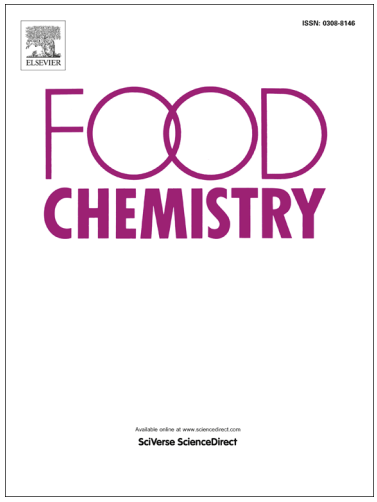

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An electricalchemical method to detect the branch-chain aminotransferases activity in lactic acid bacteria

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Abstract

In this study, an electrochemical system was established to detect the branched-chain amino acid aminotransferase (BCAT) activity in lactic acid bacteria (LAB). A nanocomposite of chitosan (CS) with multi-walled carbon nanotubes (MWCNTs) was synthesized, and the composite solution were uniformly spread over the glassy carbon electrode (GCE) surface by drop-casting to fabricate an electrochemical biosensor. The composite was characterized by scanning electron microscopy (SEM) and cyclic voltammetry (TEM). Results indicated that the MWCNTs–CS/GCE electrode exhibited higher stability and sensitivity, compared with the GCE electrode. The linear response for nicotinamide adenine dinucleotide (NADH) was 1.0–9.0 μM and the response limit was 0.12 μM. The system effectively and sensitively detected the BCAT activity by NADH concentration in the LAB culture, comparing with the optical method. The culture condition of LAB was optimized by using this system, evidencing that established method was available to detect the BCAT activity of LAB.

Key words:

Electrochemical detection system; *Lactobacillus casei 1580*; Branch-chain aminotransferases; Nicotinamide adenine dinucleotide

1. Introduction

The flavor compounds of fermented meat products are mainly derived from proteolysis, lipolysis, glycolysis, or secondary metabolites. Owing to the relatively high protein content in fermented meat products (Marušić, Aristoy, & Toldrá, 2013), proteins are transformed by microorganisms into polypeptides and further converted into various amino acids. Amino acids are identified as precursors to numerous volatile compounds including ketones, aldehydes, alcohols, acids, esters, olefins and alkanes during fermentation (Latorre-Moratalla, Bover-Cid, Bosch-Fusté, Veciana-Nogués, & Vidal-Carou, 2014). Flavor compounds derived from transamination of branch-chain amino acids (BCAAs, including leucine, isoleucine and valine) have drawn attention because of the complexity of types, high abundance, and low flavor threshold, highlighting the importance of their role in the development of fermented meat flavor (Schmidt & Berger, 1998).

In high-quality meat products, branched aldehydes were the most abundant flavor compounds, including 3-methylbutanal which was produced by transamination of leucine and further decarboxylation of α-ketoisocaproic acid (Barbieri, *et al*., 1994; Whetstine, Drake, Broadbent, & Mcmahon, 2006). The concentration of 3-methylbutanal was considered as the standard in both qualification and quantification of flavor and its production of flavor phenotypes because of its low threshold as well as malty and chocolate-like flavor (Afzal, Delaunay, Paris, Borges, Revol-Junelles, & Cailliez-Grimal, 2012; Larrouture, Ardaillon, Pepin, & Montel, 2000). In meat and meat product, 3-methylbutanal is also considered as a key flavor

compound. Therefore, it is critical to enhance the ratio of the branch-chain amino acids-derived volatile compound which makes a great contribution to the flavor of fermented meat during LAB fermentation.

BCAAs catabolism during fermented meat ripening is mainly initiated by the action of microbial aminotransferases. Many microorganisms including lactic acid bacteria (LAB) were involved in the conversion of leucine to 3-methylbutanal (Afzal, Ariceaga, Boulahya, Jacquot, Delaunay, & Cailliez-Grimal, 2016; Afzal, Delaunay, Paris, Borges, Revol-Junelles, & Cailliez-Grimal, 2012; Afzal, *et al*., 2010). Transamination is the only enzymatic system involved in the first step of amino acid degradation in LAB, accompanied by the production of nicotinamide adenine dinucleotide (NADH). In leucine metabolism, NADH is formed during the production of α-ketoisocaproate acid and isovaleryl CoA as well as 3-methylbutyric acid derived from 3-methylbutanal. If the downstream reactions of leucine metabolism are thought as NADH formation, aminotransferase activity is represented by the concentration of NADH. Therefore, the detection of NADH can represent the activity of branched-chain amino acid transaminase (BCAT) in LAB.

The conventional methods of detecting aminotransferase activity include colorimetric analysis, spectrophotometric measurement and chromatography. The BCAT activity was correlated with the concentration of final products (such as pyruvic acid; α-keto isocaproic acid) that were measured by absorbance spectral detection or chromatography. The principle of colorimetric analysis is based on the interaction between an enzyme and substrate to form and estimate colorimetrically a

colored, light-absorbing complex by adding other reagent after stopping the enzyme reaction. However, the optical method such as IFCC (International Federation of Clinical Chemistry and laboratory medicine) in detecting BCAT activity still needs some improvements, including the increase of detection accuracy and decrease of multi-step "batch" processes (Han, Song, Lee, Lee, & Yoon, 2011; Plaxco & H Tom, 2011; Plaxco & Soh, 2011). In spectrophotometric measurement, instrumentations including spectrophotometer and 3-T Signa MR scanner are required and thus not suitable for point-of-care applications. Nowadays, much more attentions have been focused on electrochemical techniques in detecting aminotransferase activity. Many biosensing studies have been reported on the electrochemical detection of NADH because of the irreversible oxidation of NADH at bare electrode substrates, which occurs at considerable overpotentials with concomitant passivation of the electrode surface and renders this step rate-limiting for the overall electrooxidation reaction (Deng, Zhou, & Li, 2013; Ge, Tan, Xie, Ma, & Yao, 2009; Gobal & Faraji, 2015; Harima, *et al*., 2011; Shin, *et al*., 2010). Notably, MWCNTs can exhibit high electrical conductivity and chemical stability (Kumar & Chen, 2008). Owing to its available reactive hydroxyl and amino functional groups, chitosan (CS) is an excellent biomaterial with desirable properties, including good film-forming ability, good adhesion, high permeability toward water, and susceptibility to chemical modifications (Maogen Zhang, Audrey Smith, & Waldemar Gorski, 2004). It is promising to develop an electrochemical method for rapid and real-time detection of BCAT activity.

