Desired soy sauce characteristics and autolysis of *Aspergillus oryzae* induced by low temperature conditions during initial *moromi* fermentation

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Revised: 21 February 2019 / Accepted: 20 March 2019 / Published online: 29 April 2019
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Abstract This is the first report on the effect of low temperature stress applied during initial *moromi* fermentation on the quality and taste of soy sauce. *Koji* was prepared to yield initial *moromi* under three comparative fermentation conditions over 9 days: (1) 4 °C and 0% brine (i.e., water) (LTSF); (2) 4 °C and 16% w/w brine (LTSH); (3) 25 °C and 16% w/w brine (the control, CRTH). Greater extent of autolysis in samples was found under low temperature stress conditions (i.e., at 4 °C, a temperature much lower than the normal temperature range like 25 °C for natural microbial growth and performance). Compared to CRTH, LTSF had a two-fold increase of glutaminase activity in dregs and 65.17% increase in supernatant, and after 60 days of *moromi* fermentation, a 5.73% and 3.47% increase, respectively, in the contents of glutamic acid (Glu) and aspartic acid (Asp). LTSF had the highest total free amino acid content due to both the low temperature stress and absence of salt. The intensity ranking of umaminess and kokumi sensation (LTSF > LTSH > CRTH) revealed by sensory analysis followed the changing trends of their umami and sweet amino acid contents with a trend reversal in the bitter amino acid content. Low temperature (4 °C) without brine for initial *moromi*

fermentation seemed beneficial, leading to a soy sauce product with desired taste and amino acid contents.

Keywords *Aspergillus oryzae* · Low temperature stress · Glutaminase activity · Mycelia autolysis · Soy sauce · Umami taste

Introduction

Soy sauce is a traditional and popular condiment worldwide, especially in oriental countries such as China, Japan and Korea. During the production of soy sauce, steamed defatted soybean flakes and baked wheat grains are often mixed with *Aspergillus oryzae* (*A. oryzae*) at 25–30 °C for 2–3 days to make a soy sauce *koji*. The *koji* is then mixed with 1.5–2.2 volumes of 22–23% saline to yield a soy sauce mash (called *moromi*) with a final sodium chloride concentration of 16–18%, prior to an aging process at room temperature for 2–3 months. During *koji* fermentation, the mold grows throughout the fermented mass and produces a number of enzymes that digest proteins and starches from soybean and wheat (Fukushima 2004). The protease and glutaminase activities of *koji* are crucial for achieving good quality and umami taste of soy sauce via fermentation (Ito and Koyama 2014). Soy and wheat proteins can be digested by endoprotease(s) and exoprotease(s) into peptides and free amino acids e.g., glutamine (Gln) and glutamic acid (Glu) during *moromi* fermentation. Gln liberated can further be hydrolyzed to Glu or synthesized γ -glutamyl peptides by glutaminase (Yang et al. 2018). If glutaminase activity is insufficient, Gln would be converted to tasteless or slightly sour pyroglutamic acid. The umaminess of soy sauce depends mainly on its Glu content. Accordingly, *moromi* fermentation is a key stage for soy sauce making,

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13197-019-03742-5>) contains supplementary material, which is available to authorized users.

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while protease and glutaminase activities affect greatly the Glu content (a main contributor to the characteristic umami taste of soy sauce).

The influence of *moromi* fermentation temperature on the quality of soy sauce was previously investigated, because traditional soy sauce *moromi* is usually produced via fermentation at room temperature and the room temperature may vary greatly with weather and season (Cui et al. 2014). Fukushima (2004) reported that a warm temperature (28–37 °C) is more favorable for the metabolism of microorganisms in *moromi*, especially lactic acid bacteria and yeasts. Jansen et al. (2003) reported that the production of fusel alcohols (important flavor compounds in soy sauce) by *Z. rouxii* depended on the fermentation temperature. Wu et al. (2010) found that increasing *moromi* fermentation temperature to 45 °C accelerated aging and led to a lower ethanol content in soy sauce. Hoang et al. (2016) indicated that *moromi* fermentation at 40.7 °C for 4.6 days with a brine content of 10% w/w resulted in a higher ammonia nitrogen content in soy sauce. Wei et al. (2013) showed the advantage of using different *moromi* fermentation temperatures at different stages for improving soy sauce quality: 15 °C for the first month and 30 °C for the remaining period. However, little research was conducted to examine the influence of a *moromi* fermentation temperature as low as 4 °C on the quality of final soy sauce products. Temperature downshift represents a significant stress microorganisms may experience, which would influence greatly their growth, performance and natural distribution, inducing biochemical and physiological changes (e.g., cellular membranes and enzymes activity) and even microorganism autolysis (Zhu et al. 2010). Upon the exposure to a low temperature below the membrane phase transition point, *Bacillus subtilis* would lose control of membrane permeability and intracellular K⁺, and suffer a reduced glucose uptake and consequently cellular lysis (Svarachorn et al. 1991).

