Vital parameters for high gamma-aminobutyric acid (GABA) production by an industrial soy sauce koji Aspergillus oryzae *NSK in submerged-liquid fermentation*

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Food Science and Biotechnology

ISSN 1226-7708

Food Sci Biotechnol
DOI 10.1007/s10068-019-00602-y

Springer

Food Sci Biotechnol
https://doi.org/10.1007/s10068-019-00602-y

Vital parameters for high gamma-aminobutyric acid (GABA) production by an industrial soy sauce *koji Aspergillus oryzae* NSK in submerged-liquid fermentation

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Received: 12 September 2018 / Revised: 4 March 2019 / Accepted: 11 March 2019

Abstract In submerged-liquid fermentation, seven key parameters were assessed using one-factor-at-a-time to obtain the highest GABA yield using an industrial soy sauce *koji Aspergillus oryzae* strain NSK (AOSNSK). AOSNSK generated maximum GABA at 30 °C (194 mg/L) and initial pH 5 (231 mg/L), thus was able to utilize sucrose (327 mg/L of GABA) for carbon source. Sucrose at 100 g/L, improved GABA production at 646 mg/L. Single nitrogen sources failed to improve GABA production, however a combination of yeast extract (YE) and glutamic acid (GA) improved GABA at 646.78 mg/L. Carbon-to-nitrogen ratio (C8:N3) produced the highest cell (24.01 g/L) and GABA at a minimal time of 216 h. The key parameters of 30 °C, initial pH 5, 100 g/L of sucrose, combination YE and GA, and C8:N3 generated the highest GABA (3278.31 mg/L) in a *koji* fermentation. AOSNSK promisingly showed for the development of a new GABA-rich soy sauce.

Keywords *Aspergillus oryzae* · Gamma-aminobutyric acid (GABA) · Soy sauce *koji* · Glutamic acid · GABA-enriched foods

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List of symbols

P_{max}	Maximum GABA concentration (mg/L)
t	Fermentation time (h^{-1})
X	Maximum cell concentration (g/L)
X_m	Maximum cell concentration (g/L)
$Y_{p/s}$	Yield factor for product on cell (mg/g)
μ_m	Maximum specific growth rate (h^{-1})
P	Overall productivity (mg/L/h)

Introduction

There have been extensive researches on fermentation using fungal species of Basidiomycetes (Wan-Mohtar et al., 2016a; 2016b) and Ascomycetes (Abdeshahian et al., 2010) to produce active compounds. An ascomycete, namely *Aspergillus* species have been broadly used in fermentation industries for large-scale production of enzymes (Kreiner et al., 2003), as well as other proteins (El-Enshasy et al., 2006), and are observed as idyllic hosts

Published online: 22 March 2019

for the active proteins production of eukaryotic origins that cannot be achieved with *E.coli* (Machida et al., 2005). *Aspergillus oryzae*, a prevalent filamentous mould, has normally been utilized for miso production (soy bean paste), sake (a rice-derived alcoholic beverage), su (vinegar) and a range of other fermented foods including soy sauce in South East Asian countries (Machida et al., 2005; Roohinejad et al., 2011; Tsushida et al., 1987). In fact, *A. oryzae* is considered as a fungus of prime importance for the soy sauce manufacture, particularly in Japan and China (Aoki et al., 2003; Tsushida et al., 1987; Wang et al., 2006). The soy sauce manufacturing process involves fermentation of soybean to produce a unique taste and aroma via the action of proteolytic enzymes from *A. oryzae* which enzymatically break down the protein into peptides (Abe et al., 2006). Other key metabolites are also produced by *A. oryzae* during fermentation, as the fungus expresses a gene capable of biosynthesizing γ-aminobutyric acid (GABA) (Kato et al., 2002).

A non-protein amino acid called GABA acts as the brain main inhibitory neurotransmitter. It is extensively dispersed in the central nervous system, and GABA deficiency in humans can lead to hypertension, migraine, diabetes, insomnia, anxiety and epilepsy (Inoue et al., 2003). As evident from the literature (Komatsuzaki et al., 2005; Siragusa et al., 2007), there is continuing interest among researchers to increase high-GABA foods, mostly via employing lactic acid bacteria.

In *A. oryzae*, glutamate decarboxylase (GAD) catalyzed GABA formation by the permanent L-glutamate α-decarboxylation in the Krebs cycle. Although culture conditions to improve GABA production have been studied extensively by various researchers using lactic acid bacteria, information on the key parameters of GABA production particularly using fungus such as *A. oryzae*, has not been reported. Our previous work proved the GABA-producing abilities of an industrial *koji A. oryzae* strain NSK (Ab Kadir et al., 2016; Hajar-Azhari et al., 2018), however the potential of this strain can be enhanced by physical (temperature and pH) and nutritional conditions (nitrogen and carbon sources) optimization. In this paper, seven selected key parameters were chosen for analyzing GABA-producing potential of *A. oryzae* NSK in submerged-liquid fermentation (SLF) include incubation temperature, initial pH, carbon sources, initial sucrose concentrations, nitrogen sources, and carbon to nitrogen ratio. These enhancements can be used to develop a novel GABA-rich soy sauce.

Materials and methods

Koji strain and spore preparation

A. oryzae strain NSK [AOSNSK] (JN 381021) isolated from a *koji* sample was previously identified (Ab Kadir et al., 2016) and used for the production of GABA. A total of 500 mg of the green coloured *koji* (Fig. 1A) samples containing a large number of spores were stored in a 500 mL glass jar and preserved in a box containing ice cubes for further use. For spore production, potato dextrose agar (PDA) was used for the agar slants (AS) preparation. A loopful of spore was inoculated into AS from the stock culture before incubation at 30 °C for 7 days to allow the development of spores. Spores produced by AOSNSK were harvested using a sterile Tween 80 [0.001% (v/v)] and counted using a haemocytometer (Copens Scientific, Assistent, Germany). A standardized spore suspension of approximately 10^4 spores/mL was used in all experiments.

