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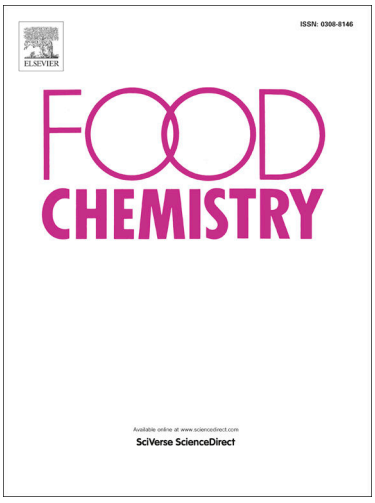

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A convenient and rapid method for detecting D-glucose in honey used smartphone

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Abstract

Information concerning food composition, including information on its glucose content, is essential for modern food industry due to greater consumer awareness and expectations. In this work, the gene encoding D-glucose dehydrogenase (GDH) from *Bacillus Natto* was expressed in *Escherichia coli* BL21(DE3) firstly. Ni-IDA column was used for the purification of GDH. Then, the purified GDH was used to construct a color system with stable and effective measurement of concentration of D-glucose. The smart phone photographing and the software Microsoft Photoshop have been used in the system for determination of the color. The enzymatic analysis system can detect the concentration of D-glucose from 5 mM to 40 mM, and other various sugars has no interference to the system. The system was used to quantitatively detect the concentration of D-glucose in honey. The system can be used for convenient and rapid detection of D-glucose in food, especially for large numbers of samples.

Keywords: Enzyme; Color reacting; Biosensor

1. Introduction

The D-glucose is one of the most widely distributed and the most important monosaccharide in nature. It is also the energy source and metabolic intermediate of living cells, so D-glucose plays an important role in biology. Different amounts of D-glucose are used directly in the food and pharmaceutical industries. According to the World Health Organization, more than 346 million people worldwide have diabetes, and prolonged high blood glucose can cause several health disorders (Krzyczmonik, Socha & Skrzypek, 2018). It is crucial to both producers and consumers to detect and know the concentration of D-glucose in food and medicine (Liu, Javvaji, Raghavan, Bentley & Payne, 2012).

At this stage, conventional methods were used to detect D-glucose include high performance liquid chromatography (HPLC) (Sato, Katayama, Arai, Sako & Tazaki, 2008), spectrophotometry (Chen, Hai, Chen & Wang, 2014), electrochemiluminescence (Xiao et al. 2017) and fluorometry (Wu, He, Wang & Yan, 2010). Although provided satisfactory accuracy and sensitivity, those methods require expensive instruments, highly qualified technical staff, and cumbersome pretreatment steps. These requirments limit the detection and application of D-glucose at on site determination (Chaichi & Ehsani, 2015). As an alternative of conventional methods, colorimetric biosensors have been considered as a promising sensing platforms for detection D-glucose due to the advantages of cost-effectiveness, ease of use, and naked-eye readout (Li et al. 2019). In the last decade, smart phones have become more and more popular in the development of low-cost and efficient biosensors (Chen et al., 2017; Guo et al., 2018; Yetisen, Martinez-Hurtado, Garcia-Melendrez, Da Cruz Vasconcellos & Lowe, 2014). Smart phones with high-resolution camera, high-speed processor and tailored APPs have been used for image collection and data analysis. They are also used to the detection of human serum albumin (Lai et al. 2017), food aging and quality (Chen et al., 2017) and amount of the bacteria (Zheng, Cai, Wang, Liao, Li & Lin, 2019). It is shown that the color information captured by smart phone can be represented using various color models, such as RGB (red, green, blue)

(Murdock, Shen, Griffin, Kelley-Loughnane, Papautsky & Hagen, 2013; Zhang, Li, Li, Song, Feng & Guan, 2014), HSV (hue, saturation, value) (Cantrell, Erenas, de Orbe-Payá & Capitán-Vallvey, 2010; Hong & Chang, 2014; Oncescu, Mancuso & Erickson, 2014), CIE (International Commission on Illumination) coordinate and CMYK (cyan, magenta, yellow, and black) (Guo, Wong, Cui, Li & Yu, 2015; Meng, Schultz, Cui, Li & Yu, 2015), CMYK as the subtractive color model has been used for color printing popularly (Wei, Yang, Luo, Wang & Li, 2019).