In this study, the effect of low temperature stress (at 4 °C) on *moromi* fermentation of soy sauce with *A. oryzae* strain in the absence and presence of salt (16% w/w brine) was examined. To the best of our knowledge, this was the first study on this aspect. The novelties of this study lie in: (1) few studies have investigated *moromi* fermentation but *koji* making process; (2) most of the previous studies focused on using high temperature or sunlight to produce soy sauce with improved quality (whilst this study explored the feasibility of applying low temperature stress to maintain the activities of enzymes and prolong their action time); (3) No study was conducted on the influence of low temperature stress on *A. oryzae* strain that is rich in enzymes essential for soy sauce fermentation such as glutaminase, protease and amylase. The correlations between the effects of low temperature stress on initial *moromi*

fermentation (including autolysis of *A. oryzae*) and the chemical composition of the resulting soy sauce, and between the amino acid profiles and taste characteristics of soy sauce, were also investigated.

Materials and methods

Materials

Commercial low temperature food grade soy flakes with 52.27% crude protein, 6.72% moisture, 0.89% fat, and 6.83% ash (all in weight percentages, w/w; dry basis), were obtained from Shandong Yuwang Industrial Co. Ltd. (Yucheng, China). An *Aspergillus oryzae* strain was provided by Jining Yuyuan Biotechnology Co. Ltd. (Shandong, China). The flour (Yuwang, Shandong, China) and salt (Yueyan, Guangdong, China) were purchased from local markets. The propidium iodide (PI) nucleic acid dye was obtained from Biofroxx GmbH (Einhausen, Germany). All other chemicals and solvents were of analytical grade or HPLC grade, and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) or Sigma Chemical Co. Ltd. (St. Louis, MO, USA).

Preparation of *koji* via solid-state fermentation

Four kilograms of food grade soy flakes were soaked in 2.0 L water for 1.0 h at room temperature, before being steamed at 121.0 °C for 15 min in a LDZX-50KBS pressure steam sterilizer (Shenan, Shanghai, China) to prepare a *koji* medium. The medium was cooled to 40 °C, inoculated with 0.03% (w/w) of *A. oryzae* spores, and incubated at 30.0 °C for 18.0 h and then 28.0 °C for another 26.0 h in a ZXJP-A1430 mould cultivation cabinet (Zhicheng, Shanghai, China). The relative humidity was about 95% during *koji* fermentation, and the internal temperature of the *koji* was kept below 40 °C through occasional turning. The preparation of *koji* was considered complete when the culture turned greenish yellow.

Preparation of *moromi* via liquid-state fermentation

The finished *koji*, which was prepared using 4.0 kg soy flakes, was divided into three parts in 4.0 L plastic tanks. The first part was mixed with water (zero brine content) at a ratio of 1:2.5 (w/w) in a *moromi* tank with the temperature controlled at 4 °C during the initial 9-day *moromi* fermentation. Then, the *moromi* was mixed with salt (to achieve a 16% w/w salt solution) with the temperature being raised to 25 °C (the resultant product was termed “LTSF”). The second part was mixed with a 22% (w/w) brine solution at a ratio of 1:3.05 (w/w) (to achieve a 16%

w/w salt solution), with the temperature controlled at 4 °C during the initial 9-day *moromi* fermentation and then raised to 25 °C (the resultant product was termed “LTSH”). The third part was mixed with a 22% (w/w) brine solution at a ratio of 1: 3.05 (w/w) (to achieve a 16% w/w salt solution), with the temperature maintained at 25 °C (the resultant product was termed “CRTH”, the “control”). The entire *moromi* fermentation process lasted 60 days with stirring once a week. Subsamples of the fermented products were withdrawn periodically on Day 1, 3, 5, 7, 9 and 60. All subsamples were filtered through double Whatman® fast qualitative filter paper (Hangzhou, China) filter papers, kept in polyethylene vials and stored in a freezer at 20 °C until analysis.

Autolysis of *A. oryzae* examined using flow cytometry

The nucleic acid content was determined according to Gandhi and Shah (2015) method with some modifications. Phosphate buffered saline (PBS) was prepared with 0.2 g of KH_2PO_4 , 2.9 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 8.0 g of NaCl, 0.2 g of KCl, and then diluted to 1000.0 mL deionized water at pH 7.4. A stock solution of propidium iodide (PI) was prepared by dissolving 1.0 mg of PI in 1.0 mL of distilled water followed by a dilution to 500 µg/mL with PBS (used as the working solution) and storage in a refrigerator at 4 °C (in dark). A aliquot (100.0 µL) of the above-mentioned filtered subsample was gently vortexed and incubated with 10.0 µL of PI dye at 25 °C for 20 min in the dark, to which 400 µL of PBS was added within 1 h prior to analysis using a CytoFLEX flow cytometry (Beckman Coulter Inc., USA). For each analysis, forward scatter (FSC), side scatter (SSC), red (PI) fluorescence was recorded, amplified and converted into digital signals. PI-stained cells emit red fluorescence at 635 nm following excitation with laser light at 488 nm, and the cells stained with PI indicate compromised membrane (Hoefel et al. 2003). The recorded signals were amplified logarithmically, and a quad-gate was created in the dot plot to select the proper region. The data were analyzed using CytExpert version 2.0 (Beckman Coulter Inc., USA).

