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Full Length Article

Factors Affecting the Fermentation Optimization of *Monascus* Strains with High γ-aminobutyric Acid Yield

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Abstract

The γ-aminobutyric acid (GABA) is one of the main functional components of red yeast rice (RYR), which has blood pressure lowering, anti-depressant, nerve cell nourishing and brain health supporting properties. This study aimed to improve GABA production from RYR, using germinated brown rice as the fermentation substrate to isolate and screen strains and optimize fermentation conditions. Six *Monascus* strains with a GABA yield of at least 20.0 mg/100 g were obtained: CICC 5008, GD-05, G9, HB-05, Z3 and FG-04. Strain CICC 5008 showed the greatest GABA yield (22.43 mg/100 g), which was 70.6% greater than the control strain CICC 5009. Using single factor optimization, Plackett-Burman design and Box-Behnken response surface analysis, the optimal fermentation process for producing GABA from strain CICC 5008 was determined as follows: brown rice soaking time 9 h, rice steaming time 15 min, fermentation bottle rice mass 80 g, fermentation temperature 25°C, brown rice soaking solution pH 5.0, 5 fermentation flask air holes and fermentation time 11 d. The GABA yield under this fermentation condition was 26.54 mg/100 g. Among the seven factors, fermentation time was the most important factor affecting GABA production from RYR, followed by fermentation temperature and flask permeability, while the other four factors had less influence.

Keywords: *Monascus*; γ-aminobutyric acid; Strain screening; Fermentation optimization

Introduction

Red yeast rice (RYR) is a product of rice fermentation by *Monascus*, which has been used in East Asian food production for thousands of years (Ma *et al*., 2000; Erdoğrul and Azirak, 2004; Chen *et al*., 2015). RYR can treat indigestion, promote blood circulation and protect the spleen and stomach. The main functional components of RYR include pigment, monacolin K (MK), γ-aminobutyric acid (GABA), active enzymes and ergosterol (Su *et al*., 2003; Lin *et al*., 2008; Shi and Pan, 2011).

GABA is a non-protein four-carbon amino acid that is the major inhibitory neurotransmitter of the mammalian nervous system (Leventhal *et al*., 2003). GABA's physiological functions are mainly involved in blood pressure lowering, anti- depressant, nerve cell nourishing, liver and kidney function improving and brain vitality improving aspects. GABA binds and activates the anxiolytic brain receptor and works synergistically with other substances to prevent anxiety-related information from reaching the brain's indicator center. Therefore, GABA can fundamentally calm nerves, creating an anti-anxiety effect (Shelp *et al*., 1999; Atack *et al*., 2006).

Chuang *et al*. (2011) used a rat model of depression (forced swimming test) to test oral administration of GABA-rich *Monascus* fermentation products (MFP) to explore possible mechanisms of their antidepressant effects. In the short-term trial, both GABA and MFP significantly decreased the duration of immobilization; in the long-term trial, the same MFP dose (2.6 mg/kg) of showed better antidepressant effects than GABA and MFP had similar efficacy to fluoxetine (Chuang *et al*., 2011).

In plants, GABA is produced during decarboxylation of L-glutamic acid, which is catalyzed by glutamate decarboxylase (Shelp *et al*., 1999). This process is related to plant stress metabolism, seed vigor and cell development (Narayan and Nair, 1990). GABA plays a physiological role in the human body, mainly through receptor mediation.

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There is a postsynaptic membrane site that can specifically recognize and bind GABA, which can cause changes in ion permeability of the cell membrane upon binding (Shelp *et al*., 1999). Studies have shown that GABA receptors can be divided into three subtypes: $GABA_A$, $GABA_B$ and $GABA_C$. The mechanism of action between GABA and the three receptors are different. $GABA_A$ is an ion receptor that mediates rapid synaptic transmission and conveys an inhibitory effect and is widely distributed in the central nervous system (Atack *et al*., 2006; Olsen and Sieghart, 2009). $GABA_B$ is distributed in the presynaptic and posterior membrane, which inhibits the release of excitatory transmitters mainly through G protein coupling, thereby playing an inhibitory role (Kaupmann *et al*., 1998; Ng *et al*., 1999). $GABA_C$ mediates melatonin (N-acetyl-5-oxytryptamine) regulation and is closely involved with prolactin (PRL) secretion. The receptor agonist cis-4-aminocrotonic acid (CACA) of $GABA_C$ increases PRL secretion in a dose-dependent manner (Greka *et al*., 2000; Prada *et al*., 2005; Nakayama *et al*., 2006).

In view of the important application value GABA has in functional foods and medicine, scholars have studied various fungi and fermentation products of GABA (Rhyu and Kim, 2002; Chen and Chen, 2009; Dikshit and Tallapragada, 2015; Hajar-Azhari *et al*., 2018). For example, Chen and Chen (2009) studied the brewing conditions of high-GABA rice vinegar using germinated rice as the raw material and identified three important factors (the amount of raw material starter compound, RYR and glucoamylase preparation) by Plackett-Burman design. Subsequently, Box-Behnken Center joint experimental design was adopted to optimize these three factors. Using alcohol fermentation, saccharification and acetic acid fermentation, rice vinegar containing 100 mg/L GABA was obtained (Chen and Chen, 2009).