Characterization of *A. oryzae* NSK *koji* strain

The isolated AOSNSK was identified for colony diameter, conidial colour, colony colour, colony texture, mycelial colour, spore masses and restructure at 7-day incubation in Czapek Yeast Extract Agar (CYA) and PDA at 25 °C. An *A. oryzae* FRR 1675 (reference strain RFS) acquired from North Ryde, Australia (Food Research Laboratory Culture Collection) (Pitt and Hocking, 1985; Robert et al., 2002) was used to compare with AOSNSK. Microscopically, the fungus was stained using lactophenol blue (BD Chemical) (Leck, 1999). Alcohol (70%) was dropped on the isolates fruiting structure and placed on the glass slide before being left to semi-dry. After that, lactophenol blue was dropped on the specimen and closed using a cover slip for observation. A light microscope (Nikon Ys-100) was used for observation at 40× magnification for microscopic characteristics, such as conidial head, conidiophores, conidia and vesicle structure. The reference strain RFS was compared with the stained samples (Sooriyamoorthy et al., 2004) with some modifications. The identification key for common *Aspergillus sp.* and teleomorphs was used to identify the isolates (Pitt and Hocking, 1985). The strains obtained were inoculated on CYA and incubated for 7 days at 30 °C (Robert et al. 2002). After the incubation period, all *Aspergillus sp.* were transferred to *Aspergillus flavus Paraciticus* agar (AFPA) in triplicate and incubated for 7 days at 30 °C and analyzed for reverse colour following a modified Pitt and Hocking (1985) method. As noted, the highest GABA producing AOSNSK was previously identified via molecular biological technique (Ab Kadir et al., 2016).

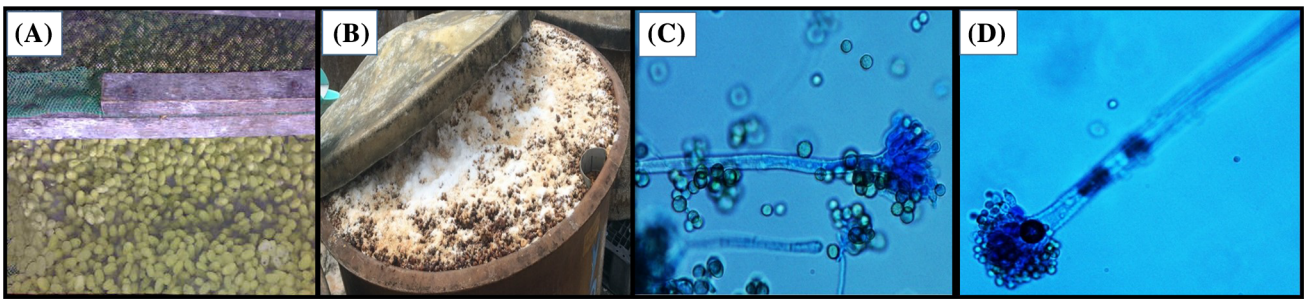

Fig. 1 Commercial soy sauce production showing (**A**) *koji*, (**B**) *moromi*, (**C**) the isolated *A. oryzae* strain NSK and (**D**) the reference strain RFS (*A. oryzae* FRR 1675)

Fermentation and seven key parameters

Batch fermentation was done in 250-mL Erlenmeyer flasks containing 150 mL prepared medium which consisted of monosodium glutamate (0.4% v/v), glucose (5% v/v), $MgSO_4{\cdot}7H_2O$ (1 g), KH_2PO_4 (1.5 g), $CaCl_2{\cdot}2H_2O$ (2 g/L) and yeast extract (0.6% v/v). The mixture was adjusted to pH 5.5 before being inoculated with 10^4 spores/mL suspension. The flask was incubated in a rotary shaker at 30 °C and 200 rpm agitation.

Effect of temperature

Initially, different temperatures at 25, 30, 35, 40 and 45 °C were tested for GABA production by AOSNSK. Other parameters including medium composition, inoculum concentration and agitation speed were fixed.

Effect of initial pH

The effect of initial pH (3.0, 4.0, 5.0, 6.0 and 7.0) on GABA production was studied through inoculating AOSNSK while medium composition, inoculum concentration, temperature and agitation remained fixed.

Effect of different carbon sources

Carbon sources of lactose, glucose, sucrose, fructose and maltose were studied by implementing the optimum temperature and pH for GABA production by AOSNSK.

Effect of different sucrose concentrations

The initial sucrose concentrations of 50 g/L were substituted with 40, 60, 80, 100 and 120 g/L to test their responses on GABA production by AOSNSK. Water activity in the fermentation broth was measured using a water activity meter (Legacy AQUALAB Series 3, METER Group, WA, USA). Three millilitre samples were placed in a disposable cup and gently shaken to cover the bottom part completely before placing it in the instrument compartment. The broth was measured three times at 30 °C and the results were recorded.

Effect of single nitrogen sources

GABA production of AOSNSK was further improved by studying the effect of single nitrogen sources on the optimized temperature, pH, carbon source, and carbon source concentrations.

Effect of different combinations of nitrogen sources

A combination of two nitrogen sources was selected when the individual nitrogen sources failed to enhance the GABA production by AOSNSK. The combinations were soybean:glutamic acid, soybean:$NaNO_3$, malt extract:glutamic acid, malt extract:$NaNO_3$ and yeast extract:glutamic acid using the previously optimized conditions.

