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Research article

Efficient production of 6-hydroxynicotinic acid by newly isolated *Pseudomonas poae* ☆

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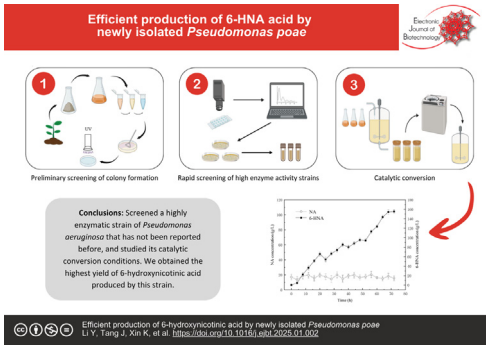
GRAPHICAL ABSTRACT

Efficient production of 6-hydroxynicotinic acid by newly isolated *Pseudomonas poae*



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ABSTRACT

Background: Nicotinic acid dehydrogenase possesses the capability to convert nicotinic acid into 6-hydroxynicotinic acid, a compound of significant research value as a pharmaceutical intermediate. The extraction of nicotinic acid dehydrogenase is primarily performed by strains. However, the enzyme activity of the strains reported currently is relatively low, and their potential to catalyze the production of 6-hydroxynicotinic acid is insufficient to meet industrial requirements.
Results: Due to the revealing properties of 6-hydroxynicotinic acid, this study proposes a technique for calculating the luminescence intensity of colonies, which is based on a fluorescence spectrometer. The developed method establishes a reliable linear relationship (88.2%) between the luminescence intensity and enzyme activity. Consequently, it has been employed to screen strains that produce nicotinate dehydrogenase. This screening approach allows for the evaluation of about 500 enzyme-producing strains daily, presenting an efficient strategy for screening.

☆ Audio abstract available in Supplementary material.

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Conclusions: Through this approach, a novel high enzyme activity strain producing nicotinic acid dehydrogenase, *Pseudomonas poae* have been obtained, which is designated as HD530. After process optimization, it was utilized to produce 6-hydroxynicotinic acid, achieving a high yield of 155.45 g/L within 72 h, meeting the requirements for industrial production. The effectiveness and potential of this technique lie in its application for strain screening and improvement.

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1. Introduction

6-Hydroxynicotinic acid (6-HNA) serves as a valuable pharmaceutical intermediate and chemical precursor. Its significance extends to the production of nitrogen-containing heterocyclic compounds crucial in chemical pesticides. Specifically, it plays a vital role in synthesizing pyridylmethyl amine insecticides like imidacloprid, known for its high efficiency, broad application range, and low toxicity [1,2,3]. In the field of medicine development, utilizing 6-HNA as the reaction substrate enables the creation of 5,6-dichloronicotinic acid, which facilitates lipid breakdown. The lipid-lowering capabilities of 6-HNA make it valuable in the formulation of weight-loss medications [4]. Additionally, in molecular microbiology, 6-HNA can function as a regulator by binding to transcriptional regulators associated with nicotinic acid metabolism, thereby exerting control over the breakdown of nicotinic acid [5]. In the realm of electrochemistry advancement, 6-HNA can be utilized to create modified electrodes, significantly influencing electrical conductivity [6]. Extensive research underscores the critical biochemical significance of 6-HNA. Biosynthesis stands as the primary method for producing 6-HNA, which involves the processing of nicotinic acid with nicotinic acid dehydrogenase. The investigation of nicotinate dehydrogenase relies on the separation and purification of this enzyme from strains involved in nicotinic acid metabolism. The endeavor to strain screening for nicotinic acid dehydrogenase commenced in the 1950s, and the recent outcomes of strain selections are documented in Table 1. However, the process of strain screening that produces nicotinic acid dehydrogenase remains laborious, constraining its widespread industrial applicability. Therefore, it is imperative to establish a sifting process that is quicker, more accurate, and more practical, with the aim of enhancing the effectiveness of the filter.

Most reported biosynthesis methods for 6-hydroxynicotinic acid involve the catalysis of nicotinic acid by nicotinic acid dehydrogenase produced by *Pseudomonas* strains [7]. The production of 6-HNA is directly influenced by the activity of nicotinic dehydrogenase [8]. In previous studies, the selection of strains producing nicotinic acid dehydrogenase was typically first fermented and cultured in a 96-well plate. The culture solution obtained was then dropped onto filter paper, after drying the filter paper, observe its development under ultraviolet irradiation. Subsequently, the selected developing strains were fermented in a 24-well plate to determine enzyme activity [9]. Although this approach allows for screening thousands of wild strains daily, it is affected by low screening accuracy, intricate sample processing, and expensive labor. Therefore, there is a need for a quick, easy, and effective approach to strain selections.

6-HNA is an aromatic hydrocarbon derivative containing multiple chromophores and is capable of absorbing light radiation. Therefore, utilizing a spectrophotometer to quantify the ability of bacterial strains to produce products has become a promising method for screening bacterial strains. In recent years, optical detection technology has found widespread use in the identification and characterization of microorganisms [10]. Optical sensors inducing colony light diffraction have been applied for the development, identification, and characterization of bacteria. These studies present a novel approach to summarizing the properties of bacterial colonies swiftly, precisely, and quantitatively. The commonality of these studies is that the colonies obtain reflectance spectra from their optical sensing devices by selecting light sources for absorption [11]. Strain parameters can be deduced from variations in light intensity, which are transformable into digital data. However, the pivotal question in employing changes in colony light intensity for strain selections is how to accurately quantify the intricate relationship between radiation levels of bacterial strains and their biological activity. Therefore, establishing a relatively accurate quantitative model is fundamental to this new method.

Several strain selection platforms have been developed for various modeling needs [12,13,14,15]. The use of FACS and microfluid devices has been reported to screen strains. For example, fluorescence-activated cell sorting (FACS) can be used to screen for novel mutants of *Fusarium graminis* spores to identify and isolate mutations that have altered DON biosynthesis [16]. In addition, Microfluidic multiple chemotactic devices (MCDs) enable high-throughput chemotactic screening of swimming microorganisms for chemical stimulation under concentration gradient conditions that may accelerate the chemotactic screening process across

Table 1
Reported nicotinic dehydrogenase-producing strains.

