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Development and evaluation of Mustard green pickled liquid as starter for *Morinda citrifolia* Linn. Fermentation

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Abstract

The selection of starter culture for preparing fermented plant beverages influences the entire production and quality of the product. The traditional way of naturally fermented mustard green pickled liquid (MGPL) has been used in northern Thailand for several decades, and the fermented *Morinda citrifolia* Linn. fruit juice is one of the primary fermented plant beverages. The current study explored the use of MGPL as a starter for the fermentation of *M. citrifolia* fruit. The MGPL was preparing as per the traditional procedure and used at a different concentration in the various formula of *M. citrifolia* fruit fermentation (MFR). Moreover, the influence of raw onion extract (ROE) in MFR has also been evaluated. The pH of the MGPL (6.5 ± 0.2 to 3.8 ± 0.1) and MFR was progressively reduced during fermentation. The physiochemical and microbiological analysis of MGPL and MFR suggested that the content of organic acids were increased upon fermentation, especially the recipes with heavy inoculation (15%) of MGPL starter. The total bacterial load, coliforms, *Lactobacillus,* yeast and mold levels were in acceptable range in naturally fermented MGPL. All the MFR recipes are microbiologically safe and enriched with Lactic acid bacteria. The ethanol content in MGPL (0.6348 ± 0.02% V/V) and MFR recipes (ranging from 0.5524 ± 0.02 to 0.9677 ± 0.04% V/V) were slightly higher than the permissible level of Thai commercial product standards (≥ 0.5%), whereas the methanol content was in acceptable level (≤ 240 ppm). The reducing sugar level in MFR recipes were gradually decreased due to the fermentation. In conclusion, the MGPL is considered as a safe starter for MFR with ROE supplementation. Hence, additional studies are recommended on traditional formulations and its applications in modern food technology, especially in the production of fermented plant beverages.

Introduction

Morinda citrifolia is, commonly known as Indian mulberry, noni, hog apple, and cheese fruit, one of the widely used as herbal juice and the commercial *M. citrifolia* products were launched in the USA during 90's (Assi *et al*., 2015). All the part (fruit, flower, leaves, bark, and root) of *M. citrifolia* plant have been used as medicine, and it is known for antimicrobial, antitumor, anthelmintic, analgesic, hypotensive, anti-inflammatory, and immune-activating effects (Nayak and Shettigar, 2010). The noni fruit extract exhibiting the antidopaminergic effect, antiemetic activity, and prevent the symptoms of nausea and vomiting (Ekpalakorn *et al*., 1987; Chuthaputti *et al*., 1996; Pandy *et al*., 2012). Many active compounds were reported in noni fruits, which includes flavonoid (3, 3', 4,' 5, 7-pentahydroxyflavone-3-rhamnoglucoside also known as rutin) and imitative coumarin compound (6-methoxy-7-hydroxycoumarin also known as scopoletin). The noni flavonoid, rutin, has been described for antioxidant, anticarcinogenic, neuroprotective, and anticonvulsant activities (Pandy *et al*., 2014).

M. citrifolia juices were prepared by crushing fresh or naturally decomposed fruits, or by fermentation processes, and the quality of the juices is depends on the method of extraction (Assi *et al*., 2015). The Thai fermented *M. citrifolia* fruits contains vitamins, alkaloids, anthraquinones, antioxidants, essential oils, flavonoids, saponins, scopoletin, and sugars, whereas the Indian *M. citrifolia* fruits contain only anthraquinones, saponins, and scopoletin (Nandhasri *et al*., 2005; Satwadhar *et al*., 2011). The fermented *M. citrifolia* fruit juice specifically enhances the growth of probiotic bacteria especially *Bifidobacterium longum* and *Lactobacillus plantarum*

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(Wang *et al*., 2009). Many factors affect the quality of the fermented *M. citrifolia* fruit juice such as processing methods, storage conditions (Yang *et al*., 2007), suspended solid particles (Joshi *et al*., 2012), fermentation duration and conditions, inoculum, raw material size (Valdes *et al*., 2009; Chaiyavat *et al*., 2013), and pasteurization (Brown 2012).

