Utilization of Pumpkin Seeds (Cucurbita Moshcata D.) in the Making of Fermented Drink

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Abstract: Pumpkin (*Cucurbita moschata* Duchesne) is a type of Indonesian plants whose flesh is widely used as traditional processed foods (dodol, kolak, chips), while the utilization of the seeds is not optimal. Pumpkin seeds have been reported to contain bioactive compounds and functional nutrients (vitamins, fiber, antioxidants) that can be increased by the fermentation process. This study aims to produce fermented pumpkin seeds. Initially, the ratio of pumpkin seeds to water (1:3, 1:4, 1:5 [w/v]) and skim milk concentration (5%, 10%) was determined on LAB fermentation of *Lactobacillus plantarum* : *Streptococcus thermophilus* 1:1. Analysis of pH, total of titrated acids (TTA), and total LAB showed a ratio of 1:3 and 10% skim milk to provide products that meet the standards, which in a sequence are 4.34, 0.84%, 1.5×10^9 cfu/ml. The selected formulation was then used to determine the ratio of *L. plantarum* and *S. thermophilus* (1:1, 1:2, 2:1). The ratio of 1:1 is selected as the ratio that yields pH, TTA, and total LAB according to the standard, which in a sequence are 4.27, 0.84%, 2.2×10^9 cfu/ml. The products with preferred formulation contains proximate according to the standard (83.62% water, 0.84% ash, 2.30% milk fat, 4.00% protein, 9.24% carbohydrate [by difference]), phenolic 643.27 mg GAE/l, flavonoid 612.00 mg GAE/l; as well as increased antioxidant activity by 55.81% after fermentation (IC_{50} 291694.47 mg/l). The product is considered acceptable by panelists in hedonic (4.05 of 7.0) with a good level of safety based on toxicity test of 786.90 ppm (low toxic) and free of coiliform microorganisms.

1 INTRODUCTION

Pumpkin (*Cucurbita moschata* Duchesne) or more commonly known by the name of "Parang" Pumpkin/Calabash originated from America (Peru and Mexico). This type of Indonesian plant is included in the *Cucurbitaceae* family which is quite potential, about 20-21 ton/hectare (Data Badan Pusat Statistik dalam Kumala and Nurlaela, 2015). Pumpkin is widely used as traditional processed foods, such as *dodol, kolak*, and chips (Usmiati, *et al*., 2005). The part of pumpkin that can be utilized in addition to the fruit flesh is its seeds.

Research by Atuonwu and Akobundu (2010); El-Aziz and El-Kalek (2011); Primawati (2007) states that pumpkin seeds potentially have antioxidant activity of 47.01%. Pumpkin seeds are a good source of protein (39.25%) and is widely used as a diabetes drug in mice in the African region (Teugwa, *et al.*, 2013), as well as worm medicines and medications for functional bladder disorder in North America and Mexico (El-Aziz and El-Kalek, 2011). Utilization of pumpkin based on its functional potential, needs to be increased again especially in the field of food such as lactic acid fermented beverage containing lactic acid and probiotics so as to provide health benefits for the body (Vasudha and Mishra, 2013). According to Wardani (2011); Mardianto, (2015); and Kencana (2015), the fermentation process also increases the antioxidant (flavonoids and phenolics) which add value to fermented beverage products.

Ratio determination research of pumpkin seeds to water. According to Ariyanto, *et al.* (2015), Primurdia and Kusnadi (2014), and Usmiati and Utami (2008), separately reveals the best ratio that can be used in the manufacture of grain fermented beverages is 1:4 and 1:5; therefore in this study tested both these ratios and also as a comparison is the ratio of 1:3. The addition of skim milk is also a factor in the successful

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manufacture of lactic acid fermented beverages. Research done by Rachelia (2014) and Leo (2013) found that 4% and 5% of skim milk can optimize the fermentation process from peanut and tolo beans, while Sari (2014) found 10% skim milk to make fermented beetroot better. Therefore, in this study, it is used 5% and 10% skim milk. The selected formulation of the ratio and concentration of skim milk was used in the next phase of the study (the determination of culture ratio of *Lactobacillus plantarum* and *Streptococcus thermophilus*) by pH analysis, total titrated acid (TTA), and total lactic acid bacteria (LAB) compared with the standard BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011).

