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Isolation, Identification, and Optimization of γ-Aminobutyric Acid (GABA)-Producing *Bacillus cereus* Strain KBC from a Commercial Soy Sauce *moromi* in Submerged-Liquid Fermentation

Wan Abd Al Qadr Imad Wan-Mohtar [1,2], Mohamad Nor Azzimi Sohedein [1], Mohamad Faizal Ibrahim [3,*], Safuan Ab Kadir [1], Ooi Poh Suan [4], Alan Wong Weng Loen [4], Soumaya Sassi [1,5] and Zul Ilham [2,5]

1 Functional Omics and Bioprocess Development Laboratory, Biotechnology Program, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; qadyr@um.edu.my (W.A.A.Q.I.W.-M.); azzimi@um.edu.my (M.N.A.S.); safuankadir1207@gmail.com (S.A.K.); sassi.soumaya94@gmail.com (S.S.)

2 Bioresources and Bioprocessing Research Group, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; ilham@um.edu.my

3 Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang 43400, Malaysia

4 Lot 3406, Jalan Perusahaan 3, Kamunting Industrial Area, Kwong Bee Chun Sdn. Bhd. Soy Sauce Factory, Kamunting 34600, Malaysia; ps.ooi_06@yahoo.com (O.P.S.); kkkbcfood@yahoo.com (A.W.W.L.)

5 Biomass Energy Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia

* Correspondence: faizal_ibrahim@upm.edu.my; Tel.: +60-397691936

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Abstract: A new high γ-aminobutyric acid (GABA) producing strain of *Bacillus cereus* was successfully isolated from soy sauce *moromi*. This *B. cereus* strain named KBC shared similar morphological characteristics (Gram-positive, rod-shaped) with the reference *B. cereus*. 16S rRNA sequence of *B. cereus* KBC was found to be 99% similar with *B. cereus* strain OPWW1 under phylogenetic tree analysis. *B. cereus* KBC cultivated in unoptimized conditions using De Man, Rogosa, Sharpe (MRS) broth was capable of producing 523.74 mg L^{-1} of GABA within five days of the cultivation period. By using response surface methodology (RSM), pH level, monosodium glutamate (MSG) concentration and temperature were optimized for a high concentration of GABA production. The pH level significantly influenced the GABA production by *B. cereus* KBC with *p*-value = 0.0023. GABA production by *B. cereus* KBC under the optimized condition of pH 7, MSG concentration of 5 g L^{-1} and temperature of 40 °C resulted in GABA production of 3393.02 mg L^{-1}, which is 6.37-fold higher than under unoptimized conditions. Overall, this study has shown that *B. cereus* KBC isolated from soy sauce *moromi* is capable of producing a high concentration of GABA together with the optimal fermentation conditions that have been statistically analysed using RSM.

Keywords: GABA; fermented food; functional food; non-protein amino acid; soy sauce fermentation

1. Introduction

Gamma-aminobutyric acid (GABA) is a non-protein amino acid produced by the α-decarboxylation of L-glutamate. The non-protein amino acid is the molecular compound that has the standard structure of an amino acid consisting of N-terminal and C-terminal. Most amino acids have at least one asymmetric carbon and are chiral [1]. Amino acids are classified as non-protein when they are not part of the 22 such

molecules that are translated into proteins by the standard genetic code [2]. These non-protein amino acids play important roles as metabolites in the organism, function as allelopathic chemicals, nutrient acquisition and in signalling as well as a stress response. Researchers also reported that they are responsible for significant medical issues in both invertebrate and vertebrate animals [3]. Biosynthesis of non-protein amino acid of GABA is catalysed by the glutamate decarboxylase (GAD) enzyme [4]. GABA is usually produced by microorganisms associated with fermented foods, such as fermented fish [5], fermented cod gut [6], fermented *tempeh* [7], fermented milk [8], fermented *adzuki* beans [9] and recently fermented soybean of soy sauce [10]. GABA plays an important role in the central nervous system (CNS) as the primary inhibitory neurotransmitter. Due to its major inhibitory functions in the brain, GABA is studied as a treatment for various neurological diseases, such as epilepsy, schizophrenia, stiff-person syndrome and anxiety disorders [11]. GABA also demonstrated hypotensive, diuretic and tranquillizer effects [12]. A high concentration of GABA can also be found in pancreatic islets, which was associated with insulin secretion, hence, can be used to treat diabetes [13,14]. Furthermore, the oral administration of GABA can effectively decrease blood pressure in a hypertensive patient [8]. These pharmaceutical applications of GABA have risen the commercial production of functional food with a high concentration of GABA as its bioactive component.

