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Evaluation of commercial soy sauce *koji* strains of *Aspergillus oryzae* for γ-aminobutyric acid (GABA) production

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Abstract In this study, four selected commercial strains of *Aspergillus oryzae* were collected from soy sauce *koji*. These *A. oryzae* strains designated as NSK, NSZ, NSJ and NST shared similar morphological characteristics with the reference strain (*A. oryzae* FRR 1675) which confirmed them as *A. oryzae* species. They were further evaluated for their ability to produce γ-aminobutyric acid (GABA) by cultivating the spore suspension in a broth medium containing 0.4 % (w/v) of glutamic acid as a substrate for GABA production. The results showed that these strains were capable of producing GABA; however, the concentrations differed significantly ($P < 0.05$) among themselves. Based on the *A. oryzae* strains, highest GABA concentration was obtained from NSK (194 mg/L) followed by NSZ (63 mg/L), NSJ (51.53 mg/L) and NST (31.66 mg/L). Therefore, *A. oryzae* NSK was characterized and the sequence was found to be similar to *A. oryzae* and *A. flavus* with 99 % similarity. The evolutionary distance (K_{nuc}) between sequences of identical fungal species was calculated and a phylogenetic tree prepared from the K_{nuc} data showed that the isolate belonged to the *A. oryzae* species. This finding may allow the development of GABA-rich ingredients using *A. oryzae* NSK as a starter culture for soy sauce production.

Keywords *Aspergillus oryzae* · Glutamic acid · γ-Aminobutyric acid · Fermentation · Soy sauce

Abbreviations
P	GABA concentration (mg/L)
S	Substrate concentration (g/L)
S_0	Initial substrate concentration (g/L)
t	Fermentation time (h^{-1})
X	Cell concentration (g/L)
X_m	Maximum cell concentration (g/L)
X_0	Initial cell concentration (g/L)
$Y_{p/s}$	Yield factor for product on cell (mg/g)
$Y_{x/s}$	Yield factor for cell on substrate (g/g)
K_s	Monod growth saturation coefficient (g/L)
μ_m	Maximum specific growth rate (h^{-1})

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Introduction

The non-protein amino acid γ-aminobutyric acid (GABA) is recognized as a principal inhibitory neurotransmitter in the mammalian central nervous system [7]. Low GABA levels in the brain are associated with several psychiatric and neurological disorders, including anxiety, depression, insomnia, and epilepsy. Clinically, GABA has also been shown to be useful for reducing blood pressure [5, 15] stimulating the immune system [26], and combating

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hypertension [7]. In addition, studies have also shown that GABA aids in relaxation, reduces anxiety, and promotes better sleep [25, 36]. Moreover, there is significant potential for GABA to be employed in functional food development. This potential has prompted researchers to explore various ways to enhance the GABA content of foods either by blending GABA directly into food production systems or by employing GRAS microorganisms to biosynthesize GABA during fermentation in food production systems. Some GABA-containing products include tea [1], red mold rice [29], germinated brown rice [30], tempeh [2], yogurt [27] and black raspberry juice [18]; however, blending GABA into food production systems is considered less acceptable to consumers. Thus, more focus has been given to the screening and exploitation of microorganisms that may be employed to produce GABA [18, 19, 33].

Fermentation is the most promising method to enhance the GABA content of food production systems. Thus, to develop effective starter cultures [40], selecting filamentous fungi that are able to produce large amounts of GABA is of prime importance. The metabolic versatility of filamentous fungi as mentioned by El-Enshasy [10] and Fazenda et al. [13] would aid the success of this study typically among *Aspergillus* species. *Aspergillus oryzae* was one of the first microbes recognized for its beneficial effects in the commercial production of foods such as *miso, hamanatto, shoyu* (soy sauce) and *tempeh.*

The fungus is known to possess a gene for glutamate decarboxylase (GAD, E.C. 4.1.1.15). GAD is a key enzyme in the biosynthesis of GABA [17] and is involved via the irreversible α-decarboxylation of ʟ-glutamic acid to γ-aminobutyric acid, which requires pyridoxal 5′-phosphate as a co-factor [20]. Because *A. oryzae* is so widely used in the production of fermented food production systems, we became interested in studying the GABA-producing capabilities of the *Aspergillus* sp. In this study, four commercial *A. oryzae* strains were obtained from *koji* samples and evaluated for their GABA-producing abilities. These investigations were conducted with the goal of enhancing GABA content in fermented foods, particularly soy sauce.