Enzymatic methods used for detecting substance have many advantages such as convenience and specificity. D-glucose oxidoreductase have been developed for using D-glucose detection in the enzymatic sensors (Haque, Nandhakumar & Yang, 2019; Yoon et al., 2019). In recent years, it has been found that D-glucose dehydrogenase (GDH) has the potential to replace D-glucose oxidase. Compared with the detection method using D-glucose oxidase, the detection method using GDH does not use oxygen as an electron acceptor and is not restricted by dissolved oxygen. Therefore, the GDH is a better alternative for detection of glucose (Fava, Silva, Prado, Moraes, Faria & Fatibello-Filho, 2019).

Honey, as a healthy and nutritious food which contains a lot of D-glucose, is often used in foods such as beverage and desserts (Da Silva, Gauche, Gonzaga, Costa & Fett, 2016). Nowadays, fake honey with cheap sweeteners such as cane sugar or refined beet sugar, corn syrup, high fructose or maltose syrup, instead of D-glucose is present on the market. So the determination of D-glucose content in honey can prove their originality and even help to identify the region of the world from which the product originates (Puscas, Hosu & Cimpoiu, 2013). Different techniques have been successfully used in the past to detect adulterated honey, e.g. thermal analysis (Amiry, Esmaiili & Alizadeh, 2017), liquid chromatography with electrochemical detection (Aliaño-González, Ferreiro-González, Espada-Bellido, Palma & Barbero, 2019), voltammetric electronic tongue (Sobrino-Gregorio, Bataller, Soto & Escriche, 2018), and optical resonance (Zainuddin et al., 2018). However, in an ordinary family, professional precision instruments are impossible. Therefore, it is necessary to find a

method of measuring the D-glucose concentration without using complicated instruments.

In this study, a color system based on enzymatic method for detecting D-glucose concentration has been established. The smartphone photographing and the software Microsoft Photoshop have been used in the system for analysis of the color.

2. Materials and methods

2.1. Reagents, plasmid and bacterial strains

Food natto and honey purchased from Nanjing supermarket market (Nanjing, China). NAD^+ was purchased from Bide Pharmatech Corporation. (Shanghai, China). The chemicals used were of highest grade and purchased from Sinopharm Chemical Reagent Corporation (Nanjing, China). Nickel-affinity chromatography resin was purchased from Bio-Rad laboratories (shanghai, China). Restriction enzymes, T4 DNA ligase, and exTaq DNA polymerase were purchased from Takara Bio Corporation (Dalian, China). The protein molecular weight marker was purchased from Nanjing Genscript Corporation (Nanjing, China). *E. coli* TG1 and pMD18-T cloning vector were used for cloning of the gene. *E. coli* BL21 (DE3) and plasmid pET30a (+) was used for protein expression.

2.2. Gene cloning, expression and GDH purification

The gene cloning and expression methods based on previous reports with slightly modified (Elbahnasawy, Farag, Mansour & El-Ghamery, 2020). To obtain the gene encoding GDH from *Bacillus Natto*, primers P1 and P2 were designed using the gene sequence of *Bacillus Natto* BEST 195 as target. The primer P1 with *Bam*H I restriction site insertion and the primer P2 with *Hind* III restriction site insertion were as follows: P1, 5'-CATTAGGATCCATGTATCCGGACTTAAAAG-3' and P2, 5'-TTCGAAGCTTACCGCGGCCTGCCTGCCTGGAATG-3'. The DNA fragment was amplified by PCR from the macrogenome of natto using P1 and P2 primers. The PCR fragment was ligated to the pMD18-T vector and transformed into *E.coli* TG1. The plasmid constructed was named pMD18-GDH. To construct an expression vector under the control of T7 promoter, pMD18-GDH was digested with *Bam*H I and *Hind*

III and the gel-purified target fragment was ligated to pET30a (+) digested with *Bgl* II and *Hind* III restriction enzymes. The resulting recombinant pET30a-GDH was transformed into *E.coli* BL21 (DE3) to express GDH.

The *E.coli* BL21 (DE3) (pET-30a-GDH) was grown in shaking incubator at 200 rpm, 37°C in 5 mL LB medium with 50 μg mL^{-1} kanamycin for 12 h. The culture was transferred into the 500 mL LB medium contained 0.2% (w/v) lactose and 50 μg mL^{-1} kanamycin, then grown in shaking incubator at 200 rpm at 37°C for 10 h. The cells were harvested by refrigerated centrifugation (4,000 × g, 5 min) at 4°C, washed with deionized water three times. The precipitate was collected for the production of GDH.

