Full Length Research Paper

Factors affecting methanol content of fermented plant beverage containing *Morinda citrifolia*

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Pectin extraction of fresh *Morinda citrifolia* Linn or Noni using HCl at various concentrations was studied. The highest pectin yield (9.89% w/w) was achieved by using 20 mM HCl. Pectinmethylesterase (PME) activity and specific activity of raw *M. citrifolia* were 0.276 (μmol/ml. min) and 1.48 (Units/mg), respectively whereas, in the ripen *M. citrifolia*, they were 1.006 (μmol/ml. min) and 2.96 (Units/mg), respectively. Four formulas (F1, F2, F3 and F4) of the fermented plant beverage (FPBs) were prepared by varying material size (blended and diced) and sterilization process [72°C and using potassium metabisulfite (KMS)] using factorial design. Soluble pectin in FPBs using blended material (F3 and F4) was higher than in FPBs using diced material (F1 and F2). Soluble pectin in FPBs using pasteurization (F1 and F3) was also higher than FPBs using KMS (F2 and F4). At 24 h fermented period, methanol concentration in FPBs using blended material (F3 and F4) was higher than FPBs using diced material (F1 and F2). Methanol concentration in FPBs using KMS (F2 and F4) was also higher than FPBs using pasteurization (F1 and F3). After 24 h fermentation time, methanol concentration in FPBs using blended material (F3 and F4) could not be detected until the end of the fermentation whereas, in FPBs using diced material, methanol was found throughout the fermentation (F1 and F2). The study indicates that the most influencial factor on methanol production in FPBs was raw material size. Other factors were sterilization, PME and pectin, respectively. Thus, the recommended procedure of the fermentation of plant beverage was the use of blended raw *M. citrifolia* and pasteurization at 72°C for 15 s. These conditions prevent methanol production in FPBs. Therefore, quality and safety of the FPBs will be accepted by consumers.

Key words: *Morinda citrifolia*, methanol, pectin, pectinmethylesterase (PME), fermented plant beverage (FPB).

INTRODUCTION

Fermented plant beverages (FPBs) are non-alcoholic beverages produced from different kinds of plants such as cereals, fruits and vegetables. The product is fermented by lactic acid bacteria (LAB) such as *Lactobacillus plantarum* and *Lactobacillus casei* (Duangjitchareon et al., 2008). In Thailand, FPBs were first produced by local Thai people for household product until they were commercially produced throughout the country. The same kinds of the beverages are produced in several countries, for example, EM•X® rice bran beverages in Japan, Vita Biosa probiotic beverage in Denmark and various FPBs in Thailand (Kantachote et al., 2010).

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The production steps of FPBs are usually mixing plant, sugar and water in the ratio of 3:1:10 (w/w/v) with or without 10% starter culture in a plastic bucket. Then, the fermentation is incubated at 30 ± 2°C for three to six months depending on plant type. The beverage is produced among the farmers throughout the country. It has been recommended for serving size at 30 ml/day. Several plants can be used to produce FPBs. *Morinda citrifolia*. Linn or Noni is the most famous raw material of FPBs in Thailand. *M. citrifolia* has been extensively used in folk and traditional medicine for over 2,000 years. The major components in the fresh plant have been determined such as scopoletin, octanoic acid, potassium, vitamin C, terpenoids, alkaloids, anthraquinones, β-sitosterol, carotene, vitamin A, flavone glycosides, linoleic acid, alizarin, amino acids, acubin, *L*-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin, and a putative proxeronine (Mian-Ying et al., 2002) but data on the *M. citrifolia* plant beverage have not been investigated. *M. citrifolia* has been reported to have broad therapeutic effects, against constipation, hypertension, stomach ulcers, rheumatism, sore throat, bacterial, viral, fungal, inflammatory, autoimmune and cancer (Wang and Su, 2001; Wang et al., 2002; Kamiya et al., 2004). People who consume FPBs believe that it is a health promoting beverage and helps in curing some diseases.