Materials and methods

Koji samples

Four *koji* samples coded *koji* NSK, *koji* NST, *koji* NSJ and *koji* NSZ were obtained from established commercial soy sauce manufacturers in Selangor, Malaysia. The green-colored *koji* samples containing *A. oryzae* strains were transported using an icebox. The master strains were subcultured and kept in Nalgene® Cryogenic Vials

(Sigma-Aldrich, Dorset, UK) and stored in −80 °C for further use. The growth of the strains was inspected regularly to avoid contamination.

Determination of GABA content in *Koji* samples

The GABA content of the *koji* samples was determined according to the method proposed by Rossetti and Lombard [31] and Yang et al. [41] with slight modification. Thirty grams of soybean *koji* were suspended in 90 mL of 50 mM phosphate buffer (pH 7.0) and blended for 10 min with a Stomacher (Stomacher® 400C Lab Blender). The suspension was kept at 40 °C for 1 h under gentle shaking (150 rpm) and centrifuged at $3000 \times g$ for 30 min at 4 °C. The supernatant was filtered through Whatman No. 2 filter paper and the suspension centrifuged again at $10,000 \times g$ for 10 min. Finally, the supernatant was filtered through a 0.22-μm pore-size nylon filter (Fisher Scientific Bishop Meadow, UK) before being analyzed with HPLC.

Isolation of *A. oryzae* strains

A sterile cotton swab was dipped into sterile distilled water before it was rolled and pressed on the surface of a *koji* sample to harvest the spores of *Aspergillus* sp. The cotton swab was then gently rolled on the surface of potato dextrose agar (PDA) and incubated at 30 °C for 2–5 days. Every colony suspected to be *A. oryzae* was transferred to a fresh PDA. The single spore isolation technique was employed to isolate pure culture fungi according to the method of Bueno et al. [3] with some modifications. A loopful of *A. oryzae* spores was grown on PDA and then transferred to a test tube containing 10 mL of sterile distilled water. The tube was shaken and poured onto a plate of 1.5 % water agar, which was prepared 24 h in advance.

The plate was rotated, and the excess liquid was poured off. It was left overnight in a slightly inclined position to allow the excess water to collect at one end. The spores were incubated at 30 °C for 5 days. The resulting pure colonies of *A. oryzae* strains were transferred to PDA medium and Czapek Yeast Agar (CYA) media slants and kept as stock cultures and at −20 °C.

Morphological characterization of *A. oryzae* strains

All of the strains were isolated and characterized for colony diameter, colony color, conidial color, mycelia color, colony texture, nature of spore masses and resting structure after 7 days of incubation at 25 °C in PDA and Czapek Yeast Extract Agar. The morphological characteristics of the isolated strains were compared with the reference strain RFS (*A. oryzae* FRR 1675), which was obtained from the Food Research Laboratory Culture Collection, North Ryde,

Australia [22, 32]. For microscopic characterization, the lactophenol blue (BD chemical) or methylene blue [37] staining process was carried out according to the method proposed by Leck [22]. An appropriate amount of each isolate was immersed in a drop of 70 % alcohol placed on the microscope slide and left to semi-dry. Then a drop of lactophenol blue was applied, and the specimen was covered with a glass slide for observation. The specimen was observed under a light microscope (Nikon Ys-100) at 40× magnification to allow the observation of microscopic structures, such as conidia, conidial head, metulae (primary stigmata) and phialides (secondary sterigmata). The entire stained sample was compared with the reference strain RFS (*A. oryzae* FRR 1675) as suggested by Sooriyamoorthy et al. [34] with some modifications.

GABA production capability of *A. oryzae* strains

A small amount of spore suspension was streaked onto the surface of a sterile potato dextrose agar (PDA) slant and incubated at 30 °C for 7 days to develop spores. To harvest the spores, an appropriate amount of a sterile aqueous solution of Tween 80 (0.001 % (v/v)) was poured into the universal bottle containing the sample and was shaken by hand for 1 min. The counting of the spores was carried out using a hemocytometer (Copens Scientific, Assistant, Germany). For inocula preparation, standardized spore suspensions (approximately 1×10^4 spores/mL medium) were used for all of the experiments. The GABA-producing capabilities of the *Aspergillus* sp. were determined using a shake flask fermentation technique. The strains (NSJ, NSK, NST and NSZ) were cultured in the fermentation broth according to the method described by Lu et al. [23] with modifications. The basal medium consisted of glucose (5 % v/v), monosodium glutamate (0.4 v/v/27.2 mM), $MgSO_4 \cdot 7H_2O$ (1 g), KH_2PO_4 (1.5 g), yeast extract (0.6 % v/v), and $CaCl_2 \cdot 2H_2O$ (2 g) in 1000 mL. The 250 mL shake flask contained a standardized 1×10^4 of spore suspension in 150 mL of medium with an initial pH of 5.5. The flask was incubated at 30 °C in a rotary shaker (Sastec ST-500R) and agitated at 200 rpm. The fermentation process was carried out for 5 days, and the GABA content was analyzed using HPLC (Shimadzu LC 20AT, Japan) according to the method described by Yang et al. [42] and Yang et al. [41] with slight modification.

