SCREENING OF GAMMA-AMINOBUTYRIC ACID-PRODUCING LACTIC ACID BACTERIA AND ITS APPLICATION IN *MONASCUS*-FERMENTED RICE PRODUCTION

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ABSTRACT

Background. Gamma-aminobutyric acid (GABA), with an antidepressant effect, and Monacolin K, with a cholesterol-lowering effect, are the main bioactive ingredients in *Monascus*-fermented rice (MFR). The simultaneous enrichment of both ingredients can effectively enhance the health benefits of MFR. However, the capacity of *Monascus* spp. to produce GABA is limited.

Material and methods. Seventeen lactic acid bacteria (LAB) strains were preliminarily screened for GABA-producing by whole-cells bioconversion of L-glutamate, followed by rescreening through fermentation with the addition of the precursor L-glutamic acid. Subsequently, the bioconversion conditions (temperature, metal ions, and pH) for the conversion of L-monosodium glutamate (MSG) were investigated. Additionally, the GABA-producing LAB was co-inoculated with a monacolin K producing strain *Monascus anka* 20-2, and the ratio of *M. anka* 20-2 to LAB in microbial consortia was optimized for MFR production.

Results. The strain *Lactobacillus plantarum* 8014 was screened out for its ability to produce GABA. At an optimal temperature of 33°C and pH 7.5, with the addition of 0.05 g/L $ZnSO_4$, the strain showed an L-glutamate conversion rate of 100%. The ratio optimization of *M. anka* 20-2 to *L. plantarum* 8014 in microbial consortia showed that when the dry cell ratio was 2:1, the content of monacolin K and GABA in the MFR simultaneously reached 2.22 mg/g and 29.9 mg/g, respectively.

Conclusion. A two-stage fermentation using microbial consortia containing *M. anka* 20-2 and *L. plantarum* 8014 was developed for the production of bioactive MFR, in which the active ingredients monacolin K and GABA were simultaneously enriched, with good consumer acceptability due to the aromatic scent produced by lactic acid bacteria.

Keywords: *Lactobacillus* spp., gamma-aminobutyric acid, *Monascus*-fermented rice, monacolin K, microbial consortia

INTRODUCTION

Monascus-fermented rice (MFR) is made by fermenting *Monascus* spp. on rice and has been used as a food ingredient and medicine in East Asia for more than 600 years (Ma et al., 2000). One of the bioactive constituents in MFR is monacolin K, which is chemically identical with lovastatin, a statin drug used for lowering cholesterol approved by the U.S. Food and Drug Administration (FDA) in 1987 (Grundy, 1988; Peng et al., 2017). Treatment with MFR has become a common alternative therapy for statin-intolerant patients who suffered statin-associated myalgia (Childress et al., 2013; Halbert et al., 2010). Hypercholesterolemia is an important risk factor for numerous diseases, include cardiovascular diseases (Nelson, 2013), hypertension (Ivanovic and Tadic, 2015), deep-vein thrombosis (Kawasaki et al., 1997), diabetes (Yang and Mousa, 2012), liver and kidney disease (Aliyev and Hajiyeva, 2018). Meanwhile, reports from the National Health and Nutrition Examination Surveys (NHANES) indicated that 60.7–64.3% of hypertensive patients also had hypercholesterolemia (Egan et al., 2013).

Another bioactive metabolite of MFR is gamma-aminobutyric acid (GABA), which has been intensively researched in pharmaceutical and medical fields (Cho et al., 2007; Ngo and Vo, 2019). The multiple physiological functions of GABA-enriched foods, such as hypotensive, neuroprotective, neurological disorder prevention, anti-diabetic, and anti-cancer effects, were also confirmed by tests in animals and human (Diana et al., 2014; Ngo and Vo, 2019; Park and Oh, 2007). Thus, biotechnological production of GABA recently has become a research hotspot, and many strategies have been employed to enhance the GABA yield, including direct fermentation by wild-type or metabolic engineered strains (Xu et al., 2017; Yu et al., 2019), enzymatic (Huang et al., 2016) or whole-cell biocatalysis (Shi et al., 2017). At present, a GABA titer of 201.18 g/L has been obtained by 10-h bioconversion using *Lactobacillus brevis* resting cells with the addition of L-monosodium glutamate (MSG) (Shi et al., 2017).