Thus, the objective of this study was to establish an electricalchemical system to detect the branch-chain aminotransferases activity in LAB, and optimize the culture conditions for *Lactobacillus casei* to increase BCAT activity by using this electrochemical detection system.

2. Experimental procedure

2.1 Materials and apparatus

Chitosan (CS) from crab shells (90% deacetylated) was purchased from Sinopharm Chemicals Co., Ltd. (Shanghai, China); Multi-walled carbon nanotube (MWCNTs) (20-40 nm diameter) was obtained from Xian Feng Nanomaterials Technology Co., Ltd. (Nanjing, China).

Leucine, pyridoxal-5'-phosphate (PLP), α-keto isocaproic acid, and lysozyme were purchased from Sangon Biotech (Shanghai, China); NADH and NAD^+ was purchased from J&K (Beijing, China). All solutions were prepared with deionized water, and all reagents were of analytical grade.

All electrochemical experiments were performed on a CHI660A electrochemical workstation (Chenhua Technology Co., Ltd., Shanghai, China) with a conventional three-electrode system in a solution containing 1.0 mM of $K_3Fe(CN)_6$ or in 0.1 M PBS solution (pH 7.0). The structure of nanomaterials was characterized using a transmission electron microscope (Tecnai 12, Philips, Netherlands) and a scanning electron microscope (S-4800 II, Hitachi, Japan).

2.2 Preparation of cell suspensions and cell-free extracts

L. casei 1580 strains were incubated for 18 h at 37°C, repeated incubation twice, harvested by centrifugation (6000 rpm, 10 min, 4°C), and adjusted to 0.2 OD_{600} in PBS solution (0.1M, pH 7.4). Aliquots of cell suspensions were stored at -80°C. The reaction mixture contained 5 mL PBS (pH 7.4), 10 mM leucine, 0.1 mM pyridoxal-5'-phosphate, 15 mM α-ketoglutaric acid (α-KG), and 0.25 mM NAD^+ at 4°C. Subsequently, cells amounting to 5 mL were incubated with a reaction mixture for 5 h at 37°C and then incubated with a 10 mM lysozyme for 3 h at 37°C. After the removal of the cell debris by centrifugation (6000 rpm, 5 min, 4°C), the resulting cell-free extracts were obtained. The concentrations of Leu, PLP, α-KG, and NAD^+ are provided in Supplementary Material Fig. S2.

2.3 Design and fabrication of electrochemica sensor

Preparation of MWCNTs/CS composite. A 0.5% CS solution (w/t) was prepared using 0.5 g CS dispersed in acetic acid (100 mL, 0.10 M HAc) by ultrasound. It was then stored at 4°C before being used. Subsequently, 0.5 mL of MWCNTs colloidal solution was added to 9.5 mL of chitosan solution and sonicated for 15 min, resulting in a MWCNTs/CS nanocomposite solution. Subsequently, 7 μL of MWCNTs/CS nanocomposite solution was uniformly dropped onto the surface of glassy carbon electrode (GCE) and then air-dried. The concentrations of MWCNTs and CS are provided in Supplementary Material Fig. S1.

2.4 Electrochemical assay

Electrochemical experiments for enzyme assays were conducted on a CHI660E electrochemical workstation with a conventional three-electrode system comprised of

a platinum wire as an auxiliary (CE), a Ag/AgCl electrode as a reference (RE), and a modified MWCNTs-CS/GCE as the working electrode (WE).

Firstly, cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) signals were recorded in the electrolyte containing 1.0 mM of $K_3Fe(CN)_6$. The CV was scanned within a potential range from - 0.2 V to + 0.6 V with a scan rate of 50 mV/s. The DPV was recorded in a potential range between -0.2 V and + 0.6 V with a pulse amplitude of 0.05 V, a pulse width of 0.05 s and a pulse period of 0.2 s. Then, CV measurements were monitored for NADH concentration, which were carried out with 10 mL of 0.1 M PBS buffer (pH=7.0) containing different concentrations of NADH.

2.5 Electrochemical determination of BCAT activity

The MWCNTs-CS/GCE was placed into the supernatants obtained by centrifugation, and the amount of NADH representing BCAT activity was detected. The detection of BCAT activity in LAB by electrochemical detection system was defined as the amount of enzyme required to produce one μmol of NADH per hour as one enzyme activity unit (1 U/L = 1 μmol NADH/h). The culture condition of *L. casei* including pH, NaCl concentration, culture temperature, the inoculum size of strains was optimized to most BCAT activity by using this electrochemical method. The pH of MRS medium was adjusted by 10 mM phosphate buffer to 6.0, 6.5, 7.0, 7.5 and 8.0. The incubation temperature was set as 27, 32, 37, 42, 47°C. The salt concentration (%, m/v) of MRS medium was adjusted by adding NaCl to 0, 2.5%, 5.0%, 7.5%, and

10.0%. The inoculation volume of *L. casei* over MRS medium was designed as 5%, 15%, 25%, 35%, 45% (v/v).

2.6 Optical determination of BCAT activity

Determination of pyruvic acid using 2,4-dinitrophenylhydrazine colorimetric method (Johnson & Scholes, 1954). Quantitative determination of cell-free extracts at 520 nm using a visible spectrophotometer. Detection of BCAT activity by using the optical method was defined as the amount of enzyme required to convert one μmol of pyruvic acid per hour as one enzyme activity unit (1 U/L = 1 μmol pyruvic acid/h).

2.7 Myofibrillar protein hydrolysis

Porcine longissimus thoracis (LT) muscles were obtained from the carcasses within 45 min post-slaughter from a commercial slaughter plant (Sushi Meat Co. Ltd, Huai'an, China). The muscle was washed 5 times with sterile physiological saline and cut into pieces on an aseptic table. The muscle of 50 g was homogenized twice with 250 ml extraction buffer (0.02 M phosphate buffer, pH 6.5) for 2 min at 5,000 rpm. The homogenate was then centrifuged at 8,000 g for 20 min for 4°C and supernatant was discarded. The pellet was washed 3 times with 4 volume of 0.03 M phosphate buffer pH and 0.1% (v/v) Triton X-100. After the centrifugation, the pellet was obtained and mixed with phosphate buffer (0.1 M, pH 6.5, containing 0.7 M KI and 0.02% (v/v) NaN_3). The homogenate was centrifuged at 8,000 g for 20 min at 4°C and the supernatant was taken and dialyzed with ultrapure water for 24 h. The myofibrillar proteins were mixed with *L. casei 1580* strains of 10^7 CFU/mL at the following conditions: optimized culture condition (OC group, pH 7.0, 37°C, NaCl concentration

of 2.8%, and inoculum volume of 15%) and natural culture condition (NC group, 37°C, inoculum volume of 15%) without adjusting the pH and NaCl. The myofibrillar protein was mixed with phosphate buffer (0.01M, pH 7.0) which was considered as a negative control (CK group). The treatment groups were cultured at 37°C, and samples were taken at 0, 2, 4, 6, 8, and 10 d. Samples (2 mL) were taken at different intervals and stored in hermetic 10 mL vials at -25 °C until analysis by headspace gas chromatography.