Effect of cultivation time on GABA production

The effect of cultivation time on GABA production was investigated in triplicate over 10 days while the culture medium and other parameters remained the same. During fermentation, the levels of glucose, cell mass, pH, GABA and glutamic acid were monitored at various time intervals.

Molecular characterization of *A. oryzae* strain

The strain with the highest GABA production was further identified through the biomolecular technique proposed by Oliveira et al. [8] with slight modification. Biological material (50 mg) was ultrasonically homogenized in 600 μL of lysis buffer (0.1 M of Tris-hydrochloric acid pH 8.0, 0.1 M Ethylenediaminetetraacetic acid (EDTA) and 0.06 M of sodium chloride) for 10 min in a 2-mL centrifuge tube. After homogenization, 60 μL of 10 % sodium dodecyl sulfate was added, and the tube was maintained in a water bath at 60 °C for 1 h. Alcalase working solution (60 μL) was added, and the tube was maintained in a water bath at 60 °C for an additional hour. Following this, 30 μL of potassium acetate (3 M) was added, and the tube was maintained for 15 min in an ice bath (0 °C). Following that, 300 μL of chloroform was added to the tube, and it was mixed gently for 10 min. The tube was centrifuged at 9168 g for 10 min, and the supernatant was transferred to a fresh microtube. The DNA/protein concentration ratio (A_{260}/A_{280}) was determined using a spectrophotometer (U2800 Hitachi, Japan). The ITS 1 and ITS 2 ribosomal DNA of the isolate was amplified by polymerase chain reaction following the method proposed by Tamura et al. [35] and Wang et al. [39]. The primer sequences employed were 5′-TCCGTAGGTGAACCTGCGG-3′ (ITS1) and 5′-TCCTCCGCTTATTTGATATGC-3′ (ITS4). The reaction was performed in a mixture (50 μL) containing 200 μM of each of the deoxynucleoside triphosphates, 1 μM of each of the two primers, 2.5 U of *Taq* DNA polymerase, and 0.2 μg of genomic DNA. The reaction mixture was overlaid with mineral oil to avoid evaporation. PCR was carried out with an Eppendorf Mastercycler gradient (Eppendorf Hamburg, Germany) programmed for the following first five cycles: a denaturation step at 95 °C for 1 min; an annealing step at 40 °C for 2 min; an extension step at 72 °C for 3 min; 25 cycles each consisting of 95 °C for 1 min, 55 °C for 2 min, and 72° C for 3 min. These cycles were preceded by an extended denaturation for 5 min at 95 °C and concluded by a final extension for 5 min at 72 °C. The PCR products were electrophoresed on a 0.9 % agarose gel containing 2 μL of ethidium bromide in 100 mL and 1 time Tris–acetate-EDTA buffer (40 mM Tris–acetate and 2 mM EDTA). The gel was visualized under a UV transilluminator and photographed.

Development of phylogenetic tree

A phylogenetic tree was prepared by calculating the evolutionary distance (K_{nuc}) between sequences of identical fungal species using the neighbor-joining (NJ) method [14] in Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Fungi with the closest evolutionary

distance (K_{nuc}) to the isolate were classified as the same species.

Analytical methods

Determination of GABA and glutamic acid

The cell suspension was separated from the fermentation broth by filtering through Whatman No. 2 filter paper, followed by centrifugation of the suspension at $10,000 \times g$ for 10 min. The supernatant was filtered through a 0.22-μm pore-size nylon filter (Fisher Scientific, Bishop Meadow, UK). A 100-uL aliquot of supernatant (or GABA standard solution) was dried under vacuum. The residue was dissolved in 20 μL of ethanol–water-triethylamine (2:2:1 v/v) and evaporated under vacuum until dry. A 30-μL volume of ethanol–water-triethylamine-PITC (7:1:1:1 v/v/v) was added to the residue and allowed to react for 20 min at room temperature to form PTC-GABA. The excess reagent was then removed under vacuum. The dry residue containing PTC-GABA was dissolved in 200 μL of the mobile phase (a mixture of 60 % solution A (aqueous solution of 8.205 g sodium acetate, 0.5 mL triethylamine and 0.7 mL acetic acid in 1000 mL) adjusted to pH 5.8, 28 % solution B (deionized water), and 12 % solution C (acetonitrile). Gradient HPLC separations were performed on a Shimadzu LC 20AT apparatus, consisting of a pump system, a CT0-10ASVP model oven with 20-μL injection loop injector, and a Model SPD-M20A PDA detector in conjunction with a DELL model DELL Optiplex integrator. A Hypersil Gold C-18 column (250 × 4.6 mm I.D., particle size 5/um; Thermo Scientific, Meadow, UK) was used for separation purposes. The mobile phase for gradient elution was pumped at 0.6 mL/min, at room temperature; detection was monitored at 254 nm.

