Identification of Yeast Species in *Sompuk*, a Fermented Vegetable from Vientiane Markets, Lao PDR

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Abstract:

Sompuk is Lao fermented vegetable made from mustard green, white cabbage, green onion, cooked skin of cow, salt and some flavoring. In this study, 27 samples comprised of three types of *Sompuk*, including *Sompukkard*, *Sompukalam* and *Sompukboua*, were obtained from three fresh markets in Vientiane, namely Khuadin, Thongkhankham, Thatluang. All *Sompuk* samples were determined for the presence of yeast in the products. The results revealed that the fermented vegetable had a range of yeast from 2.26 to 4.67 log CFU/g. After purification and identification, eight species of yeast, namely *Candida globosa, C. utilis, C. valida, Rhodotorula glutinis, Sporobolomyces salmonicolor, Saccharomyces cerevisiae, Schwanniomyces etchellsii* and *Zygosaccharomyces* spp., could be recognized from the *Sompuk* samples. Some of these yeasts have been applied in food and beverages fermentation. However, the presence of these yeasts in *Sompuk* products might have a role as spoilage microorganisms.

Keyword: *Sompuk*, Fermented vegetables, Yeasts, Enumeration, API identification

1. Introduction

Fermented vegetable is one type of fermented food products that is mainly produced through acid fermentation and/or salt addition (Perez-Dıaz *et al*., 2014). The production of this fermented vegetable is partly aimed to extend the short shelf life of the raw materials (Tamang, 2010). As other fermented food products, lactic acid bacteria (LAB) play a dominant role throughout the fermentation process (Nguyen *et al*., 2013). An example of this includes *Kimchi*, which is a Korean traditional fermented vegetable. The fermented vegetable is produced by various LAB, such as *Lactobacillus plantarum, L. pentosus, L. brevis, L. fermentum, L. casei* and *Leuconostoc mesenteroides*, after brined cabbage or radish is mixed with different spices, including red pepper, garlic, ginger, green onion and other ingredients (Lee *et al*., 2016).

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In Laos, *Sompuk* is known as a local fermented vegetable. It is popular among Lao people in which they usually consume it together with their main meals, particularly during summer season (Sing, 2007). *Sompuk* can be made from garlic, Chinese mustard green, white cabbage, green onion, cooked skin of cow, salt and glutinous rice. The production of *Sompuk* is started by mixing all ingredients together and fermented at room temperature for 12–24 h. The fermented vegetable is usually consumed within 3–5 days after production. Depending on the main raw material, *Sompuk* can be divided into three types, including *Sompukkard*, *Sompukboua* and *Sompukalam* that are produced from mustard green, green onion and white cabbage, respectively (Sing, 2007).

Yeasts are included in a unicellular fungal group that has morphology of single cell. The microorganisms can be found in natural environments as in plants, animal and food. They can adapt and survive or live in aerobic and anaerobic conditions, low pH, high sugar and salt concentration and are able to ferment sugar (Adams and Moss, 2008). Due to this reason, yeasts can be present during and/or after vegetable fermentation, when the product has a low pH value. Beside pH, salt concentration during vegetable fermentation can be increased and the presence of some nutritional compounds, such as organic acids, nitrogen and phosphorus, can support the growth of yeast (Pérez-Díaz *et al*., 2014). Since most of fermented vegetables do not receive any heat treatment, yeasts from the vegetable raw materials can continue to be presence in the final product (Breidt *et al*., 2013). It has been reported that Thai fermented vegetable was contaminated with some yeasts, including *Sacch*. *cerevisiae*, *Pichia etchell/carsonii*, *Candida krusei*, *C. tropicalis, C. jipolytica, C. rugosa, C. holmii, C. utilis, R. glutinis, Trichosporon cutaaneum* and *Zygosaccharomyces* spp. (Maneewatthana *et al.*, 2000). In *Kimchi* product, yeast was described to cause spoilage of the final product (Mheen and Kwon, 1984). Olajuyigbe *et al*. (2017) had conveyed that the presence of yeast could sometimes cause a serious public health hazard and might lead to economic losses. Since there is no available data regarding the presence of yeasts in *Sompuk*, this study is aimed to enumerate and identify yeasts in different types of *Sompuk* samples.

