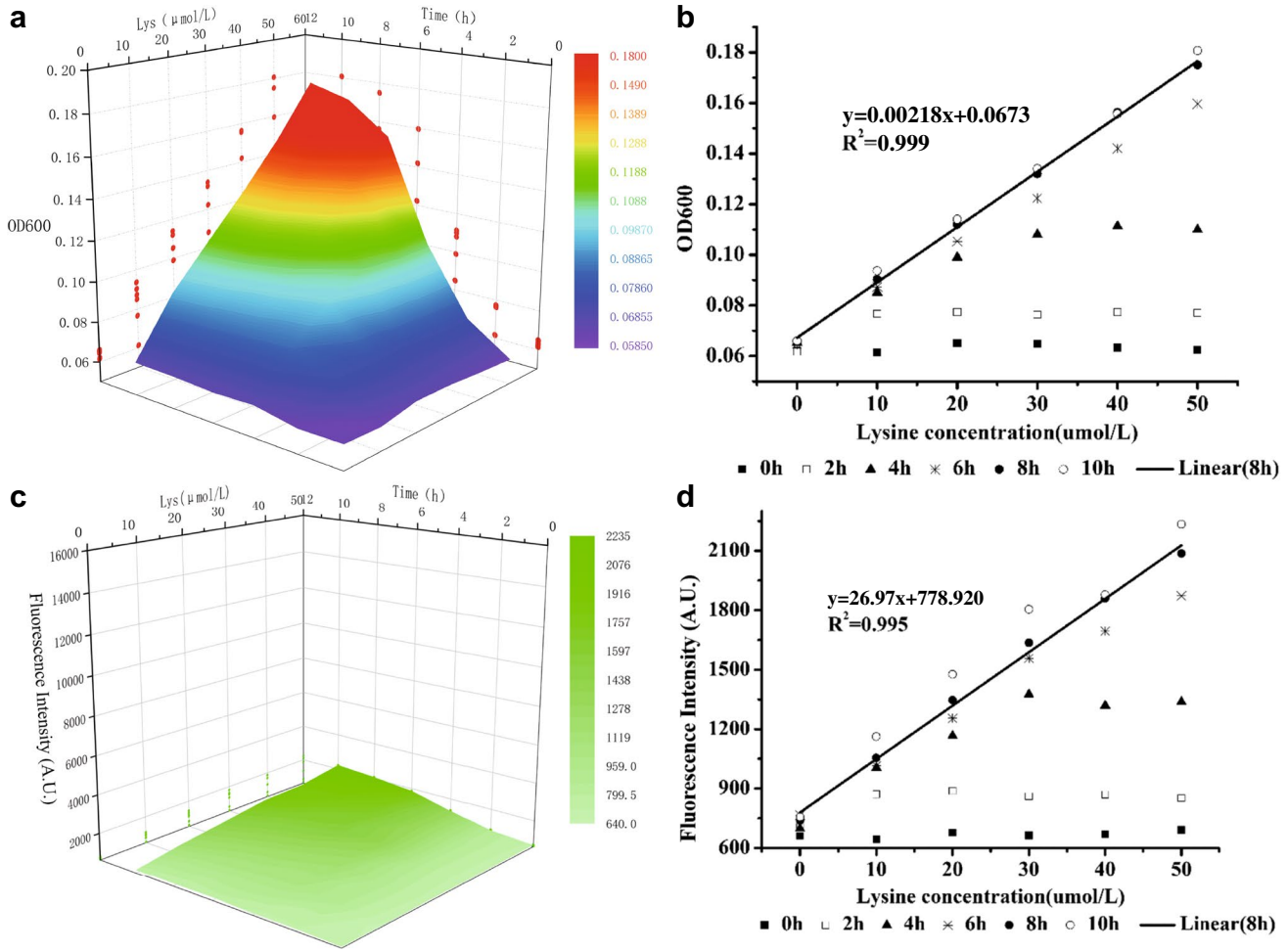

Fig. 2 Response of *E. coli ΔlysA gfp* strains to gradient concentrations of lysine at different culture times. 3D graph of the relationship between lysine concentration and OD_{600} (**a**), and fluorescence intensity (**c**) of strains at different culture time points. The fitting curve between lysine concentration and OD_{600} (**b**) and fluorescence intensity (**d**) after culture for 8 h