Determination of nucleic acid content by ascorbic acid-ammonium molybdate spectrophotometry

Phosphate determination reagent was prepared with a ratio of 1:1:1:2 for 17% (v/v) sulfuric acid, 2.5% (w/v) ammonium molybdate, 10% (w/v) ascorbic acid and distilled water. The total phosphorus (TP) and inorganic phosphorus (IP) in the sample were determined using a digestion method and dilution method, respectively (Xu et al. 2016). The absorbance at 660 nm was measured using a

spectrophotometer (YOKE Co. Ltd., PR China). Each test sample was analysed in triplicate. The organic phosphorus (OP) = total phosphorus (TP) – inorganic phosphorus (IP). The content of nucleic acid was calculated using the following equation:

$$\text{Nucleic Acid mg/g} = \frac{cv \times 11 \times 50}{m \times 100}$$

where c is the concentration of OP (µg/mL), v is the total volume of the test sample (mL), 11 is the conversion factor from phosphorus to nucleic acid, 50 is the dilution ratio of the test sample, m is the wet weight of the 1-mL sample, 100 is the unit conversion.

Glutaminase activity assay

The glutaminase activity was determined according to Nannipieri et al. (1980) method and Cui et al. (2017) method with some modifications. The supernatant/dreg was diluted with a neutral buffer (Na_2HPO_4 -citric acid, pH 7.0). A mixture of a subsample (1.0 mL), neutral buffer (1.0 mL) and 1% w/v L-glutamine (1.0 mL) was incubated at 37 °C for 10 min, before the reaction was terminated, through adding 1.0 mL of 10% w/v trichloroacetic acid (TCA) and then 6.0 mL of acidic buffer (Na_2HPO_4 -citric acid, pH 3.0). The ammonium released was measured using a Bante 321-NH4 ammonium selective electrode (Shanghai, China). The analysis of each test sample was performed in triplicate. One unit of glutaminase (U) was defined as the amount of enzyme that catalyzed the splitting of L-glutamine to yield 1 ng ammonium ion (NH_4^+) in 1.0 mL or 1.0 g of the reaction product per minute at 37 °C.

Analysis of neutral or acidic protease activity

Activities of neutral and acidic proteases in *moromi* were determined following the method of Chen et al. (2017) with some modifications. The subsample was diluted with a neutral buffer (0.1 M Na_2HPO_4 - NaH_2PO_4 , pH 7.2) or acidic buffer (0.05 M Lactic acid-sodium lactate, pH 3.0), depending on the target protease. Casein (2%, w/v) was mixed with 20.0 mL of 0.1 M NaOH prepared in the 0.1 M neutral buffer, or mixed with a few drops of concentrated lactic acid prepared in the 0.05 M acidic buffer, depending on the target protease, before a dilution to 100.0 mL at the corresponding pH. A mixture of 1.0 mL of casein solution and 1.0 mL of the diluted sample was incubated at 40 °C for 10 min, then the reaction was terminated through adding 2.0 mL of 0.4 M trichloroacetic acid (TCA). An aliquot (1.0 mL) of the filtered supernatant obtained from above reaction solution through single Whatman® fast qualitative filter paper (Hangzhou, China), was mixed with

5.0 mL of 0.4 M sodium bicarbonate, before the addition of 1.0 mL of 0.4 M Folin–Ciocalteu phenol reagent (1:3 diluted in water) and incubation at 40 °C for 20 min. The absorbance at 660 nm was measured using a YOKE UV-765 ultraviolet spectrophotometer (YOKE Co. Ltd., PR China). Analysis of each sample was performed in triplicate. One unit of protease (U) was defined as the amount of enzyme that yields the color equivalent to 1 µg tyrosine in 1.0 mL of the reaction solution per minute at 40 °C.

Analysis of quality indices of soy sauce

The pH of a soy sauce solution was measured directly with a Rex E-301-CF pH meter (INESA Co. Ltd., PR China). The total nitrogen (TN) was determined using Kjeldahl (AOAC 1990) method. The amino nitrogen (AN) and total acidity (TA) were measured by titration using the method of Cui et al. (2013). The total sugar (TS) was analyzed using the phenol–sulphuric acid method with a 6% (w/v) phenol reagent, 0.04% (w/v) D-glucose standard and YOKE UV-765 ultraviolet spectrophotometer (YOKE Co. Ltd., PR China). The intensities of darkness, red color and yellow color were determined using the method described by Li et al. (2007). All the test samples were diluted in tenfold volume of distilled water with each sample analysed in triplicate.