In order to commercially produce GABA, several biosynthetic techniques were employed, such as by using sourdough fermentation, immobilized cell technology and batch fermentation [15]. GABA produced from the fermentation of naturally-occurring microorganisms have higher demand in comparison to chemically-synthesized GABA since customers prefer a nutrient source that is naturally-produced rather than chemically-synthesized [16]. Production of GABA via naturally-occurring microorganisms is preferred as they are naturally present in the food production processes, low-cost, and can be adopted as a functional food [10] rather than painstaking and expensive chemically-synthesized GABA [17], which are more prone to antihypertensive drugs [18]. Naturally, humans have an innate sense toward natural things; therefore, food naturalness is crucial among the majority of consumers [19]. Furthermore, natural GABA also improves the taste of the food while reducing the risk of contamination with pathogenic microorganisms [20]. In order to identify a high GABA-producing strain, numerous microorganisms such as *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactococcus lactis*, *Streptococcus salivarius*, *Uonascus purpureu* and *Streptomyces bacillaris* have been isolated from various type of food like cheeses, kimchi, tea, fresh milk and fermented fish [15]. There are many studies that have reported that the culture conditions play an important role in GABA production. It can be done by (1) finding and/or developing a higher-yield GABA-producing microorganism and (2) optimizing fermentation conditions. GABA produced in fermented food is a good option as it can be directly consumed [21].

Soy sauce is a liquid seasoning used worldwide in cooking and eating [22]. It can be a potential natural functional food for GABA production since the chemically synthesized GABA are expensive compared to naturally produced-GABA [23]. In 2016, several isolated microorganisms from local Malaysian soy sauce producers were reported to be capable of producing GABA due to the abilities of the trio microorganisms, i.e., *Aspergillus oryzae* NSK (from *koji*), *Bacillus* sp. (from *moromi*) and strict anaerobic *Tetragenococcus* sp. (from *moromi*). Based on these findings, the ability of traditional solid-state fermentation of soy sauce production processes using soybean as a substrate has been explored to produce GABA by the microorganism from *moromi*. Previous studies have been done to isolate microorganisms from soy sauce *koji* for GABA production. For example, *Aspergillus oryzae* NSK isolated from the soy sauce *koji* [24] produced 73.13 ± 1.77 mg L^{-1} of GABA. However, there is no conclusive study has been done to determine the capability of microorganisms isolated from the *moromi* stage for GABA production. Therefore, a complete study from isolation, identification and optimization of a newly isolated microorganism from commercial soy sauce *moromi* for GABA production has been conducted.

2. Materials and Methods

2.1. Isolation of B. cereus KBC

Three *moromi* samples ranging from 10, 25 and 80 days were obtained from an established commercial soy sauce factory in Perak, Malaysia. Soy sauce production consists of several stages of fermentation (Figure 1). The first stage is a solid-state fermentation, in which soybeans and wheat flour are mixed with fungal species, such as *Aspergillus sojae* or *Aspergillus oryzae*, as the starter and left fermenting for several days to form *koji* [25]. During the first stage of making *koji*, the soybean was soaked overnight using tap water to allow it to be softened. The dehulled soybean was boiled at 100 °C for 3 h until the colour appeared to be slightly golden. Meanwhile, 1 kg of dried soybean was mixed with 500 g of wheat flour and 0.0005 g of *A. oryzae* that works as a starter. The mixture was loaded into four standard steel trays evenly, each loaded with 4 cm thickness. The *koji* trays were covered thoroughly by the parchment paper and incubated at around 27–35 °C for two weeks. After the incubation in a humid and aerated incubation chamber was completed, a greenish-yellow mash could be observed, known as *koji*. The second stage involves the mixing of *koji* with 20% (*w*/*v*) of sodium chloride solution (brine) and incubating for six months in a tank to produce *moromi*. *Moromi* is pressed in the final stage to collect the liquid product, which is the soy sauce.