The cell pellet was resuspended in water and then broken by ultrasonic processor for 1 h. Cell debris was removed by centrifugation for 10 min at 10,000 × g, and the supernatant was used as the crude enzyme. The crude GDH was purified by a 8 mL Ni-IDA column which was equilibrated with 5 mM imidazole, 300 mM NaCl in 50 mM sodium dihydrogen phosphate (pH 8.0), then eluted by 500 mM imidazole in the same buffer.

The SDS-PAGE methods based on previous reports with slightly modified (Guo et al., 2018). The SDS-PAGE was performed using a 5% (m/v) concentrated gel and a 12% (m/v) separated gel. Proteins in the gel were detected by Coomassie brilliant blue R-250 stain, followed by discolor treatment.

2.3. Assay of GDH activity

To a test tube, 0.2 mL of D-glucose (200 mM), 1 mL of Tris-HCl buffer (50 mM, pH 8.5), 0.5 mL of deionized water, 0.1 mL NAD^+ (5 mM) and 0.2 mL of GDH solution were added. The mixture was incubated for 10 min at 25°C. The NADH liberated was measured at 340 nm with a spectrophotometer (Hitachi U1800, Japan). One unit of the enzyme activity was defined as the amount of GDH which produce 1 μmol NADH per minute under the assay conditions.

2.4. Effects of pH on GDH

The effects of pH on enzyme activity were studied in the pH range of 7.1-10.6 at

25°C by using D-glucose (40 mM) as substrate. The buffers were used as follows: 50 mM Tris-HCl buffer (pH 7.1-9.0), 50 mM Gly-NaOH buffer (pH 9.0-10.6). The relative activity was calculated as GDH activity under different pH divided by maximum activity which was regarded as 100%. To formulate pH on the GDH stability, pH 7.0, 8.5, 9.0 and 10.0 levels were studied, and the residual activity of the enzymes were measured every 30 minutes. The residual enzyme activity was determined by comparison with original activity of the enzyme. All experiments were repeated for three times.

2.5. Effects of temperature on GDH

In order to get the optimal temperature of the GDH, the test was assayed using D-glucose (40 mM) as the substrate in the range of 20-50°C in Tris-HCl buffer (50 mM, pH 8.5). Effects of temperature on the GDH stability were conducted at 4°C, 25°C and 35°C temperature levels. The calculation methods of relative activity and residual enzyme activity were the same as above. All experiments were repeated for three times.

2.6. Substrate specificity and kinetic parameter of the GDH

An equal concentration (40 mM) of different substrates instead of the D-glucose was used to determine the substrate specificity. In the same system, sucrose, fructose, maltose, galactose and mannose were used instead of D-glucose as the substrate for GDH reaction, and then the enzyme activity was measured. In order to get kinetic parameter, the test was assayed using the range of D-glucose (0.2 mM-100 mM) as the substrate at the optimal condition. And protein content in GDH was detected by Bradford method (Krzyczmonik et al., 2018).

2.7. Establishment of the enzymatic detection system of D-glucose

In a 96-well plate, 50 μL of different concentration D-glucose (25 mM to 200 mM), 100 μL of Tris-HCl buffer (50 mM, pH 8.5), 50 μL of $K_3[Fe(CN)_6]$ (10 mM), 10μL NAD^+ (5 mM) and 10 μL of GDH (0.05 U μL^{-1}) were added. The mixture was incubated for 10 min at 25°C. Then add 30 μL of $FeCl_3$ (30 mM) to the reacting solution. Then put the 96-well plate on a white platform and place the smart phone

horizontally at a height of about 50 cm from the platform. The standard Android camera APP in the default configuration was used with smart phone (Mi8, China). The photograph were saved as JPEG files and import the original image into Microsoft Photoshop (PS). The CMYJ values of the coloured spots were detected by PS. As comparison, the wells were scanned at full wavelength from 450 nm to 850 nm with a microplate reader (SpectraMax M3, USA).

2.8. System specificity and detection standards

A series of 40 mM sugar (glucose, sucrose, fructose, maltose, galactose and mannose) as substrate was used to determine the value of C in this color reaction system. All experiments were repeated for three times. A series concentration (5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 100 mM and 140 mM) of standard D-glucose was reacted in the system for detection standards, which image was analyzed by PS, and the C value was analyzed to obtain the corresponding D-glucose concentration.