2.8 Detection of 3-methylbutanal, 3-methylbutanol and 3-methylbutyric acid

Metabolite production was assessed by headspace gas chromatography. The chromatograph (7890A-7697A, Agilent, American) was equipped with a flame ionization detector (FID). The methyl caprylate (87.70 mg/ml) was regarded as the internal standard solution by adding 10 μL to each 10 mL headspace vial. The sample was injected splitless on the column after 2 min of incubation at 90°C. The volatile compounds were separated using a capillary column (DB-WAX, Agilent, American) of 30 m×0.25 mm×0.2 μm. The carrier gas was nitrogen under a constant flow of 3.0 mL/min. The temperature of the detector was maintained at 190°C. The oven temperature was initially kept at 40°C for 4 min, and then it was increased by 5°C/min up to 130°C and then it was increased by 30°C/min up to 200°C for 1 min.

2.9 Statistical analysis

The statistical analysis was carried out using SPSS 16.0 programs (SPSS for Windows, Release 16.0.1. 2007; SPSS, Chicago, Illinois, USA). $P < 0.05$ was considered for statistical significance among treatments.

3. Results and discussion

3.1 Detection principle

The bare or pretreated GCE referenced by the paper (Ramachandran, Felix, Joshi, Raghupathy, Jeong, & Grace, 2013) and the constructed MWCNTs/CS composite solution was uniformly spread on the GCE surface by drop casting to enhance the sensitivity and stability of the GCE. The MWCNTs-CS/GCE was placed in inserted into the supernatants obtained by centrifugation to detect the amount of NADH representing the BCAT activity (Fig. 1).

3.2 Characterization of MWCNTs/CS composite by scanning electron microscope (SEM) and transmission electron microscope (TEM)

Carbon-based nanomaterials were used in electrochemical sensors for numerous analysis, such as MWCNTs. Sp2-hybridized graphite-like carbon (C=C), above 291 eV indicates the further carbonization and aromatization during the prolonged carbonization (Li, *et al*., 2015). The C=C bond that formed the MWCNTs were the most stable chemical bonds in nature, mainly due to the delocalized π bonds above and below the basal plane, thus exhibiting superior excellent electrical conductivity (Istrate, Rotariu, & Bala, 2016). However, agglomeration could occur. CS showed excellent film-forming ability, which was attributed good adhesion. They could also be easily decorated with MWCNTs because of available reactive hydroxyl and amino functional groups. In addition, CS could be entrapped with MWCNTs to reduce its agglomeration. The surface area increased, and the CS particles were completely merged into the film (Figs. 2A and 2B). The MWCNTs had a hollow tubular structure

of the same size, however, the length was different, irregular, and mutually intertwined (Fig. 2C). The CS layer was wrapped outside the MWCNTs to decrease agglomeration (Fig. 2D). The experiment showed that the MWCNTs/CS composite exhibited excellent storage stability after 10 days of storage at room temperature.

3.3 Characterization of MWCNTs/CS composite by the electrochemical method

CS could be entrapped with MWCNTs to reduce its agglomeration and increase the surface area of MWCNTs. CS particles were completely merged into the film, thus enhancing the electrocatalytic properties. Electrochemical impedance spectroscopy (EIS) studies were conducted for different electrodes in a $K_3Fe(CN)_6$ solution. The results indicated that the electron transfer resistance (Ret) values of the CS/GCE, MWCNTs/GCE, and MWCNTs–CS/GCE were lower than those of the bare GCE. The Ret value of the MWCNTs–CS/GCE was the lowest (Fig. 3A).

Differential pulse voltammetry (DPV) was employed to characterize electrodes. The peak currents of the CS/GCE, MWCNTs/GCE, and MWCNTs–CS/GCE were higher than that of the bare GCE, indicating that the sensitivity of the electrode was enhanced. The peak current of the MWCNTs–CS /GCE electrode was found to be the highest, indicating excellent sensitivity (Fig. 3B).

Both oxidation and reduction peaks were determined by cyclic voltammetry (CV) at the same scan rates. Slight differences in voltage among the peak current of the four groups of electrodes were observed: the peak current of the MWCNTs–CS/GCE was 300 μA higher than that of the bare GCE, reflecting a 2-fold increase. The MWCNTs–CS/GCE enhanced the electron transfer of $K_3Fe(CN)_6$, owing to the increased surface

area and improved electrochemical property, thereby increasing the sensitivity of electrochemical detection methods (Fig. 3C).

The irreversible oxidation of NADH to NAD^+ caused passivation at the bare electrode detecting NADH. CV was employed to investigate the determination performance of NADH. Result showed that only a NADH oxidation peak was observed, thus, it is an irreversible oxidation of NADH at the electrode surface. The peak current gradually increased with an increase in NADH concentration, resulting in a linear response ranging from 1.0 μmol/L to 9.0 μM. The linear regression equation is expressed as y=0.9797x -2.7576, with R^2 = 0.9995. Spiked recovery of 99.687%, and a detection limit of 0.12 μM are calculated according to LOD＝3 s/m, where s denotes the standard deviation of the blank sample, and m represents the slope of the NADH standard curve (Fig. 3D).

There were some reports for constructing the electrochemical methods to assess transaminase activity (Chang, Hsu, Chen, Chang, & Chen, 2003; Ohgami, Upadhyay, Kabata, Morimoto, Kusakabe, & Suzuki, 2007; Wang, *et al*., 2017; Xu, *et al*., 2015). The linear detection range and detection limit among the published reports as well as the current study were compared in Supplementary Material Table S1. It showed that the electrochemical system established in the present study possessed the lowest detection limit while the linear detection range was available in the lower range of BCAT activity. It suggested that the established electrochemical system can be used as a screening method for BCAT activity in LAB.