Although *Monascus* spp. are capable of producing both ingredients, monacolin K and GABA, previous studies primarily focused on enhancing either monacolin K or GABA content in MFR. The highest yield of monacolin K and GABA has reached 19.81 mg per gram of MFR fermented by *Monascus ruber* (Zhang et al., 2018), and 24 mg/g in solid-state fermentation of rice by *M. purpureus* MTCC 369 (Khan et al., 2019), respectively. However, the fermentation methods that simultaneously increase the content of both bioactive ingredients have not been reported.

The present study aimed to screen a GABA-producing lactic acid bacteria and optimize the conditions affecting the bioconversion of MSG to GABA. Furthermore, the MFR enriched with both monacolin K and GABA was prepared by fermentation with microbial consortia composed of *Monascus anka* 20-2 and *Lactobacillus plantarum* 8014.

MATERIAL AND METHODS

Microorganisms. Strains used in this study were *Monascus anka* 20-2, which was isolated from cooked rice and deposited to the China Center for Type Culture Collection (CCTCC No. M 2015356). Lactic acid bacteria include *Lactobacillus acidophilus* (L5), *L. bulgaricus* (L41, L48), *L. rhamnosus* (LD, 7469, L22, L20, L57), *L. reuteri* (L33, L21), *L. plantarum* (8014, L69, L24), *L. casei* (L54, LC, L49), *Bifidobacterium longum* (B28) was provided by the College of Food and Biological Engineering, Shaanxi University of Science and Technology (Xi'an, China). Stock cultures were stored at –80°C in freeze-dried powder.

Screening for GABA-producing LAB. LABs were activated on MRS agar plates (Hopebio, Qingdao, China) at 37°C for 2 days, and then a single pure colony was cultivated in MRS broth for 12 h at 37°C in the shaker at 250 rpm (Cheng et al., 2019). After cultivation, the LAB cells were harvested by centrifugation at 4,000×g for 10 min at 4°C and washed twice with 0.9% saline solution, then transferred into a 5 mL conversion system. The conversion system consisted of 2.5 g/L LAB cells, 5.75 g/L of L-monosodium glutamate (MSG; equal to 5 g/L of L-glutamic acid), 0.1 mM pyridoxal phosphate (PLP), 50 mM $CaCl_2$, 50 mM $MgSO_4$, and acetate buffer (pH 4.8; Shi et al., 2017). After 12 h of conversion, the residual L-glutamate concentration in the conversion system was determined by an SBA-40E

immobilized enzyme biosensor (Shandong, China), and the specific L-glutamate conversion rate (R_{Glu}) for each strain was calculated as follows:

$$R_{Glu} = \text{L-glutamate converted, mg / dry cell weight, g / bioconversion time, h}$$

Then LABs were divided into 3 clusters using a k--means cluster analysis algorithm, which distinguishes strains with similar R_{Glu}.

The strains clustered in the group with higher R_{Glu} were inoculated into MRS liquid medium containing 1.15 g/L of MSG (equal to 1 g/L of L-glutamic acid) and incubated at 37°C for 12 h (Li et al., 2008). The fermentation broth was centrifuged at 4°C at 12,000×g for 15 min to collect the supernatant, filtered with a 0.45 μm filter membrane, and the contents of GABA and L-glutamate were detected by high-performance liquid chromatography (HPLC).