2.9. Detection of concentration of D-glucose in honey

Honey was diluted 20 times with deionized water as the sample to be tested. At the same time, a series of glucose standards (5 mM, 10 mM, 20 mM, 30 mM, 40 mM) were reacted in the same 96-well plate. The 96-well plate was photoed by smartphone and analyzed after reaction. All experiments were repeated for three times. As comparison, the honey was diluted 200 times with deionized water and then analyzed by HPLC equipped with a Bio-rad Aminex HPX-87H column. The mobile phase of HPLC was 0.005 M sulfuric acid at 65°C with a flow rate of 0.6 mL min^{-1} (Chen, Xu, Li, Yu, Cai & Jin, 2018).

3. Results and discussion

3.1. Extraction and purification of GDH

After the purification steps, the GDH solution contains 200 μg mL^{-1} protein and the specific activity of the GDH is 235 units per one milligram protein. The molecular weight of the GDH was about 37 kDa (Fig. 1).

<<<<<Insert Fig. 1 about here>>>>>

3.2. Enzymatic properties of GDH

Under the catalysis of GDH, D-glucose and cofactor NAD^+ produce gluconolactone and NADH. Because NADH has a specific absorption peak at 340 nm, the absorbance of produced NADH was measured with a spectrophotometer to obtain the enzyme activity of GDH.

The effects of pH on the activity of the GDH were analyzed at 25°C in pH buffer range of 7.1 to 10.6. As shown in Fig. 2a, the GDH activity increased with the pH from 7.0 to 8.5, and the activity decreased significantly above pH 9.4. Although the GDH was both 100% relative enzyme activity at pH 8.5 and pH 9.4, the GDH has better tolerance at pH 8.5 than pH 9.4. Therefore, pH 8.5 was chosen for subsequent experiments. The influence of temperature on the GDH activity was investigated over a temperature range from 20°C to 50°C in pH 8.5. By the increase of the temperature, the activity decreased (Fig. 2b). The results mean that the GDH was well adapted to the temperature of natural environment. The optimal temperature of the GDH was 20°C, and the GDH maintained a high activity at 25°C. Since the room temperature always be kept at 25°C, the 25°C was chosen for subsequent experiments so that for the experiments were operability and simplicity.

As shown in Fig. 2c, the GDH revealed the high residual enzyme activity at pH 7.0 and pH 8.5, and the enzyme activity of the GDH was basically unchanged at pH 7.0 and pH 8.5 for 3 hours. Under the condition of pH 9.0, the enzyme activity decreased gradually after 30 minutes, and the enzyme activity was about 80% of the initial activity after 3 hours. Under the condition of pH 10, the GDH activity was lost significant, and only 60% of the enzyme activity remained after 3 hours. The GDH is well tolerated by pH in the environment of pH 7.0 and pH 8.5. As shown in Fig. 2d, the GDH were stable at 4°C, 25°C and 35°C. The results show that the activity of GDH was quite stable during use and storage.

<<<<<Insert Fig. 2 about here>>>>>

3.3. Substrate specificity and kinetic parameter

Among the substrate specificity test, the GDH displayed catalytic activity for D-glucose converted to gluconolactone reply on NAD^+. The GDH didn't show enzyme activity when use other sugars replaced D-glucose in reaction system (Fig. 3), suggested that the GDH is highly specific for D-glucose. The *K*m and *k*cat of the GDH for D-glucose were 32.75 mM and 34.70 s^{-1}, respectively.

<<<<<Insert Fig. 3 about here>>>>>

3.4. Procedure of colorimetric and mobile phone detection

The GDH is used to catalyze D-glucose in the presence of NAD^+ coenzyme, and the produced NADH reduce potassium ferricyanide to potassium ferrocyanide, and then the potassium ferrocyanide react with ferric chloride to produce Prussian blue. The reaction solution became cyan and visible to the naked eye. Therefore, the color of the system can be take a photo with smart phone and read the color C value to quantify concentration of D-glucose. The combination of enzyme-catalyzed reaction, smart phone imaging and color analysis might be possible to provide simple and low-cost method to quantitatively detect the concentration of D-glucose.

Under optimal assay conditions, with the change of D-glucose concentration, the color of the reaction solution gradually turning cyan was seen by eyes (Fig. 4a). It is in accord with the fact that a higher D-glucose concentration results in the generation of more Prussian blue. The absorbance spectra of the reaction solution were also recorded and shown in Fig. 4b. A readily detectable absorption peak intensity at 720 nm could be observed. The absorption peak intensity showed a good linearity with the concentration of D-glucose (Fig. 4c). Meanwhile, this good linear result provides a certain theoretical basis for subsequent detection.