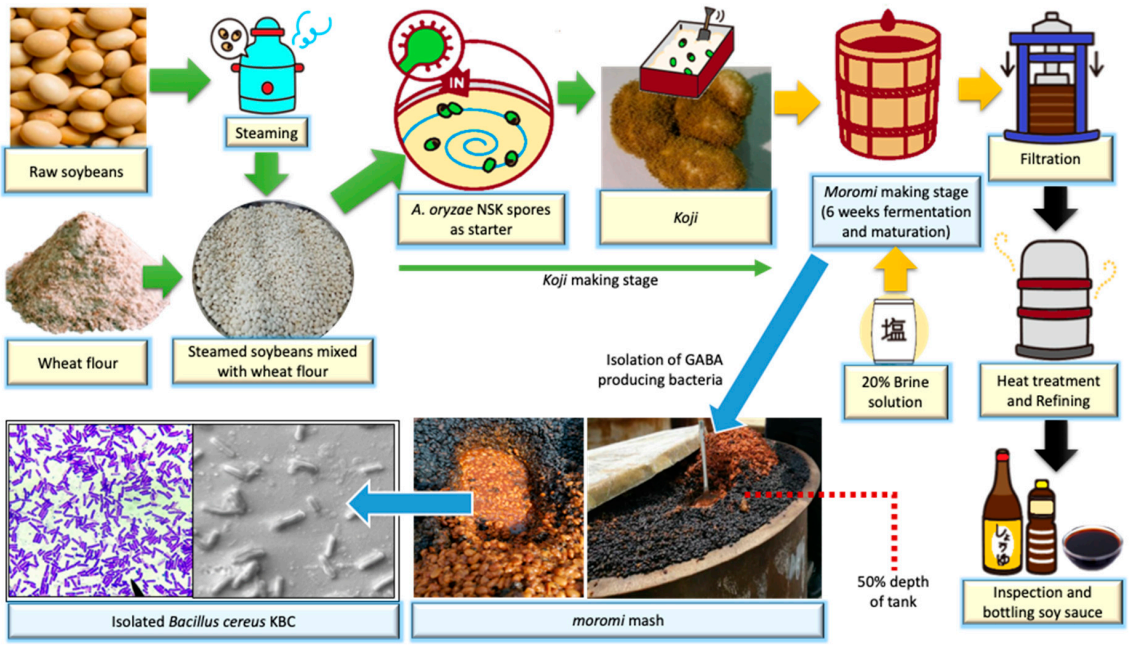

Figure 1. Soy sauce production blueprint and isolation point for *Bacillus cereus* strain KBC.

The collected *moromi* samples were stored in 500 mL Schott bottles in the cold temperature of less than 4 °C. Brine sample at the bottom of the *moromi* sample was pipetted and serial dilution was conducted. The isolation was conducted by spreading and streaking the *moromi* sample on de Man, Rogosa, Sharpe (MRS) (69964-500G, Sigma-Aldrich, Dorset, UK) agar plates. The agar plates were incubated at 30 °C for 5 days under anaerobic conditions using an anaerobic jar supplied with *Anaerocult* A (Merck) following the methods by Liu et al. [26]. After incubation, the plates were screened for colony growth and a single colony was streaked on a new MRS agar plate to obtain a pure isolate. The pure colonies were transferred to MRS media slants and kept as stock cultures at −4 °C. This pure isolate was used for morphological observation and identification. For long term preservation, the identified master strain was cultured overnight in MRS broth and 500 µL of the overnight culture was inoculated into a 2 mL glass vial containing 500 µL of 50% *v*/*v* glycerol. The glass vials containing the master strains were stored in −80 °C for future use.

2.2. Morphological Analysis

The isolated pure culture was Gram-stained and observed under a light microscope [27] to identify their morphological characteristics. This isolated pure culture was also observed under 1000× magnification using a Scanning Electron Microscope (SEM) (Brand ZEISS, Model MERLIN Compact, Oberkcohen, Germany) [28] to confirm its morphological structure.

2.3. Identification Using 16S rRNA

Identification of *Bacillus cereus* strain KBC was conducted by Apical Scientific Sdn. Bhd. (Seri Kembangan, Selangor, Malaysia) using 16S rRNA sequencing with 1.5 kb full length based on Tamura et al. [29]. The extracted bacterial DNA was amplified using universal primers 27F and 1492R, as mentioned by Jawan et al. [30]. The procedures consisted of 25 µL of total reaction volume, which consisted of genomic DNA purified using in-house extraction blueprint (0.3 pmol of each primer, 0.5 U DNA Taq polymerase deoxynucleotides triphosphates (dNTPs, 400 µM each), supplied PCR buffer and deionised water). The PCR was done strictly according to Jawan et al. [30]: 1 cycle for initial denaturation (94 °C for 2 min); 25 cycles (98 °C for 10 s; 53 °C for 30 s; 68 °C for 1 min) for annealing-amplified DNA extension via Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany). The PCR products were purified and directly sequenced with primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Co., Foster City, MA, USA). Finally, the sequences fragment (1414 bp) were compared with those deposited in the GenBank DNA database using Basic Local Alignment Search Tool (BLAST; https://www.ncbi.nlm.nih.gov/nuccore/NR_074540.1,NR_113266.1,NR_115714.1,NR_112630.1,NR_114582.1,NR_115526.1,NR_152692.1,NR_113991.1,NR_114581.1,NR_043403.1). A phylogenetic tree of Neighbour Joining (Unrooted Tree) by NCBl Blast Tree Method based on 16S rRNA 137 genes (bacteria only) was, excluding uncultured bacteria (taxid: 77133), constructed to determine the closest bacterial species by using Molecular Evolutionary Genetics Analysis (MEGA) software (Version 6.0, https://www.megasoftware.net, the software is online by Tamura et al. [29]). Distance-clustering was generated using bootstrap values based on 1000 replications. The resulting phylogenetic tree was re-run in Mega X software, as described by Wan-Mohtar et al. [10], and the isolated bacteria was identified as the same species by the closest K_{nuc}.

