SOY SAUCE DECOLORIZATION USING AUTOCHTHONOUS ISOLATES

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รัชธิดา เดชอุดม : การลดสีในซีอิ้วด้วยออโตโคนัสไอโซเลท. (SOY SAUCE DECOLORIZATION USING AUTOCHTHONOUS ISOLATES) อ.ที่ปรึกษาหลัก : รศ. ดร.ชื่นจิต ประกิตชัยวัฒนา, อ.ที่ปรึกษาร่วม : ผศ. ดร.ธนจันทร์ มหาวนิช

งานวิจัยนี้มีจุดประสงค์เพื่อสำรวจหาจุลินทรีย์ออโทโคนัสที่มีสมบัติในการลดสีน้ำตาลในโมโรมิและตรวจสอบสมบัติของไอโซเลทออโทโคนัสเพื่อใช้เป็นวิธีทางชีวภาพสำหรับลดสีในซีอิ๊ว โดยศึกษาชุมชนจุลินทรีย์ออโทโคนัสในกระบวนการหมักโมโรมิภายใต้สภาวะการผลิตที่แตกต่างกัน ด้วยวิธีการเพาะเลี้ยงบนเพลท และ Reverse transcriptase-nested PCR-DGGE (RevT-nested PCR-DGGE) ผลที่ได้จากการเพาะเลี้ยงบนเพลท พบ *Bacillus* (ร้อยละ 43.17) และ *Staphylococcus* (ร้อยละ 33.09) เป็นแบคทีเรียหลักในทุกตัวอย่างและทุกอายุการหมัก และพบ *Candida* spp. เป็นยีสต์หลักในทุกระบบการหมัก ในขณะที่ผลจากเทคนิค RevT-nested PCR-DGGE พบการแสดงออกทางพันธุกรรมของแบคทีเรียกรดแลคติก (LAB) มากที่สุด (ร้อยละ 29.73) และพบ *Bacillus* spp. กับ *Staphylococcus* spp. ในสัดส่วนที่เท่ากันที่ ร้อยละ 18.92 โดยพบแบคทีเรียที่ไม่ระบุสายพันธุ์เพิ่มเติมในบางระบบการหมัก กลุ่มยีสต์พบการแสดงออกทางพันธุกรรมของ *Candida* spp. (ร้อยละ 56.00%) *Saccharomycetales (*ร้อยละ 24.00) โดยพบ *Z. rouxii* เพียงร้อยละ 4 ผลจากการสำรวจบ่งชี้ได้ว่าการแสดงออกทางพันธุกรรมของ *Bacillus* และ/หรือ *Staphylococcus* ที่พบร่วมกับการเปลี่ยนแปลงทางเคมีกายภาพและค่าดรรชนีสีน้ำตาล มีส่วนเกี่ยวข้องกับชะลออัตราการเกิดและลดสีน้ำตาลในโมโรมิ เมื่อนำแบคทีเรียและยีสต์ไอโซเลทจำนวน 139 และ 105 โคโลนีที่คัดแยกได้จากโมโรมิมาทดสอบความสามารถในการใช้น้ำตาล ได้แบคทีเรีย 5 สายพันธุ์ที่สามารถใช้น้ำตาลไซโลสร่วมกับกลูโคส และทุกสายพันธุ์มีสมบัติในการลดสีน้ำตาลโดยรวม และ/หรือสลายเมลานอยดินได้ จึงเลือกแบคทีเรีย *Staphylococcus* sp. SSB48 และ *B. amyloliquefaciens* SSB6 ที่สามารถใช้น้ำตาลไซโลสได้ถึงร้อยละ 39.09 และ 40.08 และสามารถลดสีน้ำตาลโดยรวมได้ร้อยละ 27.04และ26.11 ตามลำดับ เป็นสายพันธุ์คัดเลือกเพื่อประเมินความสามารถในการลดสีน้ำตาลในโมโรมิ โดยพบว่า สภาวะที่เหมาะสมในการทำงานของสายพันธุ์คัดเลือกทั้งสองคือการเติมในรูปแบบของกล้าเชื้อสดเดี่ยวในช่วงสุดท้ายของกระบวนการหมักโมโรมิ และบ่มต่อภายใต้สภาวะเดิมเป็นเวลา 1 เดือน

สาขาวิชา	เทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต .....
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Rachatida Det-udom : SOY SAUCE DECOLORIZATION USING AUTOCHTHONOUS ISOLATES. Advisor: Assoc. Prof. CHEUNJIT PRAKITCHIWATTANA, Ph.D. Co-advisor: Asst. Prof. THANACHAN MAHAWANICH, Ph.D.

The aim of this study was to explore autochthonous strains having properties in reduction of brown color in moromi and evaluate their potential as biological mean for de-colorization of soy sauce. The autochthonous microbes associated to moromi fermentation under different manufacturing conditions were evaluated using both the culture plating and Reverse transcriptase-nested PCR-DGGE (RevT-nested PCR-DGGE) methods. By culture plating, *Bacillus* (43.17%) and *Staphylococcus* (33.09%) were isolated from every fermentation stages of all samples. Yeast *Candida* spp. was frequently detected (46.67%) in most samples. RevT-nested PCR-DGGE revealed the different results, as 29.73% of major bacterial DGGE band belonged to lactic acid bacteria (LAB) while equal proportion of 18.92% were *Bacillus* spp. and *Staphylococcus* spp., and including unidentified bacteria. *Candida* spp. was generally detected (56.00%) as the main fungal, following by *Saccharomycetales* (24.00%), while *Z.rouxii*, known as soy sauce yeast was identified as low as 4.00%. Through the RNA-based DGGE analysis, *Bacillus* and *Staphylococcus* demonstrated their roles in the reduction of browning generation rate. Total isolates of 139 bacteria and 105 yeast were further screened based on sugar utilization. Five isolates having co-metabolic activity of xylose and glucose in synthetic medium, in addition with containing either total browning or melanoidin degradable activities, were obtained. The two isolates of *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6 with the highest xylose utlilizability (39.09 and 40.08%, respectively), and total browning decolorizability (27.04% and 26.11%, respectively) were selected as potential strains. The ability in browning color reduction of potentials were tested in moromi in order to preliminary optimize decolorization process. The optimal condition of the potentials in de-colorization was inoculation of single candidates at final stage of moromi fermentation and incubation for another 1 month.

Field of Study:	Food Technology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER 1

INTRODUCTION

Soy sauce is one of the most representative Asian traditional condiments. Both itself and derived product had currently become increasingly used worldwide and Thailand is also one of the important exporters (Center, 2017). Its harmonious combination of flavors, distinctive aroma, and superb color resulted from the long brewing process. This subtle balance makes unique characteristic of soy sauce. The main sensory quality of soy sauce is significantly determined by color and flavor as criteria for product grading (Luh, 1995). Color is one of the major factors for product attraction. Many studies show that if the color of food product does not match the consumer expectation, the acceptance of consumer for that product is rare (Frick, 2003). Over the last decade, the color preference of soy sauce among general consumer in Japan and some other countries is change from dark brown to lighter (Miyagi, 2012; Takagi, 2005). This trend is used in one of the product marketing schemes since it influences consumer's first impression and leading to a buying decision, results in marketing success. Also, this change of consumer preference has led to the development of new soy sauce production process for brown color reduction. Japanese Agricultural Standard (JAS) has been revised the quality labeling standard of soy sauce which mainly demonstrate to a significant of color quality (Luh, 1995). High quality soy sauce would be physically defined by the vivid, clear, and shiny reddish-brown color (Wang, Sun, et al., 2018) which is depended upon raw materials used, fermentation process and particularly, microorganisms associated to the fermentations. Fermentation process includes (i) koji fermentation, to hydrolyze soybean protein into short chain peptide and/or amino acid, and reducing sugar and (ii) moromi (17-19% NaCl soybean mash) fermentation, to further hydrolyze and convert single molecules to diverse metabolites allowing for the natural development of soy sauce's characteristic. Browning in soy sauce greatly occurs

during moromi fermentation and pasteurization, and is mainly stemmed by non-enzymatic browning. Maillard is the reaction spontaneously occurred in the system containing reducing sugar and amino acid / peptide. The reaction is a complex process acts as a network containing various reaction series, through key intermediate of light brown color hydroxymethylfurfural (HMF), then further form melanoidin, nitrogenous polymers group giving a deep brown color in the product.

Mitigation of Maillard reaction and its results in soy sauce have been extensively studied by mean of preventive or corrective strategies (Rannou et al., 2016) including physical and chemical means to remove melanoidin from the final products (Miyagi, Nabetani, et al., 2013; Miyagi, Suzuki, et al., 2013; Okuhara et al., 1973; Terasawa et al., 2000). These proposed techniques could effectively reduce Maillard reaction. Nevertheless, some drawbacks such as health concerning, high production cost and low productivity were mentioned. In addition, through these process, practical application in food industry level is still limited. Considering preventive action, biotechnological technique had been applied such as using HMF oxidase from non- food bacterium to degrade HMF (Wang, Sun, et al., 2018). However, microbial application was limited by food safety regulation of being "Generally Recognized as Safe" (Food et al., 2015) or "Qualified Presumption of Safety" (QPS, EFSA) designation. Key criteria in selection of GRAS could be considered in term of source of isolates, particularly microbe associated to auto/natural food fermentation. Autochthonous microorganism is the native microflora that are normally found in the same food matrix, which is not limited by geographical factor. These microbes shown significant advantages when using as starter culture and specific use for food fermentation.

Autochthonous microorganism involving in soy sauce fermentation mainly consists of mold, yeast, and lactic acid bacteria. Some autochthonous yeast such as

Zygosaccharomyces rouxii and *Saccharomyces cerevisiae* were also reported as strains could utilize and convert HMF into 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF), a remarkable key volatile compound in soy sauce (Uehara et al., 2017). *Tetragenococcus halophila* was reported as one of the dominant lactic acid bacteria isolated from soy sauce. This bacteria was further subjected to strain development through genetic engineering to have higher activity in xylose utilization and successfully used in soy sauce decolorization prior to patent (Abe et al., 1988). However, main problem that has to be solved before application of genetic engineering microorganism in food product, apart from severe restriction or ban in many countries, is public acceptance (Gartland and Gartland, 2018). To overcome this uncertainty, soy sauce decolorization by use of efficient autochthonous isolates through their natural metabolic reactions during fermentation process reveals its potential without restriction or consumer constrains.

During autochthonous fermentation (a natural fermentation), distinctive flavor and aroma in soy sauce determining its organoleptic quality are the sequence of complex microbial profiles series. Despite the fact that main ingredients and process used in soy sauce production share common characteristics, a distinctive quality in final product from different regions can be observed (Kim et al., 2010). Therefore, better understanding of microbial community and their profiles during fermentation process is an effective quality development scheme along with isolation of autochthonous strains possibly having both de-colorizing and aromatic enhancing abilities. Many studied in microbial diversity in natural ecosystems were based on conventional plating and comparing to DNA-based methods. DNA-based technique coupling with denaturing gradient gel electrophoresis (DGGE) as PCR-DGGE has been wildly used to investigate cultural independent strains in natural and food fermentation ecosystems. The DNA profiles obtained could be used as microbial community mapping to understand their roles and/or impacts during process of

fermentation. However, through DNA-based protocol including high-throughput sequencing Miseq, metagenomic or pyrosequencing (Tang et al., 2017; Wang, Wen, et al., 2017; Wu et al., 2018), false-positive results could be caused due to detection of dead cells (Sunyer-Figueres et al., 2018). Recently, there are many reports proposing the cDNA-PCR-DGGE based protocol to detect viable microbes in fermentation ecosystems (Benítez-Cabello et al., 2016; Cardinali et al., 2018; Garofalo et al., 2017) not yet in soy sauce. In addition, the cDNA determination was also reported as a higher sensitive method relative to DNA-based. Since RNA directly links to gene expression, it could also demonstrate the possible activity of each microbe detected (Chahorm and Prakitchaiwattana, 2018). Accordingly, RNA-based coupling with nested PCR-DGGE could be as a potential gene expression map when performed a long with determination of dynamic changes of key compounds, it could help to better understand the possible microbial metabolic roles during fermentation process. The information obtained may help to easier selection of potential target microbe for further development as starter having specific function for food production.

Thus, the aims of this study were to explore autochthonous strains having properties in reduction compounds and/or reactions involved in browning during moromi fermentation and to evaluate their potential for further use as biological mean in reduction of browning color in soy sauce.

The works conducted in this study were divided into 3 Chapters.

(i) Investigation of microbial community in different moromi fermentation conditions by conventional plating and Rev-T-nested PCR-DGGE, and determination the of dominant microbes contribution in community toward key physical and chemical parameters, including browning as present in chapter 3. This work was presented in Chapter 3.

(ii) Metabolomic database together with potential autochthonous strains gained from the first part were further evaluated for their ability in mitigation of browning in moromi as shown in Chapter 4.

(iii) The candidate strains were selected and subjected to test their ability in browning reduction in moromi at laboratory-scale fermentation. Optimal condition and factors associated to de-coloring ability were investigated in Chapter 5.

CHAPTER 2

LITERATURE REVIEW

This thesis is concerned with the welled-optimized autochthonous microorganism(s) with Maillard-decolorizable activity screening from soy sauce origin and database of microbial community in relation with key physicochemical parameters affecting soy sauce quality. Key studies associated with these topics will be presented in the introduction and discussion sections of subsequent experimental chapters. This chapter will provide a brief background of autochthonous, soy sauce production along with interrelation of microbiological with physicochemical change during fermentation followed by a more detailed review of the literature describing the target browning reaction and its mitigation approach. A final section of this chapter will provide some background information of the novel analysis methods for evaluation of microbial communities in food fermentation process.

2.1 Autochthonous starter culture in food fermentation industry

Fermentation has long history usage as a preservation tool for prolonged shelf-life of perishable food products. In the earlier times, the fermentation is performed in an artisan way either following natural spontaneous fermentations, or by the back-slopping method. These traditional methods using microorganisms already present in the raw material itself. The specific participated microorganisms, environmental conditions, and type / formulation of raw materials varied markedly from region to region and even between sites within the similar area, resulting in the high specialty products Buckenhüskes (Buckenhüskes et al., 1997). Another fermentation method typically applied in this present time, especially with industrial production scale, is

well-selected starter culture inoculation. This technique allows many outcomes such as the inhibition of unwanted microorganisms including spoilage and pathogen, easy-predictable and controllable of sensory, nutritional and rheology properties. In addition, application of starter culture benefits the means of hygiene, safety and quality control. With the disclosure of characterized potential microorganisms, an ideal, well-improved products along with feasible fermentation processes are possible Hansen (Hansen, 2002).

The source of starter culture can be defined into two types, “allochthonous” and “autochthonous”. The autochthonous is the native flora isolated from and re-used on the same raw matrix. Allochthonous starters means non-native isolated from raw matrices used in fermentation of various products, indicating an alien species in that system. Commercial starter cultures using in various food fermentation industry nowadays predominantly coincide with above definition of alien-allochthonous strains (Di Cagno et al., 2008). Even though many studies report efficiently application of allochthonous starter in food fermentation (Liu et al., 2016; Özer et al., 2016; Saerens and Swiegers, 2017; Santos et al., 2014). These non-native starters also show many limitations which are, (i) sharp acidification causing unbalance fermentation environment; (ii) less complexity and/or depletion of key main sensory and functional properties of the final product; (iii) low metabolic flexibility; and (iv) the limited microbial ecology (Pérez-Díaz et al., 2017). Many studies also indicate drawbacks of allochthonous culture toward autochthonous one in food industry application (Chanprasartsuk, 2008; Di Cagno et al., 2009; Di Cagno et al., 2008; Feng et al., 2015). By these essential, authochthonous starter culture seems to show more benefit for industrial application, and verification of these two starter types in food and beverage products are reviewed as following section.

2.1.1 Viability

The viability of probiotic *Lactobacillus plantarum*, both allochthonous and autochthonous strains are examined in fermented pomegranate juice. Results indicate a sharp reduction of viable Allo- than Auto-starter which should be due to inhibitory effect of abundant polyphenolic compound in juice toward an alien species (Filannino et al., 2013). Another experiment conducting in fermented tomato juice reveals a growth in autochthonous strain while reduction was noted in allochthonous-inoculated treatment (Di Cagno et al., 2009).

2.1.2 Metabolic activity

Many experiments are conducted in order to investigate the fermentation efficiency of product with these 2 types of starter cultures. These include, but not limit to, their sensory aspect and functional properties. The advantage of using autochthonous over allochthonous in volatile compound profile which significantly contribute to the flavor and aroma characteristics of fermented food product was reported in fermented tomato juice by Cagno and coworker in 2009. The juice fermented with Allo-*Lactobacillus plantarum* was discriminated from two Auto-strains mainly by high levels of esters, alcohols and sulphur compounds along with minority of furans. Many unwanted volatiles largely generated in allochthonous treatment included butanal, pentanal and 2,4- hexadienal, characterizing as cooked flavor volatile while autochthonous strains promoted key volatile compound of 3-methyl-3-butan-1-ol. Moreover, the balance of sulphur derivatives varied between allochthonous and autochthonous strains. Linalool, one of the sulphur compound which positively influence the flavor of processed tomatoes were at the highest levels in the Auto- juice (Di Cagno et al., 2009).

The impact of Allo-strain toward flavors and aromas characteristic of probiotic juice was report by Luckow et al in year 2004. The overall liking scores for probiotic orange juice produce by an addition of *Lactobacillus paracasei* ssp. paracasei, an allochthonous isolate originated in human gastrointestinal tract, was lower than the original juice due to formation of medicinal aroma and medicinal, sour flavor (Luckow and Delahunty, 2004). In addition, effect of inoculated starter in relation with key functional properties was also investigated in this study. Tomato juice fermented with Allo- strain showed marked decreased of ascorbate and glutathione during storage. However, juice fermented with two Auto-strains maintained elevated values of these two specific features.

2.1.3 Safety aspect in term of biogenic amine

2.1.3.1 Incident of biogenic amine in fermented food product and its producer microorganisms

Biogenic amine (BA) in fermented food products produces by numerous of microorganisms including lactic acid bacteria (Baisier and Labuza) result in diverse effect of heath issue (Ladero et al., 2015; Mete et al., 2017; Poveda et al., 2017). Among this bacterial group, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus are reported as a high-efficient BA producer, and the production is mainly specific to strain-dependent rather than bacterial species (Gu et al., 2018).* These biogenic amine substances participate in diverse key function such as vascular permeability, neurotransmission and the allergic response. From this viewpoint, the various factors, especially starter culture, possibly influence the BA formation are intensively investigated nowadays. Cheese, fermented meat and soy-based product belong to the potential hazardous food.

Renes and coworker (Renes et al., 2014) study the effect of Allo- and Auto- starter cultures on the biogenic amine content of ewe's milk cheese reported significantly increase of total BA content throughout the ripening time in all tested samples. At the end of ripening, sample made by Allo-starter culture was the one with the highest concentrations of cadaverine, histamine and tryptamine indicating an unproper selection of commercial starter culture since the process predominantly focused on proteolytic capacity. This, in turn, resulted in releasing an excess quantity of free amino acids, allowing greater production of biogenic amines than others. In meat product, the BA formation by mixed Allo- and Auto- starter cultures in two type of Czech dry fermented sausage, Hercules (H) and Paprikas (P) were determined. Results indicate a massive production of biogenic amine in relation with the metabolic adaptation of Allo- culture in an unfamiliar environment, and storage condition also indirectly contributes to the BA formation through promotion of BA-producible bacteria (Komprda et al., 2004).

Incident of BA in soy-based fermented products were reported. Polyamines BA of putrescine, spermidine, spermine, tryptamine and tyramine was detected in stinky tofu spontaneously fermented by LAB and *Bacillus*. Tyramine was the predominant biogenic amine found in both stinky tofu and its fermentation brine. Since its concentration in range of 100 to 400 mg may induced a migraine or raising blood pressure, at least 20 mmHg, ≥100 mg/kg of tyramine observed in this study indicate the possibility of toxicological effects due to over-consumption of this product (Gu et al., 2018). BAs is also detected in Thai soy sauce and soybean paste with the prevalent of tyramine, putrecine, cadaverine and spermidine in all samples. Nevertheless, BAs level are broadly lower than their toxicological limit (Deetae et al., 2017). This report is similar to the previous one of BAs content in reduced-salt soy sauce. Histamine, cadavarine, putrecine, and tyramine were detected in low amounts

when mixed bacterial and yeast starter culture was applied comparing with one with yeast starter culture. Since there were reports of BA-producing ability of osmotolerant yeasts generally detected in soy sauce fermentation process such as *Candida versatilis* and *Zygosaccharomyces rouxii* due to membrane-bound decarboxylase activity, they also proposed an advantage of those mixed starter culture in counteracted with BA issue (Singracha et al., 2017).

2.1.3.2 Detection of BAs-producing strains in fermented food

Considering an importance of BA-toxicological concern in relation to public health, one feasible approach of BA mitigation is selection of proper starter culture uncapable decarboxylated target amino acids into Bas, or carrying none of Bas-encoding gene. The first approach of decarboxylase activity was reported by Mete et al (2017). They use modified decarboxylase medium for screening of BA-producing. The color changing of purple into yellow was considered as positive result. Nevertheless, results from this study along with others from pervious works point out the false negative results due to insufficient detection limit of the assay.

PCR-based technique is used for another approach for identification of BA producibility in bacteria. Multiplex PCR with the specific primers for tyrosine (*tdc*), histidine (*hdc*) and ornithine decarboxylase (*odc*) genes were proposed (Poveda et al., 2017). This method reveals an inconsistency between qualitative PCR and quantitative assay by HPLC explaining by inadequate sensitivity of HPLC, presenting of silent gene and/or biosynthesis gene mutation, and might include unappropriated environment of BA production.

2.2 Soy sauce production process

Fermented soy-based products including soy sauce are widely consumed, especially in Asiatic countries due to their specific aroma, flavor and color which are the consequence of complex two-step fermentation process leading by subsequently growth of molds, yeasts and bacteria. The complexity of soy sauce is then, partly, influenced by metabolic activities of those microorganisms. Other factors involving along with the information of production process are detailed as following.

2.2.1 Fermentation process

2.2.1.1 Koji

Koji is the first fermentation step of soy sauce production consisting raw material of cooked soybean, wheat and brine. For raw material preparation, either whole, extruded or defatted soybean are soaked in water and cooked by retort (for industrial or large scale-production) or steaming apparatus (for small-scale traditional production). Wheat is used both in form of flour or whole seed. In the latter case, it is roasted to lowering moisture content and then cracked into small pieces before mixing with prepared soybean. The general proportion of soybean and wheat is 1:1 but may be varied following each manufacturer's formulation and type of intended product such applied in Japanese soy sauce called shoyu. The first common shoyu named as koikuchi is a deep dark brown color uses equal amounts of raw materials which is similar to another shoyu of Saishikomi which brine adding after koji fermentation is replaced by unpasteurized raw soy sauce from previous batch. Usukuchi shoyu is produced from soybean with or without steamed rice thus its color is lighter. The third class called tamari uses a small quantity of

wheat, and the final group of shiro, an extra-light shoyu produced from wheat with a small proportion of soybean (Kataoka, 2005).

After the mixture is formulated, either starter culture inoculation or back-slopping technique are applied, the first microorganism group is introduced into koji. In case of commercial starter culture, approximately 0.1-0.2% starter is added. The latter case of back-slopping gains a benefit of fast fermentation, may possess desirable feature through metabolic activity of best-adapted culture(s), and economical reason (Holzapfel, 2002) even higher proportion of this kind of starter culture has to be used, may be ≥10%.

Inoculated koji mixture is spread into thin layer of 3-5 cm. thickness in tray and incubates at 25-28 ℃, or room temperature for traditional process, for 25 – 72 hours. During this incubation time, microorganisms, particularly considered as *Aspergillus* mold including *A. oryzae* or *A. sojae*) are growth by hydrolyzing soybean protein into small peptides and free amino acids, and converting gelatinized starch from wheat and soybean into simple sugars, providing nutrients for subsequently brine fermentation. Heat generated from microbial's metabolic activity is removed by mean of stirring which is one critical control point along with the selection of starter culture(s).

In industrial production, many processes including raw material preparation and fermentation are automated. Incubation of koji mixture is processed in large shallow perforated vats closing chamber equipped with automated air circulation and baffle plates for koji turning which support the proteolysis and amylolysis activities, and preventing growth of unwanted contaminated microorganisms. There is a report of around 85-90% reduction of labor cost with application of these devices comparing the manual method (Luh, 1995).

For a long history of soy sauce, it has been described as a fungal-based fermented food product since koji starter is dominant by *Aspergillus* mold. Among this species, *A. oryzae* is the most intensively used in koji starter culture due to its high efficiency production of numerous potential enzymes namely proteinases, amylase, cellulases, and phytases. Other mold found in traditional koji are *Rhizopus, Mucor, Monascus* and *Penicillium*. A determination of microbial community in soy sauce fermentation of koji stage represent a low-diversity ecology dominating with *A. oryzae* and *A. flavus* (Yan et al., 2013).

Another study of fungal ecosystem in Thai koji soy sauce informed an interesting finding about role of temperature with the dynamic shift in microbial community. It is found that cold weather promoted the growth of green koji which more preferred than black or gray koji flourished in warmer temperature. This gray koji consisted of *Rhizopus* spp., *Mucor* spp., *Absidia* spp., and a *Syncephalastrum* sp. whereas Aspergilli was predominant in the green one (Bhumiratana et al., 1980).

Few years ago, new koji mold strain of *Monascus purpureus* was proposed in term of mixed culture with *A. oryzae* and its activity toward soy sauce was compared with pure *A. oryzae* culture. The results indicated higher protease and glucoamylase activity, richer aroma profile of mixed starter. Nevertheless, deeper color was also gained from this starter as well (Chen et al., 2015).

Bacteria also presents in koji stage as a background microflora. In the study of bacterial koji by DNA-based method, 25 phylotypes including 2 major species of *Weissella* and *Staphylococcus*, and minor of *Kurthia, Corynebacterium, Enterococcus, Pediococcus* and *Lactobacillus* were identified. There is also reports the controversial distribution of *Weissella* and *Staphylococcus* population as the

former bacteria dominating the early stage and decrease through time where of the latter sharply increase and reach 40% at the last koji stage. Occurrence of other bacteria genus stated above are represented in each step but less than 10%. Predominant of *Staphylococcus* should be due to its facultative anaerobes and halotolerant properties (Guan et al., 2011). This proteolytic bacteria generates volatile fatty acid involving aroma characteristic (Yongsawatdigul et al., 2007) *Pediococcus* is a minor phytate-degrading bacteria is growth at the beginning of koji and is masked during moromi fermentation (Raghavendra et al., 2011; Yan et al., 2013).

In term of yeast, koji also contains autochthonous such as *Kluyveromyces*, *Pichia* and *Candida* spp. which the latter is the most dominant species. Generally, strains of *C. rugosa, C. glabrata and C. tropicalis* was detected in both soy sauce koji and moromi due to their fast growth rate together with acid- and salt-tolerance properties. Other yeasts such as *Saccharomycopsis. fibuligera, Pichia anomala* and *Trichosporon asahii* are observed in koji as well. These yeast are generally function in plant matter fermentation and have been associated with flavor formation when they are together with LAB. Some earlier studies revealed that *S. fibuligera* predominantly existed in starter culture involved in fermentation during an initial period before alcoholic fermentation stage. This yeast could produce various enzymes including glucoamylase and α-amylase, which support glucose accumulation (Horváthová et al., 2004). Nevertheless, only one strain is noted in an initial koji fermentation stage, supporting the possibility that this yeast is a background flora in soy sauce koji. Others yeast such as *P. anomala and C. rugosa,* the lipase producer, and *T. asahii* could tolerate acidic condition such in koji process hence become background flora playing specific roles during this soy sauce fermentation period (Tanaka et al., 2012; Yan et al., 2013).

2.2.1.2 Moromi

In the second fermentation step of soy sauce, brine (18-20%) is added into koji and the soy sauce mash mixture is called as moromi. This soy mash is transferred into fermentation tank or clay jar. One of the critical control point in this fermentation stage is maintaining viability of microorganisms (Luh, 1995). In an early moromi stage, high salt concentration acts as selective agent inhibiting unwanted microflora, and promoting the growth of specific bacterial strains such as lactic acid bacteria (Baisier and Labuza) of *Tetragenococcus halophilus*. This bacteria utilizes carbon and nitrogen source from *Aspergillus* activity and transforms into organic acid and/or volatile compounds. In order to preserve metabolically-active LAB, temperature of the mixture in industrial process should be relative low (15°C) during the first month, allowing gradually decrease of pH from 6.5 to 5.0. This acidic condition supports the growth of yeast such as *Zygosaccharomyces rouxii* and *Candida* species. After 1 month of moromi fermentation, higher temperature to the maximum of approximately 28°C is applied to initiate alcoholic fermentation, following by statically controlled temperature at 25°C (Luh, 1995). Contrastingly, temperature of traditional process is not properly control. The decline of pH during the aging of mash may be attributed to autolysis of microbial cells, accumulation of free fatty acid, amino acids, and peptides containing carbolylic side chains as a result of hydrolysis of mash constituents, as well as the microbial fermentation of carbohydrates (Chou and Ling, 1998).

The growth of osmophilic yeasts including *Torulopsis versatilis* and *T. etchelsii* are found in the late alcoholic fermentation and their metabolic activity result in generation of specific phenolic aroma compounds in final product. *Pichia farinosa, P. miso, Hansenula anomala, Cryptococcus diffluens, Candida tropicalis*, and *Trichosporon behrendii* are sporadically detected in an early moromi

fermentation while unwanted strain of *Saccharomyces rouxii* var. *halomembranis* could be observed at the late fermentaion time (Noda et al., 1980). The key soy sauce yeast of *Z. rouxii, C. versatilis, and C. etchellsii* influence the quality of soy sauce and co-inoculation of the former yeast strains with *P. guilliermondii* could largely promote formation of volatile compounds (Cui, Zheng, et al., 2014).

In term of bacteria, *Micrococcus, Bacillus, Streptococcus*, LAB, and its some related bacterial group are spontaneously marked in moromi. The 2 former bacteria groups are considered as contaminants preventing the growth of koji mold. Furthermore *Weissella cibaria, W. confusa, W. kimchii, W. salipiscis, Lactobacillus fermentum, L. plantarum, L. iners, or Streptococcus thermophilus, Staphylococcus xylosus and S. kloosii* are also noticed theoughout the fermentation process of koji and moromi (Tanaka et al., 2012). *T. halophilus*, one of LAB, reveals it biotransformation activity of aspartic acid (sour taste) into alanine (sweet taste), hence influences the taste characteristic of soy sauce (Fan and Hansen, 2012).

Microbiological interaction and diversity during fermentation process, both koji and moromi results in unique flavor of soy sauce. The dynamic changes of volatile profiles including alcohols, esters, and pyrazines alcohols, ketones, and pyrazines groups are strongly pronounced when co-inoculation of yeast with LAB is applied. These volatile compounds represent the aroma characteristics of dark chocolate, potato-like, rose-like, waxy, sweet, buttery, and phenolic odour (Cui, Zhao, et al., 2014).

2.3 Key physicochemical characters related to soy sauce quality

2.3.1 Reducing sugars

Since metabolic activities of microbial growth during soy sauce production, especially koji mold *Aspergillus* spp., are largely pronounced, free reducing sugar is released into the system. Final product of soy sauce generally contains 2-5% reducing sugar. From the overall reducing sugar, 2.05% of glucose, 0.17% of xylose, 0.08% of arabinose, 0.06% of mannose and galactose, are generally found in representative Japanese soy sauce (Luh, 1995). These sugars are predominantly used in metabolic activity of microbes especially glucose which is their preferential carbon source while the others such as xylose is primarily left in the system due to an inhibition of glucose called catabolite repression (Görke and Stülke, 2008). This pentose also goes through a spontaneous Maillard reaction promoting by acidic and optimally high temperature condition of production process, together with its high reactivity of this reducing sugar toward glucose and arabinose (Lan et al., 2010). The final deep-brown pigment product from Maillard reaction then generates both color and unique aroma in soy sauce.

2.3.2 Free amino acids (FAAs) and peptides

Good quality soy sauce normally contains 1.00-1.65% of total nitrogen (TN) consisting around 45% of simple peptides and 45% for free amino acids (Luh, 1995). Amino nitrogen value generally fluctuated during fermentation process due to microbial activities and Maillard reaction since the latter involving condensation of reducing sugars and aldehyde with free amino groups. Trimethylpyrazine (burnt) and 2,5-dimethyl pyrazine (roasted nuts) are the two desired volatile compounds generate from Maillard reaction during moromi fermentation (Devanthi et al., 2018).

Utilization of available FAAs by autochthonous microbe in soy sauce creates plentiful volatile compounds considered as specific character of this soy-based fermented product. *Saccharomyces cerevisiae* and *Z. rouxii* using Ehrlich pathway to generate higher alcohol from available amino acids. Valine, leucine, and methionine are utilized by these yeast, whose metabolites such as 2-methyl-1-propanol, 3-methyl-1-butanol and methionol giving cheesy and meaty notes to moromi (Harada et al., 2017; Udomsil et al., 2010). Some branched short-chain aldehydes (2-methyl-propanal, 2-methyl-butanal and 3-methyl-butanal), their corresponding alcohols and acids predominantly form from fungal metabolic activities toward branched-chain amino acids. Bitter almond-like of benzaldehyde and floral rose-like odor of benzeneacetaldehyde, which are considered as the potent aromas in Japanese soy sauce, are significant increase during koji fermentation and are generated from phenylalanine (Lee et al., 2006; Yunzi et al., 2013).

Apart from being the source of volatile formation by mean of microbial activity, FAAs, and peptides, themselves also devote for taste characteristic of soy sauce. The unique soy sauce flavor is partly result of different FAA species since they contain different taste characteristic such as umami (L-Glu), sweet and bitter (Pro, Gly, Ala, Val, Leu, Tyr, and Phe) (Luo et al., 2017; Tamura et al., 1990). Bitterness in di- or tripeptides is caused by hydrophobic amino acids locating at any position, while basic amino acid at N-terminus position of larger peptides contribute to its bitter taste (Kim and Li-Chan, 2006). Gutamine biotransformation product of glutamate expresses its umami taste when the concentration is higher than its taste threshold (about 1 mM), and this characteristic is enhanced by presenting of background taste of Amadori products including Fru-Val, Fru-Met and Fru-pGlu. Moreover, many Maillard reaction products (alapyridaine, N-glycosides, pyroglutamyl peptides, and N-acetylglycine) also exhibited their umami taste (Zhao et al., 2016). In

addition, essential amino acid patterns are used as one of the key parameters for soy sauce grading (Luo et al., 2017).

2.3.3 Volatile compounds

Generation of volatile compounds is reported since an initial fermentation period of koji. These compounds include aldehydes, alcohols, ketones, ester, acids, hydrocarbons, volatile phenols and sulphur-containing compounds. Among these, the dominants are 3-methyl-butanal, 1-octen-3-ol, benzeneacetaldehyde, (E)-2-octenal and benzaldehyde (Yunzi et al., 2013). Similar profiles relating to caramel-like, flowery, smoky and malty are also the taste active compound in final product. 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, 3-methyl-1-butanol and 2-methyl-1-butanol which are described as the malty note, are generated from amino acids via Strecker degradation pathway since the initial koji fermentation and stable increase throughout the process (Feng et al., 2017). Lignin pyrolysis of soy sauce substrates provides smoky, spicy and burnt flavor due to the formation of 2-methoxyphenol, 4-ethyl-2-methoxyphenol and 4-vinyl-2-methoxyphenol, which is considered as a distinct soy sauce aroma (Kaneko et al., 2013).

2.4 Color formation in soy sauce fermentation process

Color is one of the major factors for product succession. Many studies show that if the color of food product does not match the consumer expectation, the acceptance of consumer for that product is rare. Recently, the color preference of soy sauce among general consumer in Japan is change from dark brown to lighter one (Miyagi, 2012). This trend is used in one of the product marketing schemes since

it influences consumer's first impression and leading to a buying decision, results in marketing success. Also, this changing of consumer preference on soy sauce color results in the development of new soy sauce production process. Japanese Agricultural Standard (JAS) has been revised the quality labeling standard of soy sauce which mainly demonstrate to a significant of color quality (Table 1) (Luh, 1995). Thus, high quality soy sauce could be physically defined by the vivid, clear, and shiny reddish-brown color (Wang, Zhang, et al., 2018) which is directly related to raw materials, microorganisms and fermentation process.

Table 1 The sensory quality of each soy sauce grading by JAS

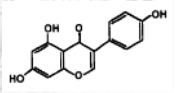
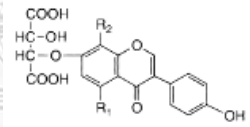
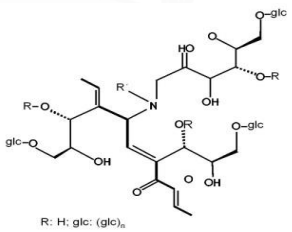
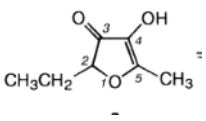
JAS grade	Name	Sensory quality
Special	Koikuchi	Pleasant aroma, complex flavor, deep reddish brown color
Upper	Usukuchi	Milder aroma and color, lighter reddish brown color
Standard	Tamari	Very light aroma (or absent), dark-brown color
Non-grade	Other shoyu	N/A

(modified from Luh, 1995)

Browning in soy sauce is largely produce during two stages of soy sauce production, the moromi and heat treatment of raw soy sauce. This browning is due to 3 main reactions (Table 2). 1) Enzymatic reaction between polyphenol oxidase (PPO) produced from koji mold and phenolic compound from raw material in aerobic condition. 2) Non-enzymatic browning Mailllard reaction between reducing sugar and carbonyl group of amino acid / peptide. 3) Microbial activity in moromi fermentation stage, which autochthonous yeast and LAB can metabolize intermediate and/or

melanoidin from Maillard reaction, result in new browned product. Moreover, various organic acid and volatile compound, which related to the complexity of soy sauce, such as HEMF, are also the result of their metabolic activity. Among these, Maillard seems to be the main reaction contributes to the browning in soy sauce since 50% of browning in soy sauce is stem from this reaction. Its products including melanoidin compound and HEMF's substrate of HMF contribute to not only color but also organoleptic properties of aroma and flavor as well (Abe et al., 1988).

