Microorganisms in sato production

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Abstract

The Thai traditional fermented rice wine is one of the most antique methods of food preservation, comprises various foods fermented with the non-pathogenic microorganisms. Loog-pang, mixed culture of fungi and bacteria, was used for sato production in a conventional method. Recently, The development of red sato production using Ang-kak red rice koji had studied. It was found that Loog-pang and Ang-kak red rice koji contained an abundant amount of bacteria. Lactic acid bacteria had significantly affected on quality of red sato. This mixed consortium forms a powerful symbiosis capable of inhibiting the growth of potentially pathogen bacteria. The quality sato was based on each sato processing. It was found that the microbial spectrum of sato consortium varied between fermentation. However, several thermoduric species remain in sato, which were *Bacillus spp.* and *Lactobacillus spp*. The phylogenetic classification showed that sato from Ang-kak red rice koji had biodiversity of bacterial strains less than sato from Loog pang. *Bacillus siamensis* was only found in the sato sample from Ang-kak red rice koji. *Bacillus subtilis, B. velesensis, B. fermentum* and *Lactobacillus harbinensis* were only found in sato sample from Loog-pang while *Bacillus amyloliquifacien* and *Lactobacillus paracasei* were found in both samples of sato. The efficiency of fermentation followed by fermentation parameters ; Brix, alcohol and pH. These values of sato from Ang-kak red rice koji and sato from Loog-pang were not significantly different, which indicated that the metabolic pathway occurred in the same way. The final sato products had 8.5-9.2 °Brix, 9 %alcohol and a pH between 3.7-3.8. It was found that sato from Ang-kak red rice koji had all fermentation metabolites (except alcohol content) less than sato from Loog-pang. However, all volatile compounds in both sato were met the standard requirement of TISI 2089-2544.

Keywords: *Ang-kak, Loog-pang, Rice wine, Sato, Red rice koji*

1. Introduction

In 2002, there were several sato factories manufacturing and several brans of sato in the market in Thailand. From the survey was found that more than half of sato was under standard (Puangwerakul, 2008). The background of research was to find out the correct and accurate manufacturing processes and quality control in each process including the method to solve the possible error in each process to maintain the standard according to the specifications of sato product. The previous research in 2004 was to compare sato quality between using Loog-pang and pure culture. It was found that sato quality was based on each sato processing step protection. The system was applied from GMP principle and HACCP framework and related texts were based on microbiology assay index, the efficiency of fermentation parameters and chemical compositions analysis which was to provide guidance to the conventional sato making method and pure culture sato making method concerned with 4 critical control points in 4 importance steps; the beginning and final of saccharification by mold and the beginning and final of alcoholic fermentation by yeast. The results showed that fermenting microbes quantity in each step related to sato chemical composition. This quality assurance of sato was then taken as guideline for the entrepreneurs to control the production of good quality sato as to be accepted by consumer (Puangwerakul and Puangwerakul,2006). The development of red sato production began in 2018; the research was based on using a pure culture of microorganisms. Nutritional quality of red sato was targeted to be improved by incorporating PathumThani1 malt replacing sticky red rice as a raw material in the conventional method. It was found that pure culture strains of ank-kak red rice koji from *Monascus purpureus* TISTR3615 showed highest cell growth, enzyme production, reducing sugar in the same level of mix culture in Loog-pang did and was selected for red sato making (Puangwerakul, 2019). However, it was found that sato production by the community still presenced of several bacteria coexisting in the final product. It is still poorly understood for the behavior and effects of these contaminations in sato making. In this experiment, microorganisms that contaminated in each step were determined comparison with the conventional method for sato making using Loog-pang and Ang-kak red rice koji.

2. Objectives

The objectives of the study were to investigate microorganisms and determine the product quality comparison with Loog-pang and Ang-kak red rice koji in sato production.