<<<<<Insert Fig. 4 about here>>>>>

The corresponding naked-eye observation of the color difference were shown in

inset of Fig. 5. As the concentration of D-glucose, the color of the solution gradually turns cyan in the 96-well plate. The results clearly demonstrated the feasibility of the colorimetric system. The color image was photographed with a smart phone, and the C value of the color was read by Microsoft Photoshop. Through data processing in Figure 5, the C value became larger as the concentration of D-glucose increases. And within a certain range (5 mM-40 mM), this study found that the C value increases linearity as the D-glucose content in the sample increased. The results mean that the glucose content in the sample can be accurately determined by taking pictures with a smartphone and software processing.

<<<<<Insert Fig. 5 about here>>>>>

3.5. Anti-interference capability of the analytical system

The specificity of the colorimetric system was also investigated by challenging this sensing system against other sugars, for example, sucrose, fructose, maltose, galactose, and mannose. As depicted in Fig. 6, 40 mM D-glucose could cause the solution to be changed to cyan. Other non-targeted analytes (40 mM) could not cause any obvious color change by comparison with the blank test. It can be visually seen from the histogram that the relative enzymatic activity of D-glucose as a substrate is very high compared to other non-targeted analytes. The results indicated that the proposed assay is good enough to selectively detect D-glucose from other control sugars which may coexist in real samples. Such a high selectivity of the system can be attributed to enzyme specificity. However, it is noting that the high concentration non-targeted sugars such as maltose, to some extent, may interfere with the detection result.

<<<<<Insert Fig. 6 about here>>>>>

3.6. Honey analysis

To validate the applicability of the developed sensor in practical sample analysis, experiment compared the D-glucose concentration of the honey sample measured by

HPLC and the system (table. s1). The concentration of D-glucose in honey was 2270 mM by HPLC, and 2169 mM by the detection system. And the relative standard deviation of two methods is 1% and 5% respectively. The result demonstrated that the possible interference from complex sample was almost negligible in the reaction system. Both methods require only a few samples, and the standard deviation is small. But this system does not require expensive HPLC devices. Therefore, the system can be used to test conveniently D-glucose in honey, especially for the analysis in many samples.

4. Conclusion

The experiment successfully extracted and purified GDH with high specificity and high enzyme activity. Because of the specificity of the enzyme, the content of the product can be measured by a spectrophotometer to accurately quantify the D-glucose content to be measured. Further research, a stable and accurate method that relies solely on smartphones was established to conveniently detect D-glucose using this enzyme and the limit of this test system is a sample containing from 5 mM to 40 mM D-glucose. The combination of enzyme-catalyzed reaction, smart phone imaging and color analysis provide simple and low-cost method to quantitatively detect the concentration of D-glucose. In order to verify the accuracy and practicability of this method, the D-glucose content of the honey sample was tested successfully and the experimental error within a reasonable range indicates the repeatability of this detection method. The convenience and rapid of the detection system motivated us to further use the technology for detecting D-glucose in food industry and pharmaceutical industry.

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Figure captions：

Figure 1. SDS-PAGE analysis of purified glucose dehydrogenase. 1. Holoprotein of the *E.coli* BL21(DE3) (pET30a); 2. Holoprotein of the *E.coli* BL21(DE3) (pET30a-GDH); 3. Precipitate of *E.coli* BL21(DE3) (pET30a-GDH) cell disruption; 4. Supernatant of *E.coli* BL21(DE3) (pET30a-GDH) cell disruption; 5. Purified GDH by Ni-changed column; M. Protein Molecular Weight.

Figure 2. a. The optimum pH of GDH. The data were measured at 25°C, the maximum activity was defined as 100% relative activity. The bule line : Tris-HCl buffer (pH 7.1-9.0). The orange line: Gly-NaOH buffer (pH 9.0-10.6). b. The optimum temperatures of GDH. The data were measured at optimum pH. The maximum activity was defined as 100% relative activity. c. The pH stability of GDH in different pH levels. d. The temperatures stability of GDH in different temperatures levels.

Figure 3. Substrate specificity under the different substrates

Figure 4. a. Color change of the reaction system by adding glucose concentrations from 0 to 50 mM. b. UV–vis absorption spectra of the reaction system and different concentrations of glucose. c. Linear calibration plot of absorbance versus glucose concentration. The absorbance spectra were measured at 720 nm.