2. Materials and Methods

2.1 Collection of *Sompuk* samples

A total of twenty seven *Sompuk* samples, mainly *Sompukkard, Sompukboua* and *Sompukalam*, were collected from three fresh markets, including Khouadin, ThokhanKham and Thatlouang markets, located in Vientiane, capital city of Laos. In each market, *Sompuk* samples were purchased from three different food sellers. All of the fermented vegetable samples were placed in an ice box with dried ice (Olajuyigbe *et al*., 2017) and transferred to the Faculty of

Agro-Industry, Chiang Mai University, Thailand within 24 h. When the samples arrived in the Faculty of Agro-Industry, the samples were directly stored in a refrigerated condition and analyzed in the following day.

2.2 Enumeration of bacteria and yeasts in *Sompuk* samples

To determine total viable count (TVC) and yeast in *Sompuk* samples, 25 g of the fermented vegetable was transferred into a stomacher bag and mixed with 225 ml of sterile 0.1% peptone water (Himedia, India). The sample was homogenized for 2 min in a stomacher (IUL Nr636/470, Spain). After homogenization, the sample was serially diluted up to 10^{-6} dilution. From appropriate dilutions, 0.1 mL of the sample solution was spread plate on Tryptic Soy Agar (TSA; Merck, Germany) or Potato Dextrose Agar (PDA; Himedia, India) containing 10% tartaric acid (Pu *et al*., 2014) for TVC or yeasts, respectively. The TSA, PDA and tartaric acid were separately sterilized at 121ºC for 15 min before used in the enumeration experiment. The inoculated plates were then incubated at 37ºC for 24–48 h (Jung *et al*., 2016) or at 30ºC for 4–5 days (Yang *et al*., 2014) for TVC and yeasts, respectively. After the incubation period, TVC or yeast colonies within a range of 30–300 colonies/plate were used in the microbial calculation using Equation 1 (Adams and Moss, 2008).

$$N_w = \frac{(C_1+C_2)}{(n_1+n_2/10)} \times \frac{1}{V} \times \frac{1}{d1}$$

Where, N_W = Number of colonies per g of *Sompuk*; C_1 = the total count on (n_1) replicates at dilution d_1; C_2 = total counts on (n_2) replicates of the next dilution; V = volume of sample plated; d_1 = first dilution factor.

2.3 Isolation, maintenance and identification of yeast colonies from *Sompuk* samples

For yeast isolation, 20 different colonies from different *Sompuk* samples that grew on TSA from TVC enumeration or PDA from yeasts counting, were separated, given specific codes, streaked on fresh solidified corresponded media and incubated at 37ºC for 24 h (Azizi-Shotorkhoft *et al.,* 2016) or at 30ºC with a similar incubation time as the enumeration method (Andrade *et al*., 2017), respectively. Purification of the colonies was done at least twice (Deschuyffeleer *et al*., 2011). A single colony from isolated yeasts was then checked using a microscope (Olympus, US) at 400 x magnification for their purity and observation for cell shapes (Yelnetty *et al*., 2013). A confirmed pure yeast colony was then grown in sterile Potato Dextrose Broth (PDB; Himedia, India) and incubated at 30ºC for 24 h (Aepinus, 2008). The young yeast culture was then mixed with sterile glycerol (Merck, Germany) at a ratio of 50:50 and stored at -60ºC as a stock culture (Andrade *et al*., 2017).

Before Analytical Profile Index (API) determination, young yeast cultures, which were obtained by growing pure yeast cultures in Potato Dextrose Broth (Himedia, India) with an incubation condition at 30ºC for 24 h (Maneewatthana *et al*., 2000), were subjected to some biochemical tests in order to do grouping some of the colonies with similar biochemical characteristics. The biochemical testing was a series of carbohydrate sources, including glucose (Ajax, Australia), lactose (Ajax, Australia), fructose (Ajax, Australia), galactose (Ajax, Australia), sucrose (Ajax, Australia), maltose (Ajax, Australia) and melibiose (Ajax, Australia) (Liu, 2011). An amount of 50 ml of carbohydrate sources (10%) was added into a biochemical medium that was composed of 10 g peptone (Himedia, India), 5 g sodium chloride (Ajax, Australia), 3 g meat extract (Himedia, India) and 10 ml Andrade's indicator (Himedia, India; Atlas, 2010). After the addition of the yeast culture, the medium was incubated at 30ºC for 24 h under aerobic condition (Chanprasartsuk *et al*., 2013). Following the incubation, the medium was observed for color changing. When the medium color changed from yellow to red, it indicated positive result or the yeast could utilize the specific carbohydrate source (Atlas, 2010).