Effect of different carbon-to-nitrogen ratios

GABA production of AOSNSK was finally improved through the study on the effect of different nitrogen sources. These concentrations were balanced by reducing the optimal levels of carbon concentration (10% w/v) and increasing nitrogen concentration (1% w/v), with a final concentration (carbon and nitrogen) of 11% w/v.

Analytical methods

Determination of glutamic acid and GABA

The fermentation culture broth was filtered using a filter paper (Whatman No. 2) and separated using 10,000×*g* of centrifugation for 10 min. A nylon filter at 0.22-μm of pore-size (Fisher Scientific, Bishop Meadow, UK) filtered the resulting supernatant, which later dried under vacuum. Next, the resulting residues was liquefied in 20 μL of ethanol–water–triethylamine (2:2:1 v/v) and vacuum-

dried. A 30-µL of PITC (ethanol–water–triethylamine) (7:1:1:1 v/v/v) was mixed with the residue and reacted to form PTC-GABA for 20 min at room temperature. The excess substance was removed under vacuum and dried PTC-GABA residue was dissolved in 200 µL of the mobile phase (60% solution A, an aqueous solution of 0.5 mL trimethylamine, 8.205 g sodium acetate, and 0.7 mL acetic acid in 1000 mL) adjusted to pH 5.8, 12% solution C (acetonitrile), and 28% solution B (deionized water). Separations of gradient HPLC were achieved using a Shimadzu LC 20AT apparatus (20-µL injection loop injector), consisting of a model oven (CT0-10ASVP) with a pump system, and a DELL Optiplex integrator with a Model SPD-M20A PDA detector. A specific separation column (Hypersil Gold C-18, 250X4.6 mm I.D., particle size 5/µm; Thermo Scientific, Meadow, UK) was used in the process. Gradient elution at 0.6 mL/min was used for mobile phase at room temperature and 254 nm detection.

Glucose determination

The concentration glucose of was analyzed using dinitro salicylicacid (DNS) technique (Frost, 2004). The DNS reagent was consisted of 200-g potassium sodium tartrate, 0.5-g sodium sulfite, 2-g phenol and in 700-mL distilled water. The mix was continuously stirred before 200 mL of sodium hydroxide and 3, 5 DNS slow addition. The mixture was stirred in a dark room for 8 h. Each fermentation broth was subjected to 10-step dilution for glucose determination. Subsequently, 1 mL of DNS reagent, 1 mL of diluted sample and 2 drops of sodium hydroxide were mixed together and boiled at 100 °C for 5 min. A 10-mL of distilled water was transferred to the solution after tap-water cooling. The solution was left at room temperature for 20 min for 540 nm absorbance determination. In the range of 0.2 to 1.2 g/L, a standard curve versus glucose concentration (expressed as g/L) was created.

Measurement of dry cell weight

The dry cell weight (DCW) of AOSNSK fermentation process was measured (Wan-Mohtar et al., 2016a) by 10-mL sample filtration via a pre-weighed GF/C filter (Whatman Ltd., U.K). The mycelial biomass was repeatedly washed (three times) with distilled water, Mili-Q® Advantage A10 (Millipore, Bedford, MA, USA) in a Buchner funnel filter set. The mycelial filter cake was predried in a food dryer for 2 h. DCW was calculated by preweighed filter subtraction from the filtrated mass and multiplied by the dilution factor in g/L of a least three independent trials.

Growth kinetics

Growth kinetics were described using the some stoichiometric parameters related to of AOSNSK batch fermentation (Hajar-Azhari et al., 2018). Maximum specific growth rate (U_{max}) is defined through Eq. 1:

$$Umax = \frac{\ln(Xt - Xi)}{t} \quad (1)$$

and can be attained by plotting ln ($X_t - X_0$) against time. The yields $Y_{p/s}$ was calculated by maximum GABA and cell concentrations measurement and the total amount of substrate consumed as in Eq. 2:

$$Yp/s = \frac{(Pmax - Pi)}{(Si - S0)} \quad (2)$$

P_{max} refers the maximum product concentration while P_i is the initial product concentration. Overall productivity, P (mg/L/h), was calculated by substracting the total amount of biomass produced over time as in Eq. 3:

$$P = \frac{(Xmax - Xi)}{T} \quad (3)$$

Statistical analysis

The analyses were performed in triplicate and respective mean ± S.D by using Statistical Analysis System (SAS University Edition, 2012). For kinetic parameters comparison, one-way ANOVA and post-test (Duncan's new multiple range test, MRT) were used.

Results and discussion

A. oryzae strain NSK characteristics

AOSNSK characteristic was compared morphologically with the reference strain RFS (Fig. 1D). The isolated strain revealed that it shared similar features in terms of the nature of spore mass, vesicle structure, colony, colony diameter, conidia and mycelia colour, and conidial head after a 7-day incubation at 25 °C on CYA and PDA. The results showed that there were no significant differences between the reference strain and AOSNSK isolates. Additional microscopic inspection revealed that the vesicle and conidial heads within the strains conidiophores were in sub-globose and radiate forms; though, the type of conidia and phialides formed on AOSNSK vesicle surface was significantly different from the reference RFS strain. AOSNSK phialides were classified as uniseriate, while its conidia were in the form of sub-globose. These discoveries are supported with the features explained by Samson et al.

(2004). Specifically, it was found that the *A. oryzae* conidiophores structure exist in two different forms. Phialides that are originated on the metulae top in conidiophores are named biseriate, while uniseriate are born directly on the vesicle surface of phialides. AOSNSK morphological features was supported by other recognized *A. oryzae* sp. by Samson et al. (2004), Sooriyamoorthy et al. (2004) and Zhong et al. (2018).