Figure 5. Plot of the relative activity versus different concentrations of glucose from 5 to 140 mM. Inset: a linear relationship with the value of glucose concentration in the range from 5 to 40 mM, and photograph of the solution against different concentrations of glucose: 0, 5, 10, 20, 30 and 40 mM.

Figure 6. a. Photograph of the reaction solution against different substrate. b. The value of C of the reaction solutions under the addition of 40 mM sucrose, fructose, maltose, galactose, mannose and DI water.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT author statement

Jie Ouyang: Validation, Data Curation, Writing - Review & Editing, Supervision

Shujin Pu: Conceptualization, Formal analysis, Writing - Original Draft

Xing Chen: Software, Visualization

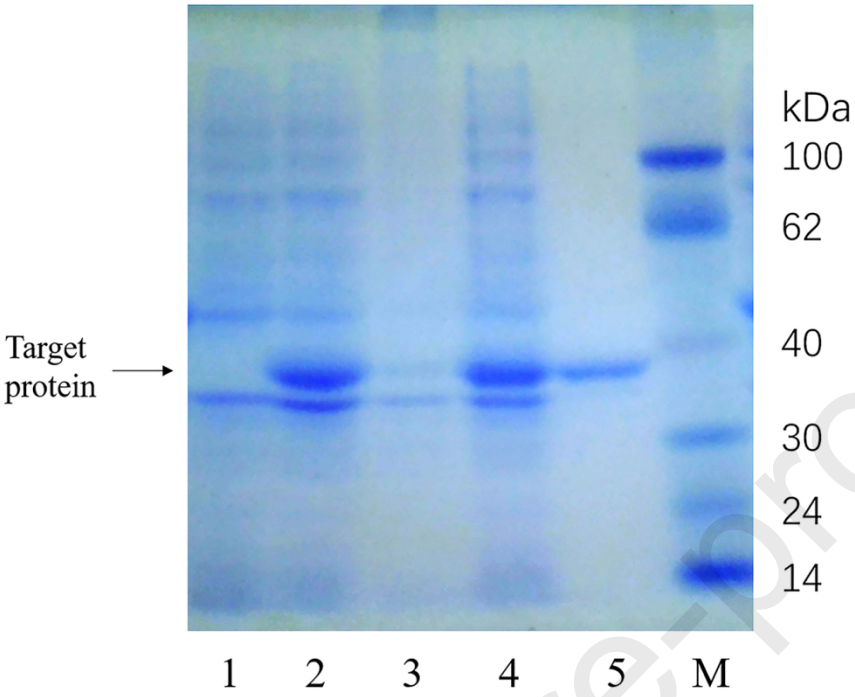
Chengli Yang: Methodology, Resources

Xuan Zhang: Investigation

Dali Li: Project administration

Highlights

- The D-glucose dehydrogenase with His-tag was expressed in *E. coli*.
- The color reaction system the enzyme catalyzed for detection of D-glucose was constructed.
- Smart phone and Microsoft Photoshop was used for analyzing color.
- The detection limit of the biosensor was 25mM to 200mM of D-glucose.



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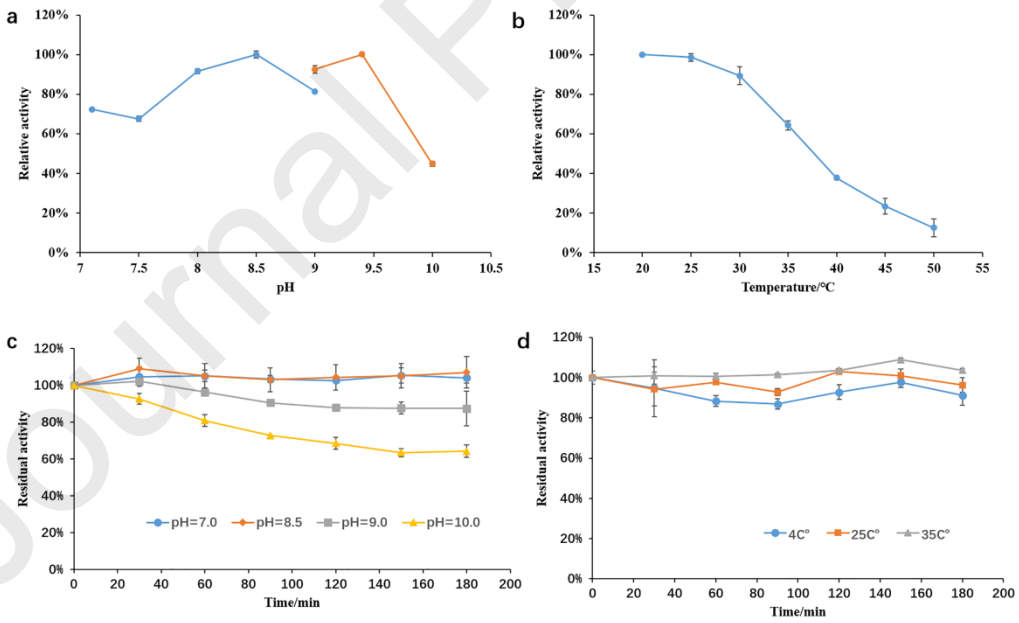