Determination of treatment level of culture ratio of *L. plantarum* and *S. thermophilus* based on research done by Sari (2014) which found the best amount of lactic acid and cell. This study used a ratio level of 1:2, 1:1, and 2:1. The selected ratio was determined through total LAB analysis, pH value, total titrated acid also compared with the standard BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011). The products with selected formulations are further chemically tested (antioxidant, phenolic, flavonoid) and microbiology (coliform), and evaluated by proximate, toxicity, and hedonic test, so that this pumpkin fermented beverage will meet the standard of fermented beverages and may be accepted by the panelists nutritionally functional and sensory.

2 MATERIALS AND METHODS

2.1 Materials

The main materials: pumpkin dried seeds, aquades, skimmed milk powder, sugar, *S. thermophilus*, *L. plantarum*. Man Rogosa Sharpe Broth (MRSB), Man Rogosa Sharpe Agar (MRSA), Butterfield's Phospate Buffered, Lauryl Tryptose Broth (LTB), and Brilliant Green Lactose Bile Broth (BGLBB). Analysis materials: NaCl, 0.1 N NaOH, phenolphtalein indicator, Folin-Ciocalteau reagent, sodium carbonate, $AlCl_3$, 25% HCl, DPPH, selenium, boric acid, 98% H_2SO_4, 35% NaOH, 0.02 N HCl, pH 4 buffer, pH 7 buffer, ethanol, dye solution, ascorbic acid standard solution, HPO_3-HOAc solution and 70% alcohol.

2.2 Methods

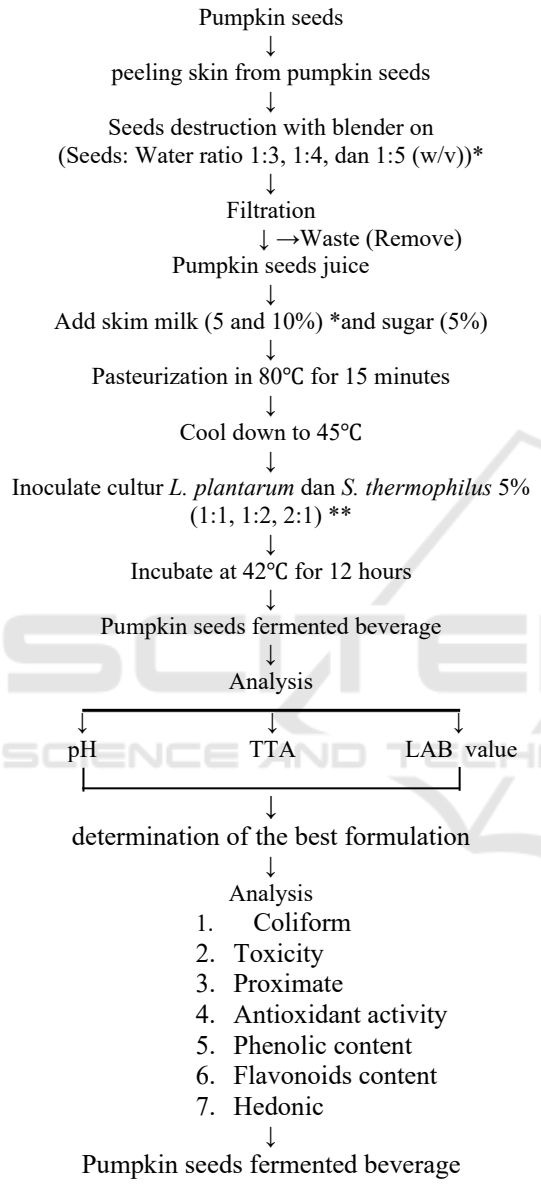
Phases of the research consisted of the phase of making pumpkin seeds juice, phase 1 and phase 2. Phases of pumpkin seeds juice extracts include peeling skin from pumpkin seeds, seeds destruction with blender, ratio of seeds:water = 1:3, 1:4, 1:5 (w/v), filtration and waste removal. Pumpkin seeds juice according to each ratio was then used in phase 1 research. Phase 1 research (Figure 1) begins with the addition of skim milk (5% and 10%) and sugar (5%) in pumpkin seeds juice according to each ratio (1:3, 1:4, 1:5 [w/v]). Pasteurization was done (80°C, 15 minutes) and cooled to 45°C. Starter *L. plantarum* (age 12 hours) and *S. thermophilus* (age 10 hours) with a ratio of 1:1 were inoculated as much as 5% and incubated at 42°C for 12 hours. Pumpkin seeds fermented beverage was then analyzed for pH (AOAC, 2005), total titrated acids (AOAC, 2005), and total LAB (Wehr and Frank, 2004). The selected formulation of the ratio of the pumpkin seeds to water and the concentration of skim milk was used in the next phase of the research through the comparison of the results of each test parameter to standard BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011).