Effect of temperature and medium initial pH on GABA production

The GABA kinetic parameters produced in batch fermentation by AOSNSK in different incubation temperatures was evaluated in Table 1A. The highest GABA production was achieved at 30 °C (194 mg/L) followed by 35 °C (171.26 mg/L), 25 °C (107.27 mg/L), and 40 °C (77.24 mg/L) after 72 h of fermentation except the condition of 20 °C (60.79 mg/L) after 48 h. Aside from GABA, a rapid growth of AOSNSK was also observed at an incubation temperature of 30 °C. As a result, the highest cell concentration (14.01 g/L) and maximum specific growth rate (μ_x= 0.034) were achieved after 72 h of fermentation. At incubation temperatures of 20, 25 35 and 40 °C, AOSNSK only produced 6.13, 7.50, 12.11 and 8.29 g/L of cells and a maximum specific growth rate of 0.022, 0.024, 0.030 and 0.027 1/h. Growth in GABA concentration and incubation temperature was observed to affect the patterns of substrate consumption by AOSNSK significantly. The rates of substrate uptake were proportional to GABA concentration and cell growth at different incubation temperatures. Only 2.22 g of glucose and 0.18 g

Table 1 Comparison of kinetic parameter values of GABA produced in batch fermentation by *A. oryzae* strain NSK with different incubation temperatures (A) and initial pH (B)

Kinetic parameter (A)	Incubation temperature (°C)				
	20	25	30	35	40
Maximum cell concentration, X (g/L)	6.13 ± 0.04^{e}	7.50 ± 0.36^{d}	14.01 ± 0.65^{a}	12.11 ± 0.20^{b}	8.29 ± 0.17^{c}
Maximum specific growth rate, μ (1/h)	0.022 ± 0.0017^{e}	0.024 ± 0.0011^{d}	0.034 ± 0.0015^{a}	0.030 ± 0.0016^{b}	0.027 ± 0.0021^{c}
Maximum GABA concentration, P_{max} (mg/L)	60.79 ± 4.48^{e}	107.27 ± 5.91^{c}	193.81 ± 2.21^{a}	171.26 ± 3.97^{b}	77.24 ± 3.52^{d}
Glucose (g glucose/g cell)	8.02 ± 0.031^{a}	6.61 ± 0.041^{b}	2.22 ± 0.029^{e}	2.57 ± 0.035^{d}	5.17 ± 0.022^{c}
Glutamic acid (g glutamic acid/g cell)	0.60 ± 0.0063^{a}	0.45 ± 0.0052^{b}	0.18 ± 0.0019^{e}	0.22 ± 0.0015^{d}	0.40 ± 0.0071^{c}
Product yield, Y*p/s* (g GABA/g substrate)	0.0011 ± 0.000082^{e}	0.002 ± 0.00011^{c}	0.0036 ± 0.0002^{a}	0.0031 ± 0.000074^{b}	0.0014 ± 0.000065^{d}
Overall productivity, P (mg/L/h)	0.84 ± 0.062^{e}	1.50 ± 0.082^{c}	2.69 ± 0.03^{a}	2.38 ± 0.055^{b}	1.07 ± 0.049^{d}
Production time, t (h)	48	72	72	72	72
Kinetic parameter (B)	Initial pH				
	pH 3	pH 4	pH 5	pH 6	pH 7
Maximum cell concentration, X (g/L)	13.42 ± 0.20^{d}	13.82 ± 0.35^{c}	15.19 ± 0.09^{a}	14.96 ± 0.18^{b}	14.96 ± 0.15^{b}
Maximum specific growth rate, μ (1/h)	0.030 ± 0.0010^{d}	0.031 ± 0.0013^{c}	0.033 ± 0.0012^{a}	0.032 ± 0.0013^{b}	0.029 ± 0.0015^{e}
Maximum GABA concentration, P_{max} (mg/L)	21.00 ± 2.36^{d}	117.46 ± 17.66^{c}	230.83 ± 5.36^{a}	169.54 ± 5.76^{b}	117.79 ± 12.57^{c}
Glucose (g glucose/g cell)	2.63 ± 0.044^{a}	2.18 ± 0.052^{b}	1.78 ± 0.027^{e}	1.91 ± 0.013^{d}	2.11 ± 0.032^{c}
Glutamic acid (g glutamic acid/g cell)	0.24 ± 0.015^{a}	$0.22^{c} \pm 0.0059$	$0.20^{e} \pm 0.0082$	$0.21^{d} \pm 0.0073$	$0.23^{b} \pm 0.014$
Product yield, Y*p/s* (g GABA/g substrate)	0.00039 ± 0.000044^{d}	0.0022 ± 0.00018^{c}	0.0043 ± 0.00088^{a}	0.0031 ± 0.00033^{b}	0.0022 ± 0.00023^{c}
Overall productivity, P (mg/L/h)	0.44 ± 0.049^{d}	1.63 ± 0.14^{c}	3.19 ± 0.35^{a}	2.35 ± 0.27^{b}	1.63 ± 0.17^{c}
Production time, t (h)	48	72	72	72	72

Values are mean ± SD of three independent experiments. Different superscript letters a, b, c, d and e within the same row indicates statistical differences ($P < 0.05$) among the temperature used

of glutamic acid were utilized by each gram of AOSNSK cells for its metabolic activity, including the production of GABA during the fermentation time phase at 30 °C. The above circumstances were again shown to be true as the maximum specific growth rate obtained at an incubation temperature of 30 °C (μ = 0.034) was statistically higher compared to others.

The initial pH likewise acts a vital role in the production of GABA; however, the optimum pH for *A. oryzae* to produced GABA is anonymous. According to Hao and Schmit (1993) and Kumar and Punekar (1997), the initial pHs ranging from 4 to 6 was found to enhance most of the fungal species GAD activity in catalyzing the GABA from glutamic acid conversion. Thus, initial pH ranging from 3 to 7 was investigated in order to enhance GABA production. The performance and kinetic parameter of GABA fermentation by AOSNSK at different initial culture pH are shown in Table 1B.