Source	Time	Optimum temperature (°C)	Optimum pH	Enzyme Activity (U/mL)	Reference
Pseudomonas fluorescens KB1	1959	25	7.2	–	[19]
Pseudomonas fluorescens TN5	1994	28	7.0	–	[20]
Comamonas testosteroni JA1	2005	30	7.0	0.42	[21]
Pseudomonas putida NA-1	2006	30	7.0	0.58	[22]
Pseudomonas putida BK-1	2007	30	7.0	0.57	[23]
Pseudomonas putida KT2440	2009	30	7.0	0.34	[24]
Pseudomonas putida H9	2017	25	7.0	0.37	[25]
Pseudomonas putida S14	2021	30	7.0	1.11	[7]
Pseudomonas entomophila L48	2009	30	7.0	0.033	[24]
Serratia marcescens IFO12648	1994	28	7.0	–	[20]

the entire sensitivity range of microorganisms [17]. However, fluorescence spectrometers have not been utilized for selection of strains. With the capability to investigate the photoluminescence, chemiluminescence, and bioluminescence of various materials, the fluorescence spectrometer can swiftly and accurately measure the fluorescence of bacterial colonies. It is noteworthy that no research has combined the fluorescence spectrometer and fluorescence intensity of bacterial colonies for strain screening.

In this study, bacteria producing nicotinic acid dehydrogenase were utilized as an example to investigate the fluorescence spectrometer based on the spectral principle in the strain screening process. Nicotinic dehydrogenase, identified as an oxidoreductase, specifically catalyzes the 6-hydroxylation of the pyridine ring of nicotinic acid, resulting in the production of 6-hydroxynicotinic acid [18]. The objective was to establish an efficient selection of strains platform by integrating the detection of colony fluorescence intensity and strain biological activity using a fluorescence spectrometer, a strain with high enzyme activity that has not been reported was screened by this method. In addition, research has been conducted on the catalytic process conditions for utilizing novel bacterial strains to produce 6-HNA. Consequently, this methodology provides valuable insights into the development of strain screening and improvement.

2. Materials and methods

2.1. Strains and culturing conditions

The original strain, identified as *Pseudomonas poae* HD530, was selected in our laboratory and is stored at the China General Microbial Species Preservation Center with CGMCC number 7.524. Before the experiment, the bacterial suspension was retrieved, and single colonies were isolated by cultivating them for 24 h on a neutral pH plate separation medium. The resulting pure strains were then preserved in the laboratory. Fig. 1 presents a comparison between workflows for the conventional screening approach and the method based on the variation in colony fluorescence intensity. The plate separation medium consisted of yeast extract (5 g/L), NaCl (10 g/L), nicotinic acid (5 g/L), and agar (20 g/L). The seed medium consisted of peptone (10 g/L), beef extract (5.0 g/L), NaCl (1 g/L), nicotinic acid (2 g/L) and agar (20 g/L). The fermentation medium was composed of yeast extract paste (10 g/L), corn steep liquor (CSL) (10 g/L), nicotinic acid (10 g/L), KH_2PO_4 (1 g/L), and $K_2HPO_4{\cdot}3H_2O$ (3.93 g/L). All media were at pH 7.0 and sterilized at 121°C for 20 min. The 1% nicotinic acid conversion solution was composed of the following: 100 mL of 20 mM buffer, 1 g of nicotinic acid; 20% nicotinic acid rehydration solution: 100 mL of 20 mM buffer, 20 g of nicotinic acid; the buffer (20 mM): 3.121 g of $NaH_2PO_4{\cdot}2H_2O$, 7.164 g of $Na_2HPO_4{\cdot}12H_2O$, pH of 7.0. All the culture media were incubated at 30°C, 200 rpm.

2.2. Chemicals

Nicotinamide dehydrogenase, with the EC number EC 1.17.1.5. The reagents, including H_3PO_4, $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}12H_2O$, $K_2HPO_4{\cdot}3H_2O$, NaCl, NaOH and KH_2PO_4, were purchased from Tianjin Kermel Chemical Reagent Co., Ltd. Methanol was procured from Tianjin Si you Fine Chemical Co., Ltd. The yeast extract, peptone, and beef extract were acquired from OXOID/REMEL (UK), while the nicotinic acid standard was obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The 6-hydroxynicotinic acid standard was purchased from J&K Scientific Technology Co., Ltd. (Beijing, China).

2.3. Plate colony development

In Kaifeng City, Henan Province, various samples of sludge, rotting fish, rotten fruit, etc., were collected from multiple locations. Fig. 2 depicts the preliminary sample processing procedure. Five g of the sample was added to 50 mL of phosphate buffer solution and an appropriate amount of glass beads. The mixture was incubated at 30°C and 200 rpm for 24 h to ensure homogeneous sample dispersion. The liquid layer was permitted to settle, following which the supernatant was filtered and diluted. Then, 200 μL of the solution was coated on a plate separation medium and incubated for 24 h at 30°C after diluting it 10^{-1}, 10^{-2}, and 10^{-3} times, respectively. Upon colony formation, the plate was subjected to UV light with a wavelength of 365 nm. Under UV light, specific colonies forming nicotinic dehydrogenase indirectly produce vibrant colors.

2.4. Fluorescent spectrometer detects colony light intensity

The fluorescence spectrometer is a sophisticated and modular spectral apparatus designed for monitoring photoluminescence. This investigation efficiently gathered photoluminescence data