Determination of glucose

Glucose concentration was determined using dinitro salicylic acid (DNS) method as described Frost [14]. The DNS reagent was prepared by mixing 2-g phenol, 0.5-g sodium sulfite and 200-g potassium sodium tartrate in 700-mL distilled water. The mixture was continuously stirred before 200 mL of sodium hydroxide and 3, 5 DNS were added slowly. The reagent was stirred in a dark room for 8 h. A 10-step dilution was made on each fermentation broth to determine glucose content. Next, the following components were mixed together and boiled at 100 °C for 5 min: 1 mL of DNS reagent, 1 mL of diluted sample and 2 drops of sodium hydroxide. A 10-mL portion of distilled water was added to the solution after it was cooled under running tap water. The solution was left for 20 min at room temperature, and the absorbance was measured at 540 nm. A standard curve of absorbance versus glucose concentration in the range of 0.2–1.2 g/L was constructed. Glucose concentration was expressed as gram per litre.

Measurement of growth

The growth of the culture (expressed in dry weight of mycelium) was measured according to Wan et al. [38]. The dry cell weight (DCW) from the harvested *A. oyrzae* fermentation process was estimated by filtering a 10 mL sample through a pre-dried and weighed GF/C filter (Whatman Ltd., UK) using a Buchner funnel filter set attached to a water pump, followed by repeated washing (three times) of the mycelial biomass with distilled water, Mili-Q® Advantage A10 (Millipore, Bedford, MA, USA). The mycelial filter cake was pre-dried for 2 min in an overnight dryer or a microwave oven (650 W). Next, it was cooled in a desiccator for 24 h before weighing. Calculation of the DCW was done by subtraction of pre-weighed filter mass from the mass with the filtrate and multiplied by the dilution factor to get DCW in gram per litre. All values are taken based on averages of a least three independent trials.

Determination of GAD enzymes activity

GAD enzyme activity of the isolates (*A. oryzae* NSK, NSJ, NST and NSZ) was determined according to the method proposed by Lu et al. [23]. One unit (U) of GAD enzyme activity from the pellets (mycelia) was defined as the formation of 1 μmol of GABA per hour at 37 °C and the results were expressed as Units/g cells.

Growth kinetics

To better describe the growth kinetics, some stoichiometric parameters related to batch fermentation have been used to evaluate the growth of *A. oryzae* strains NSK, NSZ, NSJ and NST. Maximum specific growth rate (U_{max}) is described by Eq. (1) and can be obtained by plotting ln ($X_t - X_0$) against time. The resulting slope, U_{max}. X_t refers to the cell concentration at a given time and X_i is the initial cell concentration.

$$U_{max} = \frac{\ln(X_t - X_i)}{t} \tag{1}$$

The growth yield coefficient was determined by the following equation:

$$Y_{x/s} = \frac{(X_{max} - X_i)}{(S_i - S_0)} \tag{2}$$

where S_i is the initial substrate concentration (g/L) and S_0 is the residual substrate concentration at the end of fermentation.

In addition, the yields $Y_{p/s}$ and $Y_{p/x}$ are determined by measuring the maximum GABA and cell concentrations and the total amount of substrate consumed.

$$Y_{p/s} = \frac{(P_{\max} - P_i)}{(S_i - S_0)} \quad (3)$$

and

$$Y_{p/x} = \frac{(P_{\max} - P_i)}{(X_{\max} - X_i)} \quad (4)$$

$P_{\max}$ in this equation refers to the maximum product concentration, and P_i is the initial product concentration. Overall productivity, P (mg/L/h), was determined by measuring the total amount of biomass formed over a period of time.

$$P = \frac{(X_{\max} - X_i)}{T} \quad (5)$$

Statistical analysis

All analysis were conducted in triplicate and the respective mean $\pm$ SD determined using the software, Statistical Analysis System (SAS University Edition, 2012) and shown as error bars. If the error bars do not appear, it is assumed that they are smaller than the size of the symbol. A *t*-test was used for the plotting of fermentation graphs. One-way ANOVA and post-test (Duncan's new multiple range test, MRT) were used for kinetic parameters comparison.