Table 2 Browning reaction in soy sauce

Reaction	Product	Structure	Reference
Enzymatic browning	Genistein		(Fukutake et al., 1996)
	Shoyuflavone B, C		(Kataoka, 2005)
Maillard reaction	Melaniodin		(Wang, 2011 #15
Microbial metabolic activity	HEMF		(Li et al., 1998)

2.4.1 Maillard reaction

Maillard reaction contributed to the organoleptic properties of color, taste and aroma in numerous traditional fermented food products including soy sauce. Mitigation of Maillard is difficult since it is the very complex reaction series consisting of various network reactions, and varies among environmental conditions of food process (Martins et al., 2000). The details of reaction are illustrated in the following section.

2.4.1.1 Reactions

In an early age that non-enzymatic spontaneous chemical Maillard reaction is identified, the first reaction scheme is proposed by Hodge in 1953. This reaction stems by condensation of free amino group (predominantly ∂-amino groups of terminal amino acids or **Є**-amino group of lysine) and reducing sugars, giving the first unstable intermediate of N-substituted glycosilamine, following by formation of more stable Amadori rearrangement products (ARPs). At acidic condition that pH value is below 7 such moromi, furfural or hydroxymethylfurfural (HMF) are generated. These high reactive compounds go through further reaction series including Strecker degradation, where dicarbonyl compounds are condensed with amino acids resulting in aldehydes and ∂-aminoketone formations. In Maillard advance stage, an altimate reactions of cyclisations, dehydrations, retroaldolisations, rearrangements, isomerisations and further condensations take place and final products of brown polymeric and co-polymeric melanoidin compounds are formed (Martins et al., 2000). The draft scheme of Maillard reaction depicts in Figure 1.

Considering color formation by mean of Maillard reaction, the key intermediates include 3-deoxyosuloses and 3,4-dideoxyosulos-3-enes. They can further react with amino acid to produce Strecker aldehyde (RHC=O) and this reaction is pH-dependent (Martins et al., 2000). At higher pH, sugars are mostly in acyclic aldehydic form which easily react with amino acid. Contrasingly, low pH value promotes less reactive protonated amino molecules (Lertsiri et al., 2001).

One of the main physicochemical appearance clearly observed when Maillard reaction take place is browning, which directly involving with consumer acceptance of that product. To assess an extension of Maillard reaction in food products, spectrophotometry at OD_{420} is applied. The high molecular weight, brown polymers generated from final stage of Maillard reaction could contain furan ring, nitrogen, carbonyl, carboxyl, amine, amide, pyrrole, indole, azomethine, ester, anhydride, ether, methyl and even hydroxyl groups (Martins et al., 2000).

Considering soy sauce, Maillard reaction is proposed as a main browning reaction calculating as around 40-50% (Abe et al., 1988). This reaction responses for both color, flavor and aroma characters of final products. In term of color and flavor, both aging and pasteurization affected their dynamic changes, and browning during moromi fermentation is predominantly from nonenzymatic, nonoxidative Maillard reaction (Lertsiri et al., 2001).

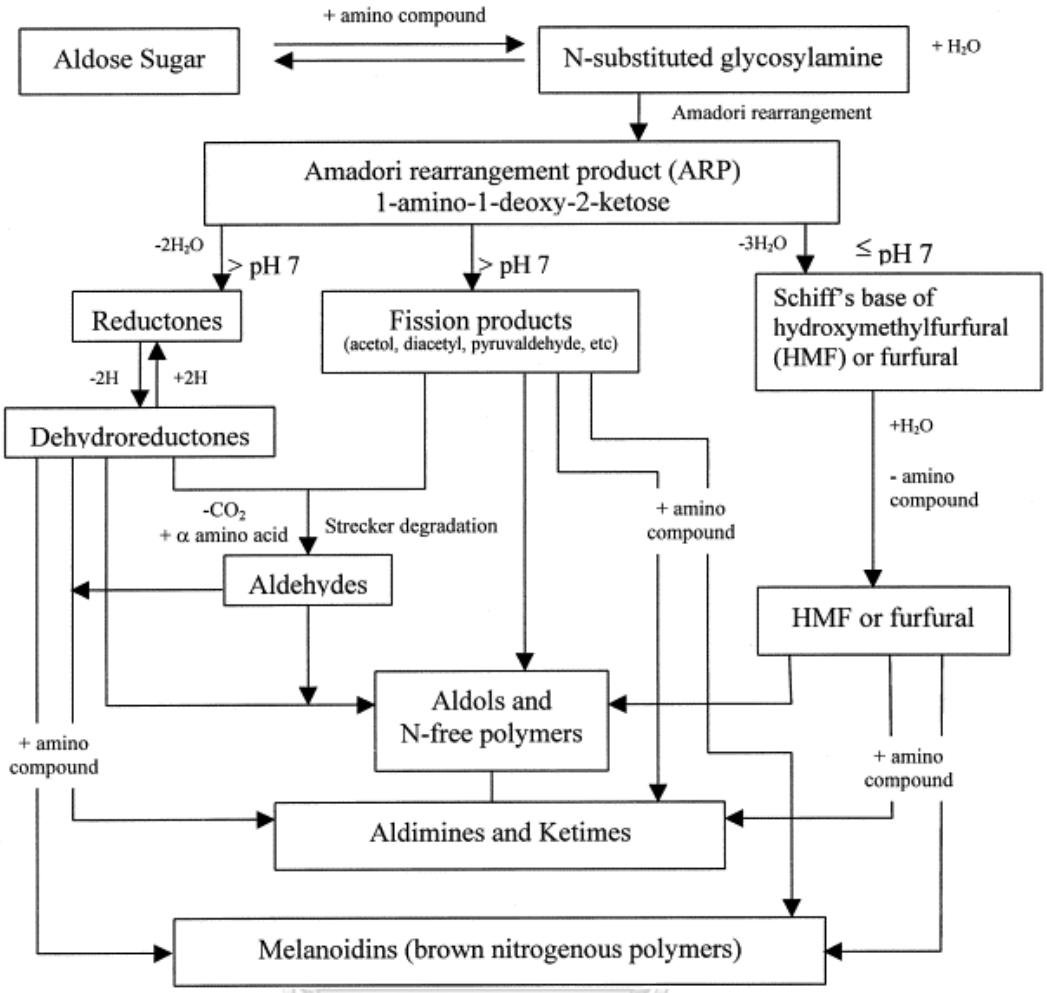


Figure 1 First scheme of Maillard reaction proposed by Hodge (Martins et al., 2000).

2.4.1.2 Factor affecting Maillard reaction

2.4.1.2.1 Temperature

Maillard is a non- enzymatic reaction that can occur spontaneously without any catalyst. Pairing with time, it results in the loss of amino groups in food matrix. For the same substrate pair, loss between high temperature-short time heating is comparable to one with low temperature foe a long period (Rufián-Henares and Pastoriza, 2016). In the same time, it was found that increase of temperature when same amount of starting materials was used resulted in an increment of both high and low molecular product (Benzing-Purdie et al., 1985).

2.4.1.2.2 pH

pH plays an important role in Maillard reaction. The effect of pH is complex as each reaction has its own optimum pH. Example is in Amadori rearrangement, furfural or hydroxymethylfurfural are produced in low pH condition, while reductones and various fission products are formed at pH >7 (Echavarría et al., 2012; Martins et al., 2000). pH also affects the configuration of reducing sugar and reactivity of amino group, results in different type and intensity of reaction (Rufián-Henares and Pastoriza, 2016).

2.4.1.2.3 Water activity

Impacts of water activity (a_w) on Maillard reaction are evidenced. It affects the mobility, dissolution, concentration and chemical reactivity of substrates (Rannou et al., 2016). When aw in the system is lower, reactants are more concentrated and reaction rate increase. If aw keeps lowering to the point that reactants are very concentrated and cannot meet each other easily so the rate decrease. Water also has a dual role in some reaction in Maillard. The glycosylamine rearrangement in Amadori product needs water to be a solvent as well as reactant (van Boekel, 2006). Optimal aw that maximized Maillard reaction is in the range of 0.5 and 0.8, depending on the system (Nursten, 2005).

2.4.1.2.4 Substrates

As Maillard starts from condensation reaction of reducing sugar with free amino groups, different reactant type is then become another influence of this complex network. **Є**- amino group of lysine shows the highest reactivity while some group such as imidazole in histidine, indole in tryptophan, and guanidil in arginine residues can react in a lower rate (de Oliveira et al., 2016). For sugar, reaction rate relies on anomerization of each sugar. Sugar with lower carbon atom and larger percent of open-chain form gives a high browning capacity via Maillard. By this, pentose is more reactive than hexose, and phosphorylated sugars react faster than non-phosphorelated one (Kwak and Lim, 2004).

2.4.1.2.5 Microbial activity

In fermented food, while physical and chemical factors directly influence the rate of Maillard reaction, existing microbial found in the process are reported as an indirect factor of this browning system. Lactic acid bacterium growth in latter stage of soy sauce fermentation produce organic acid that lowering pH of the matrix. The acidity supports the formation of furfural and furfuryl alcohol, two caramel-like brown substance produced via Maillard reaction (Harada et al., 2017). Moreover, some microbes utilize an intermediate of Maillard into other compound. This is the case of soy sauce autochthonous such as *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* (Uehara et al., 2017).

2.5 Mitigation of Maillard reaction

Browning product from Maillard reaction is undesired characteristic in wastewater treatment, and also some food products, numerous approaches have been proposed to improve this quality attribute. Apart from the mitigation strategies based on minimizing reaction rate, another interest focuses on removal of existing melanoidin from food products. Various feasible decolorization techniques were based on physical, chemical and microbiological approach. Some of these techniques were implemented in semi-pilot or pilot-scale, but mostly were limited in laboratory experiment scale. Each of novel method was reviewed as following.

2.5.1 Physical and chemical methods

2.5.1.1 Filtration

In 1973, Hashiba has been report the use of ultrafiltration (UF) technique to eliminate the browning of soy sauce such as ferric ion (Fe3+), deoxyglucosone, hydroxyfurfural, reductone, carbonyl compounds and ferricyanide-reducing substance. This decolorization technique focuses on the elimination of catalyst, intermediate compound, and browning products of Maillard reaction. The treated soy sauce shown lighter brown color even after pasteurization when compare with the non-treated one. Also, all of the browning compound and metal ion (except Cu2+) were significantly decrease after treat with UF membrane (Amicon 20E) (Hashiba, 1973).

The further study using membrane technology to decolorize soy sauce is proposed by Miyagi et al. in year 2013. Various type of nanofiltration (NF) membrane and one type of UF membrane are chosen. The difference in molecular weight cut-off partly results in difference decolorization ability. Moreover, the electric interaction between melaniodin, the main color compound in soy sauce, and membrane also involved in decolorization ability. This can be explained by the following reasons. Firstly, the isoelectric point of melanoidin is in pH range of 2.5 to 3.5, and pH of soy sauce is normally around 4.6. Thus, melanoidin shows a negative charge in this environment. Secondly, different membrane material shows the differ in the zeta potential value. NTR-74 series membrane is made from SPS, which it's zeta potential is higher than PVA/PA of NTR-72 series (-80 to -90, and -5 to -10 mV, respectively) .

In term of food product, Serpen and co-workers used a styrene-divinylbenzene copolymer-based resin to adsorb brown pigment from an apple juice (Gökmen and Serpen, 2002). Same rasin was used to treat sugar syrup (Serpen et al., 2007). Other materials used for this purpose is activated carbon, which was applied in peach juice and peach pulp (Arslanoğlu et al., 2005; Carabasa et al., 1998).

The main drawback of filtration technique is the depletion of remarkable substance contributed to the product quality. UF and NF membrane reject macromolecule, such as protein, and polyvalent ion sugar. Protein is one of the main nutritional components used in soy sauce grading. In general, good quality soy sauce contains 1.0-1.65% total nitrogen (w/v) and about 45% of the total nitrogen is found in simple peptides, and 45% in amino acids (Luh, 1995). Moreover, the 500 Da fractions free amino acids and short peptides can be isolated from soy sauce. These fractions are report as majority of the taste compounds which are the key contributors of the high intensity of umami taste in soy sauce (Lioe et al., 2010). By this, in order to achieve the light brown color with pleasant aroma and complex flavor product, further additional process has to be done.

2.5.1.2 Adsorption

For the adsorption of melanoidin from soy sauce, many adsorbents are used, including activated charcoal (AC), activated clay (ACL), diatomaceous earth (DE), magnesium oxide (MGO) and silica gel (SG) (Miyagi, Nabetani, et al., 2013). The results from the color estimation by CIELAB color space show the linearity relation with untreated soy sauce, ACL-, DE- MGO- and SG-treated soy sauce, while AC-treated reveal the deviated significantly for others. AC exhibited stronger decolorization than other adsorbents, giving the paler soy sauce than untreated one. ACL and SG processes exhibited some potential for decolorization,

but the DE process and especially the MGO process resulted in deeper brown soy sauce. The most excellent decolorization absorbent is AC, as even lower proportion of AC use, lighter soy sauce in compare with other absorbent is still observed. The adsorption mechanism for melanoidin removal can be characterized as a favorable, spontaneous and endothermic process. The reaction between adsorbed molecule and adsorbent surface involve hydrogen bond interaction and others (Nunes et al., 2015). Further study on impact adsorption on quality index of soy sauce reveal that total nitrogen (TN), unsalted soluble solid content (USSC) and reducing sugar (RS) are also absorbed by these absorbents. TN, followed by RS are known as the two highly significant substances contribute to sensory quality. Thereby, in order to maintain the essential quality of last product, further process must be implemented after adsorption-based decolorized process.

2.5.1.3 UV irradiation

UV irradiation technique is used to eliminate Maillard intermediate of HMF. Mercury lamp release wavelength between 250 and 740 nm in order to destroy HMF by photo degradation process. By this, it can reduce as high as 60% of model HMF solution (Aguilar et al., 2015). Maillard reaction products with low molecular weight such as acrylamide, furfural and HMF are successfully removed by vacuum treatment. This method was tested in hydrated biscuit and potato crisp (Anese et al., 2014), and roasted coffee (Anese et al., 2011). However, an attempt to remove furan and HMF from meat sauce and biscuit by vacuum technique was not succeeded since none of HMF was eliminated from samples (Lund and Ray, 2017).

2.5.2 Biotechnological methods

Biological approach of metabolically-active viable microorganisms provides new feasible way of mitigation of Maillard reaction. While physical and chemical techniques as reviewed in above section focus on the elimination of Maillard browned products. These are corrective action, which the resolution will take place only when the problem is already happened. However, in order to resolve some problem, the preventive action concept sounds to be the better resolution, as this concept fixes the problem at the root-cause. In this case, the preventive action is to remove the substrate of Maillard reaction, such as reducing sugar, by microbial metabolic activity. Studies of browning in soy sauce indicate that more than 50% stem from Maillard reaction (Abe et al., 1988; Lertsiri et al., 2001). The ratio of ribose and hexose in soy sauce is 1 to 10. However, these ribose, mainly xylose and arabinose, show the extremely high browning reactivity, contribute to about 40 to 50% of total Maillard reaction (Abe et al., 1988; Takeda et al., 1998). The scheme of this decolorization technique is, the microbial in moromi use hexose to produce the significant compound such as HMF and HEMF. In the same time, co-inoculated microbial culture with decolorization ability used ribose and produce organic acid and/or volatile compound without affect the organoleptic properties of soy sauce (Abe et al., 1988; Abe and Higuchi, 1998).

2.5.2.1 Preventive approach

The metabolic activity concept has been intensively developed and the research series have been proposed. In 1984, the study of decolorization ability in soy isolated microbial was done by Kanbe and Ushida. 10 soy-isolated *Pediocooci* were screened for the fermentation ability and each strain show the different fermentation pattern. The next experiment was the screening for

decolorizing ability. In the focusing of arabinose-fermentable strains, some of them were also show the color lightening activity as show in Figure 7. However, none of the *Pediococci* strains could ferment xylose (Kanbe and Uchida, 1982).

In an attempt to establish the *Pediococci* mutants that can use pentose in the high-glucose system, understanding of the carbon catabolite control is necessary. Further work was conducted by Abe and Ushida in 1998. They investigated the mechanism in sugar utilization of *Pediococcus halophilus*, and found that in presenting of glucose, the xylose fermatation ability was repressed. This corresponded to the study of xyl operon in *Tetragenococcus halophila* (former name as *P. halophilus*), which reveals the catabolite repression of glucose over xylose (Takeda et al., 1998). From these data, the mutation of *T.halophila* is established (Abe and Higuchi, 1998).

Tetragenococcus halophila mutant strain, a xylose fermentable, even in the presence of glucose, and its usage in soy sauce industrial is patented by Abe and Ushida with the assignee of Kikkoman corporation in 1988 (Abe et al., 1988). From the patent report, the remaining xylose and arabinose are remarkable low comparing with the control. The tincture test reveales the lighter color soy sauce both in raw, heat-treated, and heat-treated with 7 days storage soy sauce and of the significantly difference in organoleptic test of the *T. halophilus* - treated soy sauce, which conducts by a 28-member panels according to the triangular system.

In addition to the work of Kanbe and Ushida in 1984. All of the color-lightening strains tested in this work also show the reduction of redox potential which was further investigates by the same coworker in 1987. The purified enzymes from cell-free medium culture of *Tetragenococcus halophila* revealed NADH dehydrogenase activity with various electron acceptor such as quinones. When cell-

free extract of the color-lightening strains were compared for NADH dehydrogenase activity over the numbers of electron acceptor including quinones, the different in specificity from each *T. halophila* could be observed. The effective reduction of quinones by NADH dehydrogenase, suggest that the enzyme plays a substantial role in the depression of browning (Kanbe and Uchida, 1987) .

The decolorization by metabolic activity shows the feasible alternative as it could control the browning of soy sauce at the root of Maillard reaction. In the optimized process, yeast metabolic activity and Maillard reaction use hexose such as glucose and generate some significant compounds essential for good characteristic in soy sauce. During these, co-inoculated *Tetragenococcus halophila* consumes xylose and arabinose, the ribose presenting in the fermentation system, and produce desired volatile compound and organic acid. The output of this novel process is light-colored soy sauce with satisfied organoleptic properties.

Many studies indicate HMF removal ability in lactic acid bacteria. *Lactobacillus plantarum* reduced abundant HMF in malt from 38.3% to 27.5% (Salmeron et al., 2009). Other strains such as *L. lactis, L. cremoris* and *L. bulgaricus* can reduced HMF in both media and reconstituted milk effectively (Oral et al., 2015). Yeast also show HMF utilization ability. Taherzadeh et al in year 2000 examined an uptake rate of HMF in both aerobic and anaerobic conditions and found the main conversion product of HMF alcohol (Taherzadeh et al., 2000). *Saccharomyces cerevisiae* reveals a primary activity of HMF utilization in roasted malt, and presenting of glucose and fructose increased this metabolic activity (Akıllıoglu et al., 2011).

More attempts to minimize Maillard reaction via controlling its intermediate substances have been proposed. Amadoriase, Fructosamine-3-kinase (FN3K), Fructosamine-6-kinase coupling with fructoselysine-6-P deglycase system, and

FN3K-related protein were used as Maillard's product controlling tools(Capuano and Fogliano, 2011). Amadoriase was used to reduce the protein-bound HMF in a commercial low-lactose UHT milk (Troise and Fogliano, 2013). Applications of thermostable amadoriase for controlling Maillard reaction on single amino acid were reported in fried potatoes, baking goods and coffee (Rigoldi et al., 2018).

Another concept of using enzymatical approach to deal with Maillard reaction focus on its substrate of reducing sugars. Fructosamine oxidase (Faox) is an enzyme catalyzing the oxidation of reducing sugar and oxidative deglycation of Amadori products, thus it has been proposed as a potent enzymatic tool for browning limitation in some food (Lund and Ray, 2017). Genetic engineering was applied to produce Faox I and Faox II recombinant enzymes. The verification of enzyme activities in commercial low lactose milk was done with successive results (Troise et al., 2014). Glucose oxidase in coupling with catalase was used to remove glucose from dried egg powder, hexose oxidase for browning control in potato and mozzarella cheese (Sankaran et al., 1989; Sisak et al., 2006; Søe and Petersen, 2005).

Nevertheless, main problem that has to be solved before application of genetic engineering microorganism in food product, apart from severe restriction or ban in many countries, is public acceptance (Gartland and Gartland, 2018). To overcome this uncertainty, soy sauce decolorization by use of efficient autochthonous isolates through their natural metabolic reactions during fermentation process reveals its potential without restriction or consumer constrains.

2.5.2.2 Corrective approach

2.5.2.2.1 Adsorption of melanoidin

The mycelia of 2 white fungi, *Coriolus versicolor* IFO30340 and *Paecilomyces canadensis* NC-1, can decolorize the mixture of both model browned pigment and browned foods (Terasawa et al., 2000). In soy sauce / miso mixture, the co-inoculation of these 2 fungi results in decolorized product with the highest decolorization rate (about 40%). Investigation of the decolorization mechanism(s) indicates the reduction of adsorption rate of the latter batch which can be explained by limitation of mycelium's adsorption activity. This activity is due to the conjugation between melanoidin and mycelial surface structure (Ohmomo et al., 1988). As the adsorption begins, the conjugation between melanoidin and mycelial surface structure takes place. In the next repeat-culture experiment, the adsorption sites were almost filled with browned pigment, thus the adsorption yield in sequential replacement was decrease (Terasawa et al., 2000).

In contrast, the decolorization of *C. versicolor* IFO 30340 in the is different since only 10 % of decolorization by *C. versicolor* mycelium is bade on adsorption, while the rest is due to its enzymatic activity (Benito et al., 1997). The slow decrease in decolorization rate also can be explained as, the mycelium is protected by immobilized material, and even adsorption site is filled with melanoidin, the decolorized ability is further remained by enzymatic activity.

Apart from the adsorption ability, numbers of the studies revealed that the type of browned pigment in model foods are highly related with decolorization abilities of these fungi (Tamaki et al., 2007; Terasawa et al., 2000), and the decolorization mechanisms are different between fungi (Benito et al., 1997; Rioja

et al., 2008; Terasawa et al., 2000). In browned food which dominant by Maillard-type pigment, such as soy sauce and miso, higher ability can be observed in *C. versicolor* IFO30340 over *P. canadensis* NT-1. The same trend was also found in caramel-type pigment such as caramel. In contrast, the phenol-type browned pigment was decolorized more by *P. canadensis* NT-1. From these data, the coworker suggests the feasible method to screening type of browned pigment in food based on browning reaction.

2.5.2.2.2 Degradation of melanoidin

The second biological approach is enzymatic activity. As stated in title 2.1, the decolorization ability of *C. versicolor* IFO 30340 is mainly by this activity. The first study proposed by Watanabe et al, 1982. They screened 22 Basidiomyces for decolorization ability and found 2 *Coriolus* strains showing the highest decolorize rate (Watanabe et al., 1982). They report the 200,000kDa of purified protein with melanoidin-decolorizing activities derived from crude cell in the presence of sugars and conclude that the purified proteins were sugar oxidase, which glucose and sorbose oxidase seemed to be the two main enzymes. The decolorizing mechanism of sugar oxidase with melanoidins is as follow. The enzymes activate the oxidation of sugar, such as glucose into glucoronic acid. The reaction also produces active oxygen and peroxide species as by-products. These molecules then decolorized the melanoidin presenting in the system, resulted in lighter-colored products (Chandra et al., 2018). Apart from the melanoidin-decolorizing ability by sugar oxidase, other melanoidins decolourizing enzymes (MDEs) were isolated (Ohmomo et al., 1988). At least 5 MDEs were isolated from *C. versicolor* strain. Some of them are sugar-dependent and attack the melanoidin directly. Moreover, this coworker also reported the products from melanoidin decolorization. Four to six

percentage of nitrogen and 10.5% of carbon from melanoidin were recovered in amino acid and lactic acid, respectively.

Recently, bacterial consortium of *Klebsiella pneumoniae, Salmonella enterica, Enterobacter aerogenes* and *E. cloaceae* reveals their co-production of oxidoreductase enzymes of manganese peroxidase (MnP) in an initial bacterial growth phase and laccase in the latter phase, and could degrade up to 70% of Maillard reaction products (MRPs) after 8 days of incubation. The results also indicate incapability of these bacteria to utilize MRPS as carbon source (Kumar and Chandra, 2018).

Biodegradation of melanoidin is also reported in filamentus fungi including *Geotrichum candidum. Flavadon flavus, Trametes sp., Coriolus spp., Aspergillus flavus, Fusarium verticillioides,* and yeast *Candida tropicalis* and *C. glabrate* with high reactivity since lignolytic enzymes are generally reported as a key enzyme dealing with melanoidin degradation. These fungi could then use degrade melanoidin as carbon and nitrogen sources for their metabolic activities (Kumar and Chandra, 2018).

From reported stated in 2.5.2.2.1 and this section, the drawback of techniques is the depletion of flavors and aromas. The enzymatic side-reactions oxidize all melanoidins, including HMF and HEMF, the significant compounds in good quality soy sauce. Unbalance of amino and organic acids derived from the decolorization might also affect the organoleptic properties and complexity of soy sauce. In addition, microbial application in food products is limited by food safety regulation via GRAS designation. Since these microbes are not obtained from edible sources and/or food origin. Thus, they are not classified as autochthonous

strains, which is one of main criteria for being “Generally Recognized as Safe” (GRAS, USFDA) or “Qualified Presumption of Safety” (QPS, EFSA).

2.6 Feasible microbiological determination methods in fermented food

During autochthonous fermentation (a natural fermentation), distinctive flavor and aroma in soy sauce determining its organoleptic quality are the sequence of complex microbial profiles series. Despite the fact that main ingredients and process used in soy sauce production share common characteristics, a distinctive quality in final product from different regions can be observed (Kim et al., 2010). Therefore, better understanding of microbial community and their profiles during fermentation process is an effective quality development scheme along with isolation of isolation of autochthonous strains possibly having both de-colorizing and aromatic enhancing abilities.

2.6.1 DNA-based methods

Many studied in microbial diversity in natural ecosystems were based on conventional plating and comparing to DNA-based methods. Plate count technique shown its limitation due to specific cultivation condition needed in some microbe, and undetectable of VBNC cell by cultural plating. In harsh environmental as found in soy sauce, some microbes entered Viable But Not Culturable (VBNC) state, where they still have metabolic activity but lack ability to form colonies (Papadimitriou et al., 2016). DNA-based technique overcame these problems as all target DNA presenting in the system are detected by mean of specific primers used. Coupling with denaturing gradient gel electrophoresis (DGGE) as PCR-DGGE which is only

effective gel-based method wildly used to discriminate DNA complex in natural ecosystems. The DNA profiles obtained could be used as microbial community mapping to understand their roles and/or impacts during process of fermentation.

However, through DNA-based method including high-throughput sequencing Miseq, metagenomic or pyrosequencing (Tang et al., 2017; Wu et al., 2018), false-positive results can be caused due to detection of dead cells (Sunyer-Figueres et al., 2018).

2.6.2 RNA-based methods

Recently, there are many reports proposing the cDNA-PCR-DGGE based protocol to detect viable microbes in fermentation ecosystems (Benítez-Cabello et al., 2016; Cardinali et al., 2018; Garofalo et al., 2017) . The cDNA determination was also reported as a higher sensitive method relative to DNA-based. Since RNA directly links to gene expression, it could also demonstrated the possible activity of each microbe detected (Chahorm and Prakitchaiwattana, 2018). Accordingly, RNA-based coupling with nested PCR-DGGE could be as a potential gene expression map when performed a long with determination of dynamic changes of key compounds, it could help to better understand the possible microbial metabolic roles during fermentation process. The information obtained may help to easier selection of potential target microbe for further development as starter having specific function for food production. For example, autochthonous strains having properties in reduction compounds and/or reactions involved in browning during soy sauce fermentation.

CHAPTER 3

MICROBIAL ECOLOGY OF SOY SAUCE AND THEIR DYNAMIC CHANGE IN RELATION WITH PHYSICOCHEMICAL PROPERTIES

3.1 Introduction

Microbial community of soy sauce consists of 3 microbes namely mold, bacteria and yeast, which diversities keep change as a function of time. These microorganisms are symbiotic growth and have the metabolic and ecological interactions with each other. Metabolic activities of each microbe change their substrates into another nutrient sources suitable for other species thus dominant microorganisms are then shift by these existence substrates along fermentation process. This dynamic change affects physiochemical parameters including sensory attributes of aroma and color as microbial activities deliver variant metabolite compounds given complexity characteristic in final product (Wei et al., 2013). In term of browning, metabolic activity itself influence main browning reaction called Maillard such as bioconversion of hydroxymethylfurfural (HMF), a key intermediate in Maillard reaction, into 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) (Harada et al., 2017; Uehara et al., 2017) which both were reported as key volatile in soy sauce. Additionally, several works were report a significant of Maillard reaction in relevant to sensory attributes and its intermediates of Amadori products were both the metabolites of volatile components, and became umami taste themselves (Feng et al., 2015; Kaneko et al., 2013; Katayama et al., 2017).

By this importance of microbial quality toward physicochemical change during soy sauce fermentation, this study aimed to address these relations. Firstly, microbiological profiles of moromi from various fermentation stages of industrial and traditional process were determined based on conventional plating and RNA-based RevT-nested PCR DGGE following by identification by mean of 16s rRNA sequencing. Results from two analysis were compared and feasible determination method(s) for true microbial community playing role in fermentation was proposed.

The second experiment which was done simultaneously with the microbial analysis was elucidation of physicochemical properties including pH, sodium chloride concentration, total nitrogen (TN), total reducing sugar (RS) and their individual species, total free amino acid (FAA) and their species, and volatile profiles. Dynamic changes of physicochemical were examined concomitant with microbiological aspect, and their relations were observed to address the possible microbial diversity mapping for desired sensorial aspects including brown color.

3.2 Materials and Methods

3.2.1 Soy sauce collection

Moromi using whole soybean, wheat and brine as raw materials with a fermentation age of 2, 4 and 6 months called as initial (I), middle (M), and final (F), respectively were collected from various regions in Thailand and including both industrial and traditional fermentation process. For industrial process, 2 samples of SP1 and SP2 derived from different condition and the temperature of latter batch exceeded an optimal leval during the first 2 weeks of the process. Traditional samples from central east, central and north regions were coded as CC, NP and PL, respectively, The last one of PL was mainly for fermented soybean mash (Tao-jeaw) thus all soybean meal was separated at first 7 weeks and liquid part was then left for further fermentation resulted in secondary product of soy sauce.

Moromi in each fermentation stage was mixed by air blow (industrial process) or by manual stirring using sterile stirrer until it was homogeneous before approximately five hundred grams portion was collected in sterilized, tightly sealed plastic bag stored at room temperature away from sunlight before being transferred to laboratory. All samples were analyzed within 24 hours after collecting time.

3.2.2 Microbial population determination

3.2.2.1 Conventional technique

Ten grams of homogeneously mash samples were blended with 90 ml. of 3% sterile normal saline (Jung et al., 2016), was shook firmly for 50 times through 30 cm arc and let stand for 5 minutes before this derived 10^{-1} dilution was serially diluted to an appropriate level (Manual, 2001). Three successive dilution was selected and 1 ml of each was inoculated onto selective media of De Man, Rogosa and Sharpe (MRS) agar (Sigma) for lactic acid bacteria (Baisier and Labuza) and others bacteria while another 1 ml diluted sample was placed onto acidified Potato Dextrose Agar (PDA, Sigma) for fungal analysis.

Inoculated MRS plates were incubated at 30°C for 3 days while same temperature was applied for PDA with longer incubation time of 5 days following standard protocol of US FDA-BAM. After incubation time was finished, plates with bacterial growth in range of 25-250 colonies and fungal in range of 10-150 colonies were selected for counting. Representative colonies were selected based on Harrison's disc method (Harrigan, 1998) and were streaked onto the same growth media for 2 cycles in order to verify their purities. In addition, only bacterial sample that nutrient agar (NA) plates was used concomitantly with MRS to provide an optional growth for bacteria that might belong to other groups than LAB. All media used throughout this study was supplemented with 3% NaCl.

All pure representative isolates were collected in 2 forms, the first of agar slant by stabbing method using as working cultures, and the second of 20% glycerol stock freezing at -20°C as mother stock cultures.

3.2.2.2 RevT-nested PCR DGGE

One gram of same homogenous moromi samples used in 3.2.2.1 was mixed with 10 ml. 0.5 M iced-cool phosphate buffer pH 7.2 (Singracha et al., 2017) and centrifuged at 10,000 xg, 4°C for 10 minutes. Cell pellet was collected and washed with phosphate buffer (pH 7.2) again to eliminate any impurities affecting RNA extraction and PCR efficiencies, and promptly subjected to RNA extraction. Total RNA was extracted from moromi sample using GF-1 Total RNA Extraction Kit containing DNase (Vivantis, Malaysia), The extracted RNA was immediately used as template for cDNA synthesis by Viva 2-steps RT-PCR Kit (Vivantis, Malaysia) according to the manufacturer's instruction. All procedures were done on ice in order to minimize RNA loss as possible. Derived cDNA was directly stored at -20°C until use.

After synthesis, cDNA was amplified using 2 step nested PCR technique with universal bacterial primers specific at 16s rRNA region. The first bacterial primer pair was 27F (5'-AGT TTA GTC CTG GCT CAG-3') and 1492R (5'-GGC TAC CTT GTT ACG ACT T-3') (Kim et al., 2010) following with second pair of 357F (5′-CCT ACG GGA GGC AGC AG-3′) and 517R (5′-ATT ACC GCG GCT GCT GG-3′) with a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') attached at 5' end of 357F were used.

PCR condition for both first and nested one consisted of: denaturation at 94°C for 2 mins; annealing at 55°C for 30 sec; and extension at 72°C for 30 sec in an initial cycle. After that, denaturation time was 30 sec and amplification was done for 35 cycles. The last extension was prolonged for 10

minutes. PCR product with an expected size of 1.4 kb from the first round was verified by 1% agarose gel with standard gel electrophoresis technique before subjected to the nested round. Final PCR product (150 base pairs) was also examined for its quality using same electrophoresis technique as well.

For fungal determination, first universal primer pair namely NL1 (3'-GCA TAT CAA TAA GCG GAG GAA AAG-5') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Marshall et al., 2003), and the second for nested reaction targeting 26S rRNA gene of NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) with a GC clamp (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA-3') tagged at 5' end of NL1 were applied (Tanaka et al., 2012). PCR products of approximately 600 (1st round PCR) and 250 base pairs (nested PCR) were verified using gel electrophoresis method as stated above.

All nested PCR products were determined for DNA mapping by DGGE method. The gel was casted in condition of 10% acrylamide gel with 37.4:1 of acylamide and bis-acrylamide (Vivantis) using gradient in range of 20-40% denaturants (formamide and urea) before GC-clamped PCR products were directly applied onto gel in a running buffer (1% TAE) containing 40 mM Tris-acetate, 2 mM $Na_2EDTA\text{-}H_2O$, pH 8.5. For the DGGE condition, DCode™ Universal Mutation Detection System for DGGE (Biorad) was operated at 60°C for 4 hours at constant electricity of 120 V. After electrophoresis, acrylamide gel was stained in 1% TE buffer pH 8 containing ethidium bromide and photographed under UV translumination. DNA bands detected under UV light were precisely excised from gel using steriled razor blade and soaked into RNase-free sterile water (Vivantis) for 18 hours at 4°C to liberate DNA fragments from acrylamide gel. The eluted DNA was re-amplified by either 357F-517R bacterial or NL1-LS2 fungal primer pair, checking PCR quality by agarose gel electrophoresis and

purification using GF-1 PCR Clean-up Kit (Vivantis) before the verified one was sent to further identified by Sanger sequencing analysis (BIOBASIC, Canada).

3.2.3 Microbial identification

DNA extraction of representative isolates were done by freeze-thaw method based on procedure proposed by Silva et al (2012) with some slightly modification (Silva et al., 2012). One loop-full of pure colony of each representative derived from 3.2.2.1 were picked up by sterile loop and mixed with 500 µl RNase-free water and freezed at -20°C for at least 24 hours before incubated in boiling water for 5 minutes. After heat treatment, the mixture was vigorously homogenized by vortex for 10 seconds then immediately put on ice for 15 minutes. Cell residue was then separated from supernatant by mean of centrifugation (12,000 rpm) at 4°C for 10 minutes, supernatant was collected. The 16S rRNA V3 target region of bacterial isolates were amplified using 357F (5′-CCT ACG GGA GGC AGC AG-3′; *Escherichia coli* positions 341 to 357) and 517R (5′-ATT ACC GCG GCT GCT GG-3′; *E. coli* positions 517 to 534) primers. Conserved region 26SrRNA of fungal was also amplified using specific primer pair of NL (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′; *S. cerevisiae* positions 40 to 63) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′; *S. cerevisiae* positions 266–285) (Tanaka et al., 2012). Amplified DNA fragment was verified for their quality using agarose gel electrophoresis before sent to sequencing (BIOBASIC, Canada).

3.2.4 Physicochemical determination

For elucidation of physicochemical properties, moromi sample in each fermentation period was collect simultaneously with 3.2.2.1 and centrifuge (8,000 rpm) at 4°C for 20 minutes in order to separated liquid part from soymeal. Supernatant was then stored in zip-lock bag and frozen at -20°C until used.

