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Complete List of Authors:	Xuan, Guanhua; Ocean University of China, Lu, Xiaodong; Ocean University of China, Wang, Jingxue; ocean university of China, ; Lin, Hong; Ocean University of China, Liu, Huihui; Ocean University of China,

Determination of pyruvic acid concentration using a bioluminescence system from *Photobacterium leiognathi*

Guanhua Xuan[1], Xiaodong Lu[1], Jingxue Wang[*], Hong Lin, Huihui Liu

Food Safety Laboratory, College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China;

[*]Corresponding author: Jingxue Wang, E-Mail: snow@ouc.edu.cn; Tel.: +86-0532-8203-2389; Fax: +86-0532-8203-2389.

[1]These authors contributed equally to this work.

Abstract

A novel, highly sensitive and selective bacterial luminescence method for the detection of pyruvic acid (PA) is reported here. This method is based on a reaction system catalyzed by lactate dehydrogenase (LDH) with the bacterial luciferase-FMN:NADH oxidoreductase bioluminescence system *in vitro*. The reduced nicotinamide adenine dinucleotide (NADH) involved in the LDH reaction system could be quantitatively analyzed by the bioluminescence system. A good linear relationship between the luminescence intensity and pyruvic acid concentration was exhibited within the range of 0.00014–0.001 mol/l, and the pyruvic acid detection limit was found to be 8.537×10^{-5} mol/l. This method was successfully applied to the detection of PA in quail serum with a good recovery of over 70 %.

Keywords: pyruvic acid, luciferase, FMN:NADH oxidoreductase, lactate dehydrogenase, bioluminescence system, rapid detection

1. Introduction

Pyruvic acid (PA), the simplest alpha-keto acid, plays a central role in energy metabolism in living organisms[1]. It supplies energy to cells through the citric acid cycle when oxygen is present, or through lactic acid fermentation when oxygen is absent[2, 3]. The level of pyruvic acid plays a major role in maintaining fuel homeostasis, and it is often found to be elevated in the plasma in various pathological conditions. Therefore, determination of PA levels is important to understand the underlying metabolism and reactions in clinical analysis and bioanalysis[4, 5].

In recent years, several techniques for the determination of PA in samples have been reported, such as reversed-phase UV high performance liquid chromatography (RP/UV-HPLC) [6-9], colorimetric detection[10], nuclear magnetic resonance (NMR)-based methods[11], capillary zone electrophoresis[12,13], voltammetric techniques[14] and chemiluminescence[15]. These methods have been widely used in many laboratories because of advantages in detection sensitivity and stability. However, they also have limitations, such as expensive equipment, time-consuming and laborious sample pre-treatment. Enzymatic assays are a specific, rapid, and convenient alternative. Lactate dehydrogenase (LDH), an NADH-dependent dehydrogenase, is found in various organisms and catalyzes the inter-conversion of pyruvate and lactate with concomitant inter-conversion of NADH and NAD^+ [16]:

$$CH_3COCOOH + NADH \xrightarrow{ADH} CH_3CHOHCOOH + NAD^+$$

Currently, methods based on NADH-dependent dehydrogenase bioassays, including fluorescence detection[17], spectrophotometry[18,19] and biosensors[20], have become powerful tools for the indirect analysis of PA concentration in biological samples by using NADH as an indicator. Although these biosensor methods are highly sensitive (the detection limit of a microbial biosensor based on *Lactobacillus delbrueckii* sp. was 0.018 mmol/l NADH [16]), the instability of biosensors and lack of specificity in spectrophotometric assays has limited their wide spread use.

Bacterial luciferase and NADH:FMN oxidoreductase form an important coupled enzyme system in *in vitro* bioluminescence because they catalyze oxidation reactions to emit light in the presence of FMN, NADH, a long-chain aliphatic aldehyde, and molecular oxygen[21, 22]. Because NADH is an essential substrate for this coupled enzyme system, bacterial bioluminescence is can be to monitor the metabolism of various chemicals. Previously, a NADH-bioluminescence system has been established by our lab to estimate cell viability and monitor various analytes[23, 24]. In this study, we developed a novel method to monitor PA by combining the catalytic activity of LDH with a bacterial bioluminescent system. The amount of PA involved in the LDH reaction was equal to the consumption of NADH, and the latter can be quantitatively analyzed by a bioluminescence system. Therefore, LDH activity, and thus the

concentration of PA can be determined by measuring the luminescence intensity produced by the NADH-coupled enzyme system. By combining the LDH reaction and bacterial bioluminescence system, we validated a rapid, sensitive, simple, and reliable method for PA detection.