3. Materials and Methods

Raw materials: Pathumthani1, Ang-kak red rice koji and Yeast suspension were prepared by the method of Puangwerakul (2007). Loog-pang was purchased from Ban Tang Yao Community Enterprise, Pathumthani province.

The process of sato production in this research beginning with paddy rice, therefore the rice was polished using a high- speed rotating roller NW1000 TURBO. The outer 30% of grain was removed, leaving the central 70% that called milled rice. After milling, rice was steeped in water to remove the bran . After the grain had absorbed 30% of its weight in water, it was removed from the water and steamed for about one hour. Then, steam rice was inoculated with fungi in the form of Ang-kak red rice koji, and Loog-pang.to break down the starch in the grain to convert it to sugar for seven days at ambient temperature and called saccharification. The ratio of rice koji, yeast suspension and water placed in the 5 litres of batch size were 1.80:0.2:2.0 by weight (kilogram) The alcoholic fermentation takes five days at ambient temperature and called fermentation. It was then filtered to produce a clear liquid and pasteurized by HPP, using 400 MPa for 20 minutes at an ambient temperature called HPP and the finish sato called bottle.

3.1 The study of microorganisms in the sato process

Microbiological assay, Presumptive test by MPN, and standard plate count in plate count agar at 35°C for the total count, cook meat medium at 45°C for *Clostridium sp.*, potato dextrose agar at 25°C for yeast and mold, violet red bile agar and MPN at 35°C for coliform and Levin's eosin methylene blue agar and MPN at 45°C for fecal coliform. Confirm step by standard plate count using selective medium, trypticase sulfite neomycin selective at45°C for isolation of *Clostridium perfringens*, Yeast extract glucose chloramphenicol agar at 25°C for fungi, Coli ID at 37°C for blue coliform colonies and red for *E. coli* colonies, Staphylocoagulase detection agar at 37°C for *Staphylococcus aureus* and Cereus selective agar at 37°C for *Bacillus cereus*. In order to classify and identify mainly microorganisms in sato samples, morphological and physiological criteria were used. For yeast, colonies were picked up from potato dextrose agar and grown on a selective lysine medium to separate *Saccharomyces* and non-*Saccharomyces* yeasts. Finally, the yeast species were identified using a PCR reaction mix and RFLP-PCR of rRNA method. According to bacteria identification, GYCA medium (10% glucose, 1% yeast extract, 2% $CaCO_3$ as well as 1.5% agar supplemented with 100 mg/L of natamycin) was used as an identification media. The CTAB (cetyltrimethylammonium bromide) method was used for total DNA extraction from bacteria. All spore-forming bacteria and lactic acid bacteria (LAB) were genotyped using ERIC and (GTG) 5-rep-PCR fingerprinting techniques, and the electrophoresis was performed on 1.5% (w/v) agarose gels. The colonies surrounded by clear zone (halo) were subjected to Gram staining and catalase test. The bacteria that showed gram-positive and catalase-negative were considered to be LAB. To identify bacteria strains and determine taxonomic relationships between bacterial species, suitably accurate techniques: Enterobacterial Repetitive Intergenic Consensus-PCR and Repetitive Palindromic-PCR were used. The genomic DNA of samples has been proposed for identifying bacteria strains and for determining taxonomic relationships between bacterial species because of their high degree of polymorphism. The genomic DNA of samples were extracted, followed the method of Matsuki *et al.* (2004) by the bead-beating technique. The region containing the 16S rRNA V3-V4 variable region was amplified by PCR according to the Illumina protocol (Illumina Inc. 2013), and the sequences were analyzed using Miseq (Illumina, Inc., CA, USA). The composition of the entire bacterial community was obtained using the Miseq Reporter 16S metagenomics system. The microbial cluster tree was categorized using the NCBI taxonomy browser (MD, USA). Fermentation parameters, Brix value, pH, alcohol content, total acidity, volatile acid and reducing sugar. Volatile chemical composition by Gas Chromatography for detection of aldehyde, methyl alcohol, ethyl alcohol, propanol, ethyl acetate, isoamyl alcohol, isobutyl alcohol and pentanol. Statistic analysis had been calibrated between the mean of each item at least three times for three manufacturing lots by using ANOVA statistic.