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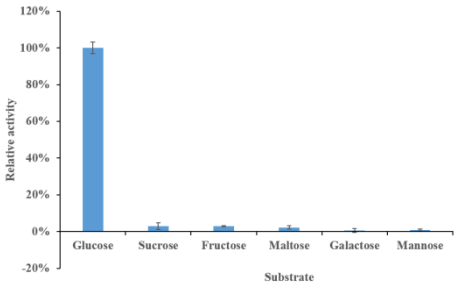

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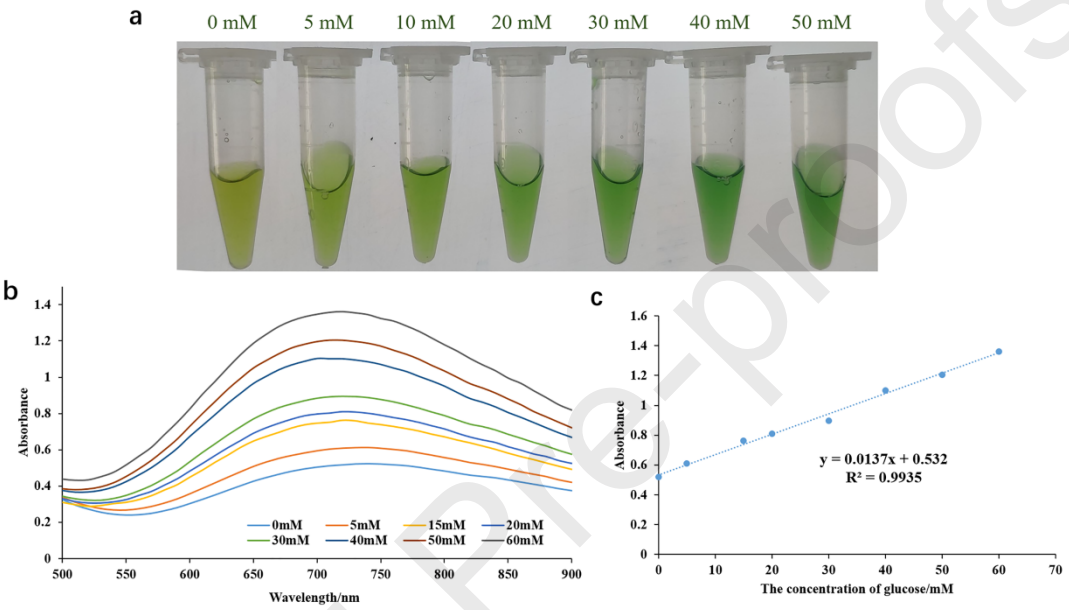