After 72 h of fermentation, the highest cell concentration of AOSNSK was achieved at initial culture pH of 5 (15.19 g/L) and decreased at higher or lower pH values. From the calculated data, a pH of 5 scored the highest maximum specific growth rate (μ), followed by pH 6 (0.032), pH 4 (0.031), pH 3 (0.030) and pH 7 (0.029). No lag phase was observed during fermentation at the different initial culture pHs (result not shown), suggesting that the pH range from 3 to 7 was favourable for AOSNSK growth. Meanwhile, the highest GABA (230.83 mg/L) and cell concentration (15.19 g/L) were produced at an initial pH of 5 between 48 and 72 h of fermentation. In initial pH of pH 3, 4, 6 and 7, AOSNSK only produce 21, 117.46, 169.54 and 117.79 mg/L of GABA and 13.42, 13.82, 14.96 g/L of cells concentration.

Due to inappropriate environmental condition provided in pH 3, 4, 6 and 7, the efficiency of AOSNSK to convert substrate into GABA was also affected. Calculated product yield ($Y_{p/s}$) showed that only 0.00039 to 0.0031 g of GABA has been produced from each gram of substrate utilized in pH higher or lower than pH 5. Weak H^+ ion formed in the medium with pH 3, 4, 6 and 7 was probably the reason for low GABA production as the supplemented substrate for converted into GABA due to the importance of H^+ ion.

Besides producing the highest amount of GABA while utilizing the lowest concentration of substrate (glucose, 1.78 g glucose/g cell), the cultivation of AOSNSK in pH 5 also resulted in the highest overall productivity value. Each gram of AOSNSK cells was found capable of producing 3.19 mg/L/h of GABA compared to initial pH 3 (0.44 mg/L/h), pH 4 (2.18 mg/L/h), pH 6 (3.14 mg/L/h) and pH 7 (2.18 mg/L/h).

Effect of different carbon and sucrose concentrations on the production of GABA

The kinetic parameters of GABA production in shake flask fermentation using different sugar types (fructose, maltose, glucose, sucrose and lactose) are shown in Table 2A. GABA concentration was the highest during fermentation using sucrose (327 g/L), followed by maltose (298 g/L), glucose (230 g/L), fructose (197 g/L), and lactose (33.7 g/L). Preference consumption of sucrose by *A. oryzae* for GABA productions could be due to the ability of the fungi to secrete invertase enzyme as reported by Dhananjay and Mulimani (2008). The enzyme which catalyzes the breakdown of sucrose into glucose from fructose was directly synthesized in the presence of sucrose without specific activation of latent enzymes. Both glucose and fructose then enter glycolysis pathways in two different entries, followed by TCA cycle and GABA shunt to the produced GABA.

The ability of sucrose monomers to enter the TCA cycle in two different entry points and mass productions of invertase enzymes are the reasons for high GABA productions. Fermentation medium containing sucrose (0.0061 g) was also found to be successful in producing the product yield (Yp/s) of GABA from each gram of substrate utilized compared to maltose (0.0055 g), glucose (0.004 g), fructose (0.0037) and lactose (0.00093 g).

Five different sucrose concentrations ranging from 40 to 120 g/L were selected for further improvement of GABA production. The time course of GABA production along with other parameters during shake flask fermentation using different concentrations of sucrose are shown in Table 2B. High growth of AOSNSK was observed during fermentation at 60, 80, 100 and 120 g/L of sucrose by attaining the highest and comparable maximum cell concentrations (X = 21.11 to 22.94 g/L) at different time intervals.

Exact cell concentration did not necessarily produce an identical maximum specific growth rate value (μ) and the highest μ was achieved during fermentation in 60 g of sucrose (0.031/h). Sucrose concentration higher or lower than 60 g significantly reduced the μ value. This can be explained through the measured water activity in Table 2B. Fungi maintains the equilibrium of water concentration between the outer and the inner environment of the cell by transporting the water molecules (osmosis). This is crucial for proper cell functions as the rate of enzyme reaction required for spore germination depends on the availability of water.

Unavailability of water affects the enzyme activity of spores by interrupting their allosteric regulation (Stadtman, 1966), association and dissociation of iso-enzymes into subunits (Wuntch et al., 1970). As a result, the time spent

Table 2 Comparison of kinetic parameter values of GABA produced in batch fermentation by *A. oryzae* strain NSK with different carbon sources (A) and sucrose concentrations (B)