3.2.4.1 Physical parameters

a) pH was determined using pH meter (Mettler Toledo). The sodium chloride concentration was determined using modified method of Zhang and Xia (2008). Twenty grams moromi samples were mixed with 4 mL 0.288 M potassium hexacyanoferrate and 4 mL. 1.196 M zinc acetate. The mixture was diluted to final volume of 250 ml. and filtered through No.1 Whatman. Three milliliters of filtrate was pipette into the mixture of 4 mL nitric acid (1:4), 5 ml gelatin colloid (5 g/ L) and 5 mL silver nitrate (0.5 %) and mixed thoroughly. The mixture was heated at 60° C for 10 minutes. Two hundred microliters of heated mixture was transferred into 96-wells plate and measured their turbidity using spectrophotometer at 385 nm.

b) Browning indexes including total browning and HMF were determined by 2 different methods as for the total and crude-free supernatant of each homogeneous moromi was directly spectrophotometry examined for their absorbance at 420 nm (Lertsiri et al., 2001). HMF browning index was determined by adding 9 ml. of absolute ethanol into 1 ml. of supernatant, vortexed and left at room temperature for 1 hour then all precipitate was removed by mean of centrifugation and clear liquid part was subjected to spectrophotometer at same wavelength as total browning (modified from (Kim, 2008 #112).

3.2.4.2 Chemical parameters

a) Total nitrogen (TN) in moromi, was determined by standard Kjeldahl method.

b) Reducing sugar (RS) was quantitatively analyzed by Nelson-Somogyi method (Nelson, 1944). Some specific reducing sugar species including glucose, arabinose and xylose was examined by HPLC equipped with Rezex RHM-monosaccharide H+ column (Phenomenex, USA) following the protocol of Chanprasartsuk, Prakitchaiwattana and Sanguandeekul (2013) with modification. Glucose standard was prepared in the working concentration of 0, 25, 50, 75, 100, 150 and 200 mg/ml. while arabinose and xylose were 0, 1, 3 and 5 mg/ml (Appendix A). Prior to HPLC analysis, solid material in sample was removed from supernatant by mean of centrifugation (12,000 rpm) at 4°C for 10 minutes along with filtration through 0.45 µM PE syringe filter.

c) Amino acid profile was analyzed by chromatography technique using Zorbax Eclipse-AAA columns and the Agilent 1100 HPLC coupled with G1315A Diode Array Detector (DAD). The derivatizing reagents were o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids. Sample were determined following a manufacturer's instruction (Agilent Technologies, USA) using methanol, acetonitrile and water in proportion of 45:45:10 as mobile phase.

d) For volatile compound, 3 grams of moromi were transferred into 20 ml. headspace vial and tightly closed by HDSP magnetic cap. Sample was then heated at 40 °C for 10 minutes before performing headspace solid-phase

microextraction (HS-SPME). The fiber (50/30μm DVB/CAR/PDMS, SUPELCO, PA) was inserted to absorb all volatile substances. The extraction time was 30 minutes, and went through desorption process using high temperature of 250 ℃ for 5 min. Gas chromatography–Mass spectrometry (GC-MS, Agilent 7890A GC-7000 Mass Triple Quad) equipped with a capillary column (DB-WAX, 60 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA) with a quadrupole mass detector, and split mode with a ratio of 5:1 were used. GC-MS conditions were as following, constant helium flow rate of 0.8 ml./minute; GC oven temperature profile series of 32 ℃ for 10 min, 40 ℃ at 3 ℃/minute and hold for 15 minutes, 160 ℃ at 3 ℃/minute, then 230 ℃ at 4 ℃/minute and hold for 5 minutes; electron ionization mode of MS with the ion source temperature of 230 ℃, and ionization energy of 70 eV. The scan mode was choosing with its range of 25 to 400 m/z. The Agilent Mass Hunter Qualitative Analysis B.04.00 software was used for data analysis. Qualification of volatile compounds was done by comparing mass spectra with NIST mass spectral libraries (National Institute of Standards, 2011 version), while peak area was calculated for qualification in term of relative abundant.

3.2.5 Statistical analysis

One-way ANOVA of univariate statistical analysis was carried out by the Duncan's test to compare mean values between treatments using IBM SPSS 22 statistics package with 95% confidence interval.

Principle component analysis (PCA) along with hierarchical cluster analysis (HCA) were performed on the quantity of amino acid and volatile substances to examine the relationship among samples from different production sites using

covariance XLSTAT™ version 2018 (Addinsoft, New York, NY) for PCA and cloud-based application Multi-Experiment Viewer (MeV Version 4.9.0) for HCA. Observations/variables were chosen as data format and PCA type of covariance matrix and Spearman's rank correlation coefficient were used in volatile and amino acid interpretation, respectively.

3.3 Results and discussions

Bacterial and yeast profile of soy sauce moromi fermented by both industrial and traditional process were accessed by conventional plating and cultural-dependent, RNA-based RevT-nested PCR-DGGE. In the same time, dynamic changes of chemical properties involving color and aroma quality such as reducing sugars, FAA and volatile compounds along with other physicochemical factors that might influence such properties during fermentation process were also evaluated. Determination was performed on samples from various sites of Thailand including of traditional produced in Central East (CC) and North part (Pariza et al.), and traditional and industrial in Central (SP1, SP2 and NP).

3.3.1 Dynamic changes of microbial communities and chemical properties during soy sauce fermentation

3.3.1.1 Industrial process

From SP1 sample collected from industrial plant during fermentation process, the bacterial population level at the Initial fermentation stage was about 6 log CFU/g. By the middle and final stages its population density had decreased to 4 to 3log CFU/g, respectively. The cultural plating also revealed *Staphylococcus* spp. and *Bacillus* spp. as the main species (Table 3, SP1I to SP1F). Only *Staphylococcus* spp. was predominantly observed (over 45% of total isolates) by this method throughout the fermentations, while *Bacillus* spp. reduced in the middle (33%) and was not prevalent in final fermentation stage. The other types of

isolates including *Bifidobacterium* sp. in SP1I, *Lactobacillus* sp. in SP1M in which also found in SP1F in addition with *Streptomyces* sp. and *Vergibacillus* sp. This observation demonstrated that the bacterial community would be more diverse when *Bacillus* population levels fell. Rev-T-PCR amplification methods supported the existence of this isolate throughout the fermentation, and thus its biphasic population levels being high at the start and late fermentation times (Table 3 and Figure 2A). The detection through RNA determination could demonstrate the role of these two species in each stage of fermentation that will be further discussed. In addition, the detection of additional species such as *Tricococcus* sp. and *Psudomonas* sp. by DGGE also could demonstrate their significant level and possible impact on fermentation properties that relatively different from the minor bacterial isolates observed by cultural plating.

The investigation of yeast, the population level at the Initial fermentation stage was about 5 log CFU/g and decreased to 4 and 2 log CFU/g at middle and final stages, respectively. By cultural plating, *Candida* sp. was one of the main of total isolates throughout fermentations. *Pichia* sp. was also significantly observed in the initial fermentation and remained prevalent in the middle stage along with *Zygosaccharomyces* sp., then their levels fell while a few numbers of other minor species were present in the last stage of fermentation. In this investigation, the information obtained by RNA analysis were relatively different. The cDNA banding as found as main species were only *Candida* spp. in the initial and final fermentation stages while only *Zygosaccharomyces* in the middle stage. Importantly, the observation of *Candida etchellsii* as found in this study indicated an important role of background microflora to significant physiochemical profiles of final product. Indeed, the same yeasts species found by RevT-nested PCR-DGGE were the main yeasts, with an occasional exception, at each fermentation stage found by

culture plating (Table 3). *Yamadazyma* sp. *Kodameae* sp., *Saccharomycdodes* sp., *Saccharomyces* sp. and *Wickerhamilla* sp. were additionally observed in these moromi samples by the plate culture approach, but not by Rev-T-PCR-DGGE (Table 3, Figure 2A), and so these are likely to be relatively rare yeasts that do not compete or survive well under these conditions.

For the physicochemical analysis, HMF significantly increased from an initial OD value of 2.20 to 5.84 by the end of the fermentation and corresponded to the increase of browning index from 5.26 to 11.49 (Figure 6 and Appendix E). Levels of RS, TN and pH were not significantly changed, whilst the level of NaCl in moromi was found significantly declined by 5% through end of the fermentation (Table3.1, Figure 3.2A) due to an immersion of salt into soybean meal (Gao et al., 2011) and precipitation (Park et al., 2016). During the first half of fermentation, arabinose was reduced by 45.50%, which was a larger decrease than that of glucose (8.56%), and xylose (6.68%). As RS value was lower for 22.23% during these periods, thus the main change seemed to relate with arabinose than others. Free amino acids (FAAs) results wth the dominants of Lys, Leu and Val along fermentation time where a higher increasing rate (18.92%) was observed during initial to middle before slowed down in the following stage (Figure 3 and Appendix B). However, profile changes in each period were noted in PCA as samples were allocated in different quadrants (Figure 4). SP1F moromi contained higher amount of ethanol (al1, Figure 5) which was used as a key index to verified fermentation efficiency, than others determined in this study. Other main compounds were ester and its derivatives of butanal, 2-methyl; 1-butanol,3-methyl and 1-propanol, 2-methyl (ad2, al3 and al9, respectively).

The SP2 moromi collected from the same manufacturing of SP1 at different processed batch in which the moromi was incubated under higher temperature (21°C instead of 16°C) at the first 3 weeks. It was found that total population numbers of both bacteria and yeast were significantly lower than SP1 throughout fermentation periods. However, every fermentation stages of the SP2 showed broadly similar microbiological and chemical results during fermentation as that of the SP1 sample outlined above, with *Staphylococcus* spp. and *Bacillus* spp. being the main bacterial species observed during the fermentation (Table 3). *Candida* sp. was also main isolate, *Pichia* sp. and *Zygosaccharomyces* sp., were also significantly prevalent in fermentation systems. An existence of *Candida* species was confirmed by RevT-DGGE results toward fermentation time, suggesting a significant role of this yeast species in the fermentation. For the minor species as additional isolated from this sample throughout fermentation were comparable with SP1. Similar dynamic changes in RS, glucose, arabinose, xylose, total nitrogen and pH, HMF and browning were also observed (Figure 3 and 6). Dominant FAAs in SP2 were still Lys and Leu with a remarkable lower proportion of all amino acids than SP1 (Figure 4 and Appendix B2). Volatile profile of SP2 also resembled to SP1 as they were clustered on PCA results (Figure 5).

Table 3 Microbial communities of soy sauce moromi from traditional and industrial processes determined by cultural plating and cultural independent RevT-nested-PCR-DGGE method.

samples (NaCl,%)	Cultural dependent						Cultural independent DGGE	
	Bacteria			Yeast			Bacteria	Yeast
	TPC	RIN*	Isolates (%)	TPC	RIN*	Isolates (%)		
SP1I (21.21)	6.54	11	*Staphylococcus* sp.[a] SB1 (45.45)	5.63	8	*Candida orthopsilosis*[b] (25.00)	*Staphylococcus* sp.[c] (B4)	*Candida etchellsii*[a] (Y1)
			Bacillus subtilis[a] (27.27)			*Candida tropicalis*[a] (12.50)	*Bacillus* sp.[b] (B2)	*Candida* sp.[b] (Y2)
			Bacillus sp.[a] (9.09)			*Candida jaroonii*[c] (12.50)	*Pseudomonas* sp.[a] (B3)	
			Bacillus amyloliquefaciens[a] (9.09)			*Kodamaea ohmeri*[a] (12.50)	Unidentified bacterium[a] (B1)	
			Bifidobacterium angulatum[a] (9.09)			*Pichia mexicana*[a] (12.50)	*Trichococcus* sp.[a] (B5)	
						Pichia sp.[a] (12.50)		
						Yamadazyma mexicana[a] (12.50)		
SP1M (18.38)	4.45	6	*Bacillus* sp.[a] (33.33)	4.32	4	*Zygosaccharomyces rouxii*[c] (50.00)	*Staphylococcus* sp.[c] (B4)	*Candida etchellsii*[a] (Y3)
			Staphylococcus camosus[a] (16.67)			*Candida orthopsilosis*[b] (25.00)	*Bacillus* sp.[b] (B2)	
			Staphylococcus condimenti[a] (16.67)			*Pichia mexicana*[a] (25.00)	Unidentified bacterium[a] (B1)	
			Staphylococcus sp.[a] (16.67)					
			Lactobacillus salivarius[b] (16.67)					
SP1F (17.92)	3.17	11	*Staphylococcus camosus*[a] (45.45)	2.69	6	*Candida halophila*[a] (33.33)	*Staphylococcus* sp.[c] (B4)	*Candida etchellsii*[a] (Y1)
			Staphylococcus condimenti[a] (18.18)			*Saccharomyces cerevisiae*[c] (16.67)	Unidentified bacterium[a] (B1)	
			Bacillus sp.[a] (9.09)			*Saccharomycodes ludwigii*[a] (16.67)		
			Virgibacillus proomii[c] (9.09)			*Wickerhamiella versatilis*[c] (16.67)		
			Lactobacillus curvatus[c] (9.09)			*Zygosaccharomyces rouxii*[c] (16.67)		
			Streptomyces sp.[a] (9.09)					

Table 3 Microbial communities of soy sauce moromi from traditional and industrial processes determined by cultural plating and cultural independent RevT-nested-PCR-DGGE method (Continue).

samples (NaCl,%)	Cultural dependent						Cultural independent DGGE	
	Bacteria			Yeast			Bacteria	Yeast
	TPC	RIN*	Isolates (%)	TPC	RIN*	Isolates (%)		
SP2I (20.01)	3.46	13	*Bacillus amyloliquefaciens* [a] (30.77)	3.21	16	*Zygosaccharomyces rouxii* [a] (25.00)	*Lactobacillus helveticus* [a] (B6)	*Candida etchellsii* [a] (Y1)
			Virgibacillus sp. [a] (23.08)			*Candida parapsilosis* [a] (25.00)	*Staphylococcus* sp. [a] (B7)	*Candida* sp. [b] (Y2)
			Bacillus sp. [a] (15.38)			*Candida mannitofaciens* [a] (6.25)		*Zygosaccharomyces rouxii* [a] (Y4)
			Bacillus subtilis [a] (15.38)			*Candida tropicalis* [a] (6.25)		
			Virgibacillus halophilus [a] (7.69)			*Debaryomyces hansenii* [a] (6.25)		
			Pseudomonas sp. [b] (7.69)			*Pichia mexicana* [a] (6.25)		
						Pichia membranifaciens [a] (6.25)		
						Pichia sp. [a] (6.25)		
						Unidentified fungal sp. [a] (6.25)		
						Yamadazyma Mexicana [a] (6.25)		
SP2M (18.38)	1.90	6	*Bacillus siamensis* [a] (16.67)	0.70	7	*Candida halophila* [a] (42.86)	*Bacillus* sp. [b] (B2)	
			Bacillus methylotrophicus [a] (16.67)			*Candida apicola* [a] (14.29)	*Staphylococcus* sp. [a] (B7)	
			Staphylococcus condiment [a] *(16.67)*			*Candida mannitofaciens* [a] (14.29)		
			Staphylococcus sp. [a] (16.67)			*Saccharomyces cerevisiae* [a] (14.29)		
			Streptococcus thermophilus [b] (16.67)			*Wickerhamiella versatilis* [c] (14.29)		
			Trichococcus sp. [b] (16.67)					

Table 3 Microbial communities of soy sauce moromi from traditional and industrial processes determined by cultural plating and cultural independent RevT-nested-PCR-DGGE method (Continue).

samples (NaCl,%)	Cultural dependent						Cultural independent DGGE	
	Bacteria			Yeast			Bacteria	Yeast
	TPC	RIN*	Isolates (%)	TPC	RIN*	Isolates (%)		
SP2F (17.92)	3.32	11	*Bacillus licheniformis* [c] (18.18)	2.96	9	*Candida apicola* [a] (22.22)	*Bacillus* sp. [b] (B2)	*Candida apicola* [a] (Y5)
			Staphylococcus camosus [a] (18.18)			*Saccharomyces cerevisiae* [a] (22.22)	*Pediococcus acidilactici* [a] (B8)	
			Bacillus megaterium [c] (9.09)			*Saccharomycodes ludwigii* [a] (22.22)		
			Bacillus sp. [a] (9.09)			*Candida halophila* [a] (11.11)		
			Staphylococcus sp. [b] (9.09)			*Candida mannitofaciens* [a] (11.11)		
			Unidentified bacterium [b] (9.09)			*Kluyveromyces marxianus* [c] (11.11)		
			Lactobacillus plantarum [C] (9.09)					
			Pediococcus sp. [b] (9.09)					
			Trichococcus sp. [b] (9.09)					
CCI (18.28)	5.48	6	*Staphylococcus* sp. [a] (50.00)	2.84	7	*Candida vrieseae* [a] (28.57)	Unidentified bacterium [b] (B9)	*Schizosaccharomyces pombe* [b] (Y6)
			Bacillus amyloliquefaciens [a] (16.67)			*Candida halophila* [a] (14.29)	*Lactobacillus delbrueckii* [a] (B10)	*Saccharomyces cerevisiae* [b] (Y7)
			Bacillus sp. [a] (16.67)			*Kodamaea ohmeri* [c] (14.29)	*Tetragenococcus halophilus* [a] (B11)	*Saccharomycopsis fibuligera* [b] (Y8)
			Bacillus subtilis [a] (16.67)			*Wickerhamiella versatilis* [b] (14.29)	*Clostridium drakei* [a] (B12)	
			Enterobacter sp. [a] (16.67)			*Zygosaccharomyces rouxii* [c] (14.29)		
						Zygosaccharomyces rouxii [a] (14.29)		

Table 3 Microbial communities of soy sauce moromi from traditional and industrial processes determined by cultural plating and cultural independent RevT-nested-PCR-DGGE method (Continue).

samples (NaCl,%)	Cultural dependent						Cultural independent DGGE	
	Bacteria			Yeast			Bacteria	Yeast
	TPC	RIN*	Isolates (%)	TPC	RIN*	Isolates (%)		
CCM (18.59)	5.31	9	*Staphylococcus* sp. [a] (44.44)	4.73	3	*Candida halophila* [a] (66.67)	*Klebsiella* sp. [a] (B13)	*Schizosaccharomyces pombe* [b] (Y6)
			Bacillus amyloliquefaciens [a] (22.22)			*Zygosaccharomyces rouxii* [c] (33.33)	Unidentified bacterium [b] (B9)	*Saccharomyces cerevisiae* [b] (Y8)
			Bacillus subtilis [a] *(11.11)*				*Lactobacillus delbrueckii* [a] (B10)	*Saccharomycopsis fibuligera* [b] (Y9)
			Bacillus sp. [a] (11.11)				*Tetragenococcus halophilus* [a] (B11)	
			Unidentified bacterium [a] (11.11)					
CCF (17.34)	7.65	12	*Bacillus subtilis* [a] (41.67)	1.22	11	*Candida vrieseae* [a] (27.27)		*Schizosaccharomyces pombe* [b] (Y6)
			Bacillus amyloliquefaciens [a] (41.67)			*Zygosaccharomyces rouxii* [a] (18.18)		*Saccharomyces cerevisiae* [b] (Y8)
			Staphylococcus sp. [a] (16.67)			*Candida halophila* [a] (18.18)		*Saccharomycopsis fibuligera* [b] (Y9)
						Wickerhamiella versatilis [b] (18.18)		
						Candida mannitofaciens [a] (9.09)		
						Zygosaccharomyces rouxii [c] (9.09)		
NPI (25.82)	9.98	10	*Bacillus subtilis* [a] *(30.00)*	8.85	3	*Zygosaccharomyces rouxii* [a] (66.67)	*Bacillus* sp. [a] (B14)	*Candida versatilis* [a] (Y10)
			Bacillus sp. [a] (20.00)			*Kodamaea ohmeri* [b] (33.33)	*Staphylococcus* sp. [b] (B15)	Candida cf. etchellsii [a] (GY9)
			Bacillus zhangzhouensis [b] (10.00)					
			Brevibacterium sp. [a] (10.00)					
			Lactobacillus curvatus [c] (10.00)					
			Staphylococcus sp. [a] (10.00)					
			Staphylococcus condimenti [a] (10.00)					

Table 3 Microbial communities of soy sauce moromi from traditional and industrial processes determined by cultural plating and cultural independent RevT-nested-PCR-DGGE method (Continue).

samples (NaCl,%)	Cultural dependent						Cultural independent DGGE	
	Bacteria			Yeast			Bacteria	Yeast
	TPC	RIN*	Isolates (%)	TPC	RIN*	Isolates (%)		
NPM (19.37)	7.86	6	*Bacillus zhangzhouensis* [b] (16.67)	1.00	5	*Candida* sp. [a] (40.00)	*Bacillus zhangzhouensis* [a] (B17)	Candida cf. etchellsii [a] (Y9)
			Staphylococcus sp. [a] (16.67)			*Kodamaea* sp. [a] (20.00)	*Paenibacillus illinoisensis* [c] (B16)	
			Lactobacillus graminis [c] (16.67)			Unidentified fungal sp. [a] (20.00)	*Staphylococcus* sp. [a] (B18)	
			Lactobacillus curvatus [a] (16.67)					
			Bacillus subtilis [a] (16.67)					
			Staphylococcus carnosus [a] (16.67)					
NPF (17.29)	4.80	11	*Bacillus amyloliquefaciens* [a] (36.36)	2.76	12	*Candida* sp. [a] (41.67)	*Bacillus* sp. [a] (B14)	Candida cf. etchellsii [a] (Y9)
			Bacillus sp. [a] (18.18)			*Kodamaea* sp. [a] (16.67)		
			Trichococcus sp. [c] (18.18)			*Kodamaea ohmeri* [b] (16.67)		
			Virgibacillus sp. [b] (9.09)			*Candida fungicola* [a] (8.33)		
			Bacillus subtilis [a] (9.09)			Unidentified fungal [a] (8.33)		
			Staphylococcus carnosus [a] (9.09)			*Torulaspora delbrueckii* [c] (8.33)		

Table 3 Microbial communities of soy sauce moromi from traditional and industrial processes determined by cultural plating and cultural independent RevT-nested-PCR-DGGE method (Continue).

samples (NaCl,%)	Cultural dependent						Cultural independent DGGE	
	Bacteria			Yeast			Bacteria	Yeast
	TPC	RIN*	Isolates (%)	TPC	RIN*	Isolates (%)		
PLI (19.15)	5.41	13	*Bacillus* sp. [a] (30.76)	1.18	5	*Candida apicola* [a] (40.00)	Unidentified bacterium [b] (B19)	*Aureobasidium pullulans* [a] (Y11)
			Unidentified bacterium [a] (23.08)			*Zygosaccharomyces rouxii* [a] (40.00)	*Lactobacillus* sp. [b] (B20)	*Candida etchellsii* [a] (Y12)
			Lactobacillus curvatus [c] (15.38)			*Candida etchellsii* [a] (20.00)		
			Bacillus amyloliquefaciens [a] (7.69)					
			Staphylococcus sp. [a] (7.69)					
			Staphylococcus epidermidis [a] (7.69)					
			Staphylococcus xylosus SB64 (7.69)					
PLM (19.01)	5.08	10	*Oenococcus oeni* [b] (30.00)	1.48	2	*Candida* sp. [a] (50.00)	Unidentified bacterium [b] (B19)	*Candida etchellsii* [a] (Y12)
			Bacillus amyloliquefaciens [a] (10.00)			*Entylomatales* sp. [c] (50.00)	*Lactobacillus fermentum* [a] (B21)	
			Bacillus sp. [a] (10.00)				*Lactobacillus* sp. [b] (B20)	
			Bacillus pumilus [c] (10.00)					
			Lactobacillus curvatus [c] (10.00)					
			Staphylococcus sp. [a] (10.00)					
			Staphylococcus epidermidis [a] (10.00)					
			Staphylococcus condiment [a] (10.00)					

Table 3 Microbial communities of soy sauce moromi from traditional and industrial processes determined by cultural plating and cultural independent RevT-nested-PCR-DGGE method (Continue).

samples (NaCl,%)	Cultural dependent						Cultural independent DGGE	
	Bacteria			Yeast			Bacteria	Yeast
	TPC	RIN*	Isolates (%)	TPC	RIN*	Isolates (%)		
PLF (18.53)	2.46	4	*Bacillus infantis* [b] (25.00)	3.22	7	*Millerozyma farinosa* [a] (42.86)	Unidentified bacterium [b] (B19)	*Candida etchellsii* [a] (Y12)
			Bacillus sp. [a] (25.00)			*Candida etchellsii* [a] (14.29)	*Lactobacillus* sp. [b] (B20)	
			Lactobacillus curvatus [c] (25.00)			*Candida* sp. [a] (14.29)		
			Staphylococcus sp. [a] (25.00)			*Kluyveromyces aestuarii* [a] (14.29)		

*RIN = Representative isolate number

a = 98 -100 %homology

b = 90-97 %homology

c = 81-89 %homology

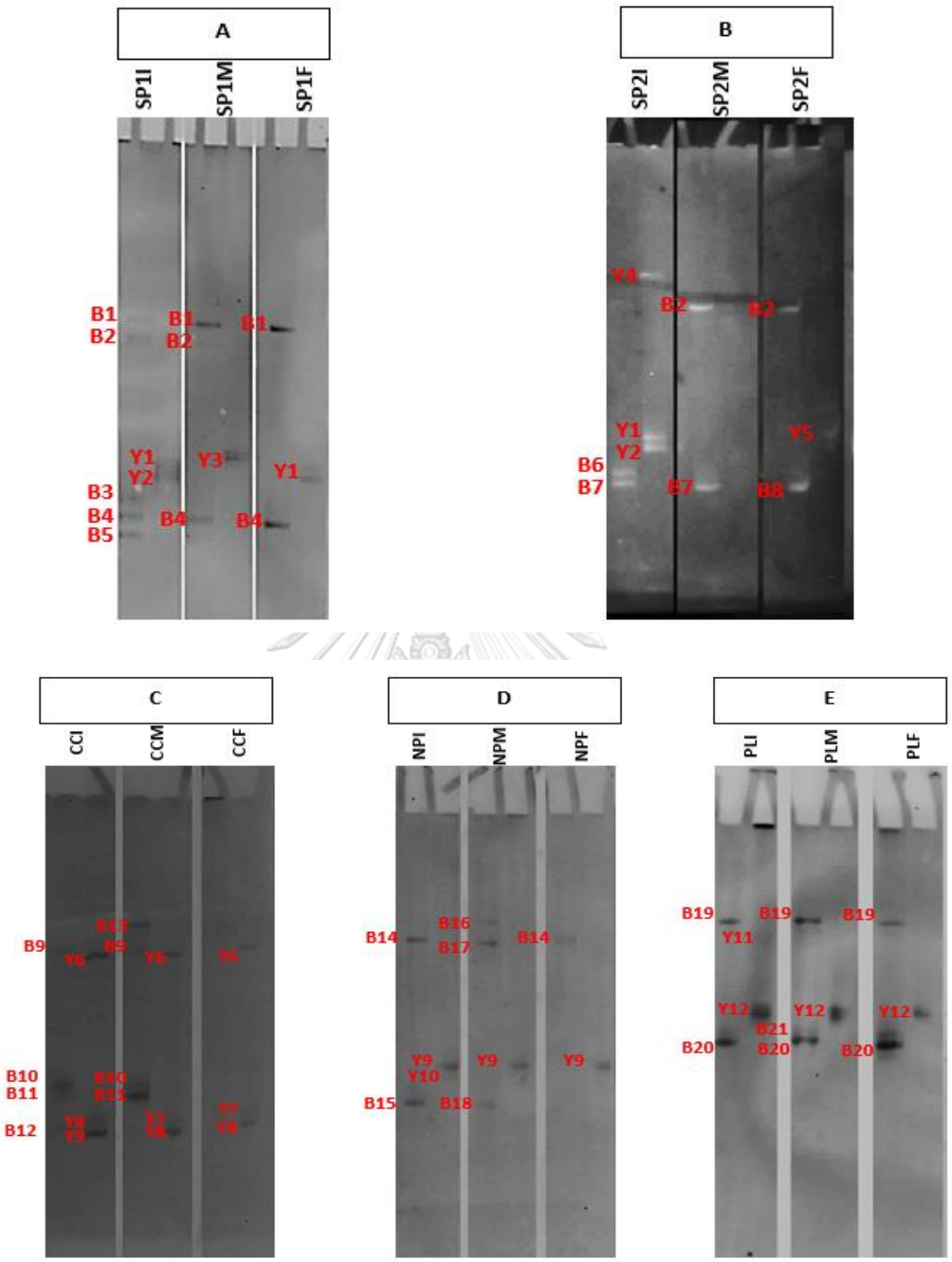


Figure 2 Bacterial and yeast DGGE profiles of moromi samples in each fermentation stage

: A = SP1 moromi, B = SP2 moromi, C = CC moromi, D = NP moromi and E = PL moromi. All DNA banding codes were detailed in Table 3.1 in cultural independent DGGE column.

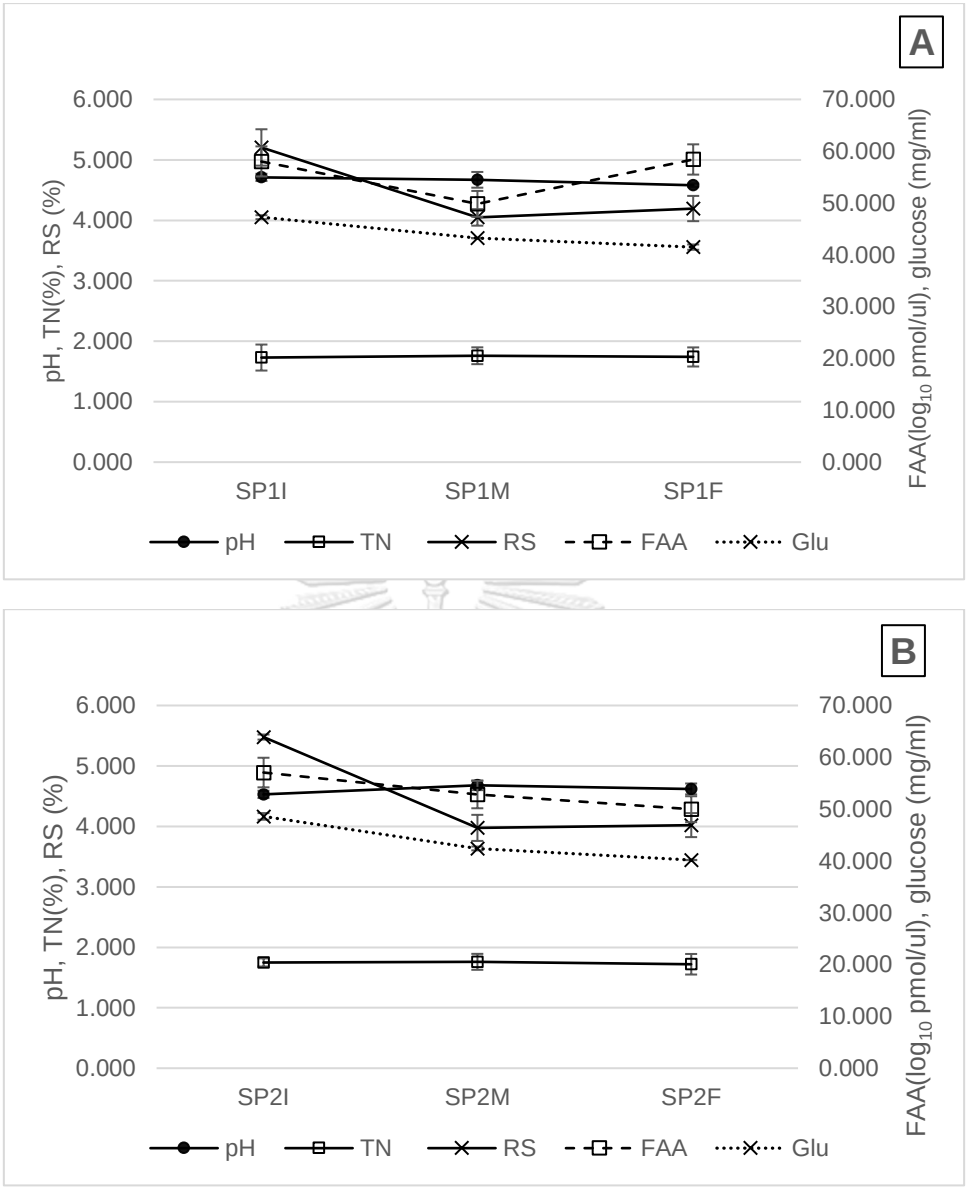


Figure 3 Physicochemical changes in moromi during fermentation

: Fig 3.2A = SP1, Fig3.2B = SP2 moromi

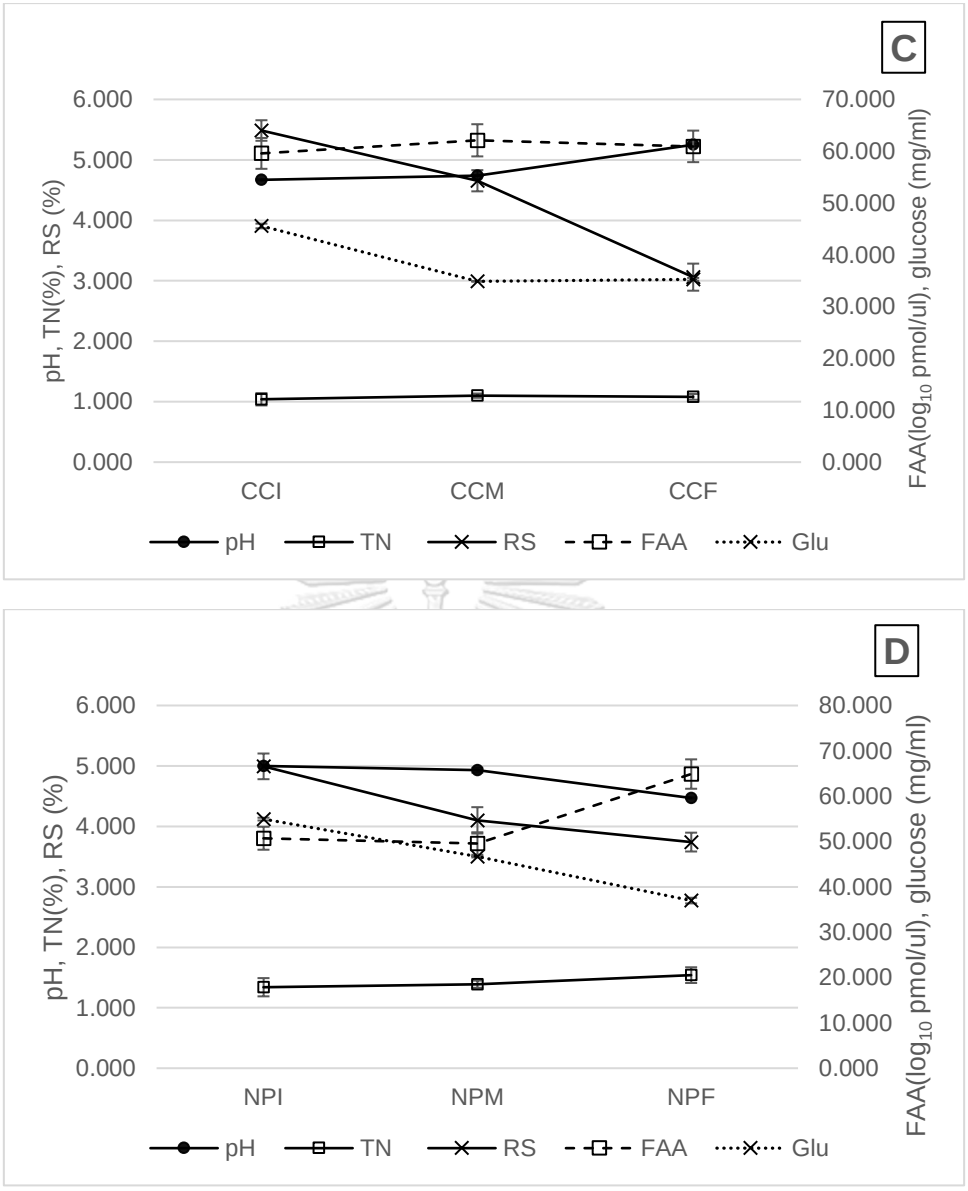


Figure 3: Physicochemical changes in moromi during fermentation (continued)

: Fig 3,2C = CC, Fig 3.2D = NP moromi.

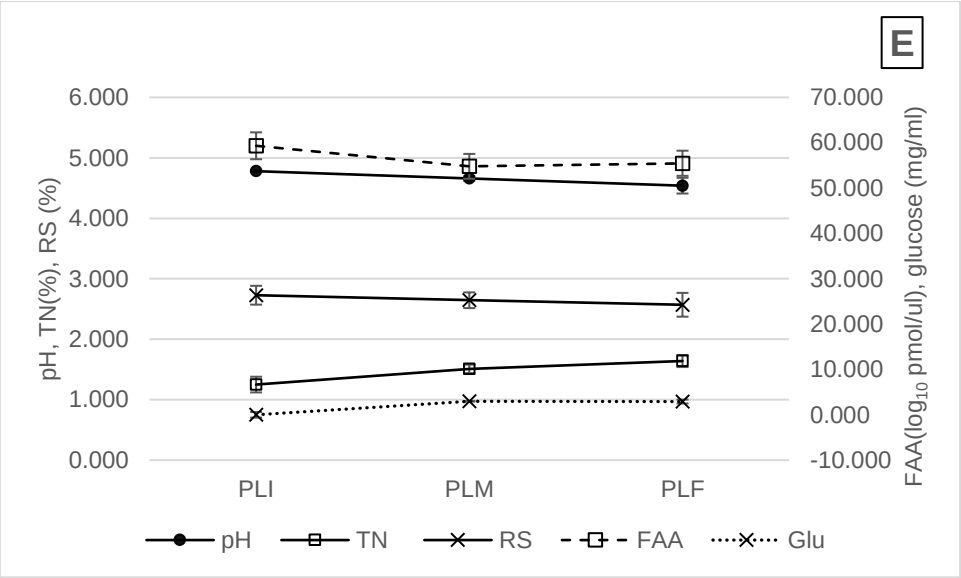


Figure 3: Physicochemical changes in moromi during fermentation (continued)

: Fig 3.2E = PL moromi.