3.2 The study of Fermentation profile and quality of the final product

Fermentation parameters of products such as brix value, pH, alcohol content, total acidity, volatile acid and reducing sugar. Volatile chemical composition was analyzed by Gas Chromatography for detection of aldehyde, methyl alcohol, ethyl alcohol, propanol, ethyl acetate, isoamyl alcohol, isobutyl alcohol and pentanol. Statistic analysis had been calibrated between the mean of each item at least three times for three manufacturing lots by using ANOVA statistic.

4. Results and Discussion

The study of microorganisms in the sato process: It was found that the number of spoilage microorganism was related to the manufacturing step, as shown Figures 1 and 2, while the number of pathogenic microorganisms was related to the manufacturing step, as shown in Figures 3 and 4.

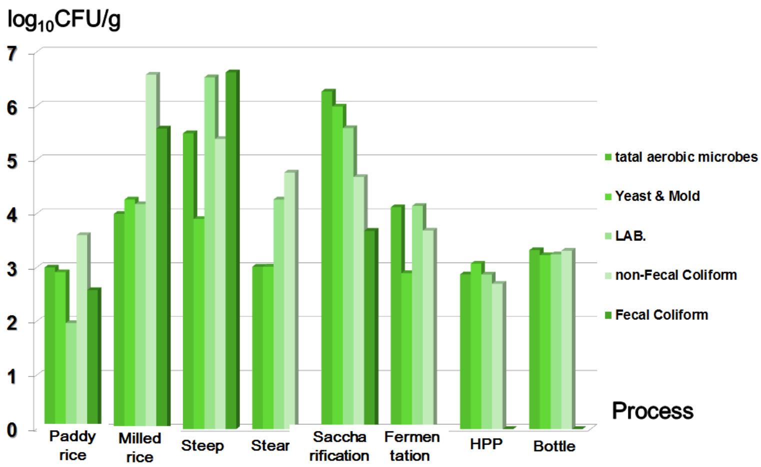


Figure 1 Spoilage number in sato processing by Loog-pang

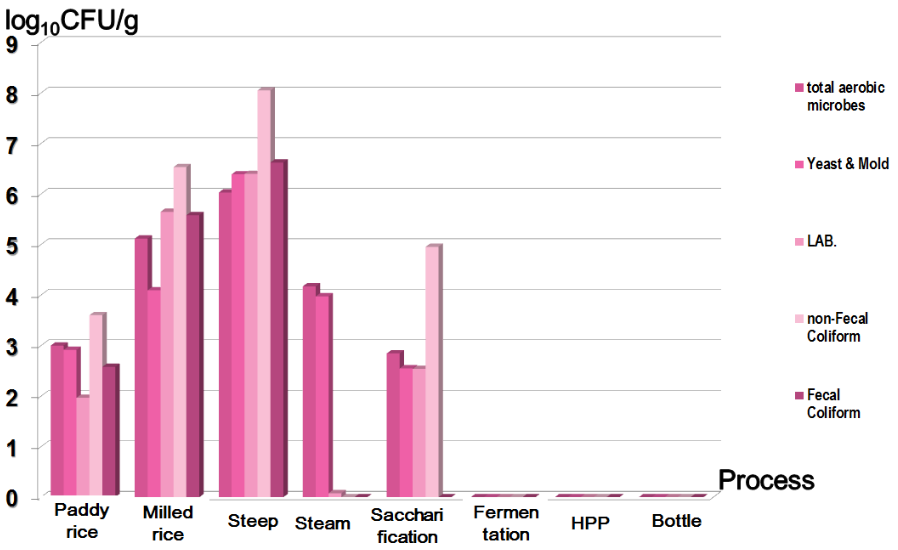