2. Materials and methods

2.1 Bacterial strains and chemicals

The luminous bacterium *Photobacterium leiognathi* YL was isolated from brine from the coastline of Qingdao, China. The strain was preserved at the China Center for Type Culture Collection and deposited under the number M206139. The 16S rRNA gene sequence of the strain was submitted to the GenBank nucleotide sequence database (http://www.ncbi.nlm. nih.gov/genbank) and recorded as EF017227.

Chemicals and enzymes used in this study, including NADH, NAD^+, dodecyl aldehyde, FMN-Na_2, pyruvic acid, and lactic dehydrogenase (Catalog No. L2500), were either analytical grade or of the highest purity and were obtained from Sigma-Aldrich Chemicals.

2.2 Preparation of luciferase-NADH:FMN oxidoreductase crude extracts from **Photobacterium leiognathi**

The luciferase-NADH:FMN oxidoreductase crude extracts (crude enzyme) were prepared as described previously[24]. A single colony of *P. leiognathi* YL was inoculated into 2216E liquid medium (300 ml) and cultured at 25 °C overnight with continuous rotation (150 $r \cdot min^{-1}$). The cells were collected by centrifugation (4000

r·min^{-1} for 15 min) and suspended in 30 ml of phosphate-buffered saline (PBS) (the ratio of cells to buffer was 1:4). Resuspension was followed by supersonic treatment at a frequency of 20 kHz for 90 min with a disrupter (JY92-II, SIENIZ, Ningbo, China) in an ice bath. After ultrasonic disruption, the supernatant was collected by centrifugation and the crude lysate was treated with ammonium sulfate. After centrifugation to obtain the insoluble fraction (precipitating between 40 and 80 % of ammonium sulfate saturation), the precipitate was concentrated by dialysis in PBS buffer at 4 °C for 24 h. Finally, the crude enzyme containing both luciferase and FMN reductase was collected and the luminescence intensity (3,200,000) was measured.

2.3 *Detection of lactic acid by fluorescence spectrophotometry*

Different concentrations of lactate (0, 0.00001, 0.0001, 0.001, 0.01, 0.1 and 1 mol/l) were added to 1 ml of 0.01 mol/l PBS, 300μl of 0.1 mmol/l NAD^+ and 30μl of 11.6 U/ml LDH. The fluorescence intensity was immediately measured with an F4600 fluorescence spectrophotometer. The fluorescence intensity of NADH (0.1 mmol/l) and NAD^+ (0.1 mmol/l) were detected as a control. Each assay was performed three times.

2.4 Effect of reaction time on PA measurement

The LDH system included 1 ml PBS (0.01 mol/l, pH 7.0), 300 μl NADH solution (0.1 mmol/l) and 30 μl LDH (11.6 U/ml) at 4 °C. To detect the effect of the length of the reaction on PA determination, 100 μl 0.01 mol/l PA standard solution was added to the LDH system, and results were collected at 0, 2, 4, 6, 8 and 10 min. Each assay was performed three times.

2.5 Establishment of the coupled enzyme system

The bioluminescence reagent included 1 ml crude enzyme, 100μl dodecyl aldehyde (27 mmol/l) and 2.5μl FMN-Na_2 (10 mmol/l). Bioluminescence assays were performed by adding 300μl of sample to the bioluminescence reagent, the light emission was then measured immediately with an Ultra-Weak Luminescence Analyzer (BPCL_K, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) at an absorption of 471 nm. Intensity values were measured for a period of 60 s at 1 s intervals.

2.6 Establishment of a standard curve for the detection of PA with bacterial luminescence

Different concentrations of PA standard solutions were prepared and added into the LDH system. After 15 seconds, 300 μl of reaction solution was taken out and added to the coupled enzyme system. The difference in the values of the luminescence intensity between the reaction solution and a baseline of 0.1 mmol/l NADH was used for quantifying the content of pyruvic acid in the sample. The standard curve was then established based on the concentration of PA solution and the increase in luminescence intensity.

2.7 Sensitivity of the detection system for pyruvic acid

In total, 100 μl of pure water, instead of PA solution, was added into the LDH system, for 15 seconds; then 300 μl of the sample was removed and mixed with the bioluminescence reagent and luminescence intensity was measured. The limit of detection was calculated based on the formula recommended by AOAC [23]. The means

A_b and standard deviation (S_b) of the results were calculated, and the lowest detected luminescence intensity was A_b+3S_b. The minimum detection content of PA was then obtained using the equation of linear regression from the linear range of the standard curve. This assay was performed 20 times.

2.8 Specificity of the bacterial luminescence method

The specificity of the bacterial luminescence method for determination of pyruvic acid was examined by monitoring its responses toward other substances, including lactic acid, acetic acid, glucose, oxalic acid, citric acid and tartaric acid. Standard solutions of each sample were prepared at concentrations of 0.01 mol/l, 0.05 mol/l, and 0.1 mol/l. The luminescence intensity difference was measured with a luminescence analyzer.

2.9 Recovery and repeatability experiments to detect pyruvic acid in samples

To evaluate the effectiveness of the method, recovery experiments in quail serum were performed. The serum samples were spiked with 0.0002 mol/l and 0.0004 mol/l of PA and then tested using the bacterial luminescence system. The recovery rate of each group and average percentage of recovery were calculated.

Each assay was performed in six parallel tests. The results were translated into corresponding PA concentrations using the linear regression equation of the standard curve. The relative SD of the results was calculated to analyze the variability of repeated experiments.

3. Results

3.1 Detection of lactic acid using fluorescence spectrophotometry

The effects of different concentrations of lactic acid on fluorescence intensity are shown in Fig. 1. Fluorescence intensity produced by lactic acid was very low, similar to that produced by the NAD^+ solution, until lactic acid concentrations reached 1 mol/l, indicating that almost no NADH is produced in the presence of low concentrations of lactic acid. Despite high concentrations of lactic acid shifting the reaction towards pyruvate and NADH production, resulting in a fluorescence intensity of 50, this fluorescence intensity is still low compared to that produced by 0.1 mmol/l NADH. These results support the specificity of the LDH reaction and support the use of our bioluminescence assay in the detection of PA.

3.2 Determination of LDH reaction system response time

The optimum duration of the LDH reaction was evaluated in this study. The luminescence intensity of the reaction mixture solution displayed no obvious increase over time, since the LDH reaction system rapidly achieved the balance (Fig. 2). We found that the luminescence intensity increased markedly at 15 s then the luminescence intensity approached to a constant value (not shown). Therefore, a reaction time of 15s was determined to be an optimal time for the bioluminescence assay.

3.3 Standard curve and limit of detection

A very good linear correlation was obtained between the luminescence intensity difference and pyruvic acid concentrations in the range of 0.00014–0.001 mol/l. The standard curve showed a linear relationship as described by y = 2.2E+9x+7.3E+5 with

an R^2 of 0.9949 (Fig. 3). The mean of the results of 20 blank samples was 626688.8, and the SD was 92578.6. According to the standard curve, the limit of detection was determined to be 8.537×10^{-5} mol/l.

3.4 Specificity of the bacterial luminescence method

To investigate the selectivity of the detection system, lactic acid was added to the bioluminescence assay. Lactic acid is not only similar to pyruvic acid in structure, but also the product of LDH reaction. Background levels of the luminescence due to lactic acid were observed in Fig. 4. Even a low concentration of PA (0.00014 mol/l), had a greater luminescence intensity than lactic acid of 0.1 mol/l. This indicated that the presence of lactic acid had little interference in the detection of PA in a sample.

Additionally, five other substances, including acetic acid, glucose, oxalic acid, citric acid, and tartaric acid, were also tested for background luminescence in the bacterial luminescence assay (Fig. 5). All have a structure similar to pyruvic acid and are likely to also be present in samples. Results showed that among the five substances, acetic acid and citric acid had the strongest bioluminescence inhibition, with luminous intensity values all below 700,000, compared to the luminescence intensity above one million, which was achieved with 0.00014 mol/l of PA standard solution. The effect of all five substances on luminous intensity was lower than that of lactic acid, and had no correlation with the concentration of any substance. The results demonstrated that the bacterial luminescence method can be specifically used for PA determination.

3.5 Recovery and reproducibility experiments in serum samples

To investigate the accuracy of the results obtained by the bacterial luminescence method, recovery experiments were performed in serum samples (Table 1). The recovery rates were all over 70 % and relative standard deviation of three groups of samples were all below 2.4 %, which indicated that this novel method can be used to determine PA in samples with high precision and good reproducibility.

4. Discussion

In this study, a novel method for the determination of PA by bioluminescence *in vitro* has been established. In this assay, PA reacts with NADH in the presence of lactate dehydrogenase (LDH) [25]. The NADH concentration in the LDH reaction was quantitatively analyzed by bioluminescence. Before testing our bioluminescence assay, we evaluated the effect of adding LDH metabolites to our reaction on NADH detection with fluorescence spectrophotometry. Results showed that there was almost no NADH produced when low concentrations of lactic acid were added to the reaction system. When high concentration of lactic acid and NAD^+ were added, only small amounts of NADH were generated. This suggests that the reaction catalyzed by LDH in this system is largely to convert PA to lactate, which supports the suitability of the bioluminescence method in PA detection.

The success of the bacterial luminescence method in this study depends on the good sensitivity of the coupled enzyme system to NADH. Mei et al. have reported that the detection limit of NADH is 10^{-10} mol/l[26]. This high sensitivity of the *in vitro* bioluminescent system makes it an appealing way to monitor various analytes. In this

study, the detection limit for PA using the luminescence method was 0.08537 mmol/l. These results are consistent with those of other reported methods, such as high-performance liquid chromatographic (HPLC) method and enzymatic fluorescence capillary analysis, with PA detection limits of 0.06125 mmol/l [27] and 0.012 mmol /l[17], respectively. A novel electrochemical sensor based on fullerene-multi- walled carbon nanotubes composite film [28] has a lower detection limit (0.1 nmol/l) than all reported methods. However, this method has several drawbacks, such as bad stability and high cost. In contrast, the bacterial luminescence method can be widely applied owing to the low cost and simplicity of the assay. The samples to be tested are added directly into the detection system without pretreatment and the apparatus are simple. Additionally, we could complete the entire detection procedure of PA within 20 min, which allows for rapid detection. Because of its speed and simplicity, the bacterial luminescence method we present here is a promising tool for the determination of pyruvic acid in clinical and industrial samples.

The detection system showed a good specificity to PA. Due to the complex composition of samples, particularly those from medical clinics or industries, the confounding effects of substances such as acetic acid, glucose, oxalic acid, citric acid, tartaric acid and lactic acid, etc. on bioluminescence assay should be considered. Our results showed that the bioluminescence system did produce some background luminescence in response to these substances. However, the magnitude of the non-specific luminescence was low compared to the luminescence produced by PA. Indeed, the luminescence intensity difference produced by 0.00014 mol/l PA was even

higher than the highest background levels produced by 0.1 mol/l lactic acid. This is in agreement with the good specificity of the bacterial luciferase-FMN: NADH oxidoreductase bioluminescent system to NADH. The high specificity of the detection system presented in this study seems superior to the chemiluminescence (CL) method, which has poor selectivity in complex samples[29, 30], despite its high sensitivity. Additionally, the obtained recoveries were more than 70 % when the system was applied to detect PA in serum samples, indicating that the co-existing substances in serum almost did not interfere with the luminescence method. Thus, our proposed procedure appears to be very specific for pyruvic acid.

The bioluminescence system we proposed in this study provides a convenient and economical assay for indirect PA determination in samples. The source of the crude enzyme is *Photobacterium leiognathi* YL, which is easy to cultivate, and 30 ml of crude enzyme can be produced in a single preparation from 300 ml YL cultures. And the preparation of crude enzyme and optimization of bioluminescence system has been made by our laboratory and steadily applied in the detection of a variety of pollutants. The detection system in this study is expected to be an attractive substitute technology because of its rapidity, low cost, and operational ease. In future research, we will work to further improve this technology via a reduction in the detection limit by purifying the coupled enzyme, increasing the amount of enzyme produced, and simplifying the detection protocol, ultimately to broaden the applicability of this method.

5. Conclusion

In this paper, a novel method for PA determination using bacterial luminescence was established. The detection procedure was based on an enzymatic conversion system involving concentration changes of reduced nicotinamide adenine dinucleotide (NADH), which was an essential substance for the bioluminescent systems *in vitro*. A good linear relationship between the luminescence intensity and PA concentration was found in the range of 0.00014–0.001 mol/l, with a detection limit of 8.537×10^{-5} mol/l. Finally, the bacterial luminescence method was reliably able to detect PA in quail serum. This method is simple, sensitive, and easy to generalize, and has good potential as a tool in biological and clinical analysis of PA.

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Figure legends

Table 1. Results of recovery experiment from serum samples (n=6)

Fig. 1. Effect of lactic acid concentration on fluorescence intensity. Average fluorescence intensity and standard deviation (error bars) were calculated from three replicates (n = 3) per assay.

Fig. 2. Effects of reaction time of LDH system on the luminescence intensity

Fig. 3. Standard curve of detecting pyruvic acid by the bioluminescent method. Dots represent the experimental data and solid line, the linear fit. Average luminescence intensity and standard deviation (error bars) were calculated from three replicates (n = 3) per assay.

Fig. 4. Luminescence intensity of different lactic acid concentrations

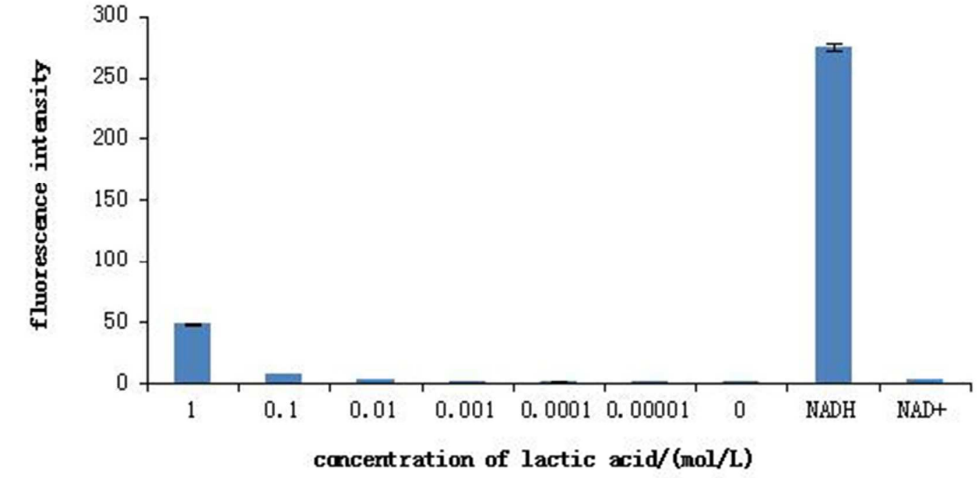
Fig. 5. Luminescence intensity difference values of acetic acid, glucose, oxalic acid, citric acid and tartaric acid. Average luminescence intensity difference values and standard deviation (error bars) were calculated from three replicates (n = 3) per assay.

Table 1

Number	Added (10^{-4} mol/l)	Measured (10^{-4} mol/l)	Recovery %	RSD %
1	4	2.890±0.055	72.26±0.014	1.92
2	2	1.410±0.004	70.51±0.002	0.26
3	4	3.002±0.070	75.04±0.018	2.33

Every assay was performed six times and the data represent the average value ± SD.

Fig. 1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150
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NADH
NAD+
concentration of lactic acid/(mol/L)

Fig. 2

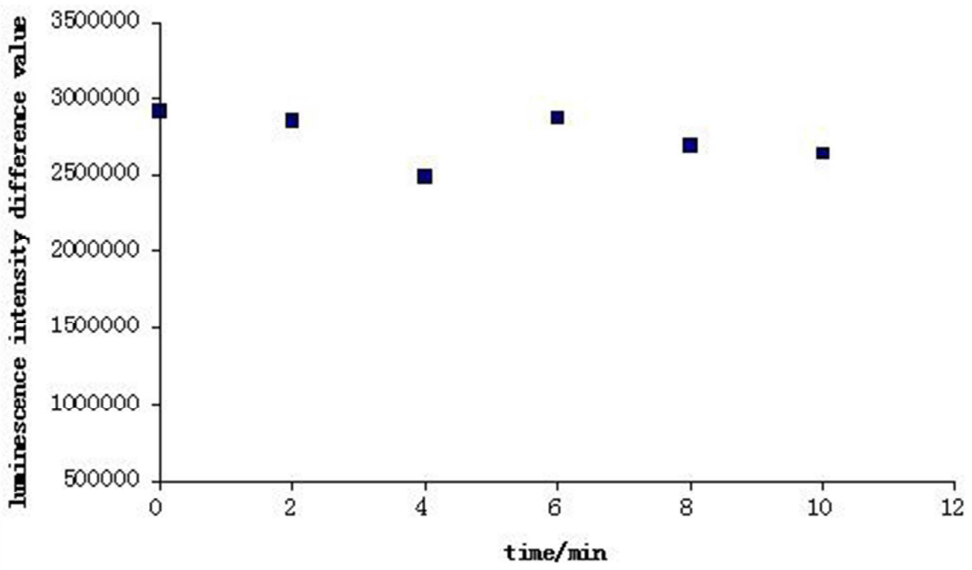
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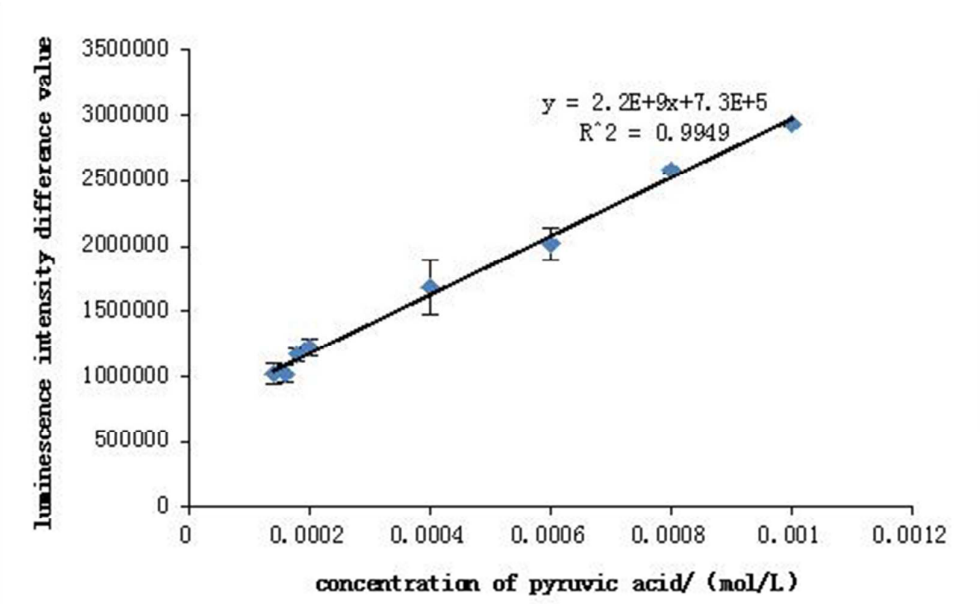
Fig. 3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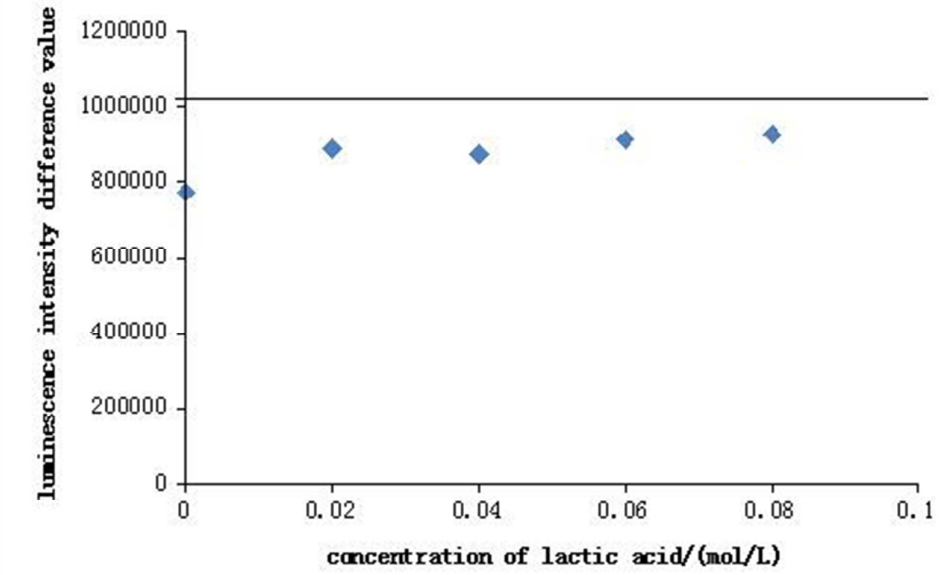
concentration of pyruvic acid/ (mol/L)

Fig.4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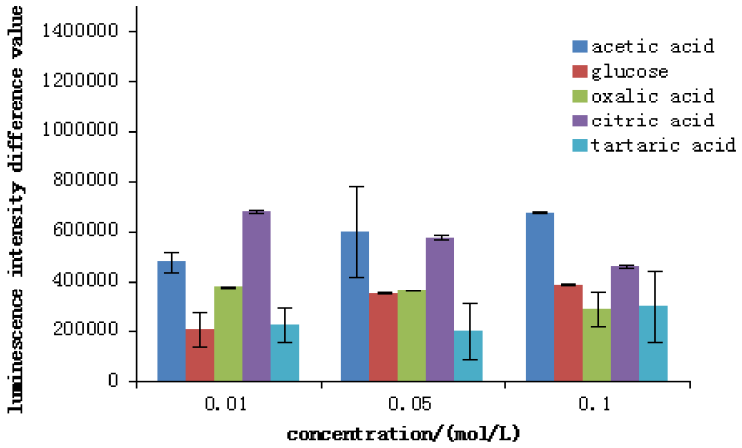
concentration of lactic acid/(mol/L)

Fig. 5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glucose
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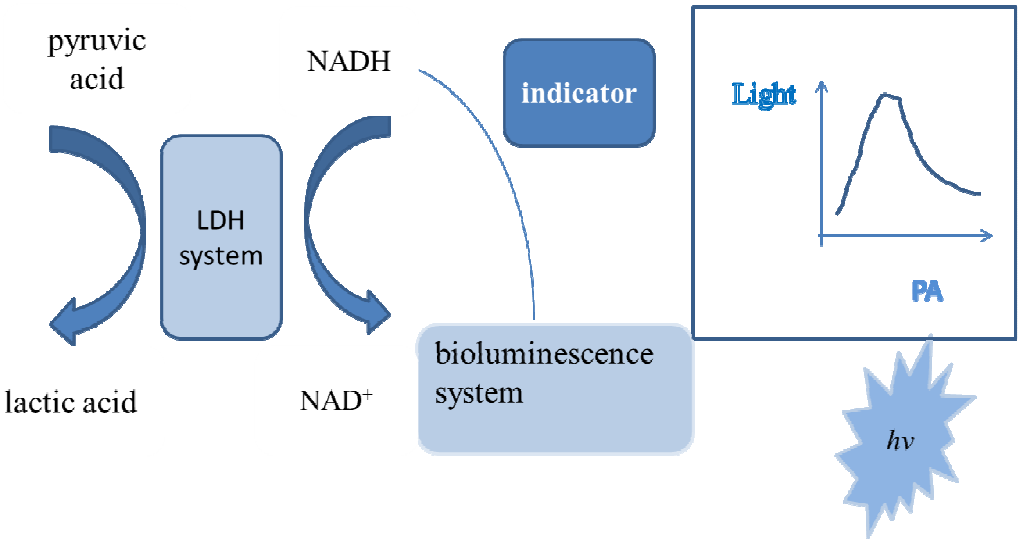
citric acid
tartaric acid



A novel method combined the catalyzing by LDH with bacterial bioluminescence system was developed for pyruvic acid detection. The detection system was expected to be an attractive substitute technology that can be applied rapidity, low cost and operational ease.