Kinetic parameter (A)	Carbon sources				
	Glucose	Sucrose	Lactose	Fructose	Maltose
Maximum cell concentration, *X* (g/L)	16.07 ± 0.31^{c}	16.91 ± 0.12^{b}	6.18 ± 0.07^{e}	17.56 ± 0.05^{a}	15.78 ± 0.07^{d}
Maximum specific growth rate, μ (1/h)	0.030 ± 0.00058^{b}	0.032 ± 0.0020^{a}	0.021 ± 0.0013^{e}	0.027 ± 0.0025^{d}	0.028 ± 0.0023^{c}
Maximum GABA concentration, P_{max} (mg/L)	230.83 ± 5.36^{c}	327.68 ± 4.09^{a}	33.79 ± 7.44^{e}	197.86 ± 19.36^{d}	298.62 ± 11.86^{b}
Glucose (g glucose/g cell)	1.99 ± 0.028^{de}	2.25 ± 0.025^{c}	5.69 ± 0.023^{a}	2.89 ± 0.030^{b}	2.21 ± 0.027^{d}
Glutamic acid (g glutamic acid/g cell)	0.22 ± 0.0031^{c}	0.19 ± 0.0026^{d}	0.66 ± 0.0029^{a}	0.29 ± 0.0030^{b}	0.20 ± 0.0032^{d}
Product yield, Y*p/s* (g GABA/g substrate)	0.004 ± 0.00037^{c}	0.0061 ± 0.0004^{a}	0.00093 ± 0.0002^{e}	0.0037 ± 0.00036 ^{cd}	0.0055 ± 0.00022^{ab}
Overall productivity, P (mg/L/h)	3.21 ± 0.14^{ab}	3.41 ± 0.18^{a}	0.75 ± 0.15^{d}	2.06 ± 0.20^{c}	3.11 ± 0.12^{b}
Production time, *t* (h)	72	96	48	96	96
Kinetic parameter (B)	Sucrose concentration (g/L)				
	40	60	80	100	120
Maximum cell concentration, *X* (g/L)	14.90 ± 0.86^{c}	21.11 ± 0.37^{a}	22.94 ± 0.07^{a}	22.94 ± 0.68^{a}	22.73 ± 0.42^{a}
Maximum specific growth rate, μ (1/h)	0.026 ± 0.0015^{d}	0.031 ± 0.0013^{a}	0.030 ± 0.0016^{b}	0.030 ± 0.0012^{b}	0.028 ± 0.0010^{c}
Maximum GABA concentration, P_{max} (mg/L)	150.54 ± 12.79^{d}	369.28 ± 9.18^{c}	464.34 ± 9.53^{b}	646.78 ± 24.20^{a}	464.24 ± 0.07^{b}
Glucose (g glucose/g cell)	2.86 ± 0.061^{c}	1.90 ± 0.042^{e}	2.58 ± 0.016^{d}	3.24 ± 0.091^{b}	3.82 ± 0.082^{a}
Glutamic acid (g glutamic acid/g cell)	0.35 ± 0.0074^{a}	0.14 ± 0.012^{b}	0.14 ± 0.0085^{b}	0.14 ± 0.0017^{b}	0.12 ± 0.0097^{c}
Product yield, Y*p/s* (g GABA/g substrate)	0.0038 ± 0.00032^{c}	0.0062 ± 0.00015^{ab}	0.0058 ± 0.00012^{b}	0.0065 ± 0.00024^{a}	0.0039 ± 0.00026^{c}
Overall productivity, P (mg/L/h)	2.09 ± 0.18^{e}	5.12 ± 0.13^{ab}	4.84 ± 0.10^{c}	5.39 ± 0.20^{a}	3.87 ± 0.32^{d}
Production time, *t* (h)	72	72	96	120	120
Water activity	0.993	0.990	0.987	0.983	0.981

Values are mean ± SD of three independent experiments. Different superscript letters a, b, c, d and e within the same row indicates statistical differences ($P < 0.05$) among the temperature used

for maximum cells concentration at each a_w level increased with decreasing a_w, (120 h for 0.993, 216 h for 0.990 and 240 h for 0.987).

The remaining 19.89 g/L, 20.81 g/L, 25.67 g/L and 33.17 g/L of sucrose after fermentation time showed that at least 50 g/L of sucrose was required for development of AOSNSK cells. It was noted that inadequate carbon source supplied during fermentation in 40 g/L of sucrose could induce the fungi to use other alternatives to sustain their metabolic activities. Consuming additional glutamic acid is one of the outcomes (Evers et al., 2004). In the present study, a two-fold value for glutamic acid was obtained (0.35 g/L) compared to other concentrations and depleted after 3 days of fermentation. Rapid sucrose and glutamic acid utilization could be the reason for low GABA production (150 mg/L) during fermentation in 40 g/L sucrose.

The highest GABA concentration was produced in 100 g/L (646 mg/L) of sucrose followed by 80 g/L (464 mg/L), 60 g/L (369 mg/L), 120 g/L (464 mg/L) and 40 g/L (150 mg/L) at different time intervals (Table 5). Sucrose concentrations higher than 80 g/L extend the maximum GABA production from 3 to 5 days of fermentation and these phenomena correlated with AOSNSK cell growth. Because GABA production by AOSNSK depends on growth, extended lag and log phases generated from water activity reduction are the reason for delayed maximum GABA biosynthesizing activities. Sucrose concentration above 100 g/L significantly reduced GABA production as high concentration may repress the secretion

of GAD enzyme for GABA biosynthesis. In a concentration of 100 g/L, each gram of substrate consumed (sucrose and glutamic acid) gave product yield (Yp/s) of 0.0065 and the value was significantly higher compared to concentrations of 40, 60, 80 and 120 g/L. Overall productivity showed that GABA was biosynthesized productively during fermentation in 100 g/L of sucrose compared to other concentrations. Therefore, it is concluded that the combination between sucrose and its initial concentration (100 g/L) was the best for GABA production.

Effect of single and different combination of nitrogen sources on GABA production

A preliminary fermentation process to screen GABA production using glutamic acid (GA), sodium nitrate (SN), yeast extract (YE), soybean hydrolysate (SH) and malt extract (ME) as a single source of nitrogen negatively produced high amounts of GABA (Table 3A). AOSNSK showed poor growth in broth containing SN (1.05 g/L cell, 3.37 mg/L GABA) and GA (3.69 g/L cell, 18.36 mg/L GABA) as a single source of nitrogen, moderate growth in ME (5.65 g/L cell, 9.65 mg/L GABA) and excellent growth in YE (17.06 g/L cell, 23.54 mg/L GABA) and HS (14.43 g/L cell, 25.58 mg/L GABA). Supplying an individual amino acid as a sole nitrogen source in growth media does not always produce a good growth since some of the components have been reported to have inhibitory effects upon fungi growth. *Amanita muscaria*, are the fungi species that are extensively inhibited in the presence of L-methionine, L-cystine and L-tryptophane while *Hebeloma crustuliniforme* in L-tyrosine and L-valine as a single nitrogen source (Abuzinadah and Read, 1988). Different from AOSNSK, both fungi showed a remarkable growth in a medium containing L-aspartic and L-glutamic acid (Abuzinadah and Read, 1988).

