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Original Article

On-line near-infrared spectroscopy optimizing and monitoring biotransformation process of γ-aminobutyric acid ☆

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ABSTRACT

Near-infrared spectroscopy (NIRS) with its fast and nondestructive advantages can be qualified for the real-time quantitative analysis. This paper demonstrates that NIRS combined with partial least squares (PLS) regression can be used as a rapid analytical method to simultaneously quantify L-glutamic acid (L-Glu) and γ-aminobutyric acid (GABA) in a biotransformation process and to guide the optimization of production conditions when the merits of NIRS are combined with response surface methodology. The high performance liquid chromatography (HPLC) reference analysis was performed by the *o*-phthaldialdehyde pre-column derivatization. NIRS measurements of two batches of 141 samples were firstly analyzed by PLS with several spectral pre-processing methods. Compared with those of the HPLC reference analysis, the resulting determination coefficients (R^2), root mean square error of prediction (*RMSEP*) and residual predictive deviation (*RPD*) of the external validation for the L-Glu concentration were 99.5%, 1.62 g/L, and 11.3, respectively. For the GABA concentration, R^2, *RMSEP*, and *RPD* were 99.8%, 4.00 g/L, and 16.4, respectively. This NIRS model was then used to optimize the biotransformation process through a Box-Behnken experimental design. Under the optimal conditions without pH adjustment, 200 g/L L-Glu could be catalyzed by 7148 U/L glutamate decarboxylase (GAD) to GABA, reaching 99% conversion at the fifth hour. NIRS analysis provided timely information on the conversion from L-Glu to GABA. The results suggest that the NIRS model can not only be used for the routine profiling of enzymatic conversion, providing a simple and effective method of monitoring the biotransformation process of GABA, but also be considered to be an optimal tool to guide the optimization of production conditions.

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1. Introduction

γ-aminobutyric acid (GABA) is an important non-protein component amino acid that is applied in various fields such as food [1], medical healthcare [2], and chemical engineering [3]. GABA has received growing attention in the medical healthcare field due to its inhibitory effects on central nervous system, including sedation, anti-depression, anti-insomnia, anti-hypertensive and diuretic effects [4–6]. It can be produced by several methods such as plant tissue enrichment [7], microbial fermentation [8] and biotransformation [9], and can be transformed by *Escherichia coli* (*E. coli*), genetically engineered strain expressing high levels of glutamate decarboxylase (GAD, EC4.1.1.15). By the enzymatic synthesis strategy (Fig. 1), the production of GABA has higher concentration and purity. Although the amino acid enzymatic conversion strategy is easy to operate, it is not possible to ensure that the enzymatic activity remains constant, as strain passage and culture conditions can lead to some differences. If the enzymatic activity is too high, it will be a waste of the enzyme using the same biomass. When the enzymatic activity is too low, the transformation rate will decrease, which decreases the product purity and production efficiency due to the unclear endpoint of the biotransformation process [10]. Therefore, a proper process-monitoring method is beneficial for the programmed production of GABA.

Processional analytical technology (PAT) is defined as “systems for continuous analysis and control of manufacturing processes based on real-time, or rapid measurements during processing, of quality and performance attributes of raw and in-process materials and processes to ensure end product quality at completion of the process” by the United States Food and Drug Administration (USFDA). To ensure product quality, FDA suggests that the introduction of PAT can provide quality control during processing,

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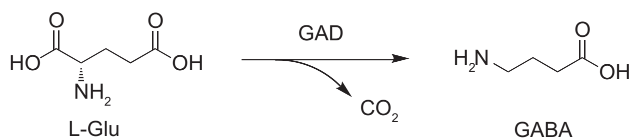

Fig. 1. Reaction equation of biotransformation from L-Glu to GABA.

including control over raw and in-process materials and processes to ensure end product quality at the end of the process. In particular, near-infrared spectroscopy (NIRS) is an advanced analytical technology that has recently been highly developed in the PAT field [11–13]. Because NIRS has many merits such as simple operation (no sample pretreatment), rapid measurement, no pollution, providing a large amount of information (physical and chemical properties can be determined with simple one time scanning and without damaging samples) [14]. Due to these advantages, NIRS has been introduced to the pharmaceutical, food, chemical engineering, textile, petroleum, and vintage industries [15–21]. For example, NIRS was investigated as a PAT to monitor amino acid concentration profiles during the hydrolysis of Cornu Bubali [22]. In this research, a NIRS model was developed using interval partial least squares and synergy interval partial least squares to monitor 11 different amino acids. A new parameter desirability index and multivariate quantification limit (MQL) values were used to evaluate the NIRS model. Excellent accuracies and low MQL values were obtained for L-proline, L-tyrosine, L-valine, L-phenylalanine and L-lysine. The results confirmed that these models are suitable to improve hydrolysis efficiency, and enabled a reduction in hydrolysis time, which directly affects process productivity.