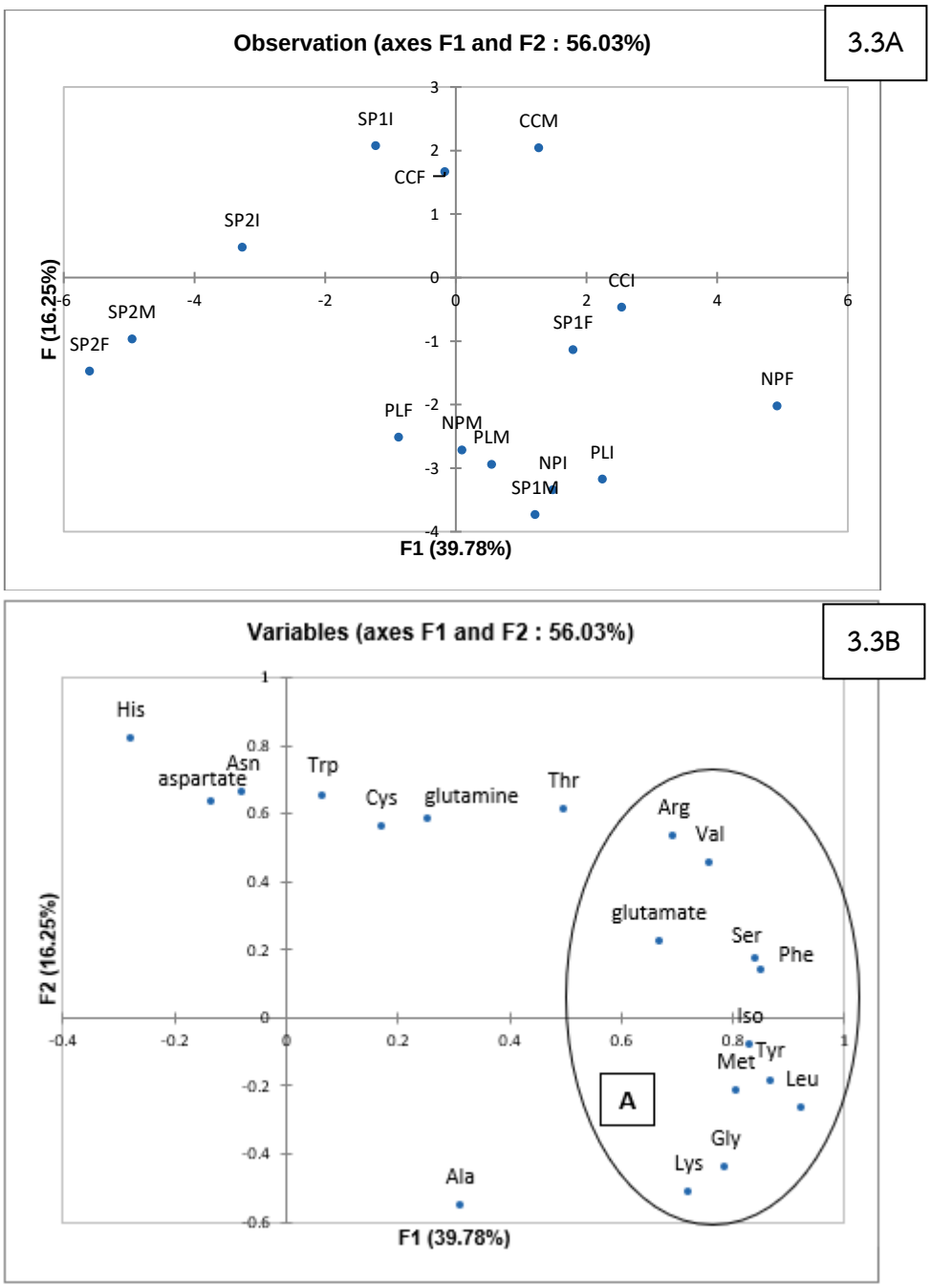


Figure 4 Principle component analysis performed on free amino acid during moromi fermentation

: A = Observations and B = Variables

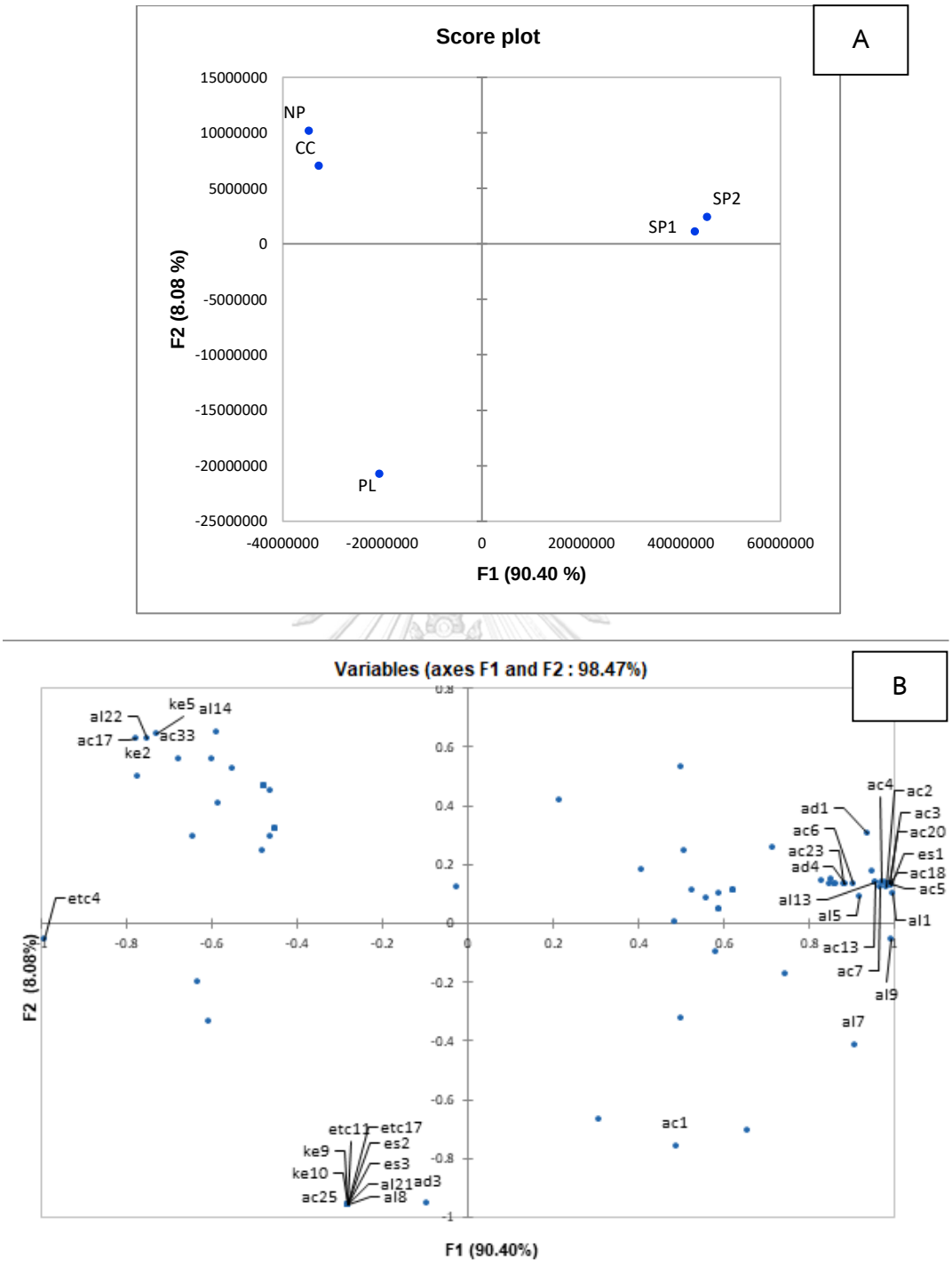


Figure 5 Principle component analysis performed on volatile compounds during moromi fermentation

: A, Observations and B, Variables. Abbreviation of each volatile code as following

ac1	=	Acetic acid	al5	=	1-Butanol, 3-methyl-, acetate
ac2	=	Propanoic acid, 2-hydroxy-, ethyl ester, (S)-	al6	=	Phenylethyl alcohol
ac3	=	Hexadecanoic acid, ethyl ester	al7	=	1-Octen-3-ol
ac4	=	Benzoic acid, ethyl ester	al8	=	2-Furanmethanol
ac5	=	9,12-Octadecadienoic acid, ethyl ester	al9	=	1-Propanol, 2-methyl-
ac6	=	Octanoic acid, ethyl ester	al13	=	1-Butanol, 2-methyl-, acetate
ac7	=	Nonanoic acid, ethyl ester	al14	=	2,3-butanediol, [R-(R*, R*)]-
ac13	=	Butanedioic acid, diethyl ester	al21	=	cyclohexanol, 1-propyl-
ac17	=	butanoic acid, 3-methyl	al22	=	4,5-Octanediol, 2,7-dimethyl-
ac18	=	Butanedioic acid, monomethyl ester	es1	=	Ethyl Acetate
ac20	=	Oleic acid, ethyl ester	es2	=	furfural
ac23	=	Butanoic acid, ethyl ester	es3	=	hexanal
ac25	=	butanoic acid, 4-hydroxy	etc4	=	phenol, 2-methoxy-
ac33	=	propanoic acid, 2-methyl-	etc11	=	hexane
ad1	=	Benzaldehyde	ke1	=	2-butanone
ad2	=	Butanal, 2-methyl-	ke2	=	2-butanone, 3-hydroxy-
ad3	=	Butanal, 3-methyl-	ke4	=	acetone
ad4	=	3-Furaldehyde	ke5	=	2,3-butanedione
al1	=	Ethanol	ke9	=	2-propanone, 1-hydroxy-
al3	=	1-Butanol, 3-methyl-	ke10	=	1-octen-3-one

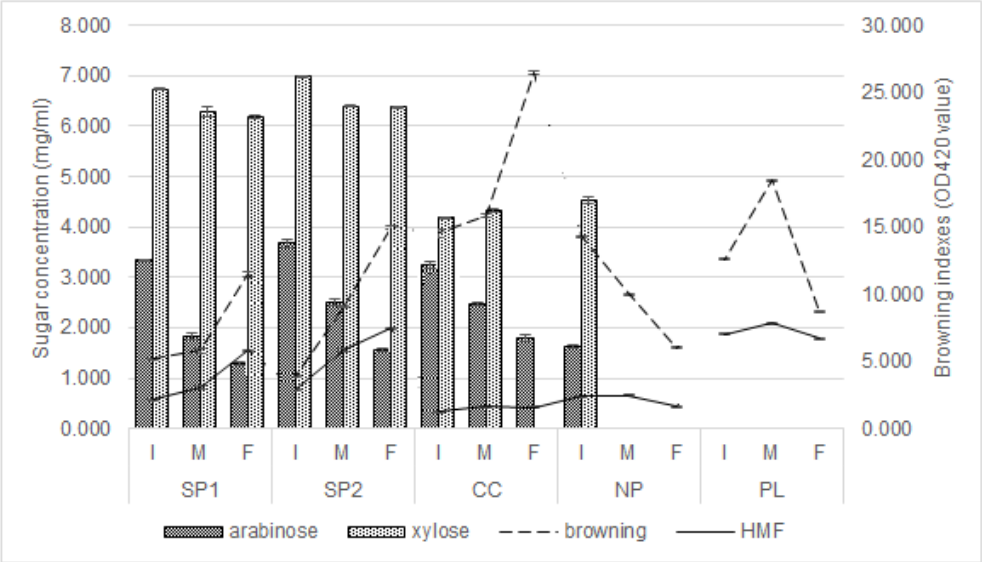


Figure 6 Reducing sugar profiles of glucose, arabinose and xylose in relation with browning indexes in SP1, SP2, CC, NP and PL moromi.

3.3.1.2 Home/traditional production

CC, a traditional moromi in tightly-closed clay jar with optimal incubation temperature of 30 - 32°C, consisted of main microbial species of *Bacillus* and *Staphylococcus* spp. as reported in commercial process excepting a remarkable increase of bacteria to nearly 8 logCFU/g. was observed in CCF. Minor bacterial species were found in CCI and CCM, where *Bacillus* spp. was ≤ 50%. *Candida* and *Zygosaccharomyces* were 2 dominant yeast found at all stages. A contrast of diversity with conventional plating were noticed in DGGE analysis where bands of bacterial *Lactobacillus* and *Tetragenococcus*, and yeast in Saccharomycetales group and *Schizosaccharomyces pombe* were identified from all fermentation periods (Table 3.1). Salinity in CCs relative stable in range of 17.34 – 18.59% while pH increased through fermentation, giving the highest value of 5.25 at CCF (Table 3). In

the second half, some remarkable changes in physicochemical were monitored including browning and reducing sugar profiles. Comparing with those industrials, TN, whose values were the lowest among others in every fermentation stages (Figure 3), and HMF values were relatively stable during these periods in contrast with a notable rising of browning observed. Another set of contradictions were in RS and its constituents as the former shown a slightly decrease with the changes of: glucose was slightly higher and pentoses notable reduced (Figure 3 and 6). A strong reduction pronounced in xylose than arabinose was depicted in Figure 3.5, calculated as -100% and 26.56% reduction, respectively. Leu and Lys still be the dominant free amino acids over all CC samples, but a remarkable glutamine content was noted in CCF as the highest among all tested moromi (Figure 4 and Appendix B3). CCF was plotted in upper left quadrant opposite the industrial cluster when PCA was applied, which the result of the inversion aroma profiles from SP1 and SP2 was illustrated (Fig 5).

The second traditional sample was NP which its storage temperature was relatively lower (25-28°C) from others in same group. Bacterial and yeast population of NPI started with highest value among other tested samples of 9 and 8 logCFU/g., respectively, before declining. Although *Bacillus* and *Staphylococcus* still were the major, also an equal ratio of these two species and *Lactobacillus* spp. was marked in NPM. These plating results were supported by DGGE where *Bacillus* or *Staphylococcus* were detected. Despite the fact that *Candida* was not isolated from NPI plates (Table 3), a similar profile with conventional result of CC could be found as this species was noted in DGGE instead. Thus, *Candida* seemed to be a potential yeast in this production due to a confirmation of DGGE results (Figure 2). In term of physicochemical, salinity of NP starting with the highest of 25.82% then rapidly and continuously plunged to final

value of 17.29%. Sharp reduction of pH in second half stages might related to microbial metabolic activity and would be discussed in the last section. Only one increment trend was found in TN determination during the second half period (Figure 3). Apart of this properties, interesting matter displayed during middle stage including significantly decrease of both browning indexes, HMF (-32.00%) and browning (-66.67%), from NPM to NPF simultaneously with arabinose and xylose content which reached zero at NPM (Figure 6). Glucose normally declined as well as RS values and free hexose still presented in the system at NPF (Figure 3). Free amino acid profiles of NPs were similar, but an exceptional amount of Lys, and Leu were examined in NPF allowing the highest increase of 48.18% in total when compared with others (Appendix B4). Negative relation of volatile profile of CC with industrial samples was also observed in NP as they shared same cluster, thus NP had similar profile with CC.

The last sample of PL having different aging process as soy mash was removed at 7th week and remaining liquid was further incubated outdoor where temperature reached as high as 38°C. Microbial profiles in PL moromi were generally similar to the previous samples, consisting of bacterial *Bacilli* and *Staphylococci*, and yeast *Candida*. Background fungal *Aereobasidium pullulans* noticed in PLI as a smear, thin DGGE band might be an effect of this different process conducting at early time before PLI was collected. In addition, *Lactobacillus* sp. was isolated as minor species in every collecting moromi along with its band in DGGE analysis (Figure 2). This suggest an important role of this bacteria to PL microbial community. Profile changing of pH, NaCl, TN, RS and HMF in PL resembled to those derived from NP (Figure 3). Nevertheless, their values were not comparable such as HMF results were at least 3 times higher in PL than same fermentation time of NP. At the middle to final point, same browning reduction trends were found in both samples. And again, with 2 times higher rate in PL (-112.07%, compared with -66.67% in NP, Figure 3.5).

Available glucose and 2 pentose could not be detected in PL as it shown a relative zero value due to the detection limit of 2.85 mg/ml. for glucoses (Figure 3), 0.01 mg/ml. for arabinose and 0.11 mg/ml. for xylose (Figure 6), since the beginning of fermentation. Dominant FAA of PL contrasted with one from SP1, while total change was completely contrast as a 24.33% reduction of PL against 28.61% increment of SP1 (Figure 4). An isolation of PL to an extinct area of lower left quadrant was depicted in PCA of volatile compounds analysis as a result of high butanal, 3-methyl (ad3), 2-furanmethanol (al8) and furan,2,5-dimethyl (etc17) (Figure 5).

3.3.2 Relationships among/between microbial levels and community structures, and key compounds involved in aromatic compounds and browning reactions

3.3.2.1 Sugars and browning indexes

Increasing trend of browning indexes observed in 2 industrial moromi seemed to directly relate with rising of HMF value then resulting in elevation of browning, which was stemmed by reducing sugar especially arabinose as illustrated in Figure 3A, 3B and 6. Determination of reducing sugars and browning indexes in traditional samples revealed many interesting points needed further investigation. First of all, even the browning increment to the peak and arabinose decrement were noted similarly as found in industrial samples, remarkable disappearance of xylose during the second half of CC was detected which might indicate the significant influence of this sugar to the browning of moromi. Nevertheless, HMF was only slightly increase toward fermentation time (Figure 6). Microbial population shift including higher dominant bacteria (*Bacillus* and

Staphylococcus spp.) to the significant scale of nearly 8 logCFU/g., and lower *Z. rouxii* which was a second major yeast were also detected in the same periods (Table 3).

Interestingly, diminutions of two pentose since the middle stage should related to the decrease of browning indexes in NP, especially an extreme declining rate of the browning (Figure 6). This also could reflect a significant role of pre-dominant microbes playing under those conditions as their bacterial populations containing *Bacillus, Staphylococcus* and *Lactobacillus* spp. reached significant level in range of 7 to 9 logCFU/g. Similar *Z. rouxii* population level in NPI and *Candida* spp. presenting in latter stages might also played an important role with de-coloring as well. A supportive evidence was found in PL samples as reduction of browning and HMF during middle to final period concomitated with similar microbial diversity found in NP.

3.3.2.2 FAAs and browning indexes

FAA also one important factor influenced on browning of moromi as it, together with reducing sugar, are substrates of either Maillard reaction or advance steps of melanoidin. Microbial containing proteolytic activity degraded N-containing macromolecule into smaller peptide and FAAs which both could undergo non-enzymatic browning thus change in FAA profile and its further spontaneous chemical reaction resulted in browning indexes differences directly related to microbial activities. Principle component analysis was then used to investigate their relations. Shift in FAA profiles via PCA linking with browning indexes were identified toward fermentation process (Figure 4) and some interesting data were revealed.

Diminished of aspartate, asparagine, glutamine, His and Trp was found along with the increment of browning indexes in industrial samples, and divergent case observed in PL. 11 FAAs (cluster A, Figure 4B) including Leu, Met, Lys, Gly and Tyr seemed to also play an important role in browning as shown in CC and NP which higher of FAAs in cluster A found during an extreme increase of browning was observed, as vice versa in the latter batch. This FAA in cluster A should also responded for the higher browning indexes of SP2 toward SP1 as well. Traditional process of CC, which browning were extremely increase in CCF, had less of these 11 FAAs including Leu, Met, Lys, Gly and Tyr when compared with CCI. Nevertheless, they were higher in NPF than NPI, while browning reduction was observed. Another color decrement found in PL might be due more amount of Lys and Gly from PLF than PLI was noticed (Figure 4).

3.3.2.3 Volatile compound profiles and browning

Moromi from final stage fermentation were analyzed for their volatile compounds by SPME-GC-MS technique. Different in the most abundant compound found in samples were observed. In final fermentation period of five moromi, 95 volatile compounds were detected and grouped as acids (41.05%), alcohol (23.16%), ketone (12.63%), pyrazine and aldehyde (7.37%), ester (3.16%) and others (15.79%). PCA results clustered samples into 3 groups of industrial (upper right), CC and NP (upper left) and traditional process PL (lower left quadrant) (Figure 3.4). Dominant volatile in each group was ethanol (al1) in SP1 and SP2, 2-furanmethanol (al8) in PL, and butanoic acid (ac17) in CC and NP (Figure 5, Appendix D). Divergent profiles of other dominant or background aroma compounds were also observed in each sample indicating impacts of microbiological and/or

physicochemical parameters toward this scheme. In term of microbial, SP1 and SP2 samples, which *S. cerevisiae* and *C. etchellsii* were used as starter culture, contained some specific yeasts species such as alcohol producer *S. cerevisiae* and *Sac. ludwigii* and were not isolated from any stages of traditional process, and they had extremely positive interaction with al1 locating on far right of upper quadrant. A contrast profiles of CC and NP toward industrial samples were obviously noticed by PCA such as al1 which was detected only in CC but with the lowest amount while it was not detected in NP. As *Z. rouxii* could not detected via both cultural plating and DGGE of NP moromi thus this yeast should respond for al1 production in these samples. Maillard reaction's product of pyrazine group (py1 – 8) was detected only in NPF moromi, while none of notable microbial species was marked therefore some potential microbe growth in this sample might responded for this metabolite concomitant with Maillard reaction. Acetic acid (ac1) along with furan derivative compounds of al8 and furan, 2,5-dimethyl (etc17) were shown highly positive relation with PLF as they all located in negative F2 area (Figure 5). Ac1 produced of LAB metabolic activity identified both from conventional and RevT-nested PCR-DGGE (Table 3) promoted acidity in the system containing glucose and high temperature, thus Maillard reaction was promoted and its key intermediate of HMF was then largely used for formation of aroma furan derivative compounds as a final product of PL.

Many differences linking with FAA were noted in this study. industrial moromi especially SP2 displayed negative interaction with FAA Phe (Figure 4) but shown a positive with benzaldehyde (ad1), a volatile situated on upper right quadrant in Figure 5. As all traditional samples revealed the reverse trend of high Phe and low ad1 compound, thus this FAA should play a role in ad1 formation. Notable positive relations between SP2 with 1-butanol, 3 methyl-; phenylethyl

alcohol and 1-propanol, 2-methyl- (al3, al6 and al9, respectively) were also noticed and could be linking with its highly negative interaction with FAA such as Val, Iso, Leu and Phe. Same interaction type of these FAAs was detected in PL, but high amount of butanal,2-methyl- (ad2) and butanal, 3-methyl (ad3) were marked instead of those alcohols. Nevertheless, even extraordinary level of these two aldehydes were noted in PL, they were also detected in other sample in lower proportion, pointing their significant role as aroma compound in soy sauce.

3.3.3 A potential microbial community and their roles in browning reduction

In the fermentation of moromi evaluated here were done by both the culture plating and Rev-PCR–DGGE methods Culture plating from moromi samples identified the total of 139 bacterial and 105 yeast representative isolates. It illustrated the dominant of *Bacillus* (43.17%) and *Staphylococcus* (33.09%) from every fermentation stages of all samples. *Bacillus* isolates included *Bacillus* sp. (13.67%), *B. amyloliquefaciens* (13.67%), *B. subtilis* (12.23%), and a few of *B. siamensis, B. methylotrophicus, B. licheniformis, B. megaterium, B. zhangzhouensis, B. pumilus* and *B. infantis. Staphylococcus* isolates included *Staphylococcus* sp. (15.83%), *S. carnosus* (8.63%), *S. condiment* (4.32%) while *S. epidermidis* and *S. xylosus* were occasionally found. Lactic acid bacteria (Baisier and Labuza) was detected as minor strain (10.79%) which included the generally found *Lactobacillus* spp. and *Trichococcus* spp., and sporadically detected species of *Streptococcus thermophilus*, *Pediococcus* sp. and *Oenococcus oeni*. In term of fungal, from total of 105 representative isolates, 15 yeast species were identified. *Candida* spp. was frequently detected (46.67%) in most samples. Apart from this species, 16.19% of *Zygosaccharomyces* (all were *Z.rouxii*), 7.62% of *Pichia,* 6.67% of *Kodamaea* and

4.76% of *Saccharomycetales (*except *Candida*) were observed throughout moromi samples.

RevT-nested PCR-DGGE revealed the difference results as 29.73% of major bacterial DGGE band belonged to LAB while equal proportion of 18.92% were *Bacillus* spp., *Staphylococcus* spp. and including unidentified bacteria. *Candida* spp. was generally detected (56.00%) as main fungal following by Saccharomycetales (24.00%) while *Z.rouxii*, known as soy sauce yeast was identified as low as 4.00%. Variation of microbial diversity data derived by cultural dependent and independent technique could be due to the fact that around 25-50% of microbes presenting in fermented food were unculturable (Benítez-Cabello et al., 2016) and/or sometime could not compete with their neighbors' strains to grow on agar media. This finding was interesting, as *Bacillus* were generally reported as dominant bacteria found in moromi fermentation and in these literatures, there were done based on cultural plating and/or DNA-based protocol (Tanaka et al., 2012; Wang, Wen, et al., 2017) and also as found in this study when determined by the same methods but not in RNA-based method. The different profiles of each microbial group as found could help in extending the information about the role of the microorganisms during moromi fermentations and their possible role associated in browning color reduction as focused in this study.

Bacillus spp. were reported as strong proteolytic and amylolytic bacteria that present as autochthonous flora having important role in salt-protein-rich fermented foods due to those of enzyme generation and hemophilic/halotolerant properties (Seo et al., 2018). Some strains were therefore developed as soy-based dried starter culture (Kim et al., 2018) and sometime were added in koji in combination with *Aspergillus* strains in some industrial soy-sauce productions. This

bacteria can survive well during koji aging and seemed to be strain-specific to soybean fermentation (Roslan et al., 2018). However, their activity and role in soybean digestion at this step has not been well known/reported relative to *Aspergillus*. Generally, in many manufacturing, particularly, traditional making, this bacteria was not intentionally added into any stage of fermentation system but the presence to roll out the activity to the fermentation of this bacteria always found in the systems (Hong et al., 2016). Many bacteria exist as natural flora on the surfaces of grains in form of spore including *Bacillus*. Consequently, soybean grains are a primary source of *Bacillus* associated with soy sauce fermentation. In this study, all moromi samples were manufactured without adding of *Bacillus.* Although this bacteria became the main population observed throughout fermentation steps in all batches, an expression of RNA in their cells did not mainly observed. This could demonstrate the activity of *Bacillus* that could be divided into two points. (i) *Bacillus* spores from primary source might germinate well in koji. When the koji was submerged in high salt condition (moromi), some vegetative cell of *Bacillus* strains (*B. zhangzhouensis, Bacillus* sp.) could adapt well, still further express and playing role in fermentation along with the other bacteria and yeasts as observed in DGGE. The major activity of this bacteria included furfure hydrolyzed protein and polysaccharide in soy mesh (Lee et al., 2017) instead of *Aspergillus* that still survive in moromi but could not express activity. In this study, the *Aspergillus* was mainly observed on agar plate but totally not observed on DGGE (data not shown). The second important role of *Bacillus* was production of key secondary metabolites which were 1-octen, 3-ol, a cooked soybean flavor alcoholic volatile produced (Lee et al., 2018) detecting in all moromi determined in this study, especially in industrial samples (al7 in Figure 5 and Appendix D), indicated gene expression of this species such as the viable population results illustrated in Table 3. The other vegetative *Bacillus* cell that could not adapt in high osmotic pressure and acidic environmental like moromi might sporulate that

would not produce any significant enzyme in moromi. These strains would be *B. subtilis, B. licheniformis* and of *B. amyloliquefaciens* as previous reports (Lee et al., 2017; Yang et al., 2017). (ii) On the other hand, *Bacillus* might enter to moromi step in form of spore and some of them partially germinate and express activity as detected on DGGE as described above. Thus, in cultural plating, the *Bacillus* shown as main population since the colony forming included both vegetative cell and the rest of spores always remained in every moromi fermentation stages. In addition, there were some reports demonstrate an impact of *Bacillus* in cultural plating assay on the detection of the other strains. Since *Bacillus* had specific feature of Quorum Quenching (QQ) which was the process of interrupting communication molecules used by numerous bacteria including LAB (Yin et al., 2012) this might inhibit colony formation of the near-neighbor strains and entirely could not compete to grow on agar surface. These evidences indicated a positive consolidation effect using two analytical methods of conventional plating and RNA-based DGGE to evaluate and extend an information about the metabolic-active microbe and their influences toward dynamic changes during fermentation of soy sauce as each technique could not fulfill the complete database required.

Apart from *Bacillus*, a contradiction population profiles of *Staphylococcus* spp. were also observed as cultural plating revealed a higher proportion than RNA-based in which LAB expressed as one of major bacteria in all moromi samples. Similarly, The Staphylococci was also reported as another strain having same QQ process as Bacilli, particularly, *Staphylococcus* strains present in moromi (Chong et al., 2012) therefore, the overrated resulted could be explained.

In this study, ReVT-nested PCR-DGGE revealed an abundant of LAB toward Bacilli and Staphylococci which was similar to the report of Tang et al (2017)

finding Lactobacillus as a main species in initial stage of Chinese liquor fermentation by DNA-based-Miseq technique. In addition, this study also found that LAB proportion detected by cultural plating was in the third rank following *Bacillus* and *Staphylococcus* spp. This could support the impact of neighbor strains on cultural plating results. Although, the number of LAB could be assayed on specific media, intensive information obtained might not benefit from this investigation. In addition, the changes of physicochemical property such as pH and TTA of moromi resulting from key metabolite of LAB particularly lactic acid along with the expression of LAB (Table 3) assayed by DGGE cultural plating could support the real action of this bacteria. LAB was generally considered as one of acid and volatile producers in soy sauce during brine fermentation. Moreover, some specific properties of LAB member were reported such as exopolysaccharide liberated from *S. thermophilus* (Yang et al., 2011) and *O.oeni* (Gänzle, 2015) along with roasted coffee aroma diacetyl production of the latter bacteria (Sternes et al., 2017), and generation of bacteriocin by *Pediococcus* strains (Porto et al., 2017) that could be a criteria for further development of specific strains.

In fungal investigation, in industrial samples, *Candida* and *Zygosaccharomyces* were mainly observed from both cultural plating and DGGE assays since they were starter cultures inoculated for sugars (glucose) reduction and aroma generation. Amount of cell and culture conditions were optimized prior to seeding to allow/help them to grow well and express their activity in moromi fermentation as observed. These inoculated yeasts seemed to produce volatile compounds responsible for specific aroma profile of this moromi that were significantly deferent from all traditional manufacturing as illustrated by PCA analysis (Figure 5). In traditional moromi, fermentations were conducted by yeasts naturally available in the systems. Thus, yeast profiles observed from both analytical methods

were relatively different. This finding might be resulted from the same factors as found in bacteria. *Saccharomycetales* were noticed in much higher proportion by RevT-nested PCR-DGGE than cultural plating in which only *Candida* observed as main yeast. For instance, main *S. cerevisiae* DGGE bands (Y8, Table 3) were observed in CC samples along with detection of two sterioisomers of higher alcohols 2/3-butanediol (al14 and al16), and 2-butanone, 3-hydroxy- (ke2, Figure 3.4) that were key metabolites of this yeast previously reported (Harada et al., 2018). The generation of these metabolites indicated/reflected the role of this yeast that would certainly express its activity during moromi fermentation over *Candida* species.

Disappearance of the *S. cerevisiae* from plating also might be a possible inhibitory effect of its own metabolite (i.e; octanoic acid (ac35) producing when cell entering harsh environment of high osmophilic stress such moromi (González et al., 2018). In addition, it was found that volatile profile of moromi samples from the similar yeast Rev-T-DGGE pattern certainly located in the same PCA cluster (Figure 5). This finding could support that genetic expression of yeast in the fermentation system possibly/mainly responsible for generation of specific moromi aroma characteristic. This point could be a criterion in further development of starter culture for use as volatile generation strains determining to aroma/flavor quality.

Even though the data of dominant species along with microbial community obtained from this study were similar to other previous report (Dolci et al., 2013), RNA-based DGGE technique could directly reflect metabolic activity of some microbes during moromi fermentation. Therefore, actual key microorganisms playing potential fermentation role(s) were fully depicted via combination of conventional plating and RevT-nested PCR DGGE determination. Derived databases of microbial gene expression could be used as DNA mapping in order to predict key

physicochemical properties of final soy sauce product. This included the generation of desired aroma profiles and browning mitigation scheme, which highly influenced by bacterial activity as previously explained in the results and would be discussed below. Accordingly, for the best of knowledge, this was the first time that microbial diversity in soy sauce was assessed by RevT-nested PCR-DGGE and exact metabolically active key microorganisms was illustrated.

To better understand the role of microbes in the traditional and industrial fermentation profile of moromi, chemical determinations were done and the relationship between microbes and the chemical changes during the fermentation were investigated. The results revealed that, general physiochemical properties of all moromi samples at initial stage of fermentation were closely similar. Their pH, TN and RS levels were in ranges of 4.8-5.0, 1.0-1.5 and 5.0-5.5 %, respectively, and also contained predominantly similar amount of initial FAA at 5.0 pmol/µl and glucose at 40.0 mg/ml, approximately. In exception, only PL sample contained RS and glucose with lower to around 1% and < 2.85 mg/ml, respectively.

The dynamic change patterns of all parameters in all samples were relatively similar in which TN and pH remained stable throughout the fermentation periods. Glucose in all samples excepted PL was reduced from initial level to middle phase by 8.56 – 23.45% through the final stage while FAA changes seemed to vary with both linear reduction and wave pattern were observed. From this investigation, it demonstrated significant role of microorganisms in moromi in term of population size and types, particularly, type of main microbe as observed on DGGE. Both factors likely to associate to significant/dynamic changes of glucose, RS and also FAA which all were the key factor involving in browning reaction.

As mentioned above, *Bacillus* and *Staphylococcus* were likely to associated moromi fermentation. In the batches that TPC of both bacteria and yeasts reduced throughout the fermentation stages, a slight reduction of glucose would be observed as seen in SP1 and SP2. While in the batches contained higher initial viable populations (NP) and/or slightly increased (CC) (Table 3), a shaper glucose reduction would be displayed (Figure 3A-E). This indicated that glucose in these batches could be utilized by the viable cells and/or the enzymes released that remained in the system to generate some metabolites rather than for cell generation, the same as microbial roles in aging process (Stahnke, 1999).

The microbial profiles as found on DGGE and their relation to dynamic changes of FAAs and sugars as found in this study rolled out a significant information to extend the knowledge in browning reaction taken place during moromi fermentation. It could be well observed that in the moromi fermentation with *Bacillus* and *Staphyloococcus* expressed as the first two main bacteria of the rank on DGGE, the fluctuated pattern of FAAs change would be observed (SP1 and NP, Appendix B) while the fermentation conducted with different bacterial pattern, particularly, leaded by lactic acid bacteria, FAAs would be displayed as linear reduction throughout the fermentation. It seemed that *Bacillus* and/or *Staphyloococcus* played role as main microbes to initiate moromi fermentation by further digestion of protein in soy mesh and yielded a certain amino acid profile predominant with Lue and Lys (Figure 4). The amino acids generated were further utilize along with re-generated throughout the fermentation as their concentration observed as fluctuated patterns. The metabolites obtained from these amino acid catabolisms of the microbes in moromi could be pyrazines (py1 – 8, Figure 5), 1-butanol, 3-methyl (al3), 1-butanal, 2/3-methyl (ad2, ad3), benzyl alcohol (ac17) (Lee et al., 2013; Song et al., 2015; Watanabe et al., 2015). However, while amino acids

were uptaken into microbial cell during the fermentation, partial FAAs were possibly reacted with sugar via Maillard to form browning products driving by several factors.

(i) The results as shown in Figure 3.5, reflected these relations and some interesting points, under similar fermentation condition (i.e, SP samples) in the fermentation batches that FAAs were reduced and re-generated throughout the fermentation (SP1), browning indexes level would be significantly lower relative to the batch with FAAs reduced linearly (SP2). These relations were also observed in traditional fermentation as browning index level of NP was lower than CC and PL. This demonstrated the significant role of *Bacillus* and/or *Staphyloococcus* expressed in the moromi system. In the system without an expression of these two bacteria, all FAAs were likely to freely (react to sugar via) enter to Maillard reaction and formed larger number of browning molecules while the batch containing active *Bacillus* and/or *Staphyloococcus*, Maillard reaction would generate from partial FAAs rested from catabolism process. As found in this investigation, maintaining of amino acids balance by utilization of microbial cell throughout fermentation could be one of principle in retarding and/or prevention of Maillard reaction. Thus, one of methodology in reduction or prevention of browning reaction, the conditions of moromi should be optimized for these autochthonous bacteria to well express along with maintaining their population to dominate the system throughout the fermentation in order to maintain FAA balance.