Hajar-Azhari *et al*. (2018) studied GABA-producing *Aspergillus oryzae* strains and isolated a GABA-rich strain of Malaysian *A. oryzae* NSK from soy sauce koji. Using this strain, optimal GABA yield was achieved and a new functional GABA soy sauce was developed by providing sugarcane molasses as the fermentation substrate (354.08 mg/L) followed by sugarcane syrup (320.7 mg/L) and nipah syrup (232.07 mg/L) (Hajar-Azhari *et al*., 2018).

Pyo (2008) studied the GABA content from soybean fermentation from different *Monascus* strains. Among the five experimental strains, soybeans fermented by the *Monascus pilosus* strain IFO 480 had the highest GABA content; the GABA content of *Monascus* fermented soybeans (MFS) (78.5 mg/100 g DW) was 1.5 times greater than unfermented soybeans (Pyo, 2008).

Isato and Himeno (2000) studied the change of GABA content during *beni-koji* production by using *M. pilosus* strain IFO 4520. After the beginning of starter propagation, as the proportion of *tomo koji* (10%, 30% and 50%) increased, glutamic acid decarboxylase (GAD) activity and GABA production increased steadily, peaking at 5 d after fermentation and decreasing significantly afterwards (Isato and Himeno, 2000).

Su *et al*. (2003) adopted solid fermentation to screen *M. purpureus* CCRC 31615 (a strain with high MK and GABA yields) from 16 strains of *Monascus* and found that after adding sodium nitrate or potassium dihydrogen phosphate to fermentation medium, MK and GABA in the fermentation product could significantly increase (Su *et al*., 2003).

In this study, we targeted GABA production in *Monascus* solid-state fermentation products using germinated brown rice as the fermentation substrate. Isolation and screening of *Monascus* strains and optimization of the fermentation process was performed. This would provide experimental basis for the preparation of GABA-rich RYR, and thus lays the foundation for improved RYR application in the fields of functional foods and medicine.

Materials and Methods

Experimental Materials

Red yeast rice: Following nine varieties (JXDD1203, JXDD1205, XFD1202, XFD1204, YGZ1201, GT11-10, GT11-12, GT12-02 and ZA12003) were purchased in Beijing, Gutian County and Zhao'an County of Fujian Province.

Monascus strains: 13 species (ACCC 30341, ACCC 30501, ACCC 30352, CICC 5004, CICC 5008, CICC 5009, CICC 5016, CICC 5024, CICC 5025, CICC 5026, CICC 5027, CICC 5037 and GIM 3.239) were purchased from Agricultural Culture Collection of China (ACCC), China Center of Industrial Culture Collection (CICC), and Guangdong Culture Collection Center (GCCC).

Brown rice germination: The brown rice variety N84 was immersed in sterile water at pH 5.0 for 9 h at 20°C and then spread on a sterile Petri dish and covered with 0.5 mmol/L $CaCl_2$ solution-soaked gauze. The brown rice was then placed in a 35°C thermostatic incubator for use after 32 h germination.

Isolation and Purification of *Monascus* Strains

One gram of commercially available red yeast rice powder was taken, then placed in a Petri dish. Sterile physiological saline was added until the sample was just immersed, then it was gently shaken for 1 min and 1 mL of the bacterial suspension was pipetted onto the PDA plate. The bacterial concentration in solution was recorded as 10^{-1}; 1 mL of bacterial suspension was taken, and a dilution solution with a concentration gradient of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} was prepared in physiological saline, then separately coated on a PDA plate and incubated at 28°C for 3-5 d. Single colonies with *Monascus* characteristics were selected, and then spread in "Z" shape on a new PDA plate, cultured at 28°C for 3-5 d, then transferred

to a new PDA plate. After multiple isolation and purification cycles, it was transferred to PDA slant medium, cultured at 28°C for 5 d and stored at 4°C for use.

Medium Preparation, Inoculation and Culture

Preparation of wort agar medium: 150 mL of wort and 3 g of agar powder were diluted to 1000 mL with water then autoclaved at 121°C for 20 min.

Preparation of seed solution medium: the solution containing glucose (50 g/L), peptone (5 g/L), yeast extract (1 g/L), KH_2PO_4 (1 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L) was autoclaved at 121°C for 20 min.

Preparation of germinated brown rice medium: After steaming germinated brown rice for 20 min, 60 g of each rice was placed in a 300 mL plastic fermentation flask and autoclaved at 121°C for 20 min.

After activating the *Monascus* strain with PDA slant culture medium, the slanted surface was scraped using sterile water and 10% of bacterial suspension was inoculated into the seed solution medium then cultured at 200 rpm at 28°C for 3 d. The inoculated seed solution medium was then inoculated at a ratio of 10% to the fermentation medium with N84 germinated brown rice as the substrate, and cultured at 25°C for 15 d. During the culture period, the substrate was shaken every 3 d to disperse the substrate, so that the substrate was in full contact with the strain. Three repetitions were used for each treatment.