Almost all types of compounds and mixtures can be quantified by NIRS with the chemometrics method. In this study, the optimization of the GABA enzymatic synthesis and monitoring of the biotransformation process both required multiple points testing and rapid results feedback. NIRS with its fast and nondestructive advantages will be qualified for the real-time quantitative analysis. It can provide guidance to determine whether the production process of GABA biotransformation is performing consistently with expectations, and rapid analysis at the end of the biological reaction. In this study, multivariate models were developed to quantify L-Glu and GABA levels that had previously been determined by pre-column derivatization high performance liquid chromatography (HPLC), and then partial least squares (PLS) regression prediction models with NIRS were constructed. After evaluating the stability, a new batch of samples was used for external validation to monitor biotransformation. Then, a Box-Behnken experimental design was used to optimize the process parameters using the NIRS model. Finally, the best process conditions were used for the GABA biotransformation process with on-line NIRS monitoring.

2. Materials and methods

2.1. Instruments

Analysis was carried out on an Agilent–1200 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump (G1311C), on-line degasser (G1322A), autosampler (G1229A), thermo column compartment (G1316A) and photo-diode-detector (G4212A). Near-infrared diffuse reflectance spectra were acquired using a Bruker TENSOR 37 FT-NIR spectrometer (Bruker Optik, Ettlingen, Germany) with an InGaAs detector and an Integrating Sphere Module over the wavenumber range of 12,000–4000 cm^{-1}.

The biotransformation process was carried out in a 15 L glass tank bioreactor (Applikon Biotechnology, Delft, the Netherlands). The GAD genetically engineered super producer *E. coli* strain was cultivated in a 150 L fermenter (Biotech–2002 Bioprocess Controller, Baoxing, Shanghai, China).

2.2. Chemicals and materials

HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Deionized water was prepared by a Milli-Q water system (Millipore, Bedford, MA, USA) to prepare samples and the mobile phase. Other reagents were of analytical grade, and were purchased from Concord Technology (Tianjin, China). All solvents were filtered through 0.22 µm membrane filters before analysis.

The reference standards (GABA and L-Glu) and the coenzyme pyridoxal 5′-phosphate (PLP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The purities of all of the standards were not less than 98%. The L-Glu, yeast extract and tryptone used in fermentation and biotransformation were purchased from Teda Letai Chemical Co., Ltd (Tianjin, China).

2.3. Cultivation of genetically engineered strain

The GAD high-expression strain of *E. coli* BL21 (NK-GAD402) was obtained from earlier work in our laboratory. The *E. coli* strain (NK-GAD402) was grown at 37 °C in a fermenter with 100 L of nutrient medium with the following composition: 5.0 g/L of yeast extract, 10 g/L of NaCl, 10 g/L of tryptone and up to 65 L of tap water (pH 7.2). The mixer rotation speed was 300 rpm, and the flow rate of sterile air was 15 L/min. When the optical density of the culture medium reached 1.0 at 600 nm, approximately 5 L of a 200 g/L lactose solution was fed into the fermentation broth. After 4 h of lactose induction, approximately 500 g of the GAD high-expression strain was obtained through centrifugation (3000 rpm). The obtained biomass was redissolved in water to obtain a concentration of 200 g/L and frozen at −20 °C.

The enzymatic activity of GAD was expressed in U/g cells (wet weight) and determined according to a previously developed method with slight modification [23]. One unit (U) of GABA-forming activity was defined as the amount of enzyme that liberates 1 µM GABA per minute in the following activity assay mixture. To start the reaction, 5 mL of 10 mg/mL biomass (wet weight) was added into the reaction mixture containing 35 g/L sodium glutamate, 200 mM Macilvaine buffer and 0.02 mM PLP at pH 4.35. After incubation at 37 °C for 30 min, the reaction was terminated by dilution in boric acid buffer (0.4 M, pH 10.2). The supernatant was obtained by centrifugation at 12,000 rpm for 10 min at room temperature and was then subjected to HPLC analysis.

2.4. Biotransformation of L-glutamic acid

Improving the cell wall permeability can increase the chance of substrate access to intracellular enzymes, increasing the conversion efficiency. To undergo thermal activation, the strain NK-GAD402 biomass was stored in a −20 °C freezer and then pre-incubated in a 37 °C water bath before use. L-Glu (2 kg) and PLP (50 mg) were then added into 10 L of water. Initially, 1200 U/L GAD high-expression strain (NK-GAD402) and 1 mL organic foam suppressor were added to the reaction environment. One hour later, fed-batch processing began at 660 U/L/h biomass, lasting for 8 h. The first sample was collected after 20 min. From that time on, samples were obtained every 10 min up to 12 h from the beginning of reaction. A total of 141 samples were obtained from two fermentation trials. The first batch of samples was used for

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