FPBs contain bioactive compounds derived from the raw material and LAB activities (Duangjitchareon et al., 2008). It was claimed to relieve constipation, relieve diabetes, treat colitis, enhance specific and nonspecific immune response, inhibit pathogen growth and translocation, stimulate gastrointestinal immunity, reduce chance of infection from common pathogens, reduce risk of certain cancers (colon, bladder), detoxify carcinogens, suppress tumors, lower serum cholesterol concentrations, reduce blood pressure in hypertensives (Duangjitchareon et al., 2009). Most of consumers are elderly people. Therefore, safety is the most important issue. Chemical quality control of the product is very important for consumer safety, especially methanol, a colorless volatile compound with a mild alcohol odor. It is toxic to humans and is readily absorbed by ingestion and inhalation and more slowly, through the skin. In the body, methanol is metabolized at the liver, and converted first to formaldehyde and then to formate. High levels of formate after excessive methanol intake can cause severe toxicity and even death (Lamiable et al., 2004; Zocca et al., 2007). Therefore, lowest level of methanol in food is preferable. Maximum level of methanol in FPBs according to Thai community product standard (TCPS 481/2004) was 240 mg/L whereas Australia New Zealand Food Authority (ANZFA) permitted the maximum concentration of methanol in spirit beverages at 8 g/L of ethanol in the beverage [maximum ethanol level in Thai FPBs is 3% (v/v)].

Methanol is an undesirable component in the production of FPBs. Methanol is naturally produced in the anaerobic metabolism of many varieties of bacteria and is ubiquitous in the environment (Vries, 1986; Schrader et al., 2009). It is also presented as a consequence of enzymatic degradation of pectins. Pectinmethylesterase (PME, EC: 3.1.1.11) de-esterifies pectins to low-methoxyl pectins, resulting in the formation of methanol (Micheli, 2001).

In this paper, we studied the factors that contribute to methanol production in FPBs containing *M. citrifolia*. Raw material size, sterilization process, PME of raw material and soluble pectin at various fermentation times were studied. Knowing factors which affect the formation of methanol during the fermentation will be useful for the assessment of safety and quality of FPBs.

MATERIALS AND METHODS

Determination of pectin in fresh *M. citrifolia*

Sample preparation

M. citrifolia sample was purchased from a local market. It was washed with distilled water and cut into small pieces. The sliced samples were then dried to constant weight at 60°C for 48 h. Then, they were grinded with grinder and stored in closed container at 4°C until use.

Removal of impurity

1 g of sample was mixed with 2 ml 95% ethanol. After that, the mixture was boiled in water bath at 70°C for 10 min. The mixture was then filtered through four layers of cheesecloth. The pomace was collected, and 20 ml of 30% ethanol was added to the pomace. It was kept at room temperature for 30 min and the pellet was collected.

Pectin extraction using hydrochloric acid (modified from Bhatia et al., 1959)

The *M. citrifolia* pellet was mixed with HCl with the ratio 1:30 (w/v). Various concentrations of HCl (5, 20, 50 and 100 mM) were used. The mixture was then boiled in water bath for 1 h. The mixture was immediately filtered through cheesecloth.

Pectin precipitation (modified from Alexander and Sulebele, 1980)

The filtrate was precipitated with cooled 95% ethanol (40:45, v/v) and continuously stirred until pectin gel occurred. The sample was then cooled in refrigerator for 12 h. Pectin pellet was then filtered under vacuum and washed with 80% ethanol (1:15 v/v), followed by 95% ethanol. Finally, pectin pellet was dried to constant weight at 40°C.

Determination of pectinmethylesterase (PME) in fresh *M. citrifolia*

PME Extraction

The extraction of the enzyme was performed by modifying method of Verlent et al. (2004). 60 g of *M. citrifolia* in 100 ml cooled distilled water was blended and filtered through cheesecloth to remove

Table 1. Description of fermented plant beverage formulation containing *M. citrifolia.*

Formula	Process	
	Raw material preparation	**Sterilization process**
1 (F1)	Diced	Pasteurization (72°C for 15 s) and cool shock
2 (F2)	Diced	Potassium metabisulfite
3 (F3)	Blended	Pasteurization (72°C for 15 s) and cool shock
4 (F4)	Blended	Potassium metabisulfite

remove solid particles. The filtrate was then centrifuged at 10,000 xg at 4°C for 20 min. The pellet was kept in 100 ml of 0.003 M phosphate buffer pH 7.5. 1% (w/v) polyvinylpolypyrolidone (PVPP) was then added. The mixture was stirred using magnetic stirrer at 4°C for 24 h. Then, it was centrifuged at 10,000 x *g* for 20 min. For PME activity determination, the pH of obtained supernatant was adjusted to 7.5.