HPLC Determination of GABA Content in RYR

Pretreatment of red yeast rice samples: Red yeast rice samples were and dried to constant weight in a 50°C oven. After grinding into a powder and passing through a 100 mesh sieve, 5 g of sample was added to 30 mL 60% ethanol in a water bath at 55°C for 6 h. Samples were shaken well for 30 s every 30 min, then centrifuged for 15 min at 3000 g. The supernatant was concentrated to 5 mL and filtered through a 0.45 μm membrane.

Derivatization of samples: 1 mL Acetonitrile solution containing 1% 2,4-dinitrofluorobenzene (FDNB) was used as a derivatizing agent for samples. 1 mL of the sample concentrate was added to a 10 mL volumetric flask, then 0.5 mol/L $NaHCO_3$ solution (pH 9.0) and 1 mL of derivatizing agent were added and incubated in a water bath at 60°C in the dark for 1 h. Samples were then cooled and phosphate buffer (pH 7.0) was added until the mark of 10 mL, then shaken well and filtered through a 0.45 μm membrane before injection.

HPLC chromatographic conditions: A Hypersil ODS2 C18 (250 nm × 4.6 nm, 5 μm) chromatographic column was used. The mobile phase was acetonitrile : water : phosphate buffer (pH = 7.2) at 25:20:55 (v/v), flow rate 1.0 mL/min, detection wavelength 360 nm, column temperature 28°C and injection volume 10 μL.

Fermentation Process Optimization for RYR Rich in GABA

Brown rice was used as the substrate according to the method in Section 1.2.2. The effect of seven fermentation process parameters on GABA production were studied (rice soaking time, soaking liquid pH, rice steaming time, fermented bottled rice mass, fermentation temperature, number of fermentation flask air holes and fermentation time). Plackett-Burman design and Box-Behnken response surface analysis were then adopted to optimize the fermentation process of strain CICC 5008 for high GABA yield from RYR, and the optimal fermentation conditions were determined. The quadratic regression model obtained from response surface analysis was verified by experiments. The recorded data were statistically analyzed using SPSS 18.0 software.

Results

Isolation and Purification of *Monascus* Strains

95 strains of *Monascus* were isolated and purified from 9 varieties of commercially available red yeast powder. These strains were inoculated on wort agar medium for cultivation. Morphological identification and microscopic observations indicated that these strains included *M. purpureus*, *M. ruber*, *M. anka* and *M. barker*.

The total number of strains obtained by the above isolation and purification and those strain purchased from the culture collection center was 108. After solid fermentation products of each strain were pretreated, the GABA content was determined by HPLC. Results (Table 1) show that the six strains with the highest GABA yield were CICC 5008, GD-05, G9, HB-05, Z3 and FG-04 and their GABA yields were all above 20.0 mg/100 g. The strain CICC 5008 had the highest GABA yield of 22.43 mg/100 g, which was 70.6% higher than that of the control strain CICC 5009.

Optimization Results of Fermentation Conditions for High-Yield GABA from RYR

Effect of brown rice soaking time on GABA production from RYR: The soaking times of N84 brown rice were selected as 0, 3, 6, 9, 12 and 15 h. It can be seen that brown rice soaking time has a significant effect on GABA production of red yeast rice (Fig. 1). When soaking time was below 9 h, GABA yield increased with soaking time;

Table 1: GABA content in red yeast rice fermented by different *Monascus* strains (mg/100 g)