(ii) In Maillard reaction, sugars are also key compounds since they react to amino acids to form browning agents. In this study, glucose and xylose seemed to main sugars playing role in the Maillard reaction, as observed in CC and industrial samples and NP in which the initial glucose significantly reduced more than the other

batches, the significant increase of total browning were found. Xylose seemed to be another sugar impacted on Maillard reaction in moromi. As seen in Figure 6, reduction patterns of the xylose were related to the sharp increase of browning. This sugar might have more impact on browning intensity, since it was reported that reactivity of this pentose toward Maillard reaction was significantly higher than glucose (Laroque et al., 2008). Thus, removal of xylose from the system could be another methodology in decolorization of soy sauce. For arabinose, there were no relation between this sugar and Maillard reaction observed in this sugar.

(iii) In this study, temperature seemed to be another key factor catalyzing Maillard reaction. In figure 3.5, browning indexes as observed in traditional samples (NP, CC and PL) fermented under higher temperature (≥30°C) were totally much higher than industrial samples (SP1 and SP2) (21°C at initial stage, and 28-30°C afterward). This reflected temperature could drive reaction leading to rapidly formation of melanoidin compounds via promotion of active open-chain form reducing sugars (van Boekel, 2001) and cross-linking rate of amino with Maillard intermediates resulting in high molecular weight complex melanoidin (Lan et al., 2010). Thus, in prevention and/or retarding the Maillard reaction in moromi, apart from FAA balance, temperature control was also important. In addition, there were some interesting point observed in the traditional moromi fermentation NP. Although, browning compounds were largely initiated in the traditional moromi fermentation, sharp reduction of browning during middle throughout fermentation were occurred in which was not found in industrial process. In this process, browning compounds likely to be decomposed that could be promoted by enzymatic reactions from autochthonous flora present in the system (Kumar and Chandra, 2018). This finding seemed to correspond with the previous reports as just those

described, the significant decomposition of melanoidin was found in the traditional batch in which *Bacillus* and *Staphylococcus* spp. mainly expressed in the system.

Comparing to industrial production, strains of *Bacillus* and *Staphylococcus* in traditional moromi was certainly more diverse, it was therefore high potential to contain strains producing enzymes in oxidoreductase group such as laccase, and this enzymatic reaction was therefore performed to decompose melanoidin as observed. Considering of other key characteristics pf soy sauce, volatile profiles of NP was comparable with CC and its glutamate, an umami taste FAAs, was relatively higher than others. This finding is relatively novel and could be used as an efficient mean to simple decolorization of moromi. This point was further investigated by isolation of microbes from these moromi samples to find the microbes having key enzymatic properties as mentioned. Moromi-isolated *Bacillus* SSB6 and *Staphylococcus* SSB48 that could decompose melanoidin were obtained from these moromi batch that could support our observation and discussion (information shown in Chapter 4 and 5). These isolations potential to further develop as autochthonous starter for soy source de-coloring and/or light color soy sauce production.

CHAPTER 4

SCREENING OF POTENTIAL ISOLATES DECOLORIZABLE BROWNING IN SOY SAUCE

4.1 Introduction

As important information obtained from Chapter 1, various physicochemical including free amino acids (FAAs), reducing sugar species and temperature were significantly associated with browning in moromi. The Maillard mitigation approach, as proposed in Chapter 3, were FAA balancing and control of key reducing sugar through a specific activity of some microbes. Substantial findings from previous chapter of FAA utilization in generation of key aromas, and consumption of pentose simultaneously with glucose were noticed in traditional-process moromi containing as a result of metabolic activities of potential autochthonous microbe(s). Additionally, reduction of Maillard's product of browning pigment (melanoidin) found in NP samples indicated an unexpected activity of these autochthonous in degradation of melanoidin as well.

Considering feasibility of this microbial-based approach, this study aimed to screen potential autochthonous isolate(s) having xylose-utilization ability and/or melanoidin degradation activity from soy sauce origin for further application in soy source fermentation process to reduce browning color. All representative isolates from 3.2.2.1 were screened for their salt-tolerance and carbohydrate fermentation before some additional specific properties were examined including volatile profiles and biogenic amine productions.

4.2 Materials and methods

4.2.1 Screening for candidate strains

Representative colonies randomly picked by Harrison's disc method from 3.2.2.1 were further tested for their salt tolerance and carbohydrate utilization. Firstly, working culture of all isolates were transferred from agar tube into 1 ml of 1% peptone water supplemented with 3% NaCl and incubated overnight at 30°C. Absorbance of activated culture suspension at optical density of 600 (OD_{600}) was adjusted to 0.1 and further diluted for ten-times resulted in ready-to-use working culture suspension using in 4.2.1.1 and 4.2.1.2.

4.2.1.1 Salt tolerance properties

100 µl of diluted suspension was inoculated into 900 µl of 1% peptone broth supplemented with NaCl in range of 5, 10, 15, 20 and 25%. The mixtures were incubated at 30°C for 2 days and their growth was determined at OD600, both just before and after incubation (modified from (Feng et al., 2012). The experiment was done into 2 replicates and 1% peptone broth was used as a blank for spectrophotometry determination.

4.2.1.2 Carbohydrate utilization

Prepared working culture suspension was further serially diluted for 2 series and 10 ul of it was inoculated into 190 ul of phenol red carbohydrate broth (US FDA - BAM online: https://www.fda.gov/Food/FoodScienceResearch/Laboratory

Methods/ucm063494 .htm) supplemented with 0.5% of either glucose, arabinose or xylose using as screening media in 96-wells plate. Growth was observed by color changing from red to yellow after 2 days of incubation at room temperature.

4.2.1.3 Ability in browning compounds degradation

Representatives shown their growth in at 15% NaCl along with any pentose fermentable ability were selected as candidate isolates. All selected candidates from agar tube stock were activated in fresh 1% peptone water supplemented with 3% NaCl overnight before OD_{600} were adjust to 0.1 and used as working culture suspension.

Soy sauce broth (SSB) formulation and determination: SSB consisted of 1% peptone, 10% raw soy sauce from 6-months moromi, and 3% NaCl was applied as tested media for screening of total browning degradation ability. 10 µl of prepared working culture was mixed with 1,000 µl SSB in 2.0 ml Eppendorf tube and incubation at room temperature for 2 weeks before any solid part was separated from liquid by mean of centrifugation at 12,000 rpm, 4°C for 10 minutes. 200 ul of supernatant was pipetted into 96 wells-plate and spectrophotometry determined at absorbance of 420 nm.

Melanoidin synthesis and determination: melanoidin solution was synthesized from 2-hours refluxed mixture of 2 M D-xylose, with 2M glycine and 0.2 M $NaHC0_3$ and filtration sterilization (Murata et al., 1992). After that, melanoidin broth (MB) containing 1% peptone water, 3% NaCl and 1% synthesized melanoidin was prepared and screening of melanoidin degradation activity was examined using mixture of 10 µl working culture suspension and 1,000 µl of MB following same protocol as SSB.

4.2.2 Fermentation properties of candidate strains

4.2.2.1 Sugar utilization profiles

Candidates shown highest decolorization of browning indexes were selected as potential isolates and were determined for pentose - fermentable in the presence of hexose in term of glucose in the system. 0.45 µM filter-sterile of either glucose, xylose or arabinose were diluted into 1% peptone water to their final concentration of 1%. 3 ml of each broth were then inoculated with 30 µl of activated cell suspension of potential isolates prepared as stated in 4.2.1 and incubated at room temperature for 2 days then subjected to HPLC analysis of reducing sugar species following protocol stated in 3.2.4.2.

4.2.2.2 Volatiles profiles

Ten grams of moromi from industrial process was mixed with 100 µl of freshly prepared following 4.2.1 procedure in 20 ml amber and tightly sealed bottle and incubated at room temperature for 2 weeks. 3 grams of inoculated moromi was then transferred into 20 ml. headspace vial sealing by HDSP magnetic cap and volatile profile was determined by SPME GC-MS following 3.2.4.2. Chromatogram profiles were analyzed and illustrated in heat map visualization (4.2.4).

4.2.3 Safety test of candidate strains

For food safety information, biogenic amine (BA) genes were targeted in this study. DNA extracted from 5 potential isolates were prepared by freeze-thawing method following 3.2.3 and use as templates for multiplex PCR. Seven primer pairs targeting various biogenic amine encoding genes were listed as shown in Table 4. Two multiplex PCR reactions were applied for this test. The first one consisted of 4 primer pairs mixture (TD2/5 + TD-F/R + PUT2-F/R + HDC 3 / 4) and the second of 3 pairs (PUT1-F/R + TDC 1 / 2 + JV16HC/17HC). PCR reaction was as stated in 3.2.2.2.2

4.2.4 Statistical analysis

Data of Table 7, were subjected to two-way analysis of variance (Carabasa et al.) to evaluate significant difference of mean within factors using the IBM SPSS® software (version 20, IBM, USA). The Fisher Least Significant Difference (LSD) test along with Duncan were used for multiple comparisons of the factors with the level of significance fixed at 5%.

Hierarchical Clustering (HCL) with distance metric of Pearson correlation and agglomerative type were used to calculating clustered dendrogram and results were displayed by heatmap. Online application Multi Experiment Viewer (MeV) version 4.8.0 were used to analyze HCL of volatile profiles in this study (http://mev.tm4.org/#/welcome).

Table 4 Primers used in detection of biogenic amine-encoding genes

No.	Size (BPs)	Primers	Sequence (5'- to -3')	Target BA	References
1	367	JV16HC	AGA TGG TAT TGT TTC TTA TG	Histamine	(Landete et al., 2007)
		JV17HC	AGA CCA TAC ACC ATA ACC TT	Histamine	
2	435	HDC3	GAT GGT ATT GTT TCK TAT GA	Histamine	(Coton et al., 2010)
		HDC4	CCA AAC ACC AGC ATC TTC	Histamine	
3	1100	TD2	ACA TAG TCA ACC ATR TTG AA	Tyramine	
		TD5	CAA ATG GAA GAA GAA GTA GG	Tyramine	
4	720	TDC1	AAC TAT CGT ATG GAT ATC AAC G	Tyramine	(Landete et al., 2007)
		TDC2	TAG TCA ACC ATA TTG AAA TCT GG	Tyramine	
5	825	TDC-F	TGG YTN GTN CCN CAR ACN AAR CAY TA	Tyramine	
		TDC-R	ACR TAR TCN ACC ATR TTR AAR TCN GG	Tyramine	
6	1440	PUT1-F	TWY MAY GCN GAY AAR CAN TAY YYT GT	Putrescine	
		PUT1-R	ACR CAN AGN ACN CCN GNG GRT ANG G	Putrescine	
7	624	PUT2-F	ATH WGN TWY GGN AAY ACN ATH AAR AA	Putrescine	
		PUT2-R	GCN ARN CCN CCR AAY TTN CCD ART C	Putrescine	

4.3 Results and discussions

Representative from cultural plating of moromi during fermentation selected by Harrison's disk method included 139 bacteria and 105 yeast colonies were further screened for their salt tolerance, reducing sugar utilization and melanoidin degradation ability by means of selective medium to verify decolorization activities based on substrate utilization and degradation of final product. The selected potential isolates were tested for safety aspect before evaluation of autochthonous decolorization process by co-inoculation technique were done in moromi simultaneously with determination of key chemical elements.

4.3.1 Growth in various salt concentrations

The growth of representatives in various salt concentration were observed by turbidity increment of growth media via spectrophotometry. When series of media without inoculation were set as blank, samples shown OD_{600} value of 0.001-0.009 were interpreted as +, 0.01 – 0.05 were ++, and values higher than 0.05 were considered as +++ in order to approximately grouping their growth into 3 classes of 0 to 4 logCFU/g., 4 – 6 logCFU/g., and higher than 6 logCFU/g., respectively (Dalgaard et al., 1994).

By this interpretation, results from Table 5 revealed 121 bacteria, calculated as 87.05%, and Table 6 shown 97 yeast (92.38%) that could grow in high NaCl concentration of 15%. The isolates obtained from both soya sauce were mainly highly halophilic bacteria showing growth at 5% salt concentration and over (DasSarma, 2006). When salt concentration increased, lower number of representatives survived especially at 25% where more than half of bacteria broadly

died. Contrastingly, yeast that could growth in this extreme salt concentration were detected in much higher percentage (60.00%) than bacteria (30.94%). In fact, from 15 moromi samples, 12 were contained 25% NaCl growthable yeast with the percentage ≥ 50. It was clearly seen from Table 5 that all *Zygosaccharomyces rouxii*, together with other yeast of *Candida halophila* and *Millerozyma farinosa* could tolerate extreme NaCl which was the same as previously report of Dakal, Solieri and Giudici in 2014 which classified these yeast as halotolerance . The overall representatives selected due to their salt tolerance at concentration ≥15% were 122 bacteria and 97 yeast.

4.3.2 Key carbohydrate utilizations

Ability in utilization of tested strains in each reducing sugars (glucose, xylose and arabinose) was tested. Results were illustrated in Table 5 and 6. All tests showing shift in color from orange red to yellow were marked as “+”.

Glucose was generally fermented by almost bacteria and yeast representatives. Nevertheless, *Bacillus subtilis* strain SSB21 isolated from NPI and NPM moromi do not shown its color changing to yellow toward 1% glucose but became bright pink indicated the negative (-) result or no ability in glucose utilization. This color was the result of phenol red indicator in its conjugate base form and indicated ≥ 8.2 pH condition which should be due to metabolites produced and/or released by this *B. subtilis*.

Bacteria shown broadly good pentose fermentable activity comparing with yeast where only half of them revealed this property. Total of 111 (79.86%) and 107 (76.98%) bacterial representatives obtained arabinose and xylose utilization ability,

respectively, while it was 55 representative yeast for arabinose (52.38%) and 56 (53.33%) for xylose. As this experiment was done in 96 wells microplate and static incubation where oxygen was limited (Duetz et al., 2000) to promote anaerobic fermentation was promoted. This low oxygen environment affected enzyme cofactor catalyzing xylose metabolism in yeast and fermentation was inhibited (Harner et al., 2015). Therefore, lower amount of xylose (and might include arabinose)-fermentable yeast was noticed.

Among bacteria, percentage of pentose – fermentable bacterial in PL moromi were remarkable low than others which should be due to condition that representatives were isolated. Pl process included removal of soybean mash at 7 weeks after moromi fermentation resulted in very low concentration of these pentose in PL moromi, thus microorganisms isolated from this environment might not familiar with pentose.

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates.

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP1I	1.1	G	G				+	+	+	N/A	N/A	*Bacillus* sp. strainSSB13
SP1I	1.2	G	G	R	R	R	+	+	+	2.405 ± 0.08	2.215 ± 0.04	*Bacillus amyloliquefaciens* strain SSB2
SP1I	1.3	G	G	R			+	+	+	2.507 ± 0.03	NG	*Bifidobacterium aoulatum*
SP1I	1.4	G	G	F	F	F	+	+	+	2.271 ± 0.08	2.306 ± 0.32	*Staphylococcus* sp. SSB43
SP1I	1.5	G	G	G	R		+	+	+	2.976 ± 0.03	2.173 ± 0.12	*Staphylococcus* sp. SSB47
SP1I	1.6	G	G	F	F	F	+	+	+	2.271 ± 0.03	2.306 ± 0.38	*Staphylococcus* sp. SSB43
SP1I	1.7	G	F	R	F	F	+	+	+	2.408 ± 0.03	NG	*Staphylococcus* sp. SSB42
SP1I	1.8	G	G				+	+	+	N/A	N/A	*Staphylococcus* sp. SSB50
SP1I	1.9	G	F	R			+	+	+	2.329 ± 0.03	NG	*Bacillus subtilis* strain SSB19
SP1I	1.10	G	F	R			+	+	+	2.329 ± 0.07	NG	*Bacillus subtilis* strain SSB19
SP1I	1.11	G	F	R			+	+	+	2.329 ± 0.09	NG	*Bacillus subtilis* strain SSB19
SP1M	3.1	G	G	R			+			N/A	N/A	*Lactobacillus salivarius*
SP1M	3.2	G	R				+	+	+	N/A	N/A	*Bacillus* sp. strainSSB13
SP1M	3.3	G	G	G	F		+	+	+	2.388 ± 0.02	2.455 ± 0.08	*Bacillus* sp. strainSSB13
SP1M	3.3.1	G	G	F	F	F	+	+	+	2.535 ± 0.03	2.878 ± 0.03	*Staphylococcus carnosus* strain SSB32
SP1M	3.4	G	F	R			+	+	+	2.203 ± 0.02	2.314 ± 0.02	*Staphylococcus condimenti* strain SSB36

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP1M	3.4.2	G	F	R	F	F	+	+	+	2.408 ± 0.05	2.511 ± 0.03	*Staphylococcus* sp. SSB42
SP1F	5.1	G	F	R			+	+	+	2.271 ± 0.09	2.362 ± 0.07	*Staphylococcus carnosus* strain SSB30
SP1F	5.2	G	G	G	F		+	+	+	2.388 ± 0.11	2.455 ± 0.08	*Bacillus* sp. strainSSB13
SP1F	5.2.1	G	F				+	+		N/A	N/A	*Virgibacillus proomii*
SP1F	5.2.2	G	G				+			N/A	N/A	*Staphylococcus condimenti* strain SSB40
SP1F	5.3	G	F	F	F	F	+	+	+	2.842 ± 0.20	2.405 ± 0.14	*Staphylococcus carnosus* strain SSB34
SP1F	5.4	G	F	F	F	F	+	+	+	2.842 ± 0.06	2.405 ± 0.04	*Staphylococcus carnosus* strain SSB34
SP1F	5.5	G	F	R			+	+	+	2.277 ± 0.06	2.352 ± 0.04	*Staphylococcus carnosus* strain SSB33
SP1F	5.6	G	G	F	F	F	+	+	+	2.535 ± 0.03	2.878 ± 0.02	*Staphylococcus carnosus* strain SSB32
SP1F	5.8	G	G	G	F	F	+	+	+	2.158 ± 0.05	2.414 ± 0.03	*Staphylococcus condimenti* strain SSB41
SP1F	5.9	G	G	G	F	F	+			N/A	N/A	*Lactobacillus curvatus* SSB26
SP1F	5.10	G	F				+	+	+	N/A	N/A	*Streptomyces* sp.
SP2I	8.1	G	G	R			+	+	+	2.388 ± 0.01	2.455 ± 0.01	*Bacillus* sp. strainSSB13
SP2I	8.2	G	G	F	R		+	+	+	2.154 ± 0.07	2.424 ± 0.05	*Bacillus amyloliquefaciens* strain SSB3
SP2I	8.3	G	F	F	R		+	+	+	3.004 ± 0.02	2.979 ± 0.01	*Virgibacillus* sp. strain SSB54

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP2I	8.4	G	G	R			+	+	+	3.126 ± 0.09	2.386 ± 0.07	*Virgibacillus halophilus*
SP2I	8.5	G	G	F	R	R	+	+	+	2.405 ± 0.07	2.125 ± 0.04	*Bacillus amyloliquefaciens* strain SSB2
SP2I	8.6	G	G	R			+	+	+	2.773 ± 0.08	NG	*Bacillus subtilis* strain SSB16
SP2I	8.7	G	G	F	R		+	+	+	2.154 ± 0.05	2.424 ± 0.05	*Bacillus amyloliquefaciens* strain SSB3
SP2I	8.8	G	G	F	R	R	+	+	+	2.405 ± 0.07	2.125 ± 0.04	*Bacillus amyloliquefaciens* strain SSB2
SP2I	8.9	G	G	R			+	+	+	2.889 ± 0.04	2.406 ± 0.04	*Virgibacillus* sp. strain SSB55
SP2I	8.10	G	G	R	F		+	+	+	2.271 ± 0.02	2.418 ± 0.08	*Virgibacillus* sp. strain SSB56
SP2I	8.11	G	G	R			+	+	+	NG	NG	*Pseudomonas sp.*
SP2I	8.12	G	G	R			+	+	+	2.388 ± 0.02	2.455 ± 0.08	*Bacillus* sp. strainSSB13
SP2I	8.13	G	G	R			+	+	+	2.773 ± 0.08	NG	*Bacillus subtilis* strain SSB17
SP2M	10.1	G	G	G	F	F	+			N/A	N/A	*Bacillus siamensis*
SP2M	10.2	G	G				+	+	+	N/A	N/A	*Bacillus methylotrophicus*
SP2M	10.3	G	G				+			N/A	N/A	*Streptococcus thermophilus*
SP2M	10.4	G	G	F			+			N/A	N/A	*Trichococcus* sp. strain SSB52
SP2M	10.4.1	G	F	R			+	+	+	2.203 ± 0.02	2.314 ± 0.02	*Staphylococcus condimenti* strain SSB37
SP2M	10.5	G	R	R			+	+	+	2.081 ± 0.08	2.291 ± 0.11	*Staphylococcus* sp. SSB51

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP2F	27.1	G	G	F	R		+	+	+	2.447 ± 0.10	NG	*Bacillus licheniformis*
SP2F	27.2	G	G	F	F	F	+			N/A	N/A	*Trichococcus* sp. strain SSB52
SP2F	27.3	G	G	F	R		+	+	+	2.447 ± 0.10	NG	*Bacillus licheniformis*
SP2F	27.4	G	G	R			+	+	+	2.298 ± 0.11	2.496 ± 0.09	*Staphylococcus* sp. SSB44
SP2F	27.5	G	G	G	G	G	+	+		2.279 ± 0.07	2.269 ± 0.08	*Staphylococcus carnosus* strain SSB31
SP2F	27.6	G	G	R			+	+	+	2.683 ± 0.19	3.426 ± 0.10	*Bacillus* sp. strainSSB12
SP2F	27.7	G	G	R			+	+	+	2.605 ± 0.12	NG	Unidentified bacterium strain SSB25
SP2F	27.8	G	G	G	G	F	+	+	+	2.295 ± 0.05	2.283 ± 0.10	*Staphylococcus carnosus* strain SSB35
SP2F	27.9	G	G	G	F	F	+	+	+	2.459 ± 0.10	NG	*Lactobacillus plantarum*
SP2F	27.10	G	G				+	+	+	N/A	N/A	*Bacillus megaterium*
SP2F	27.11	G	G	F	F		+			N/A	N/A	*Pediococcus* sp.
CCI	12-1	G	F	R			+	+	+	2.277 ± 0.11	2.364 ± 0.07	*Bacillus amyloliquefaciens* strain SSB5
CCI	12-2	G	G	G	G		+	+	+	2.602 ± 0.14	2.405 ± 0.19	*Staphylococcus* sp. SSB51
CCI	12-3	G	G	F			+	+		2.311 ± 0.11	2.423 ± 0.09	*Staphylococcus* sp. SSB49
CCI	12.4	G	G	F	R		+	+	+	2.471 ± 0.06	3.287 ± 0.13	*Bacillus* sp. strainSSB10
CCI	12.5	G	G	R			+	+	+	2.984 ± 0.13	2.283 ± 0.12	*Bacillus subtilis* strain SSB22

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
CCI	12.6	G	G	R			+	+	+	3.143 ± 0.12	NG	*Enterobacter* sp.
CCM	13.1	G	G	F	R	R	+	+	+	2.405 ± 0.07	2.125 ± 0.04	*Bacillus amyloliquefaciens* strain SSB2
CCM	13.2	G	G	R			+	+	+	2.984 ± 0.13	2.125 ± 0.04	*Bacillus subtilis* strain SSB22
CCM	13.3	G	G	F	R	R	+	+	+	2.405 ± 0.07	2.125 ± 0.04	*Bacillus amyloliquefaciens* strain SSB2
CCM	13.4	G	G	G	G		+	+	+	2.602 ± 0.14	2.405 ± 0.19	*Staphylococcus* sp. SSB51
CCM	13.5	G	G	F			+			N/A	N/A	*Staphylococcus* sp. SSB51
CCM	13.6	G	F	F			+			N/A	N/A	*Bacillus* sp. strain SSB14
CCM	13.7	G	G	F			+			N/A	N/A	*Staphylococcus* sp. SSB51
CCM	13.8	G	G	F	R	R	+	+	+	1.657 ± 0.08	2.424 ± 0.08	*Staphylococcus* sp. SSB48
CCM	13.9	G	G	F			+			N/A	N/A	Unidentified bacterium strain SSB24
CCF	14.1	G	F	R			+	+	+	2.255 ± 0.14	2.358 ± 0.19	*Bacillus subtilis* strain SSB20
CCF	14-2	G	G	G	G		+	+	+	2.602 ± 0.14	2.405 ± 0.19	*Staphylococcus* sp. SSB51
CCF	14.3	G	G	R			+			N/A	N/A	*Staphylococcus* sp. SSB51
CCF	14.4	G	G	F			+	+	+	2.724 ± 0.03	2.424 ± 0.07	*Bacillus subtilis* strain SSB18
CCF	14.5	G	F	R			+	+	+	2.277 ± 0.11	2.364 ± 0.07	*Bacillus amyloliquefaciens* strain SSB5
CCF	14.6	G	G	R			+	+	+	2.984 ± 0.13	2.291 ± 0.13	*Bacillus subtilis* strain SSB22

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
CCF	14.7	G	G	F			+			N/A	N/A	*Bacillus subtilis* strain SSB22
CCF	14.8	G	G	F			+			N/A	N/A	*Bacillus amyloliquefaciens* strain SSB1
CCF	14.9	G	G	F	R	R	+	+	+	2.405 ± 0.07	2.462 ± 0.23	*Bacillus amyloliquefaciens* strain SSB6
CCF	14.10	G	G	G	G		+	+	+	2.602 ± 0.02	2.405 ± 0.05	*Staphylococcus* sp. SSB51
CCF	14.11	G	F	R			+	+	+	2.684 ± 0.06	2.234 ± 0.07	*Bacillus subtilis* strain SSB18
CCF	14.12	G	G	F	R	R	+	+	+	2.405 ± 0.07	2.125 ± 0.04	*Bacillus amyloliquefaciens* strain SSB2
NPI	15-1	G	G	R	R	R	+	+	+	2.507 ± 0.03	3.275 ± 0.08	*Bacillus* sp. strainSSB11
NPI	15-2	G	G	R			+	+	+	2.66 ± 0.04	2.291 ± 0.10	*Bacillus zhaozhouensis* strain SSB23
NPI	15.3	G	G	R	R	F	+	+	+	2.634 ± 0.04	3.502 ± 0.02	*Bacillus pumilus* strain SSB8
NPI	15.4	G	G	F			+			N/A	N/A	*Staphylococcus* sp. SSB45
NPI	15.5	G	G	F			+	+	+	2.376 ± 0.04	3.32 ± 0.09	*[Brevibacterium] frigoritolerans*
NPI	15.6	G	F	R			+	+	+	2.203 ± 0.02	2.314 ± 0.02	*Staphylococcus condimenti* strain SSB38
NPI	15.7	G	G	G	F	R		+	+	2.211 ± 0.07	NG	*Bacillus subtilis* strain SSB21
NPI	15.8	G	G	G	F	R		+	+	2.211 ± 0.07	NG	*Bacillus subtilis* strain SSB21
NPI	15.9	G	G	G	F	R		+	+	2.211 ± 0.07	NG	Bacillus subtilis strain SSB21
NPI	15.10	G	G	F	R		+			N/A	N/A	*Lactobacillus curvatus* SSB29

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
NPM	16.1	G	G	R			+	+	+	2.66 ± 0.04	2.291 ± 0.10	*Bacillus zhaozhouensis* strain SSB23
NPM	16.2	G	G	F	F	F	+	+	+	2.904 ± 0.08	2.276 ± 0.06	*Bacillus subtilis* strain SSB17
NPM	16.3	G	G	F			+			N/A	N/A	*Staphylococcus* sp. SSB45
NPM	16.4	G	G	G	G	F	+	+	+	2.295 ± 0.05	2.283 ± 0.10	*Staphylococcus carnosus* strain SSB35
NPM	16.5	G	G	F			+	+	+	2.784 ± 0.03	2.387 ± 0.13	*Lactobacillus graminis*
NPM	16.6	G	G	F			+	+	+	2.777 ± 0.03	2.54 ± 0.07	*Lactobacillus curvatus* SSB28
NPF	17.1	G	G	F	F	F	+	+	+	2.271 ± 0.02	2.356 ± 0.05	*Trichococcus* sp. strain SSB53
NPF	17.2	G	F	F	R		+	+	+	3.004 ± 0.01	2.979 ± 0.14	*Virgibacillus* sp. strain SSB54
NPF	17.3	G						+	+	N/A	N/A	*Bacillus subtilis* strain SSB21
NPF	17.3.1	G	G	F	F	F	+	+	+	1.678 ± 0.15	2.419 ± 0.02	*Bacillus amyloliquefaciens* strain SSB6
NPF	17.4	G	G	F	F	F	+			N/A	N/A	*Trichococcus* sp. strain SSB52
NPF	17.5	G	G	G	G	F	+	+	+	2.241 ± 0.02	2.356 ± 0.07	*Bacillus amyloliquefaciens* strain SSB4
NPF	17.6	G	G	G	G	F	+	+	+	2.295 ± 0.05	2.283 ± 0.10	*Staphylococcus carnosus* strain SSB35
NPF	17.7	G	G	F	F	F	+	+	+	2.864 ± 0.06	2.306 ± 0.03	*Bacillus* sp. strain SSB15
NPF	17.8	G	G	F			+	+	+	2.277 ± 0.11	2.364 ± 0.07	*Bacillus amyloliquefaciens* strain SSB5
NPF	17.9	G	G	R	R		+	+	+	2.864 ± 0.06	2.306 ± 0.03	*Bacillus* sp. strain SSB15

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
NPF	17.10	G	G	F	R	R	+	+	+	2.405 ± 0.07	2.125 ± 0.04	*Bacillus amyloliquefaciens* strain SSB2
PLI	18.1	G	G	R			+	+	+	2.271 ± 0.07	NG	*Lactobacillus curvatus* SSB27
PLI	18.2	G	G				+	+	+	N/A	N/A	*Bacillus coagulans*
PLI	18.3	G	G	R			+	+	+	2.354 ± 0.05	2.301 ± 0.03	*Bacillus* sp. strain SSB9
PLI	18.4	G	G	R			+			N/A	N/A	*Bacillus marisflavi*
PLI	18.5	G	G				+	+	+	N/A	N/A	*Bacillus coagulans*
PLI	18.6	G	G	G	F	F	+	+	+	3.106 ± 0.07	NG	*Lactobacillus curvatus* SSB27
PLI	18.7	G	G	R			+	+	+	2.199 ± 0.11	3.074 ± 0.07	*Bacillus aerolacticus*
PLI	18.8	G	G	G	R		+	+	+	3.246 ± 0.06	2.344 ± 0.08	*Staphylococcus* sp. SSB46
PLI	18.9	G	F	R	R		+	+	+	2.267 ± 0.02	2.364 ± 0.09	*Staphylococcus epidermidis*
PLI	18.10	G	G	G	F	F	+	+	+	3.106 ± 0.07	NG	*Staphylococcus xylosus*
PLI	18.11	G	G	R			+			N/A	N/A	Unidentified bacterium strain SSB25
PLI	18.12	G	G	R			+			N/A	N/A	Unidentified bacterium strain SSB25
PLI	18.13	G	G	R			+			N/A	N/A	Unidentified bacterium strain SSB25
PLM	19.1	G	G	G	R		+	+	+	3.246 ± 0.06	2.344 ± 0.08	*Staphylococcus* sp. SSB46
PLM	19.1.1	G	F	R	R		+	+	+	2.267 ± 0.02	2.364 ± 0.09	*Staphylococcus epidermidis*

Table 5 Halotolerant and carbohydrate fermentation proportion of representative bacterial isolates (Continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
PLM	19.2	G	F	R			+	+	+	2.203 ± 0.02	2.314 ± 0.02	*Staphylococcus condimenti* strain SSB39
PLM	19.3	G	F				+	+	+	N/A	N/A	*Bacillus marisflavi*
PLM	19.4	G	R	R			+	+	+	2.354 ± 0.05	2.301 ± 0.03	*Bacillus* sp. strain SSB9
PLM	19.5	G	G				+	+		N/A	N/A	*Oenococcus* sp.
PLM	19.6	G	G				+			N/A	N/A	*Oenococcus* sp.
PLM	19.7	G	G				+			N/A	N/A	*Oenococcus* sp.
PLM	19.8	G	F	F			+			N/A	N/A	*Bacillus pumilus* strain SSB7
PLM	19.9	G	G	G	F	F	+			N/A	N/A	*Lactobacillus curvatus* SSB27
PLF	20.1	G	G				+	+	+	N/A	N/A	*Staphylococcus succinus*
PLF	20.1.1	G	G				+	+	+	N/A	N/A	*Bacillus* sp. strain SSB9
PLF	20.3	G	G	G	F	F	+			N/A	N/A	*Lactobacillus curvatus* SSB27
PLF	20.4	G	G	F	R		+	+	+	2.354 ± 0.05	2.354 ± 0.05	*Bacillus infantis*

N/A = not available NG = no growth

* + = rarely growth ++ = fair growth +++ = good growth

**+ = positive results; G= glucose; A=arabinose; X= xylose ; P = bright pink color

Table 6 Halotolerance and carbohydrate fermentation proportion of representative yeast isolates.

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP1I	1.1	G	F	R	F		+			N/A	N/A	*Pichia mexicana* strain SSY15
SP1I	1.1.1	G	G		F	R	+			N/A	N/A	*Pichia* sp.
SP1I	1.2	G	G	G			+			N/A	N/A	*Yamadazyma mexicana*
SP1I	1.3	G	G	G	R	R	+		+	2.836 ± 0.09	2.418 ± 0.03	*Kodamaea ohmeri* strain SSY12
SP1I	1.4	G	G	F	F	F	+	+	+	2.451 ± 0.03	2.699 ± 0.02	*Candida* sp. strain SSY5
SP1I	1.5	G	G	R	R	R	+	+	+	2.206 ± 0.02	2.1985 ± 0.15	*Candida atlantica*
SP1I	1.6	G	G	G	F	R	+	+	+	2.26 ± 0.14	2.302 ± 0.00	*[Candida] amphicis*
SP1I	1.7	G	G	G	F	R	+	+	+	2.26 ± 0.14	2.302 ± 0.00	*[Candida] amphicis*
SP1M	3.1	G	G	R	R	R	+	+	+	2.61 ± 0.35	2.79 ± 0.06	*Zygosaccharomyces rouxii* strain SSY27
SP1M	3.2	G	G	G	F	F	+	+	+	2.347 ± 0.07	2.397 ± 0.09	*Zygosaccharomyces rouxii* strain SSY24
SP1M	3.3	G	G	G	F	R	+	+	+	2.26 ± 0.14	2.302 ± 0.00	*[Candida] amphicis*
SP1M	3.4	G	G	F		R	+			N/A	N/A	*Pichia mexicana* strain SSY16
SP1F	5.1	G	G	G	G	G	+	+	+	2.5 ± 0.15	2.7515 ± 0.05	Candida versatilis strain SSB6
SP1F	5.2	G	G	F	F	R	+			N/A	N/A	*Candida versatilis* strain SSB7
SP1F	5.3	G	G	F	F	R	+			N/A	N/A	*Wickerhamiella versatilis* strain SSY21
SP1F	5.4	G	G	R	R		+	+	+	2.61 ± 0.35	2.79 ± 0.06	*Zygosaccharomyces rouxii* strain SSY27

Table 6 Halotolerance and carbohydrate fermentation proportion of representative yeast isolates (continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP1F	5.5	G	G				+	+	+	N/A	N/A	*Saccharomyces cerevisiae* strain SSY17
SP1F	5.6	G	G				+			N/A	N/A	*Saccharomycodes ludwigii*
SP2I	8.1	G	G	R			+	+	+	2.274 ± 0.06	2.284 ± 0.03	*Candida parapsilosis* strain SSY1
SP2I	8.1.1	G	G	R			+	+	+	2.274 ± 0.06	2.284 ± 0.03	*Candida parapsilosis* strain SSY1
SP2I	8.1.2	G	G	R	R		+	+	+	2.713 ± 0.13	2.7435 ± 0.01	*Candida pseudojiufeoensis*
SP2I	8.2	G	G	R			+	+	+	2.274 ± 0.06	2.326 ± 0.04	*Candida parapsilosis* strain SSY2
SP2I	8.3	G	G	F	R	R	+	+	+	2.289 ± 0.06	3.048 ± 0.07	*Zygosaccharomyces rouxii* strain SSY23
SP2I	8.4	G	G	F	R	R	+	+	+	2.289 ± 0.06	3.048 ± 0.07	*Zygosaccharomyces rouxii* strain SSY23
SP2I	8.5	G	G	F	R	R	+	+	+	2.356 ± 0.35	2.282 ± 0.03	*Zygosaccharomyces rouxii* strain SSY26
SP2I	8.6	G	G	F	R	R	+	+	+	2.61 ± 0.35	2.79 ± 0.06	*Zygosaccharomyces rouxii* strain SSY27
SP2I	8.7	G	G	R			+			N/A	N/A	Unidentified fungal sp. strain SSY9
SP2I	8.8	G	G	G	R		+	+	+	3.174 ± 0.07	2.85 ± 0.03	*Candida mannitofaciens*
SP2I	8.9	G	G	R			+	+	+	2.274 ± 0.06	2.284 ± 0.03	*Candida parapsilosis* strain SSY1
SP2I	8.10	G	F	F	F		+			N/A	N/A	*Pichia mexicana* strain SSY15
SP2I	8.11	G	G	R	R	R	+	+	+	N/A	N/A	*Debaryomyces hansenii*
SP2I	8.12	G	G	R	R		+			N/A	N/A	*Pichia membranifaciens*

Table 6 Halotolerance and carbohydrate fermentation proportion of representative yeast isolates (continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP2I	8.13	F	G	G			+			N/A	N/A	*Pichia* sp.
SP2I	8.14	G	G		F	R	+			N/A	N/A	*Yamadazyma mexicana*
SP2M	10.1	G	G	G	G	G	+			N/A	N/A	*Candida versatilis* strain SSB6
SP2M	10.2	G	G	G	R		+	+	+	3.174 ± 0.07	2.85 ± 0.03	*Candida mannitofaciens*
SP2M	10.3	G	G	F	F	R	+			N/A	N/A	*Wickerhamiella versatilis* strain SSY21
SP2M	10.4	G	G	F	F	R	+			N/A	N/A	*Candida halophila*
SP2M	10.5	G	G	F	F	F	+			N/A	N/A	*Candida halophila*
SP2M	10.6	G	G	G	R	R	+			N/A	N/A	*Saccharomyces cerevisiae* strain SSY18
SP2M	10.7	G	G	G	F	R	+			N/A	N/A	*Candida apicola*
SP2F	27.1	G	G	G	F	R	+		+	2.78 ± 0.04	2.3525 ± 0.04	*Candida apicola*
SP2F	27.2	G	G	G	F	R	+			N/A	N/A	*Candida apicola*
SP2F	27.3	G	G	F	F	F	+			N/A	N/A	*Candida halophila*
SP2F	27.4	G	G	G	R		+	+	+	3.174 ± 0.07	2.85 ± 0.03	*Candida mannitofaciens*
SP2F	27.5	G	G	G	R	R	+			N/A	N/A	*Saccharomycodes ludwigii*
SP2F	27.6	G	G				+			N/A	N/A	*Saccharomyces cerevisiae* strain SSY18
SP2F	27.7	G	G	R		R	+			N/A	N/A	*Saccharomyces cerevisiae* strain SSY18

Table 6 Halotolerance and carbohydrate fermentation proportion of representative yeast isolates (continued).