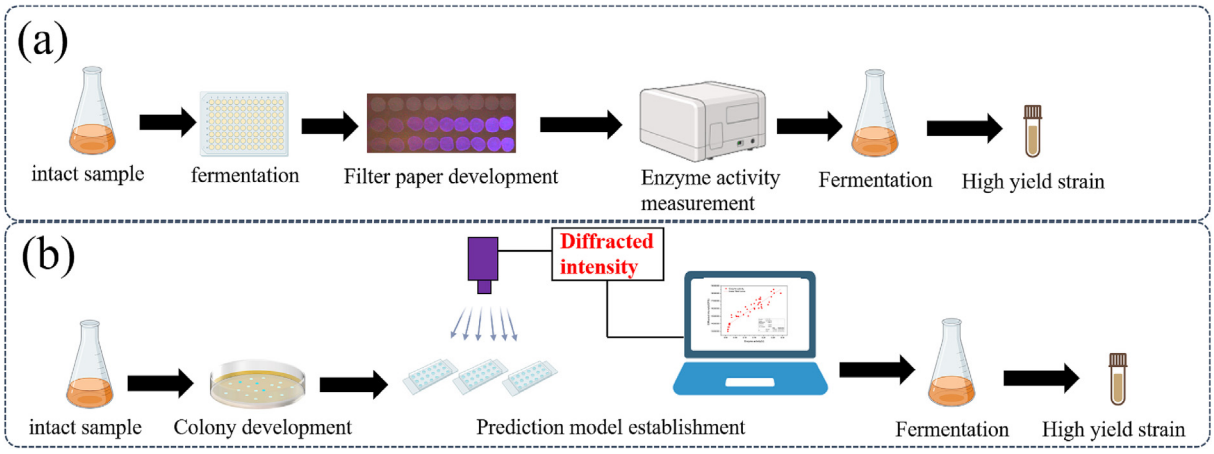

Fig. 1. Comparing methods of sifting based on colony fluorescence intensity with conventional screening techniques. (a) Traditional sifting methods. After culturing the bacterial strains in well plates, the culture liquid is dripped onto filter paper. After the filter paper is dried, observation is conducted for color development. Subsequently, the bacterial liquid showing coloration is transferred back to the well plate for further cultivation, and the enzyme activity of the fermentation broth is measured. (b) Screening based on colony luminescence intensity. After the growth of colony on the agar plate, the specific light intensity of the developed colonies is determined using a spectrophotometer. By combining these data with a proportional relationship model established beforehand, the enzyme activity can be assessed. This method bypasses liquid cultivation, thus enhancing screening efficiency.

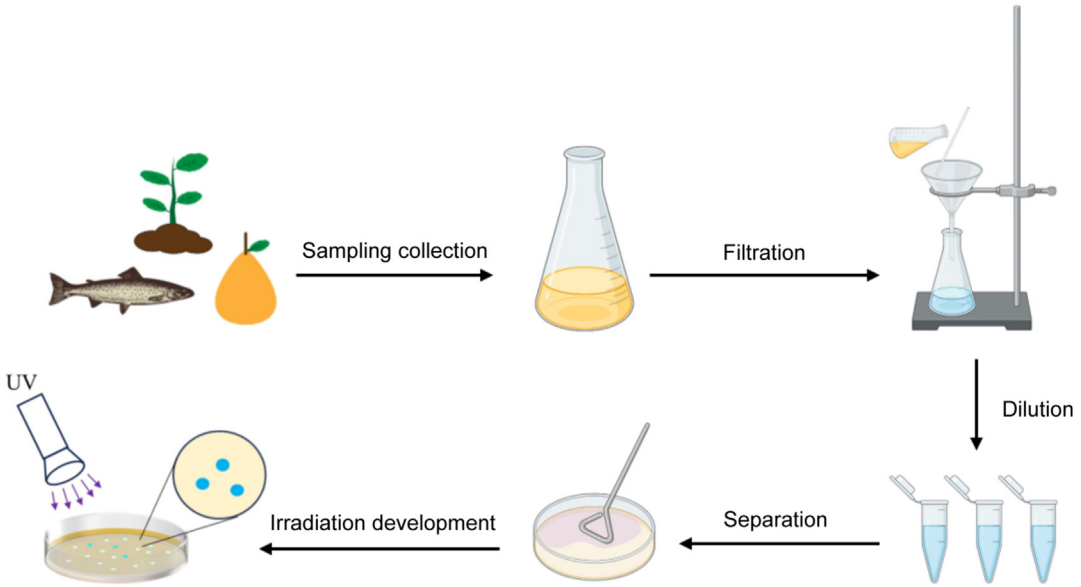

Fig. 2. Screening of nicotinic dehydrogenase-producing strains.

from bacterial colonies using a fluorescence spectrometer (JY HORIBA FluoroLog-3, Horiba Scientific). The strain's light intensity was assessed by measuring the diffraction light's intensity (Count Per Second (CPS)) generated by the developed colonies. The excitation wavelength is precisely set to 360 nm and the emission wavelength to 400 nm to detect luminescent colonies. Following the conversion of photocurrent, the recorder transmitted a digital signal to the computer interface. The Origin 8.5 software program (Origin Lab, USA) was utilized to measure and record the volume of light intensity.

2.5. Establishing an Origin screening model

The Origin software was utilized to develop the sifting model for nicotinic dehydrogenase-producing strains. This model aimed to enhance the sieving efficiency of enzyme-producing strains. The sieving efficiency was defined as the ratio of the number of enzyme-producing strains to the total number of strains. The formula was as follows in **Equation 1**:

$$\eta_s = n_e/n_t \times 100\% \quad (1)$$

where, η_s is the sieving efficiency, n_e is the number of colony-producing enzyme, n_t is the total number of colonies.

2.6. Biocatalytic production of 6-hydroxynicotinic acid

2.6.1. Growth cell-based nicotinic acid catalytic conversion

First, we placed single colonies in 96-well plates with 0.2 mL of seed culture media after separating and purifying them. After 24 h of shaking and culturing at 30°C and 200 rpm, 20 μL of the bacterial suspension was poured into a 24-well plate with 1.2 mL of fermentation media. Both the 96-well plate and the 24-well plate are covered with breathable sealing membranes and lids. All operations are conducted under sterile conditions. The enzyme activity of the fermentation solution was tested when the culture was agitated for 24 h at 30°C and 200 rpm.

2.6.2. Resting cell-based nicotinic acid catalytic conversion

A single colony of the screened nicotinate dehydrogenase-producing bacteria was chosen and inoculated with 50 mL of seed culture medium. It was then incubated for 24 h at 30°C and 200 rpm to obtain the first-level seed liquid. Subsequently, 5% of the inoculum was transferred into 500 mL of seed culture medium and cultivated at 30°C and 200 rpm for 20 h to obtain the secondary seed liquid. Transfer the entire 500 mL seed culture into a 20 L fermenter containing 10 L of fermentation broth. The fermentation culture conditions were as follows: 1 VVM of ventilation per minute, 0.04–0.06 MPa of tank pressure, 30°C of tank temperature, 7.0 pH, and 300 rpm of rotating speed. The fermentation broth was centrifuged in the high-efficiency centrifuge (Beckman Coulter, Avanti J-E, USA) to collect the biomass. Centrifugation was performed at 4°C for 20 minutes at a relative centrifugal force (RCF) of 14,000 × *g*. The cells were washed twice with 500 mL of phosphate buffer (20 mM, pH 7.0). The obtained resting cells were resuspended in a 3L shake flask containing 500 ml of 1% nicotinic acid conversion solution, and the conversion process was conducted at 30°C and 200 rpm. Every 4 h, nicotinic reflux solution was added to keep the substrate concentration at 10–20 g/L. Finally, high-performance liquid chromatography was used to determine the product concentration and record the product conversion rate. The product conversion rate was defined as the ratio of the product yield to the substrate input. The formula was as follows in **Equation 2**:

$$\eta_p = n_p/n_s \times 100\% \quad (2)$$

where, η_p is the product conversion rate, n_p is the product yield, n_s is the substrate input.

