Isolation, identification and application on soy sauce fermentation flavor bacteria of CS1.03

Xuewei Jiang^{1,2} · Yantao Xu¹ · Jing Ye¹ · Zijiang Yang^{2,3} · Shouen Huang^{1,2} · Yongle Liu^{1,2} · Shangting Zhou^{2,3}

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Abstract *Bacillus* is a common bacterial genus in Chinese traditional sun-brewed moromi. Some *Bacillus* species can metabolize sauce flavour and aroma substances. To address the insufficiency of flavour microbes in soy sauce fermentation, we used 16S rDNA sequence analysis to identify the CS1.03 strain, which was isolated from moromi produced through the traditional and natural sun brewing methods. A Box–Behnken design was used to optimize the addition technology of CS1.03, and ammoniacal nitrogen content and sensory evaluation were adopted as determination indices. GC–MS analysis was also performed to analyse the flavour components. Results identified CS1.03 as *Bacillus subtilis*. The optimal conditions yielded 10.7 g/L ammoniacal nitrogen in fermented soy sauce with rich aroma and flavour, and a sensory evaluation score of 90.5. GC–MS analysis revealed that the contents of phenol, aldehyde and ketone are all increased. These results demonstrated that CS1.03 could improve the flavour of soy

sauce and shows good potential for application in soy sauce fermentation.

Keywords Flavour microorganism · Soy sauce fermentation · 16S rDNA sequence analysis · *Bacillus subtilis* · Box–Behnken design · GC–MS analysis

Introduction

Soy sauce is a traditional condiment (Feng et al. 2012b; Kim et al. 2015) with rich nutrients, such as ammoniacal acid and other special flavour substances, from Southeast Asia. This condiment is formed by various biochemical reactions of enzymes secreted by various microbes. In soy sauce fermentation, *Aspergillus oryzae* is the main fermentation microbe (Kim et al. 2017; Xu et al. 2010), but other related microbes, such as bacteria and yeasts, and their enzyme systems have a crucial effect on the quality and special flavour of soy sauce. Therefore, soy sauce with an excellent flavour profile is the result of a multi-strain mixed fermentation (Feng et al. 2012a).

Natural sun-brewed fermentation is an ancient soy sauce fermentation method used in Southeast Asia, and its earliest recorded use in China was in approximately A.D. 550 (Bao 2011). Soybean and malt are boiled and mixed with *A. oryzae* and then placed in a jar and fermented in an open natural environment. During fermentation, koji becomes naturally contaminated by microorganisms, such as *Lactobacillus pobuzihii*, *Aspergillus flavus*, and *Aspergillus parasiticus* (Shukla and Kim 2016; Yang et al. 2017). After close to 1 year of fermentation, soy sauce is obtained because of the contribution of various strains, such as *Aspergillus oryzae*, *Aspergillus niger*, *Lactobacillus* spp., *Zygosaccharomyces rouxii*, et al. (Zhu and

The strain of CS1.03 has been collected in China Center for Type Culture Collection (CCTCC, Wuhan University, Wuhan City, China), and its collection number is M 2015791.

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✉ Xuewei Jiang
jxw_72@sina.com

- ¹ School of Chemistry and Food Engineering, Changsha University of Science and Technology, Changsha 410114, Hunan, China
- ² Hunan Provincial Engineering Technology Research Center for Condiment Fermentation, Changsha 410600, Hunan, China
- ³ Jiajia Food Group Co. Ltd., Changsha 410600, Hunan, China

Cai 2014). However, natural contamination remains difficult to be controlled and poses a risk to quality and safety. Such a risk becomes intensified because of the low efficiency in production. Thus, sun-brewed fermentation has gradually been replaced by closed high-salt liquid-state fermentation.

Modern high-salt liquid-state fermentation technology in closed fermentation equipment is essential for the prevention of contamination by infectious microbes (Feng et al. 2015). However, certain microorganisms that contribute to the soy sauce flavour are consequently lost. Therefore, bacteria beneficial to the formation of flavour substances should be screened. The main flavour microorganisms in soy sauce fermentation are yeasts (Liu et al. 2015) and lactic acid bacteria (Masuda et al. 2008; Qi et al. 2016), although other microorganisms have also been reported. Alcohols, aldehydes, and other substances are provided by the metabolism of yeasts, while lactic acid is produced by the metabolism of lactic acid bacteria (Harada et al. 2017). Li et al. (2015) and Yao et al. (2010) demonstrated that a *Bacillus subtilis* strain isolated from the pit mud of Maotai-flavoured liquor, which is a famous Chinese liquor, possesses a flavour similar to that of soy sauce, and its metabolism yields the main flavour substances, such as guaiacol, 4-vinyl guaiacol, and other phenolic substances, in Maotai-flavoured liquor. These substances are also the significant components of sauce flavour. Thus, *Bacillus subtilis* can promote the formation of sauce flavour, but this ability has been rarely reported in the field of soy sauce fermentation. Hence, in this work, we isolated and identified a strain from the sauce moromi produced by traditional and natural sun-brewed fermentation to optimize the addition conditions and determine the volatile flavour components in soy sauce fermented using this strain. This study elucidates the mechanism by which this strain contributes to the flavour of soy sauce and reveals its potential application. This study also aims to obtain a new type of microorganism beneficial for soy sauce flavour.