To ensure that the number of yeast colonies was high enough to do the API testing, pure yeast cultures were proliferated in PDB for 24 h at 30ºC (Maneewatthana *et al*., 2000). The enumeration of these yeast colonies revealed that after the incubation period, the number of yeasts was 8.29±0.02 log CFU/mL. These yeast cultures were then subjected for identification using API ID 32C (bioMérieux, France) method according to the manufacturer's instructions (Liu, 2011). This identification method had been reported to have extensive database and accuracy for common yeast (Wang *et al*., 2008; Santos *et al*., 2017).

2.5 Statistical analysis

Collected data were statistically analyzed using Completely Randomized Design of Analysis of Variance in SPSS version 17.0 software (SPSS, Chicago, IL, USA). A significance level was determined at 0.05 (p<0.05). Mean comparison was carried out using a Turkey's test (Jalil and Zenad, 2016).

3. Results and Discussion

3.1 Enumeration of bacteria and yeasts in *Sompuk*

Sompuk samples were enumerated for their total microbial counts using TSA medium. The results found that all of the fermented vegetables had a microbial in the range of 7.34±0.06 to 7.96±0.06 log CFU/g (Table 1). This finding was similar to a report of Rhee *et al.* (2011), who showed that the number of total bacteria in *Kimchi* after 1 day fermentation at 20ºC was 3.6×10^{7} CFU/mL. The high number of total microorganisms in *Sompuk* could contribute to the presence of lactic acid bacteria as suggested by Rhee *et al*. (2011) and Buckenhuesk (2015). According to Marco *et al*. (2017), many microorganisms found in fermented food products, including lactic acid bacteria, were either identical to or share physiological traits with species relevant to promoting gastrointestinal tract health. However to ensure the safety of *Sompuk* products, further identification of different bacteria in the femented vegetable needed to be carried out. Liu *et al*. (2018) found Gram negative rods, including Enterobacteriaceae, in *Rubing* milk cake and *Yan-cai* vegetable pickles. The Enterobacteriaceae family would include *Escherichia coli*, *Salmonella* and *Shigella*, which were known as pathogenic bacteria.

Table 1 Total viable count (log CFU/g) of *Sompuk* samples from Vientiane markets.

Fresh markets	*Sompuk* samples	Total viable count (log CFU/g)
	SPK*	7.40 ± 0.05^{e}
Khuadin	SPL	7.34 ± 0.06^{e}
	SPB	7.42 ± 0.06^{e}
	SPK	7.45 ± 0.05^{e}
Thongkhankham	SPL	7.96 ± 0.06^{a}
	SPB	$7.58 \pm 0.04b^{c}$
	SPK	7.90 ± 0.03^{a}
Thatluang	SPL	7.56 ± 0.05^{cd}
	SPB	7.61 ± 0.04^{b}
	SPK*	4.31 ± 0.07^{b}

Note: *SPK: *Sompukkard*, SPL: *Sompukalam* and SPB: *Sompukboua*.

$^{a-e}$ Different letters indicate significantly different at a statistical level of 0.05.

To investigate whether some yeast cultures were grown in the TSA medium, 20 different colonies were separated and streaked on fresh solidified TSA medium. The isolated colonies were derived from 10 colonies from *Sompukkard* samples, 2 colonies from *Sompukalam* samples and 8 colonies from S*ompukboua* samples. After purification of all of

these colonies, investigation of the colony morphology under a microscope revealed that there was only one yeast colony under a specific code of TSPB221 (Table 3). The colony was isolated from *Sompukboua* sample that was bought from Thongkhankham market. This colony was then subjected to biochemical characterization and also identified using an API kit.