2.4. Inoculum Preparation

The stock culture of *B. cereus* KBC in an MRS media slant was thawed to room temperature before being streaked on MRS agar plates under sterile conditions. The *B. cereus* KBC on the MRS agar plates was cultured for two days to check their viability, then, a loop of the bacterial colony was transferred into 100-mL Erlenmeyer flask containing 50 mL of MRS broth. The MRS broth culture was incubated for 24 h and 1% inoculum with the cell's viability count of 10^6 CFU mL^{-1} was used for the fermentation to produce GABA.

2.5. Medium Preparation

MRS agar and broth (Sigma-Aldrich, Dorset, UK) were used in these experiments. This media composed of (in g/L) peptone (10.0), yeast extract (4.0), glucose (20.0), dipotassium hydrogen phosphate (2.0), sodium acetate (5.0), triammonium citrate (2.0), magnesium sulphate (0.2), manganese sulphate (0.05), and meat extract (8.0). The MRS agar and broth were prepared by measuring a suitable amount of media (31 g of MRS powder in 500 mL distilled water) and autoclaved at 121 °C for 15 min. Monosodium glutamate (MSG) was added into the media for the optimisation of GABA production according to the amount specified by the response surface methodology (RSM).

2.6. Production of GABA by an Isolated Strain

GABA production was conducted by transferring 150 mL of MRS broth into 250-mL Erlenmeyer flasks. The flasks were then inoculated with 1.5 mL of 1% inoculum with the cells viability count of 10^6 CFU mL^{-1}. The inoculated flasks were incubated at 30 °C, 100 rpm for 7 days using a shaker incubator (Binder, Bohemia, NY, USA).

2.7. Optimisation of GABA Production Using RSM

The GABA production by the isolated strain was optimised using RSM with central composite design (CCD) in Design Expert 7.0 software (Version 7, Godward St NE, Suit 6400, Minneapolis, MN, USA). The fermentation for optimisation was conducted in a 100-mL Erlenmeyer flask containing 50 mL MRS broth. The variables being tested are temperature, pH and MSG concentrations with the ranges shown in Table 1. The experimental design constructed by CCD with the α-value set at 1.0 generated a total of twenty runs as listed in Table 2. All the experimental runs were conducted accordingly, and the GABA concentration was measured and inserted as a response for the RSM analysis.

Table 1. The selected variables, range and levels inputted for optimisation study.

Variables	Range and Levels		
	−1	**0**	**1**
pH	3.0	5.0	7.0
MSG (g L^{-1})	1.0	3.0	5.0
Temperature (°C)	20	30	40

Table 2. Central composite design (CCD) design with studied variables and actual responses for the gamma-aminobutyric acid (GABA) production (mg L^{-1}) of *B. cereus* KBC.

Run No.	Variables			Actual Response
	pH	**MSG (g L^{-1})**	**Temperature (°C)**	**GABA (mg L^{-1})**
1	3	1	40	1989.54
2	5	3	20	3303.81
3	5	3	30	2178.9
4	7	5	40	3393.02
5	5	3	30	1990.2
6	5	5	30	2141.45
7	3	3	30	1857.36
8	5	3	30	2181.97
9	3	5	20	1965.97
10	5	3	30	2063.18
11	3	5	40	2060.37
12	5	3	30	2146.49
13	7	5	20	3585.29
14	7	1	40	3018.52
15	5	3	40	2379.37
16	5	1	30	2520.9
17	7	3	30	3303.19
18	3	1	20	1969.95
19	7	1	20	1766.99
20	5	3	30	2047.25

2.8. Analytical Procedures

2.8.1. Determination of Dry Cell Weight

Centrifuge tubes of 1.5-mL were pre-dried in a dehydrator overnight and weighed. A 1 mL of *Bacillus* strain culture was collected aseptically and centrifuged at 4000 rpm for 15 min. The supernatant

was collected and stored in another 1.5 mL tube for the determination of GABA. The pellets were washed with distilled water and centrifuged again. The water was discarded, and the pellets were dried in a dehydrator overnight before the biomass was weighed.

2.8.2. Determination of GABA

The supernatant was filtered through a 0.22-μm pore-size nylon filter (Fisher Scientific, Brecon, UK). The supernatant was injected into an HPLC equipped with a Hypersil Gold C-18 column (250 × 4.6 mm I.D., particle size 5/um; Thermo Scientific, Meadow, UK). Mobile phase (a mixture of 60% solution A (aqueous solution of 100.02 millimolar (mM) sodium acetate, 3.59 mM triethylamine and 12.49 mM acetic acid in 1000 mL) adjusted to pH 5.8, 28% solution B (deionized water), and 12% solution C (acetonitrile) was used during the separation processes. The flow rate of the mobile phase was set at 0.6 mL/min. The separation process was conducted at room temperature, and the detection was monitored at 254 nm. 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.