Analysis of free amino acids (FAAs)

An aliquot (4.0 mL) of soy sauce sample was mixed with 1.0 mL of 15% (v/v) sulfosalicylic acid solution, before an incubation at 4 °C for 1.0 h. The mixture was centrifuged at 10,000×g and 4 °C for 15 min, and the resultant supernatant was filtered through a micropore film (pore size 0.22 µm; Millipore, Merck Millipore Inc., MA, USA). The filtrate was used for the determination of FAA composition using the amino acid analyzer A300 (membraPure GmbH, Germany), based on the method of Hughes and Frutiger (1990). Briefly, amino acids were separated by liquid chromatography with an ion exchange column (TS263, membraPure) and colored by ninhydrin. The absorbance at 570 nm for most AAs or at 440 nm for proline was measured. The concentration of each amino acid was determined by using an external standard through calibration. The data were analyzed using iControl and iPeak software.

Sensory evaluation

Quantitative descriptive analysis (QDA) was applied to evaluate the differences in taste characteristics among the test samples following the method of Gao et al. (2010). QDA was carried out with a panel consisting of five

women and seven men (healthy, aged 25–45 years) in the sensory laboratory of a soy sauce company (Foshan Haitian flavoring & food Co., Ltd, Guangdong, China). Nine panelists were trained to perform a triangle test to evaluate the samples at room temperature (23 ± 2 °C) (Carpenter, Lyon and Hasdell 2012). Five basic taste qualities were defined using the following reference solutions: 0.7% (w/v) NaCl for saltiness, 0.35% (w/v) monosodium glutamate (MSG) for umaminess, 1% (w/v) saccharose for sweetness, 0.08% (w/v) citric acid for sourness, and 0.5% (w/v) L-isoleucine (Ile) for bitterness. All soy sauce samples (10 mL each) were put into 50-mL disposable paper cups coded with random three-digit numbers. These cups were covered with lids prior to the sensory evaluation. After each sample (2 mL) was placed in the mouth for 10 s, the panelists were asked to rinse their mouths with cool boiled tap water before further evaluation. The number of samples were controlled within 6 and the tasting time was controlled in 2 h, to avoid evaluation error caused by sensory fatigue. In addition, samples were maintained their temperature in a range of 15–30 °C (at which human tongues are the most sensitive). The intensities of different tastes and the degree of taste-enhancement (kokumi) were scored using a 9-point linear scale from 0 (lowest intensity) to 9 (highest intensity).

Statistical analysis

All the test samples were prepared in duplicate in this study with each test sample analyzed in triplicate except for the sensory evaluation. Data were reported as mean $\pm$ standard deviation (SD). Analysis of variance (ANOVA) and significance of the differences among means were tested by one-way ANOVA using SPSS software (version 16.0 for Windows, SPSS Inc., Chicago, IL).

Results and discussion

Autolysis of *Aspergillus oryzae* mycelia

Autolysis is often induced by the hydrolysis of intracellular biopolymers with endogenous hydrolytic enzymes, causing cell death and broken cell membranes (Babayan and Bezrukov 1985). Nucleic acid is one of the major substances from *A. oryzae* mycelia and largely comes out of mycelia during the process of autolysis, thereby can be used to indicate autolysis. As a nucleic acid dye, PI is impermeant to intact cell membranes, but stains dead cells with red fluorescence and binds tightly to the nucleic acids inside the cells. Thus, the intensity of fluorescence reflects the degree of autolysis of *A. oryzae*. In this study, the changes in fluorescence intensity during the initial *moromi*

fermentation were monitored by flow cytometry (scatter plots as shown in Fig. S1). The fluorescence intensity for the samples under low temperature stress (LTSF and LTSH) was much higher than the control (CRTH) especially after 3 days (Fig. 1a), suggesting that low temperature stress induced greater autolysis of *A. oryzae*. The images of *A. oryzae* mycelia during the initial *moromi* processing were obtained by a Research-Grade Upright microscope (Fig S2), which show the influence of low temperature stress on the morphological changes of mycelia after fermentation for 1 day and 7 days at 4 °C. The number and length of mycelia decreased significantly for LTSF (a), LTSH (b) and CRTH (c) samples as *moromi* fermentation proceeded, suggesting that the degree of autolysis of mycelia increased over the initial *moromi* fermentation. Compared to the mycelia in CRTH, the LTSF and LTSH samples obtained under low temperature stress had less mycelia on the 1st day and smaller sized mycelia on the 7th day. These results implied that low temperature stress applied during the initial *moromi* fermentation could significantly promote the autolysis of *A. oryzae*. Zhao et al. (2018) also reported that autolysis of mycelia was induced in *Volvariella volvacea* (a temperature-sensitive fungi) under low temperature stress (0–5 °C), due to the degradation of proteins in cells by proteases and glutaminases.

Under the same low temperature stress condition, the sample with high sodium chloride involved (LTSH) had a significantly higher fluorescence intensity than the sample without sodium chloride (LTSF). van Niel et al. (2003) indicated that a high sodium chloride content would influence greatly the bacterial growth and cause cell autolysis. Jennifer et al. (2011) also reported that

Lactococcus lactis strains (MA16, PK04, KK05, ATCC) exhibited the greatest autolysis at a basic pH (7.0) and a high salt concentration (0.51 M), because of the highly significant ($P > 0.001$) impact caused by the interaction between salt concentration and pH. Accordingly, the use of high sodium chloride concentration would facilitate the autolysis of *A. oryzae*, due to the associated high osmotic pressure effect and synergistic effect with low temperature stress.