2.6.3. Optimizing catalytic conditions

For the optimization of seed culture conditions, prepare 50 mL seed culture solutions in 250 mL Erlenmeyer flasks containing different nitrogen sources, carbon sources, and metal ions. After incubating for 24 h, measure both the biomass and enzyme activity. For the optimization of biocatalytic conditions, the optimization of initial substrate concentration is conducted as per **Method 2.6.2**, the obtained biomass is collected, weighed for wet weight, and divided equally. Subsequently, each portion is added to 50 mL of conversion liquid containing 10–50 g/L of nicotinic acid in 250 mL conical flasks. After 24 h of incubation, the yield of the substrate-determined derived product is measured. The optimization of biomass involved mixing bacterial cultures of different volumes, specifically 10, 20, 30, 40, and 50 mL, with 1 mL of a 20 g/L nicotinic acid conversion solution, followed by the assessment of product yield. For the optimization of pH and temperature, 50 mL of

20 g/L nicotinic acid conversion solution was prepared in 250 mL conical flasks with pH values set at 6.0, 6.5, 7.0, 7.5, and 8.0, and temperatures adjusted to 20, 25, 30, 35, and 40°C. The substrate yield was then measured after 24 h. All cultivation processes were conducted at a constant temperature of 30°C and a shaking speed of 200 rpm.

2.7. Enzyme activity determination method

Three mL of the fermentation broth was centrifuged (Eppendorf, Centrifuge 5254R, Germany) at 4°C for 3 min with a centrifugal force of 17000 × *g*. Remove fermentation supernatant, the collected bacterial cells were washed twice with 1 mL of 20 mM phosphate buffer (pH 7.0). Following that, 1 mL of a 1% nicotinic conversion solution, prepared with 20 mM phosphate buffer (pH 7.0), was added, and the mixture was agitated at 30°C and 200 rpm for 60 min. The conversion solution was centrifuged at 12,000 rpm for 1 min after the reaction, and the supernatant was diluted to a specific fold. The concentration of 6-HNA produced by the enzyme catalytic reaction in the supernatant was determined using a microporous plate enzyme-linked immunosorbent assay; the wavelength is measured to be 295 nm. The enzymatic activity of nicotinamide dehydrogenase was indirectly calculated based on the measured concentration of 6-HNA [9]. One unit of the enzyme activity toward nicotinic acid was defined as the amount of enzyme required to produce 1 μmol of 6-HNA in 1 min. The formula was as follows in **Equation 3**:

$$1U = \left(n \times c \times 10^3\right)/(M \times t) \quad (3)$$

where, n is the dilution factor, c is the mass concentration of 6-hydroxynicotinic acid (g/L), M is the molar mass of 6-hydroxynico-tinic acid, and t is the time of the catalytic reaction (min).

2.8. Strain identification

The screened strains were subjected to PCR amplification of the 16S rRNA sequence using universal primers under the following conditions: Pre-denaturation at 94°C for 5 min, followed by 30 s of cyclic denaturation at 94°C, 30 s of cyclic renaturation at 55°C annealing temperature, 30 s of cyclic extension at 72°C extension temperature, and then fully extended at 72°C for 2 min before storing at 4°C. (Primers F: 5′-CAGAGAGGAGTTTGATCCTGGCT-3′, Primers R: 5′-AGGGGTGATCCCCCGCA-3′). The PCR product was subjected to 16S rRNA full-sequence sequencing of the strain by Sheng Gong Biotechnology Co., Ltd. (Shanghai, China). The 16S rRNA sequences were blasted with reported sequences from NCBI (National Center for Biotechnology Information).

2.9. High-performance liquid chromatography analysis

6-Hydroxy nicotinic acid was analyzed and characterized using HPLC (high-performance liquid chromatography, Agilent LC 1100, USA) on an Agilent 5 HC-C18 (5 μm, 4.6 × 250 mm) at 30°C. The eluent was 20% methanol (pH = 3.0), with an elution time of 20 min, a total flow rate of 0.4 mL/min, and a detection wavelength of 260 nm.

3. Results and discussion

3.1. Establishment of a strain screening platform

6-HNA is a type of aromatic hydrocarbon derivative containing a conjugated structure. The molecule comprises chromophores and multiple auxochromes, which exhibit a strong absorption capacity under specific wavelength ultraviolet irradiation, resulting in a distinct fluorescence bright color. However, without ultraviolet irradiation, it does not display a fluorescent bright color. In this study, the initial sample was deposited onto a flat, solid-growth medium with nicotinic acid as the sole carbon source. The plate was then exposed to UV light. It was observed that some wild strains exhibited a noticeable fluorescent bright color, indicating the presence of the target enzyme-producing strain (Fig. 3a). In previous studies, screening samples were enriched and fermented, and the fermentation droplets were subsequently observed and developed on filter paper. Compared to previous investigations, this study eliminates the need for labor-intensive fermentation liquid culture, allowing for the rapid identification of target enzyme-producing strains. The methodology ensures uniform growth time for each wild colony (24 h), with controlled colony sizes ranging from 1 to 2 mm. By observing the development of colonies under standardized time conditions, a preliminary identification of target enzyme-producing strains is achieved, enabling a rough estimation of enzyme activity. Subsequently, a more precise re-screening of the target strain is conducted. In this study, we introduce a novel approach that circumvents the laborious liquid culture processes commonly employed in conventional methodologies [26].