2.9. Statistical Analysis

All experimental runs were done in triplicates and the respective mean ± SD was determined using the software, GraphPad Prism 7 (GraphPad Software Inc., 2016, San Diego, CA, USA) and presented as error bars in the graph or ± symbol in the table. It should be noted the error bars might not appear in the graph if SD is smaller than the size of the symbol for the data point.

For the optimisation of GABA production by *B. cereus* KBC using RSM, the statistical tool in Design Expert 7.0 software was used to conduct an analysis of variance (ANOVA) for the experimental responses. The significance of the model and variables being studied was determined based on p-value < 0.05.

3. Results and Discussion

3.1. Identification of B. cereus KBC

3.1.1. Morphological Characteristics of *B. cereus* KBC

Morphological characteristics of the bacterial strain isolated from the soy sauce *moromi* are shown in Figure S1. It can be observed that the isolated strain exhibited round, opaque and milky-coloured colonies when grown on MRS agar plates. Gram staining also clearly showed that this strain is a Gram-positive with rod-shaped morphology and has been confirmed under 1000× magnification using an SEM.

3.1.2. 16S rRNA Identification of *B. cereus* KBC

Molecular identification was performed on the microorganism isolated from *moromi* in order to identify the species of the strain. The base pairs of the isolated strain were estimated using agarose gel electrophoresis under ultraviolet (UV) light (Figure S2). The marker in Lane 1 was used to construct the standard curve for base pairs length determination. The base pairs of BCSNSK were estimated to be 1414 bp. The phylogenetic tree (Figure 2) confirmed the first isolated strain to be closely related with the species *Bacillus cereus* and thus will be designated as *Bacillus cereus* strain KBC (*B. cereus* KBC) in further discussion.

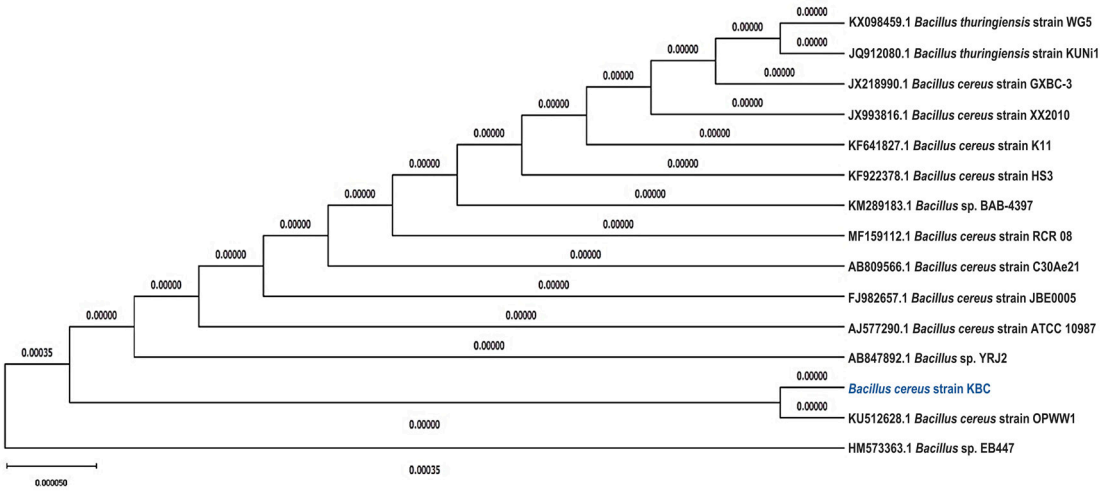

Figure 2. Phylogenetic tree of *B. cereus* strain KBC with evolutionary distance. The *B. cereus* KBC was closely related to *B. cereus* strain OPWW1.