In this study, the fluorescence results were generally consistent with the changing trends detected by the ascorbic acid-ammonium molybdate spectrophotometry (Fig. 1b), although the differences in nucleic acid concentration among LTSF, LTSH and CRTH were found smaller. The nucleic acid concentration of the samples under low temperature stress (LTSF and LTSH) was higher than that for CRTH. This result was in agreement with the finding reported by Gong et al. (2015) who found that low temperature at 4 °C for 24 h induced the autolysis of *Volvariella volvacea* mycelia. Chang (1978) already reported that storage of *V. volvacea* at a low temperature (4 °C) caused autolysis of mycelia. In the mid-1990s, autolysis of *A. oryzae* was already found in the presence of high temperatures but low pHs (Arima et al. 1965), biochemical inhibitors under aerobic conditions (Horikoshi 1958), and/or organic solvents (Horikoshi 1958).

Glutaminase activity

In this study, the glutaminase activities in the fermented supernatants and dregs of LTSF, LTSH and CRTH samples decreased ($P < 0.05$) gradually with fermentation time (Fig. 2a, b). The glutaminase activities of samples under

Fig. 1 Fluorescence intensity (by flow cytometry (a)) or nucleic acid concentration (by ascorbic acid-ammonium molybdate spectrophotometry (b)) as a function of time during the initial *moromi* processing

(“open bar”, “stripped bar”, “boxed bar” represent LTSF, LTSH and CRTH, respectively)

Fig. 2 The glutaminase activity of the *moromi* supernatant (a) and dreg (b) obtained during the initial *moromi* processing (“square”, “circle”, “triangle” represent LTSF, LTSH and CRTH, respectively)

low temperature stress (LTSF and LTSH) were significantly higher than that for CRTH (from the 1st day for the supernatants or from 2nd day for the dregs) during the initial *moromi* fermentation, suggesting that low temperature stress and/or high salt concentration promoted the glutaminase activities in both supernatants and dregs. On the 9th day, low temperature stress applied during initial *moromi* fermentation was found to cause a twofold increase in glutaminase activity in dregs and a 65.17% increase in supernatant. In general, the LTSF sample had the highest glutaminase activity i.e., after Day 3 for the supernatants and from Day 1 for the dregs, probably due to low temperature stress and absence of salt during the initial *moromi* fermentation. The glutaminases of *A. oryzae* might be markedly inhibited by a high salt content in soy sauce during fermentation (Nandakumar et al. 2003).

Glutaminase is an important enzyme for soy sauce fermentation and hydrolyzes Gln to Glu and ammonia (Fukushima 2004). Glu is critical for the unique flavor of soy sauce. Approximately 60% of the Glu in soy sauce is produced through the glutaminase hydrolysis reaction (Ito et al. 2013). In comparison, Gln resulted from the degradation of proteins may change in the absence of enzymatic action and is quickly turned into tasteless pyroglutamic acid (Machida et al. 2007). Therefore, research efforts have been directed at the increase of glutaminase activity to convert Gln into Glu in order to achieve an enhancement of the umami taste of soy sauce. Based on the location, the glutaminases of *koji* molds are classified into extracellular, cell surface and intracellular species (cell membrane and cytoplasm) (Machida et al. 2007). Only a limited number of reported microbial glutaminases are extracellular enzymes, with most located on the cell surface and

intracellularly (Machida et al. 2007; Binod et al. 2017). The intracellular fractions of *A. oryzae* can be further divided into two groups: soluble and insoluble (Shikata 1978). Under normal conditions, mycelia autolysis occurs only when the fermentation is prolonged, causing the release of the extracellular, cell surface and soluble intracellular glutaminases into the supernatant with the insoluble fraction remaining in the dreg.

Protease activity

Based on the required optimal pH, the proteases of *A. oryzae* are classified into neutral protease, alkaline protease and acidic protease. Protease activity in the *moromi* is critical for the release of peptides and free amino acids (Gao et al. 2011). During the *moromi* fermentation, neutral protease and acidic protease play dominant roles because of the neutral or slightly acidic pH of the *moromi* fermentation system.