PME activity determination

PME activity was spectrophotometrically determined using modified method of Hagerman and Austin (1986). 2 ml of pectin, 0.15 ml of bromothymol blue and 1.35 ml of phosphate buffer were mixed. The solution was heated at 60°C for 2 min. The activity was monitored by adding 0.5 ml of sample and the absorbance was read at 620 nm for 1 min afterwards. PME activity was calculated as that for D-galacturonic acid 1 μmol per 1 min as follows:

$$\text{PME activity (unit/ml)} = \frac{\text{D-galacturonic acid (}\mu\text{mol/ml) x Total solution volume (ml)}}{\text{Enzyme added (ml) x Reaction time (min)}}$$

Total protein determination

The protein contents were determined according to Bradford (1976). 0.50 ml of sample was mixed with 0.10 ml of distilled water, 0.10 ml of 0.15 M NaCl and 4.30 ml of dye (0.01% w/v Coomassie brilliant blue G-250). The solution was kept for 10 min. Then absorbance was read at 595 nm (using bovine serum albumin as standard). PME specific activity (units/mg) was calculated according to the following equation:

$$\text{PME specific activity (Units/mg)} = \frac{\text{Total PME (Units)}}{\text{Total protein (mg)}}$$

Determination of pectin in fermented plant beverage (FPBs)

Preparation of fermented M. citrifolia beverage

FPBs containing *M. citrifolia* was prepared by mixing fresh *M. citrifolia*, sugar, water and *Lactobacillus plantarum* starter culture (10^6 cfu/ml) with the ratio 3:0.26:10:1.3 by weight. The experimental FPB was assigned into four formulas by varying raw material preparation (diced or blended) and sterilization process (pasteurization and cool shock or using potassium metabisulfite (KMS)) using factorial design as shown in Table 1. The FPBs was kept in close and sterile fermentor at 37°C. The samples were collected at 0, 12, 24, 36, 42, 72, 120, 240, 360, 480, 600 and 720 h, respectively.

Determination of pectin as polygalacturonic acid using colorimetric method

200 μl sample was added to 1.2 ml of 0.125 M sulfuric acid/tetraborate. The mixture was cooled on ice for 2 min then heated at 100°C for 5 min and cooled again. Afterwards, the sample was mixed with 20 μl of 3-phenylphenol (using acetate-EDTA buffer as blank). Then, the absorbance was read at 520 nm. Galacturonic acid (0.1 mg/ml) was used as a standard. Pectin determination was modified from Monsoor et al. (2001).

Determination of methanol using gas chromatography (GC)

Methanol concentration was determined by modified method from Wang et al. (2004) and Okunowo and Osuntoki (2007). A Perkin-Elmer GC (Sigma 3B) equipped with a flame ionization detector and an automatic injection system was used. Column: Carbowax 20M column, was set at 120°C. The injector was set at 180°C and the detector at 200°C. Isobutanol (0.5% v/v) and isopropanol (0.5% v/v) were used as internal standards. The standard methanol was prepared in the range of concentrations of 10 to 1000 mg/L. Helium was used as carrier gas at a flow rate of 0.9 ml/min.

Statistical analysis

All experiments were performed in triplicate. One-way analysis of variance was used followed by the Duncan' multiple comparison test to analyze significant differences between treatments at significant level of 95% (p<0.05). Pearson's correlation was analyzed for relation between pectin and methanol level in FPBs (only F1 and F2). SPSS version 17.0 for Windows was used.

RESULTS

Determination of pectin in fresh *M. citrifolia*

Pectin extracted from *M. citrifolia* using HCl at various concentrations was determined. Pectin yields from each HCl concentration were significantly different. Figure 1 shows that pectin yield increased when HCl concentration was changed from 5 to 20 mM. However, using higher HCl concentrations (50 and 100 mM), pectin yield decreased. The result showed that the highest pectin yield, using 20 mM HCl was 9.89% and the lowest pectin yield, using 100 mM HCl was 6.55%.