From the fitting curves between ΔOD_{600} and gradient concentrations of lysine of three engineering bacterial strains (*E. coli ΔlysA*, *E. coli ΔlysA gfp*, and *E. coli ΔlysA luxI-luxR-gfp*) after culture for 8 h, it was found that with the increase of lysine concentration, all OD_{600} values of three engineering bacteria increased, and all showed a good linear relationship. It can be also found that even the strain was genetically modified with the *luxI/luxR* cyclic amplification circuit; the detection sensitivity for lysine cannot be significantly improved by monitoring bacterial growth (OD_{600}).

The fitting curves of lysine concentration versus fluorescence intensity of the engineered bacteria with and without signal-amplified circuits were obtained and are shown in Fig. 4; sensitivity was described as the ability of the circuit to respond to the external stimulus, lysine, herein. It can be found that the signal amplification circuit amplified the fluorescence intensity by roughly one order of magnitude. In other words, at the same concentration of lysine, the fluorescence intensity of the circuit involving positive feedback was roughly ten times higher than that in the circuit lacking positive feedback. Compared with the detection of lysine through bacterial growth (OD_{600}), the positive feedback circuit combined with the *luxI/luxR* system can significantly improve the sensitivity of lysine detection.

Table 1 shows the comparison of the strain constructed in this study with other biosensors for lysine detection. It can be found that the obtained sensitivity herein was comparable to or higher than that of optical, fluorescent, electrochemical, and other microbial biosensors based on lysine oxidase, lysine decarboxylase, etc. Moreover, the detection time of the present study was also comparable to or shorter than that of similar microbial biosensors.