Some amino acids induce the germination of AOSNSK spore and to cell development resulted in high cell concentration. Besides amino acid, ME, YE and HS are rich in vitamin B as well. El-Zaher et al., (2011) reported that the addition of vitamins (B1, B6 and B12) at minimum concentration of 0.1% w/v significantly enhanced *Fusarium oxysporum*, *Hormodendrum bergeri* and *A. flavus* var. *coulmnaris* growth. Since GABA production is associated with growth, therefore, nitrogen sources inducing both GABA production and fungal growth are required. However, a single source of nitrogen to stimulate both of these

Table 3 Comparison of the kinetic parameter values of GABA produced in batch fermentation by *Aspergillus oryzae* strain NSK with single (A) and different combination (B) of nitrogen sources

Kinetic parameter (A)	Single nitrogen sources				
	Yeast extract	Malt extract	Hydrolysate soybean	Sodium nitrate	Glutamic acid
Maximum cell concentration, X (g/L)	17.06 ± 0.71^{a}	5.65 ± 0.27^{c}	14.43 ± 0.66^{b}	1.05 ± 0.10^{e}	3.69 ± 0.22^{d}
Maximum GABA concentration, P_{max} (mg/L)	23.54 ± 0.64^{b}	9.65 ± 1.05^{d}	25.58 ± 0.76^{a}	3.37 ± 0.93^{e}	18.36 ± 0.55^{c}

Kinetic parameter (B)	Combination of nitrogen sources				
	Soybean and glutamic acid	Soybean and $NaNO_3$	Malt extract and glutamic Acid	Malt extract and $NaNO_3$	Yeast extract and glutamic acid
Maximum cell concentration, X (g/L)	15.88 ± 1.34^{c}	18.04 ± 1.26^{b}	7.04 ± 0.51^{d}	6.27 ± 0.42^{e}	22.94 ± 0.68^{a}
Maximum specific growth rate, μ (1/h)	0.028 ± 0.0012^{b}	0.028 ± 0.0015^{b}	0.016 ± 0.0014^{c}	0.016 ± 0.0010^{c}	0.030 ± 0.0016^{a}
Maximum GABA concentration, P_{max} (mg/L)	88.76 ± 12.61^{b}	50.01 ± 9.18^{c}	20.92 ± 5.05^{e}	26.10 ± 5.78^{d}	646.78 ± 24.20^{a}
Glucose (g glucose/g cell)	5.88 ± 0.11^{b}	5.35 ± 0.13^{c}	8.93 ± 0.16^{a}	8.99 ± 0.15^{a}	3.24 ± 0.078^{d}
Glutamic acid (g glutamic acid/g cell)	0.62 ± 0.067^{c}	0.55 ± 0.021^{d}	1.23 ± 0.15^{b}	1.33 ± 0.33^{a}	0.44 ± 0.055^{e}
Product yield, Yp/s (g GABA/g substrate)	0.00085 ± 0.00005^{b}	0.00048 ± 0.00003^{c}	0.00020 ± 0.00004^{e}	0.00025 ± 0.00004^{d}	0.0062 ± 0.0003^{a}
Overall productivity, P (mg/L/h)	1.23 ± 0.13^{b}	0.69 ± 0.029^{c}	0.29 ± 0.033^{e}	0.31 ± 0.058^{d}	5.39 ± 0.61^{a}
Production time, t (h)	72	72	72	84	120

Values are mean ± SD of three independent experiments. Different superscript letters a, b, c, d and e within the same row indicates statistical differences ($P < 0.05$) among the temperature used

factors is not available; thus a combination of two or more nitrogen sources is needed.

The combination of two nitrogen sources successfully enhanced the growth and GABA production of AOSNSK (Table 3B). The growth was notably enhanced in a medium containing a combination of YE and GA (22.94 g/L) as a nitrogen source followed by soybean (S) and SN (18.04 g/L), S and GA (15.88 g/L), ME and GA (7.04 g/L) and ME and SN (6.27 g/L). As a result, a combination of YE and GA (0.030 1/h) scored the highest specific growth rate value (μ) and the highest concentration of GABA (646.78 mg/L) compared to other combinations.

Other types of nitrogen sources could not produce a high concentration of GABA even though the combination effectively enhanced the growth of *A. oryzae*. Different from S and ME, YE not only rich in various water-soluble amino acids but also a free form of vitamin B6 (Copping, 1936). The metabolically active co-enzyme form of vitamin B6 is pyridoxal 5′-phosphate (PLP) (Oka, 1999). This co-factor stimulates the catalytic activity of the GAD enzyme in catalyzing permanent α-decarboxylation of supplemented glutamic acid to GABA which resulted in high GABA production. SH and ME vitamin B6 is probably not water-soluble and exists in a bonded form. Therefore, the stimulation process is impossible. The presence of a co-factor in AOSNSK growth media (YE and GA) affected the overall productivity of GABA production by generating the highest overall productivity value (5.39 mg/L/h) and such media produced maximum GABA production of 646.78 mg/L as compared to the other types of nitrogen combinations.