3.4 Comparison of aminotransferase activity by traditional and electrochemical methods

In the optical method, aminotransferase activity was determined by measuring the substrate consumption. In the electrochemical method, aminotransferase activity was calculated based on the production of NADH in transaminase enzymatic reactions. According to the mechanism of enzyme catalysis, detection of enzymatic activity using both methods should exhibit a similar trend. Therefore, by comparing the results obtained using both methods under different substrate concentrations, we verified that the electrochemical method can be used to detect the BCAT activity.

The optical method showed a linear range of 1.0–24.0 μM, the linear regression equation was $y=0.0111x+0.2025$, with $R^2 = 0.9994$ (Fig. 4A). After LAB was incubated in the reaction solution with different concentrations of leucine, the BCAT activity detected using the electrochemical method was consistent with that detected using the optical method (Fig. 4B). The BCAT activity first increased and then decreased with increasing concentration of leucine. The BCAT activity reached the highest value at 2 M of leucine by using both methods. Therefore, the established electrochemical detection system was used to determine the BCAT activity in LAB.

The optical method for detecting BCAT activity is widely used and applied for decades (Neeley, O'Classen, & Gruber, 1985). In the present study, the optical method was regarded as a standard method in comparison with established electrochemical system to evidence the accuracy and applicability of the electrochemical system. The optical method had its advantage for a liner regression in a larger range in comparison

with electrochemical system in the current study. The liner range of electrochemical system in our study was 1.0 to 9.0 μM which was available for detecting BACT activity in LAB. In addition, the electrochemical system has its own advantages, including a higher stability and sensitivity, reagent-free detection, real-time and continuous detection of BACT activity, when compared with the optical method (Han, Song, Lee, Lee, & Yoon, 2011). In addition, modifications of the electrode seemed to be the most difficulties to construct the electrochemical system. In order to meet high reproducibility, iterative tries and skilled techniques will be needed to establish the electrochemical system.

3.5 BCAT activity of *L. casei 1580* by electrochemical detection

The BCAT activity was affected by temperature, pH, NaCl concentration, and inoculum volume. To enhance the BCAT activity, we optimized the culture conditions to increase the concentration of volatile compounds. With an increase in culture pH, the enzymatic activity increased and then decreased, achieving the maximum enzymatic activity at pH 6.5–7.5. The BCAT activity decreased when pH was considerably high or low, which might have affected the growth of LAB and the absorbance of nutrients (Fig. 5A). At a higher pH, the enzyme became denatured and lost its function, which was consistent with the rapid decrease in BCAT activity when the pH increased in this experiment. Thus, pH 7.0 was optimal for culturing *L. casei 1580*. Sinz, Freiding, Vogel, & Schwab (2013) showed the optimal pH for *L. sakei* of the most BCAT activity was also 7.0, supporting that optimized pH of *L. casei* for BCAT activity by the electrochemical detection was plausible.

The BCAT activity of *L. casei 1580* varied with different temperatures. Enzymatic activity increased from 27°C to 37°C, with the highest enzymatic activity achieved at 37°C. The BCAT activity sharply decreased from 37°C to 42°C and gradually decreased from 42°C to 47°C. The temperature generally exerted a greater effect on enzymatic activity: BCAT activity at 47°C was only 30% that at 37°C. Compared with low temperature, high temperature had a larger effect on enzyme activity, which could be attributed to the enzyme denaturation or the sensitivity of *L. casei 1580* to high temperature. The result of our study was consistent with the most suitable temperature for most reported strains producing aminotransferase (Fig. 5B). The effect of NaCl concentration on the BCAT activity of *L. casei 1580* was significant ($P < 0.05$). High concentrations of NaCl could decrease the BCAT activity, with a difference of 1.1 U/L between the highest and the lowest concentrations. Meanwhile the optimal amount of NaCl (2.5%) promoted the activity of BCAT to 1.63 U/L. Studies indicated that high concentrations of Na^+ could strongly inhibit transamination (De la Torre, Salibián, & Ferrari, 1999), thus, the most suitable concentration of NaCl was 2.5% (Fig. 5C). When the volume of the inoculum was increased, BCAT activity increased and then decreased, reaching the maximum at 15%. Overnutrition occurred and enzyme production lagged when inoculation was too low; with a higher volume of inoculum, enzyme production decreased because of malnutrition in the later stages. Compared to different inoculum volume, the optimal inoculum volume was 15% (Fig. 5D).

3.6 Production of 3-methylbutanal, 3-methylbutanol and 3-methylbutyric acid

Pre-cultivation conditions of the starter culture is one factor that could influence the catabolic activity of LAB in the fermented products (Olesen & Stahnke, 2003). In order to evidence the optimized conditions for *L. casei 1580*, the products of transamination and the further reactions including 3-methylbutanal, 3-methylbutanol and 3-methylbutyric acid in meat myofibrillar protein hydrolysis system between the optimized conditions and natural fermentation condition for *L. casei 1580* pre-cultivation were investigated (Fig. 6). The content of 3-methylbutanal, 3-methylbutanol and 3-methylbutyric acid in optimized condition were significantly higher than that in natural condition and the control group which were not add the *L. casei 1580* ($P < 0.05$).

Olesen & Stahnke (2004) showed that adding *S. carnosus* in reaction media leaded to an increase in pH from 5.4 to 8.6 markedly enhancing the degradation of leucine and accumulation of 3-methylbutanoic acid. In addition, the maximum production of 3-methylbutanal for *L. sakei CTC 494* was observed with at pH 7.2 under static conditions (Larrouture-Thiveyrat & Montel, 2003). The current study suggested that the pre-culture conditions which was optimized by the established electrochemical system can promote the increase of BACT activity, resulting in more metabolites production in myofibrillar protein hydrolysis system.

4. Conclusion

In summary, an efficient electrochemical electrode for the rapid and sensitive determination of NADH was fabricated by uniformly spreading MWCNTs/CS composite on the GCE by drop casting. An electrochemical system for the

determination of BCAT activity was established. This system combined the electrochemical detection of NADH and optimization of culture conditions for *L. casei 1580.*

We demonstrated that the MWCNTs–CS/GCE electrode exhibited higher sensitivity to NADH that is, 3-fold higher than that for of the bare electrode. The linear response of the biosensor for NADH was 1.0–9.0 μM, and the response limit of NADH was 0.12 μM. The detection system effectively and sensitively detected the BCAT activity by using the NADH in the LAB culture.

Compared the traditional optical method, the electrochemical method obtained consistent BCAT activity. This finding indicated that the established electrochemical detection system detected the BCAT activity of LAB. The culture conditions for *L. casei 1580* were optimized using the electrochemical detection system under the following conditions: pH, 7.0; inoculum volume, 15%; concentration of NaCl, 2.8%; concentration of leucine, 10 M.