Materials and methods

Strains

The CS1.03 strain (CCTCC NO: M 2015791) used in this work was derived from natural sun-brewed fermentation moromi (Longpai Food Co. Ltd., Xiangtan, China). The culture medium was beef extract peptone medium with 16% salinity. *Torulopsis* CS2.01 (Jiajia Food Group Co., Ltd., Changsha, China) was cultured using the malt extract medium.

Preparation of media

Beef extract peptone medium was prepared by dissolving 3.0 g of beef extract, 10.0 g of peptone, and 5.0 g of NaCl in 1000 mL of water. The pH was adjusted to 7.2 ± 0.1 .

Malt extract medium was produced by dissolving 130 g of malt extract powder and 0.1 g of chloramphenicol in 1000 mL of water, and the pH was adjusted to 5.6 ± 0.2 .

Both the above two media were sterilized at 121 °C for 20 min (BXM 30R, Shanghai Boxun Industrial Co., Ltd., Medical Equipment Factory, China). All of the reagents were purchased from the Sinopharm Group in China.

Morphological identification and 16S rDNA sequence analysis

The CS1.03 strain was grown for 24 h at 37 °C and then subjected to Gram staining. The shape and staining results were observed under a microscope (Eclipse E200, Nikon Instruments (Shanghai) Limited Co., Ltd., Japan). The bacteria grown in a shake-flask culture for 24 h were used as a PCR template. The universal primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') was adopted for PCR. The PCR reaction system had a total volume of 50 µL, which comprised 25 µL of PCR Premix (Sangon Biotech Co., Ltd., Shanghai, China), 2 µL of template, 2 µL of 10 pmol/µL forward primer, 2 µL of 10 pmol/µL reverse primer, and 19 µL of ddH₂O. After predenaturation was performed for 5 min at 94 °C, amplification was carried out in 30 cycles as follows: denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, and elongation at 72 °C for 90 s; final run with ultimate elongation at 72 °C for 5 min (Jiang et al. 2016). The PCR product was detected through agarose gel electrophoresis at a constant voltage of 80 V for 20–30 min. The target DNA was cut and placed in a 1.5 mL EP tube and then sequenced (Sangon Biotech (Shanghai) Co. Ltd.). The sequencing result was analysed with sequence homology via the NCBI database. A phylogenetic tree was constructed with MEGA version 5.0.

Soy sauce fermentation

The produced koji (50 g of dry koji per conical flask) was mixed with 50 mL of 18% salinity brine. After 10 days of fermentation at 40 °C, 1 mL of CS1.03 and CS2.01 spore suspension (10^7 CFU/mL) was added respectively to samples of S₁ and S₂ (Control group represented by S₀ without CS1.03 and CS2.01). The moromi extraction cycle involved fermentation once per day for 10 days at 40 °C after the solution was mixed with 50 mL of brine with 18% salinity. Finally, the soy sauce was extracted (Zhang et al. 2009), then the contents of ammoniacal nitrogen (AN),

total acid (TA) and total ester (TE) were determined, and the sensory evaluation (SE) was performed.

Optimization of the fermentation process for CS1.03 by the Box–Behnken design (BBD) response surface method

Soy sauce fermentation was applied. The BBD model of Design-Expert version 8 was used to optimize the design of the response surface. The inoculum sizes of CS1.03 and CS2.01 were used, and the initial pH was set as the independent variable. The level of coding was as follows. Factors A and B referred to the inoculum sizes of CS1.03 and CS2.01, respectively. The levels of -1 , 0 , and $+1$ corresponded to 1×10^5 , 1×10^6 , and 1×10^7 CFU/g dry koji, respectively. The initial pH was expressed as Factor C, and the levels of -1 , 0 , and $+1$ denoted pH 5, 6, and 7, respectively. AN content and SE were used as response values. Then, a validation test was performed based on the obtained response value-optimal condition to validate the reliability of the obtained optimal condition.

Determination of ammoniacal nitrogen and total acid

The content of AN was determined in accordance with the formaldehyde method as follows. Soy sauce (5.0 mL) was placed in a 100 mL volumetric flask, diluted with water to obtain 100 mL, and mixed. Then, 20 mL of the dilution was transferred from the volumetric flask to a 200 mL volume beaker. Subsequently, 60 mL of water was added to the beaker. The diluted mixture was titrated with NaOH standard solution (0.050 mol/L) to pH 8.2. Formaldehyde (10 mL) was placed in the beaker and mixed with the contents. The mixture was again titrated in NaOH standard solution to pH 9.2. The consumption of NaOH standard solution was observed. The same step was repeated to titrate 80 mL of water as the control group. The content of TA was expressed as Eq. (1), and the content of AN was expressed as Eq. (2):

$$X_1 = \frac{(V_1 - V_0) \times c \times 0.09}{5 \times V_4/100} \times 100 \quad (1)$$

$$X_2 = \frac{(V_3 - V_2) \times c \times 0.014}{5 \times V_4/100} \times 100 \quad (2)$$

where X_1 is the content of TA (g/100 mL); V_1 is the soy sauce dilution consumption of NaOH standard solution (mL) before adding formaldehyde; V_0 is the consumption of NaOH standard solution (mL) in the control group before adding formaldehyde; X_2 is the content of AN (g/100 mL); V_3 is the soy sauce dilution consumption of NaOH standard solution (mL) after adding formaldehyde;

V_2 is the consumption of NaOH standard solution (mL) in the control group after adding formaldehyde; V_4 is the usage amount of the soy sauce dilution (mL); and c is the concentration of NaOH solution (mol/L). The constant of 0.09 expresses that the TA is converted to lactic acid. The constant of 0.014 is equivalent to the ratio of 1.00 mL of NaOH standard solution to the weight of nitrogen (g).

Determination of total ester

Soy sauce (2 mL) was diluted to 20 mL then added to a 500 mL conical flask. Two drops of 0.5% phenolphthalein reagent were added into the flask. Then the dilute solution was titrated to a reddish colour with a 0.1 mol/L concentrate of NaOH solution. 25 mL of 0.1 mol/L NaOH was added accurately. Reflux saponification to the dilute solution was performed in boiling water for 30 min. The cooling dilute solution was immediately titrated with 0.1 mol/L HCl until the red colour just disappeared. The content of TE is expressed by Eq. (3):

$$X_3 = \frac{(C_{\text{NaOH}} \times 25 - C_{\text{HCl}} \times V_{\text{HCl}}) \times 0.08812}{2} \times 100 \quad (3)$$

where X_3 is the content of TE (g/100 mL), and the constant of 0.08812 expresses the milligram equivalent of ethyl acetate. C and V express concentration and volume of the solution corresponding to their subscript, respectively.

Methods of sensory evaluation

In accordance with the basic principle of sensory evaluation (Liu 2004; Wu 2002), the colour, lustre, aroma, taste, and consistency of the soy sauce were scored out of 100 marks by 21 trained scorers (20–60 years old; 14 males and 7 females; living in different districts in China). The score standard was listed as follows. The soy sauce aroma was scored from 0 to 40, with 0 as the strongest ammonia smell without alcohol and 40 as the best alcohol smell without ammonia. Similarly, colour and lustre were scored from 0 to 30, where 0 represented the lowest saturability, luminance, and transparency, and 10, 15, and 5 corresponded to their best scores, respectively. Likewise, 0–15 marks were given to taste and consistency, where 0 indicated the worst mouth sensation, forceful salinity, acidity, bitterness and astringency, or unqualified consistency, and 15 denoted the best score. Water was provided between intakes of different samples to cleanse the palate of the scorers, and a separate scoring form was provided for each sample. The sum of the average values for each index was used as the total score of the SE.

Analysis of volatile components

The two sauce samples used for volatile components analysis were fermented by the obtained optimal condition (Optimal sample, S_{op}), and fermented without any flavour microorganisms (Control sample, S_c). 200 mL of S_{op} and S_c were placed in a separating funnel, and 50 mL of anhydrous ether was added. Sodium chloride was also added until saturation after the mixture was violently agitated and allowed to stand for 30 min to allow layering. The supernatant liquid was obtained, and anhydrous sodium sulfate was added for filtration. The extraction process was repeated twice. The extracts were combined, and concentrated vacuum distillation with rotary evaporation was performed at 40 °C. Eventually, approximately 0.5 mL of orange concentrated liquid with a thick aroma was obtained (Zhu et al. 2007).

GC–MS (Trace GC/Palariis Q, Thermal Fisher Scientific, USA) analysis was performed to analyse the volatile flavour substances on a DB-WAX (Agilent Technologies Inc., USA) elastic quartz capillary column (30 m, inner diameter of 0.25 mm). The carrier gas was helium and the flow velocity was 0.8 mL/min. The initial temperature was 50 °C and was held for 5 min. The heating rate was 5 °C/min, and the final temperature was 230 °C, which was held for 15 min. Splitless injection was carried out. The temperature of the injection port was 250 °C, and the sample volume was 0.02 µL. Electron ionization mass spectrometry (EI-MS) was performed at 70 eV. The emission current was 200 A and the electron multiplier voltage was 350 V. The ion source temperature was 200 °C and the scanning m/z range was 30–450. The retrieval spectrum library was NIST14. The volatile components were detected by the matching degree of more than 800 (1000 maximum) of the compounds.

Statistical analysis

The SE scores and the determined content of AN in BBD were then subjected to ANOVA. Significant differences were determined with LSD multiple comparison method. All analyses were performed using SPSS version 18.0 (International Business Machines Corporation, USA). A P -value of < 0.05 was considered significant.

Results and discussion

Identification of CS1.03

The colonial morphology and the Gram staining results of CS1.03 are shown in Fig. 1. The observed colony surface was relatively dry and became wrinkled after 24 h of

culturing in plates (Fig. 1a). The strain was preliminarily identified as *Bacillus* sp. in accordance of some features, including Gram-positive staining, spore production, rod shape, and approximately $0.5\ \mu\text{m} \times 2\text{--}3\ \mu\text{m}$ size (Fig. 1b).

A 1464 bp fragment was obtained after 16S rDNA sequence amplification. The fragment was sent to Sangon for sequencing, and the result was uploaded to NCBI. The sequence homology was then analysed using the BLAST Search Database, and a phylogenetic tree was constructed with MEGA version 5.0. Figure 1c shows that CS1.03 was the most closely related to *Bacillus subtilis* with 100% similarity. Therefore, the strain was confirmed to be *Bacillus subtilis*.

Optimization of the addition conditions of CS1.03 in soy sauce fermentation by response surface methods

AN is an important classification index of soy sauce and is often used as the parameter for investigating the fermentation level of soy sauce (Kim et al. 2017). The content of AN directly explains the degradation level of proteins in soy sauce fermentation; meanwhile, it is also directly related to the content of the umami amino acids. The production of AN is closely related to the metabolism of microorganisms in soy sauce fermentation, therefore, AN can be used as the optimization index to investigate the property of flavour microorganism. *Bacillus subtilis* has a good ability to secrete amylase and protease, thus, starches and proteins can be decomposed by adding *Bacillus subtilis* CS1.03 in soy sauce fermentation; furthermore, this provides a good foundation for the substances able to be used in the formation of soy sauce flavour. Yeasts are early flavour microorganisms used in soy sauce fermentation. Certain yeasts, such as *Torulopsis* spp., *Candida* spp., and *Zygosaccharomyces rouxii*, are often included in the fermentation process. The addition of yeast helps improve the production of alcohol, aldehyde, and ester in fermentation (Devanthi et al. 2018). When CS1.03 and CS2.01 were added to the fermentation respectively, the results of SE indicated that the flavour of soy sauce was improved; meanwhile, the contents of AN, TA, and TE were all changed at different levels (Fig. 2a). The formation of flavour substances in fermentation is considered to be the action result of complex microbial flora; thus, a species of *Torulopsis* CS2.01 was mixed with CS1.03 and added to the soy sauce fermentation. The inoculum sizes of CS1.03 and CS2.01, the initial pH, and other process parameters were optimized by using the BBD. The AN content and the SE scores were used as response values for the experiment. The left section of Table 1 lists the design and results of the experiment.

The quadratic regression equation fitting of all the variables was obtained with Design-Expert 10 by

Fig. 1 Morphological identification and 16S rDNA phylogenetic tree of CS1.03. **a** Colonial morphology of CS1.03. **b** Gram stain of CS1.03 (10×100). **c** 16S rDNA phylogenetic tree of CS1.03

subjecting the test results to regression analysis. Equations (4) and (5) express the regression results of AN content determination and SE, respectively.

$$Y_1 = 10.42 - 0.30A + 0.28B - 0.021C + 0.80AB + 0.088AC + 0.10BC - 1.55A^2 - 0.015B^2 - 1.85C^2 \quad (4)$$

$$Y_2 = 90.32 + 1.28A + 0.84B + 0.16C - 1.03AB + 0.075AC + 0.45BC - 8.19A^2 - 4.41B^2 + 0.74C^2 \quad (5)$$

The results of the regression coefficient significance and variance analyses of the regression equation are shown in the right section of Table 1. The models used in this experiment showed highly significant results ($p < 0.01$) and a lack of fit greater than 0.05 indicated no significance. These two models were meaningful and exhibited a high fitting degree. Moreover, an important correlation existed between the predicted value and the experimental value (correlation coefficient $R_1^2 = 0.936$, $R_2^2 = 0.989$). Therefore, these two models could be used to optimize the parameters and predict the experimental results. From significance

Fig. 2 Influence of CS1.03 and CS2.01 in soy sauce fermentation and its optimization of addition condition. **a** SE and determination of the contents of AN, TA and TE on single-strain addition experiment. **b** and **c** Influence of mixed addition of CS1.03 and CS2.01 on content

analysis, the addition of CS1.03 and initial pH showed a significant effect on the content of AN (A^2 and C^2 were very significant); while for SE, the addition of CS1.03 and CS2.01 both had a significant effect (A , A^2 , B^2 were very significant), which also indicated the importance of multi-strain fermentation for an excellent flavour profile of soy sauce.

Design Expert was used for the polynomial regression fitting of the experimental data. The influence of the

of AN. **d** and **e** Influence of mixed addition of CS1.03 and CS2.01 on content of SE. The inoculum size scale of CS1.03 and CS2.01 of five, six and seven mean 10^5 , 10^6 and 10^7 , respectively in b, c, d and e

inoculum sizes of CS1.03 and CS2.01 on the determination of AN content and SE are presented in Fig. 2. These two strains significantly influenced the AN content. In particular, the AN content maintained a relatively high value, whereas the inoculum size of CS1.03 was 10^5 – 10^6 CFU/g dry koji. This content inconspicuously changed as the inoculum size of CS2.01 was varied (Fig. 2b and c), mainly because CS1.03 is a *Bacillus subtilis* strain with a relatively high protease secretion ability. The protease produced from

Table 1 BBD design and regression coefficient and variance analysis

No.	Factor			Response value		Source	Y ₁			Y ₂			Sig.			
	A	B	C	Content of AN L) ^α	Y ₁ (g/ SE Y ₂ ^β		Sum of square	Mean square	F value	p < F	Sig.	Sum of square		Mean square	F value	p < F
1	0	1	−1	8.20 ± 0.05 ^f	86.6 ± 2.14 ^b	Mode	30.11	3.35	11.43	0.0020	**	404.89	44.99	73.40	<0.0001	***
2	−1	−1	0	9.50 ± 0.20 ^e	73.6 ± 1.66 ^d	A	0.74	0.74	2.52	0.1563		13.00	13.00	21.22	0.0025	**
3	1	1	0	9.80 ± 0.05 ^b	79.8 ± 1.47 ^c	B	0.61	0.61	2.07	0.1936		5.61	5.61	9.15	0.0192	*
4	−1	0	−1	7.30 ± 0.05 ^g	81.8 ± 3.11 ^{b,c}	C	0.003	0.003	0.012	0.9146		0.21	0.21	0.34	0.5756	
5	−1	1	0	9.30 ± 0.10 ^d	78.3 ± 2.36 ^c	A B	2.56	2.56	8.75	0.0212	*	4.20	4.20	6.86	0.0345	*
6	0	−1	−1	8.70 ± 0.10 ^e	86.8 ± 3.11 ^b	A C	0.031	0.031	0.10	0.7558		0.022	0.022	0.037	0.8535	
7	1	−1	0	6.80 ± 0.05 ^j	79.2 ± 2.05 ^c	B C	0.040	0.040	0.14	0.7225		0.81	0.81	1.32	0.2881	
8	1	0	−1	7.01 ± 0.15 ^h	83.2 ± 2.87 ^{b,c}	A ²	10.15	10.15	34.69	0.0006	***	282.08	282.08	460.22	<0.0001	***
9	0	0	0	10.43 ± 0.15 ^a	90.4 ± 1.05 ^a	B ²	0.0009	0.0009	0.003	0.9995		81.89	81.89	133.60	<0.0001	***
10	0	0	0	10.42 ± 0.10 ^a	90.3 ± 0.86 ^a	C ²	14.45	14.45	49.39	0.0002	***	2.31	2.31	3.76	0.0936	
11	0	0	0	10.43 ± 0.05 ^a	90.4 ± 1.16 ^a	Residual	2.05	0.29				4.29	0.61			
12	1	0	1	6.90 ± 0.20 ⁱ	84.1 ± 2.36 ^b	Lack of fit	2.05	0.68	4015.1	<0.0001	***	4.26	1.42	201.98	<0.0001	***
13	−1	0	1	6.84 ± 0.15 ^{i,j}	82.4 ± 2.06 ^{b,c}	Pure error	0.0008	0.0001				0.028	0.007			
14	0	0	0	10.41 ± 0.05 ^a	90.2 ± 1.43 ^a	Total error	32.15					409.18				
15	0	1	1	8.60 ± 0.15 ^e	87.4 ± 2.15 ^b											
16	0	−1	1	8.70 ± 0.15 ^e	85.8 ± 1.98 ^b											
17	0	0	0	10.40 ± 0.05 ^a	90.3 ± 1.21 ^a											

Data denoted by different superscript lower-case letters are significantly different ($p < 0.05$)^aContents of AN are expressed as the mean ± standard error (n= 3)^bSE is expressed as the mean ± standard error (n= 21)* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

the fermentation process played an important role in promoting protein degradation and remarkably improved the AN content in the fermented soy sauce. Figure 2d and e show the substantial contributions of CS1.03 and CS2.01 to the SE because of the various metabolic pathways and abundance of the products of these two microorganisms. Furthermore, most of the metabolic products were relevant to the flavour substances of soy sauce.

Given these two indices, two sets of optimal parameters and their predicted values were obtained. The two corresponding fermentation conditions were compared through a validation experiment. The relative error between the experimental results and the predicted values of the two indices was less than 2%. The regression models excellently fitted to the actual situation. The following optimal conditions of fermentation with CS1.03 were eventually determined after these two indices were considered: the inoculum sizes of CS1.03 and CS2.01 were 1.1×10^6 and 1.4×10^6 CFU/g dry koji, respectively; the initial pH was 6.0; the AN content was 10.7 g/L, which increased by 21.2% under this condition; the SE score was 90.52, which increased by 13.7% compared with that of the control group.

Validation test of response surface method optimization experiment

To validate the reliability of the obtained optimal addition condition of CS1.03, the validation test was performed according to Table 2. No.1 was control group without inoculation of CS1.03 and CS2.01, No.2 was the optimum

of Y_1 (Content of AN) and No.3 was the optimum of Y_2 (SE). We observed that the results of No.2 and No.3 were very close to their own predicted values, thus, it was inferred that the obtained optimal addition condition was credible.

Volatile substance analysis of soy sauce

The flavour of the CS1.03-inoculated fermented soy sauce was obviously better than that of the soy sauce without CS1.03. The chemical structure of each flavour component was determined by artificial analysis and NIST14 mass spectra database retrieval. As a result, 42 and 37 different components were identified from S_{op} and S_c , respectively. The area normalization method was used to determine the relative contents of the flavour components. The contents of phenols in S_{op} increased by 105% compared with that of S_c ; the aldehyde and ketone contents increased by 75.6%; moreover, the acid and ester contents slightly increased, but the alcohol and heterocyclic compounds decreased (Table 3). The improvement of the contents of phenols, aldehydes, and ketones arose from the generation of guaiacol, 4-vinyl guaiacol, maltol, furfural, and other compounds.

The aroma of soy sauce is mainly formed by the synergistic effect of alcohols, phenols, acids, esters, aldehydes, ketones, and other compounds (Seo et al. 1996; Song et al. 2015). In our study, 15 characteristic volatile substances were selected for content comparison and analysis (Table 4). In soy sauce, the acids play two main roles, namely, as a flavour-blending material and synthetic

Table 2 Results of validation test

No.	Inoculate size of CS1.03	Inoculate size of CS2.01	Initial pH	Content of AN (g/L)		SE	
				Predictive value	Measured value	Predictive value	Measured value
1	0	0	6.00	–	8.83 ± 0.03	–	79.62 ± 1.96
2	1.4×10^6	1×10^7	6.00	10.72	10.78 ± 0.13	–	89.23 ± 1.12
3	1.1×10^6	1.4×10^6	6.00	–	10.70 ± 0.10	90.04	90.52 ± 0.57

Table 3 Comparison of relative content of volatile flavour compounds

Category of components	S_{op}		S_c	
	Number of components	Relative content (%)	Number of components	Relative content (%)
Acids	10	35.94	9	32.68
Phenols	6	3.59	3	1.75
Alcohols	7	18.14	11	26.31
Esters	6	23.03	5	21.97
Aldehydes and Ketones	9	10.38	4	5.91
Heterocycles	4	8.92	5	11.38
Total	42	100	37	100

Table 4 Main volatile flavour compounds in soy sauce

Sort	Retention time/min	Compound	Molecular formula	Relative molecular mass	Relative content (%)	
					S _{op}	S _c
Acid	17.77	Acetic acid	C ₂ H ₄ O ₂	60.05	19.42	15.44
	21.58	Isobutyric acid	C ₄ H ₈ O ₂	88.105	2.44	1.26
	41.27	Benzeneacetic acid	C ₈ H ₈ O ₂	136.15	4.75	3.45
Phenols	28.33	Guaiacol	C ₇ H ₈ O ₂	124.14	1.06	0.67
	34.99	4-Vinyl phenol	C ₉ H ₁₀ O ₂	150.175	0.52	0.35
	30.71	Methyl maltol	C ₆ H ₆ O ₃	126.11	0.91	ND ^a
Alcohols	12.12	Isoamyl alcohol	C ₅ H ₁₂ O	88.15	2.87	1.56
	4.2	Ethanol	C ₂ H ₆ O	46.07	3.43	1.87
	21.99	2,3-Butanediol	C ₄ H ₁₀ O ₂	90.121	2.13	1.28
	23.91	2-Furanmethanol	C ₅ H ₆ O ₂	98.1	1.13	1.09
Esters	3.33	Ethyl acetate	C ₄ H ₈ O ₂	88.1	12.29	10.31
	15.82	Ethyl lactate	C ₅ H ₁₀ O ₃	118.13	1.41	0.76
Aldehydes	18.92	2-Furaldehyde	C ₅ H ₄ O ₂	96.08	0.29	ND
	24.06	Phenylacetaldehyde	C ₈ H ₈ O	120.15	0.70	ND
Ketones	36.59	3-phenylmethyl-2 (3H) furanone	C ₁₁ H ₁₂ O ₂	176.212	6.99	ND

^aND means not detected

substrate of fragrant materials. Acetic acid is one of the main components of soy sauce flavour, it can be synthesized into acetate substances, increasing the smell and flavor of soy sauce (Feng et al. 2009); 2-Methyl propionic acid (isobutyric acid) is the main metabolic product of CS1.03 and an important flavour material in the Chinese liquor Maotai (Yang et al. 2010). Table 4 show the acidity and its richness of soy sauce could be enhanced by the improvement of acetic acid, phenylacetic acid, and 2-mythel propionic acid in soy sauce fermentation arising from the addition of CS1.03. Furthermore, acids with a certain concentration can inhibit the growth of some contaminant microorganisms. Phenols are the main source of soy sauce-like aromas, in particular, the aroma of soy sauce is influenced directly by guaiacol and 4-vinyl guaiacol, and other similar substances can offer a unique aroma to soy sauce (Zhao et al. 2013). The relative contents of guaiacol and 4-vinyl guaiacol increased by 58.2% and 48.6%, respectively, compared with those in S_c; maltol, with a strawberry fragrance in dilute solution state, was also detected in S_{op} but not in S_c. Alcohol plays an indirect role in the formation of flavour substances by serving as the substrate of ester (Xu et al. 2016). 2,3-Butanediol, as a characteristic metabolic product of *Bacillus subtilis* with a cream aroma (Yang et al. 2011), can be converted to 3-hydroxy-2-methyl ethyl ketone, which is an internationally recognized aromatic substance in foods (Yang et al. 2010). Although the alcohols decreased integrally in S_{op}, the contents of certain characteristic alcohols, such as isoamylol, 2,3-butanediol, ethanol, and furfuryl alcohol,

obviously increased; in addition, the content of furfuryl alcohol (2-furanmethanol) was also improved slightly, which is a burnt sugar substance (Lee et al. 2006). Generally, esters are formed after the reactions of acids and alcohols in soy sauce fermentation, thereby enhancing the sensory fragrance of soy sauce (Feng et al. 2009). As representative volatile esters in soy sauce, ethyl lactate and ethyl acetate are fragrant and highly volatile (Meng et al. 2017; Wah et al. 2013; Zhang et al. 2016). In this work, the relative contents of both ethyl acetate and ethyl lactate were increased in S_{op}, which meant that CS1.03 was effective to improve the fruity aroma of soy sauce, and also could eradicate the irritating smell caused by excessive acid, and improve the richness and softness of the soy sauce aroma. As a characteristic component of the soy sauce aroma, 2-furaldehyde, with a strong grain aroma (Meng et al. 2014) and a coffee aroma with a low aromatic threshold was detected in S_{op} but not in S_c; similarly, phenylacetaldehyde, a honey-like aroma material (Shu et al. 2013), was also detected in S_{op}. In addition, 3-phenylmethyl-2(3H)-furanone was detected in S_{op}, and this furanone-like substance has rarely been reported previously.

The analysis of volatile flavour components indicate that the count and richness of which are significantly contributed for soy sauce flavour in S_{op} are obviously higher than S_c. This result is highly accord with the result of SE, therefore, CS1.03 is proposed to be a positively effective strain for the flavour of soy sauce.

Conclusion

Bacillus subtilis CS1.03 strain significantly enhances the flavour of soy sauce and shows potential for application in soy sauce fermentation. Recent research on genomics and metabolomics of *Bacillus subtilis* have annotated more than 4000 genes and 180 metabolism pathways, which indicate that *Bacillus subtilis* has a strong metabolism ability and relatively complete amino acid metabolism pathway. The further development of genomics and metabolomics imply that the metabolism ability of CS1.03 on soy sauce flavour substances will be improved more, and it will result in a more flavour application value of CS1.03 in soy sauce fermentation.

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Compliance with ethical standards

Conflict of interest The author declares that they have no conflict of interest.

References

- Bao QA (2011) Science and brewing technology of soy sauce. China Light Industry Press, Beijing
- Devanthi PVP, Linforth R, Onyeaka H, Gkatzionis K (2018) Effects of co-inoculation and sequential inoculation of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* on soy sauce fermentation. *Food Chem* 240:1–8. <https://doi.org/10.1016/j.foodchem.2017.07.094>
- Feng XJ, Wu HQ, Huang XL, Li L, Gao YM (2009) Comparison of substances in natural fermented soy sauce and blended soy sauce by GC–MS discriminant analysis. *J Instrum Anal* 28:661–665
- Feng J, Zhan XB, Wang D, Zhang LM, Lin CC (2012a) Identification and analysis of the metabolic functions of a high-salt-tolerant halophilic aromatic yeast *Candida etchellsii* for soy sauce production. *World J Microbiol Biotechnol* 28:1451–1458. <https://doi.org/10.1007/s11274-011-0945-6>
- Feng J, Zhan XB, Zheng ZY, Wang D, Zhang LM, Lin CC (2012b) A two-step inoculation of *Candida etchellsii* to enhance soy sauce flavour and quality. *Int J Food Sci Technol* 47:2072–2078. <https://doi.org/10.1111/j.1365-2621.2012.03071.x>
- Feng YZ, Su GW, Zhao HF, Cai Y, Cui C, Sun-Waterhouse DX, Zhao MM (2015) Characterisation of aroma profiles of commercial soy sauce by odour activity value and omission test. *Food Chem* 167:220–228. <https://doi.org/10.1016/j.foodchem.2014.06.057>
- Harada R, Yuzuki M, Ito K, Shiga K, Bamba T, Fukusaki E (2017) Influence of yeast and lactic acid bacterium on the constituent profile of soy sauce during fermentation. *J Biosci Bioeng* 123:203–208. <https://doi.org/10.1016/j.jbiosc.2016.08.010>
- Jiang XW, Zhou ST, Ye J, Xu YQ, Chen S, Wu C (2016) Identification of contamination microbes separated from finished deterioration sauce and the analysis of the deterioration reason. *Food Mach* 32:46–50
- Kim HW, Hwang KE, Song DH, Kim YJ, Ham YK, Yeo EJ, Jeong TJ, Choi YS, Kim CJ (2015) Effect of soy sauce type on the quality characteristics of emulsion sausages. *Food Sci Biotechnol* 24:1309–1315. <https://doi.org/10.1007/s10068-015-0168-y>
- Kim KM, Lim J, Lee JJ, Hurh BS, Lee I (2017) Characterization of *Aspergillus sojae* isolated from Meju, Korean traditional fermented soybean brick. *J Microbiol Biotechnol* 27:251–261
- Lee SM, Seo BC, Kim YS (2006) Volatile compounds in fermented and acid-hydrolyzed soy sauces. *Food Chem Toxicol* 71:146–156
- Li ZM, Chen L, Bai ZH, Wang DL, Gao LP, Hui BD (2015) Cultivable bacterial diversity and amylase production in two typical light-flavor Daqus of Chinese spirits. *Front Life Sci* 8:264–270
- Liu RQ (2004) Sensory evaluation of soy sauce. *China Condiment* 29:39–40
- Liu JJ, Li DS, Hu Y, Wang C, Gao B, Xu N (2015) Effect of a halophilic aromatic yeast together with *Aspergillus oryzae* in koji making on the volatile compounds and quality of soy sauce moromi. *Int J Food Sci Technol* 50:1352–1358. <https://doi.org/10.1111/ijfs.12789>
- Masuda S, Yamaguchi H, Kurokawa T, Shirakami T, Tsuji RF, Nishimura I (2008) Immunomodulatory effect of halophilic lactic acid bacterium *Tetragenococcus halophilus* Th221 from soy sauce moromi grown in high-salt medium. *Int J Food Microbiol* 121:245–252. <https://doi.org/10.1016/j.ijfoodmicro.2007.10.011>
- Meng Q, Hatakeyama M, Sugawara E (2014) Formation by yeast of 2-furanmethanethiol and ethyl 2-mercaptopropionate aroma compounds in Japanese soy sauce. *Biosci Biotechnol Biochem* 78:109–114
- Meng Q, Imamura M, Katayama H, Obata A, Sugawara E (2017) Key compounds contributing to the fruity aroma characterization in Japanese raw soy sauce. *Biosci Biotechnol Biochem* 81:1–6
- Qi CC, Wang M, Shen AB (2016) The impact of adding salt-tolerant lactic acid bacteria on the flavor of high-salt liquid soy sauce. *China Condiment* 41:102–105
- Seo JS, Chang HG, Ji WD, Lee EJ, Choi MR, Kim HJ, Kim JK (1996) Aroma components of traditional Korean soy sauce and soybean paste fermented with the same meju. *J Microbiol Biotechnol* 6:278–285
- Shu K, Kenji U, Osamu I (2013) Studies on the key aroma compounds in raw (unheated) and heated Japanese soy sauce. *J Agric Food Chem* 61:3396–3402. <https://doi.org/10.1021/jf400353h>
- Shukla S, Kim M (2016) Determination of biogenic amines and total aflatoxins: quality index of starter culture soy sauce samples. *Food Sci Biotechnol* 25:1221–1224. <https://doi.org/10.1007/s10068-016-0194-4>
- Song YR, Jeong DY, Baik SH (2015) Effects of indigenous yeasts on physicochemical and microbial properties of Korean soy sauce prepared by low-salt fermentation. *Food Microbiol* 51:8
- Wah TT, Walaisri S, Assavanig A, Niamsiri N, Lertsiri S (2013) Co-culturing of *Pichia guilliermondii* enhanced volatile flavor compound formation by *Zygosaccharomyces rouxii* in the model system of Thai soy sauce fermentation. *Int J Food Microbiol* 160:282–289. <https://doi.org/10.1016/j.ijfoodmicro.2012.10.022>
- Wu MC (2002) Food analysis and sensory evaluation. China Agricultural Press, Beijing
- Xu Y, Wang D, Fan WL, Mu XQ, Chen J (2010) Traditional Chinese biotechnology. *Adv Biochem Eng/Biotechnol* 122:189–233
- Xu N, Liu YQ, Hu Y, Zhou MZ, Wang C, Li DS (2016) Autolysis of *Aspergillus oryzae* mycelium and effect on volatile flavor

- compounds of soy sauce. J Food Sci 81:C1883–C1890. <https://doi.org/10.1111/1750-3841.13396>
- Yang F, Wang HY, Yao CP, Lin L, Wang L, Lv YH, Ji KL (2010) The difference & the similarities in metabolites of *Bacillus subtilis* strains by different techniques. Liquor-Making Sci Technol 31:104–106
- Yang F, Lin L, Wang HY, Wang L, Yang DY, Lv YH, Ji KL (2011) Comparative analysis of the metabolites of three *Bacillus* strains in Maotai Daqu starter. Liquor-Making Sci Technol 8:42–46
- Yang Z, Yang HL, Yao H, Zhou XW, Tang SZ, Wu XY (2017) Gene sequencing and traditional microbial culture for the detection of aerogen in swelled packing soy sauce. Food Ferment Ind 11:197–201
- Yao CP, Wang HY, Yang F, Lin L, Wang L (2010) Optimization of liquid culture conditions for *Bacillus subtilis* and analysis of its metabolites. Liquor-Making Sci Technol 194:43–45
- Zhang HZ, Jiang YJ, Chen M, Hong WY, Zhang JP (2009) The effect of spraying-extraction fermentation on soy sauce flavor. China Condiment 34:91–94
- Zhang JR, Du GC, Chen J, Fang F (2016) Characterization of a *Bacillus amyloliquefaciens* strain for reduction of citrulline accumulation during soy sauce fermentation. Biotech Lett 38:1723–1731
- Zhao GZ, Yao YP, Wang XH, Hou LH, Wang CL, Cao XH (2013) Functional properties of soy sauce and metabolism genes of strains for fermentation. Int J Food Sci Technol 48:903–909
- Zhu LX, Cai X (2014) Application of natural drying method in improving the flavor of low-salt solid soy sauce. China Condiment 39:78–81
- Zhu ZX, Wu HQ, Huang XL (2007) Identification of volatile compounds in soy sauce. Food Res Dev 28:135–138

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