Bioconversion conditions. 0.5 mL of an overnight culture of *L. plantarum* 8014 (approx. $1×10^8$ CFU/mL) was inoculated into 50 mL of MRS liquid medium containing 1.15 g/L of MSG. The effect of temperature on the bioconversion activity of *L. plantarum* 8014 was evaluated by performing reactions at 25, 29, 33, 37, and 41°C, respectively. To estimate the effect of metal ions on bioconversion, metal solutions ($CaCl_2$, $ZnSO_4$, $FeCl_3$, $FeCl_2$, $CoCl_2$, $MgSO_4$, KH_2PO_4, $CuSO_4$) were added separately to MRS broth at a final concentration of 0.05 g/L (Gheisari et al., 2016), and the concentrations of $ZnSO_4$ (0.01, 0.03, 0.05, 0.07 and 0.09 g/L) were further optimized. Moreover, the influence of pH was investigated by conducting the bioconversion in a 3-L fermentor (BioFlo 110, USA) containing 1.0 L MRS medium with the addition of 0.05 g/L $ZnSO_4$. *L. plantarum* 8014 (approx. $1×10^8$ CFU/mL) was inoculated at 5% (v/v), and cultured at 37°C, pH 7.0, agitation speed 300 rpm. When the culture reached an OD_{600} of 1.0, a final concentration of 1.15 g/L MSG was supplied to each fermentor, and the culture pH was maintained at 3.5, 4.5, 5.5, 6.5, and 7.5, respectively, by feeding with either HCl (5 M) or NaOH (5 M). After 24 h incubation, the bioconversion solution was centrifuged at 12,000×g for 15 min, and the supernatant was filtered with a 0.45 μm filter membrane for detection of the contents of GABA and L-glutamate.

Production of bioactive MFR by microbial consortia. *M. anka* 20-2 was activated three times in 250 mL shake flasks containing 50 mL malt extract medium, inoculated with 2.5 mL of the spore suspension ($1×10^5$ spores/mL), and incubated at 28°C for 48 h prior to use as a starter culture for preparation of MFR (Khan et al., 2019). Then 2 mL of starter culture with $1×10^6$ spores/mL was fermented in 250 mL shake flasks containing 40 mL fermentation medium for 7 d on a shaker at 28°C, 200 rpm. The fermentation medium contained the following components (g/L): rice flour – 70, NH_4Cl – 15, K_2HPO_4 – 1.5, $MgSO_4·7H_2O$ – 0.5. Subsequently, 10 mL of different biomass concentrations of *L. plantarum* 8014 suspensions were inoculated into the 40 mL *Monascus*-fermented broth to adjust the biomass ratios of *M. anka* 20-2 and *L. plantarum* 8014 of 3:1, 2:1, 1:1, 1:2, and 1:3, respectively. *L. plantarum* 8014 suspensions were obtained by centrifuging collected 12-h cultured cells at 4°C, 4000×g for 5 min, and resuspended in phosphate buffer (pH 7.5). Microbial consortia fermentation was carried out in 3-L fermentor (BioFlo 110, USA) at 33°C, pH 7.5, 300 rpm, and a final concentration of 1.15 g/L MSG and 0.05 g/L $ZnSO_4$ was supplied accordingly after inoculation. The concentration of L-glutamate in the fermenter was determined periodically, and when the L-glutamate concentration decreased to 0.2 g/L, MSG was fed to a final concentration of 1.15 g/L MSG. After 40 h of bioconversion, the substrate and solution were vacuum lyophilized at –85°C using a FreeZone 2.5 Plus freeze dry system (Labconco, USA), and ground into a fine powder using a mortar and pestle. Hereafter, the GABA and monacolin K content in the MFR powder was measured by high-performance liquid chromatography (HPLC).

Detection of biomass. In this experiment, the dry weight of *M. anka* 20-2 was determined by measuring mycelial biomass. The liquid fermentation medium (40 mL) was filtered through four layers of gauze and washed with sterile water until the liquid was colorless, then dried at 60°C until reaching a constant weight. For determination of dry cell weight of *L. plantarum* 8014, 30 mL of cell culture was centrifuged at 4°C, 4000×g for 5 min, and the cell pellet was washed twice with 10 mL of distilled water, then dried

at 60°C until a constant weight attained. All samples were determined at least three times.