Figure 2 Spoilage number in sato processing by Ang-kak red rice koji

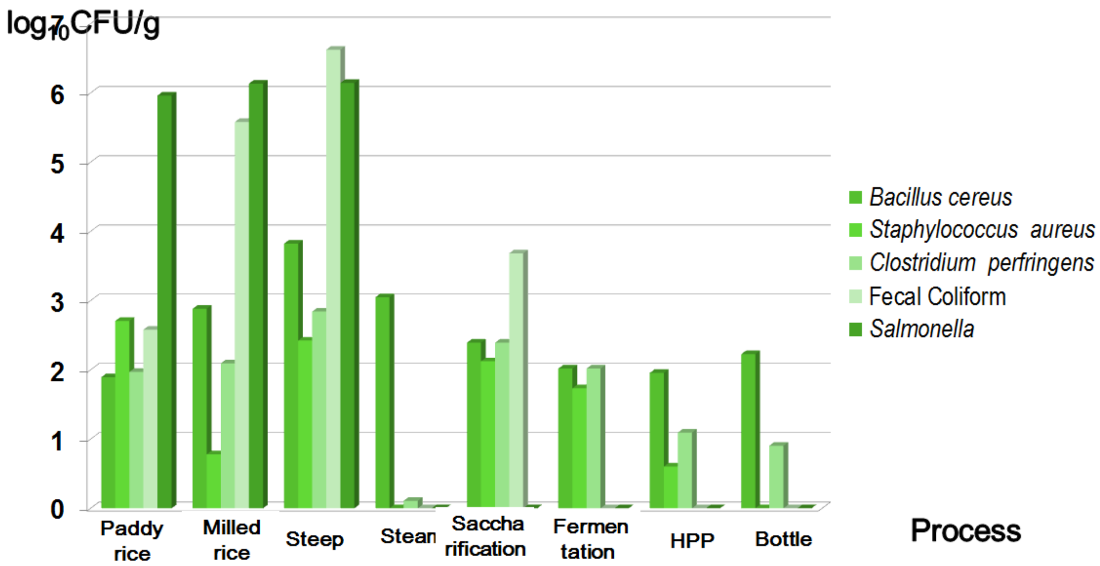


Figure 3 Pathogenic number in sato processing by Loog-pang

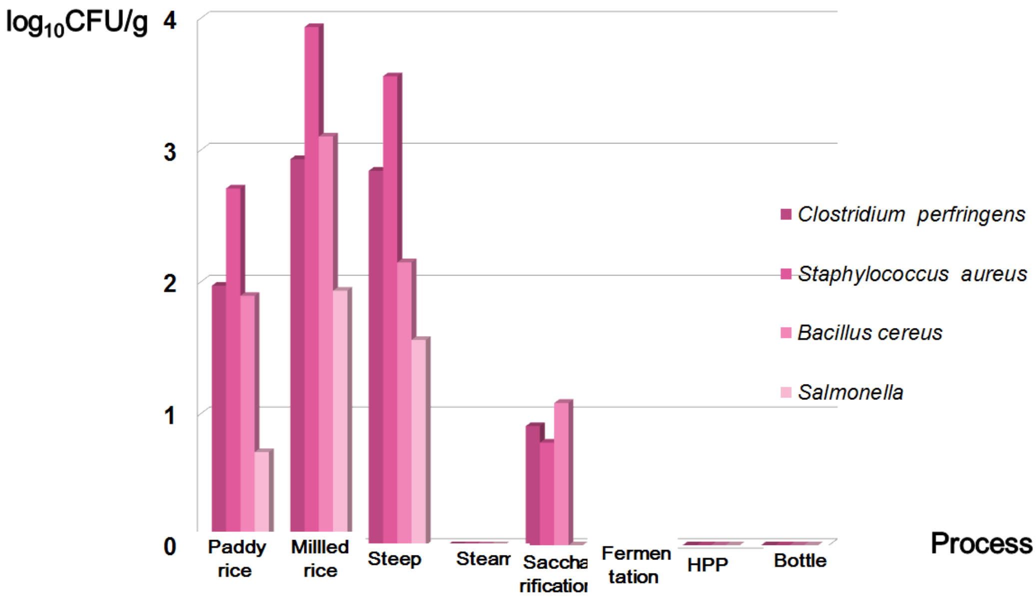


Figure 4 Pathogenic number in sato processing by Ang-kak red rice koji

From Figure.1-4 , great variation in microbial flora among several steps were observed. The results above suggested that Ang-kak red rice koji reached a lower number than Loog-pang. To identify the microbial flora of the products, genomic DNA was extracted from samples. The phylogenetic classification studied was shown in Table 1-7. The results showed that sato from Ang-kak red rice koji (sample no.1) had biodiversity of bacterial strains less than sato from Loog-pang (sample no.2). *Bacillus siamensis* was only found in *the* sato sample from Ang-kak red rice koji. *Bacillus subtilis, B. velesensis, B. fermentum and Lactobacillus harbinensis* were only found in sato sample from Loog-pang while *Bacillus amyloliquifacien* and *Lactobacillus paracasei* were found in both sample of sato