3.2. GABA Production by B. cereus KBC

The biomass and GABA-producing capability of *Bacillus cereus* strain KBC was studied by measuring its biomass and GABA content in MRS broth for seven days. The graph in Figure 3 shows biomass and GABA production during the fermentation period. The biomass increased until it reached the highest value of 0.57 ± 0.07 g L^{-1} on day two, then decreased until day seven. The biomass concentration could demonstrate the viability of the cells. *B. cereus* KBC displayed rapid growth and reached maximum cell concentration on day two. Nutrient limitation, such as a limited amount of carbon source, might cause a decrease in the biomass of *B. cereus* KBC. The GABA content increased each day until day five in which it reached the maximum value of 532.74 ± 5.89 mg L^{-1} before decreasing. GABA could improve the growth of bacteria by acting as a growth factor. The previous study has shown the importance of GABA as a growth factor for a gut microorganism known as KLE1738 [31], as the isolated *B. cereus* strain KBC may also produce GABA to stimulate their growth. On day five of fermentation, the GABA production was the highest while the biomass is the lowest. On the next day, the biomass suddenly increases while the GABA content decreases. *Bacillus cereus* strain KBC could utilise the GABA for survivability and result in this unbalanced growth. More research is needed to be done to study the existent of GABA-dependent metabolism mechanism in *B. cereus* strain KBC. In comparison with a study by Ab Kadir et al. [24], GABA is produced mostly during the log and stationary phase, while, in this present study, GABA was produced during stationary phase. This situation might be caused by nutrient limitation such as peptone (nitrogen source) in the MRS broth. These components are vital elements for the microorganism in order to synthesize amino acids for growth and secondary metabolite production such as GABA [24]. The result also showed GABA concentration of 286 mg L^{-1} at day zero, which could be originated from the inoculum culture as GABA introduced in the fermentation broth, and it was continuously produced by *B. cereus* KBC while growing during the fermentation period. It was observed that there is an increment of 247 mg L^{-1} of GABA from day zero until day five (maximum amount of GABA production). In the study by Ab Kadir et al. [24], maximum GABA concentrations of 194 mg L^{-1} were achieved around day two to day four, using *Aspergillus oryzae* strain NSK isolated from soy sauce *koji*, which grows and behaves differently from *B. cereus* KBC. Technically both *A. oryzae* strains NSK (from *koji*) and *B. cereus* KBC (from *moromi*) are responsible in metabolizing the soybean while producing GABA but at different stages and rates.

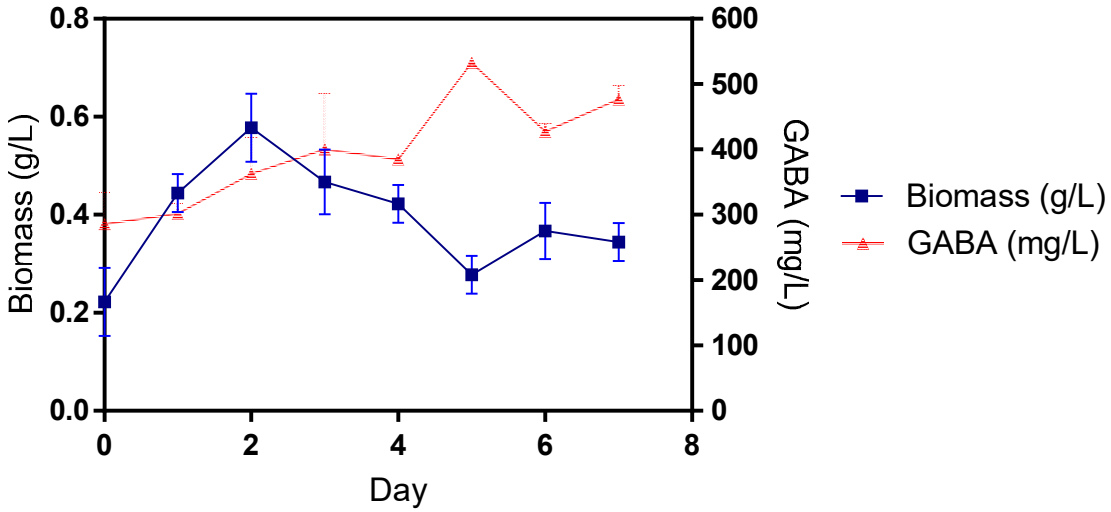

Figure 3. Biomass and GABA production of *Bacillus cereus* strain KBC in a 7-day cultivation period.

3.2.1. Optimisation of GABA Production by *B. cereus* KBC

The ANOVA results for GABA production of *B. cereus* KBC are shown in Table 3. It was found that 48.21% (R^2 = 0.4821) of the variability in the actual response could be described using the CCD linear model. The *p*-value was 0.0127, indicating that the model was significant ($p < 0.05$). In Figure 4, the effect of a single factor on GABA production was demonstrated. Among the three variables, pH (A, *p*-value = 0.0023) showed significant effect on the GABA production at $p < 0.05$ (Figure 3a). pH value is an important factor for GABA production [21], which affect the cells growth and glutamate decarboxylase (GAD) activity. GABA production usually being conducted in acidic pH conditions range between 3.5 and 5.0, depending on the types of microorganism and different properties of GADs. However, higher initial pH could trigger the cells to produce more GABA into the system.

Table 3. Analysis of variance (ANOVA) results for the actual responses using the CCD linear model for GABA production of *B. cereus* KBC.

Source	Sum of Squares	Mean Square	DF	*F* Value	Prob > *F*	
Model	3,088,531.300	1,029,510.433	3	4.965	0.0127 *	significant
A: pH	2,728,832.702	2,728,832.702	1	13.161	0.0023 *	significant
B: MSG	353,508.535	353,508.535	1	1.705	0.2101	
C: Temperature	6190.063	6190.063	1	0.030	0.8650	
Residual	3,317,449.610	207,340.600	16			
Pure Error	31,289.248	6257.850	5			
Lack of Fit	3,286,160.362		11	47.739	0.0002 *	significant
Cor Total	6,405,980.910	298,741.851	19			
Standard Deviation = 455.35		Mean = 2393.19		Adequate Precision = 7.221		
R^2 = 0.4821				Adjusted R^2 = 0.3850		

* Significant value.