In this study, the neutral protease activities were much higher than the acidic protease activities for the same time point and the same type of soy sauce sample (Fig. 3). These findings likely resulted from the nature of the current fermentation system (a neutral or slight acidic *moromi* condition) and different optimal pH ranges of the neutral and acidic proteases (Basu et al. 2008; Siala et al. 2012). The neutral protease activities of LTSF, LTSH and CRTH exhibited a sharp drop (with the same decreasing rate) from Day 0 to Day 1, and then a slow decline from Day 1 to Day 9 (at slightly different rates: LTSH = LTSF > CRTH for Day 1–3, and LTSH > LTSF > CRTH for Day 3–9) (Fig. 3a). Since a high concentration of sodium chloride would inhibit the protease activity (Su et al. 2005), the

Fig. 3 The neutral protease activity (a) and acidic protease activity (b) of the *moromi* supernatant obtained during the initial *moromi* processing (“square”, “circle”, “triangle” represent LTSF, LTSH and CRTH, respectively)

protease activity in this study was determined using a serial dilution method. Only about 0.2% of salt was retained in the diluted sample, thereby the impact of such a low salt concentration on the protease activity was minimal. For the acidic protease activities, LTSF, LTSH and CRTH exhibited the same changing trend i.e., a sharp drop (over Days 0–1) followed by a slow and then slight increase (over Days 1–9) (Fig. 3b). LTSH had the greatest acidic protease activity after Day 3, probably due to the presence of salt-tolerant acidic protease and the slightly increased pH after fermentation for 3 days (Lee et al. 2010). It was not surprising to see such changes in the enzymatic activities with time. Proteases may exert their effect on protein hydrolysis in a dynamic manner, and a decreased hydrolysis rate occurring at the latter stage was probably due to the reduced concentration of the susceptible sites of peptide bonds with the hydrolysis time and the inhibition of enzymatic activity by certain hydrolysis products (Feng et al. 2018).

Quality indices of soy sauce

The contents of TN and AN are important quality indices for soy sauce products (Su et al. 2005). As shown in Table 1, the TN contents after the 60-day fermentation were 1.77 (LTSF), 1.80 (LTSH) and 1.79 (CRTH) g/100 mL, respectively, indicating no significant difference ($P > 0.05$) among LTSF, CRTH and LTSH. This result confirmed the finding of Wu et al. (2010) that the fermentation temperature was not the important factor influencing the TN of final products. The AN contents after the 60-day fermentation were 0.89 (LTSF), 0.89 (LTSH) and 0.88 (CRTH) g/100 mL, respectively, with no significant

differences ($P > 0.05$) found in the AN contents of the three samples after the 60-day fermentation. Similar TN and AN values were reported by Li et al. (2007) who investigated the physical–chemical properties of raw soy sauce. Moreover, the TA and TS contents after fermentation for 60 days showed no significant differences ($P > 0.05$) among LTSF, CRTH and LTSH. TA was a critical quality index for a fermented food, which is closely associated with the production of organic acids during fermentation. In this study, the pH of CRTH decreased rapidly to 4.90 at room temperature after 60 days of fermentation, compared to LTSF and LTSH (whose pH values decreased to 5.06 and 5.04, respectively). The pH of CRTH was significantly different ($P < 0.05$) from that of LTSF or LTSH. Previous studies (Devanthi et al. 2018; Singracha et al. 2017) reported that the pH values decreased from ~ 5.3 to ~ 4.8 in reduced-salt *moromi* fermentation of soy sauce. A traditional Korean soy sauce was found to have a pH around 4.80 (Song et al. 2015). A decrease of pH during fermentation was probably due to the release of free fatty acids, organic acids and free amino acids. Low temperature stress likely induced a slightly elevated pH, leading to a neutral or slight acidic *moromi* (in which glutaminases and neutral proteases became most active). Color intensity, red and yellow indices are commonly used to evaluate the color of soy sauce industry. Chen et al. (2015) reported a lower color intensity but similar red and yellow indices for soy sauce prepared with *A. oryzae koji* compared to those for the three samples in this study. Red and yellow indices were significantly higher for CRTH than for LTSH and LTSF, suggesting that low temperature stress facilitated a decrease in color indices of soy sauce. LTSH was slightly darker than LTSF, indicating that a

Table 1 The physico-chemical properties of soy sauce during the initial moromi fermentation at different temperatures and salt concentrations

Analysis	Low temperature stress process		Conventional process
	LTSF	LTSH	CRTH
Total nitrogen (g/100 mL)	1.77 ± 0.02a	1.80 ± 0.02a	1.79 ± 0.01a
Amino nitrogen (g/100 mL)	0.89 ± 0.01a	0.89 ± 0.01a	0.88 ± 0.01a
pH	5.06 ± 0.01a	5.04 ± 0.02a	4.90 ± 0.02b
Total acidity (g/100 mL)	1.48 ± 0.04a	1.50 ± 0.03a	1.63 ± 0.04a
Total sugar (g/100 mL)	3.50 ± 0.07a	3.60 ± 0.05a	3.49 ± 0.13a
Dark color intensity	5.04 ± 0.02a	5.06 ± 0.02a	5.08 ± 0.04a
Red index	1.95 ± 0.04c	2.05 ± 0.06b	2.26 ± 0.05a
Yellow index	3.57 ± 0.03c	3.90 ± 0.03b	4.22 ± 0.02a

¹All parameters are given as the mean ± standard deviation (n = 3)²Means within the same row followed by different letters are significantly different at $P < 0.05$

higher salt content would lead to a slightly darker color. Accordingly, it is of practical importance to investigate further how to improve the color intensities under low temperature stress.