Results and discussion

Morphological characteristics of *A. oryzae*

The morphological characteristics of the isolated strains were compared with the reference strain, RFS. The results revealed that all of the isolates shared similar features in terms of the colony, conidia and mycelia color, nature of spore mass, colony diameter, conidial head, and vesicle structure (observed after 7 days of incubation at 25 °C on PDA and CYA). In addition, there were no significant differences between the *A. oryzae* isolates and the reference strain. Further microscopic examination showed that the conidial heads and vesicle within the conidiophores of the strains were in radiate and sub-globose forms; however, the type of phialides and conidia formed on the surface of *A. oryzae* NSK vesicle was significantly different from the other strains (NSJ, NSZ, NST) and from the reference strain. *A. oryzae* NSK phialides were classified as uniseriate, while its conidia were in the form of sub-globose. These findings are in agreement with the features described by Samson et al. [32]. Specifically, it was found that the conidiophores structure of *A. oryzae* exist in two different

forms. Phialides that are born directly on the vesicle surface are called uniseriate, while phialides that are born on the top of metulae in conidiophores structure are called biseriate. The morphological features of *A. oryzae* NSK, *A. oryzae* NSJ, *A. oryzae* NSZ and *A. oryzae* NST were in agreement with Samson et al. [32], Sooriyamoorthy et al. [34] and Elbashiti et al. [11]; therefore the entire strains were identified as *A. oryzae* sp.

GABA concentration of *Koji* samples

It has been reported that *A. oryzae* possess the glutamate decarboxylase (GAD) gene, which is capable of producing γ-aminobutyric acid (GABA) [17] by irreversible decarboxylation of glutamic acid. It has also been postulated that the presence of GABA in certain fungi-derived fermented foods could be the results of GABA-biosynthesizing activity of the fungi present. Hence, in this study, four commercial *koji* samples were analyzed for GABA content. The results revealed that all of the *koji* samples contained appreciable amounts of GABA (223–443 mg/kg). Since only fungal species are able to survive in low water activity of solid state *koji* fermentation, the presence of GABA in all the samples strongly indicated that GABA was biosynthesized by the fungi during *koji* fermentation. As the raw material (soybean) for *koji* contains substantial amounts of protein and carbohydrate, these substrates could have been hydrolyzed by the endogenous and exogenous enzymes present in the fungus. The fungus may have converted these proteins and carbohydrates into simple building blocks that were subsequently converted into GABA by the naturally present glutamate decarboxylase enzyme produced by the *A. oryzae*.
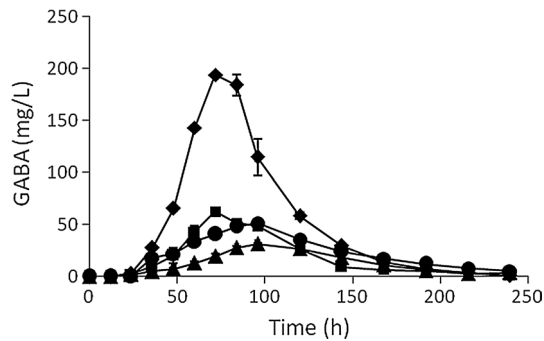
GABA-producing capabilities of A. oryzae strain isolates

A. oryzae strain isolates were further examined for GABA-producing capability by culturing the isolates in broth media containing 0.4 % (w/w) of glutamic acid. Spore suspension was used as inoculum because it may be simply prepared and allows for consistent inoculum size. The fermentation process was carried out for 5 days; all of the strains showed rapid growth after they were subcultured on PDA and CYA. As shown in Table 1, the batch fermentation showed that *A. oryzae* NSK produced the highest GABA concentration (73.13 ± 1.77 mg/L). The GABA concentrations produced by *A. oryzae* NSJ (29.87 ± 1.80 mg/L), *A. oryzae* NST (25.75 ± 25.75 mg/L) and *A. oryzae* NSZ (26.78 ± 2.17 mg/L) were all similar and significantly ($P < 0.05$) lower than *A. oryzae* NSK. The results also showed that the glutamic acid concentrations in the *A. oryzae* NST and *A. oryzae* NSJ fermentation broths