In addition, optimising MSG concentration is aimed to stimulate the production of GABA by GAD via the GABA shunt way. However, it was reported that excessive MSG could inhibit cell growth and decrease GABA production [32,33]. In this present study, MSG (B, *p*-value = 0.2101) and temperature (C, *p*-value = 0.8650) were observed to be less influential on the GABA production of *B. cereus* KBC (Figure 4b,c, respectively). It should be noted that the optimal concentrations of MSG are different for various microorganisms in GABA production. Figure 5 shows the merged effect of pH, MSG concentration, and temperature on GABA production displayed as the response surface profiles. Figure 5a shows the effect of pH (A) and MSG concentration (B), Figure 5b shows the effect of MSG

concentration (B) and temperature (C), and Figure 5c shows the effect of pH (A) and temperature (C) on GABA production of *B. cereus* KBC. The GABA production can be seen heavily influenced by the pH level, while the temperature and the MSG concentration only provide small effects on the GABA production of BSCKBC. Although some studies reported that temperature is a crucial parameter in GABA production [21], maintaining the temperature at the range of that *B. cereus* KBC could sustain its metabolic pathway could retain GABA production at a high level.

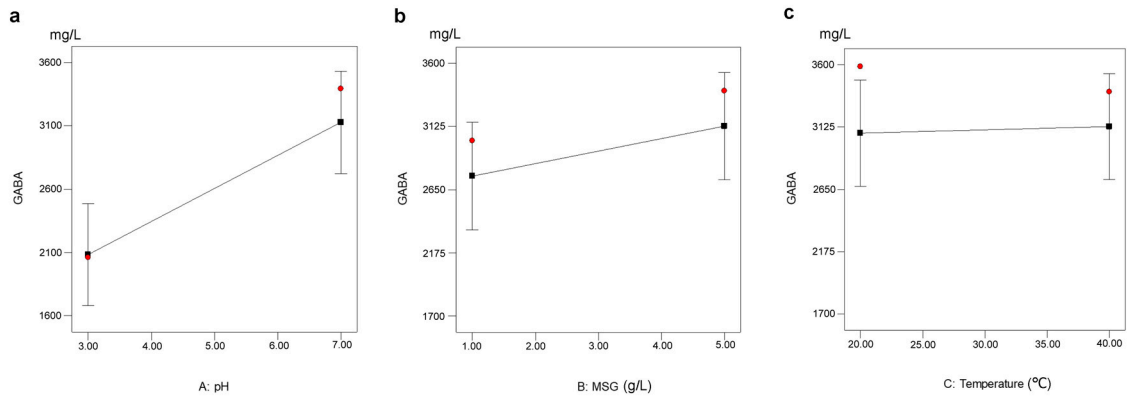

Figure 4. One factor profiles, which demonstrated the effects of (**a**) pH, (**b**) MSG concentration (g L^{-1}), and (**c**) temperature (°C) on GABA production of *B. cereus* KBC.

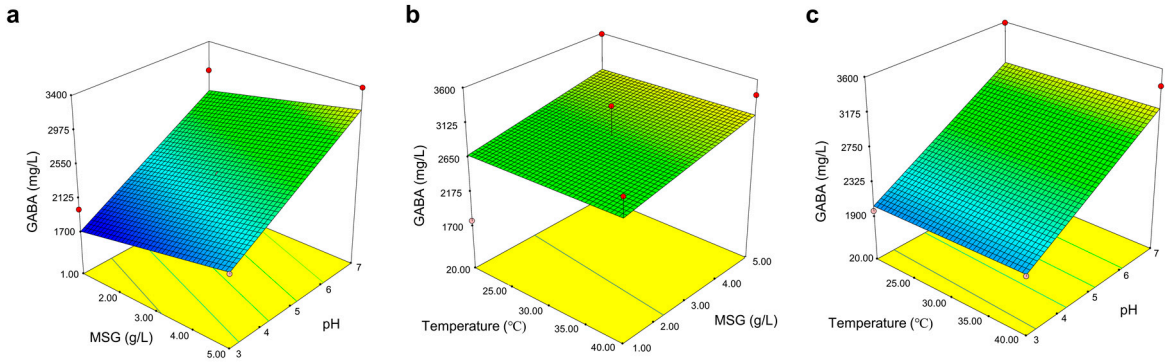

Figure 5. Response surface profile of GABA production from *B. cereus* KBC indicated the effects between (**a**) MSG and pH, (**b**) temperature and MSG, and (**c**) temperature and pH.