Phase 2 research (Figure 1) was done by inoculating Starter *L. plantarum* (age 12 hours) and *S. thermophilus* (age 10 hours) with a ratio of 1:1, 1:2, and 2:1. The incubation conditions in the fermentation process and the test analytical parameters were performed the same as in the previous phase. The culture ratios results that meet the standard BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011) determined as the selected ratio. The fermented beverages of the selected formulation and ratio were then tested chemically (antioxidantt activity [Nahak and Sahu, 2011], phenolic [Conde, *et al*., 1997], flavonoids [Lamien-Meda, *et al*., 2008]) as well as microbiological tests (coliform [BSN, 2006]). Furthermore, it was evaluated proximately (water, ash, fat, protein, carbohydrate by difference [AOAC, 2005]), toxicity [Lisdawati, *et al*., 2006], and hedonic (scale 1-7).

2.3 Experimental Design

The experimental design of the Phase 1 research was Completely Randomized Design of two factors. The first factor (the ratio of pumpkin seeds to water) contains three levels (1:3 [A1], 1:4 [A2], 1:5 [A3]) with three repetitions. The second factor (skim milk concentration) contains two levels (5% [B1] and 10%

[B2]) with three repetitions. In the Phase 2 research culture ratios of *L. plantarum* and *S. thermophilus* was Completely Randomized Design one factor that contained three levels (1:2 [A1], 1:1 [A2], and 2:1 [A3]) with repetition three times.



Note:

* = Phase I

** = Phase II

Figure 1. Research Flowchart

3 RESULT

3.1 Phase 1

The first phase research was conducted to determine the best ratio of pumpkin seeds to water (1:3, 1:4. 1:5) and skim milk concentration (5% and 10%) through pH value analysis, total titrated acid value, and total LAB based on comparison with standard BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011). *L. plantarum* and *S. thermophilus* cultures were 5% inoculated at optimum age of 12 hours (1.6×10^9 cfu/ml) and 10 hours (1.8×10^8 cfu/ml), respectively.

The result of statistical test between ratio of pumpkin seeds and skim milk concentration showed that both interact ($p<0.05$) influenced the pH value of pumpkin seeds fermented. A number of nutrients in the pumpkin seeds and milk lactose decompose as the fermentation process becomes lactic acid and other organic acids are formed (Wood, 1997; Retnowati and Kusnadi 2014), thus lowering the pH value from 6.65 (control) to 4.09-4.49 (Table 1). The pH value of all samples were still within the maximum standard of 4.5 (FSANZ, 2014).

Results of the statistical test ratio of pumpkin seeds and the concentration of skim milk showed no interaction ($p>0.05$) influenced the TTA value of pumpkin seeds fermented beverage, but each had significant effect ($p<0.05$). Table 2 shows the lactic acid and other organic acids are increasing with the concentration of the pumpkin seeds juice and the increasing concentration of skim milk. Energy sources of pumpkin seeds and skim milk nutrients increase the formation of lactic acid and other organic acids during fermentation (Dewi, *et al*., 2013; Mulyani, *et al*., 2013; Retnowati and Kusnadi, 2014; Yanuar and Sutrisno 2015). During fermentation, the TTA control value (0.33%) increased to 0.58-0.84%, the value is still included in the standard of BSN (2009) 0.2-0.9%; the sample ratio of 1:5 does not meet the standards of PNS (2007) and CODEX (2003) which more than 0.6%; and only 1:3 ratio and skim milk 10% that meets KEBS standard (2013) 0.7-0.9%.

Results of statistical test between ratio of pumpkin seeds and skim milk concentration showed no interaction to total LAB ($p>0.05$). The pumpkin juice ratio did not affect the total LAB ($p>0.05$), whereas the concentration of skim milk affected the total LAB ($p<0.05$). Table 2 shows the total log value of LAB during fermentation having increased from log 6.68 to ~log 8-9 both in the seeds ratio sample and on the skim milk concentration sample. According to

Vasudha and Mishra (2013), the fermentation process will increase total lactic acid bacteria. Li, *et al*. (2012) added that fermentation for 18-24 hours with more than one culture can result in total lactic acid bacteria of about 10^8-10^9 cfu/ml (log 8-9). All test samples meet the standard minimum of 10^7 cfu/ml (CODEX, 2003; PNS, 2007) and minimum 10^6 cfu/ml (BSN, 2009; FSANZ, 2015; JETRO, 2011).

Based on the analysis of pH value, TTA, and total LAB compared with BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011), then the best formulation of pumpkin seeds fermented beverages is the ratio of pumpkin seeds to water and skim milk 1:3 and 10%. This formulation has the results of analysis of each parameter that meets all standards that is, pH 4.34, TTA 0.84%, total LAB 1.5×10^9 cfu/ml; and will used for phase 2 research in determining the best culture ratio between *L. plantarum* and *S. thermophilus*.

Table 1. Phase 1 Test Results (pH)

Pumpkin Seeds : Water	Skim Milk (%)	pH
1:3	5	4.09±0.02[a]
	10	4.34±0.03[c]
1:4	5	4.13±0.01[b]
	10	4.36±0.02[c]
1:5	5	4.37±0.03[c]
	10	4.49±0.00[d]

Note: - Different notation showed there was significant difference ($p<0.05$)

Table 2. Phase 2 Test Results (TTA and Total LAB)

	TTA (%)	Total LAB (Log)
Pumpkin Seed : Water		
1:3	0.84±0.08[a]	9.12±0.05[a]
1:4	0.69±0.11[b]	9.01±0.11[a]
1:5	0.58±0.15[c]	9.05±0.06[a]
Skim Milk (%)		
5	0.62±0.15[a]	8.99±0.05[a]
10	0.78±0.10[b]	9.15±0.04[b]

Note: - Different notation showed there was significant difference ($p<0.05$)
- No comparison between parameter analysis

3.2 Phase 2

Phase 2 research was conducted to determine the best culture ratio between *L. plantarum* and *S. thermophilus* through analysis of pH value, TTA, and total LAB based on comparison with standard BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011). The results of statistical test of culture ratio *L. plantarum* and *S. thermophilus* showed an influence on TTA pumpkin seeds fermented beverage ($p<0.05$). Table 2 shows that the 1:1 ratio yields the highest TTA value (0.84%) and meets all TTA value standards that is, BSN (2009) 0.2-0.9%; CODEX (2003) and PNS (2007) more than 0.6%; KEBS (2013) 0.7-0.9%. The ratio of 1:2 and 2:1 both have no significant effect on TTA value (0.60% and 0.66%) and significantly affect the 1:1 ratio. According to Chandan and Kilara (2013), the amount of *Lactobacillus* and *Streptococcus* should be balanced so that *Lactobacillus* can constantly provide peptide supply decomposition result of *Lactobacillus* to *Streptococcus*.

Results of statistical test of *L. plantarum* and *S. thermophilus* showed that there was no effect on pH value ($p>0.05$). Table 3 shows the entire sample yielding pH values 4.27-4.3 and meeting the pH standard FSANZ (2015) of 4.5. According to Zhang, *et al*. (2011), pH of skim milk which tend to be neutral and can serve as buffer can cause pH value of three culture ratios not significantly different. In addition, the amount of H^+ ions only shows the acid content dissociated in the sample and does not measure all the acid content contained in the product such as total titrated acids, so it can happen that the pH value is significantly different but the TTA value is not (Primurdia and Kusnadi, 2014).

The result of statistic test of culture ratio of *L. plantarum* and *S. thermophilus* also did not show any influence to total LAB ($p>0.05$). Table 2 shows the three culture ratios result total LAB in the range of log 9.28-9.34 that include in standard of CODEX (2003) and PNS (2007), that is more than 10^7 cfu/ml.

Based on the analysis of pH, TTA, and total LAB compared with BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011), then the best ratio of *L. plantarum* and *S. thermophilus* as culture of pumpkin seeds fermented beverage is a 1:1 ratio. This ratio has the results of analysis of each parameter that meets all standards, that is pH 4.27, TTA 0.84% (significantly different from 1:2 and 2:1 ratio), total LAB 2.2×10^9 cfu/ml. Pumpkin seeds fermented beverage with the selected formulation of seeds ratio and the concentration of

skim milk (1:3; 10%) and the selected culture ratio (1:1) were then chemically analyzed (antioxidant, phenolic, flavonoid activity), microbiologically (coliform), proximate, toxicity, and hedonic.

Table 3. Phase 2 Test Result (pH, TTA, and Total LAB)

Culture Ratio (ST : LP)	pH	TTA (%)	Total LAB (Log)
1:1	4.27 ± 0.02^{a}	0.84 ± 0.04^{b}	9.34 ± 0.02^{a}
1:2	4.30 ± 0.05^{a}	0.60 ± 0.03^{a}	9.30 ± 0.04^{a}
2:1	4.30 ± 0.05^{a}	0.66 ± 0.04^{a}	9.30 ± 0.04^{a}

Note: - Different notation showed there was significant difference ($p<0.05$)
- No comparison between parameter analysis

Based on the analysis of pH, TTA, and total LAB compared with BSN (2009), CODEX (2003), KEBS (2013), PNS (2007), FSANZ (2014), and JETRO (2011), then the best ratio of *L. plantarum* and *S. thermophilus* as culture of pumpkin seeds fermented beverage is a 1:1 ratio. This ratio has the results of analysis of each parameter that meets all standards, that is pH 4.27, TTA 0.84% (significantly different from 1:2 and 2:1 ratio), total LAB 2.2×10^{9} cfu/ml. Pumpkin seeds fermented beverage with the selected formulation of seeds ratio and the concentration of skim milk (1:3; 10%) and the selected culture ratio (1:1) were then chemically analyzed (antioxidant, phenolic, flavonoid activity), microbiologically (coliform), proximate, toxicity, and hedonic.

The antioxidant activity test is performed to find out how much antioxidant compound in the pumpkin seeds fermented beverage can prevent radical formation, inhibit oxidation reaction, decrease oxygen concentration, metal chelating agent, and act as bond breaker to prevent hydrogen removal from the substrate (Winarsi, 2007). The analysis was performed by DPPH method which measures the sample ability in binding to free radicals expressed in IC_{50}. The lower the IC_{50} value, the higher the antioxidant activity which means is a few amount of sample is needed to bind 50% of the free radicals. (Somawathi, *et al*., 2014). According to Putri and Hidajati (2015), antioxidant activity has five categories: very strong (IC_{50}<50 mg/l), strong (IC_{50} 50-100 mg/l), moderate (IC_{50} 100-250 mg/l), weak (IC_{50} 250-500 mg/l), and very weak (IC_{50}>500 mg/l). The results of antioxidant activity test (IC_{50}) pumpkin seeds fermented beverage before and after fermentation were 660142.2 mg/l and 291694.7 mg/l; although relatively weak, antioxidant activity increased due to increased lactic acid levels during fermentation. In addition, probiotic bacteria can produce vitamin C and E which can act as an antioxidant (Kusumaningrum, 2011).