Source	Code	Salt tolerance					Carbohydrate utilization*			Decolorization		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
SP2F	27.6	+++	+++				+			N/A	N/A	*Saccharomyces cerevisiae* strain SSY18
SP2F	27.9	+++	+++				+			N/A	N/A	*Saccharomycodes ludwigii*
CCI	12.1	+++	+++	+++	+	+	+		+	NG	NG	[Candida] gorgasii
CCI	12.2	+++	+++	++	++		+	+		2.333 ± 0.21	2.273 ± 0.11	*[Candida] gorgasii*
CCI	12.3	+++	+++	++	++		+	+	+	2.333 ± 0.21	2.273 ± 0.11	*Candida halophila*
CCI	12.4	+++	+++	++	++	++	+			N/A	N/A	*Wickerhamiella versatilis* strain SSY22
CCI	12.5	+++	+++	++	++	++	+			N/A	N/A	*Zygosaccharomyces rouxii* strain SSY28
CCI	12.6	+++	+++	+	++	++	+	+	+	2.339 ± 0.04	2.379 ± 0.02	*Zygosaccharomyces* sp.
CCI	12.7	+++	+++	+		+	+	+	+	2.377 ± 0.07	2.342 ± 0.04	*Kodamaea ohmeri* strain SSY11
CCM	13.1	+++	+++	++	++	++	+			N/A	N/A	*Candida halophila*
CCM	13.2	+++	+++	++	++	++	+			N/A	N/A	*Candida halophila*
CCM	13.3	+++	+++	+	++	++	+	+	+	2.339 ± 0.04	2.379 ± 0.02	*Zygosaccharomyces rouxii* strain SSY28
CCF	14.1	+++	+++	++	++		+	+		2.333 ± 0.07	2.273 ± 0.05	*[Candida] gorgasii*
CCF	14.1	+++	+++	++	++	++	+			N/A	N/A	*Candida mannitofaciens*
CCF	14.1	+++	+++	++	++	+	+			N/A	N/A	*[Candida] gorgasii*

Table 6 Halotolerance and carbohydrate fermentation proportion of representative yeast isolates (continued).

Source	Code	Salt tolerance					Carbohydrate utilization*			Decolorization		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
CCF	14.2	+++	+++	+++	+		+	+	+	3.174 ± 0.06	2.850 ± 0.04	*Candida halophila*
CCF	14.3	+++	+++	++	++		+	+		2.333 ± 0.04	2.273 ± 0.02	*Zygosaccharomyces rouxii* strain SSY28
CCF	14.4	+++	+++	++	++	++	+			N/A	N/A	*Zygosaccharomyces* sp.
CCF	14.5	+++	+++	+	++	++	+	+	+	2.339 ± 0.07	2.379 ± 0.04	*Zygosaccharomyces* sp.
CCF	14.6	+++	+++	+		+	+	+	+	2.377 ± 0.21	2.342 ± 0.11	*[Candida] gorgasii*
CCF	14.7	+++	+++	+		+	+	+	+	2.377 ± 0.06	2.342 ± 0.04	*Candida halophila*
CCF	14.8	+++	+++	++	++		+	+	+	2.333 ± 0.21	2.273 ± 0.11	*Wickerhamiella versatilis* strain SSY22
CCF	14.9	+++	+++	++	++	++	+			N/A	N/A	*Wickerhamiella versatilis* strain SSY21
NPI	15.1	+++	+++	++	+	+	+	+	+	2.289 ± 0.06	3.048 ± 0.07	*Zygosaccharomyces rouxii* strain SSY23
NPI	15.2	+++	+	++	++	+	+	+	+	2.423 ± 0.09	2.475 ± 0.06	*Zygosaccharomyces rouxii* strain SSY25
NPI	15.3	+++	+++	+++	+	+	+		+	NG	NG	*Kodamaea ohmeri* strain SSY10
NPM	16.1	+++	+++	+++	+++		+	+	+	NG	NG	*Candida* sp. strain SSY3
NPM	16.2	+++	+++	++	++		+	+	+	2.733 ± 0.06	2.804 ± 0.03	*Candida* sp. strain SSY4
NPM	16.3	+++	+++	++	++		+	+	+	2.733 ± 0.06	2.804 ± 0.03	Saccharomycetales sp. strain SSY19
NPM	16.4	+++	+++	+	++		+			N/A	N/A	Unidentified fungal sp. strain SSY8
NPM	16.5	+++	+++	+++	++		+	+		2.610 ± 0.08	2.855 ± 0.02	*Kodamaea* sp.

Table 6 Halotolerance and carbohydrate fermentation proportion of representative yeast isolates (continued).

Source	Code	Salt tolerance					Carbohydrate utilization*			Decolorization		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
NPF	17.1	+++	+++	+++	+++		+	+	+	NG	NG	*Kodamaea* sp.
NPF	17.10	+++	+++	+++	+++		+	+	+	NG	NG	*Kodamaea ohmeri* strain SSY10
NPF	17.11	+++	+++	+++	+	+	+		+	NG	NG	*Candida* sp. strain SSY4
NPF	17.12	+++	+++				+			N/A	N/A	*Candida* sp. strain SSY4
NPF	17.2	+++	+++	+++	+	+	+		+	NG	NG	*Saccharomycetales* sp. strain SSY19
NPF	17.3	+++	+++	++	++		+	+	+	2.733 ± 0.06	2.804 ± 0.07	*Saccharomycetales* sp. strain SSY20
NPF	17.4	+++	+++	++	++		+	+	+	2.733 ± 0.01	2.804 ± 0.09	*Saccharomycetales* sp. strain SSY20
NPF	17.5	+++	+++	+	++		+			N/A	N/A	Unidentified fungal sp. strain SSY8
NPF	17.6	+++	+++	+	++		+			N/A	N/A	*[Candida] fuoicola*
NPF	17.7	+++	+++	++			+			N/A	N/A	*Kodamaea* sp.
NPF	17.8	+++	+++	+++	++		+	+		NG	NG	*Kodamaea ohmeri* strain SSY10
NPF	17.9	+++	+++	+++	+	+	+	+	+	2.403 ± 0.11	2.347 ± 0.03	*Torulaspora delbrueckii*
PLI	18.1	+++	+++	+++	++	++	+	+	+	2.347 ± 0.07	2.397 ± 0.09	*Zygosaccharomyces rouxii* strain SSY24
PLI	18.2	+++	+++	++	+	+	+	+	+	2.289 ± 0.06	3.048 ± 0.07	*Zygosaccharomyces rouxii* strain SSY23
PLI	18.3	+++	+++	+++	++	+	+	+	+	2.403 ± 0.01	2.459 ± 0.05	*[Candida] etchellsii*
PLI	18.4	+++	+++	+++	+++	+	+			N/A	N/A	*Candida apicola*
PLI	18.5	+++	+++	+++	++	+	+			N/A	N/A	*Candida apicola*
PLM	19-1	+++	+++	++	++	++	+	+	+	2.605 ± 0.03	2.786 ± 0.03	*Candida sp.* strain SSY3

Table 6 Halotolerance and carbohydrate fermentation proportion of representative yeast isolates (continued).

Source	Code	Salt tolerance (%)*					Carbohydrate utilization**			Absorbance (OD420)		Sequencing
		5	10	15	20	25	G	A	X	SSB	MB	
PLM	19-2	G	G	G	F		+			3.033 ± 0.08	3.1425 ± 0.09	*Entylomatales sp.*
PLF	20-1	G	G	G	F	R	+	+	+	2.403 ± 0.01	2.4585 ± 0.05	*[Candida] etchellsii*
PLF	20-1.1	G	G	G	F	R	+	+	+	NG	NG	*Millerozyma farinosa* strain SSY13
PLF	20-2	G	G				+			N/A	N/A	*Candida pseudofarinosa*
PLF	20-3	G	G	F	F	F	+			N/A	N/A	*Candida sp. strain SSY3*
PLF	20-4.1	G	G	G	R	R	+	+	+	NG	NG	*Millerozyma farinosa* strain SSY14
PLF	20-5	G	G	R			+			N/A	N/A	*Kluyveromyces aestuarii*
PLF	20-6	G	G	G	F	R	+	+	+	2.403 ± 0.01	2.4585 ± 0.05	*[Candida] etchellsii*

N/A = not available NG = no growth

* + = rarely growth ++ = fair growth +++ = good growth

**+ = positive results; G= glucose; A=arabinose; X= xylose

4.3.3 Screening for potential isolates

Results obtained from 4.3.1 experiment screened out many representative colonies and the remaining, called as candidates, were equal to 59 bacterial and 29 yeast strains by the sequencing results (Table 5 and 6). These candidates were further screening for potential isolates with specific decolorization activity of Maillard's reaction product.

Candidate strains were inoculated into SSB in order to determine their decolorization activity of total browning in synthetic soy sauce media containing 10% soy sauce liquid derived from 6 months moromi. It was found that 18 bacterial and 2 yeast candidates could reduce total browning as high as 27.04% in bacteria and 4.05% in yeast. The different of decolorization activity could clearly displayed a strain-specific characteristic. Bacteria group shown better activity than yeast. Considering previous report of removal of brown pigment resulting from Maillard reaction by use of microorganisms, key metabolites produced by melanoidin decolorizing microbe including oxidoreductase enzymes were reported (Georgiou et al., 2016). And also lignolytic multi-enzymatic complex (Ferreira et al., 2010), and cell itself with melanoidin adsorption mechanism (Sirianuntapiboon and Prasertsong, 2008) were proposed. All of these bioremediation approach dealing with melanoidin were clearly based on growth efficiency of the microbes. Due to this essential, the slow growth rate of yeast limited its efficiency of degrading melanoidin and bacteria become promising alternative with higher growth rate and higher environmental adaptability (Kumar and Chandra, 2018).

Among all tested strains, an interestingly occurrence was found during SSB test in which media inoculated with *Staphylococcus* sp. SSB48 and *Bacillus amyloliquefaciens* strain SSB6 contained brown precipitate observed at the bottom of vials (Figure 7). This could possibly be due to specific metabolic activity of inoculated cultures reacting with compounds in SSB matrix including N-containing complex substance of melanoidin. Even exact structure of melanoidin is still not confirmed, it consisted of polymeric and high dispersed colloids with negative charge (Miyagi, Suzuki, et al., 2013) and was able to react with available free amino acid such as Arg or Lys to form larger structures (Singla et al., 2018). Such extending reaction could be found in most food where amino acids presented mostly as part of protein so majority of melanoidin compounds were bounded to the protein (Troise et al., 2018). Microbial enzymes of oxidoreductase groups, laccase for instance, revealed their degradation activity toward these complex molecules resulted in breaking down of high-molecular-weight (>500,000 Da) to the smaller fragments (<1000 Da) (Singh et al., 2015). Strains of *Bacillus* and *Staphylococcus* could produce melanoidin degradation enzyme of ligninolytic laccase (Chowdhary, 2018). Accordingly, deep brown precipitate detected via SSB determination should be the consequence of unique enzymatic activity of *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6. These potential isolates were subject to investigate melanoidin degradation were done in Chapter 5.

Finally, three highest total-browning decolorizable microbe of *Staphylococcus* sp. SSB48 (27.04%), *B. amyloliquefaciens* strain SSB6 (26.11%) and *S. condimenti* strain SSB41 (4.98%) isolated from middle fermentation of CC final period of NP and final of SP1 moromi, respectively, were selected as potential isolates.

In order to determine the activity with pure melanoidin, xylose-glycine brown pigment model was applied. Synthesized melanoidin spiked into MB was synthesized from Maillard reaction of xylose and glycine. These two substrates were selected based on their high reactivity in Maillard reaction (Bertrand et al., 2018; Laroque et al., 2008) along with significant amount of these two molecules were also observed in moromi (Figure 3 and Figure 6). The synthesis procedure was modified from the reference as soy sauce melanoidin, alike with other natural forming, contained mixture of various compounds producing during complex reaction series of Maillard (Kumar and Chandra, 2018). Thus, dialysis process was omitted in order to gain as many diverse molecular weight melanoidin compounds as possible. When experiment was done, any samples revealed their OD_{420} lower than 2.299, which was the value of control MB, were marked as xylose-glycine melanoidin (XGM) decolorizable strains.

Figure 7 Examples of SSB inoculated with either Staphylococcus sp. SSB48 or B. amyloliquefaciens strain SSB6 for 2 weeks, static condition at room temperature.

For overall microbes, 12 bacteria shown their XGM reduction in range of 0.35 – 7.57% and 0.65 – 4.35% reduction were noticed from 4 yeast strains. Better XGM decolorizable activity observed in bacteria toward yeast candidates should be from higher growth rate of the former microbial group as discussed previously in 4.3.2.1.1. It was noted that most of SSB decolorizable strains did not revealed same activity toward MB, and vice versa, which should be due to different balance of carbon/nitrogen ratio in these two mediums since organic nitrogen excessing in the system reduced the induction of melanoidin-degradable peroxidase enzyme (Miyata et al., 2000).

Two potential XGM-decolorizable isolates, namely *Staphylococcus* sp. SSB47 (5.48% reduction) and *Bacillus amyloliquefaciens* strain SSB2 (7.57%) from initial period of SP1 fermentation were selected by their highest decolorization in MB media (Table 5).

4.3.4 Fermentation properties of candidate strains

Five potentials, three with total browning reduction (*Staphylococcus* sp. SSB48, *B. amyloliquefaciens* strain SSB6 and *S. condimenti* strain SSB41) and 2 with XGM-decolorizable activity (*Staphylococcus* sp. SSB47 and *B. amyloliquefaciens* strain SSB2) were determined for other key characteristics needed in soy sauce in order to evaluate the possibility of using these potentials in industrial soy sauce production

4.3.4.1 Sugar utilization profile

Previous experiment of carbohydrate fermentation (4.3.2) was tested in broth containing single reducing sugar species which was not the real system. As multiple reducing sugars was present in soy sauce fermentation and various reports of catabolite repression of sugars in relation with metabolic activity shuffling were proposed (Abdel-Rahman et al., 2015; van den Bogaard et al., 2000), thus broth contain mixed sugar was also tested in this step.

Table 7 illustrated the reduction of glucose, arabinose and xylose concentrations after 5 potentials were growth in peptone water (1%) containing three sugars (1% each) which glucose utilization in each isolate was not significantly different among others at the 0.05 level. Contrastingly, using same alpha of 0.05 and Duncan test, isolates utilizing arabinose and xylose with the highest reduction could be identified. They were *S. condimenti* strain SSB41 with its 16.33% reduction of arabinose, and *Staphylococcus* sp. SSB48 (24.38%) and *B. amyloliquefaciens* strain SSB6 (25.22%) of xylose sugar.

Considering the reduction of glucose simultaneously with arabinose and xylose as shown in Table 7, it was clearly that all of potentials obtained co-metabolized activity of these 3 sugars. Their metabolic controls termed as catabolite repression (CCR), an economical switch controlling enzyme utilized less-favored carbon substrate such as arabinose and xylose over preferential one (mostly glucose) (Adnan et al., 2018), was a strain-dependent property (Abdel-Rahman et al., 2015; Kim et al., 2009; Wang et al., 2014). Therefore, it possible that CCR in potentials were in loosely state, or no CCR in their carbohydrate metabolism and all sugar utilization-encoding genes were all independent for others. In term of mix sugar utilization efficiency, all potentials revealed an outstanding consumption of xylose toward glucose. Comparing with previous studies, special techniques of fed-batch fermentation (Abdel-Rahman et al., 2015) or even genetical improvement of bacterial

culture (Fu et al., 2017) were applied in order to induce bacterial strains to co-metabolized glucose and xylose, that glucose consumption still be approximately 3 times higher than xylose. Hence, results from this experiment disclosed the excellent properties of isolate strains for co-fermentation in soy sauce. In addition, the potential properties based on the ability in consumption of key reducing sugars, glucose has positive effect on the melanoidin decolorization by sugar-oxidase and peroxidase reaction due to an activation of hydrogen peroxide reaction which in turn, oxidized melanoidin molecule (Kumar and Chandra, 2006). Hence, co-metabolizing properties of glucose with pentose reveals the novel properties of these strains in controlling Maillard reaction.

The most efficient isolates shown remarkable consumption of pentose, arabinose and xylose were *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6 with C-5 sugars reduction of 39.09 and 40.08%, respectively. Together with good glucose co-metabolization, the two potential cultures revealed their capability for biological mitigation of possible browning Maillard reaction browning based on i) reduce substrate for Maillard reaction and ii) degrade melanoidin.

Table 7 Reduction percentage of three reducing sugars by potential isolates

Potential isolates	Reduction (%)		
	Glucose^{ns}	Arabinose	Xylose
Staphylococcus sp. SSB48	19.26 ± 0.09	14.71^{ab} ± 0.33	24.38^{b} ± 0.21
B. amyloliquefaciens strain SSB6	19.27 ± 0.44	14.86^{ab} ± 0.32	25.22^{b} ± 0.18
S. condimenti strain SSB41	17.63 ± 0.34	16.33^{b} ± 0.28	22.29^{ab} ± 0.17
Staphylococcus sp. SSB47	21.44 ± 0.13	12.87^{ab} ± 0.23	19.72^{a} ± 0.20
Bacillus amyloliquefaciens strain SSB2	18.98 ± 0.38	9.61^{a} ± 0.11	21.50^{ab} ± 0.25

Different superscript letters in the same column indicate significant different at the level of 0.05 (n=3); ns = no significant

4.3.4.2 Volatile profiles

Cluster analysis using HCL and heatmap visualization were applied for volatile profile data derived from SPME GC-MS determination. Comparing with uninoculated industrial moromi SP1, all potentials were clustered away from the control (Figure 8). Nevertheless, 9 out of ten key volatile compounds in SP1 samples (Appendix C) of ethanol; ethyl acetate; phenol,4-ethyl,2-methoxy; 1-butanol,3-methyl; acetic acid; benzaldehyde; butanal,2-methyl and 1-butanol,2-methyl were all detected in inoculated samples with predominantly relative amount comparing with uninoculated SP1.

Total 73 volatiles detected in this experiment including control sample were consisted of 7 furan derivatives. Furan was one of the heterocyclic, colorless compound mainly stemmed by Maillard reaction of specific amino acid and reducing sugars, and considered as a hazardous chemical and possibly carcinogen by the international Agency for Research on Cance (Huang and Barringer, 2016). Relative abundant of 5 furans (3-furaldehyde; 2-furanmethanol; furfural; furan,2-pentyl and 2-furancarboxaldehyde,5-methyl-) were comparable among inoculated samples. However, 2 furan derivatives of furan-tetrahydro-2,2-dimethyl-5-(1-methylpropyl)- and furan, 2,2'-(oxybis (methylene) bis- were lower in *B. amyloliquefaciens* strain SSB6, and both *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6, respectively. This might be due to unique characteristic of furan's substrates

utilization of these two potentials toward other isolates since fermentation of all strains were conducted under similar condition. Thus, only factor impacted on furan formation could be from the specific function of *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6 as previous mentioned.

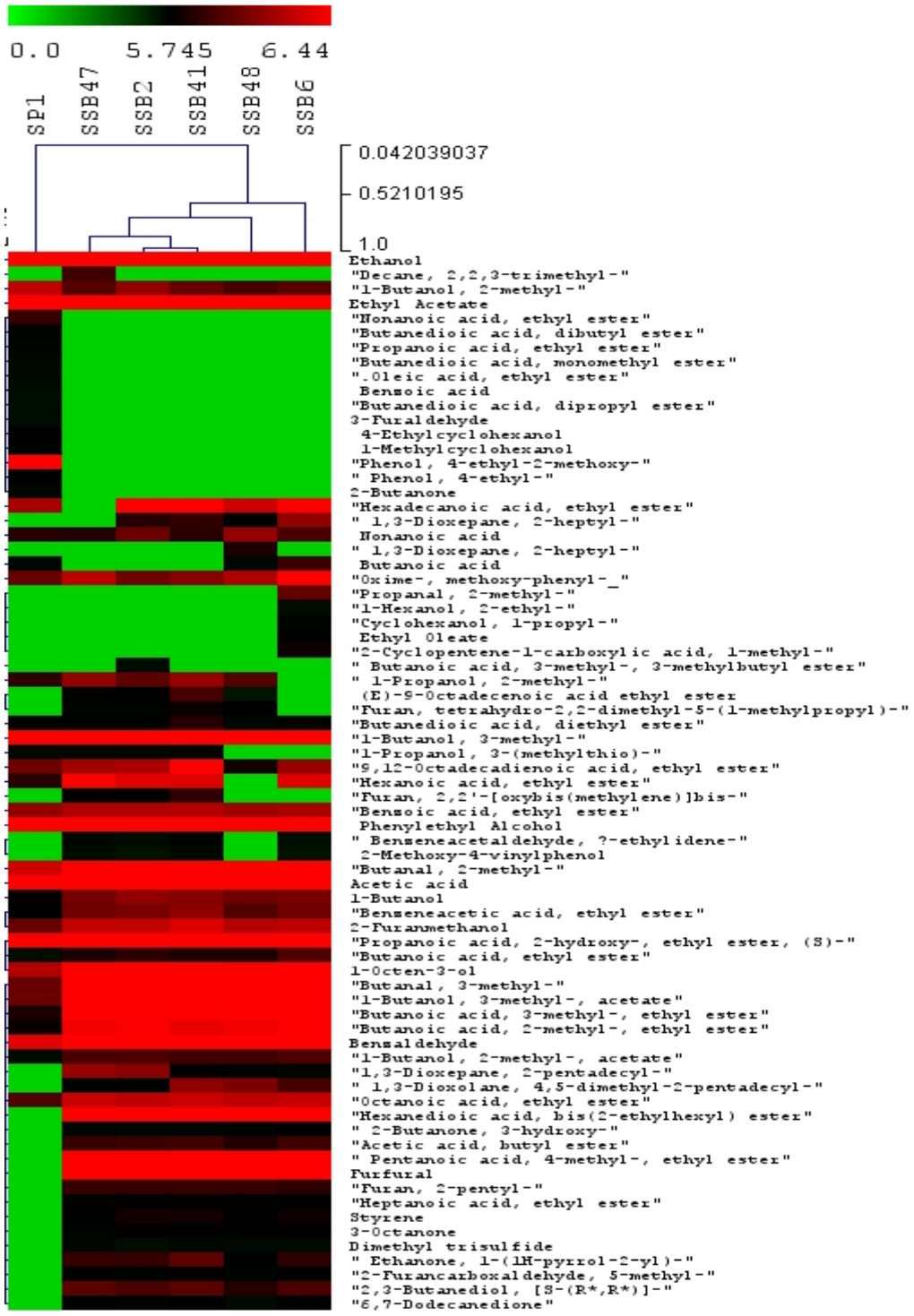


Figure 8 HCL heatmap analysis of volatile profiles derived from moromi inoculated with Staphylococcus sp. SSB48 (S1), B. amyloliquefaciens SSB6 (S2), S. condimenti strain SSB41 (S3), Staphylococcus sp. SSB47 (1M) and B. amyloliquefaciens strain SSB2 (2M).
SP1 was 6 months commercial moromi from industrial process.

4.3.4.3 Biogenic amine (BA) gene-carrying isolates

BAs is nitrogenous molecule causing significant toxicity effects including headache, high blood pressure and severe case of death (Deetae et al., 2017) due to their carcinogenic and mutagenic nature, and could be produced by some microorganisms during fermentation process of various food including soy sauce Reduction percentage of three reducing sugars by potential isolates (Shukla and Kim, 2016). By this importance to consumer's health, all 5 potential cultures from this study were verified for their BA producing capability based on determination of BAs-encoding genes namely histamine, tyramine and putrescine, whose generally detected in food and beverage (Landete et al., 2007).

Two reactions of Multiplex PCR indicated the safety of 5 tested cultures based on histamine, tyramine and putrescine formation ability as none specific DNA band of BA-producing genes was shown (Figure 9).

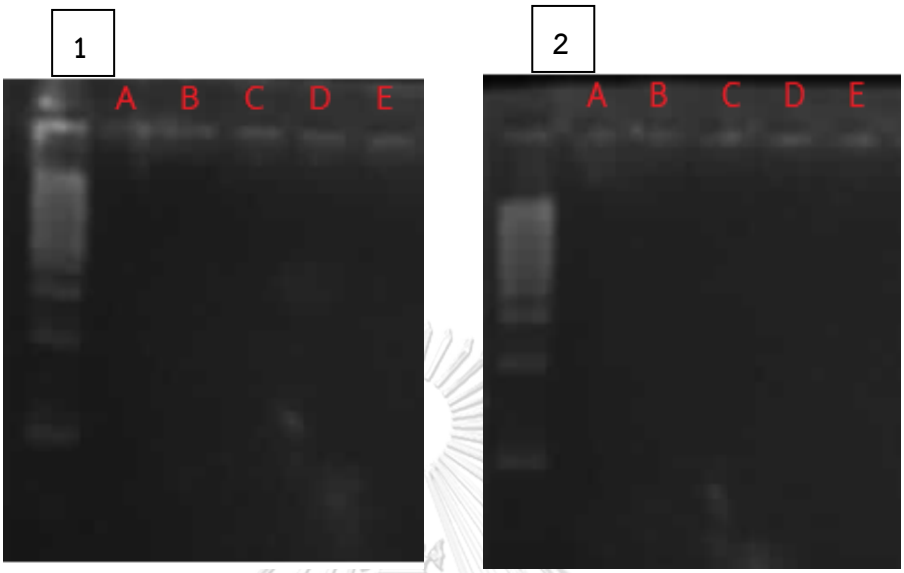


Figure 9 Verification of BAs-encoding genes in potential isolates

; A= Staphylococcus sp. SSB48, B= B. amyloliquefaciens strain SSB6, C= S. condimenti strain SSB41, D= Staphylococcus sp. SSB47 and E= B. amyloliquefaciens strain SSB2.

Figure 9-1: Multiplex PCR consisted 4 primer pairs mixture (TD2/5 + TD-F/R + PUT2-F/R + HDC 3 / 4).

Figure 9-2: Multiplex PCR of 3 primer pairs (PUT1-F/R + TDC 1 / 2 + JV16HC/17HC).

From all of the results stated above, 2 potential strains of *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6 were selected since they revealed extreme pentose reduction ability both in single or mixed sugar which glucose still simultaneously consumed with good tolerance in optimal NaCl concentration found in soy sauce (15-25%). Interestingly, these two isolates also reveal good decolorization of total browning in SSB model containing complex mixture of browning index compounds comparable with one in soy sauce. In addition, these 2 bacteria were safety strains as none of target Bas-encoding

genes was detected, and their metabolic activity also preserved since 9 key volatiles were noted in comparable amount with the major compounds detected from industrial - process soy sauce moromi of SP1F.

CHAPTER 5

PROCESS OPTIMIZATION OF POTENTIAL ISOLATES

5.1 Introduction

Strains optimization is important processes that have to be done to evaluate an ability of microbial isolates for development as starter culture and/or for specific use in fermented food productions as numerous critical factors including fermentation medium and condition affected both of starter's growth and yield (Panda et al., 2007) are subjected to investigation. The conditions as optimized is an important step in scaling-up experiment used in optimization could lead to less process constancy, reproducibility of key metabolites and increase of unwanted by products disturbing product quality, a key issue in industrial food fermentation.

The aim of this chapter was to test potential strains isolates; *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6 for browning reduction in moromi at 250 grams laboratory-scale fermentation. Optimized conditions included inoculation types (single and mixed strains), fermentation time (1 and 2 months) and ages of moromi (2,4 and 6-month).

Inoculated moromi was determined for total viable count along with dynamic changes of key parameters including browning indexes, reducing sugars and volatile profiles comparing with non-inoculated sample. Melanoidin profile of each treatment was also determined in order to evaluate oxidoreductase producibility in potential isolates. Selected key parameter(s) derived from this experiment could be applied for the further optimization process in semi-pilot and pilot scales.

5.2 Materials and methods

5.2.1 Preparation of isolates(s)-inoculated moromi

Ten microliters of glycerol stock cultures *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* strain SSB6 from 3.2.2.1 were inoculated into 50 ml nutrient broth (NB) supplemented with 3% NaCl and incubated at room temperature for 2 days. Activated crude cell (Figure 10) was collected by mean of centrifugation before amount of 3% NaCl solution was added to resuspend the culture until its final absorbance at OD_{600} reached 0.1.

Industrial moromi in fermentation age of 2, 4 and 6 months were selected as a fermentation medium in this experiment in order to represent the condition of initial, middle and final moromi fermentation stages. Samples were collected aseptically from the production site in tightly-closed bottles, were same-day transferring to laboratory and then stored in -20C until use. Before usage, moromi was thaw at room temperature in same storage container until all ice crystal was melted and moromi was mixed thoroughly by gently flipping the container.

Two hundred and fifty grams of homogeneous moromi was transferred into 500 ml flask and inoculated with 2.5 ml of prepared culture suspension. The flask was then sealed with cotton cap and stored at room temperature for total of 2 months.

5.2.2 Investigation of strains viability in moromi

Moromi in each tested condition was sampled for total viable count (TVC) at fermentation time at 0 (initial count), 1 and 2 months. Conventional plating with spreading technique using nutrient agar (NA) supplemented with 3% NaCl was applied with the protocol as stated in 3.2.2.1. Agar plates were incubated at room temperature for 2 days before plates with bacterial growth in range of 25-250 colonies were selected for counting. The colonies of each strain with its typical morphology as shown in Figure 10 were counted

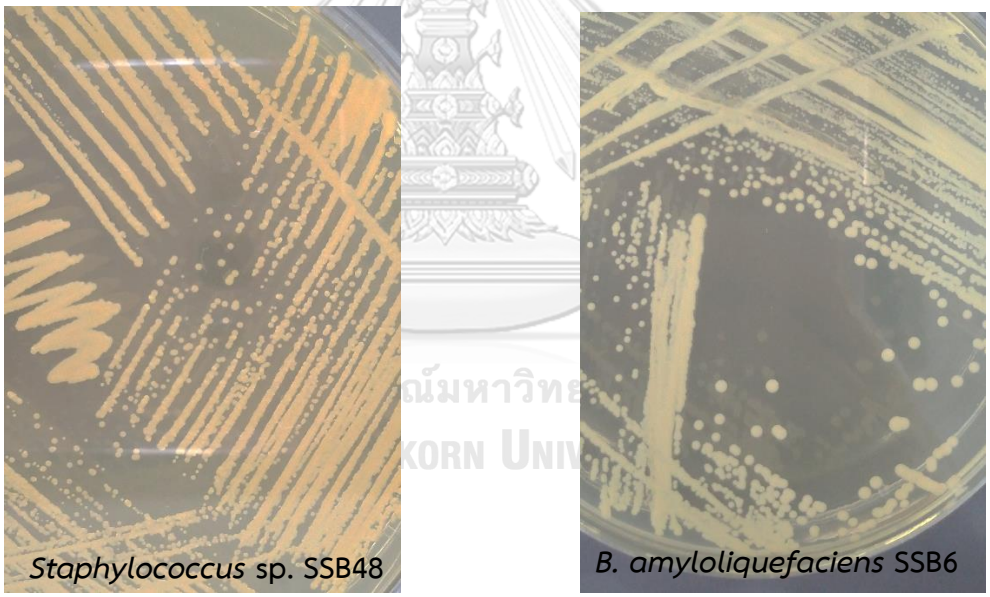


Figure 10 colony morphology of potential isolates used in process optimization at 24 hours growth

: SSB48 was a small, circular, glistening, light-orange yellow colony with a convex elevation; SSB6 was a raised, dull, light cream colony with entire margin at ≤ 24 hours-growth on NA and became lobate with more deep color at longer incubation time.

5.2.3 Determination of browning indexes

Ten grams of homogeneous moromi was transferred into tube and centrifuged at 8,000 rpm, 4℃ for 20 minutes before supernatant was determined for browning indexes. Total browning was directly determined at absorbance of 420 nm while additional procedure of sample preparation as stated in 3.2.4.1 using ethanol extraction was applied for HMF determination.

5.2.4 Key sugars utilization

One microliter of momomi-liquid prepared as 5.2.3 was filtered through 0.45 µM using syringe filter and stored in 0.5 ml amber-glass HPLC vial, tightly capped at -20℃ before HPLC analysis. Rezex RHM-monosaccharide H+ column (Phenomenex, USA) with HPLC and refractive index detector were used for glucose, xylose and arabinose following previous protocol of (Chanprasartsuk et al., 2013) with a modification as stated in 3.2.4.2.

5.2.3 Volatile profiles

Prior the determination, 3 methyl-2 heptanone solution using as internal standard was prepared by diluting 20 µl of 3 methyl-2 heptanone in 50 ml deionized water. 5 milliliters of moromi spiked with 2 µl of 3 methyl-2 heptanone was added into 20 ml GC-MS headspace vial containing 3 grams of NaCl and capped. Sample was pre-heated at 40 ℃ for 15 min with agitator 250 rpm, and then volatile compounds were extracted by SPME fiber (50/30µm DVB/CAR/PDMS, SUPELCO, PA) for 30 minutes before the fiber was desorbed in GC injector port at 250 ℃ for 4 min and post desorbed for 5 min.

Gas chromatography (Agilent 7890B)–Mass spectrometry (Agilent 5977 B) equipped with a capillary column (DB-WAX, 30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA) and injector operation mode of Splitless Helium gas with constant flow rate of 1 mL/min. of carrier gas were applied. GC-MS condition for separation of the desorbed volatiles using a The GC oven temperature at 40 °C was, in sequential, 15 minutes; increased at 3 °C/minute to 160 °C; increased to 230 °C at 4 °C/minutes and final hold for 5 minutes. MS was set in the electron ionization mode with the ion source temperature at 230 °C, and ionization energy set at 70 eV. The scan mode was used in the scan range of 25 to 400 m/z.

Data analysis of GC-MS signals was done by The Agilent Mass Hunter Qualitative Analysis B.04.00 software. Identification of volatile compounds was performed by comparing mass spectra with Wiley mass spectral libraries (National Institute of Standards, 2011 version). The content of volatile compound was calculated from peak area.

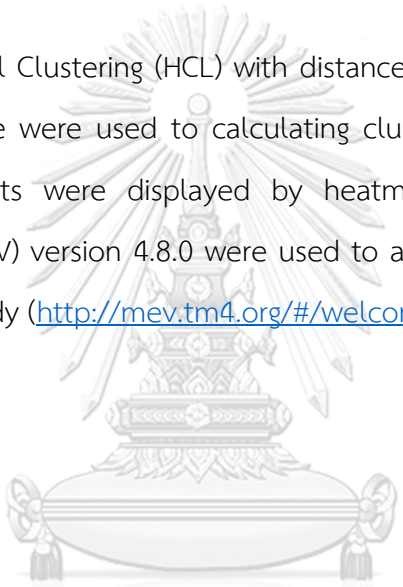
5.2.4 Qualification of melanoidin

One microliter of momomi-liquid prepared as 5.2.3 was filtered through 0.45 μM using syringe filter and stored in 0.5 ml amber-glass HPLC vial and tightly capped. Melanoidin profile was examined by HPLC (Shimadzu) equipped with detector of Diode-Array Detection (DAD) and C-18 reverse phase column of 5 μM, 4.6x150mm. (Eclipse XDB-C18, Agilent, USA). Mobile phase consisted of acetonitrile and methanol in 45:55 ratio, supplemented with 1 ml glacial acid and 0.5 ml sodium acetate with a flow of 1 ml/minute. Melanoidin peaks were analyzed at 475 nm. (Tiwari et al., 2012).

5.2.5 Statistical analysis

One-way ANOVA of univariate statistical analysis was applied to the browning indexes results to compare mean between treatments by the Duncan's test using IBM SPSS 22 statistics package with 95% confidence interval (IBM, USA).

Hierarchical Clustering (HCL) with distance metric of Pearson correlation and agglomerative type were used to calculating clustered dendrogram of volatile compounds and results were displayed by heatmap. Online application Multi Experiment Viewer (MeV) version 4.8.0 were used to analyze HCL of volatile profiles (title 4.2.5.2) in this study (http://mev.tm4.org/#/welcome)



5.3 Results and discussions

5.3.1 Initial bacterial population

Working bacterial cultures with their OD_{600} value of approximately 0.1 revealed their TVC in single culture of 8.57 (*Staphylococcus* sp. SSB48, colony morphology shown in Figure 10A) and 8.46 (*B. amyloliquefaciens* strain SSB6, colony in Figure 10B), and of 8.78 $\log_{10}$CFU/g. for the 1:1 ratio mixed culture (Table 8). Inoculation of culture suspensions into moromi resulted in 2 serial-dilution thus the initial population of potentials in each moromi should be, in relatively, 6 $\log_{10}$CFU/g of moromi in all treatments.