Table 1 GABA-producing capability of *A. oryzae* (NSK, Z, J and T) isolates from soy sauce *koji*

Compound	*A. oryzae* strain			
	NSK	NST	NSJ	NSZ
γ-Aminobutyric acid (mg/L)	73.13 ± 1.77^a	25.75 ± 1.40^b	29.87 ± 1.80^b	26.78 ± 2.17^b
Remaining glutamic acid (mg/L)	8.82 ± 1.97^b	122.01 ± 4.12^a	110.25 ± 6.26^a	29.43 ± 5.04^b

* Data are mean ± standard deviation values of three determinations. Data in a column followed by the same superscript letter are not significantly different ($P < 0.05$)

Fig. 1 Comparison of GABA-producing abilities of *A. oryzae* NSK (*filled diamond*), NSZ (*filled square*), NSJ (*filled circle*) and NST (*filled triangle*) strains in batch fermentation

remained at 122.01 and 110.25 mg/L; however, the glutamic acid concentrations in the *A. oryzae* NSK and *A. oryzae* NSZ samples were 8.82 and 29.4 mg/L, respectively. It can be concluded that all the *A. oryzae* isolates possessed GABA-producing activity albeit at different levels.

Effect of cultivation time on GABA production

All the strains were further evaluated for their ability to produce GABA during 10 days of fermentation by cultivating the isolates in media containing glucose (5 % v/v), monosodium glutamate (0.4 v/v/27.2 mM), $MgSO_4 \cdot 7H_2O$ (1 g), KH_2PO_4 (1.5 g), yeast extract (0.6 % v/v), and $CaCl_2 \cdot 2H_2O$ (5.5 g). The samples were held at a constant

temperature of 30 °C and an initial pH 5.5; they were subjected to batch fermentation using 250-mL shake flasks. During fermentation, the behavior of all the isolates was monitored for GABA formation, substrate consumption (glutamic acid and glucose), cell formation and change in pH. The results are shown in Fig. 1 and are summarized in Table 2. GABA was generally produced at the maximum level either during the exponential phase or the stationary phase. As shown in Table 2, all the *A. oryzae* isolated strains showed a similar growth pattern and maximum cell concentration (X_{max}); however, these strains had different glucose and glutamic acid consumption rates ($Y_{x/s}$) even though glucose and glutamic acid were consumed rapidly by all the *A. oryzae* strains and depleted after 2–4 days of fermentation.

However, the calculated $Y_{x/s}$ values showed that *A. oryzae* NST consumed the largest amounts of glucose and glutamic acid for each gram of cells (0.2664 g/g) compared to *A. oryzae* NSJ (0.2639 g/g), *A. oryzae* NSZ (0.2614 g/g) and *A. oryzae* NSK (0.2596 g/g). Exhaustion of glucose and glutamic acid was observed to be the main contributor to their short log phases. The compounds such as glucose and glutamic acid as carbon and nitrogen sources, respectively, are the crucial elements required by microorganisms to synthesize the amino acids and proteins for cell growth and energy maintenance as well as the production of metabolites during fermentation [4, 12, 24, 28]. For food processing and fermentation processes, fungi that consume the smallest amount of substrate while producing the largest amount of desirable end- products are preferable.

Table 2 Comparison of GABA biosynthesizing activity of *A. oryzae* (NSK, Z, J and T) in batch fermentation using 250 mL shake flask

Kinetic parameter	*A. oryzae* strain			
	NSK	NST	NSJ	NSZ
P_{max} (mg/L)	194 ± 2.21^a	31.66 ± 0.97^d	51.53 ± 1.20^c	62.65 ± 2.21^b
X_{max} (g/L)	14.010 ± 0.65^a	14.380 ± 0.227^a	14.246 ± 0.253^a	14.056 ± 0.477^a
U_{max} (h^{-1})	0.0336^b	0.0284^c	0.0277^d	0.0345^a
$Y_{p/x}$ (mg/g)	13.61^a	2.20^d	3.62^c	4.43^b
$Y_{p/s}$ (mg/g)	3.59^a	0.59^d	0.96^c	1.16^b
$Y_{x/s}$ (g)	0.2596^d	0.2664^a	0.2639^b	0.2614^c
Productivity (mg/L/h)	2.69^a	0.33^d	0.54^c	0.87^b

* Data are mean ± standard deviation values of three determinations. Data in a column followed by the same superscript letter are not significantly different ($P < 0.05$)

It is clear that *A. oryzae* NSK is significantly different from the other strains in terms of GABA yield and substrate consumption. A Duncan Multiple Range Test showed that *A. oryzae* NSK not only used less substrate (0.2596 g/g) but also synthesized the highest amount of GABA (194 mg/L) compared to the other strains. Overall, *A. oryzae* NSK produced 3.59 mg GABA per gram of substrate ($Y_{p/s}$) used, and each gram of NSK cells yielded 13.61 mg of GABA ($Y_{p/x}$). Therefore, *A. oryzae* NSK was found to be the most productive strain in terms of GABA production compared to *A. oryzae* NSZ, NSJ and NST. It has also been hypothesized that GABA production is related to the formation of conidia. This was reported by Kumar et al. [21], who observed high levels of GAD enzyme in mature conidia compared to mycelia.