Table 1 *Bacillus amyloliquefaciens*

Species	*Bacillus amyloliquefaciens*
Classification	Bacteria
Colony type	Rough
Morphotype	Rod-shaped

Medium	Colony picture	Micrograph
YPD Sample No.1 Colony ID 1-3		
Medium	**Colony picture**	**Micrograph**
YPD Sample No.1 Colony ID 2-3		

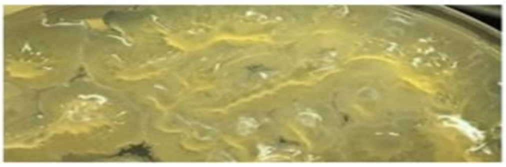
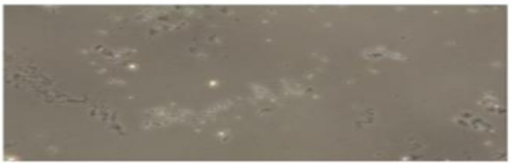
Medium	Colony picture	Micrograph
YPD+CHX		
Sample		
No.2		
Colony ID		
3-5		

Table 2 *Bacillus siamensis*

Species	***Bacillus siamensis***
Classification	Bacteria
Colony type	Rough
Morphotype	Rod-shaped

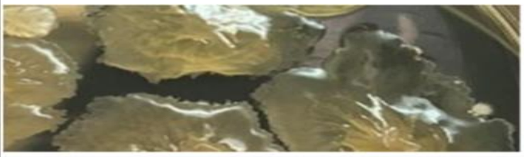
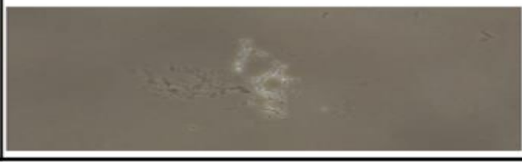
Medium	Colony picture	Micrograph
YPD+CHX		
Sample		
No.1		
Colony ID		
3-2		

Table 3 *Bacillus subtilis*

Species	***Bacillus subtills***
Classification	Bacteria
Colony type	Rough
Morphotype	Rod-shaped

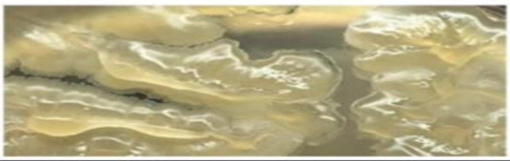
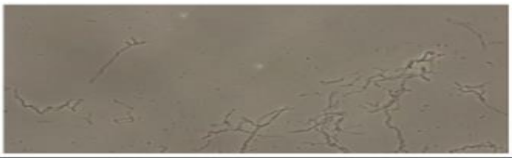
Medium	Colony picture	Micrograph
YPD		
Sample		
No.2		
Colony ID		
2-7		