Free amino acids (FAAs) of soy sauce

The actions of protease and glutaminase would lead to protein hydrolysis and the formation of free amino acids during the soy sauce fermentation. FAAs are important contributors to the taste of soy sauce (Lioe et al. 2007). The basic tastes, and their concentrations and combinations influence the ultimate taste of foods. Depending on their concentrations, amino acids exhibit umami and sour tastes (L-glutamic acid and L-aspartic acid), sweet taste (L-alanine, L-glycine, L-serine, L-threonine and L-glutamine), bitter taste (L-arginine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tyrosine, L-valine and L-tryptophan), or tasteless (L-proline and L-lysine) (Kawai et al. 2012). Some amino acids may have multiple tastes. L-valine, which is generally considered as a bitter amino acid, also has a slightly sweet taste; L-serine, which often tastes sweet, also exhibits minor umami and sour tastes at a high concentration.

As shown in Table 2, the total free amino acid content after the 60-day fermentation decreased in this order: LTSF > CRTH > LTSH, although no significant difference between CRTH and LTSH was found at $P > 0.05$. The concentrations of all taste FAAs were higher than their threshold values except for proline and tyrosine, which agreed with that described previously by Chen et al. (2015). Glu, Leu, Ile, Asp and Thr were the major amino acids in the samples of this study, which were in agreement with the finding of Gao et al. (2010).

In this study, Glu and Asp, accounting for over 23.94% of the total FAA content, were the dominant umami FAAs in soy sauce. Enzymatic hydrolysis could lead to a rapid

increase of free amino acids (Yang et al. 2011). The highest umami amino acid content (1517.66 mg/100 mL; 24.88% of total free amino acids) was detected in LTSF, followed by LTSH (1500.42 mg/100 mL; 24.87% of total free amino acids), and then by CRTH (1445.57 mg/100 mL; 23.94% of total free amino acids). The contents of Glu in LTSF and LTSH were significantly higher than that in CRTH ($P < 0.05$, increased by 5.73% and 4.19%, respectively). The content of Glu in LTSF was anticipated higher than that in LTSH, as a higher salt concentration could decrease the activities of glutaminase during the initial *moromi* fermentation. Similarly, the contents of Asp in LTSF and LTSH were increased by 3.47% and 2.99%, respectively ($P < 0.05$), compared to that in CRTH. Interestingly, LTSF and LTSH had significantly higher ($P < 0.05$) contents of sweet FAAs but significantly lower ($P < 0.05$) contents of bitter FAAs, compared with CRTH.

Aguirre et al. (2008) indicated that the free amino acid content could reflect intuitively the enzymatic hydrolysis of soybean protein. The formation of free amino acids is associated with product organoleptic characteristics, nutrition value and bioactivity (Yang et al. 2011). In this work, relatively high amounts of umami and sweet amino acids indicated that low temperature process could help promote the desirable tastes and reduce the bitterness of soy sauce products. Accordingly, the preparation of soy sauce under low temperature stress during the initial *moromi* fermentation may be beneficial, as the desired umami and sweet tastes increased while the bitterness was reduced.

Sensory evaluation

The flavor of soy sauce is complex and depends on both ingredient type and amount as well as processing methods (including the conditions of fermentation). The overall taste of soy sauce is predominated by salty and umami

Table 2 Free amino acid content in soy sauce during the initial moromi fermentation at different temperatures and salt concentrations

Taste	Amino acid (mg/100 mL)	Low temperature stress process		Conventional process	Threshold values (mg/100 mL) in water
		LTSF	LTSH	CRTH	
Umami	Asp	492.31 ± 0.57a	490.07 ± 0.54a	475.82 ± 0.52b	100
	Glu	1025.35 ± 1.23a	1010.35 ± 1.18b	969.75 ± 1.14c	30
	Subtotal	1517.66 ± 1.80a	1500.42 ± 1.72b	1445.57 ± 1.68c	
Sweet	Ala	392.13 ± 0.48b	387.42 ± 0.45c	397.84 ± 0.54a	60
	Gly	330.23 ± 0.44a	315.65 ± 0.39c	325.68 ± 0.41b	130
	Ser	154.62 ± 0.23b	155.68 ± 0.25a	156.42 ± 0.27a	150
	Thr	408.67 ± 0.48c	416.56 ± 0.55b	419.86 ± 0.58a	260
	Pro	195.46 ± 0.32a	188.45 ± 0.24b	189.42 ± 0.27b	300
	Gln	260.12 ± 0.37a	215.45 ± 0.30b	180.34 ± 0.28c	ND
	Subtotal	1741.23 ± 2.32a	1679.21 ± 2.18b	1669.56 ± 2.35c	
Bitter	Arg	298.36 ± 0.36c	299.93 ± 0.37b	304.77 ± 0.39a	50
	His	95.48 ± 0.17c	98.12 ± 0.19b	102.51 ± 0.20a	20
	Ile	495.15 ± 0.58b	504.33 ± 0.60a	504.32 ± 0.61a	90
	Leu	645.34 ± 0.74b	625.45 ± 0.72c	648.85 ± 0.75a	190
	Met	119.86 ± 0.23a	110.58 ± 0.20c	115.23 ± 0.22b	30
	Phe	384.68 ± 0.46c	388.95 ± 0.47b	394.26 ± 0.50a	90
	Tyr	89.97 ± 0.14c	97.81 ± 0.18b	106.44 ± 0.21a	91
	Val	403.55 ± 0.49c	412.20 ± 0.51b	432.19 ± 0.58a	40
	Trp	90.13 ± 0.14a	88.45 ± 0.12c	89.54 ± 0.13b	82
	Subtotal	2622.52 ± 3.31b	2625.82 ± 3.36b	2698.11 ± 3.59a	
Tasteless	(Cys)2	2.19 ± 0.01c	3.65 ± 0.01b	5.06 ± 0.01a	ND
	Lys	219.56 ± 0.31c	226.48 ± 0.36a	224.91 ± 0.33b	50
	Subtotal	221.75 ± 0.32b	230.13 ± 0.37a	229.97 ± 0.34a	
Sum	Subtotal	6103.16 ± 7.75a	6035.58 ± 7.63b	6043.21 ± 7.96b	