Determination of pectinmethylesterase (PME) in fresh *M. citrifolia*

PME activity in raw and ripen *M. citrifolia* was measured.
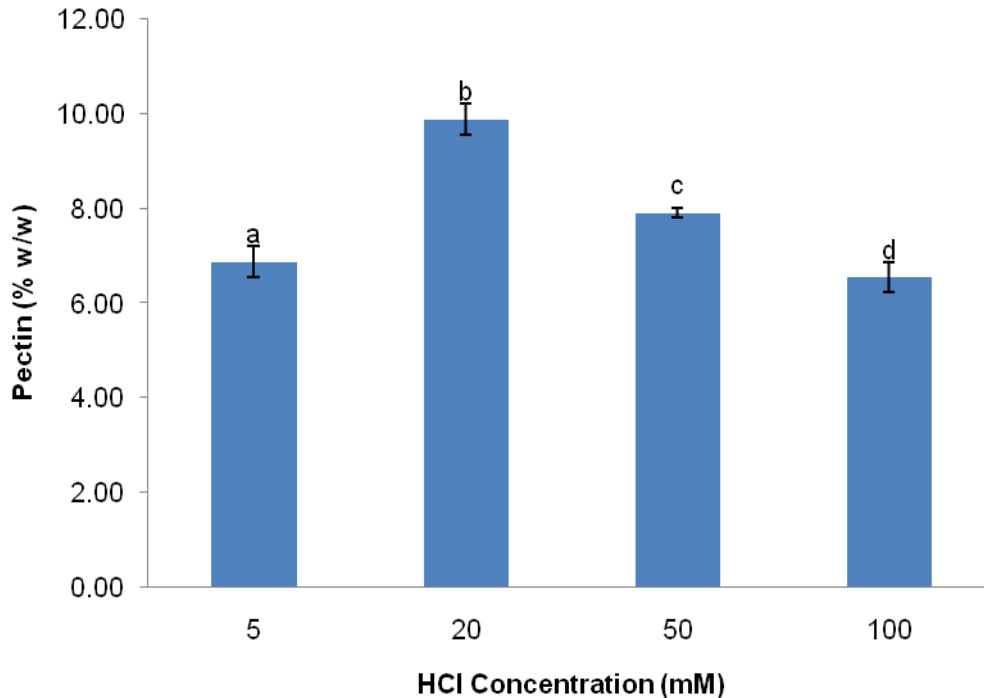
Figure 1. Pectin yields (% w/w of *M. citrifolia* fresh weight) extracted by HCl at concentrations 5, 20, 50 and 100 mM. Bars indicate standard deviation of triplicate experiment. Means different with small characters indicate significant different (p<0.05) between treatment.

The result showed that PME activity of raw *M. citrifolia* was 0.276 µmol/ml. min with specific activity of 1.48 Units/mg. PME activity of ripen *M. citrifolia* was 1.006 µmol/ml. min with specific activity of 2.96 Units/mg.

Determination of pectin in fermented plant beverage

Changes in pectin content in FPBs, determined as soluble pectin, were significantly different in all treatments. Figure 2 shows the soluble pectin in FPBs containing *M. citrifolia* with different production process. Pectin was found within the range of 0.5 to 3.5 mg/ml. Soluble pectins of all formula were exponentially decreased in the early fermentation. After 72 h fermentation period, soluble pectin in F1, F3 and F4 were slightly decreased along the fermentation time but soluble pectin in F2 greatly decreased. At the end of fermentation (30 days, 720 h), soluble pectin in FPBs using blended material (F3 and F4) was higher than FPBs using diced material (F1 and F2). When compared with the sterilization process between using pasteurization and using KMS, the soluble pectin of the formulas using pasteurization was higher than for the other one. It was observed from Figure 2 that soluble pectin of F3 was higher than that of F4. In the same manner, soluble pectin of F1 was higher than that of F2. At the end of fermentation, the highest soluble pectin was found in F3

(2.015 mg/ml) and the lowest soluble pectin was found in F2 (0.789 mg/ml).