Table 4 *Bacillus velesensis*

Species	***Bacillus velesensis***
Classification	Bacteria
Colony type	Rough
Morphotype	Rod-shaped

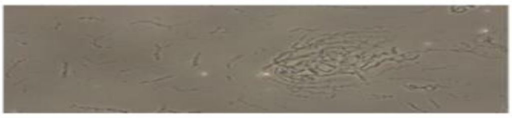
Medium	Colony picture	Micrograph
YPD		
Sample		
No.2		
Colony ID		
1-8		

Table 5 *Lactobacillus fermentum*

Species	*Lactobacillus fermentum*
Classification	Bacteria
Colony type	White/Round
Morphotype	Rod-shaped

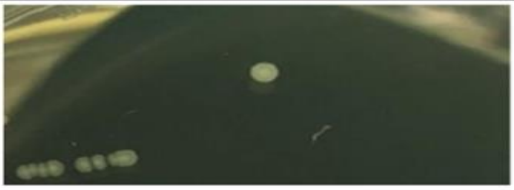
Medium	Colony picture	Micrograph
YPD		
Sample		
No.2		
Colony ID		
2-5		

Table 6 *Lactobacillus herbinensis*

Species	*Lactobacillus harbinensis*
Classification	Bacteria
Colony type	Rough
Morphotype	Rod-shaped

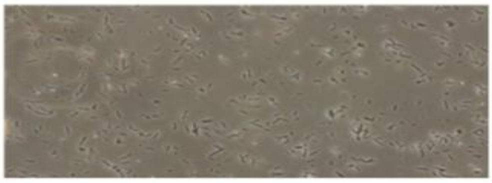
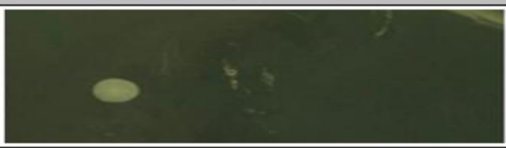
Medium	Colony picture	Micrograph
MRS+CHX		
Sample		
No.2		
Colony ID		
2-33		

Table 7 *Lactobacillus paracasei*

Species	*Lactobacillus paracasei*
Classification	Bacteria
Colony type	White/Round
Morphotype	Rod-shaped

Medium	Colony picture	Micrograph
YPD		
Sample		
No.1		
Colony ID		
1-4		

Medium	Colony picture	Micrograph
YPD		
Sample		
No.2		
Colony ID		
1-6		

The study of Fermentation profile and quality of the final product

Any microorganisms responsible for fermentation might affect the final sato product. However, only the main components and some key metabolite parameters produced in the fermented sato were followed and presented in Figures 5-7. The 5-litre batch size of fermentation for five days indicated that sato preparing from Ang-kak red rice koji and Loog-pang had the same level of Brix, alcohol and pH. The quality of sato as Brix content, percent alcohol and pH were 8.5-9.2, 9.2, and 3.7-3.8, respectively.