The medium used may have favorably stimulated the formation of *Aspergillus* sp NSK conidia to an optimum level, which subsequently generated more GABA. It was also observed that, during the production of GABA, the pH became more acidic, and GABA was still produced even after the pH increased. The increase and decrease in pH occurred for all of the strains tested. The fluctuation in pH is probably due to the generation of ammonia by the deamination of amino acids derived from medium composition [16]. In addition to pH, time also affected the rate of GABA production. Normally, maximum GABA production was achieved after 72–96 h of fermentation [6]. For *A. oryzae* NSK and NSZ, the maximum concentrations of GABA were reached after 72 h, while *A. oryzae* NSJ and NST reached their highest levels after 96 h.

This result was due to high GAD enzyme activity of *A. oryzae* NSK (26.10 units/g cells), which led to higher GABA production compared to *A. oryzae* NSZ (8.44 units/g cells), *A. oryzae* NST (3.23 units/g cells) and *A. oryzae* NSJ (5.54 units/g cells) after 72–84 h of fermentation times. High GAD enzyme activity aided in the rapid conversion of supplemented glutamic acid to GABA before being gradually utilized as a source of nitrogen to support the increasing cell growth. After GABA achieved maximum levels, its concentration decreased naturally; GABA was no longer detected after 240 h for all *A. oryzae* strains investigated. The increase and decrease in GABA concentration over time could be related to the status of nitrogen levels present during fermentation in which glutamic acid supply is not only required for GABA production but also for new cell development and maintenance.

Depletion of glutamic acid after 72 and 96 h caused the isolate to utilize its own secondary metabolites i.e. GABA by catabolizing the GABA produced via the GABA shunt where GABA is transaminated to succinic semialdehyde (SSA) and succinate by 4-aminobutyrate transaminase (GABAT) and succinic semialdehyde dehydrogenase to produce α-ketoglutarate following the TCA cycle [9]. The
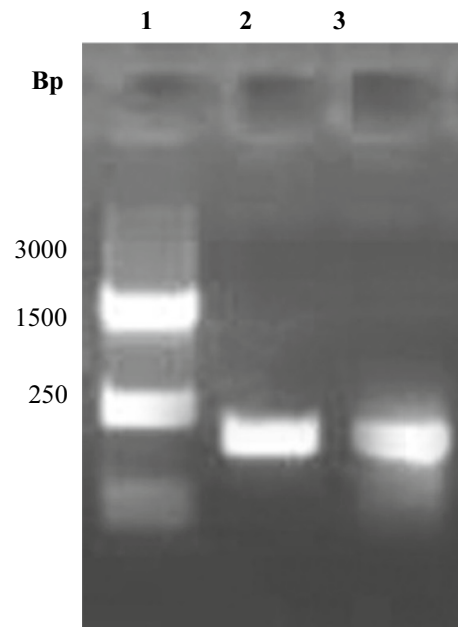

Fig. 2 Ethidium bromide fluorescence image showing electrophoresis of *A. oryzae* NSK PCR product. The gel consists of 0.9 % agarose and run with 1 % TE buffer at 80 V. *Lane 1* 3 kb DNA marker; *lanes 2 and 3* PCR product

release of both enzymes is under the control of UGA1 and two genes [9]. After the removal of α-ketoglutarate amino group by the transamination steps, the ammonia formed is undergoing the nitrification process before being absorbed as a nitrogen source in the form of nitrate [21]. The catabolism process to release nitrogen compound from GABA is thermodynamically unfavorable since it involves an extensive and complex pathway i.e.: GABA shunt and TCA cycle; hence, GABA is not utilized immediately after it has been produced [20, 21].