Analytical methods. The GABA and the monacolin K concentrations were determined by reverse-phase high-performance liquid chromatography (HPLC) using a Thermo U-3000 HPLC system (Thermo Fisher Scientific, USA) equipped with a fluorescence detector (FLD-3400RS) and DAD detector (DAD-3000). The stationary phase was a reverse-phase column Agilent 5 TC-C18(2) (250 mm × 4.6 mm × 5 μm) (Agilent, Netherlands). The GABA concentration was determined by pre-column phenylisothiocyanate (PITC) derivatization following a previously described procedure (Cho et al., 2007). The mobile phase was a mixture of water (solvent A) / acetonitrile (solvent B) / 0.125 M sodium acetate (pH 5.8) (solvent C) flowing under gradient elution at 1 mL/min for the mobile phase A/B/C with ratio of 28:12:60 (0 min), 28:12:60 (6 min), 10:30:60 (6.10 min), 10:30:60 (20 min), and 28:12:60 (25 min). The injection volume was 10 μL, and the temperature of the column was 30°C, and the compound was detected through a DAD detector at 254 nm. The concentration of monacolin K was determined by HPLC as previously described (Wang et al., 2011). The mixture of acetonitrile and water (55:45, v/v), acidified with phosphoric acid to pH 2.5, was used as a mobile phase with a fixed flow rate of 1 mL/min. UV detection was at 238 nm, and the column temperature was maintained at 28°C. The injection volume was 10 μL.

Statistical analysis. Experiments were conducted in triplicate. The values shown represent the mean ±standard deviations (SD). The experimental data were analyzed by one-way analysis variance (ANOVA), followed by Tukey's post-hoc analysis; $P < 0.05$ represented significant differences. The k-means cluster analysis was conducted using SPSS 15.0 for Windows (SPSS Inc., USA).

RESULTS AND DISCUSSION

Screening for GABA-producing LAB

GABA is the direct decarboxylation product of L-glutamate catalyzed by the L-glutamate decarboxylase (GAD). Thus, the activity of GAD is critical for a GABA-producing LAB. Therefore, the activity of
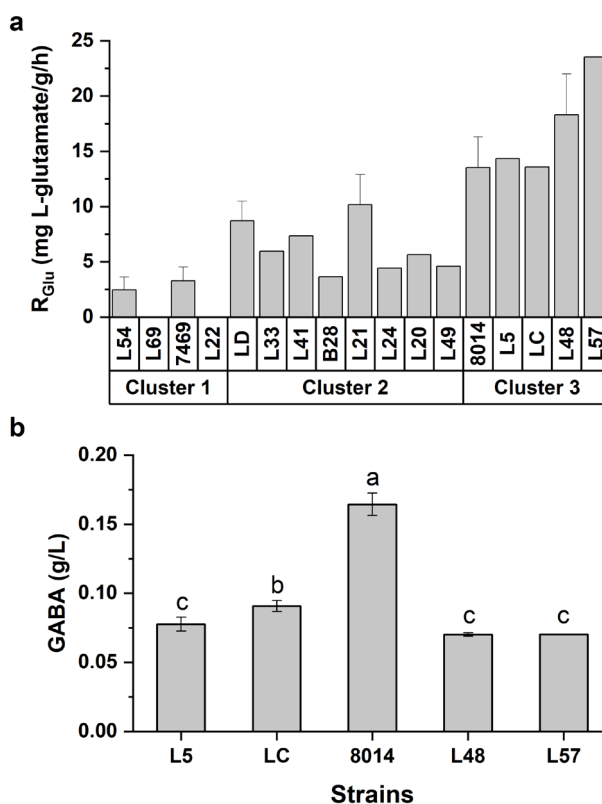

Fig. 1. The specific L-glutamate conversion rates (R_{Glu}) of seventeen LAB strains (a) and the GABA yield of five re-screening strains (b). Error bars indicate standard deviation, as determined from triplicate experiments (three independent bacterial cultures)

L-glutamate decarboxylase of seventeen LAB strains was evaluated using whole-cells bioconversion of L-glutamate, followed by k-means cluster analysis. As shown in Figure 1a, the specific L-glutamate conversion rates (R_{Glu}) of the strains were significantly different ($P < 0.001$), and the result of k-means cluster analysis showed that strains *L. plantarum* 8014, *L. acidophilus* L5, *L. casei* LC, *L. bulgaricus* L48, and *L. rhamnosus* L57 were clustered in a group with the high specific L-glutamate conversion rates. The five strains were rescreened through fermentation with the addition of the precursor L-glutamic acid. As shown in Figure 1b, the GABA yield of *L. plantarum* 8014 was significantly higher than that of the other four strains ($P < 0.001$), and reached 0.16 g/L after 12 h fermentation in an MRS broth containing 1.15 g/L of MSG (equal to 1 g/L of L-glutamic acid).