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References

Afzal, M. I., Ariceaga, C. C., Boulahya, K. A., Jacquot, M., Delaunay, S., & Cailliez-Grimal, C. (2016). Biosynthesis and role of 3-methylbutanal in cheese by lactic acid bacteria: Major metabolic pathways, enzymes involved, and strategies for control. *Critical Reviews in Food Science and Nutrition, 57(2), 399-406.*

Afzal, M. I., Delaunay, S., Paris, C., Borges, F., Revol-Junelles, A. M., & Cailliez-Grimal, C. (2012). Identification of metabolic pathways involved in the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism by *Carnobacterium maltaromaticum LMA 28*. *International Journal of Food Microbiology, 157*(3), 332-339.

Afzal, M. I., Jacquet, T., Delaunay, S., Borges, F., Millière, J. B., Revoljunelles, A. M., & Cailliezgrimal, C. (2010). Carnobacterium maltaromaticum: identification, isolation tools, ecology and technological aspects in dairy products. *Food Microbiology, 27*(5), 573-579.

Barbieri, G., Bolzoni, L., Careri, M., Mangia, A., Parolari, G., Spagnoli, S., & Virgili, R. (1994). Study of the volatile fraction of parmesan cheese. *Journal of Agricultural & Food Chemistry, 42*(5), 1170-1176.

Chang, K.S., Hsu, W.L., Chen, H.Y., Chang, C.K., & Chen, C.Y. (2003). Determination of

glutamate pyruvate transaminase activity in clinical specimens using a biosensor composed of immobilized L-glutamate oxidase in a photo-crosslinkable polymer membrane on a palladium-deposited screen-printed carbon electrode. *Analytica Chimica Acta*, *481*(2), 199-208.

De la Torre, F. R., Salibián, A., & Ferrari, L. (1999). Enzyme activities as biomarkers of freshwater pollution: Responses of fish branchial (Na^+ K) $^-$ATPase and liver transaminases. *Environmental Toxicology: An International Journal*, *14*(3), 313-319.

Deng, K. Q., Zhou, J. H., & Li, X. F. (2013). Direct electrochemical reduction of graphene oxide and its application to determination of L-tryptophan and L-tyrosine. *Colloids & Surfaces B: Biointerfaces, 101*, 183-188.

Ge, B., Tan, Y., Xie, Q., Ma, M., & Yao, S. (2009). Preparation of chitosan–dopamine-multiwalled carbon nanotubes nanocomposite for electrocatalytic oxidation and sensitive electroanalysis of NADH. *Sensors & Actuators B: Chemical, 137*(2), 547-554.

Gobal, F., & Faraji, M. (2015). Electrochemical synthesis of reduced graphene oxide/TiO 2 nanotubes/Ti for high-performance supercapacitors. *Ionics*, *21*(2), 525-531.

Han, Y. D., Song, S. Y., Lee, J. H., Lee, D. S., & Yoon, H. C. (2011). Multienzyme-modified biosensing surface for the electrochemical analysis of aspartate transaminase and alanine transaminase in human plasma. *Analytical and bioanalytical chemistry, 400*(3), 797-805.

Harima, Y., Setodoi, S., Imae, I., Komaguchi, K., Ooyama, Y., Ohshita, J., Mizota, H., & Yano, J. (2011). Electrochemical reduction of graphene oxide in organic solvents. *Electrochimica Acta*, *56*(15), 5363-5368.

Istrate, O.M., Rotariu, L., & Bala, C. (2016). Electrochemical determination of NADH using

screen printed carbon electrodes modified with reduced graphene oxide and poly (allylamine hydrochloride). *Microchimica Acta*, 183(1), 57-65.

Johnson, G., & Scholes, G. (1954). Micro-determination of acetaldehyde as its 2: 4-dinitrophenylhydrazone. *Analyst, 79*(937), 217-219.

Kumar, S. A., & Chen, S. M. (2008). Electroanalysis of NADH Using Conducting and Redox Active Polymer/Carbon Nanotubes Modified Electrodes-A Review. *Sensors, 8*(2), 739-766.

Larrouture-Thiveyrat, C., & Montel, M.C. (2003). Effects of environmental factors on leucine catabolism by *Carnobacterium piscicola*. *International Journal of Food Microbiology, 81*(3), 177-184.

Larrouture, C., Ardaillon, V., Pepin, M., & Montel, M. C. (2000). Ability of meat starter cultures to catabolize leucine and evaluation of the degradation products by using an HPLC method. *Food Microbiology, 17*(5), 563-570.

Latorre-Moratalla, M. L., Bover-Cid, S., Bosch-Fusté, J., Veciana-Nogués, M. T., & Vidal-Carou, M. C. (2014). Amino acid availability as an influential factor on the biogenic amine formation in dry fermented sausages. *Food Control, 36*(1), 76-81.

Li, X., Fang, Y., Lin, X., Tian, M., An, X., Fu, Y., Li, R., Jin, J., & Ma, J. (2015). MOF derived Co_3O_4 nanoparticles embedded in N-doped mesoporous carbon layer/MWCNT hybrids: extraordinary bi-functional electrocatalysts for OER and ORR. *Journal of Materials Chemistry A, 3*(33), 17392-17402.

Maogen Zhang., Audrey Smith, A., & Waldemar Gorski. (2004). Carbon nanotube−chitosan system for electrochemical sensing based on dehydrogenase enzymes. *Analytical*

Chemistry, 76(17), 5045-5050.

Marušić, N., Aristoy, M. C., & Toldrá, F. (2013). Nutritional pork meat compounds as affected by ham dry-curing. *Meat Science, 93*(1), 53-60.

Ohgami, N., Upadhyay, S., Kabata, A., Morimoto, K., Kusakabe, H., & Suzuki, H. (2007). Determination of the activities of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase in a microfluidic system. *Biosensors and Bioelectronics, 22*(7), 1330-1336.

Olesen, P. T., & Stahnke, L. H. (2003). The influence of precultivation parameters on the catabolism of branched-chain amino acids by *Staphylococcus xylosus* and *Staphylococcus carnosus*. *Food Microbiology, 20*(6), 621-629.

Olesen, P. T., & Stahnke, L. H. (2004). The influence of environmental parameters on the catabolism of branched-chain amino acids by *Staphylococcus xylosus* and *Staphylococcus carnosus*. *Food Microbiology, 21*(1), 43-50.