3.2.2. Validation of the Optimised Conditions

After the construction of the RSM linear model for GABA production by *B. cereus* KBC, the Design Expert 7.0 software was used to conduct optimisation for high GABA production. The software calculated the optimum conditions to be at pH 7, MSG concentration of 5 g L^{-1} and temperature of 40 °C. The predicted response was 3128.42 mg L^{-1}, while the actual response generated from the experiment was 3393.02 mg L^{-1}. The actual response was comparatively higher than the predicted response calculated by the software. Interestingly, the GABA production by *B. cereus* KBC under optimised conditions appeared to be 6.37-fold higher than the GABA production by *B. cereus* KBC under unoptimised conditions (532.74 mg L^{-1}). By just specifically controlling the pH, temperature and MSG concentration, six times more GABA can be produced from the same microorganism. In functional food production, this could mean more GABA can be produced in each fermentation run and save more production cost. The final product will also contain a much higher concentration of GABA and provide more benefit to the consumer.

Comparison between GABA production from different microorganisms isolated from various food sources is shown in Table 4. The GABA concentration produced by *B. cereus* strain KBC (532.74 mg L^{-1})

isolated from Malaysian soy sauce *moromi* was relatively higher than the GABA concentration (73.13 mg L^{-1}) produced by *A. oryzae* NSK isolated from the Malaysian soy sauce *koji* [24]. Under optimized conditions, *B. cereus* strain KBC demonstrated slightly higher GABA concentration (3393.02 mg L^{-1}) than *A. oryzae* NSK (3278.31 mg L^{-1}) [10]. In addition, the GABA production by several lactic acid bacteria was several folds higher than GABA produced by *B. cereus* strain KBC. Examples of lactic acid bacteria with high GABA production are *Lactobacillus paracasei* NFRI 7415 isolated from fermented fish (31145.3 mg L^{-1}), *Lactobacillus brevis* BJ20 isolated from kimchi (2465 mg L^{-1}), and *Lactococcus lactis* subsp. *lactis* B isolated from kimchi and yoghurt (6410 mg L^{-1}). However, GABA production by *B. cereus* strain KBC was slightly higher than the GABA produced by *Lactobacillus plantarum* DSM19463 isolated from cheeses (498.1 mg L^{-1}).

Table 4. GABA concentration by microorganisms isolated from various food sources.

Isolated Strain	Source	GABA Concentration (mg L^{-1})	Reference
Lactobacillus paracasei NFRI 7415	Fermented fish	31,145.30	[5]
Lactobacillus brevis BJ20	Kimchi	2465.00	[6]
Lactococcus lactis subsp. *lactis* B	Kimchi and yoghurt	6410.00	[34]
Lactobacillus plantarum DSM19463	Cheeses	498.10	[35]
Aspergillus oryzae NSK	Soy sauce *koji*	73.13	[24]
Aspergillus oryzae NSK	Soy sauce *koji*	354.08	[36]
Aspergillus oryzae NSK (unoptimized)	Soy sauce *koji*	3278.31	[10]
Bacillus cereus strain KBC (unoptimized)	Soy sauce *moromi*	532.74	This study
Bacillus cereus strain KBC (optimized)	Soy sauce *moromi*	3393.02	This study

4. Conclusions

A novel bacteria strain was successfully isolated from the soy sauce *moromi* by using MRS media under anaerobic conditions. The 16S rRNA sequencing and phylogenetic tree analysis revealed that the strain belongs to *B. cereus* species. The GABA-producing capability of this strain was studied, and the results demonstrated that *B. cereus* KBC managed to produce 532.74 mg L^{-1} GABA. The optimisation of GABA production using RSM also demonstrated that pH significantly influenced the GABA production of *B. cereus* KBC. The optimized conditions for high GABA production of *B. cereus* KBC were found to be at pH 7, 5 g L^{-1} MSG concentration and temperature of 40 °C. These optimized conditions resulted in a GABA production of 3393.02 mg L^{-1}, which is 6.37-fold higher than in unoptimised condition (532.74 mg L^{-1}). This study will be useful in isolating and optimizing potential GABA-producing strains for the production of soy sauce with a high concentration of GABA in the future.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/6/652/s1, Figure S1: (a) Fermentation tank containing soy sauce *moromi* at a commercial soy sauce factory in Perak, Malaysia, (b) 80 days soy sauce *moromi* sample in which the bacterial strain was isolated, (c) Morphologies of *B. cereus* KBC on MRS agar plate, (d) *B. cereus* KBC under light microscope (400x magnification) after Gram staining, (e) *B. cereus* KBC at 1000x magnification under Scanning Electron Microscope (Bar = 10 µm) and Figure S2: Agarose gel electrophoresis of 16S rRNA isolated from *B. cereus* KBC culture plate. Lane 1 and 3 correspond to 10kb marker. Lane 2 corresponds to the sample (*B. cereus* KBC). Lane 4 corresponds to negative control (-ve) and Lane 5 corresponds to positive control (+ve).

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