Determination of methanol using GC

Figure 3 shows the chromatogram with given GC conditions. Methanol (A), iso-propanol (B; internal standard) and iso-butanol (C; internal standard) gave retention time at 2.63, 7.46 and 12.52 min, respectively. Various manufacturing process was performed as described in Table 1. Methanol concentration of FPBs was determined as shown in Figure 4. Methanol concentration in F1 and F2 gradually increased from 0 h to the end of the fermentation (30 days, 720 h) and has the significantly highest concentration at 40.90 and 264.80 mg/L, respectively. Methanol in F3 and F4 rapidly increased and had the highest concentration at 24 h, at 475.28 and 853.41 mg/L, respectively. Then, the methanol concentrations dropped to undetectable level and could not be detected until the end of the fermentation. The highest methanol concentration throughout the fermentation was found in F2, using diced *M. citrifolia* as raw material and using KMS as sterilization agent. The lowest methanol concentration was found in F3 and F4. Both used blended *M. citrifolia* as raw material. The Pearson's correlation between soluble pectin and methanol in F1 and F2 were -0.4502 (p =
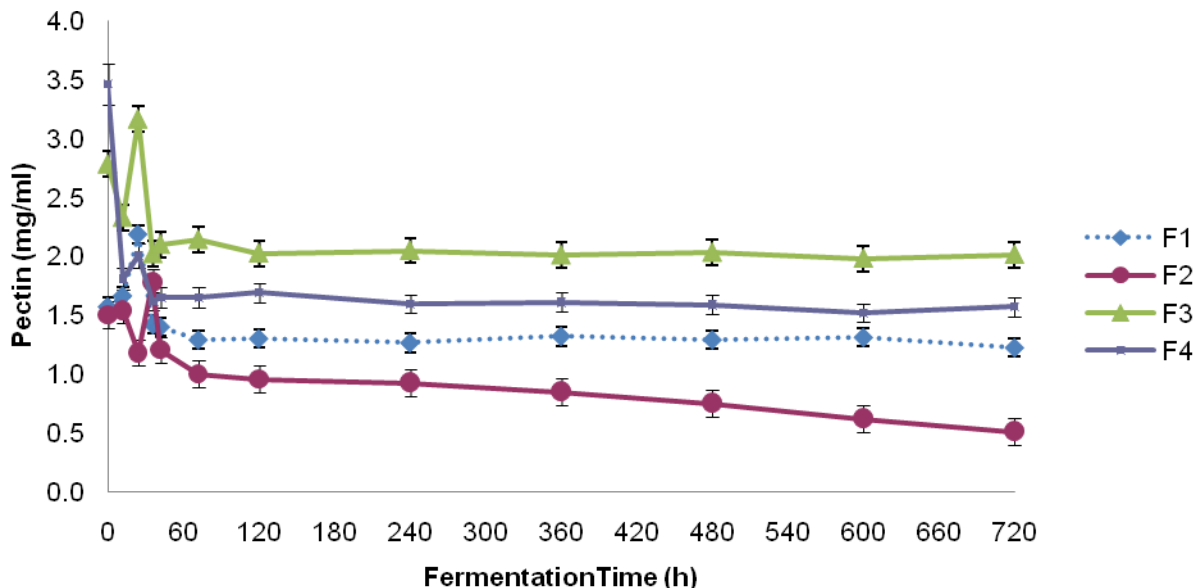
Figure 2. Soluble pectin in fermented plant beverage containing *M. citrifolia* at various fermentation time. Bars indicated standard deviation of triplicate experiment. See Table 1 for F1 to F4.
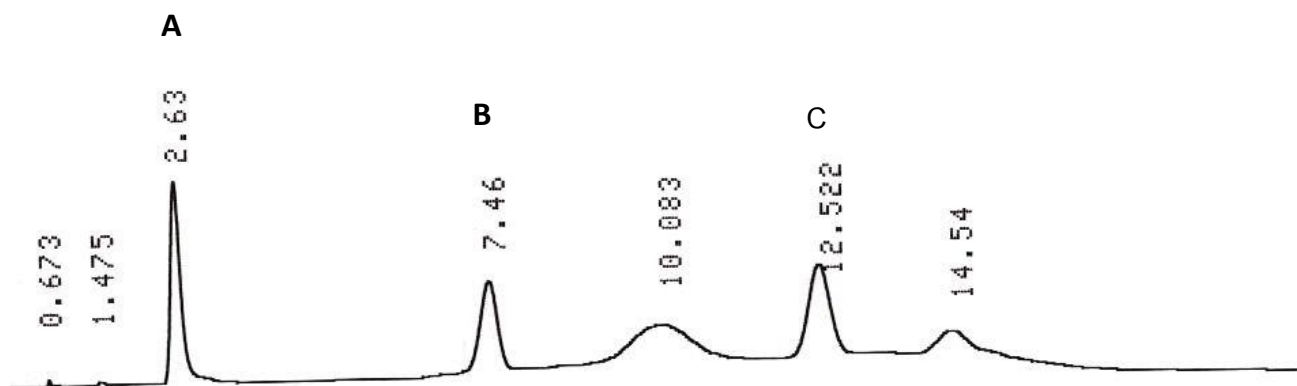

Figure 3. Gas chromatogram of fermented plant beverage containing *M. citrifolia* sample. A = Methanol; B = iso-propanol; C = iso-butanol.