Effect of carbon-to-nitrogen ratios on GABA production

Observations conducted during 9 days of the fermentation process showed that the highest cell concentration (X, 24.01 g/L) was achieved in the broth containing carbon-to-nitrogen concentration ratio of C8:N3 ($P < 0.05$) in Table 4. Likewise, the same ratio generated a maximum specific growth rate (μ) value of C8:N3 (0.036 1/h) followed by C9:N2 (0.033 1/h), 7:4 (0.032 1/h), 10:1 (0.030 1/h) and 6:5 (0.030 1/h). Imbalance of C and N concentration in the fermentation broth affected both AOSNSK growth and GABA production. This can be observed with 646.78 mg/L and 1802.59 mg/L of GABA production by AOSNSK in fermentation broth containing C10:N1 and C6:N5. The result was expected as AOSNSK was capable of maintaining the pH of the broth within the optimum range for GAD activity as shown Table 4. In this study, developing GABA-rich functional foods requires only minimal fermentation time of 216 h without manipulating the standard soy sauce production process.

Evaluation of the existing work with related literature

Table 5 displays the present GABA-producing capabilities of AOSNSK in *koji* submerged-liquid fermentation (SLF) for soy sauce production using ten different parameters. There were only two previous studies utilizing *A. oryzae* for this purpose, which both utilized Malaysian *koji* for different incubation times (Ab Kadir et al., 2016) and native sugars (Hajar-Azhari et al., 2018). To the best of our

Table 4 Evaluation of the kinetic parameter values of GABA produced in batch fermentation by *Aspergillus oryzae* strain NSK with different carbon-to-nitrogen ratios

Kinetic parameter	Carbon to nitrogen source (g/L)				
	C10N1	C9N2	C8N3	C7N4	C6N5
Maximum cell concentration, X (g/L)	22.94 ± 0.68^{c}	22.94 ± 0.85^{c}	24.01 ± 0.112^{a}	23.40 ± 0.70^{b}	21.93 ± 2.16^{d}
Maximum specific growth rate, μ (1/h)	0.030 ± 0.0014^{d}	0.033 ± 0.0012^{b}	0.036 ± 0.0011^{a}	0.032 ± 0.0014^{bc}	0.030 ± 0.0010^{d}
Maximum GABA concentration, P_{max} (mg/L)	646.78 ± 24.20^{e}	2607.65 ± 64.39^{b}	3278.31 ± 59.38^{a}	2297.37 ± 61.72^{c}	1802.59 ± 135.09^{d}
Glucose (g glucose/g cell)	3.242 ± 0.18^{b}	3.478 ± 0.15^{a}	3.060 ± 0.17^{c}	2.644 ± 0.12^{e}	2.715 ± 0.12^{d}
Glutamic acid (g glutamic acid/g cell)	0.141 ± 0.032^{e}	0.234 ± 0.048^{a}	0.186 ± 0.025^{c}	0.175 ± 0.018^{d}	0.219 ± 0.036^{b}
Product yield, Y*p/s* (g GABA/g substrate)	0.0062 ± 0.00037^{e}	0.025 ± 0.00058^{d}	0.038 ± 0.0006^{a}	0.03 ± 0.00078^{b}	0.026 ± 0.0022^{c}
Overall productivity, P (mg/L/h)	5.39 ± 0.61^{e}	18.10 ± 0.45^{a}	15.18 ± 0.27^{b}	10.636 ± 0.37^{c}	8.343 ± 0.75^{d}
Production time, t (h)	120	144	216	216	216

Values are mean ± SD of three independent experiments. Different superscript letters a, b, c, d and e within the same row indicates statistical differences ($P < 0.05$) among the temperature used

Table 5 Comparison of the current work on GABA-producing capabilities of *Aspergillus oryzae* strain NSK in *koji* submerged-liquid fermentation

No.	Parameters	Optimized condition	GABA concentration (mg/L)	References
1	Effect of *A. oryzae koji* strains	*A. oryzae* strain NSK	194	Ab Kadir et al. (2016)
2	Effect of incubation times	72 h		
3	Effect of native sugars	Cane molasses	354.08	Hajar-Azhari et al. (2018)
4	Effect of incubation temperatures	30	193.81	*Current study*
5	Effect of initial pH	pH 5	230.83	
6	Effect of carbon sources	Sucrose	327.68	
7	Effect of sucrose concentrations	100 g/L	646.78	
8	Effect of single nitrogen sources	Hydrolysate soybean	25.58	
9	Effect of combinations of nitrogen sources	Yeast extract and glutamic acid	646.78	
10	Effect of carbon to nitrogen ratios	C8N3	3278.31	

[1] Ab Kadir et al. (2016). [13] Hajar-Azhari et al. (2018)

knowledge, the optimized key parameters reported here is the latest *A. oryzae* application for GABA production using SLF. Comparatively, the current work was most effective in producing GABA (3278.31 mg/L) compared to the nearest counterpart (646.78 mg/L) by imposing the carbon to nitrogen ratio strategy in Table 5. In traditional Malaysian two-step soy sauce production, *koji* fermentation was initially done prior the *moromi* fermentation (Wu et al., 2010). The current enhancement provides an alternative strategy to increase GABA compound prior *moromi* fermentation. The study opens up an efficient technique to boost soy sauce production containing GABA from *koji* to *moromi* fermentation in a bioreactor.

To conclude, a commercial Malaysian *koji A. oryzae* strain NSK produces the highest GABA-producing capability at 3278.31 mg/L using seven optimized key parameters (30 °C, pH 5, sucrose as carbon source, 100 g/L of sucrose, hydrolysate soybean, YE and GA, and carbon-to-nitrogen C8N3) in a controlled shake-flask fermentation method. The strategy offers an economical approach in increasing the value of soy sauce without any additives during *koji* fermentation.

Acknowledgements Highest gratitude to the Ministry of Education (MOE) Malaysia for the funding given to Dr Wan-Mohtar under the FRGS (FPP066-2018A) and Research University (RU) UM Geran (RU003I-2017).

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