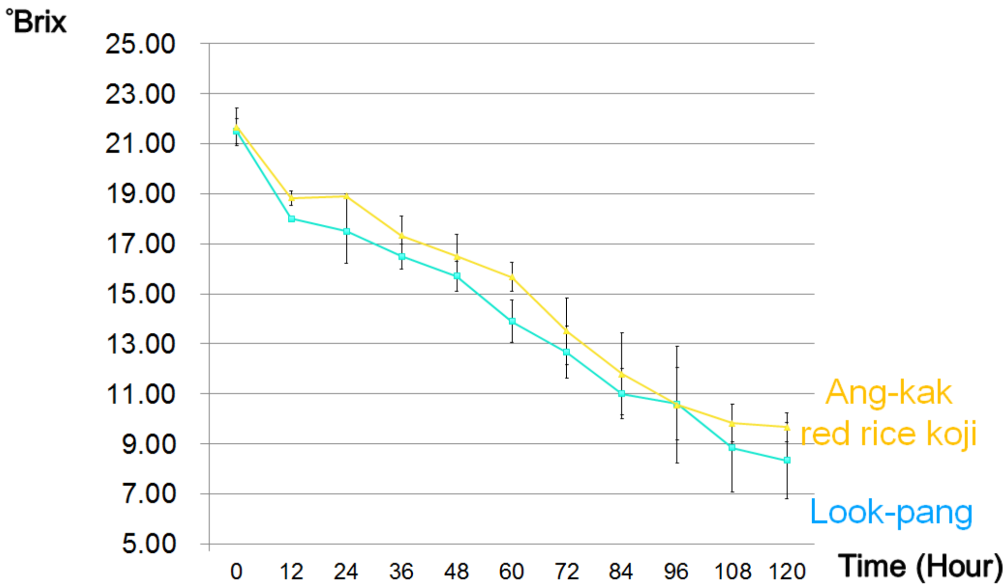


Figure 5 Chang of °Brix throughout 5-day fermentation period

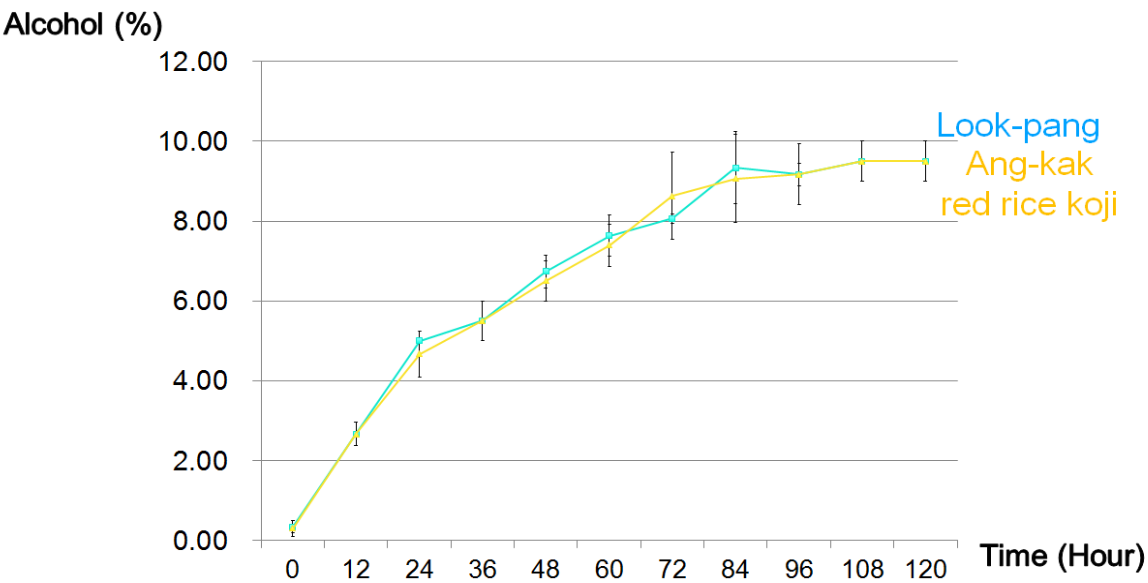


Figure 6 Chang of %alcohol throughout 5-day fermentation period

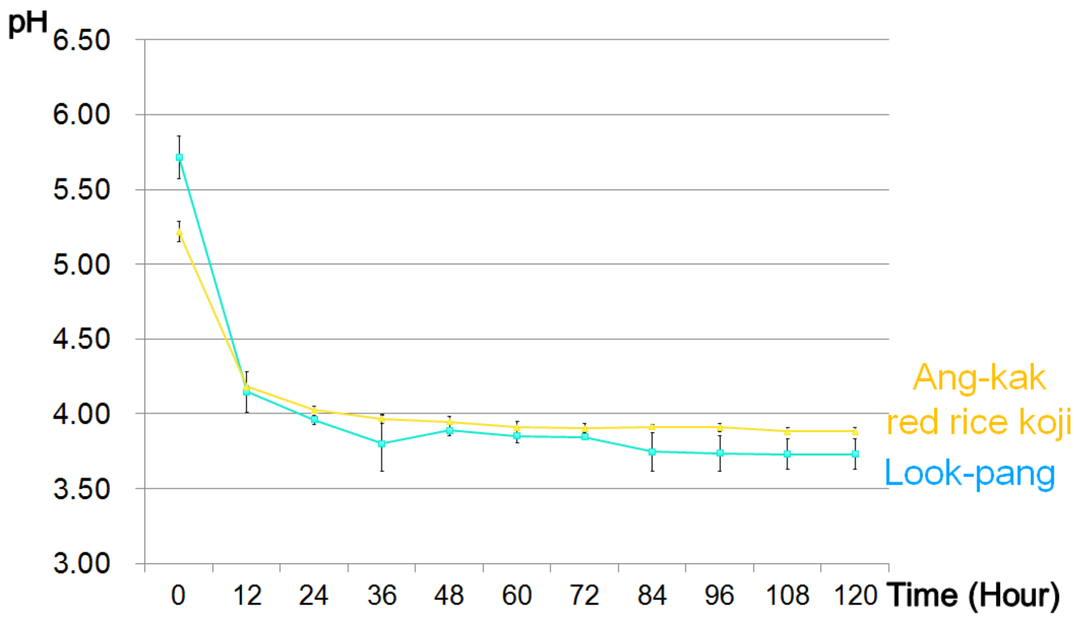


Figure 7 Chang of pH throughout the 5-day fermentation period

From Figures 5-7, key fermentation parameter profiles of sato from Ang-kak red rice koji was not different from another, which indicated that the metabolic pathway did always occur in the same way. Moreover, this sense, interaction of yeast strains in sato had been proven to be contributing factors that generated final desirable characteristics, and these results agreed with a previous study by Chen and Liu (2000).

Fermentation metabolites such as acetaldehyde, ethyl alcohol, n-propyl alcohol, ethyl acetate, isobutyl alcohol, isoamyl alcohol, isoamyl acetate, and ester; ethyl caproate are all known as flavor constituents in Japanese sake and were particularly abundant among several flavor constituents (Ichikawa and Hirata,2019; Yazawa et al., 2019). For chemical properties analysis in sato products, it was found that sato from Ang-kak red rice koji had all fermentation metabolites (except alcohol content) less than sato from Loog-pang. However, all volatile compounds in both sato were met the standard requirement of TISI 2089-2544 defined as standard quality without defect. The results showed in Table 8.

Table 8 Fermentation metabolites in sato comparison with standard criteria

Volatile compound		Ang-kak red rice koji	Loog-pang	Standard of TISI 2089-2544
Acetaldehyde	mg/L	56.11b	118.20a	≤ 160
Methyl alcohol	mg/L	28.12b	52.54a	≤ 420
Ethyl alcohol	%v/v	9.20a	9.20a	≤ 15
Fusel oil	mg/L	158b	252a	≤ 5,500
Ester	mg/L	133b	453a	≤ 1,200

5. Conclusion

The problem of sato production is caused by the internal factor, which means the quality of sato still lack physical, chemical, and microorganism standard in each manufacturing lot. For external factor, it is the lack of scientific knowledge of the manufacturer and manufacturing without understanding. Microorganisms were related to fermentation quality and sato quality as it is important both of its type and volume including contaminated period. It should be controlled at the beginning and in each manufacturing stage. The fermentation processes should be corrected to produce good quality sato. Contamination control could be made by changing from using Loog-pang to use pure microorganism or maintain the former method with strictly control the manufacturing sanitation.

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