Effect of temperature on L-glutamate bioconversion

MRS medium supplemented with 1 g/L L-glutamic acid was inoculated with 1% (vol/vol) overnight culture of *L. plantarum* 8014 (approx. 1×10^8 CFU/mL), and incubated for 24 h at 250 rpm at 25°C, 29°C, 33°C, 37°C and 41°C, respectively. As shown in Figure 2,
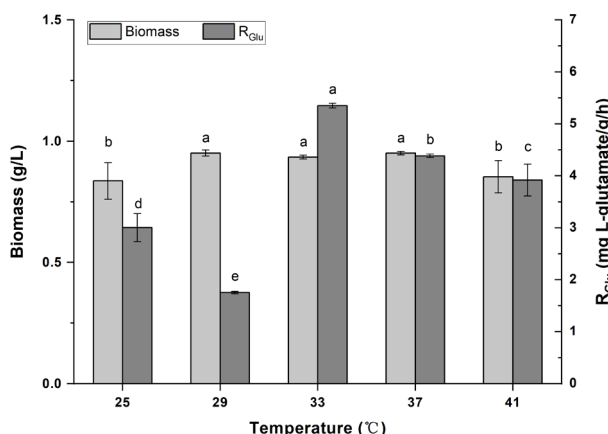
Fig. 2. Effect of temperature on L-glutamate bioconversion. All values indicated are means ±SD ($n = 3$)

the incubation temperature had a significant effect on the growth of the bacteria. The biomass of cells cultured between 29–37°C was significantly higher than that of 25°C and 41°C ($P = 0.002$). To avoid the influence of variation in the number of bacteria on the L-glutamate conversion rate, the specific L-glutamate conversion rate (R_{Glu}) was evaluated experimentally. Accordingly, a significant difference in R_{Glu} was observed among different incubation temperatures ($P < 0.0001$), the highest R_{Glu} was detected at 33°C, and reached 5.35 mg L-glutamate/g/h. The results suggested that temperature may be influencing the conversion capacity of *L. plantarum* 8014 by affecting the activity of L-glutamate decarboxylase (GAD) in the bacteria. Since the optimum temperature of GAD varies in different strains, the optimum bioconversion temperature differed from one strain to another as well. For example, the optimal temperature for fermentation production of GABA by *L. plantarum* Taj-Apis362 was 36°C (Tajabadi et al., 2015), whereas for *L. brevis* TCCC 13007, it was 33°C (Zhang et al., 2012).