In accordance with previous research on microbial colonies [27], the variation in fluorescence intensity of the nicotinic acid dehydrogenase-producing strain under ultraviolet irradiation appears to be associated with its enzyme activity. In this study, building upon the earlier plate colony development, we subjected the specific fluorescence intensity of the developed colonies to quantitative analysis. The fluorescence intensity of the developing colonies was measured using a fluorescence spectrometer. The luminous colonies identified during the initial screening were transferred to a small slide of the spectrometer and positioned in a sample cell. The spectrometer furnished a UV excitation light source for the sample. During the absorption of excitation light, the molecule was prompted to the excited electronic state, releasing energy in the form of light. The emitted fluorescence was directed onto the photomultiplier tube. The photocurrent generated by it was amplified and conveyed to the recorder through the amplifier. (Fig. 3b). Simultaneously, the enzyme activity of the developing colony was assessed. In Fig. 3c, a distinct proportional relationship is observed between the fluorescence intensity of the colony and its enzyme activity, with a remarkably high correlation of 88.2%, representing previously unreported findings.

The proportions derived from this relationship were utilized for a subsequent round of strain selections to validate the viability and accuracy of this proportional relationship. A total of 20 developing strain samples were employed to verify this relationship. The correlation between the projected enzyme activity of strains and the enzyme activity determined by the catalytic approach exhibited high consistency (Fig. 3d), the correlation coefficient between the two is 0.95, and the RMSE (Root Mean Square Error) is 0.03. Therefore, the established relationship model between colony fluorescence intensity and enzyme activity in this study can effectively be applied for screening strains producing nicotinic acid dehydrogenase. This innovative approach offers advantages over other widely used methods in addressing practical challenges such as complex samples, non-linearity, excessive labor, and low accuracy commonly encountered in most screening methods.

3.2. Applying the built high throughput screening method

A colony selection model was developed in this study for the production of nicotinic dehydrogenase, establishing a platform for efficient strain screening without requiring extensive personnel and resources. The wild-developing strains screened on the plate were analyzed using a spectrometer to measure their color inten-

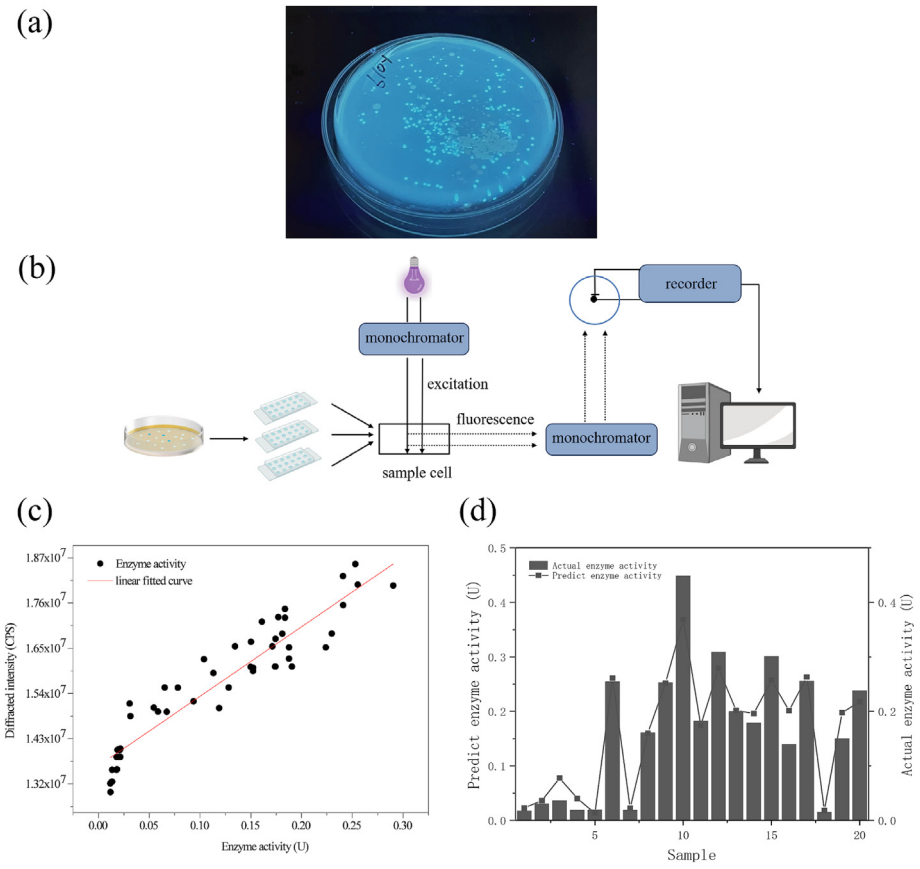

Fig. 3. Establishment process of strain selections model. (a) Plate screening physical map. (b), A scheme of colony fluorescence intensity measurement principle. (c), Establishment of selection model. (d), selection model validation.

sity. The enzyme activity of the strains was rapidly determined using the selection model, enabling the rapid selection of highly active nicotinate dehydrogenase-producing strains. This model allows for the screening of approximately 500 developing strains per day. Table 2 presents a selection of the numerous enzyme-producing strains that were screened using this model. The proposed screening method comprises several key components (Fig. 4).

This model was employed to screen multiple novel strains producing nicotinic dehydrogenase. The identification confirmed their affiliation with *P. putida*, *P. fluorescens*, *Aeromonas salmonicida*, *P. poae*, etc. Notably, strains such as *P. putida* and *P. fluorescens* have been previously reported in studies [25,28,29]. However, this study identified novel strains like *A. salmonicida* and *P. poae* that have not been documented before. Notably, during the screening process (Strain 6 in Table 2), a new strain named *P. poae* HD530 was recognized for its high enzyme activity.

Table 2
Some strains selected by the prediction model. The data are the average values of triplicate measurements with standard deviations.

Strain number	Colony light intensity (CPS)	Enzyme activity (U/ml)	Yield (%)
Strain 1	$(1.73 \pm 0.09) \times 10^7$	0.22 ± 0.01	77.5
Strain 2	$(1.80 \pm 0.09) \times 10^7$	0.26 ± 0.01	82.3
Strain 3	$(1.40 \pm 0.07) \times 10^7$	0.12 ± 0.004	52.6
Strain 4	$(1.63 \pm 0.08) \times 10^7$	0.16 ± 0.007	65.6
Strain 5	$(1.79 \pm 0.09) \times 10^7$	0.25 ± 0.01	81.1
Strain 6	$(1.99 \pm 0.08) \times 10^7$	0.37 ± 0.01	91.2

3.3. Efficient conversion process construction based on screening strains

To investigate 6-HNA production using *P. poae* HD530, the catalytic process conditions were optimized. Initially, the nitrogen source, carbon source, and inorganic salt required for seed growth and enzyme production were optimized (Fig. 5). The optimal conditions were determined by monitoring the biomass and enzyme activity of the strain over a specific time. The aim was to achieve a high concentration and activity of bacteria, maximizing the utilization of the bacterial enzyme system for biocatalysis. Subsequently, the substrate concentration, pH, temperature, and biomass required for the biocatalysis of the strain were optimized. The optimal conditions for the strain to catalyze were determined by monitoring the product conversion rate (Fig. 6).