0.1227) and -0.4952 (p = 0.002), respectively.

DISCUSSION

Determination of pectin in fresh *M. citrifolia*

Pectins occur as structural polysaccharide in higher plants cell walls. During ripening, pectin is broken down by PME to low-methoxyl pectin (Micheli, 2001), resulting in the formation of methanol. The result show high content of pectin in *M. citrifolia* (9.89 %) extracted with HCl at concentration of 20 mM. This concentration allowed suitable pH for pectin precipitation (Faravash and

Ashtiani, 2006). When compared with another pectin plant sources such as guava (4.36%), tomato (0.3%), apple (0.5%), carrot (0.8%), cherries (0.4%) (Holloway et al., 1983), *M. citrifolia* has much more pectin contents (9.89%). The aim of the determination of pectin content in this plant is to indicate possibility of methanol production in FPBs related pectin content.

Determination of pectinmethylesterase (PME) in fresh *M. citrifolia*

The result indicate that age of *M. citrifolia* is related to PME activity. The enzyme is present in most plant
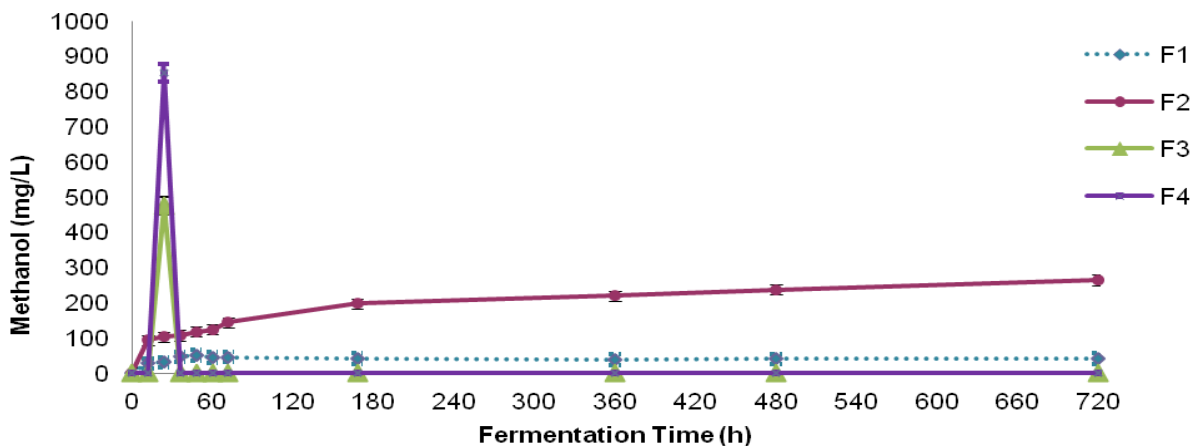
Figure 4. Methanol content (mg/L) of fermented plant beverage containing *M. citrifolia* at various fermentation time. Bars indicate standard deviation of triplicate experiment. See Table 1 for F1 to F4.

tissues. It likely plays an important role in plant metabolism. PME removes methyl groups from the pectic component of cell wall during fruit ripening which can then be depolymerized by polygalacturonase, decreasing the intercellular adhesivity and tissue rigidity (Assis et al., 2001). There was a close correlation reported between PME activity and methanol levels in fruit tissues from both wild-type and a PME antisense mutant tomato, indicating that PME is on the primary biosynthetic pathway for methanol production in tomato fruit (Frenkel et al., 1998). For PME activity determination in *M. citrifolia*, the result show that PME activity and specific activity in ripen *M. citrifolia* was higher than in raw *M. citrifolia*. This may result in higher methanol production in FPBs.

Thus, age of plant (raw material) is an important factor for methanol production in FPBs according to PME activity. Therefore, choosing raw *M. citrifolia* that contain low PME will be able to prevent methanol production in FPBs. Besides, pasteurization of fermentation is regularly using high temperature at 72°C for 15 s. PME has high activity at the temperature between 50 and 60°C. The enzyme loss activity is at the temperature higher than 70°C (Tijskens et al., 1999; Amaral et al., 2005). Sterilization using temperature higher than 80°C can complete inactivate PME but may cause decomposition of bioactive component in FPBs. pH of FPBs is normally in the range of 3 to 4. These pH range also affect PME activity. In general, PME has been found to have an optimum pH ranging from 7.5 to 9.0 (Amaral et al., 2005; Arotupin et al., 2008).