Strain number	GABA yield	Strain number	GABA yield	Strain number	GABA yield
CICC 5008	22.43±0.26	J-02	15.04±0.16	Z7	13.30±0.14
GD-05	21.83±0.22	G3	14.74±0.15	GD-25	13.28±0.13
G9	21.66±0.24	HB-14	14.74±0.15	FX-05	13.21±0.13
HB-05	21.39±0.23	GS-10	14.63±0.14	CICC5009(CK)	13.15±0.14
Z3	21.12±0.22	FG-13	14.61±0.14	GD-04	13.04±0.14
FG-04	20.89±0.21	GD-14	14.54±0.15	FG-05	12.91±0.13
GD-06	20.06±0.21	HB-11	14.49±0.14	BG-03	12.87±0.13
ACCC 30501	19.90±0.22	BG-07	14.48±0.15	GD-21	12.82±0.12
CICC 5024	19.83±0.20	Z1	14.45±0.13	HB-01	12.81±0.13
FG-07	19.82±0.20	FX-04	14.42±0.15	GD-26	12.79±0.13
GS-02	19.75±0.21	GD-02	14.41±0.14	GD-03	12.74±0.15
GS-04	19.36±0.20	Z6	14.33±0.16	FX-01	12.70±0.13
BG-01	19.30±0.20	Z5	14.32±0.14	GIM 3.239	12.62±0.12
HB-03	19.22±0.21	GD-16	14.25±0.14	FX-07	12.61±0.13
GS-05	19.19±0.20	G7	14.10±0.15	BG-06	12.56±0.13
GD-07	19.04±0.20	FG-02	14.03±0.14	GS-06	12.51±0.12
FG-03	18.87±0.19	BG-10	13.99±0.13	Z4	12.49±0.14
HB-13	18.75±0.19	G2	13.97±0.15	FG-10	12.41±0.13
G5	18.04±0.18	J-01	13.96±0.14	CICC 5016	12.40±0.13
FG-06	17.98±0.19	CICC5037	13.95±0.14	GD-15	12.39±0.12
FG-01	17.96±0.20	BG-11	13.93±0.15	HB-06	12.35±0.12
HB-09	17.93±0.18	G6	13.89±0.14	FX-02	12.34±0.13
HB-07	17.69±0.18	BG-04	13.88±0.13	GD-10	12.30±0.14
GS-01	17.65±0.16	BG-08	13.87±0.14	GD-12	12.23±0.12
G1	17.49±0.18	GS-03	13.82±0.13	GD-13	12.23±0.13
Z2	17.26±0.18	GD-23	13.81±0.12	ACCC 30341	11.96±0.13
A-01	16.52±0.17	GD-19	13.71±0.14	GD-22	11.91±0.12
FG-12	16.43±0.17	CICC5025	13.70±0.14	CICC 5026	11.89±0.11
G8	16.28±0.16	FG-08	13.68±0.13	HB-08	11.88±0.12
FX-06	16.27±0.17	BG-12	13.63±0.14	GD-11	11.66±0.12
ACCC30352	16.10±0.16	FG-09	13.59±0.13	GD-18	11.65±0.11
BG-05	15.87±0.16	GD-24	13.58±0.12	CICC 5027	11.59±0.13
HB-10	15.65±0.18	A-02	13.57±0.14	CICC 5004	11.57±0.12
HB-12	15.46±0.16	FG-11	13.48±0.13	GD-08	11.57±0.11
HB-02	15.42±0.16	GD-01	13.43±0.15	GD-09	11.24±0.12
BG-09	15.35±0.16	GD-17	13.41±0.14	FG-14	11.19±0.12

when the soaking time exceeded 9 h, GABA yield decreased significantly. Therefore, when the soaking time was 9 h, red yeast rice had the highest GABA yield (15.06 mg/100 g).

Effect of brown rice soaking solution pH on GABA production from RYR: The pH of the brown rice soaking solution was selected at six values (3.0, 4.0, 4.5, 5.0, 6.0 and 7.0). Soaking solution pH directly affects the initial pH of the fermentation medium, which in turn affects *Monascus* mycelial growth. pH of the soaking solution had a significant effect on GABA production of red yeast rice (Fig. 2). The suitable pH value for GABA production by *Monascus* was 4.5-5.0, which is consistent with the finding that *Monascus* grows best in a slightly acidic environment. When soaking solution pH was 5.0, red yeast rice had the highest GABA yield (15.73 mg/100 g); as pH of the soaking solution increased or decreased from this point, GABA yield decreased accordingly.

Effect of rice steaming time on GABA production from RYR: The rice steaming times were selected as 5 min, 10 min, 15 min, 20 min, 25 min and 30 min. The rice steaming duration directly affected the cooking degree of germinated brown rice. Rice steaming time had a significant effect on GABA production in red yeast rice (Fig. 3). When rice steaming time was 15 min, red yeast rice had the highest GABA yield (13.94 mg/100 g); as the rice steaming time increased or decreased, the GABA yield was significantly less.

Effect of fermentation bottled rice mass on GABA production from RYR: The fermentation bottled rice mass was selected as 20 g, 30 g, 40 g, 50 g, 60 g, 70 g, 80 g and 90 g. The bottled rice mass is related to the matrix number and gas permeability of the fermentation flask. Fermentation bottled rice mass had a significant effect on GABA production from RYR (Fig. 4). When bottled rice mass was below 70 g, GABA production increased with the increase of bottled rice mass; when the bottled rice mass was 70 – 90 g, there was no significant difference in GABA production. Therefore, a suitable bottled rice mass for the production of GABA by *Monascus* is 70 g of germinated brown rice.

Effect of fermentation temperature on GABA production from RYR: The fermentation temperatures were selected at 20, 25, 28, 30, 35, and 40°C. The fermentation temperature was closely related to *Monascus* mycelial growth, and good hyphal growth is a prerequisite for accumulating GABA. Fermentation temperature had a significant effect on GABA production in red yeast rice (Fig. 5). When the fermentation temperature was 30°C, red yeast rice had the highest GABA yield (12.30 mg/100 g); as fermentation temperature increased or decreased from this value, GABA yield was significantly less.