Table 8 TVC of inoculated moromi at fermentation time of 1 and 2 months

Samples	Strains*	TVC (log10 CFU/ml)		Colony similar to target strain(s) (log10 CFU/ml)	
		1M	2M	1M	2M
working cultures	SSB48	8.57		A* (8.57)	
	SSB6	8.46		B* (8.46)	
	mixed	8.78		A* (8.57) / B* (8.36)	
2 month moromi	SSB48	5.28	5.40	A* (≤2.00)	A* (≤2.00)
	SSB6	5.20	5.34	B* (4.47)	B* (4.63)
	mixed	5.32	5.32	A (≤2.00) / B* (4.04)	A* (≤2.00) / B* (4.53)
4 month moromi	SSB48	4.90	5.15	A* (≤2.00)	ND
	SSB6	4.90	5.04	B* (4.90)	B* (5.04)
6 month moromi	SSB48	5.08	5.15	A* (≤2.00)	ND
	SSB6	5.54	5.54	B* (≤2.00)	ND

* SSB48 = *Staphylococcus* sp. SSB48; SSB6 = *B. amyloliquefaciens* SSB6

A*= colony morphology of small, circular, glistening, light-orange yellow colony with a convex elevation (Figure 10).

B*= colony morphology of raised, dull, light cream colony with lobate margin (Figure 10).

5.3.2 Two-month moromi age samples

After incubation for 1 month, TVC of all inoculated treatments were remarkable decreased from an expected value at the initial point of 6 $\log_{10}$CFU/g to value of 5 $\log_{10}$CFU/g. Colony with the morphological property similarly with the inoculated strains as depicted in Figure 5.1 were counted as similarly colony shown in Table 8. This result demonstrated the state of *Staphylococcus* sp. SSB48 that cells partially became VBNC and/or die off due to stress condition in moromi as TVC reduction larger than 76.66% observed throughout the fermentation time. However, it was found that browning index of HMF in *Staphylococcus* sp. SSB48 inoculation was 2.345 at first month then increased by 49.47% to 3.505. This reflected that in moromi, if this reaction performed by this bacteria, the *Staphylococcus* sp. SSB48 candidate, would naturally play role during fermentation with VNBC state same as information obtained in Chapter 3. In addition, its performance was also further confirmed by its displaying in sugar utilization property as observed in Chapter 4. It was found that all key sugars; glucose, xylose and arabinose in *Staphylococcus* sp. SSB48 inoculated moromi were disappeared, which were the same as all treatments tested in this experiment after 2 months of incubation (Table 10). Volatile profiles of this treatment revealed high proportion of phenol,4-ethyl-2-methoxy; phenylethyl alcohol; 1-butanol, 3-methyl and organic acid such as propanoic, 2-methyl; butanoic, 2-methyl along with its derivatives while styrene was relatively low (Figure 11). A specific property of melanoidin-degradable in this treatment was examined and the chromatogram was shown in Figure 13A. Relative abundant ($\log_{10}$) of major peaks during first 2.200 minutes (RT ≤ 2.200) was 4.747, calculated as 89.52% of total peak area, containing of 2 peaks at RT 1.496 and 1.918. Compounds detected after that RT depicted a convex band of small peaks which lasting to the final RT of 10.785 (Figure 13A). The observation demonstrated the degradation of melanoidin that might conducted by enzymatic reaction of this candidate strains.

In *B. amyloliquefaciens* SSB6 fermentation batch, a plunge of colony similarly to *B. amyloliquefaciens* SSB6 was also marked similarly to those in *Staphylococcus* sp. SSB48 describing above with an approximately 47.16% reduction at the first month while the value at the second fermentation month was slightly increased from 4.47 to 4.63 logCFU/g. (Table 5.1). HMF index of moromi was increase 24.24% from first to the second month while total browning was decrease for 2.35% (Table 9). Volatile profiles of this *B. amyloliquefaciens* SSB6 treatment was comparable with *Staphylococcus* sp. SSB48 despite the fact that clustering analysis projected this treatment into another cluster away from others due to more positive interaction with pentanal and 1-octen, 3-ol (Figure 11). Melanoidin profiles revealed the 3 major unretained peaks given the peak area of 4.747, attaching by 2 tailed peaks, and along with small 14 minor peaks released before RT 5.700 (Figure 13B) which reflected the degradation of melanoidin

The mixed co-culture treatment of *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* SSB6 which was optimized only in 2-month moromi was shown same viability trend comparable with single inoculation treatment as *Staphylococcus* sp. SSB48 was rarely detected while *B. amyloliquefaciens* SSB6-similarly colony was lightly flourished between second incubation month. Interesting results of browning indexes were noted in this treatment since HMF and total browning was exceptional stable throughout fermentation period. The values were approximately 2.6 for HMF and 34.5 for total browning (Table 9). Since HCL analysis as depicted in Figure 11 clustered this mixed inoculation treatment in same group as single *Staphylococcus* sp. SSB48 inoculation, their volatile profiles were comparable, excepted the mixed had more negative relation with furan derivative of anti-2-(alpha-phenyl II-tosylaminoethyl)-2,5-dihydrofuran. Numerous small peaks were found after RT of 2.222 and lasting to 12.249 minute, along with 5 major peaks eluted before 2.222

minutes of injection time. The relative abundant of this major peak area was 4.65 in log_{10}-based (Figure 13D). Comparing with its control, this sample revealed the largest biodegradation and biotransformation

Browning indexes changing in 2-month moromi-control samples illustrated in Figure 9 revealed a significantly increased of HMF with slightly decrease of total browning during the first and second fermentation month. Its volatile profile was separated from three inoculation treatment as shown in Figure 11 with a low relation with 1-octen, 3-ol and butanoic acid, 3-methyl-, 3-methyl butyl ester. Melanoidin profile consisted of 3 major peaks (relative abundant area = 4.755) and minor convex peak band predominantly eluted before RT 6.230 (Figure 12A).

5.3.3 Four-month moromi

TVC of 4-month moromi inoculated with either *Staphylococcus* sp. SSB48 or *B. amyloliquefaciens* SSB6 were identical at 4.90 logCFU/g. at the first month before slightly increase at the second. Nevertheless, *Staphylococcus* sp. SSB48-similarly colony diminished after 2 months. Browning indexes of HMF and total browning in this treatment rose from first month in value of 13.70 and 8.52%, respectively while benzeneacetaldehyde volatile was generated in a significantly low proportion.

The growth of colony likely-to-be *B. amyloliquefaciens* SSB6 during the second half period was noted with the percentage of 2.86% with the increment of HMF (36.01%) and total browning (4.56%). A relatively low interaction with disulfide, dimethyl and high relation with pyrazine, 2,6-dimethyl were depicted in this treatment. Profiles of melanoidin compound consisted of 4 major peaks with 2 mains

at RT 1.496 and 1.840 along with various small peaks spreading during RT 2.648 to 19.803 (Figure 13C).

The control treatment of 4-month moromi had lower HMF value at the second month while total browning was higher comparing to the first month value, and revealed a chromatogram of 2 dominant peaks at RT 1.497 and 1.844 in major peak area while a few minor small peaks were observed (Figure 12B).

5.3 4 Six-month moromi

There were no growth of neither *Staphylococcus* sp. SSB48 nor *B. amyloliquefaciens* SSB6-similarly colony was detected in cultural plating after fermentation for 2 months, and the results at 1 month was ≤2.00 logCFU/g. The significant lower of HMF than the control was noticed in both inoculated treatments at the first half period before became extremely higher at the second half (Table 9).

Total browning of *Staphylococcus* sp. SSB48 was statistically different with the others only in the 1 month incubation, which was also the lowest value of all tested moromi. It was noted that the change in browning index of 6-month control treatment was similar to 4-month results in which HMF decreased while total browning increased at the second determination time. Melanoidin profiles of *Staphylococcus* sp. SSB48 treatment had significant amounts of small peaks during RT of 2.653 – 5.415 (Figure 13E) while minor peaks in control sample dispersed from 4.388 to 13.150 area (Figure 12C).

Table 9 Browning indexes of 2-month moromi inoculated with potential isolate(s)

Browning indexes	Fermentation time (month)	2 months moromi				4 months moromi			6 months moromi		
		Control	SSB48	SSB6	Mixed	Control	SSB48	SSB6	Control	SSB48	SSB6
HMF	1	2.485^{def} ± 0.00	2.345^{f} ± 0.00	2.455^{ef} ± 0.01	2.640^{cde} ± 0.02	2.750^{c} ± 0.00	2.700^{cd} ± 0.01	2.555^{cdef} ± 0.00	3.600^{a} ± 0.00	3.055^{b} ± 0.00	3.025^{b} ± 0.00
	2	3.035^{c} ± 0.01	3.505^{ab} ± 0.01	3.050^{c} ± 0.01	2.635^{d} ± 0.01	2.555^{d} ± 0.01	3.070^{c} ± 0.00	3.475^{b} ± 0.02	3.140^{c} ± 0.00	3.835^{a} ± 0.00	3.525^{ab} ± 0.00
Total browning	1	35.135^{a} ± 0.24	34.025^{ab} ± 0.05	34.835^{a} ± 0.03	34.510^{ab} ± 0.00	33.540^{abc} ± 0.04	31.790^{bc} ± 0.03	32.750^{abc} ± 0.00	34.510^{ab} ± 0.00	30.770^{c} ± 0.02	31.800^{bc} ± 0.03
	2	35.125 ± 0.06	34.015 ± 0.05	34.015 ± 0.05	34.500 ± 0.00	35.750 ± 0.00	34.500 ± 0.15	34.245 ± 0.15	35.125 ± 0.06	35.750 ± 0.00	35.750 ± 0.00

Different superscript letters in the same row indicate significant different at the level of 0.05 (n=2)

Table 10 Quantification of key reducing sugars in moromi before and after 2 months fermentation

Moromi age	Fermentation time	Reducing sugars (mg/ml)		
		glucose	arabinose	xylose
2-month	start	47.270 ± 0.38	3.350 ± 0.012	6.734 ± 0.018
	2 months	ND	ND	ND
4-month	start	43.224 ± 0.02	1.826 ± 0.072	6.284 ± 0.105
	3 months	ND	ND	ND
6-month	start	41.495 ± 0.51	1.278 ± 0.033	6.169 ± 0.036
	4 months	ND	ND	ND

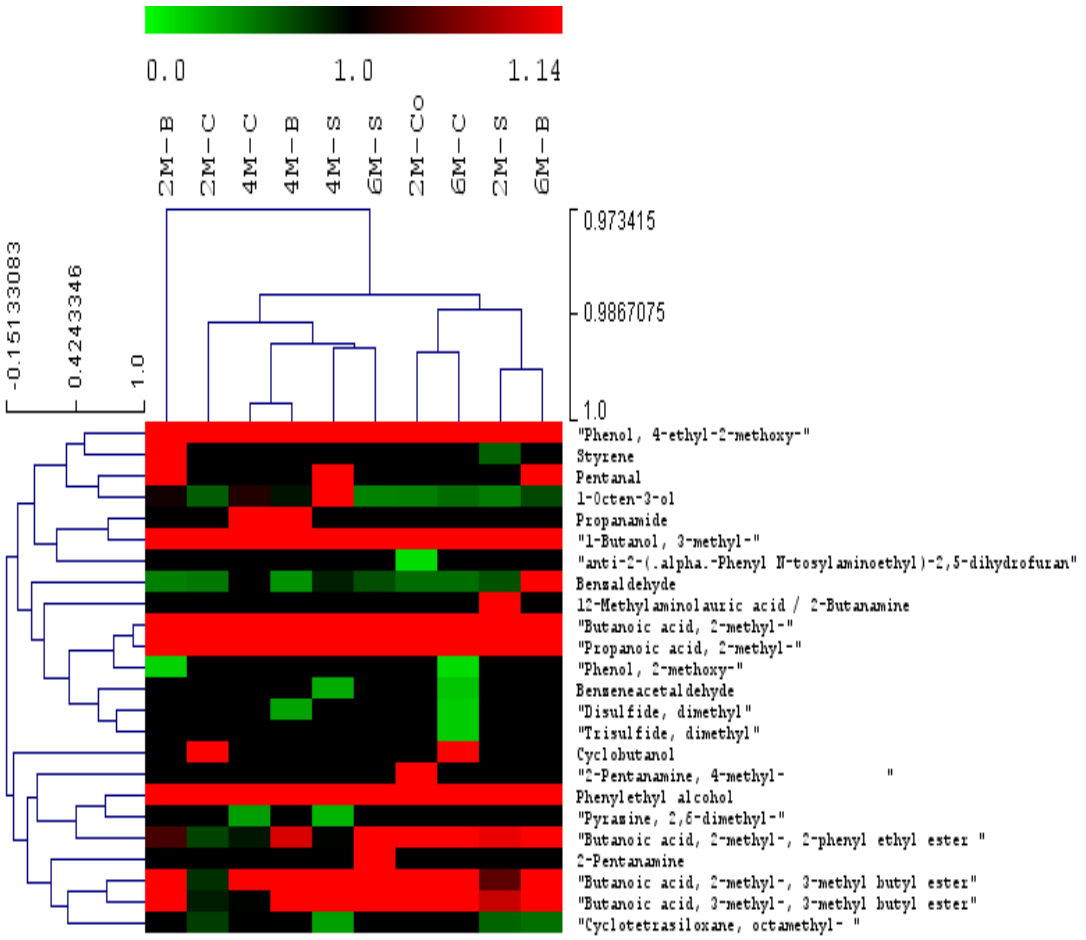


Figure 11 HCL analysis of volatile compound from uninoculated and inoculated moromi fermented for 2 month

: 2M-C = 2-month moromi, uninoculated; 2M-S = 2-month moromi, inoculated with Staphylococcus sp. SSB48; 2M-B = 2-month moromi, inoculated with B. amyloliquefaciens SSB6; 2M-Co = 2-month moromi, inoculated with mixed culture of Staphylococcus sp. SSB48 and B. amyloliquefaciens SSB6; 4M-C = 4-month moromi, uninoculated; 4M-S = 4-month moromi, inoculated with Staphylococcus sp. SSB48; 4M-B = 4-month moromi, inoculated with B. amyloliquefaciens SSB6; 6M-C = 6-month moromi, uninoculated; 6M-S = 6-month moromi, inoculated with Staphylococcus sp. SSB48; 6M-B = 6-month moromi, inoculated with B. amyloliquefaciens SSB6.

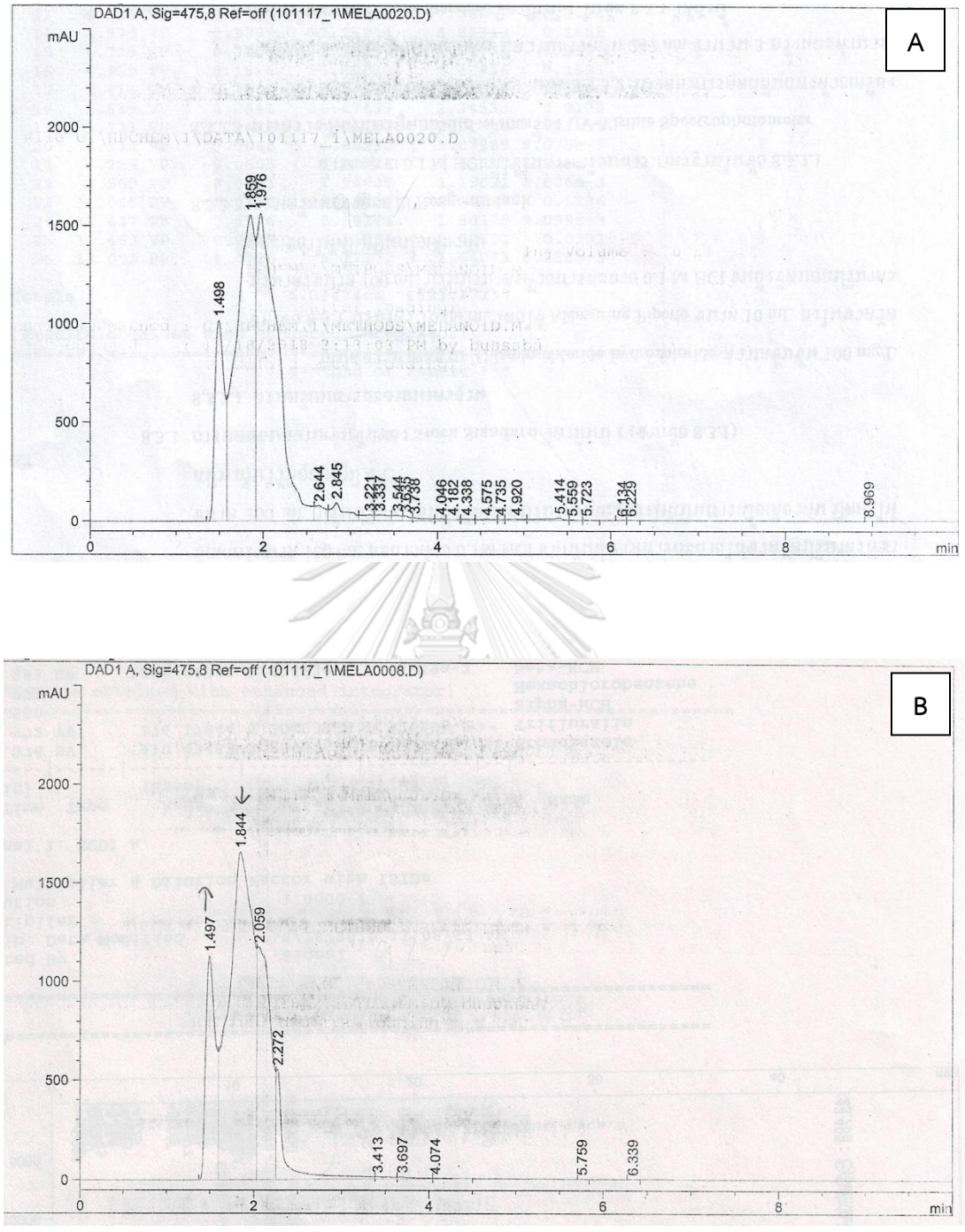


Figure 12 Chromatogram of melanoidin compounds in uninoculated moromi after 2 months incubation

; 12-A = 2-month moromi, 12-B = 4-month moromi.

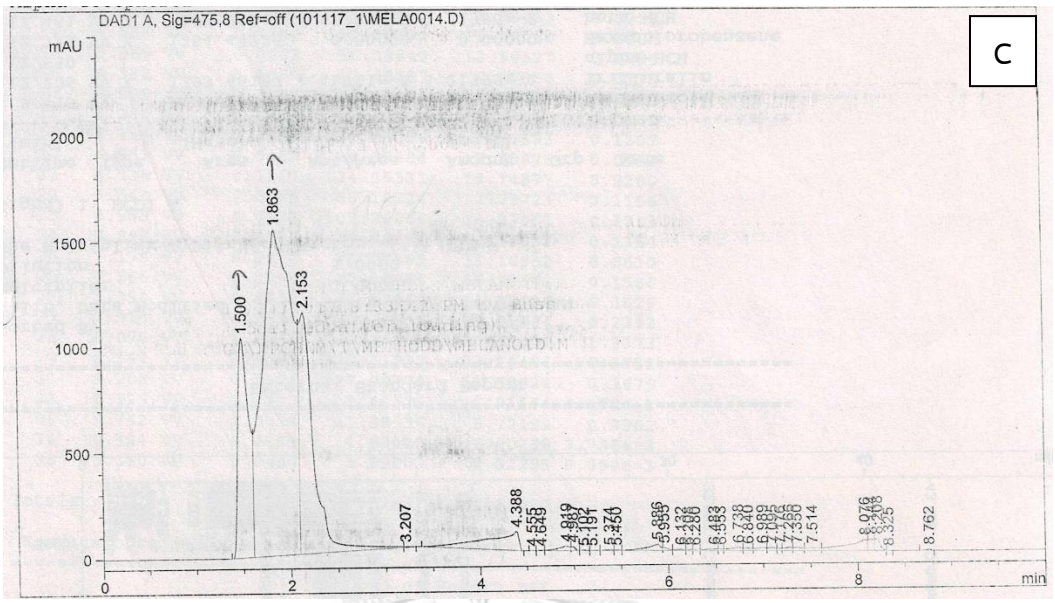


Figure 12 Chromatogram of melanoidin compounds in uninoculated moromi after 2 months incubation (continue)

; 12-C = 6-month moromi.

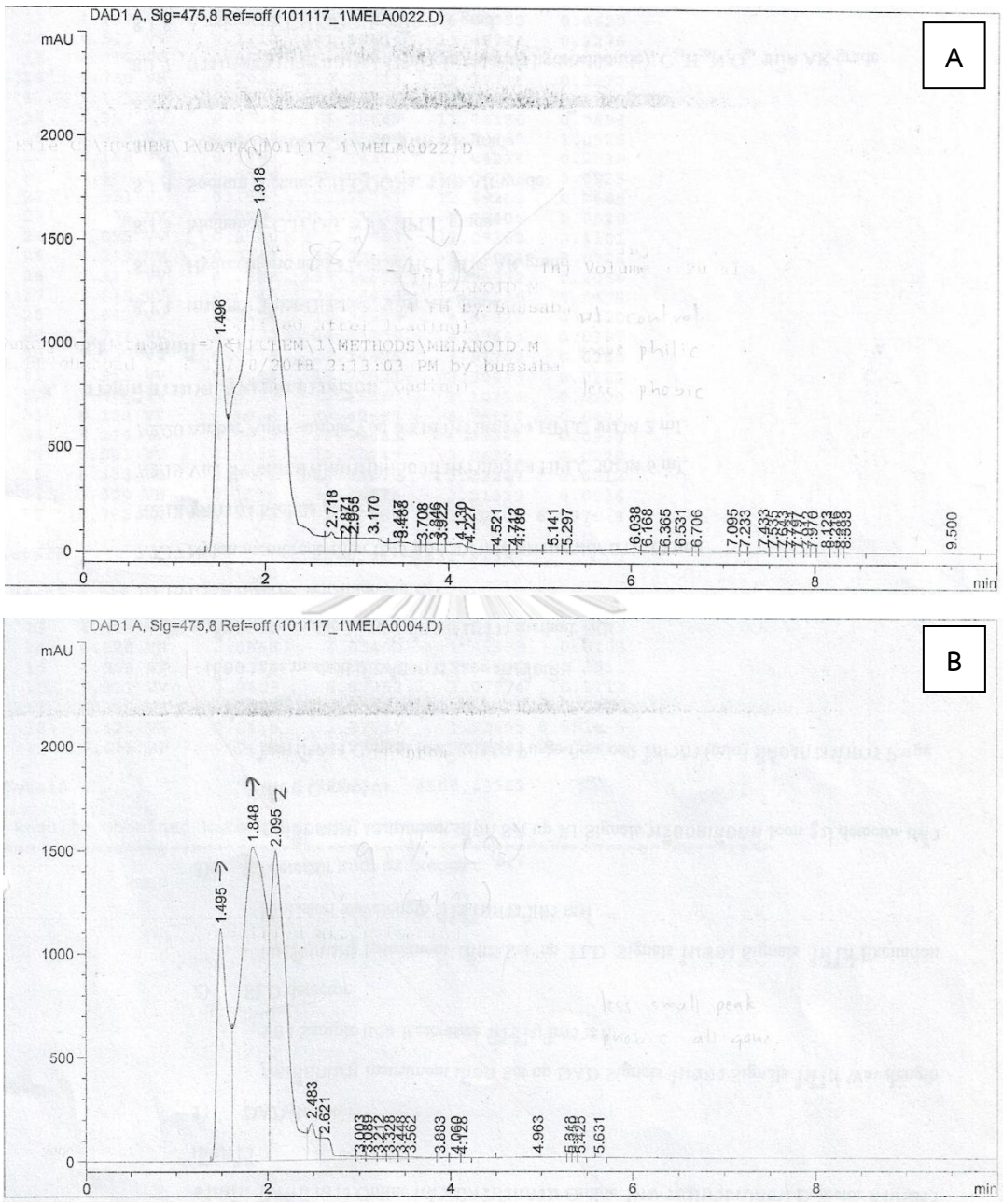


Figure 13 Chromatogram of melanoidin compounds in inoculated moromi after 2 months incubation

; 13A = 2-month moromi with Staphylococcus sp. SSB48, 13B = 2-month moromi with B. amyloliquefaciens SSB6.

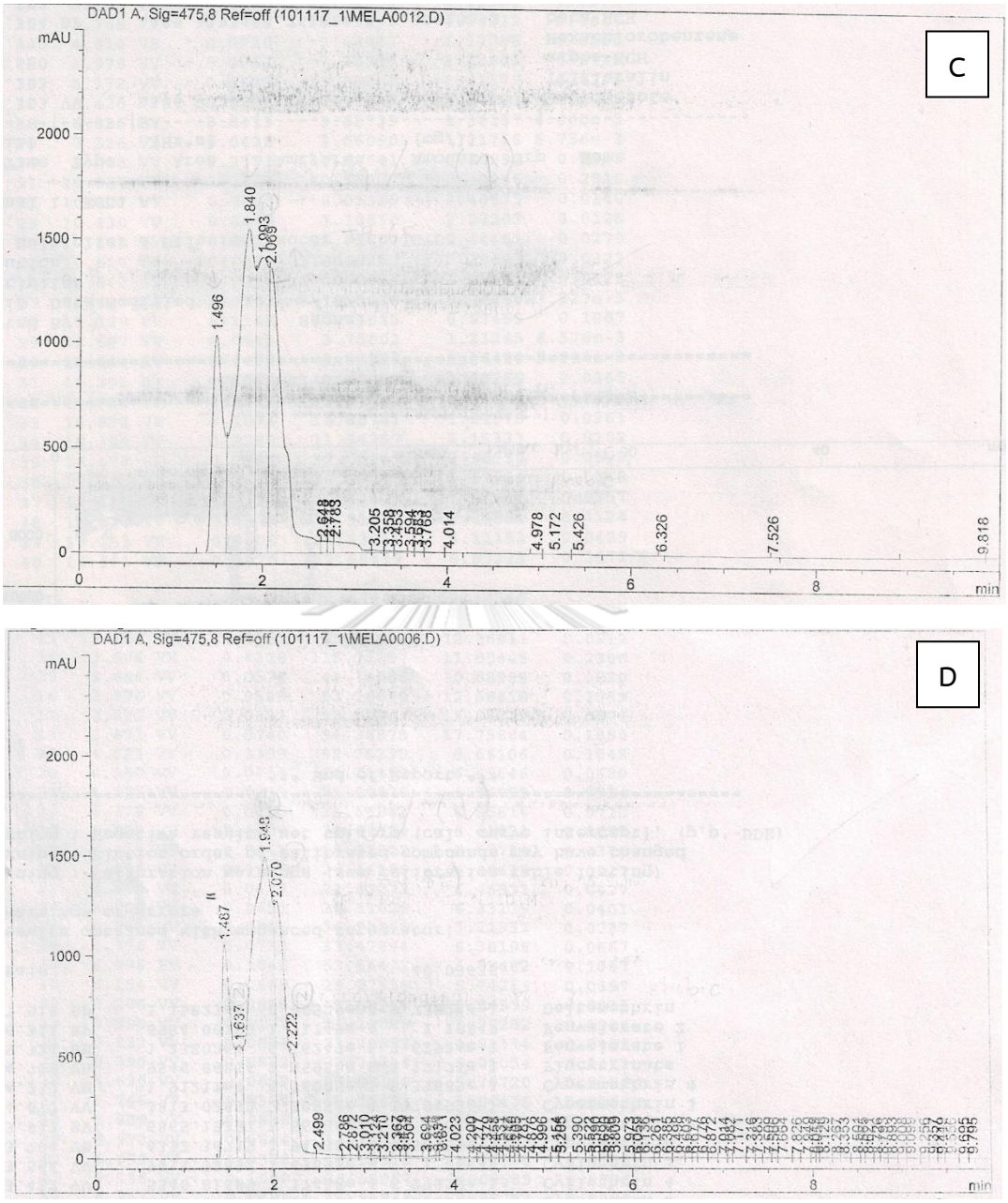


Figure 13 Chromatogram of melanoidin compounds in inoculated moromi after 2 months incubation (continue)

; 13C = 4-month moromi with *B. amyloliquefaciens* SSB6, 13D = 2-month moromi with mixed cultures.

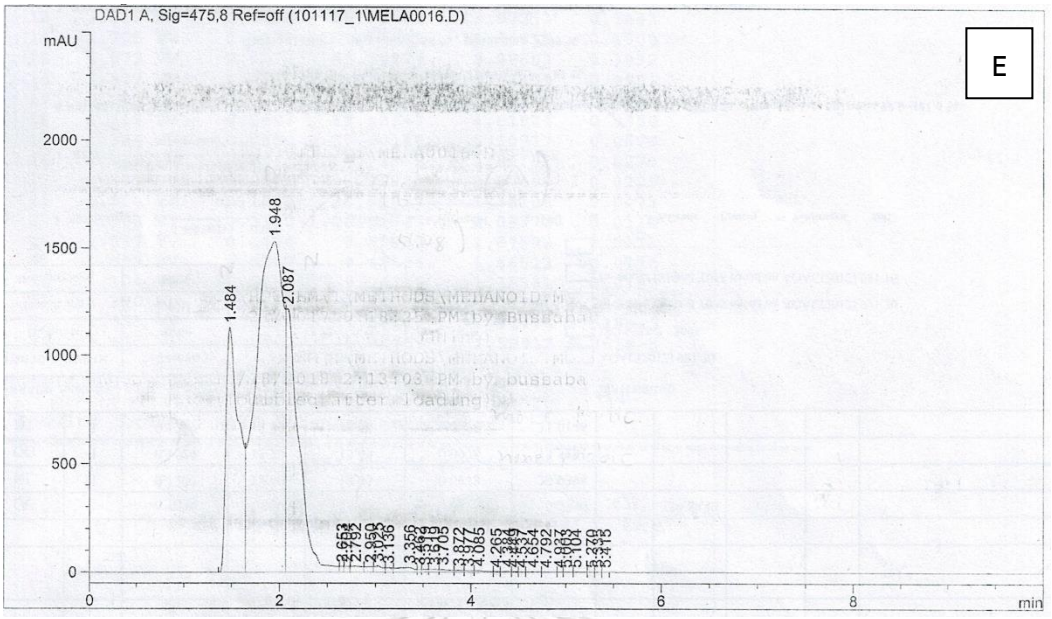


Figure 13 Chromatogram of melanoidin compounds in inoculated moromi after 2 months incubation (continue)

; 13E = 6-month moromi with *Staphylococcus* sp. SSB48.

5.3.5 Influence of moromi condition

Considering results of dynamic shift of browning indexes in each sample during 2 months fermentation, a dynamic change of 4-month and 6-month control samples illustrated the progressive increase of total browning along with the decrement of HMF. Thus, Maillard reactants should be not available since 1 month after inoculation and HMF was continuously transformed into brown pigment. Contrastingly, HMF values from 2-month control treatment, and including its single inoculation treatments, were significantly raised but total browning remained stable. Since determination of HMF-type browning index based on removal of high molecular weight-low polarity compounds, protein and protein-bound compounds including melanoidin by mean of ethanol precipitation thus the remaining, even were mainly HMF, could include other colorless or light-color reactive Maillard reaction intermediate compounds such as 3-deoxyosuloses, cis-and trans-forms of 3,4-dideoxyosulos-3-ene (Echavarría et al., 2012), furfural and reductones (Lund and Ray, 2017). These intermediates, called as Amadori products, were the first stable compounds in Maillard reaction and could be further reacted with others and / or free amino and small peptide into high-molecular weight deep color of melanoidin (Troise et al., 2018). Since high-Maillard reactivity free amino acids in 2-month control including Typ, Tyr and Gly were remarkable lower than those in 4 and 6-month (Figure 4A and B; 2, 4, 6-month moromi as SP1I, SP1M and SP1F, respectively) hence advance melanoidin formation could be retarded and most of Maillard reaction products were in ethanol-dissolvable Amadori products resulting in remarkable stable of total browning.

In order to quantify key reducing sugars in moromi, samples fermented for 2 months were subjected to HPLC analysis. However, reducing sugars determined in this experiment of glucose, arabinose and xylose, were totally disappeared from

all samples. This should be due to either biotransformation or spontaneous chemical reaction of browning, or their co-reaction. Considering of total browning difference of start samples (Figure 6, SP1) against all 2 month inoculation control treatments (Table 9), there were increased in ranged of 58.75 – 85.01% which indicated an extreme browning occurred during fermentation period. Concomitantly with substantially TPC values, it seemed like vanishing of reducing sugars should be from chemical reaction rather than biotransformation, and this depletion was then, in turn, influenced the growth of bacteria in the system.

HCL analysis of volatile compound with moromi treatments depicted a big cluster of inoculated and inoculated moromi while 2-month moromi inoculated with *B. amyloliquefaciens* SSB6 was excluded (Figure 11). In the big cluster, all control samples were detached into small subgroup referring the shift in volatile compound which should be due to metabolic activity of autochthonous native microorganisms survived from sub-zero storage condition along with progressive reaction of Maillard products. Two key volatile compounds found in industrial-process soy sauce of phenol, 4-ethyl-2-methoxy (etc12) and phenylethyl alcohol (al6) (Figure 3.4 and Appendix B and D), along with others previously reported as major volatiles such as 1-butanol,3-methyl (al3) ; benzeneacetaldehyde ; phenol, 2-methoxy (etc4) and pentanal (Devanthi et al., 2018; Sun et al., 2010) still broadly remain in moromi with high proportion. Hence, inoculation of potential cultures did not affect key quality in term of aroma.

Qualitative analysis of melanoidin generated in 2 months fermentation of moromi was conducted and chromatograms of control samples revealed the different patterns including peak shifting (example were peaks at RT of 1.859 of Figure 12A with at RT 1.844 of Figure 12B and 1.863 of Figure 12C, occurrence of new peaks and clearance of some peaks such as detection of peak at RT 4.338 and 4.388

in 2 and 4-month samples and deletion of small peaks during RT 4.500 to 5.500 observed in 6-month moromi. These indicated dynamic shift of melanoidin during soy sauce fermentation of, at least, industrial-process moromi of SP1. Considering peaks eluted during first 2.200 minutes, these compounds revealed high hydrophilicity due to its early released from C18 column by dissolving with polar solvent of acetonitrile-methanol mixture and were the major melanoidin compounds detected by HPLC as illustrated in Figure 11A – C. These unretained peaks were noted by characteristic of brown compound (Bailey, 1996 #373). After this RT, numerous small peaks were serially eluted based on their polarities and were named of minor peaks in this experiment. The same result of these convex broad bands was also report in the work of Bailey, Ames and Monti (1996) and their diode-array spectra reflected a formation of brown polymer designated as polymeric melanoidin. Thus results in this work depicted the variation of melanoidin profiles mainly relying on generation and deletion of small polymeric than large hydrophilic brown compounds through fermentation period. The fluctuation of dominant polymeric melanoidin began with high hydrophilic compound at the 2-month moromi, disappeared in the middle of 4-month and became more hydrophobic at the final period.

5.3.6 Influence of inoculation condition

In order to determine the expression of inoculated potential strains toward browning, viable cell with metabolically-active function had to be firstly identified. Comparing with total population of moromi before frozen storage (Table 3, where SP1I= 2-month, SP1M= 4-month and SP1F= 6-month moromi), all moromi were dominated by autochthonous Staphylococci and Bacilli, which the latter mainly presented in spore form rather than vegetative cell (Sakaguchi, 1958). When food

matrix such as moromi was stored at -20°C, Staphylococci merely survived due to sub-zero freezing coupling with acidity effects (Archer, 2004). Thus, after moromi was thaw and storage at room temperature, spore of Bacilli might flourish and dominated the system, however, since TVC values of all samples fermented for 1 and 2 months were comparable, therefore, most of the Bacillus bacteria presenting in moromi likely to be in spore phase and turned to vegetative cell only on plating procedure. This was also supported by observing of colony morphology on plates, which crowned by large, lobate margin, raised, dull, light cream color colonies with an occasionally minor population of other including small, round, raised, light orange yellow colony (Table 8). The metabolically-inactive status of dominant bacteria also affected other key properties which were discussed below.

5.3.6.1 Influence of single inoculation: Staphylococcus sp. SSB48

Single inoculation of potential *Staphylococcus* sp. SSB48 was strongest pronounced toward browning index in 6-month moromi treatment as the reduction of HMF at 15.14% and of total browning at 10.84% were calculated. These decrements were noticed even *Staphylococcus* sp. SSB48-similar colony was detected just only in the first month, Considering reduction of total browning, it was noted that bacterial oxidoreductase were generally produced intracellular (Rodríguez-Couto, 2018) and/or immobilized on spore surface (Dwivedi et al., 2011) indicating the possible activity even after cell lysis. This hypothesis was supported by 3.81% reduction of major peak area, disappearance of high hydrophobic brown polymer eluting after RT 5.450 and generation of many hydrophilic convex band of *Staphylococcus* sp. SSB48-6-month treatment toward its control (Figure 13E and 12C).

Staphylococcus sp. SSB48-2-month moromi treatment also reveal shift of melanoidin compound. This chromatogram contained lower proportion of major peak area from the control with 2 shifting peak at RT 1.496 and 1.918, and vanishing of one peak at RT 1.859 (Figure 13A). More of high-polar polymeric small peaks were detected along with deletion of all low-polar compounds eluted after 10 minutes of injection, which was the same result as depicted in *Staphylococcus* sp. SSB48-6-month moromi treatment (Figure 13E). These represented the possible biotransformation and biodegradation function (Kumar and Chandra, 2018) of this strain. Comparing with the stable total browning value and remarkable increasing of HMF (49.47%) during first and second fermentation month of 2-month moromi, and less activity (raise of 25.53% HMF) in 6-month treatment the possibility of bacterial degradation of high molecular weight melanoidin into small hydrophilic compounds in *Staphylococcus* sp. SSB48 was supported.