Effect of metal ions on L-glutamate bioconversion

The cell growth and specific L-glutamate conversion rate of *L. plantarum* 8014 cultured with the supplement of different metal ions were examined, and the results are shown in Figure 3a. The addition of most types of ions except Mg^{2+} had no significant effect on the growth of *L. plantarum* 8014 ($P = 0.097$); only the supplement of $MgSO_4$ led to a 34% reduction in biomass, compared with the unsupplemented group (CK). In contrast, all the ions positively affected R_{Glu}, especially with the addition of Zn^{2+}, which led to a 4.16-fold increase in R_{Glu} compared with CK. Therefore, the concentration of Zn^{2+} was modified, and the results (Fig. 3b) showed the bioconversion ability of *L. plantarum* 8014 increased with the increase of Zn^{2+} concentration at 0.01–0.05 g/L and then decreased dramatically with further elevating Zn^{2+} concentration.
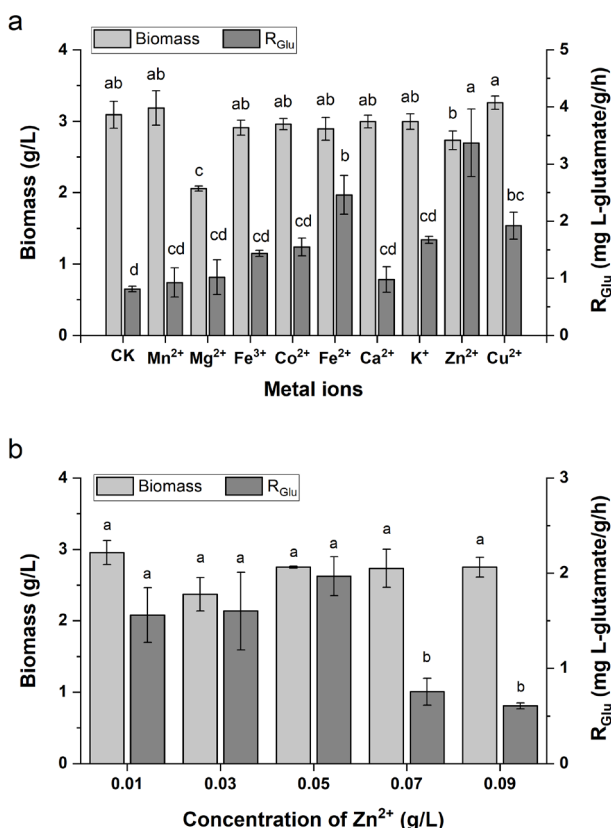
Fig. 3. Effect of metal ions (a) and the concentration of Zn^{2+} (b) on cell growth and L-glutamate bioconversion. All values indicated are means ±SD ($n = 3$)

The enhancement in bioconversion ability appeared to be closely related to the increase in GAD activity. Previous studies showed that the activity of GAD could be increased by $MnCl_2$ (152%), $CoCl_2$ (118%), and $ZnCl_2$ (115%) (Sa et al., 2015), which was consistent with increased R_{GLu} detected in this study (114%, 191%, and 416%, respectively). Besides, a positive effect of $MgSO_4$ (125%) was also observed, as described by Ueno et al. (1997). Nevertheless, a discrepancy in the effect of metal ions on GAD activity has also been discovered (Xu et al., 2017), and this implies that the GAD enzymes in different species can vary widely in function and structure.

Effect of pH on L-glutamate bioconversion

The experiments were conducted in 3-L fermentors, and the pH of the conversion system was adjusted in real-time by feeding with either HCl (5 M) or NaOH (5 M). As shown in Figure 4, cell growth was significantly affected by pH ($P < 0.0001$), and the optimal pH was between 5.5 and 7.5. After incubation in an MRS medium containing 1 g/L L-glutamic acid for 24 h, the conversion ratio of L-glutamic acid was above 80% at all pH conditions, especially at pH 7.5, it was significantly higher than other pHs ($P = 0.027$), with a GABA yield of 0.71 g/L and 100% L-glutamate conversion. Although R_{Glu} was highest at pH 3.5, reaching 12.4 mg L-glutamate/g/h, the growth of the LAB was severely limited, which led to a decrease in GABA yield and did not meet the requirements of large-scale product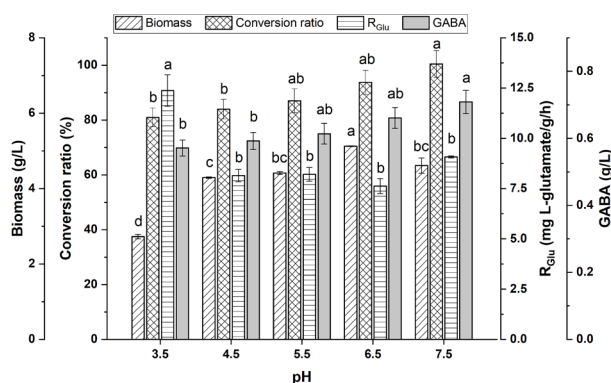ion. Therefore, the pH of the conversion system was finally chosen to be 7.5, since the bacteria can grow naturally, the R_{Glu} is moderate, and the L-glutamate can be fully converted without any residue.