Effect of fermentation flask ventilation properties on GABA production from RYR: The number of fermentation flask air holes was selected as 0, 1, 2, 3, 4 and 5. Fermentation flask permeability was adjusted by changing the number of air holes. The number of fermentation flask air holes has a significant effect on GABA production of red yeast rice (Fig. 6). When the number of air holes was below 3, GABA production increased with the increase in of air hole number. This is because *Monascus* is an aerobic fungus with a high oxygen demand during the fermentation process. When the number of air holes was 3-5, there was no significant difference in GABA production. Therefore, to reduce the possibility of bacterial contamination of the fermentation process, the number of fermentation flask air holes suitable for GABA production from *Monascus* fermentation was 3.

Effect of fermentation time on GABA production from RYR: Fermentation times were selected as 5, 7, 9, 11, 13, 15, and 17 d. Fermentation time had a significant effect on GABA production in red yeast rice (Fig. 7). When the fermentation time was below 15 d, GABA yield increased with fermentation time prolongation; when the fermentation time exceeded 15 d, GABA yield decreased significantly. Therefore, a suitable fermentation time for GABA production by *Monascus* was 15 d (GABA yield 15.88 mg/100 g).

Plackett-Burman design results for high-yield GABA

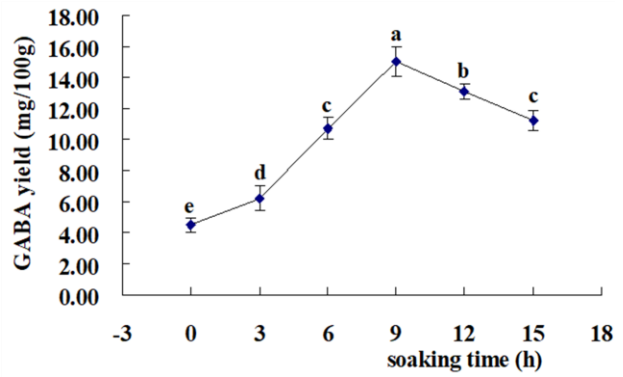

Fig. 1: Effects of brown rice soaking time on GABA production. Different letters in different treatments indicate significant differences ($P < 0.05$), the same below

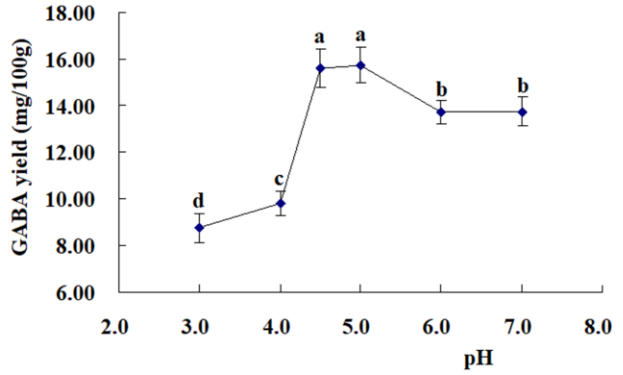

Fig. 2: Effects of brown rice soaking solution pH on GABA production

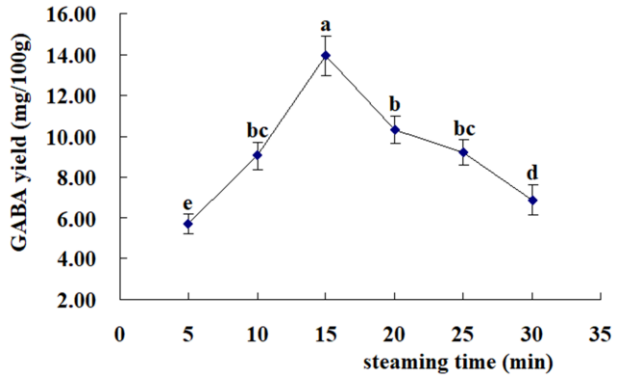

Fig. 3: Effect of rice steaming time on GABA production

fermentation process using *Monascus*: According to single factor optimization results, the optimal value for each factor was determined and this value is referred to here as the reference value. Those higher than the value level were set to 1, or otherwise set to -1 and then Plackett-Burman design (Table 2 and 3) of the seven factors and two levels was carried out. Twelve groups were needed and each set had three repetitions. The results (Table 3) showed that the