Additionally, metabolic activity of *Staphylococcus* sp. SSB48-2-month moromi toward volatile profiles reduced styrene, a benzene derivative with pungent smell (Tischler, 2015), which might be related to extracellular oxidoreductase activity (Braun-Lüllemann et al., 1997). In the same time, most of key volatile compound presenting in 2-month control moromi, namely phenol, 4-ethyl-2-methoxy; phenylethyl alcohol; 1-butanol,3-methyl; benzeneacetaldehyde; phenol, 2-methoxy and pentanal was preserved in *Staphylococcus* sp. SSB48-inoculated treatment thus indicating the feasibility of this strain.

5.3.6.2 Influence of single inoculation: B. amyloliquefaciens SSB6

Even the presenting of metabolically- active *B. amyloliquefaciens* SSB6 could not clearly identified by morphological determination as discussed in 5.3.6, results of browning indexes along with volatile and melanoidin profiles

indicated an expression of this bacteria. Firstly, there was a statistically reduction of HMF in *B. amyloliquefaciens* SSB6-6-month moromi (15.97% from its control) concomitantly with the similarly colony of this bacteria was noticed (Table 9).

The second evidence was from dynamic shift of volatile profiles from the control samples. In 4-month moromi, *B. amyloliquefaciens* SSB6 had less positive relation with benzaldehyde and disulfide dimethyl, volatile generated by spontaneous Maillard reaction (Liu et al., 2015) than its control sample, illustrating its notable shift in Maillard reaction product. Higher proportion of pyrazine, 2,6-dimethyl also evidenced the growth and metabolic activity of *B. amyloliquefaciens* SSB6 since this volatile, along with other derivatives, was generated by *Bacillus* species (Kim et al., 1996). As the same format, a *Bacillus*-generating volatile metabolite of 1-octen-3-ol (Lee et al., 2018) could support an existing of *B. amyloliquefaciens* SSB6 in 2-month moromi as well. Thus, expressing of other metabolic activities such as pentose utilization and formation of oxidoreductase should be pronounced in these 2 treatments. It was noted that *B. amyloliquefaciens* SSB6 in 2-month moromi had volatile profiles of key aroma compound as stated in 5.3.5 in a comparable proportion toward its control, addition with higher pentenal therefore application of this strain as soy sauce starter culture was promising.

Metabolic activity of *B. amyloliquefaciens* SSB6 toward melanoidin from 2-month moromi seemed to be affected mostly with polymeric melanoidin with hydrophobicity character as all of minor peaks with RT more than 5.631 were all degraded, leaving system dominated by hydrophilic brown compounds (Figure 13B). Contrastingly, the culture growth in 4-month sample depicted biodegradation property of major peaks as 2 compounds at RT 1.496 and 1.840 shown the reduction of their peak area (Figure 13C). Additionally, the remarkable formation of minor polymeric peaks particularly those hydrophobic

eluting after 10 minutes was noted in this treatment and might be the related to the aggregation phenomenon found previously in micro fermentation of *B. amyloliquefaciens* SSB6 with soy sauce broth as stated in 4.3.2.1.1 and Figure 7. Low polarity browning substance generated in 4-month moromi could cluster with others by hydrophobic interaction and further fermentation might be finally led to precipitation of large browning polymeric compounds. The same reduction of total dissolved solids in microbial treated-melanoidin was also reported (Kumar and Chandra, 2018; Yadav and Chandra, 2012).

5.3.6.3 Influence of mixed culture inoculation

Mixed culture of *B. amyloliquefaciens* SSB6 and *Staphylococcus* sp. SSB48 inoculated into 2-month moromi shown the lowest HMF value of 2.635 toward all tested sample at the second month. From the hypothesis proposed in 5.3.5, the stable total browning values throughout fermentation time should be, at least partly, due to Maillard retarding condition as found in control sample. In term of HMF, it was possible that some co-metabolic activities involving browning degradation were expressed and resulted in elimination of Amadori products thus HMF value resembled stable. This proposed scheme might relate to the lower proportion of anti-2-(alpha-phenyl ll-tosylaminoethyl)-2,5-dihydrofuran in this treatment. This furan derivative product of Maillard reaction (Umano et al., 1995) could be degraded by bacterial activity (Wang et al., 1994) resulted in2,3-butanediol such detected in verification of candidate strains toward volatile compound generation (Figure 11).

An interesting activity of bacteria with melanoidin was noticed in mixed culture treatment where biodegradation of major peak at RT 1.948, a reduction of total major peak area, and formation of new polymeric compounds, both hydrophilic and hydrophobic, were marked (Figure 13D). Comparing with single culture treatments of either *Staphylococcus* sp. SSB48 or *B. amyloliquefaciens* SSB6, a greatly increase of small polymeric peaks after RT 2.200 was noted in mixed culture treatment, proposing the better formation of simple molecules from the complex melanoidin via co-metabolic activity of inoculated strains than the single culture (Yadav and Chandra, 2012), and evidence melanoidin degradable properties in these strains which should be via oxidoreductase enzyme of laccase in *B. amyloliquefaciens* (Wang, Lu, et al., 2017) and *Staphylococcus* sp. (Chauhan et al., 2018; Velayutham et al., 2018). Those previous literatures indicated laccase production as intracellular for *B. amyloliquefaciens* (Chen et al., 2015) was extracellular and secreted into the system in *Staphylococcus* sp. (Chauhan et al., 2018) thus enhancement of melanoidin degradation should rely on, at least partly, these 2 different characteristics along with the specific degradation pathway of laccase which was reported as β-O-4 and Cβ-Cγ bonds, and Cα-oxidation in *B. amyloliquefaciens* (Yang et al., 2018), and as o-phenols oxidation in *Staphylococcus* sp. (Chauhan et al., 2018).

From overall results, the best condition for decolorization by either *Staphylococcus* sp. SSB48 or *B. amyloliquefaciens* SSB6 were revealed in 6-month moromi together with fermentation time of 1 month. Metabolic activity of *Staphylococcus* sp. SSB48 reduced HMF and total browning as high as 15.14% and 10.84%, respectively, along with the biodegradation activity with minor peaks of hydrophobic melanoidin compound. *B. amyloliquefaciens* SSB6 decreased HMF to 15.96% and degrade major melanoidin peaks (14.55% reduction). HMF decolorization and melanoidin degradation efficiencies of mixed cultures in 2-month moromi was

lower than the single treatments (13.18% and 4.53% reduction, respectively) hence co-inoculation seemed to inhibit negative effect with Maillard-based decolorization.

Further research will be focused on semi-pilot scale optimization of approximately 700 kg moromi fermentation in fermenter vat with similar biological and physicochemical determinations. Among these, additional methods for organoleptic properties examination should be applied in order to fully and correctly evaluation of flavors and aromas in final products. Since all information related to this event was done only in chemical analysis, which both of its own technique's bias and matching derived results with multisensory perception still be major challenges in this research's field (Regueiro et al., 2017). Hence implementation of sensory analysis by mean of trained panels and might be supplementary chemical-based methodology such as should be done to depict the better accurate picture of flavor and aroma profiles. In addition, balance of volatile toward none and semi-volatile compounds, and proportions of dominant with background volatiles are also imparted in complexity in soy sauce (Kong et al., 2018; Yanfang and Wenyi, 2009), thus comparative analysis of these characters with qualified sample should be verified.

Two potential isolates *Staphylococcus* sp. SSB48 or *B. amyloliquefaciens* SSB6 were selected based on their functionality in decolorization of Maillard reaction related-substances. The safety concerning was also taking place in this starter selection as biogenic amine-encoding genes were determined. However, further standard toxicology evaluation of clinical trails must be evaluated for commercial application of these isolates following national and international regulation. The safety evaluation process included toxicity identification by

comparing genome sequencing with databases, history of safe use and origin of isolates (Pariza et al., 2015).

CHAPTER 6
CONCLUSION

Microbial population and diversities assessed by culture dependent and independent method based on RevT-nested PCR DGGE depicted different dynamic shift of microbial communities through moromi fermentation. Through the RNA-based DGGE analysis, the role of *Bacillus* and *Staphylococcus* in the reduction of browning generation speed was reflected. In addition, within a group of these two bacteria, some potential strains that could play a significant role in the reduction of browning color were observed. This possibility is due to their enzymatic reaction that was capable of degrading melanoidin compounds without interfering with the moromi aromatic properties. This information fulfills the objective that metabolomic database together with autochthonous profiles could help to simple selection of potential strains to further develop as autochthonous culture for specific use in browning color reduction in soy sauce as proposed.

Total of 139 bacteria mainly *Bacillus* and *Staphylococcus* and 105 yeast isolates, mainly, *Bacillus* sp., *B. amyloliquefaciens, B. subtilis, Staphylococcus sp., S. carnosus, S. condiment, Candida* spp. and *Z.rouxii* were obtained from moromi fermentation process. The isolates were screened for their potentials in browning

color reduction through an evaluation of salt tolerance properties, sugars utilization and browning substances degradation. All tested strains were highly halophillic that tolerated up to ≥15% salt. Fifty-nine bacterial and 29 yeast isolates had arabinose and/or xylose fermentable ability and were selected to determine extra-feature of browning degradation. Five bacterial strains having ability in degradation of browning substances when tested in soy sauce model and/or synthetic melanoidin, In addition with having ability in co-metabolization of xylose and glucose were obtained.

After safety evaluation of the isolates, two candidate strains were selected, *Staphylococcus* sp. SSB48 and *B. amyloliquefaciens* SSB6 to strains optimization process. The best condition for decolorization by either *Staphylococcus* sp. SSB48 or *B. amyloliquefaciens* SSB6 were revealed in 6-month moromi together with fermentation time of 1 month. HMF decolorization and melanoidin degradation efficiencies of mixed cultures in 2-month moromi was lower than the single treatments hence co-inoculation seemed to inhibit negative effect with Maillard-based decolorization of moromi.

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APPENDICES

APPENDIX A

CHROMATOGRAM AND STANDARD CURVE OF STANDARD SUGARS

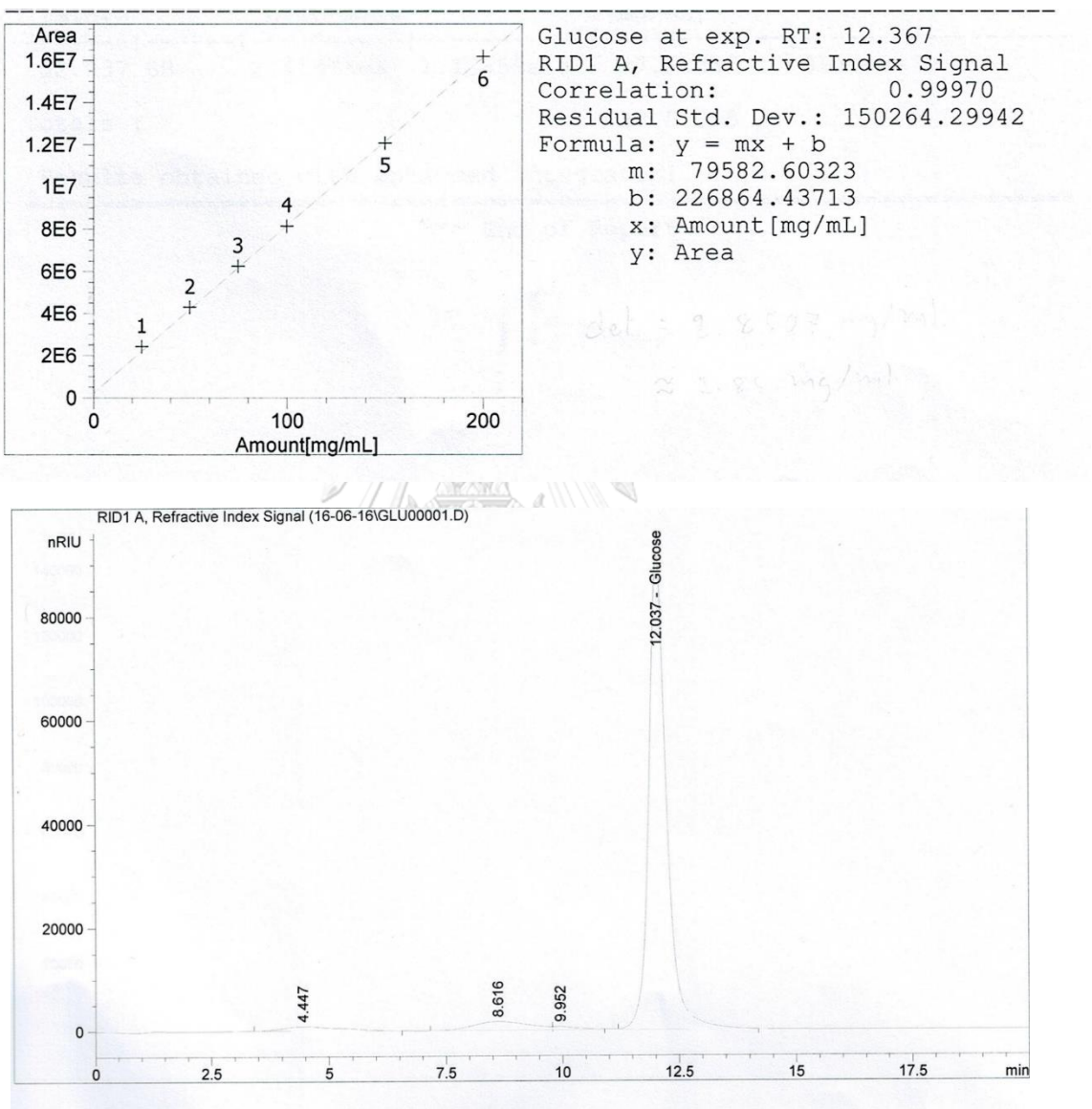


Figure A1 Standard curve of glucose along with its chromatogram

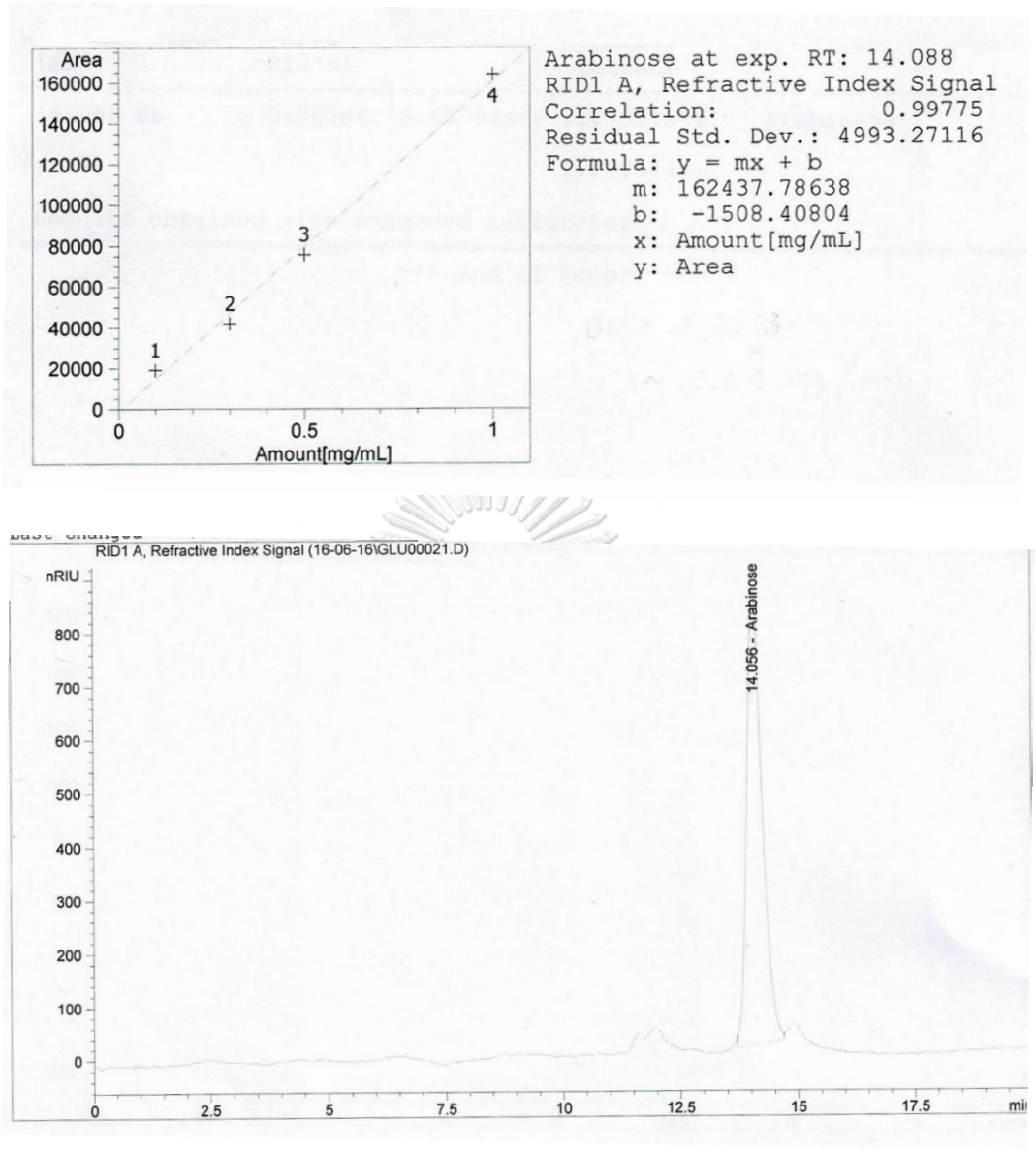
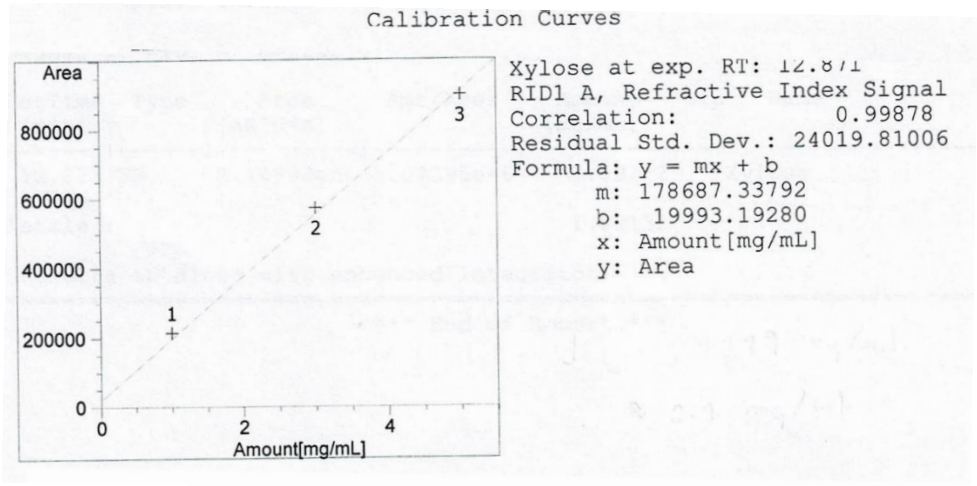


Figure A2 Standard curve of arabinose along with its chromatogram



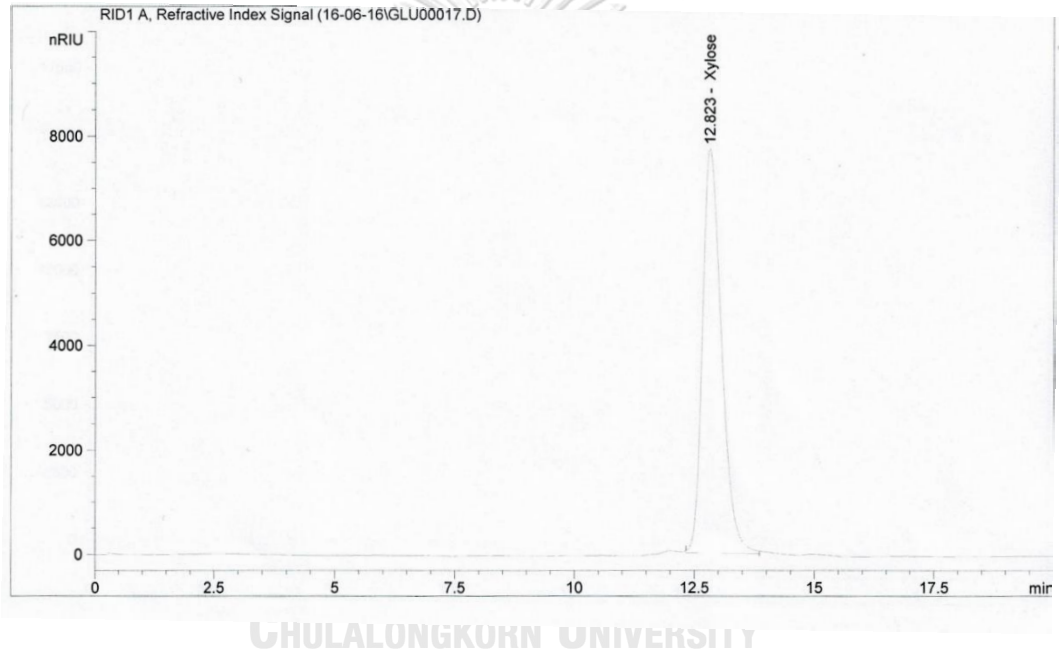


Figure A3 Standard curve of xylose along with its chromatogram

APPENDIX B

AMINO ACID PROFILES IN INDUSTRIAL AND TRADITIONAL-PROCESS MOROMI

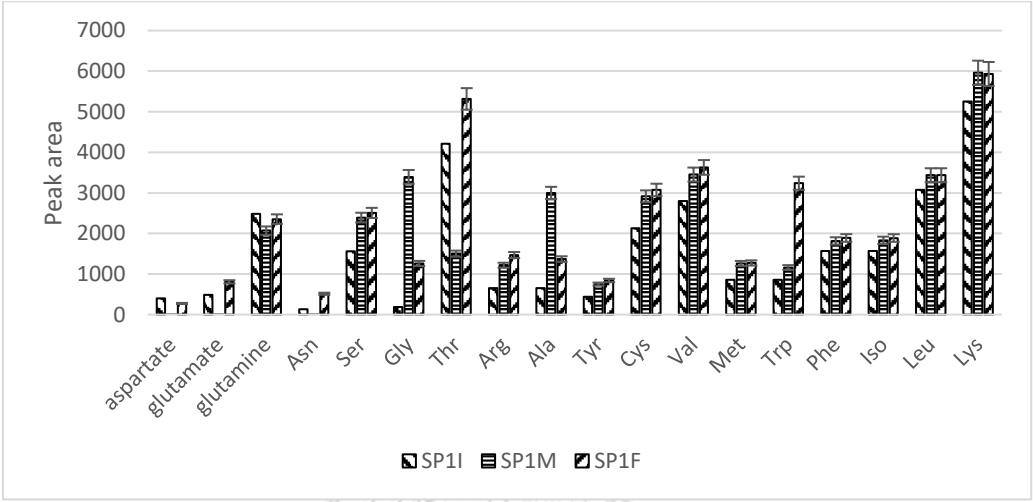


Figure B1 Free amino acids of SP1 moromi

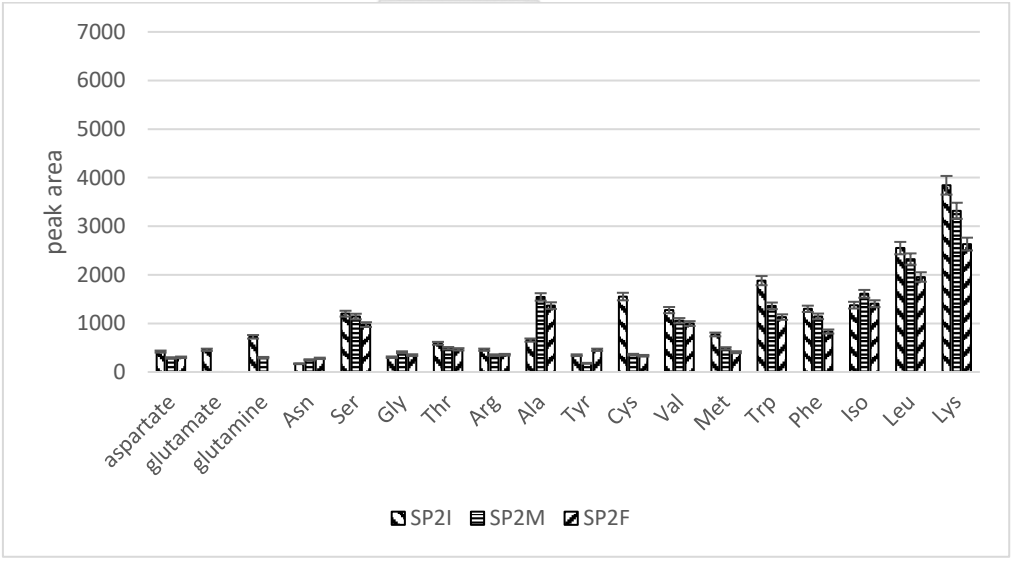


Figure B2 Free amino acids of SP2 moromi

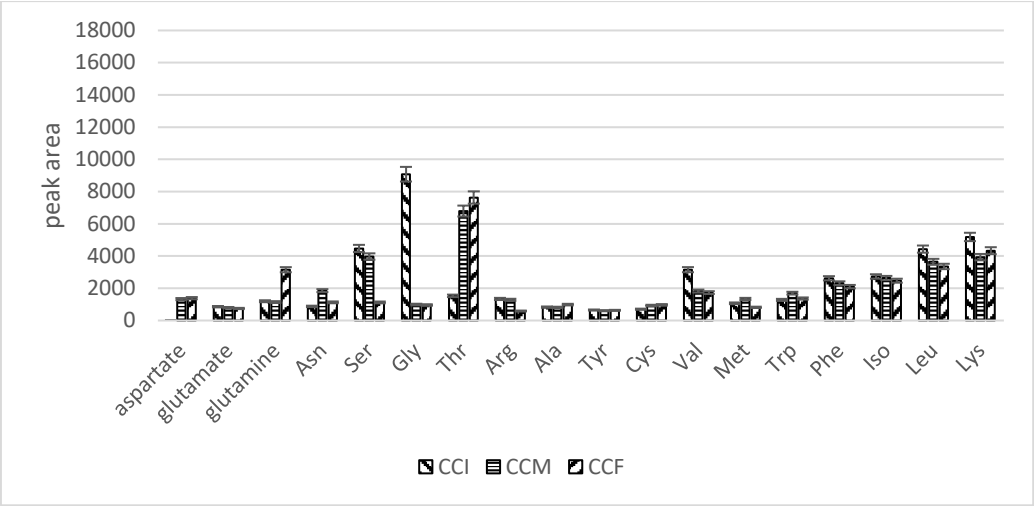


Figure B3 Free amino acids of CC moromi

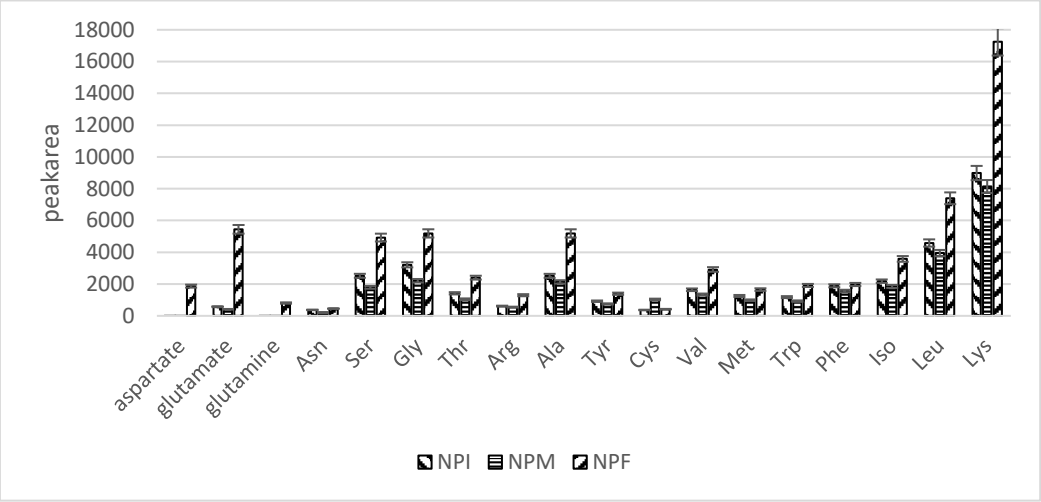


Figure B4 Free amino acids of NP moromi

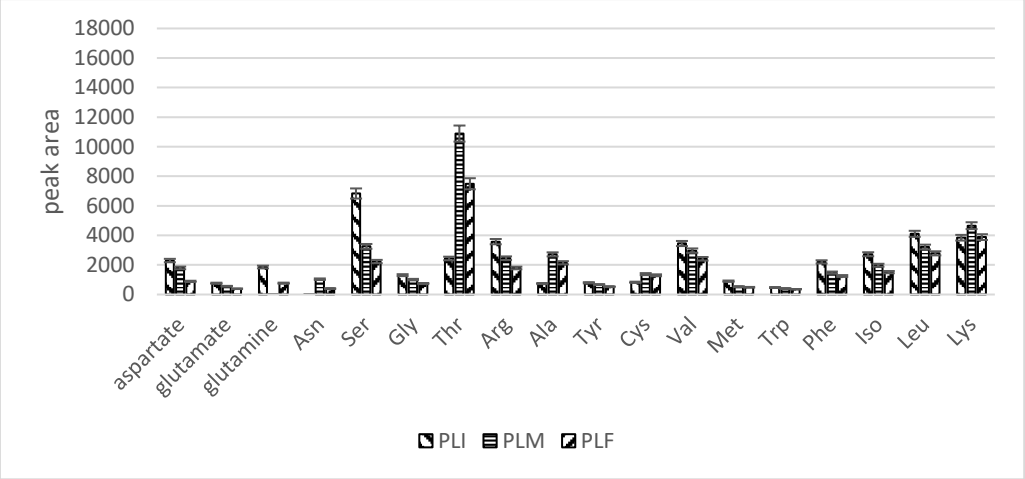


Figure B5 Free amino acids of PL moromi

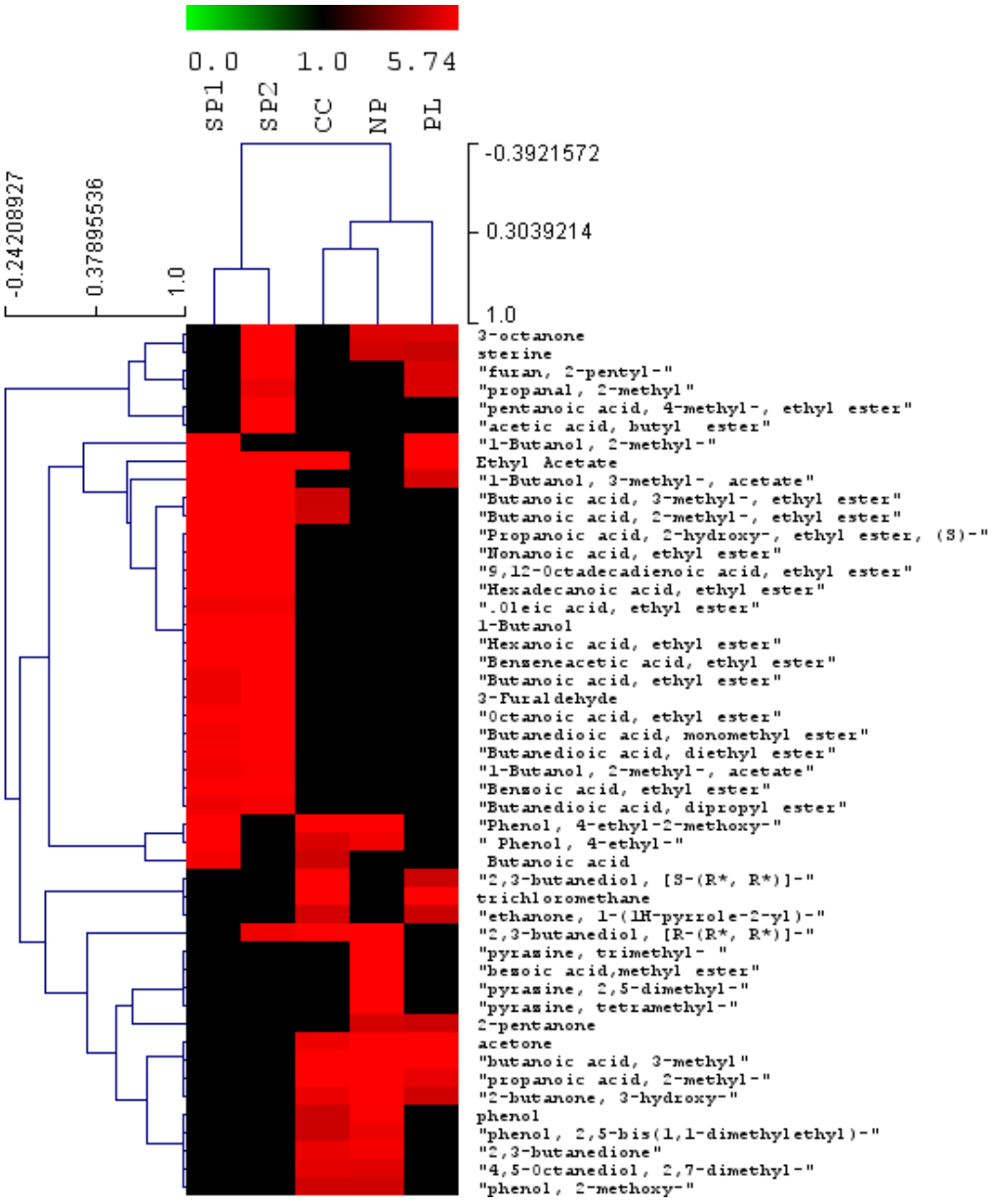
APPENDIX C

THE MOST 10 ABUNDANT VOLATILE COMPOUNDS IN MOROMI

Order	SP1	SP2	CC	NP	PL
1	Ethanol	Ethanol	Butanoic acid, 3-methyl-	Butanoic acid, 3-methyl-	Acetic acid
2	Ethyl Acetate	Ethyl Acetate	Trichloromethane	Propanoic acid, 2-methyl-	1-Butanol, 3-methyl-
3	Phenol, 4-ethyl-2-methoxy-	1-Butanol, 3-methyl-	Phenylethyl Alcohol	1-Butanol, 3-methyl-	Ethanol
4	1-Butanol, 3-methyl-	Phenylethyl Alcohol	Acetic acid	Benzoic acid	Butanal, 3-methyl-
5	Acetic acid	Acetic acid	2,3-Butanediol, [S-(R*,R*)]-	Acetone	Butanal, 2-methyl-
6	Phenylethyl Alcohol	Propanoic acid, 2-hydroxy-	Propanoic acid, 2-methyl-	Pyrazine, trimethyl-	1-Butanol, 2-methyl-
7	Propanoic acid, 2-hydroxy-,	1-Butanol, 3-methyl-,	Ethyl Acetate	Phenylethyl Alcohol	1-Octen-3-ol
8	Benzaldehyde	Benzaldehyde	2,3-Butanediol, [R-(R*,R*)]-	2-Butanone	Butanoic acid, 3-
9	Butanal, 2-methyl-	Butanoic acid, 3-methyl-,	Benzaldehyde	Benzoic acid, methyl	Oxime-, methoxy-
10	1-Butanol, 2-methyl-	Hexanoic acid, ethyl ester	Oxime-, methoxy-phenyl-_	Benzaldehyde	Benzaldehyde

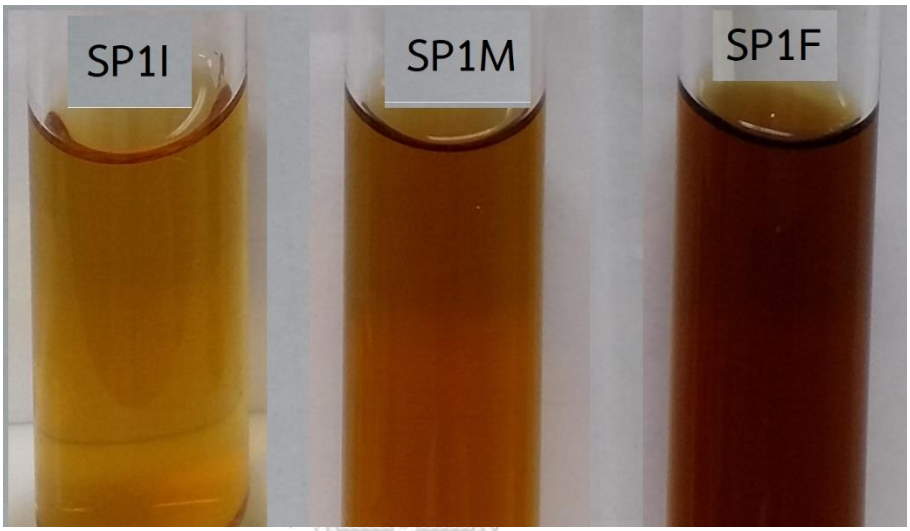
APPENDIX D

HCL ANALYSIS OF VOLATILE COMPOUNDS IN MOROMI



APPENDIX E

PHYSICAL APPEARANCES OF SOYMEAL-FREE MOROMI SUPERNATANT FROM INDUSTRIAL SP1 PROCESS



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