Plaxco, K. W., & Soh, H. T. (2011). Switch-based biosensors: a new approach towards real-time, in vivo molecular detection. *Trends in Biotechnology, 29*(1), 1-5.

Ramachandran, R., Felix, S., Joshi, G. M., Raghupathy, B. P. C., Jeong, S. K., & Grace, A. N. (2013). Synthesis of graphene platelets by chemical and electrochemical route. *Materials Research Bulletin, 48*(10), 3834-3842.

Schmidt, S., & Berger, R. G. (1998). Aroma compounds in fermented sausages of different origins. *LWT-Food Science and Technology, 31(6), 559-567*.

Shin, H. J., Kim, K. K., Benayad, A., Yoon, S. M., Park, H. K., Jung, I. S., Jin, M. H., Jeong, H. K., Kim, J. M., & Choi, J. Y. (2010). Efficient reduction of graphite oxide by sodium borohydride and its effect on electrical conductance. *Advanced Functional Materials,*

19(12), 1987-1992.

Wang, J., Xu, Z., Zou, H.Q., Hu, S.L., Wang, D.Z., Liu, J.S., & Wang, L.D. (2017). Electrochemical determination of glutamic pyruvic transaminase using a microfluidic chip. *Microfluidics and Nanofluidics, 21*(2), 27.

Whetstine, M. E. C., Drake, M. A., Broadbent, J. R., & Mcmahon, D. (2006). Enhanced nutty flavor formation in cheddar cheese made with a malty *Lactococcus lactis* adjunct culture. *Journal of Dairy Science, 89*(9), 3277-3284.

Xu, Z., Hu, S.L., Tian, W.F., Wang, D.Z., Liu, J.S., Liu, C., & Wang, L.D. (2015). Cyclic voltammetric determination of glutamic-pyruvic transaminase activity based on transdeamination. *Analytical Methods, 7*(22), 9421-9425.

Figures

Figure 1. Schematic illustration for the preparation of electrochemical system. Chitosan (CS) was combined with carbon nanotubes, and the composition was dropped on the electrode surface by one-step dispensing to increase the sensitivity and stability of the glassy carbon electrode (GCE). The multi-walled carbon nanotubes (MWCNTs)-CS/GCE was placed into the supernatants obtained by centrifugation, and the amount of NADH representing BCAT activity was detected.

Figure 2. Characterization of multi-walled carbon nanotubes (MWCNTs)/chitosan (CS) composite by scanning electron microscope (SEM) and transmission electron microscope (TEM). (A) SEM patterns MWCNTs, (B) SEM patterns MWCNTs / CS nanomaterials, (C) TEM patterns MWCNT and (D) TEM patterns MWCNTs / CS nanomaterials.

Figure 3. Electrochemical characterization of the electrochemical biosensor. (A) Electrochemical impedance spectroscopy (EIS), (B) Differential pulse voltammetry (DPV) and (C) Cyclic voltammograms (CV) of (a) bare glassy carbon electrode (GCE), (b) chitosan (CS) / GCE, (c) multi-walled carbon nanotubes (MWCNTs) / GCE, (d) MWCNTs - CS / GCE. (D) CV curves for different concentrations of NADH in PBS and Linear regression curve between NADH concentration and current value.

Figure 4. Effects of different leucine concentrations on the production of NADH and the consumption of pyruvate. (A) Linear regression curve between pyruvate concentrationand absorbance values by optical method. (B) The comparision of electrochemical method with red line and optical methods with black line of different leucine concentration.

Figure 5. Effect of different culture conditions including pH (A), temperature (B), NaCl concentration (C), and inoculation volume (D) on Lactobacillus casei BCAT activity detected by electrochemical methods. Lower-case letters (a~c) indicate significant differences between the different treatment groups ($P < 0.05$).

Figure.6. Changes in leucine metabolites including 3-methyl butyraldehyde (A), 3-methylbutanol (B), 3-methylbutyric acid (C) of myofibrillar proteins which was inoculated with *L. casei 1580* strains under optimized culture condition (OC group, pH 7.0, 37℃, NaCl concentration of 2.8%, and inoculum volume of 15%) and natural

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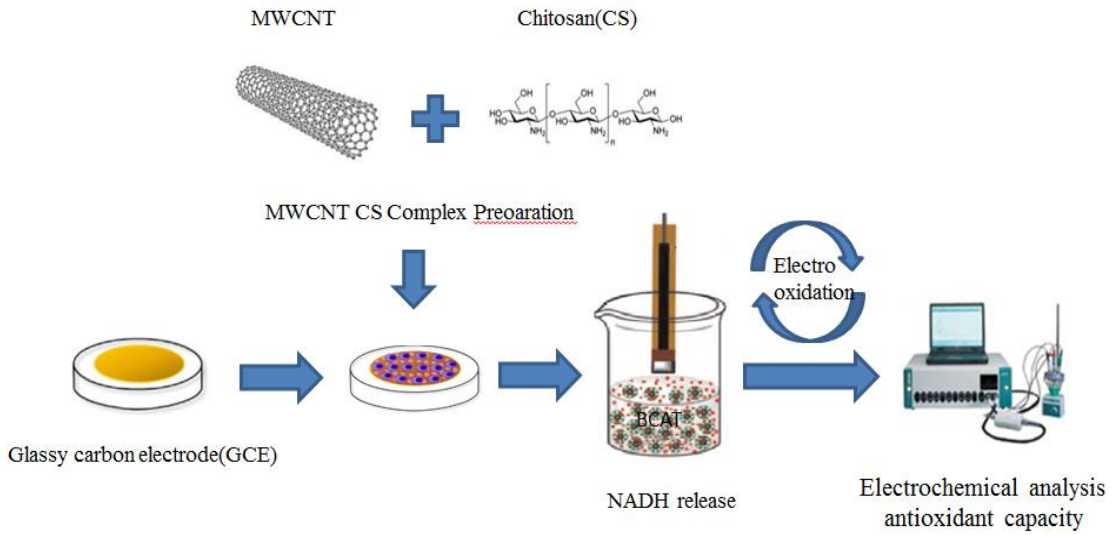