Yeasts in *Sompuk* samples were enumerated on PDA and the results are shown in Table 2. From this Table, it could be seen that all the *Sompuk* samples significantly had different amounts of yeast (p<0.05). The range of yeast in the fermented vegetable was between 2.26±0.05 and 4.67±0.01 log CFU/g. The highest number of yeast was determined in SPK2, which was *Sompukkard* from Thongkhankham market. On the other hand, the lowest numbers of yeast were found SPB2 and SPB1, which *Sompukboua* collected from Thongkhankham and Khuadin markets, respectively. Finding of yeasts in this study was in an agreement with the review of Lee *et al*. (2015), who conveyed that yeasts, which could utilize lactic and acetic acids as carbon sources, were also presence at the end of *Kimchi* fermentation. Interestingly, different types of vegetable used to make *Sompuk* produced different amount of yeasts in the final product. There was a possibility that different raw materials, in this case mustard green, green onion and white cabbage, harbored different initial microbial population. For green onion, it was previously reported that the vegetable were hand-bunched and tended to involve more intensive hand labor during harvesting and packing processes than most fruits and vegetables, which could increase the probability of microbial contamination (Neetoo *et al*., 2011; Xu *et al*., 2015). Higher initial microbial load in this vegetable source might affect the growth of yeast in the final product. Maneewatthana *et al.* (2000) had reported that they found many types of yeast in brine vegetable fermentation, which were found in a range from 2.70 to 7.23 log CFU/g. Those yeasts in the brine included *Sacch*. *cerevisiae*, *C*. *rugosa*, *C. utilis, R*. *glutinis*, and *Zygosaccharomyces* spp. Some of these yeasts were also identified in this study (see the next section). It was previously conveyed that yeast could contaminate fermented food products from environments, producers, unhygienic conditions during processing and commercial markets (Olajuyigbe *et al*., 2017). According to Maneewatthana *et al*. (2000), the sources of yeast contamination in fermented vegetable products could be from producers, sources of vegetable, pets, water, soil and insects. In this study, some of yeasts in *Sompuk* samples might originate from the surrounding of the fermented vegetables during selling in the wet markets. Previous studies showed that commercial selling of *Sompuk* was carried out in open containers on market tables that were offered together with fresh vegetables (Giawang *et al*., 2014; Emmanuel and Hontoyon, 2016). Due to this selling method, the possibility of contamination from fresh produces and buyers could not be ruled out.

Table 2 Enumeration of yeasts (log CFU/g) in *Sompuk* samples

Fresh markets	*Sompuk* Samples	Number of Yeasts (log CFU/g)
Khuadin	SPL	3.28±0.06[bd]
	SPB	2.26±0.05[e]
	SPK	4.67±0.01[a]
Thongkhankham	SPL	4.25±0.29[bc]
	SPB	2.33±0.04[e]
	SPK	4.39±0.08[b]
Thatluang	SPL	3.99±0.10[c]
	SPB	3.28±0.11[d]

Note: *SPK: *Sompukkard*, SPL: *Sompukalam*, SPB: *Sompukboua.*

[a-e] Different letters indicate significantly different at a statistical level of 0.05.

Table 3 Morphology, biochemical tests and identification of yeast from *Sompuk* samples

Codes	Colony characteristic	Morphology	Ga*	G	F	S	L	M	Me	Identity (%)	Yeast species
YSPL21	Red, round, big	Pseudohyphae	-	-	-	-	-	-	+	99.8	*Candida utilis*
YSPB34221											
YSPB321	White, smooth, round, big	Pseudohyphae	-	-	-	-	-	-	-	98.5	*Candida globosa*
YSPL311	White, smooth, black center, round, big	Pseudohyphae	-	+	-	-	-	+	+	97.7	*Candida valida*
YSPK331	White, smooth, black center, round, big	Pseudohyphae	-	-	-	-	-	-	+	96.0	*Zygosaccharomyces spp*
YSPK321, YSPK111 YSPK22	White, smooth, round, big	Pseudohyphae	-	-	-	-	-	-	-	95.7	*Rhodotorula glutinis*
YSPK112	White, round, big	Pseudohyphae	-	+	+	-	-	-	-	95.3	*Saccharomyces cerevisiae*
YSPK212	White, smooth, round, small	Pseudohyphae	-	+	-	-	-	-	+	90.3	*Sporobolomyces salmonicolor*
TSPB221	White, smooth, round, Big	Pseudohyphae	-	+	+	+	+	+	-	97.2	*Schwanniomyces etchellsii*

Note: *Ga = galactose, G = glucose, F = fructose, S = sucrose, L = lactose, M = maltose, Me = melibiose, Y = Yeast, T = Total Viable Count, SPK = *Sompukkard,* SPL = *Sompukalam,* SPB = *Sompukboua*, (-) means cannot ferment sugar and (+) can ferment sugar