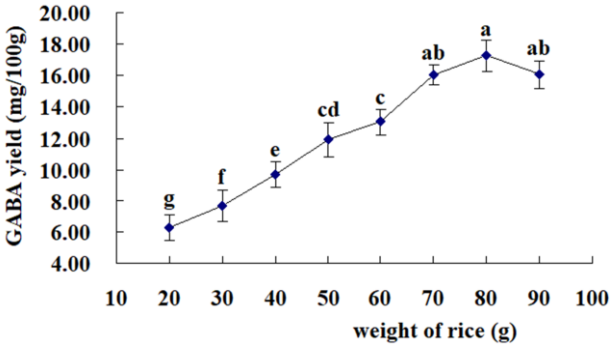

Fig. 4: Effects of fermentation bottled rice mass on the production of GABA

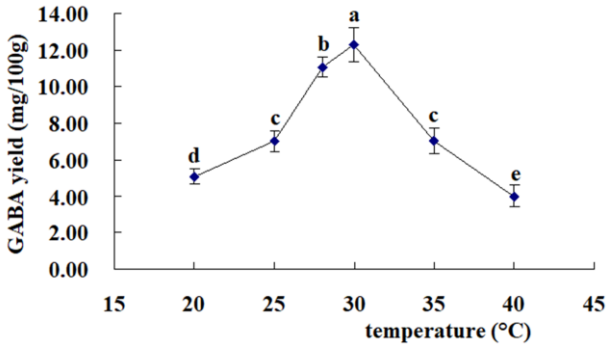

Fig. 5: Effects of fermentation temperature on GABA production

fifth group has the highest GABA yield in red yeast rice, and the corresponding fermentation process was: brown rice soaking time 9 h, rice steaming time 15 min, fermentation bottled rice mass 80 g, fermentation temperature 30°C, brown rice soaking solution pH 5.0, number of fermentation flask air holes 5, fermentation time 15 d. Under this fermentation condition, GABA yield was 16.19 mg/100 g.

Univariate analysis of the Plackett-Burman design variance was made using SPSS 18.0 software to test the inter-subject effect of influence factors. Results (Table 4) showed that fermentation time, fermentation temperature and flask permeability were the three main factors affecting the GABA yield of RYR. Among them, fermentation time is the most important factor affecting GABA production in red yeast rice, and its effect reaches a very significant level ($P < 0.01$). Fermentation temperature and flask permeability have secondary influence, which does not reach a significant level ($P > 0.05$). The other four factors (brown rice soaking time, brown rice soaking solution pH, rice steaming time and fermentation bottled rice mass) have less influence than all of the above three main factors.

Box-Behnken response surface analysis of high GABA yield fermentation by *Monascus*: On the basis of single factor optimization and Packett-Burman design, three main factors affecting the fermentation process of *Monascus* strain CICC 5008 with high GABA yield were determined: fermentation temperature, number of

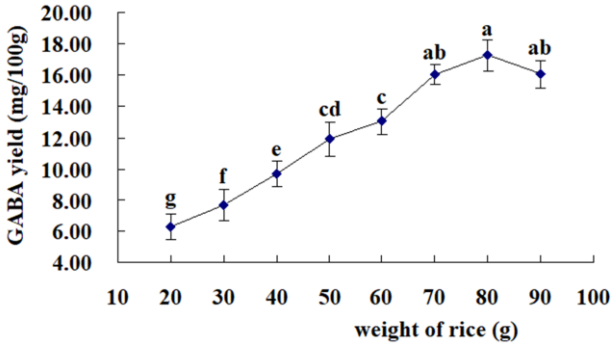

Fig. 4: Effects of fermentation bottled rice mass on the production of GABA

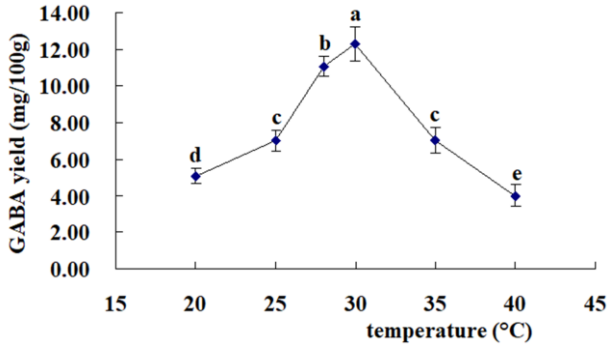

Fig. 5: Effects of fermentation temperature on GABA production

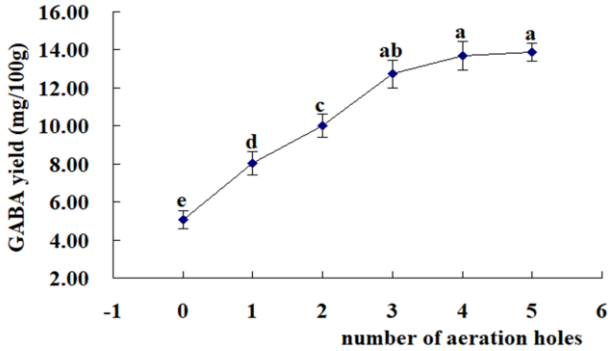

Fig. 6: Effects of fermentation flask ventilation properties on GABA production

aeration holes of the fermentation bottle and fermentation time. Box-Behnken response surface design (Table 5 and 6) of the three main factors was carried out according to the schedule given by software Design-export 7.1.3. The results (Table 6) showed that the GABA yield of RYR was the highest in the second group, reaching 22.61 mg/100 g. The corresponding fermentation process was as follows: the number of aeration holes in fermentation bottles was set 5, and the fermentation culture lasted for 15 days at 25°C.