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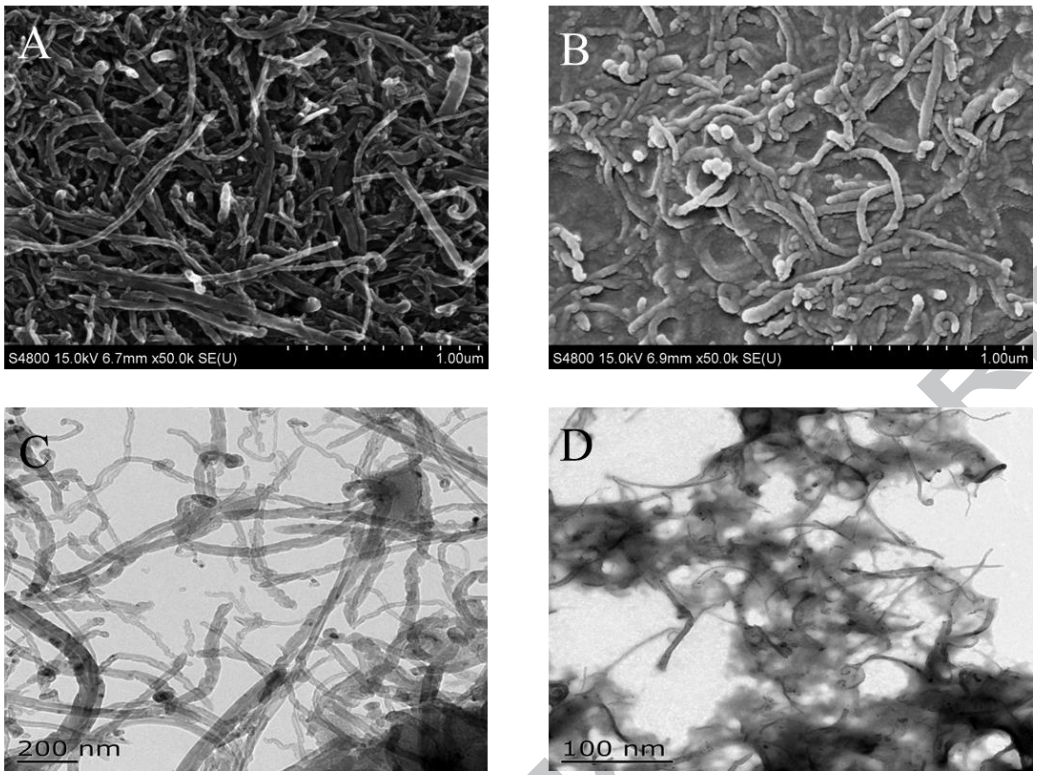

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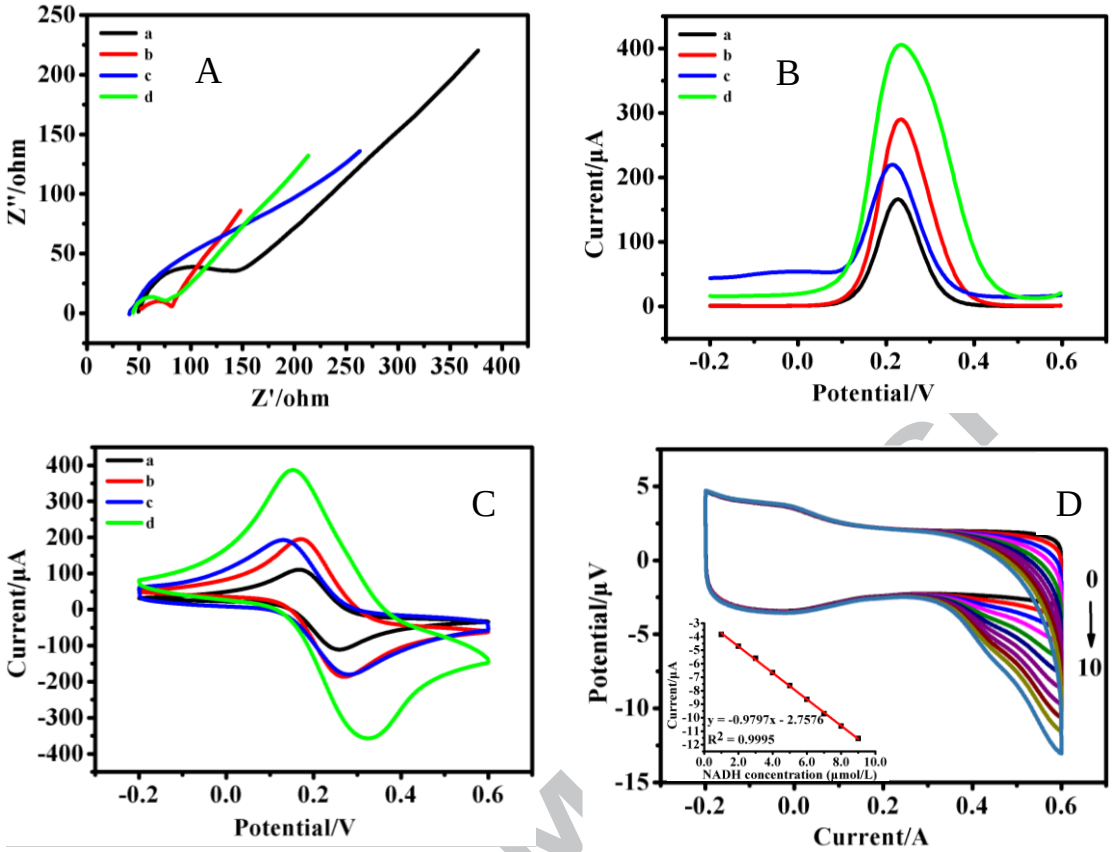

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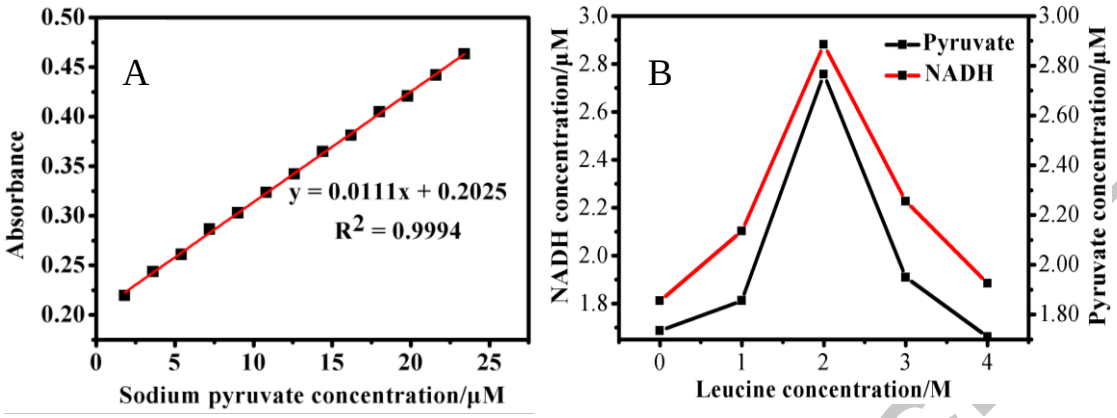