3.2 Purification and Identification of yeasts from *Sompuk*

From different yeasts colonies growth on PDA, a number of 20 colonies, based on the color, size, and appearance, were selected to be further investigated. These 20 colonies were originated from 6 colonies from *Sompukkard*, 6 colonies from *Sompukalam* and 8 colonies from *Sompukboua*. All of the selected colonies were further purified by streaking at least two more times on fresh PDA medium and observing under a microscope. The pure colonies were also subjected to carbohydrate testing, namely galactose, glucose, fructose, sucrose, lactose, maltose and melibiose. Based on the colony characteristic, morphology and result of carbohydrate fermentation of yeast, the 20 yeast colonies were classified into 10 yeast groups. In Table 3, it is displayed the 10 yeast groups that had different capabilities to utilize carbohydrate sources. There were seven groups of yeast that could use glucose, four groups for fructose, one group for lactose, one group for maltose, six groups for melibiose and one group for sucrose. For the one yeast culture from TVC isolation, it could metabolize glucose, fructose, sucrose, lactose and maltose. All of the isolated yeasts from *Sompuk* were found to be unable to utilize galactose as the main carbohydrate source. It has been reported that yeast utilized carbohydrate sources to be changed into alcohol and carbon dioxide during fermentation (Walker and Stewart, 2016).

Isolated yeast colonies were cultured in fresh PDB and incubated at 30ºC for 24 h before being subjected to API identification using API ID 32C. The identification results can be seen in Table 3. All of the yeasts were identified as good to very good identification with ID percentage of not less than 90.2%. From 11 yeast groups, there were eight identified yeast species, including *C. utilis, C. globose* (the species was identified from colony codes YSPB34221 and YSPB321)*, C. valida, R. glutinis* (the species included colony codes YSPK321, YSPK111, and YSPK22)*, Spor. salmonicolor, Sacch. cerevisiae, Schwan. etchellsii* and *Zygosaccharomyces* spp. In a previous study, it has been reported that there were some species of yeast found in fermented vegetables, such as *Debaryomyces, Torulopsis, Brettanomyces, Hansenula, Endomycopsis*, *Candida*, *Pichia* and *Saccharomyces* (Fleming *et al*., 2013). *R. glutinis* and *Sacch. cerevisiae* were also discovered in Spanish olives green fermentation (Lara-Hidalgo *et al*., 2017). Based on Mheen and Kwon (1984) report, they informed that some molds and yeast could decrease the quality of *Kimchi* by softening fermented vegetable texture and imparting undesirable flavor. A similar note was also stated by Dang *et al*. (2009). These researchers stated that spoilage of food products from the growth of yeast *Zygosaccharomyces* could cause considerable economic losses in the food industry. A work by Gadaga *et al*. (2000) informed that *Candida lusitaniae* was recognized as an important spoilage yeast in yoghurt. On the other hand, pink yeasts, such as *Rhodotorula* spp.,

could change olive colour and produce softening in the olive fermentation (Arroyo-López *et al*., 2006). Beside causing spoilage, yeasts could also contribute to development of flavor in fermented food products (Gadaga *et al*, 2000; Tsuyoshi *et al*., 2005). Due to this reason, deeper understanding for the role of yeasts in *Sompuk* would be interested to be further investigated. Regarding *Sompuk* safety from the presence of different yeast species, previous workers had reported that *Candida* spp., *Lodderomyces elongisporus*, *Kodamaea ohmeri*, *Meyerozyma guilliermondii* and *Trichosporon ovoides* could cause serious infections in immunocompromised hosts, whereas *Sacch. cerevisiae* and *Cryptococcus* were opportunistic agents (Maciel *et al*., 2013; La Anh, 2015; Palková and Váchová, 2016). Since *Candida* spp. and *Sacch. cerevisiae* were identified in *Sompuk*, the product may not be suitable to be directly consumed by certain consumer segments. Reducing the presence of these yeasts through hygiene practices during production and distribution of *Sompuk* would improve the product safety.

4. Conclusion

This study investigated the presence of yeasts in *Sompuk*, mainly *Sompukkard*, *Sompukalam* and *Sompukboua*. The yeasts in the fermented vegetable were in the range of 2.26 to 4.67 log CFU/g. From these numbers, eight yeast species had been identified, particularly *C. globosa, C. utilis, C. valida, R. glutinis, Spor. salmonicolor, Sacch. cerevisiae, Schwan. etchellsii* and *Zygosaccharomyces* spp. The presence of these yeasts might affect the quality of the fermented vegetable during storage.

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