Molecular characteristics of *A. oryzae* strain NSK

A. oryzae NSK was selected as the best GABA-producing strain for biomolecular identification and construction of the genetic tree. Therefore, genomic DNA of *A. oryzae* NSK was extracted, and a polymerase chain reaction process was conducted to amplify the partial region of *A. oryzae* NSK rDNA using ITS 1 and ITS 4 primers. The amplified sequence was a combination of two complete genes of 5.8S ribosomal RNA, an internal transcribed spacer 2 and two partial sequences of an internal transcribed spacer 1 and 28S ribosomal RNA. From the result, a product of approximately 1200 bp was successfully amplified (Fig. 2), sequenced and aligned with the related species based on NCBI BLAST. *A.oryzae* NSK sequence was found to be similar to *A. oryzae* and *A. flavus* with 99 % similarity. Subsequently, the evolutionary distance (K_{nuc}) between
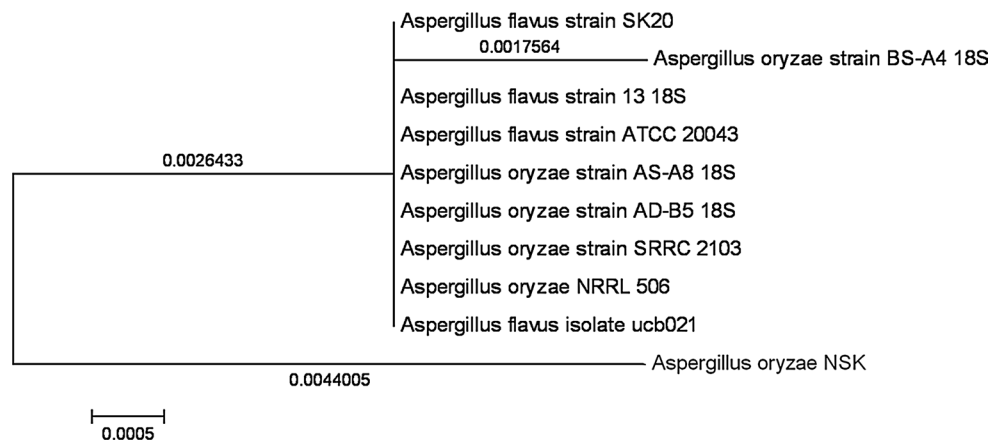
Fig. 3 Neighbor-joining phylogenetic tree showing the relationships of *Aspergillus* sp. isolate (strain NSK) and related species based on 18S rRNA gene sequences. *Aspergillus flavus* strain SK20 = DQ467973.1, *Aspergillus oryzae* strain BS-A4 = HQ285531.1, *Aspergillus flavus* strain 13 = DQ467970.1, *Aspergillus flavus* strain ATCC 20043 = AY939782.1, *Aspergillus oryzae* strain AS-A8 = HQ285525.1, *Aspergillus oryzae* strain AD-B5 = HQ285522.1, *Aspergillus oryzae* strain SRRC 2103 = AY373857.1, *Aspergillus oryzae* NRRL 506 = AF459735.1, *Aspergillus flavus* isolate ucb021 = EF409787.1. *Bar* 0.0005 K_{nuc}

sequences of identical fungal species was calculated using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 for further identification [35]. A phylogenetic tree was prepared using the neighbor-joining (NJ) method [35] from the K_{nuc} data using the same software (Fig. 3). The K_{nuc} values showed that the isolate was closely related to the *A. oryzae* BS-A4 compared to the *A. flavus* strain. The morphological characteristics confirmed the identity of this fungus as belonging to *A. oryzae* (strain NSK).

Conclusion

In this study, we evaluated the GABA-producing abilities of four commercial strains of *Aspergillus* sp, namely *A. oryzae* NSK, NSJ, NST and NSZ isolated from *koji*. All the strains shared similar morphological characteristics in terms of colony color, texture and diameter as well as mycelia color, conidial color, conidial head structure and vesicle structure; however, they had different GABA-producing capabilities; highest GABA productivity was demonstrated by *A. oryzae* NSK compared to *A. oryzae* NSJ, NSZ and NST. Molecular identification was performed to confirm the *A. oryzae* NSK species and to construct its phylogenetic tree. The results showed that the isolated DNA scored the highest percentage of sequence coverage's similarity (77 % coverage) to the *A. oryzae* strain FH4 (EU409806.1). Further analysis was conducted by calculating the evolutionary distance (K_{nuc}) between sequences using Molecular Evolutionary Genetics Analysis (MEGA); the results confirmed that the isolate belonged to the species *A. oryzae*. The GABA-production potential of *A. oryzae* NSK can be further improved through

optimization of its physical (pH and temperature) and nutritional conditions (carbon and nitrogen sources). These improvements may allow the development of an innovative functional food material rich in GABA particularly soy sauce.

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