The experimental data of Table 6 were fitted by multiple quadratic regression analysis using the software

Table 2: Factors and levels of the Plackett-Burman design

number	factors	levels	
		-1	1
A	soaking time of brown rice/h	6	9
B	steaming time/h	10	15
C	rice weight loaded in a fermentation flask/g	50	80
D	fermentation temperature/°C	25	30
E	pH of soaking solution	4.0	5.0
F	vent hole numbers of fermentation flask	3	5
G	fermentation time/d	11	15

Table 3: Arrangement and results of the Plackett-Burman design

group	A	B	C	D	E	F	G	GABA yield (mg/100 g)
1	1	-1	-1	-1	1	-1	1	8.36 ± 0.11
2	-1	-1	-1	1	-1	1	1	5.21 ± 0.08
3	-1	1	1	-1	1	1	1	6.37 ± 0.09
4	-1	-1	-1	-1	-1	-1	-1	10.45 ± 0.13
5	1	-1	1	1	1	-1	-1	16.19 ± 0.16
6	-1	1	-1	1	1	-1	1	7.21 ± 0.09
7	-1	1	1	1	-1	-1	-1	15.18 ± 0.10
8	-1	-1	1	-1	1	1	-1	8.80 ± 0.06
9	1	1	1	-1	-1	-1	1	6.13 ± 0.06
10	1	-1	1	1	-1	1	1	7.11 ± 0.08
11	1	1	-1	-1	-1	1	-1	9.55 ± 0.09
12	1	1	-1	1	1	1	-1	12.10 ± 0.11

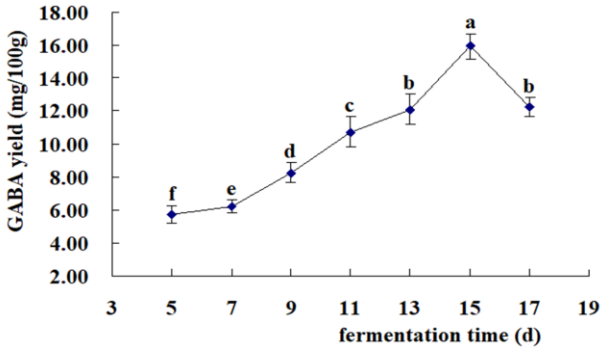

Fig. 7: Effects of fermentation time on GABA production

Design-export 7.1.3. The GABA yield was set Y, fermentation temperature was D, the number of aeration holes in fermentation bottle was F and fermentation time was G. The quadratic regression equation was obtained as follows:

$$Y=85.21068-3.15995\cdot D-5.18568\cdot F+17.14042\cdot G+0.12208\cdot D\cdot F-0.42292\cdot D\cdot G+3.75000E-003\cdot F\cdot G+0.027269\cdot D^2+0.048307\cdot F^2-0.49865\cdot G^2$$

The variance analysis results of regression model (Table 7) showed that the regression of the quadratic regression model was highly significant ($P < 0.01$), and the missing items were not significant ($P > 0.05$); the effects of fermentation time and fermentation temperature on GABA production of RYR were highly significant ($P < 0.01$), and the effects of the number of aeration holes in fermentation bottles reached a significant level ($P < 0.05$); the interaction between the three factors was not significant ($P > 0.05$). The decision

Table 4: Analysis results of the Plackett-Burman design

origin	type III square sum	df	mean square	F	Sig.
calibration model	126.392[a]	7	18.056	6.915	0.040
intercept	1057.690	1	1057.690	405.046	0.000
A	3.224	1	3.224	1.235	0.329
B	0.015	1	0.015	0.006	0.944
C	3.967	1	3.967	1.519	0.285
D	14.830	1	14.830	5.679	0.076
E	2.430	1	2.430	0.931	0.389
F	17.232	1	17.232	6.599	0.062
G	84.695	1	84.695	32.434	0.005
error	10.445	4	2.611	—	—
total	1194.527	12	—	—	—
corrected total	136.838	11	—	—	—

Table 5: Factors and levels of the Box-Behnken response surface design

factors	levels		
	-1	0	1
fermentation temperature/°C	25	28	31
vent hole numbers of fermentation flask	1	3	5
fermentation time/d	11	15	19

Table 6: Arrangement and results of the Box-Behnken response surface design

group	fermentation temperature	vent hole numbers of fermentation flask	fermentation time	GABA yield (mg/100 g)
1	1	-1	0	12.46
2	-1	1	0	22.61
3	0	0	0	13.91
4	1	0	1	10.91
5	0	1	1	17.36
6	1	1	0	11.45
7	0	0	0	14.06
8	0	-1	1	18.27
9	0	-1	-1	8.35
10	0	1	-1	7.32
11	-1	0	-1	8.61
12	0	0	0	14.17
13	-1	-1	0	21.61
14	1	0	-1	7.06
15	-1	0	1	14.74

Table 7: Analysis of variance for the quadratic regression model of Box-Behnken response surface design

regression	sum of squares	df	mean square	F	P (Pr>F)
total model	325.88	9	36.21	35.62	0.0005
D	82.50	1	82.50	81.15	0.0003
F	12.05	1	12.05	11.86	0.0184
G	178.70	1	178.70	175.78	<0.0001
DF	8.58	1	8.58	8.44	0.0636
DG	25.76	1	25.76	25.33	0.40
FG	3.600E-003	1	3.600E-003	3.541E-003	0.9549
D^2	0.22	1	0.22	0.22	0.6597
F^2	2.21	1	2.21	2.17	0.2007
G^2	14.69	1	14.69	14.45	0.0626
missing item	5.05	3	1.68	98.81	0.1
pure error	0.034	2	0.017	—	—
total error	330.97	14	—	—	—

coefficient of the model is 0.9846 *i.e*., the model can explain 98.46% of the GABA content change.

Based on the above analysis results, the model fits well with the experimental results. It can be used to predict the optimization of GABA production by solid-state fermentation of *Monascus* strain CICC 5008 using N84 germinated brown rice as substrate.

The software Design-export 7.1.3 was used to predict the optimum fermentation process of GABA production by strain CICC 5008. The maximum value of GABA production was 26.21 mg/100 g after the optimum value of each factor was obtained. The optimum fermentation process corresponding to this value was as follows: the number of aeration holes in fermentation bottles was set 5, and the fermentation time was 11 days at 25°C.

The solid-state fermentation experiment was carried out with the optimal fermentation process obtained from the above prediction, and GABA content of the RYR was 26.54 mg/100 g. The actual value was only 1.26% higher than the predicted value of the model, which showed that the model could accurately predict the fermentation process of the designed experiment. The GABA yield of RYR under the optimal fermentation conditions was 11.8% higher than the highest GABA yield (22.43 mg/100 g) detected during the above strain screening process. The results showed that the optimal fermentation parameters of RYR with high GABA yield obtained by Box-Behnken response surface analysis were accurate and reliable.

Discussion

Monascus is a traditional Chinese fermenting fungus. RYR is a natural dietary supplement (Ma *et al*., 2000) and its metabolite MK has proven to be an effective cholesterol-lowering drug (Istvan and Deisenhofer, 2001). Compared with MK, there are few literature reports of GABA production from *Monascus* fermentation, but GABA is also an important metabolite of red yeast (Lin *et al*., 2008). GABA has important physiological functions, such as antihypertension (Shelp *et al*., 1999) and antidepressant properties (Chuang *et al*., 2011) and has been proven to be an effective antihypertensive agent (Rhyu and Kim, 2002). Therefore, it is necessary to screen the *Monascus* strains for GABA production and optimize fermentation process parameters to increase the GABA yield of RYR, thus broadening the application range for RYR in functional foods and medicine.

From our studies, the pH suitable for GABA production by *Monascus* fermentation was 4.5-5.0 (Fig. 2). The results of this study are consistent with the finding that *Monascus* grows best in a slightly acidic environment (Panda *et al*., 2009), which is also in line with the appropriate pH for production of MK by *Monascus* fermentation (Lee *et al*., 2007). This shows that fermentation medium pH exerts a similar effect on GABA production and MK production from MFP. A similar situation exists for fermentation time: results of this study (Fig. 7) that the suitable fermentation time for GABA production by *Monascus* fermentation is 15 d, while

the suitable fermentation time for MK production is also approximately 15 d (Suraiya *et al*., 2018).

Fermentation temperature is also an important factor affecting GABA production in MFP (Su *et al*., 2003). From this study, the highest GABA production was achieved by *Monascus* fermentation at 30℃ (Fig. 5), which is consistent with the results of Su *et al*. (2003). The MK yield of MFP reached the highest at 30℃. The MK and GABA yields decreased significantly when the fermentation temperature was lower than 30℃ or higher than 30℃ (Wang *et al*., 2003). Therefore, the optimum fermentation temperature for simultaneous production of MK and GABA by *Monascus* fermentation is 30℃. The above similarities in the GABA and MK production from *Monascus* fermentation processes can help to simultaneously increase GABA and MK yields of MFP. Therefore, we can use GABA and MK yield simultaneously as indicators to study on the screening and fermentation optimization of *Monascus* strains.

In this study we used germinated brown rice (variety N84) as the fermentation substrate, and 6 strains of *Monascus* with a GABA yield of at least 20.0 mg/100 g were obtained: CICC 5008, GD-05, G9, HB-05, Z3, and FG-04. Strain CICC 5008 had the highest GABA yield of 22.43 mg/100 g, which was 70.6% greater than the control strain CICC 5009.

By combing single factor optimization, Plackett-Burman design and Box-Behnken response surface analysis, the optimal fermentation process for producing GABA from strain CICC 5008 was determined as follows: 9 h brown rice soaking, 15 min rice steaming, 80 g fermentation bottled rice mass, fermentation temperature 25℃, brown rice soaking solution pH 5.0, 5 fermentation flask air holes, and 11 d fermentation time. The GABA yield under this fermentation condition was 26.54 mg/100 g.

Conclusion

The fermentation time, fermentation temperature and flask permeability were the three main factors affecting the GABA yield of RYR. Among them, fermentation time was the most important factor affecting GABA production from RYR, and its effect was very significant ($P < 0.01$). Fermentation temperature and fermentation flask permeability had a secondary influence, which was not significant ($P > 0.05$). The other four factors had less influence than all of the above three main factors.

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