¹All parameters are given as the mean ± standard deviation (n = 3)

²Means within the same row followed by different letters are significantly different at $P < 0.05$

³Chen et al. (2015)

tastes, with moderate sweetness, slight bitterness and kokumi sensation. The sensory results of LTSH, LTSF and CRTH were shown in Fig. 4. Based on the QDA test, the

Fig. 4 Sensory evaluation of soy sauce samples

three types of samples exhibited different taste profiles and taste-enhancing properties but had the same sweetness and bitterness (without significant differences) ($P < 0.05$). In general, the overall taste of soy sauce prepared under low temperature stress (LTSF and LTSH) was dominated by umami, followed by saltiness and kokumi sensation, with slight sweetness and bitterness. The decreases in the intensities of salty and bitter tastes during fermentation were desirable for achieving a well-balanced characteristic taste of soy sauce (Gao et al. 2011). The umami taste seemed to be the most prominent taste for LTSF and LTSH. The intensity of umami taste followed this order: LTSF > LTSH > CRTH, which was in agreement with the findings on the glutaminase activities (Fig. 2) and contents of umami FAAs (Table 2) in the three analyzed samples. For the salty taste, the trend was reversed: Compared to CRTH, LTSF and LTSH exhibited a slightly weaker salty taste, resulting in a moderate and balanced taste with

increased umaminess. Moreover, the samples obtained under low temperature stress (LTSF and LTSH) exhibited a marginally greater kokumi effect than CRTH did. Kokumi sensation described sensory characteristics such as thickness, continuity, richness, mouthfulness, and long-lasting harmony in taste. The kokumi sensation of soy sauce mainly come from γ -glutamyl peptides such as γ -Glu-Val, γ -Glu-Met and γ -Glu-Val-Gly (Yang et al. 2018). Therefore, higher contents of umami FAAs in LTSF and LTSH upon the actions of the enzymes in the microorganism (such as γ -glutamyltransferase) might be associated with higher contents of γ -glutamyl peptides.

Conclusion

This study clearly demonstrated the beneficial outcome of performing initial *moromi* fermentation at 4 °C while the remaining process at room temperature, as well as the influence of salt (16% w/w brine), in terms of the quality and taste of final soy sauce product. Low temperature stress induced quicker autolysis of *A. oryzae* mycelia (evident as stronger fluorescence and higher nucleic acid concentration), causing greater release of glutaminases and proteases during *moromi* fermentation. These enzymes broke down the proteins in the raw material into amino acids (including Glu, one of the most important contributors to the umami flavor). Compared to the control, low temperature stress generally increased the activities of enzymes (including glutaminase) and the contents of umami and sweet amino acids which agreed with the desirable changes in soy sauce taste profiles (especially umaminess and kokumi sensation) revealed by sensory evaluation. There existed interplay between low temperature stress and salt (16% w/w brine) from *moromi* fermentation (e.g. microbial autolysis and enzymatic activities) to formation of the final soy sauce product (with characteristic composition and taste). The intensity ranking of umaminess and kokumi sensation (LTSF > LTSH > CRTH) revealed by sensory analysis followed the changing trends of their umami and sweet amino acid contents with a trend reversal in the bitter amino acid content. To enable the practical application of low temperature stress to commercial soy sauce production, further work is required to optimize all the quality attributes (including colour) of final soy sauce under the proposed temperature regime in this study (i.e., initial *moromi* fermentation at 4 °C and the remaining process at room temperature).

Acknowledgements The authors are grateful for the financial support from National key Technologies R&D Program for 13rd Five-year Plan (2016YFD0400803), the Fundamental Research Funds for the Central Universities (2017ZD093), the Science and technology

plan project of Guangdong Province (2017A010105002) and National Natural Science Foundation of China (31201416 and 31571821).

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