Tests of total phenolic compounds were performed using the Folin-Ciocalcetau colorimetric method (standard phenolic curve), while for total flavonoids using the aluminum chloride colorimetric method (standard flavonoid curve). The total test results of phenolic and flavonoid pumpkin seeds fermented beverage sequentially were 643.27 mg GAE/l and 612 mg QE/l. The total phenolic and flavonoid contained are interconnected with the antioxidant activity obtained (Primurdia and Kusnadi, 2014).

Testing of coliform contamination (Gram negative, not forming spores, rod shape, producing acid and gas) is stated negatively on pumpkin seeds fermented beverage, while the amount of coliform allowed in fermented beverages is a maximum of 10 APM/ml (Badan Standarisasi Nasional, 2009). The organic acid produced by LAB inhibits the growth of coliform bacteria (Soccol, *et al.,* 2013).

Proximate test in the form of moisture content, ash content, fat, protein, and carbohydrate (by difference) can be seen in Table 3. All test parameters include in the standard of SNI except fat content but still fulfill requirement of CODEX (2003) that is <15%.

Product toxicity test is performed as a preliminary test in more complex toxicity testing. The Brine Shrimp Lethality Test (BSLT) method is used to produce LC_{50} (*Lethal Concentration* 50) which is the number of products needed to kill 50% of shrimp larvae. According to Onzago, *et al.* (2014), LC_{50} 0-100 ppm: strong toxic, LC_{50} 100-500 ppm: moderate toxic, LC_{50} 500-1000 ppm: low toxic, and LC_{50}>1000 ppm: non toxic. The pumpkin seeds fermented beverage has LC_{50} 786,90 ppm (low toxic). According to Prasetia and Intan (2013), LC_{50}<1000 ppm can be caused by components such as flavonoids contained in the sample so that the product is still safe for consumption.

The hedonic test of pumpkin seeds fermented beverage was conducted on 70 panelists to determine the level of product acceptance. The test scale consists of 7: extremely dislike (1), dislike (2), moderately dislike (3), neutral (4), moderately like (5), like (6), extremely like (7). Average yields of each parameters results is 3.29, taste 4.01, texture 4.4, and overall 4.05. Overall the panelists evaluate the product in the neutral category (4.05), that means the product can still be accepted by the panelists.

Table 4. Phase 2 Test Result (Proximate)

Parameters	Pumpkin seeds fermented beverage	SNI Standard (BSN, 2009)	CODEX Standard (CODEX, 2003)
Water (%)	83.62	-	-
Ash (%)	0.84	max. 1.00	-
Fat (%)	2.30	0.50-0.60	max. 15%
Protein (%)	4.00	min. 1.00	min. 2.7
Carb. [by diff] (%)	9.24	-	-

4 CONCLUSIONS

Preparation of pumpkin seeds fermented beverage with a ratio of pumpkin seeds to water of 1:3, addition of skim milk 10%, and culture ratio of *L. plantarum* : *S. thermophilus* 1:1 result pH, TTA, and total LAB that meet the standard. The pH, TTA, and total LAB are respectively 4.27; 0.84%; 2.2×10^9 cfu/ml.

The products of this selected culture formulation and ratio contains 83.62% water, 0.84% ash, 2.30% fat, 4.00% protein, 9.24% carbohydrate, total phenolic 643.27 mg GAE/l sampel, and total flavonoids 612 mg QE/l. The product increased the antioxidant activity by 55.81% after the fermentation process, with IC_{50} value after fermentation were 291694.7 mg/l. In hedonic, fermented beverage of pumpkin seeds are still acceptable to consumers (4.05 of 7.00), and declared safe for consumption with low toxicity and coliform-free microorganisms.

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