Determination of pectin in fermented plant beverage

Four formulas of FPBs with different material size and sterilization process were studied. When comparing soluble pectin between FPBs containing blended and diced material, it was found that soluble pectin in FPBs containing blended material (F3 and F4) was higher than FPBs containing diced material (F1 and F2). This might be explained thus: mechanic force from blender might break long-chain pectin to lower chain pectins allowing lower carbon pectins to dissolve more. Surface area of small pectin size might also affect pectin releasing in FPBs.

For sterilization process, FPBs using pasteurization (F1 and F3) had more soluble pectin than FPBs using KMS (F2 and F4). FPBs using KMS, soluble pectin might be used as PME substrate. In contrast, in FPBs using high temperature during pasteurization (72°C), PME was inactivated, resulting in the remaining soluble pectin which is higher than the other one. Moreover, high temperature more than 60°C enhanced pectin solubility (Fox, 1994). These could be a reason for higher soluble pectin in FPBs using high temperature sterilization.

Determination of methanol using GC

Considering factors affecting methanol production in FPBs, it was found that raw material size has effect on methanol concentration. FPBs using blended material and sterilization using either pasteurization (F3) or KMS (F4) had high methanol concentration in the early fermentation period (24 h).

After that, the methanol concentration dropped to undetectable level until the end of fermentation. This might be explained that blended material released whole pectin and PME into FPBs, resulting in high production of methanol within 24 h. When comparing between F3 and F4, FPBs using pasteurization (F3) inactivated PME activity resulting in lower methanol production than FPBs using KMS (F4). In FPBs using diced material, methanol

was found throughout the fermentation period. It might be implied that pectin and PME were gradually released along the fermentation. Methanol concentration in FPBs using KMS (F2) was gradually increased along the fermentation. Nevertheless, methanol in FPBs using pasteurization (F1) was found constant after 72 h. When comparing the sterilization process between using pasteurization (F1) and using KMS (F2), PME activity in FPBs using pasteurization (F1) was inactivated. Therefore, the methanol concentration of the formula using KMS (F2) was higher than the other one. The result indicate that the most effective factor on methanol production was material size. For the formula using blended material, methanol could not be detected at the end of the fermentation (720 h) even if high methanol concentration was found within 24 h.

In FPBs using diced material, methanol was found throughout the fermentation. The next factor is sterilization process. FPBs with blended material using KMS (F4) has higher methanol concentration than FPBs using pasteurization (F3) at 24 h. Afterwards, methanol could not be detected until the end of the fermentation. Among FPBs using diced material, methanol concentration of FPBs using KMS was greatly higher than FPBs using pasteurization and tended to increase along the fermentation time. Pectin and PME also had effect on methanol production. Higher pectin in FPBs using blended material (F3 and F4) had higher methanol concentration in the early fermentation than FPBs using diced material (F1 and F2). When comparing the sterilization process using the same material size. FPBs using KMS (F2 and F4) had higher pectin yield than FPBs using pasteurization (F1 and F3) but had lower methanol concentration. The Pearson's correlation between soluble pectin and methanol level in FPBs (F1 and F2) significantly showed low relation between the factors. This could indicate that PME has more effect on methanol production than soluble pectin in FPBs. Maximum methanol concentration according to Thai community product standard (TCPS 481/2004) was 240 mg/L. It was found that FPBs with diced material and using KMS (F2) had methanol concentration higher than the standard regulation.

Conclusion

This study indicates that the main factors which affect methanol production in FPBs containing *M. citrifolia* was raw material size. FPBs using blended material had lower methanol concentration at the end of fermentation than FPBs using diced material. Next factor is the sterilization process. FPBs using KMS had higher methanol concentration than FPBs using pasteurization. PME activity in raw and ripen, as well as soluble pectin in FPBs had effect on methanol production as well.

From our experiment, we recommend appropriate con-

dition for the fermentation of plant beverage by using blended raw *M. citrifolia* and pasteurization with temperature higher than 70°C in order to produce FPBs containing low methanol with the characteristic of the FPBs been: clear brown solution, sour flavor and a smell originating from plant with little smell of ester from fermentation. Good Manufacturing Practice (GMP) and Good Hygiene Practice (GHP) could not also be negligible for high quality and safety of the products that is acceptable for domestic and international consumer.

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