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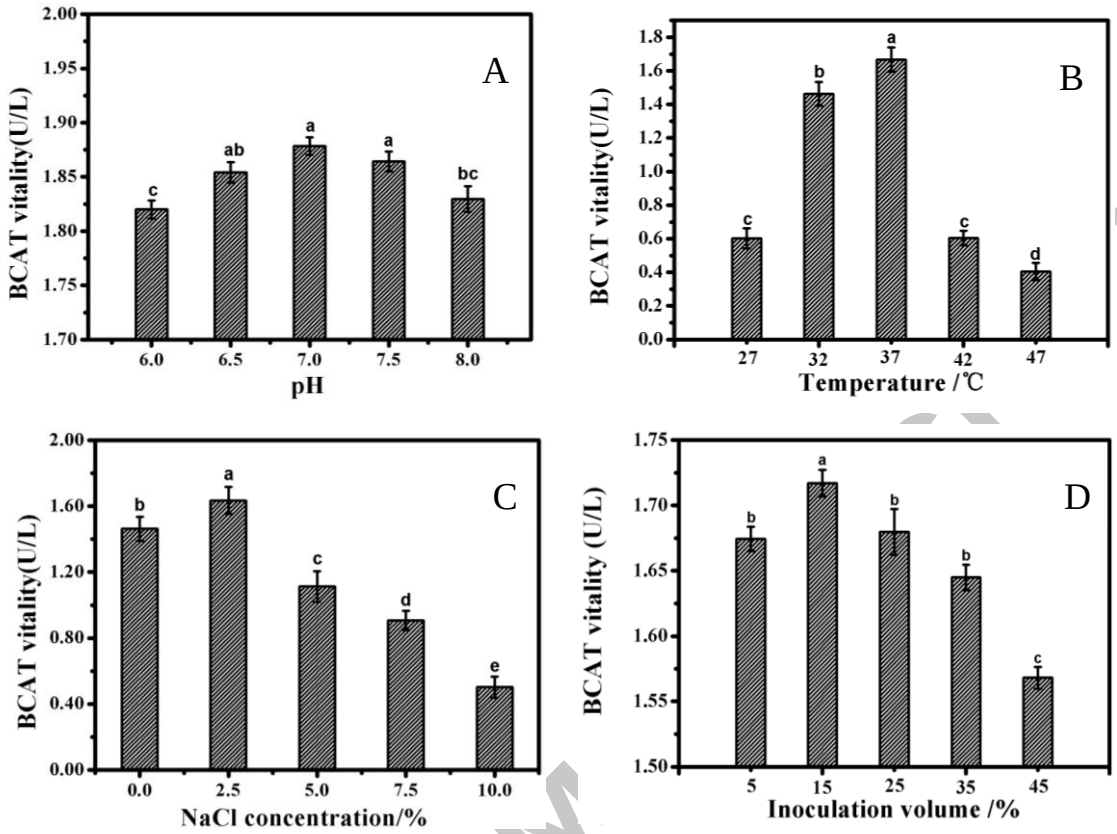

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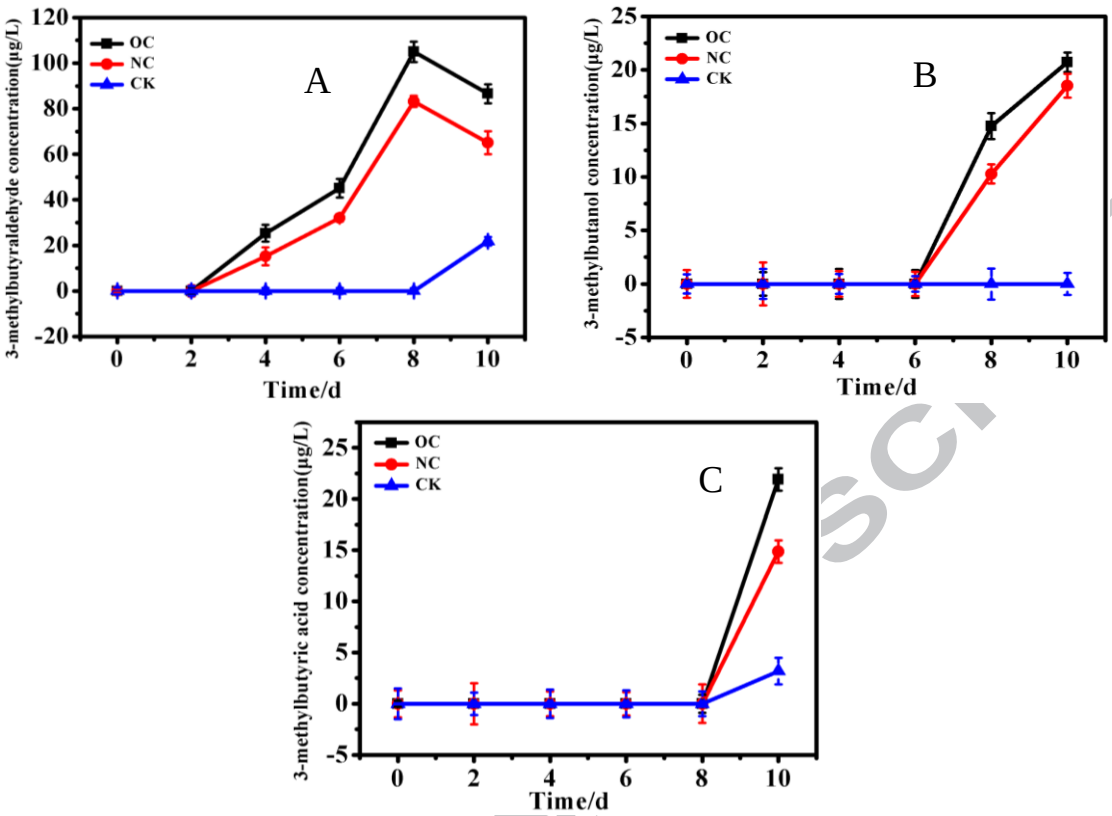

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1. A novel electrochemical detection system to detect BCAT activity in LAB was initially established.
2. Determination of NADH with excellent sensitivity and selectivity.
3. Powerful analytical tool for monitoring the BCAT activity in LAB.

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: