

Screening and Gas Production Capacity Analysis of a Halotolerant *Zygosaccharomyces rouxii* Strain Isolated from the Swollen Packages of Pixian Broad Bean-chili Paste

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Abstract: Pixian broad bean-chili paste (PBCP) is a traditional condiment in Southwest China, and package swelling is one of the major quality flaws in commercial PBCP. In this study, PBCP and its two semi-finished products, namely broad bean *moromi* (BM) and pickled chili (PC) paste were collected. Salt contents and water activities (a_w) of the samples were determined and the halotolerant microorganisms were isolated. The results showed that the gas-producing samples had relatively lower salt contents and higher a_w values. By inoculating the diluted sample into YM broth media containing 12% (*m/m*) of NaCl, *Zygosaccharomyces rouxii*, a halotolerant yeast, was isolated. The production of gas could be induced through inoculating the yeast in YM broth medium, or by re-inoculating the yeast in the autoclaved BM, PC and PBCP with salt contents lower than 16% (*m/m*). The optimal growth conditions for *Z. rouxii* were 4% (*m/m*) NaCl, pH 4~6 and 30 °C, with the gas production being largest at 8% (*m/m*) NaCl and pH 5~7. Gas production by *Z. rouxii* was inhibited when the salt content increased in the culture medium or the sterilized samples. *Z. rouxii* can grow under acidic and high-salt conditions and can produce gas in PBCP, causing package swelling.

Key words: quality defect; salt content; gas production; halotolerant; control

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胀包郫县豆瓣中耐盐鲁氏接合酵母的筛选和产气能力分析

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摘要: 郫县豆瓣是我国西南地区的传统调味品, 胀包是市售郫县豆瓣最主要的质量缺陷。该研究采集了郫县豆瓣和其两种半成品, 甜瓣子和辣椒醅, 测定了其食盐含量和水分活度 (Water Activities, a_w), 并筛选了其中的耐盐微生物。结果表明, 产气的样品食盐含量相对较低, a_w 相对较高。将样品稀释液接种于含 12% (*m/m*) 氯化钠的 YM 肉汤培养基中, 可分离出一种耐盐微生物, 鲁氏接合酵母 (*Zygosaccharomyces rouxii*)。鲁氏接合酵母在 YM 肉汤培养基, 或者回接入食盐含量低于 16% (*m/m*) 的灭菌甜瓣子、辣椒醅和郫县豆瓣中, 均可导致产气现象发生。其最适生长条件为含盐量 4% (*m/m*), pH 值 4~6 及 30 °C。其在含盐量 8% (*m/m*)、pH 值 5~7 的情况下产气量最大。提高培养基或灭菌样品的盐含量, 鲁氏接合酵母的产气受抑。鲁氏接合酵母可在酸性高盐条件下生长, 并能够在郫县豆瓣样品中代谢产气, 导致其胀包。

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关键词: 质量缺陷; 盐含量; 产气; 耐盐; 控制

Pixian broad bean-chili paste (PBCP), also known as horse bean-chili-paste and *Pixiandouban*, is a traditional fermented condiment originated from *Pixian* county. As an important spicy ingredient for Sichuan cuisine, recent years saw the rapid growth of PBCP industry due to the popularity of Sichuan restaurants among Chinese people. PBCP production consists of three solid-state fermentation phases: *moromi* making, red chili pickling and post fermentation^[1,2]. In *moromi* making, *koji* is made by inoculating *Aspergillus oryzae* on blanched broad bean (*Vicia faba*) cotyledons covered with flour and fermented for 3 months after being mixed with saline water. Meanwhile, dry pickled red chilis (*Capsicum annuum*) were cut into small pieces. Subsequently, chopped pickled chilis and broad bean *moromi* were mixed for post fermentation according to customers' requirements before broad bean-chili paste was obtained.

Similar to other traditional fermented condiments such as soy paste, *doenjang* and *sufu*, high amount of salt is added in PBCP to prevent microbial spoilage and promote the growth of some halotolerant and halophilic microorganisms^[2-4] that contribute to the formation of flavor. Generally, PBCP is not pasteurized in order to keep the intact shape of broad bean cotyledons as well as to prevent undesirable scent produced in heat treatment. In this occasion, microbial activities sometimes may cause gas production of PBCP. Although gas production is a long existing phenomenon that may happen in any of the three fermentation phases in PBCP making according to our experience, it usually leads to package swelling of vacuum sealed PBCP during storing and retailing when temperature is high. Swollen package of PBCP is generally considered as an unacceptable quality flaw, which has become the most frequent reason for rejection by retailers and caused economic loss.

Some previous studies have focused on the aerogenic microorganisms in traditional fermented condiments. For example, Liu, et al.^[5] reported that *Clostridium butyricum* caused the bottle leakage and explosion in packaged soy sauce. Cheng, et al. compared the microbial communities between normal and swollen canned soy sauce and concluded that contamination of several LABs and bacillus were involved in the swelling

of cans^[6]. Niu, et al. screened a *Bacilluslicheniformis* strain that caused gas producing spoilage of broad bean paste after heat treatment^[7]. For PBCP, however, microorganisms that are involved in gas production is still unknown. It is essential to study the microbial reason underlies the package swelling of PBCP before control measures were taken.

In this study, PBCP and its two major ingredients, broad bean *moromi* (BM) and pickled chilis (PC) were investigated for their salt contents and water activities (a_w). Meanwhile, an aerogenic yeast, *Zygosaccharomyces rouxii*, was isolated and gas production capacity of this strain was measured.

1 Materials and Methods

1.1 Sample collection

In a local production plant in *Pidu* district, Chengdu, 13 bags (approximately 2 kg for each bag) of broad bean *moromi* (BM), 26 bags of pickled chili (PC) and 34 bags of *Pixian* broad bean-chili paste (PBCP) were sampled at several timepoints through the year and from different areas of fermentation pools. Samples were directly put into sterile polyethylene ziplock bags and stored at 4 °C before analysis.

1.2 Determination of salt contents, a_w and gas production

Salt contents in BM, PC and PBCP were determined according to the AgNO₃ titration method from Chinese National Standard GB/T 5009.44-2016^[8]. Water activities (a_w) were measure by putting 5g of samples in a HD-3A water activity meter (Huake equipment Co., China) at 25 °C in a thermostatic chamber. Gas production was observed by vacuum sealing 300 g sample in a polyamine bag and incubated at 30 °C for 15 d.

1.3 Screening and identification of halotolerant microorganisms

Aerogenic samples from different groups (5 g) were suspended in 250 mL sterilized water followed by placing

1 mL of suspension on the sterile Petri dish. Melted agar culture was then poured into the plates and mixed with sample suspensions. Yeast-maltose (YM) medium and De Man, Rogosa and Sharpe (MRS) medium supplemented with 12% NaCl were used for screening of yeasts and lactic acid bacteria. Plates were incubated at 30 °C (for yeasts) or 37 °C (for lactic acid bacteria) for 7 d in a 2.5 L Anaerobar (Oxoid, UK) with AnaeroGen 2.5 L atmosphere generation system (Oxoid, UK) and anaerobic indicator (Oxoid, UK). For screening of thermotolerant sporeformers, sample suspensions were heated to 85 °C for 5 min followed by inoculating into reinforced clostridium medium (RCM) agar plates (supplemented with 12% NaCl) and aerobically incubating at 37 °C for 7 d. After incubation, single colonies from agar plates were picked and inoculated in 20 mL corresponding media broth (containing 12% NaCl). Genomic DNA purification kits (Sangon Biotech, China) for yeasts and bacteria were used to extract genomic DNA according to the instructions. Samples were sent to TSINGKE Co. Ltd. for nucleotide sequencing of its ITS or 16s rRNA gene after being amplified in a T960 PCR machine (Heal Force, China). Sequences were compared in NCBI-BLAST to identify the species of microorganisms.

1.4 Aerogenic capacity evaluation of screened isolates in culture broth and autoclaved samples

Halotolerant microorganisms isolated from aerogenic samples were incubated in test tubes with 20 mL media broths (containing 12% NaCl) and inert Durham tubes for 7 d to test their aerogenic responses. Meanwhile, isolates were incubated in 500 mL media broths containing 12% (*m/m*) NaCl for 3 days before centrifugation at 4 000×*g* for 5 min. Cell pellets were suspended with 0.8% sterile saline solution. 3 mL of cell suspension with proper concentration was reinoculated in 300 g autoclaved samples to obtain initial inoculation concentration of 10⁶ CFU/g. Samples were vacuum packed in polyamide bags and incubated at 30 °C for 5 d. Autoclaved samples without inoculation were incubated in the same environment as blank.

1.5 Growth curves of aerogenic yeast

Aerogenic yeast (about 10⁴ cell/mL) was inoculated into YM broth with different NaCl contents, different pH or incubated at different temperatures. 2 mL of broth culture was taken out and measured for its optical density at 620 nm (OD₆₂₀) at a certain time interval after shaking the broth thoroughly.

1.6 Volume measurement of gas produced by aerogenic yeast

Volume of gas produced by aerogenic yeast was measured according to the method previously reported^[5] with some modifications. YM broth (100 mL) was inoculated with approximate 10⁴ cell/mL aerogenic yeast and mixed by shaking thoroughly. 1 mL of broth media was immediately drawn in a 5 mL sterile syringe with the luer slip sealed by Vaseline and the bevel needle was inserted in a rubber block. Syringes were placed upwardly in a sterile glass container and incubated at 30 °C for 72 h to observe the volume of gas produced.

1.7 Statistic analyses

All physiochemical analyses were triplicated for a single sample and the results were averaged to obtain the mean value. The data were visualized using Origin 2018 software.

2 Results and discussion

2.1 Salt contents, *a_w* and aerogenic capacity evaluation of samples

Production of traditional fermented condiments requires salt as an essential ingredient to control the fermentation process as well as activities of microorganisms by lowering *a_w* value. According to our investigation in a fermentation plant, 14%, 16%~18% (*m/m*) salt is added in the *moromi* fermentation and pickling of red chilis, respectively. Another 1% (*m/m*) salt is added at post fermentation stage when *moromi* and chopped chilis are mixing. Moreover, part of water in PBCP was evaporated during the post fermentation. For these reasons, PBCP is usually very high in salt content. In our study, salt contents and *a_w* varied dramatically among three groups of samples (Fig.1a). The average salt

contents for BM, PC and PBCP were $12.20\% \pm 1.24\%$ (m/m), $18.99\% \pm 4.98\%$ (m/m) and $18.90\% \pm 2.81\%$ (m/m), respectively. This is generally higher than the salt contents in Chinese soybean paste ($9.71\% \sim 16.71\%$) and Korean *doenjang* ($9.01\% \sim 18.78\%$) that are produced with similar processes^[3,9]. In *moromi* making phase, BM was fermented indoors for 3 months with frequent stirring to guarantee the uniformity in the fermentation pools. Therefore, the salt content of BM was stable. Also, BM group showed lowest salt content and highest a_w value (0.795 ± 0.008). Compared with BM, salt content and a_w value of PC and PBCP showed larger in-group variations. PC was made by covering salt directly on fresh or dried red chilis for months without any mixing. PBCP, on the other hand, was usually made through post fermentation in the open-air for more than 3 months after BM and CP being mixed. These are in fact very arbitrary operation processes that were influenced by many factors like weather condition or quality of ingredients^[1], making salt contents varied greatly among different samples.

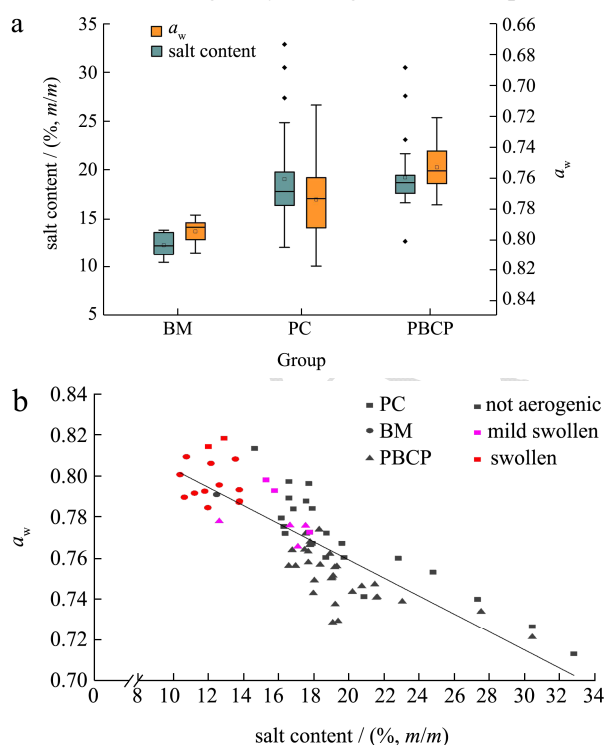


Fig.1 Salt contents, water activities (a_w) and gas production of samples

Notes: (a) is boxplot of salt contents and a_w in different samples, (b) is salt contents, a_w and gas production of samples.

Although there are various low molecular sugars, organic acids and amino acids exist in samples, salt contents may be the decisive factor to a_w values

considering relatively low amounts of other components (data not shown). Fig.1b showed that a_w was negatively correlated with salt content ($R=-0.83$). BMs were distributed on top left in the plot, in which salt contents were lower than 14% (m/m) and a_w higher than 0.78 . Most samples in PBCP group were higher on salt content and lower on a_w and appeared on central and right bottom. As for PCs, they scattered on the whole plot from top left to right bottom since salt contents and a_w values were widely dispersed in this group.

Based on the amount of gas produced in vacuum sealed bag after 15 days of incubation, samples were divided into three types, namely, not aerogenic, mild swollen and swollen (Fig.1b). Almost all samples in BM group were “swollen”, which produced large amount of gas that made package bags expand significantly. However, only few samples produced gas in PC and PBCP groups. There were 4 “mild swollen” samples in PBCP group that only made package expand mildly. Interestingly, all aerogenic samples distributed on top left area in the scatter plot which stands for low salt content under 18% (m/m) and high a_w value above 0.76 . This trend was seen particularly in BM group in which almost all samples scattered on top left corner while made vacuum bags swollen. For aerogenic samples in PC and PBCP groups, they were also lower on salt content and higher on a_w compared with the average level in the group. It can be deduced that activity of aerogenic microorganisms were strongly related with high a_w value of samples that may be caused by low salt content.

2.2 Screening and identification of halotolerant microorganisms

Despite lower salt content of aerogenic samples compared with the average level in each group, they still contained over 10% (m/m) salt, which is not suitable for growth of most microorganisms. Some samples even produced gas with salt content higher than 16% (m/m), indicating strong halotolerance of aerogenic microorganisms in samples. Therefore, we used culture media supplemented with 12% (m/m) NaCl to screen halotolerant microorganisms from samples. According to NCBI-BLASTn comparison, *Zygosaccharomyces rouxii*, *Tetragenococcus halophilus* and *Oceanobacillus*

timonensis were identified. *Z. rouxii* were screened from aerogenic samples of all three groups. Colonies of *Z. rouxii* were found to be round, yellowish white and smooth on the surface with size vary from 0.5~2.5 mm in diameter (Fig.2a). Light microscope observation showed that cells from colonies were spherical or ellipsoidal with buds attached to the parent cells. *T. halophilus* was a kind of Gram-positive coccus isolated from BM and PBCP, which formed round yellowish white and smooth-surfaced colonies less than 1 mm on MRS plates (Fig.2b). *O. timonensis*, a Gram-positive bacillus that formed round and whitish colonies on RCM plates, was found in BM and PBCP (Fig.2c). All of 3 microbial species were halotolerant and could grow on media with 12% (m/m) NaCl.

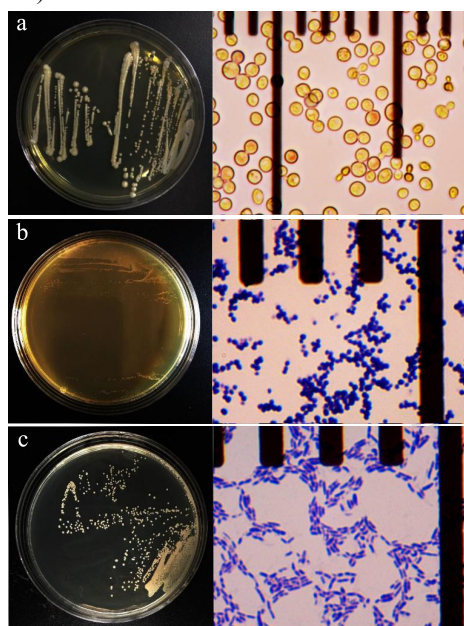


Fig.2 Colony and cell morphology of halotolerant microorganisms screened from aerogenic samples

Notes: (a) is *Zygosaccharomyces rouxii*, (b) is *Tetragenococcus halophilus*, (c) is *Oceanobacillus timonensis*. *T. halophilus* and *O. timonensis* were Gram stained. Microscopic photos were taken on a glass calibration slide, 1 div.=10 μm .

According to previous studies, *T. halophilus* is a halotolerant lactic acid bacterium that produces proteinase and flavor compounds in traditional fermented condiments such as soy sauce, *doenjang* and fish^[10-12]. However, *T. halophilus* seems unlikely to induce package swelling since it is not able to produce CO_2 from glucose^[13]. *Oceanobacillus timonensis* was first isolated by Senghor, et al. from human stool and reported as a Gram-positive, rod-shaped bacteria that was able to grow

with 0.5% to 20% (m/V) NaCl^[14]. It was also screened by Lu, et al. from PBCP^[15]. As for *Z. rouxii*, it is strongly osmotolerant and can grow not only in high salt content but also in environment with sugar content as high as 70° Brix^[16]. *Z. rouxii* metabolizes sugar and amino acids into various of volatile compounds such as fusel alcohols, esters, and furan derivatives^[17-20], which contributes to the flavor formation of traditional fermented condiments. However, *Z. rouxii* was also frequently reported as a spoilage yeast in concentrated fruit juice, syrup, and bakery products^[20-22], which usually lead to the alcohol or ester odors and evident gas production^[21,22]. Therefore, we suspected that *Z. rouxii* caused the package swelling of PBCP.

2.3 Aerogenic test of screened strains in culture broth and autoclaved samples

To test whether the isolated strains can produce gas, *Z. rouxii*, *T. halophilus* and *O. timonensis* were inoculated into broth media and autoclaved samples. After inoculation of *Z. rouxii* strains in test tubes with Durham tubes, significant gas production was observed at 6th or 7th day of incubation. However, *T. halophilus* and *O. timonensis* did not produce gas in corresponding media broths. Strains were then reinoculated into autoclaved samples with different salt contents adjusted by adding NaCl or sterile deionized water. It is showed that reinoculation of *Z. rouxii* induced package swelling of BM, PC and PBCP. However, volume of gas produced by *Z. rouxii* decreased with the increasing of salt content (Fig.3). Packages of BM and PBCP with 10% (m/m) salt expanded greatly after 5 d incubation, while there was only little expansion for samples with 16% (m/m) salt (Fig.3). For PC samples, only small bubbles were observed in packages after inoculation of *Z. rouxii*, though amount of bubble was larger in low salt samples. There was no gas observed in samples of all three groups when salt content exceeded 16% (m/m). On the other hand, *T. halophilus* and *O. timonensis* strains were not able to produce gas in samples of any salt content. Therefore, *Z. rouxii* was the aerogenic microorganism that induces gas production and swollen packages in PBCP with relatively lower salt contents.

Gas production in foods caused by *Z. rouxii* was

previously known in bakery products and fruit juice^[21,23]. However, *Z. rouxii* was scarcely reported to be responsible for package swelling of fermented condiments. This may be due to the prevalent application of pasteurization and preservatives or the low content of substrates such as glucose in these products. Study by Liu, et al. demonstrated that bottle leakage and explosion of soy sauce was caused by *Clostridium butyricum*, which was also isolated from PBCP in our lab by incubating

sample dilutions in *Gifu* anaerobic medium (GAM) agar plates in Anaerobar with AnaeroGen 2.5 L atmosphere generation system^[5]. However, aerogenesis of *C. butyricum* may only occurs in sodium reduced products since in our study *C. butyricum* was totally inhibited when salt content of media higher than 5% (m/m), which was confirmed by previous study^[24]. This indicated that *C. butyricum* is unable to induce package swelling in PBCP of which the salt content was over 16% (m/m).

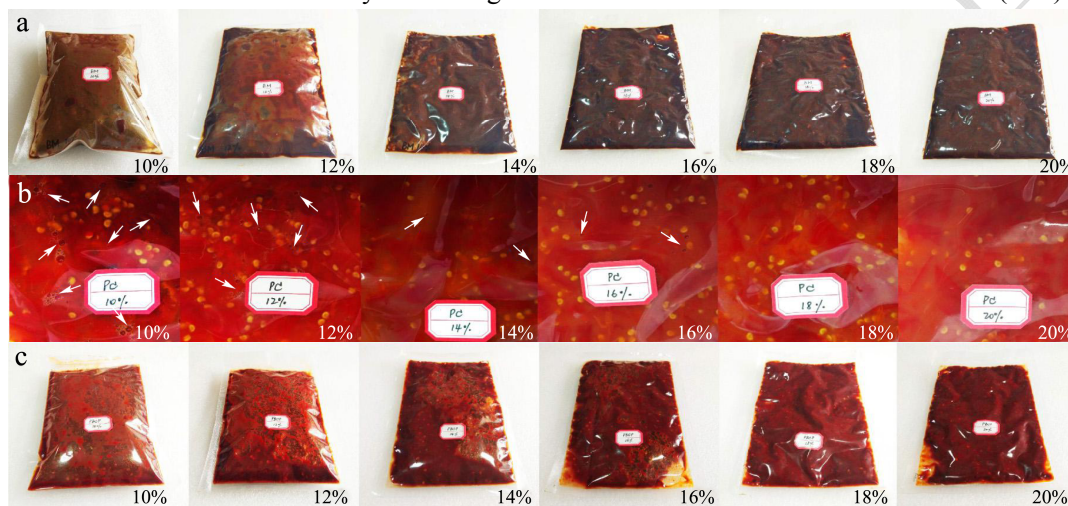


Fig.3 Aerogenic test of *Zygosaccharomyces rouxii* in BM (a), PC (b) and PBCP (c) with different salt contents

2.4 Growth curves of aerogenic yeast in different salt contents, pH and temperatures

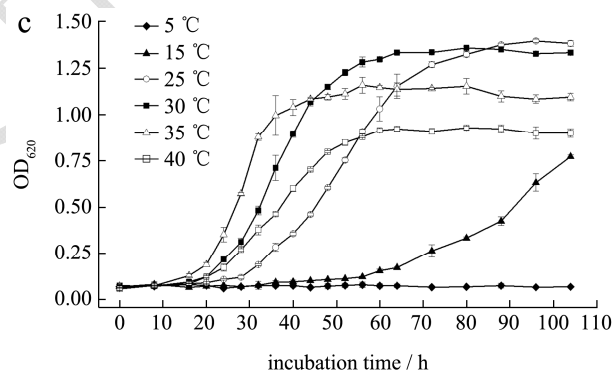
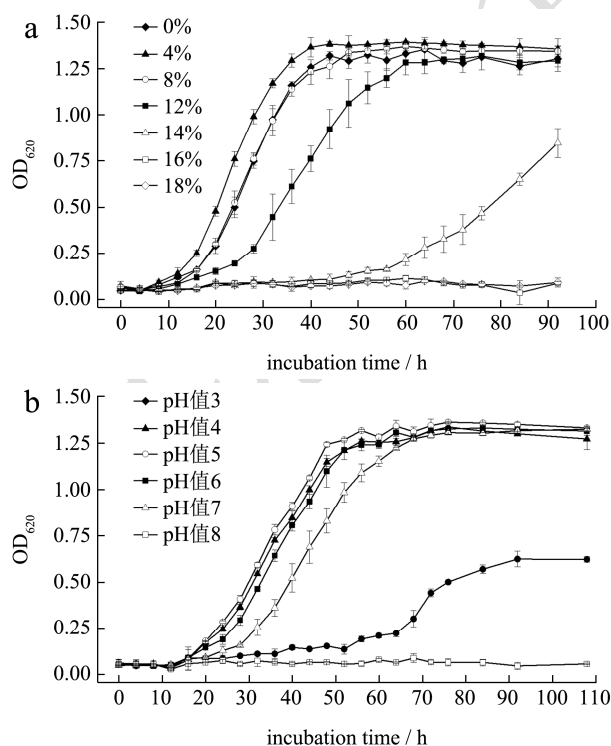


Fig.4 Growth curves of *Zygosaccharomyces rouxii*

Notes: A: *Z. rouxii* (about 10^4 cell/mL) was inoculated in YM broth media with different salt concentrations and incubated at 30 °C. B: pH of YM broth media supplemented with 12% (m/m) salt were adjusted by 1 mol/L HCl or NaOH solution after autoclaving. *Z. rouxii* (about 10^4 cell/mL) was inoculated in YM broth media and incubated at 30 °C. C: *Z. rouxii* (about 10^4 cell/mL) was inoculated in YM broth media supplemented with 12% salt and incubated at different temperature.

As shown in Fig.4a, *Z. rouxii* was strongly halotolerant and evident increasing of OD₆₂₀ values was observed in YM media with 0~14% (m/m) NaCl. Highest growth rate at exponential stage (Fig.4a) were found in

4% (*m/m*) NaCl although there was little difference on OD₆₂₀ values at stationary phase among groups between 0% and 12% (*m/m*) NaCl. The increasing of NaCl content prolonged time of lag phase and decreased OD₆₂₀ value in stationary phase. This is consistent with a systematic study by Yong, et al.^[25]. In fixed NaCl content (12%, *m/m*) and incubation temperature (30 °C), pH range of 4~6 was optimal for the growth of *Z. rouxii* while it could not multiply in media with pH of 2 or higher than 8 (Fig.4b). In some previous studies, pH range for *Z. rouxii* growth seemed to be wider. For example, Yong, et al. investigated dry cell weight of *Z. rouxii* incubated in YM broth containing 10% (*m/m*) NaCl and found that yeast was able to grow in pH of 2 and 9^[25]. Restaino, et al. also reported a wider pH range of 1.5~10.5 for *Z. rouxii* incubated in 2×potato dextrose agar (PDA) media supplemented with 12% (*m/m*) glucose^[20]. The discrepancy may be due to the different culture media, pH regulator and incubation time. For yeast incubated in different temperatures, growth rate at exponential stage increased with the rise of incubation temperature under 35 °C. However, OD₆₂₀ value at stationary stage seemed to decrease when the temperature was above 30 °C (Fig.4c). This was also in line with study by Yong, et al., in which showed that doubling time of *Z. rouxii* was less at 37 °C during the exponential growth while total amount of growth was greater at room temperature (28 °C)^[25]. In summary, *Z. rouxii* strains screened from aerogenic samples were able to grow in wide ranges of salt contents, pH and temperatures.

2.5 Volume of gas produced by *Z. rouxii*

After 72 h incubation, volume of gas produced by *Z. rouxii* was measured. As shown in Fig.5, obvious gas production (gas volumes over 0.7 mL) was seen when *Z. rouxii* was incubated in broth with 0~16% (*m/m*) NaCl and pH ranging from 3~8, while gas production was significantly inhibited when the pH of broth higher than 9 or lower than 2. Broths with 8% (*m/m*) NaCl and pH of 5~7 were optimal for gas production of yeast, in which volumes of gas produced were more than 1 mL. Although similar OD₆₂₀ values at stationary phase were observed at salt content of 0~12% and pH of 4~7 (Fig.4), volumes of gas produced by yeast were different, which may be related with their adaptive behaviors in different salt

contents. Also, it is showed that sub acidity environment (pH value 4~5) promoted gas production of the yeast in higher salt contents. However, not like inoculation in the samples, gas production was completely inhibited when inoculating *Z. rouxii* in BM, PC and PBCP with salt content more than 16% (Fig.3). This is probably due to the different sugar contents in samples and broth media. Moreover, no changes of OD₆₂₀ values were detected after seeding *Z. rouxii* in YM media with more than 16% (*m/m*) NaCl (Fig.4). It is indicated that in high salt environment, fermentation of *Z. rouxii* sustained even though their proliferation was inhibited. According to our preliminary studies, pH value of BM, PC and PBCP were 4.67, 4.06 and 4.20, respectively (data not shown), making the package swelling induced by *Z. rouxii* happen when there was enough substrate exists.

Traditionally, salt content in PBCP were not strictly controlled. Some previous studies have also described wide ranges of salt content in BM, PC and PBCP^[1,26]. Nevertheless, gas production by *Z. rouxii* in PBCP was scarcely reported. It may be because the production of PBCP was used to comprise a longer fermentation time in which the activity of *Z. rouxii* was inhibited when water was lost and salt content was increased. However, with the increasing demand in the market, post fermentation time of PBCP have been shortened greatly in order to reduce the lead time. In some production plants, post fermentation of PBCP was less than 2 months. Moreover, introduction of vacuum packaging highlighted the gas production and swollen packages that are considered as “spoilage” by customers.

A culture dependent and independent study by Lu, et al. on PBCP reported that *Z. rouxii* was one of the dominant microorganisms in all three fermentation phases^[1]. In accordance with this result, our study isolated *Z. rouxii* from BM, PC and PBCP samples despite their aerogenic properties. It seemed that unlike the accidental contamination in PBCP production, *Z. rouxii* was an inherent part of fermentation system and plays an important role in flavor formation. Therefore, activity of *Z. rouxii* should not be eliminated or totally inhibited. By reinoculating *Z. rouxii* in autoclaved samples, it was found that high salt contents significantly deterred gas production. This is also demonstrated by incubating *Z. rouxii* in YM media. However, salt content

may not be the only factor that influence the activity of *Z. rouxii*, since some samples did not produce gas even their salt contents were below 16% (*m/m*). Studies on other physiochemical properties (such as contents of fermentable sugar and protein) of samples and their relationships with aerogenic properties are needed for prediction of gas production.

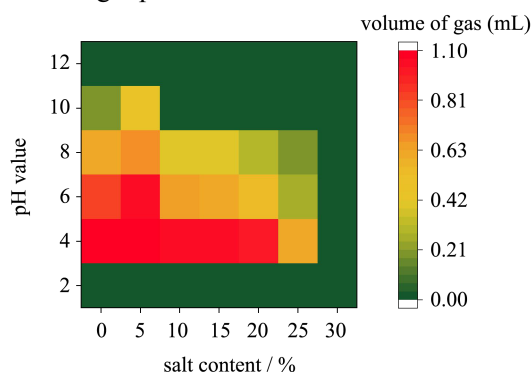


Fig.5 Volume of gas produced by *Zygosaccharomyces rouxii* in YM broth media with different salt contents and pH

3 Conclusions

The results from study indicated that activity of halotolerant *Zygosaccharomyces rouxii* lead to the package swelling of Pixian broad bean-chili paste as well as pickled chili and broad bean *moromi*. Aerogenic samples were lower on salt content (*m/m*) and higher on a_w value compared with the non-aerogenic ones. Optimal growth conditions for *Z. rouxii* were 4% (*m/m*) NaCl, pH value of 4~6 and 30 °C, while largest volume of gas was produced in media with 8% (*m/m*) NaCl and pH of 5~7. Gas production by *Z. rouxii* was be inhibited when salt content increased both in autoclaved samples and in culture media.

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