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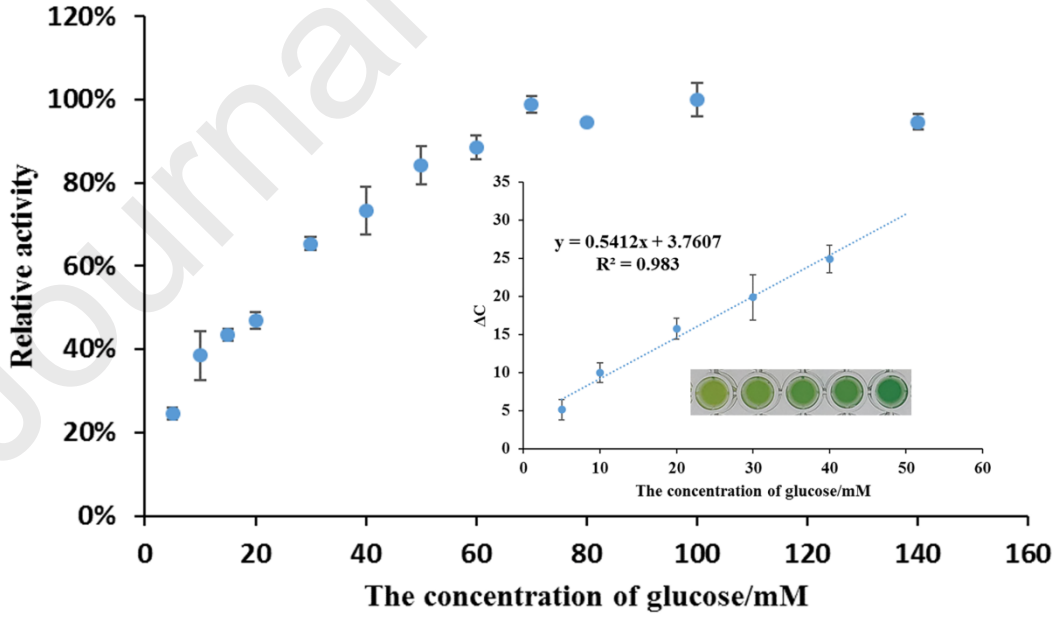

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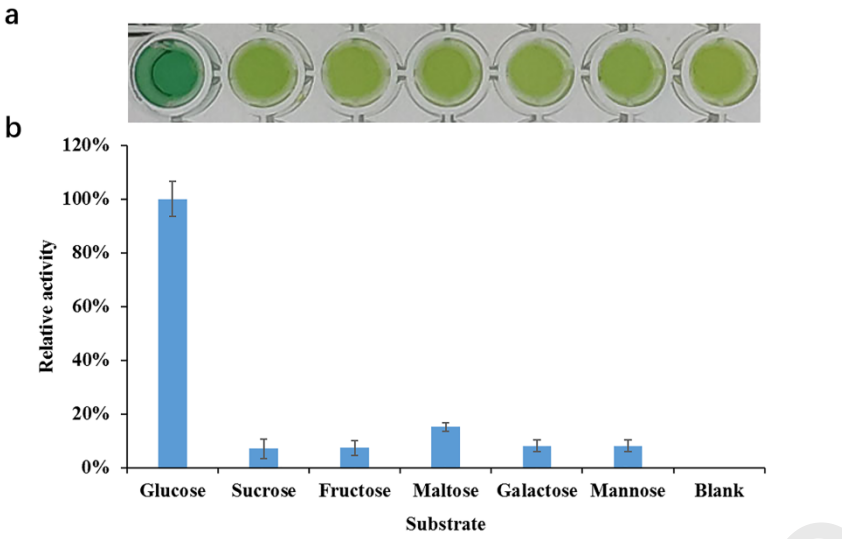

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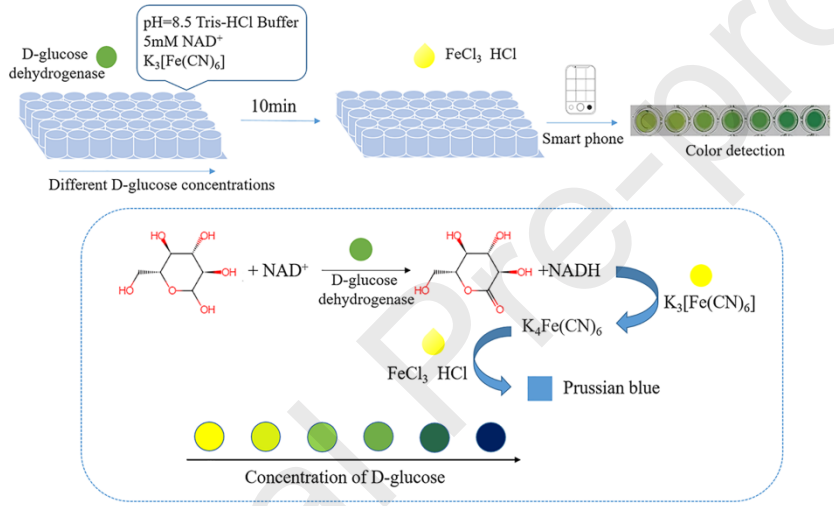

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