Production of bioactive MFR by microbial consortia

A two-stage microbial consortia fermentation process was developed to improve the GABA content in MFR, and the yield of monacolin K and GABA produced by microbial consortia containing different ratios of *M. anka* 20-2 and *L. plantarum* 8014 were investigated. As shown in Figure 5, there were significant differences in the yields of both monacolin K ($P < 0.0001$) and GABA ($P = 0.008$) by fermentation with different *M. anka* 20-2/*L. plantarum* 8014 (dry weight ratio). The highest yields of both bioactive ingredients monacolin K and GABA were obtained when the ratio of *M. anka* 20-2 to *L. plantarum* 8014 was 2:1, reached 2.22 mg/g, and 29.9 mg/g, respectively. Moreover, compared with the bitter-moldy taste of MFR produced by single *Monascus* sp. strain, MFR fermented with the microbial consortia is more acceptable to consumers because of the aromatic scent and sour taste of lactic acid bacteria.

In comparison, the content of GABA in MFR (29.9 mg/g) prepared in this study was slightly higher than that of a previous study, in which a GABA content of 24 mg/g was produced by *M. purpureus* MTCC 369
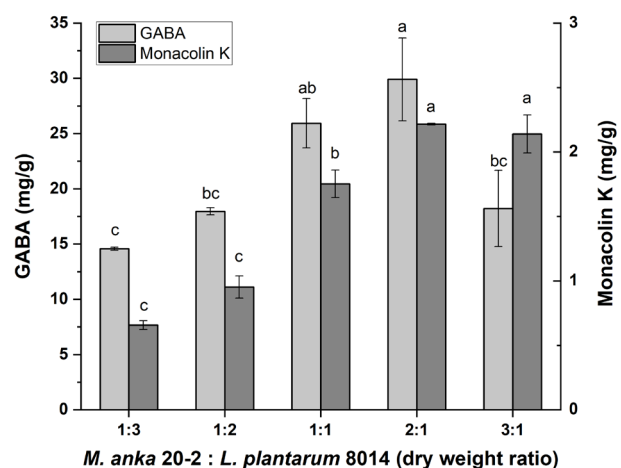
Fig. 4. Effect of pH on cell growth and L-glutamate bioconversion. All values indicated are means ±SD ($n = 3$)

Fig. 5. The yield of GABA and monacolin K at different ratio of *Monascus* to *Lactobacillus*. All values indicated are means ±SD ($n = 3$)

(Khan et al., 2019), but the monacolin K content in MFR (2.22 mg/g) was lower than that reported 19.81 mg/g by Zhang et al. (2018) and 12.9 mg/g by Lu et al. (2013). However, the monacolin K content in MFR could be improved by prolonging the first-stage of fermentation with *M. anka* 20-2, and a higher GABA yield can also be achieved by extending the second-stage of the bioconversion process with *L. plantarum* 8014.

CONCLUSION

In this study, a GABA-producing strain *L. plantarum* 8014 was screened, and the optimum temperature, ions, and pH for the conversion of L-glutamate were investigated. Furthermore, the monacolin K high-yielding strain *M. anka* 20-2 was co-inoculated with *L. plantarum* 8014 at different ratios to develop a two-stage fermentation process for the production of bio-active MFR. The results showed that the conversion ratio of L-glutamate by *L. plantarum* 8014 could reach 100% at 33°C, pH 7.5, with the addition of 0.05 g/L ZnSO$_4$ and 1.15 g/L of MSG in the MRS medium. After 40 h fermentation with the microbial consortia of *M. anka* 20-2 and *L. plantarum* 8014 at a dry weight ratio of 2:1. The contents of monacolin K and GABA in MFR reached 2.22 mg/g and 29.9 mg/g, respectively. These results provide a reference for the industrial production of MFR with simultaneous enrichment of monacolin K and GABA. The approaches developed in this study can be applied to prepare other food with multiple bioactive compounds based on microbial consortia fermentation.

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