3.3.1. Effect of different nitrogen sources

For optimizing nitrogen sources, the impact of various inorganic and organic nitrogen sources (equivalent in percentage) was compared with yeast extract in shake flasks. The nitrogen sources included soybean powder, $(NH_4)_2SO_4$, CSL, and beef extract, with each nitrogen source added at a concentration of 10 g/L. As illustrated in Fig. 5a, CSL emerged as the most favorable nitrogen source for the growth of *P. poae* HD530, followed by yeast extract.

Lu et al. [30] reported that inorganic nitrogen sources were not conducive to the growth of bacteria and the formation of enzymes. Beef extract was identified as the most favorable nitrogen source for the formation of hydroxylase, exhibiting the highest enzyme activity per unit volume of fermentation broth. In contrast to the above findings, this study observed that the nicotinic acid dehydro-

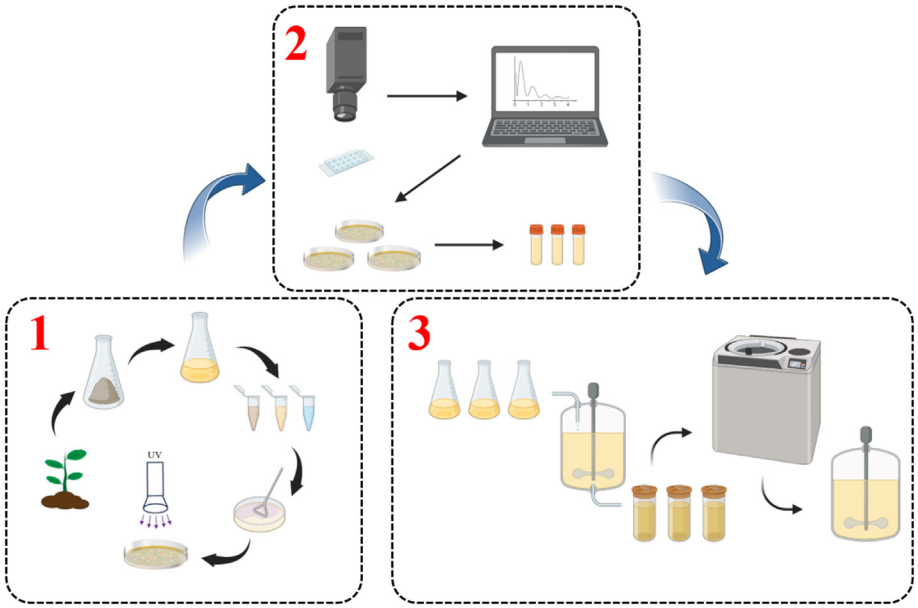

Fig. 4. The workflow of the proposed method is as follows: Step 1: Preliminary screening of colony formation identifies numerous bacteria producing nicotinic dehydrogenase. Step 2: A fluorescence spectrometer promptly measures the brightness intensity of the initially screened strains. The brightness data are then combined with the selection model to rapidly determine the enzyme activity of the strains. Step 3: The resting cell method and process optimization verify the transformation ability of the screened strains.

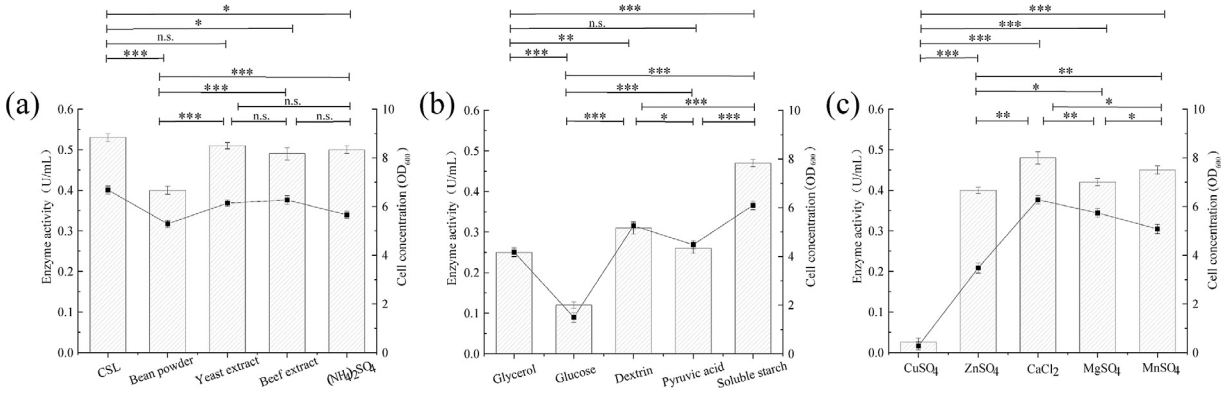

Fig. 5. Effects of different nutrients on the strain HD530. Optimization of (a) nitrogen sources, (b) carbon sources, and (c) metal ions. All experiments were performed in three biological repeats. Values and error bars represent the mean values and standard deviations of biological repeats (* represents $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. = no significance).

genase activity using CSL was higher than that of other tested nitrogen sources, and beef extract and soybean powder were not considered favorable nitrogen sources. Taking into account issues of price and availability, this study selected the most effective and cost-effective CSL as the preferred nitrogen source.

3.3.2. Effect of different carbon sources

The impact of various carbon sources on the production of 6-HNA was compared in shake flasks. Glycerol, glucose, dextrin, pyruvic acid, and soluble starch were chosen as carbon sources in this study, with the nitrogen source added at a concentration of 10 g/L. As depicted in Fig. 5b, different carbon sources exhibit varying effects on cell growth. It is evident that soluble starch was identified as the optimal carbon source for 6-HNA production by *P. poae* HD530.

Shang et al. [7] reported that organic acids such as pyruvate and fumaric acid, when used as fermentation carbon sources, can enhance the increase in nicotinic acid dehydrogenase activity. In contrast to these findings, the nicotinic acid dehydrogenase activity using soluble starch in this study was higher than that of other carbon sources, and pyruvate was not considered a favorable carbon source. Additionally, glucose was found to be the least favorable for the growth of *P. poae* HD530.

3.3.3. Effect of different metal ions

Metal ions exert a certain influence on the catalytic effect in the fermentation process. In this study, zinc sulfate, calcium chloride, copper sulfate, magnesium sulfate, and manganese sulfate were introduced to the medium to investigate their promoting effects on cells, aiming to screen metal ions that enhance the activity of nicotinic dehydrogenase. The concentration of added metal ions was 2 g/L. As illustrated in Fig. 5c, the catalytic effect of metal ions on the strain is suboptimal. It is observed that the nicotinic acid dehydrogenase activity is higher when calcium ions are added compared to other metal ions. Notably, the addition of copper ions results in almost no catalytic activity in strain HD530.

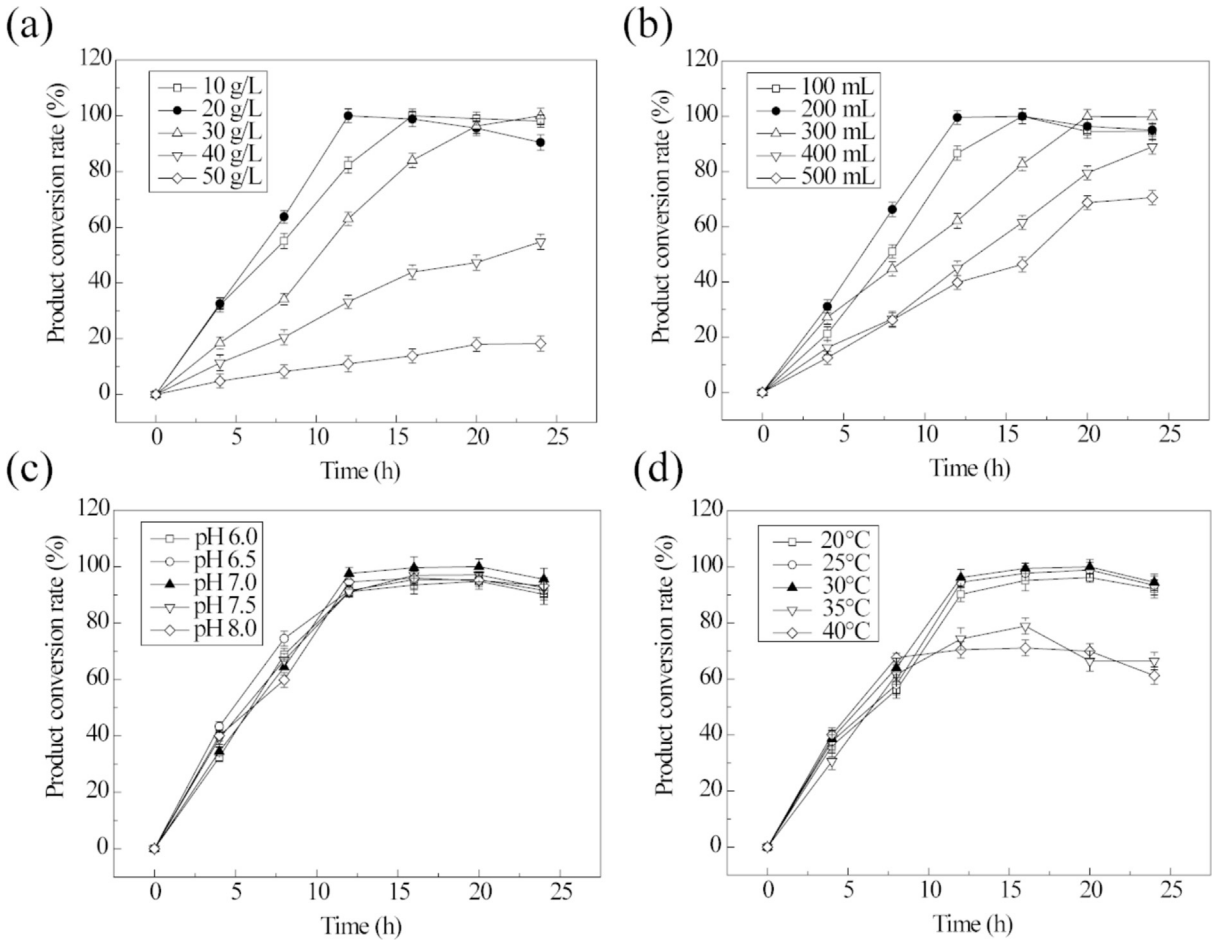

Fig. 6. Optimization of crucial process parameters for screening out strain HD530. Optimization of (a) substrate concentration, (b) bacterial biomass, (c) pH, and (d) temperature. All experiments were performed in three biological repeats. Values and error bars represent the mean values and standard deviations of biological repeats.

3.3.4. Initial substrate concentration optimization

The initial substrate concentration is crucial in biocatalytic processes. If the initial substrate concentration is too low, the reaction cannot produce the best outcomes. However, excessively high initial substrate concentrations can lead to substrate inhibition effects. Previous reports have indicated that an initial high substrate concentration exerts an inhibitory effect on product production [31,32]. Therefore, the catalytic production of 6-HNA by nicotinic acid at different initial concentrations was preliminarily studied. In this investigation, five initial substrate concentrations ranging from 10 to 50 g/L were established for the biocatalytic production of 6-HNA, and the conversion rate of the product was monitored over the same time period (24 h). As depicted in Fig. 6a, it can be observed that when the initial substrate concentration was 20 g/L, complete conversion was achieved within 12 h, with the effect slightly inferior at an initial substrate concentration of 10 g/L. The conversion efficiency significantly decreased when the initial substrate concentration exceeded 20 g/L, and the higher the initial substrate concentration, the less obvious the conversion efficiency.

The above results showed that initial substrate (nicotinic acid) concentration inhibition was a clear limitation to commercial production. Therefore, in order to expand the industrial production of 6-HNA, the method of fed-batch fermentation can be tried.

3.3.5. Bacterial biomass optimization

In the optimization of the initial substrate concentration, the impact of resting cell biomass on the transformation was investigated. Bacterial biomass ranging from 100 to 500 mL was added along with 10 mL of substrate nicotinic acid for the conversion (concentration of bacterial mass 10–50 times). The conversion rate of nicotinic acid varied with bacterial biomass concentrations, as depicted in Fig. 6b. The results indicate that the production intensity at a cell concentration of 10 times was comparable to that at 20 times. However, the product conversion rate at a cell concentration of 20 times was higher and peaked within 12 h. Subsequently, the product conversion rate declined with the increase of cell concentration. When exceeding 30 times, the product could not be completely converted within 24 h. This trend may be attributed to an excessive bacterial concentration, saturating the bacterial biomass and impeding the enzyme activity of the strain.

3.3.6. The pH optimization

The nicotinic acid conversion was conducted at varied pH values to investigate the effect of pH on 6-HNA conversion. Building on the previous optimization of catalytic conditions, five pH concentration gradients of 6.0, 6.5, 7.0, 7.5, and 8.0 were established for optimization experiments. As illustrated in Fig. 6c, the effect of pH on the biocatalytic conversion of the strain was not highly significant. However, it can be observed that at pH 7.0, the biocatalytic conversion of the strain was relatively more conducive. The final conversion of nicotinic acid reached the maximum value of 99% at pH 7.0 (Fig. 6c), established as the optimum condition.

3.3.7. Temperature optimization

Nicotinic acid was subjected to conversion at different temperatures to assess the impact of temperature on 6-HNA conversion. As depicted in Fig. 6d, in this temperature study, five temperature

gradients of 20°C, 25°C, 30°C, 35°C, and 40°C were established for optimization experiments. The effect of temperature on the biocatalytic conversion of the strain was more pronounced than that of pH. It is evident that at a temperature of 30°C, the biocatalytic conversion of the strain was relatively more conducive. Almost 100% nicotinic acid conversion was achieved at 30°C. Thus, 30°C was designated as the optimum temperature.

3.3.8. Producing 6-hydroxynicotinic acid using resting cells

The microbial transformation of foreign substrates utilizing microbial entire cells as reaction catalysts is known as the resting cell transformation technique. Its benefits include low cost, minimal byproducts, excellent selectivity, pollution-free operation, and gentle reaction conditions. The primary method for producing 6-hydroxynicotinic acid is using resting cells [33,34]. In recent years, the industrial production of 6-hydroxynicotinic acid has primarily involved the catalytic conversion of nicotinic acid by the free cells of *P. s putida* [35,36]. There have been no instances of *P. poae* producing 6-hydroxynicotinic acid in recent years. Fig. 7 depicts the feed batch catalytic conversion of *P. poae* HD530 with the nicotinic acid concentration maintained at an appropriate level (10–20 g/L) during the catalytic process. Strain HD530 cells were collected based on previous optimization conditions, and 500 mL of substrate nicotinic acid conversion solution was added. The catalytic conversion was carried out in a reactor, resulting in the highest yield of 6-hydroxynicotinic acid, reaching 155.45 g/L within 72h (Fig. 7). Table 3 provides a list of the yield of 6-HNA reported in different studies in recent years, with most involving *P. putida*. The *P. poae* HD530 used in this study is a new strain suitable for the industrial production of 6-HNA. This accomplishment has expanded the avenues for the industrial production of 6-hydroxynicotinic acid.

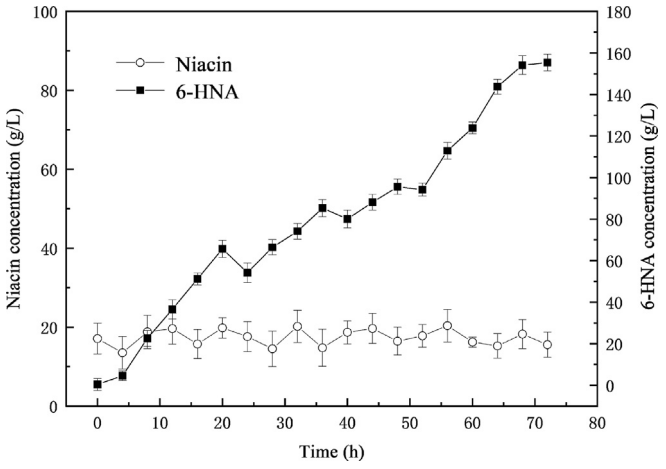

Fig. 7. Production of 6-HNA via resting cell method. All experiments were performed in three biological repeats. Values and error bars represent the mean values and standard deviations of biological repeats.

Table 3
Yield of 6-HNA reported in different reports.

Strain	Time (h)	6-HNA yield (g/L)	Year	References
Pseudomonas poae HD530	72	155.5	2024	–
Pseudomonas putida S14	30	176	2021	[7]
Pseudomonas putida H9	48	124.77	2017	[25]
Pseudomonas putida NA-1	36	108.39	2005	[30]

4. Conclusions

This study introduced a novel strain screening method based on a fluorescence spectrometer. Through spectral analysis tools, this method parameterized the fluorescence brightness of colonies, analyzed the photoluminescence phenomenon of colonies, measured the specific luminescence values of various colonies, and expanded the research avenues for strain selections. In this context, we validated the correlation between the fluorescence brightness of nicotinic acid dehydrogenase-producing colonies and their enzyme activity, confirming its accuracy and applicability. These benefits position this newly developed approach as ideal for application in other similar strain selection research. Through the above method, we screened a highly enzymatic strain of *Pseudomonas poae* that has not been reported before, and studied its catalytic conversion conditions. We obtained the highest yield of 6-hydroxynicotinic acid produced by this strain, providing insights and ideas for subsequent related research.

CRediT authorship contribution statement

Yi Li: Writing – review & editing, Writing – original draft. **Jiacheng Tang:** Visualization, Validation. **Kaixiang Xin:** Software. **Zongda Chen:** Resources, Project administration. **Lele Zhao:** Supervision, Software. **Yifan Zhao:** Investigation. **Yinbiao Xu:** Data curation. **Pei Zhou:** Methodology, Investigation. **Yang Sun:** Funding acquisition, Formal analysis. **Yupeng Liu:** Resources, Project administration. **Hua Li:** Data curation, Conceptualization.

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Declaration of competing interests

The authors declare no competing interests.

Supplementary material

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Data availability

Data will be made available on request.

Preprint statement

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