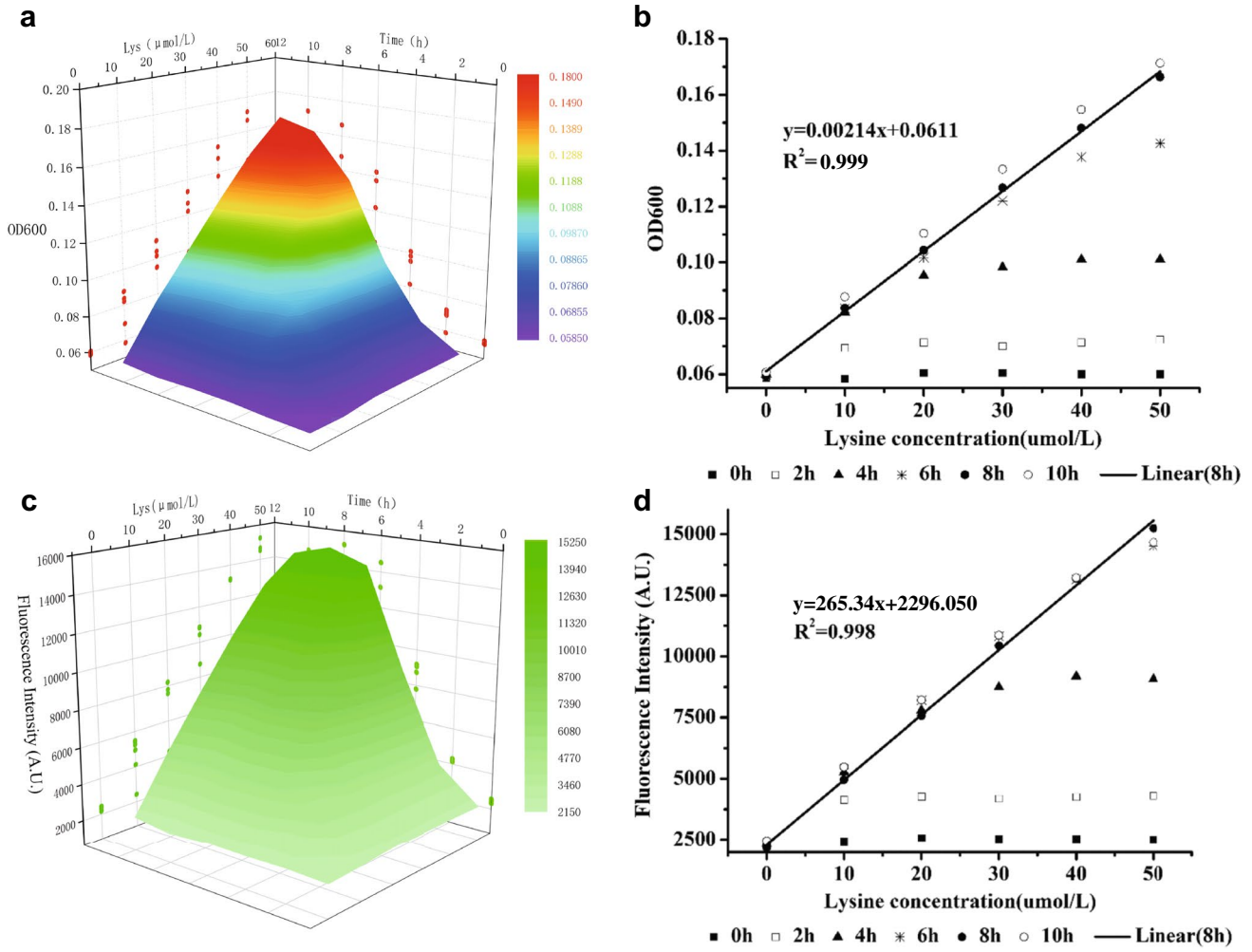

Fig. 3 Response of *E. coli ΔlysA luxR-luxI-gfp* strains to gradient concentrations of lysine at different culture times. 3D graph of the relationship between lysine concentration and OD_{600} (**a**), and fluorescence intensity (**c**) of strains at different culture time points. The fitting curve between lysine concentration and OD_{600} (**b**) and fluorescence intensity (**d**) after culture for 8 h

Specificity of *E. coli ΔlysA luxI-luxR-gfp* biosensor for lysine detection

In order to evaluate the specificity of the engineered bacteria *E. coli ΔlysA luxI-luxR-gfp* for lysine detection, several essential amino acids such as arginine, phenylalanine, and tryptophan, as well as NEAA and BSA, were used to induce the engineered bacteria. After adding different additives to the M9 medium, the bacterial growth (OD_{600}) and the expressed fluorescence intensity of *E. coli ΔlysA luxR-luxI-gfp* strains are shown in Fig. 5. It can be found from Fig. 5a that with the presence of glycine, arginine, tryptophan, phenylalanine, NEAA, and BSA, the engineered bacteria cannot grow and express normally, and only with the presence of lysine can the engineered bacteria grow normally. Accordingly, only when lysine was added did the engineering bacteria express fluorescence (Fig. 5b). After 8 h of culture, the expressed fluorescence of the lysine-added

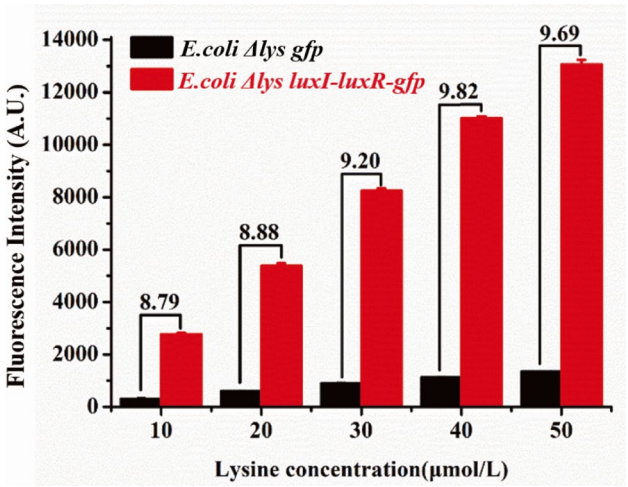

Fig. 4 Comparison of the fluorescence intensity of *E. coli ΔlysA gfp* and *E. coli ΔlysA luxI-luxR-gfp* strains to different concentrations of lysine after culture for 8 h

Table 1 Comparison of the constructed strain with other biosensors for lysine detection

No.	Methods	Principle	LOD (μM)	Linear range (μM)	Detection time	References
1	Optical	Chemiluminometric L-lysine determination	5	10–1000	30–50 s	[29]
2	Fluorescence	(R)-1,1′-b-2-Naphthylamine probe	0.453	0–500	/	[30]
3	Electrochemical	Dissolved oxygen (DO) electrode	6.7	6.7–670	/	[31]
4	Electrochemical	GC electrode	0.39	0.5–5.5	/	[32]
5	Electrochemical	Lysine decarboxylase	25	25–400	1–3 min	[21]
6	Microbial	*E. coli ΔlysA gfp*	/	0–20.52	6 h	[33]
7	Microbial	*E. coli ΔlysA gfp*	0.342	/	/	[34]
8	Microbial	*E. coli ΔlysA gfp*	/	/	8 h	[24]
9	Microbial	*E. coli ΔlysA luxI-luxR-gfp*	0.256	10–50	6 h	This study

The sensitivity of the constructed strain was comparable to or higher than that of some biosensors, and the detection time of the constructed strain was comparable to or shorter than that of similar microbial biosensors

group was significantly higher than that of the other groups, which only expressed the background fluorescence and did not increase (Fig. 5c).

Accuracy of *E. coli ΔlysA luxI-luxR-gfp* biosensor for lysine detection

Tryptone was used as the matrix to determine the accuracy and robustness of the *E. coli ΔlysA luxR-luxI-gfp* biosensor. The results of the recovery experiments are shown in Table 2. A standard curve was established according to the lysine concentration versus fluorescence intensity. As for the M9 medium containing 30 μg/mL tryptone, the lysine concentration was calculated to be 14.12 ± 0.73 μM. The lysine content in 30 μg/mL tryptone was calculated to be 6.87 ± 0.36%, and was consistent with the total amount of lysine (7.13 ± 0.34%) determined by Chalova et al. [33]. After additionally adding 10, 30, and 50 μM lysine, the lysine concentration was calculated to be 25.21 ± 1.51 μM, 45.37 ± 2.64 μM, and 67.15 ± 3.54

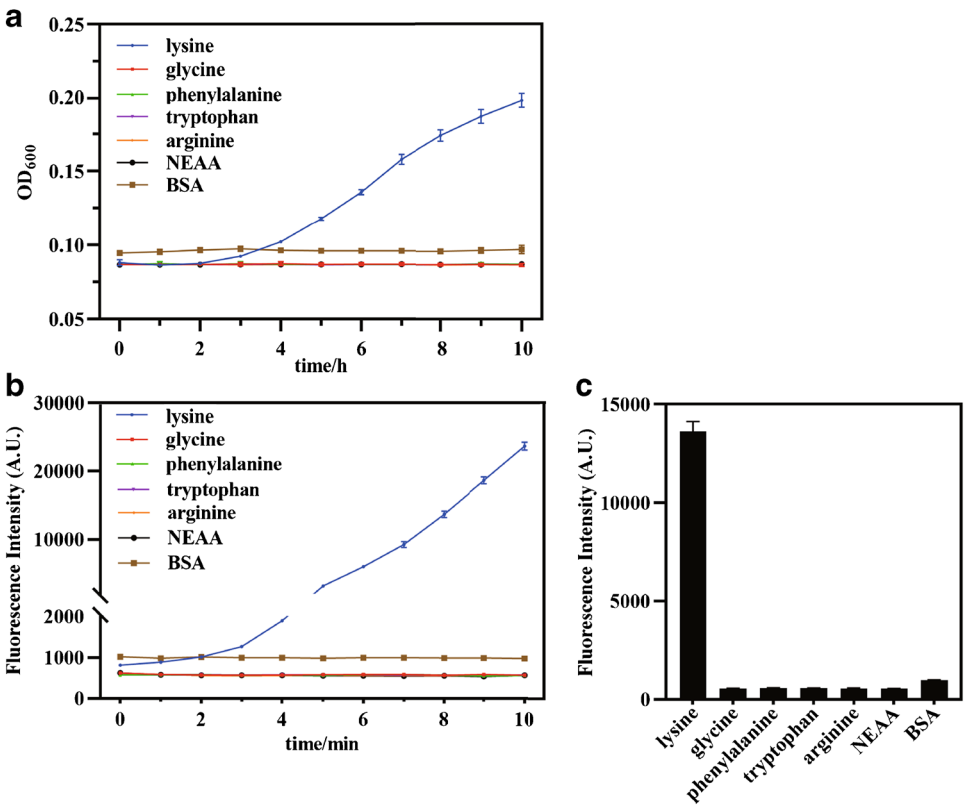

Fig. 5 Lysine interference experiment. **a** Growth of *E. coli ΔlysA luxR-luxI-gfp* strains after adding different additives. **b** Fluorescence intensity of *E. coli ΔlysA luxR-luxI-gfp* strains after adding different additives. **c** Fluorescence intensity of *E. coli ΔlysA luxI-luxR-gfp* strains after 8 h of culture with the presence of different additives

Table 2 Lysine recovery from the artificial samples

Group	Additions to culture medium		Estimated lysine (μM)	Recovery (%)	CV (%)
	Tryptone (μg/mL)	L-Lysine (μM)			
1	30	0	14.21 ± 0.73	/	/
2	30	10	25.21 ± 1.51	109.98 ± 10.44	9.49
3	30	30	45.37 ± 2.64	103.88 ± 7.66	7.37
4	30	50	67.15 ± 3.54	105.89 ± 6.34	5.99

High recovery and low coefficient of variation can be obtained by the developed biosensor for lysine detection in artificial samples

μM, respectively. Thus, the recoveries were calculated to be 109.98 ± 10.44%, 103.88 ± 7.66%, and 105.89 ± 6.34%, respectively. Accordingly, the coefficient of variation (CV) was 9.49%, 7.37%, 5.99%, respectively. The obtained high recovery and low coefficient of variation indicated the developed biosensor based on the fluorescence of auxotrophic engineered bacteria was specific, stable, and accurate for lysine detection.

Conclusion

Three engineering bacterial strains (*E. coli ΔlysA*, *E. coli ΔlysA gfp*, and *E. coli ΔlysA luxI-luxR-gfp*) were successfully constructed and used for lysine detection based on bacterial growth (OD_{600}) and fluorescent protein expression. Quantifying lysine by fluorescent protein expression was more sensitive than that by bacterial growth (OD_{600}). By embedding the *luxI/luxR* cyclic amplification gene sequence into the fluorescent protein expression gene circuit, the LOD of lysine can be reduced to 256 nM, which was lower than those of previously reported whole-cell fluorescent microbial biosensors. The quantification of lysine based on nutrient-deficient engineered bacteria *E. coli ΔlysA luxI-luxR-gfp* was specific, stable, and accurate. The introduced positive feedback lines of *luxI/luxR* can amplify any transcriptional signal, which only depend on a single gene and do not require other modulation. As the design is modular, it can conceivably be utilized as a component to design more complex synthetic gene circuits without any changes to the amplifier and reporter system.

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Author contribution W. W. and Z. J. conceived and designed the research. Z. J., T. H., and W. W. conducted the experiments and analyzed the data. W. W. wrote the manuscript. L. X. and L. X made important revisions to the paper. D. Y. approved the final paper. All of the authors read and approved the manuscript.

Data availability The data used to support the findings of this study are included within the article. The original data values of the charts generated during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This article does not contain any study with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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