The naturally fermented mustard green (*Brassica juncea* L var. rugosa) juice is one of the popularly consumed fermented vegetable, especially sour pickled mustard green, among the Thai people. The natural fermentation of mustard green (MG) attributed to the exsisting microbes of raw materials, thus the quality of the product is varied from batch to batch (Kamdee *et al*., 2014). The lactic acid bacteria (LAB), based fermentation of MG, provided the high quality fermented MG than naturally fermented juice on pH, and probiotic values, further it achieves the consistency in the quality of the product and *L. fermentum* have the vital role in MG fermentation (Kamdee *et al.*, 2014).

The fermentation of *M. citrifolia* Linn. fruit with naturally fermented MG juice as a starter has not been explored in detail. There is no in-depth knowledge about the changes in the organic acid content, alcoholic content, and microbial load during the fermentation of *M. citrifolia* Linn. fruit using fermented MG juice starter. Thus, the current study was conceived to develop and evaluate the MG pickled starter for *M. citrifolia* Linn. fruit fermentation on microbial content, organic acids, and alcohol content.

Materials and Methods

Raw materials

The mustard green (MG), *M. citrifolia* Linn, and cane sugar samples were collected from local market of Chiang Mai, Thailand.

Preparation of pickled liquid

The mustard green (MG) were selected for preparing the naturally fermented pickled liquid. Briefly, MG was collected and washed with sterile water to remove the dust, and soil particles, and soaked in potassium permanganate solution (0.01%) for 10 min to remove excess contaminants. Then, MG was dried under sunlight for one day after the second wash with sterile water. The dried MG were cut into small pieces and about 250 g of chopped MG was mixed with 15 g of salt (6%), and packed in polyethylene storage box (800 ml capacity). Then, boiled sticky rice water (1 Kg of rice, *Oryza sativa* var. glutinosa, was soaked in 2 L of water for overnight, then boiled and cooled to room temperature) was poured to submerge the pre-processed MG and incubated at room temperature for ten days. The described MG pickling method is the traditional way of making the naturally fermented pickled liquid in Thailand.

Table 1. The experimental MFR recipes and its composition.

Recipe No.	Ingredients
R1	MCW*
R2	MCW* + 5% of Pickled starter
R3	MCW* + 10% of Pickled starter
R4	MCW* + 15% of Pickled starter
R5	MCW* + 5% of Pickled starter + Raw onion extract
R6	MCW* + 10% of Pickled starter + Raw onion extract
R7	MCW* + 15% of Pickled starter + Raw onion extract

**Morinda citrifolia* Linn (M) + Cane sugar (C) + Water (W) at the ratio of 3:1:10.

Fermentation of M. citrifolia *Linn*

The fermentation of *M. citrifolia* Linn was carried out with pickled liquid as a starter. *M. citrifolia* Linn, cane sugar, and water were mixed at the ratio of 3:1:10, and pickled starter and raw onion extract were added. Total seven recipes were prepared and incubated at room temperature for 45 days. The composition and a specific number of the recipes were detailed in Table 1. The samples were collected at different time points of fermentation and analyzed.

Physical observation

All the recipes were physically examined for color, odor, taste, turbidity, and gas by the researcher during fermentation.

Quantification of microbial load

The total bacterial load, *Lactobacillus, Bacillus cereus, Staphylococcus aureus, Clostridium perfringens, Salmonella* spp., coliform and *Escherichia coli*, yeast and mold content of pickled liquid, and fermented *M. citrifolia* Linn (FMCL) were determined by standard serial dilution and plating techniques. The samples were collected at different time points and serially diluted with sterile distilled water plated on respective solid media. The plate count agar (PCA), potato dextrose agar (PDA), phenol red egg-yolk kanamycin agar (PREYK), Mannitol Salt Phenol Red Egg-Yolk Agar (MSEY), MacConkey Agar (MAC) and *Salmonella-Shigella*

Table 2. The physical properties of MG pickled liquid (MGPL) and *M. citrifolia* fermentation recipes (MFR) during fermentation process. ROE means raw onion extracts.

Color		odor		Taste		turbidity		Gas formations		Remarks
MGPL	MFR	MGPL	MFR	MGPL	MFR	MGPL	MFR	MGPL	MFR	• Color of the MFR was more intense in the recipes without ROE. • Sweetness in MFR is due to the cane sugar
Cloudy to pale	Light to dark Brown	Sour to pickle like smell	Aroma of *M. citrifolia* (until 7 days) to sour (7 to 45 days) in all the recipes	Sour	Sweet (until 7 days) to sour (7 to 45 days) in all the recipes	Reduced	Increase d (at 30-45 days) in all the recipes	Reduced	Reduced and no gas after 30 days in all the recipes	

Agar (SS), Cooked Meat Medium, and Man Rogosa Sharpe (MRS) Agar medium were used for the specific growth and enumeration of total bacteria, yeast and mold, *B. cereus, S. aureus, Salmonella* spp. *C. perfringens*, lactic acid bacteria (LAB), respectively (Pattananandecha *et al*., 2016). The coliform and *E. coli* content of the samples were determined by multiple tube fermentation technique (Eckner 1998). The concentration of microbial populations was denoted as log CFU/ml of sample or most parable number (MPN) /100 ml of sample.

Determination of pH and organic acid

The organic acids in the collected samples, both pickled liquid and fermented *M. citrifolia* Linn, were measured. The pH was measured as per our previous report (Peerajan *et al*., 2016), and the organic acid content was evaluated by titration method as described by Sinclair *et al*., 1945.

Determination of Ethanol and Methanol content

The ethanol and methanol contents of pickled liquid and fermented *M. citrifolia* Linn were determined by gas chromatography (GC-14B, Shimadzu, Japan) as per our previous report (Chaiyasut *et al*., 2016).

Estimation of reducing sugar level

The sugar content of the samples was determined by Dinitrosalicylic acid (DNS) method (Miller, 1959). Briefly, 3 ml of sample and 3 ml of DNS solution (10 g of 3,5 Dinitrosalicylic acid, 2 g of phenol, 0.5 g of sodium sulfite and 10 g of NaOH were dissolved in 1000 ml of distilled water) was mixed and boiled in hot water for 10 min. After cooling to room temperature, 1 ml of 40% Potassium sodium tartrate was added and read at 562 nm. The concentration of sugar in the sample was extrapolated from glucose standard curve (0-400 ppm or 0-150 mg/ml).

Statistical analysis

All the experiments were performed in triplicates except pickled liquid preparation and values were represented as mean ± SD.

Results and Discussion

The physical properties like color, odor, taste, turbidity, and gas formations were observed personally by the researcher and found that the color of MG pickled liquid (MGPL) was cloudy at the beginning of the fermentation, when the duration of the fermentation increases the color become pale. The odor of the MGPL was like sour smell until the second day then it became pickle like smell. The turbidity and gas formation of the MGPL was reduced while the fermentation time increases and gas formation was not observed after the third day of fermentation. The taste of the MGPL was sour during fermentation (Table 2). The pH of the MGPL was gradually decreased from 6.5 ± 0.2 to 3.8 ± 0.1 during fermentation (Table 3).

The color of the *M. citrifolia* fermentation recipes (MFR) was brown, and the brown color was more intense in the recipes without raw onion extract (ROE). The odor of the MFR recipes was from the smell of *M. citrifolia* Linn. (until six days of process) to sour (from 7 to 45 days), due to the resultant of fermentation and production of organic acids. The turbidity of MFR was increased during fermentation period due to the increase in dissolved solid particles, whereas the gas formation was reduced and no gas was observed after 30 days of fermentation. The taste of the MFR was slightly sweet, because of the presence of cane sugar, and astringent up to seven days of the process and become sour (Table 2). The pH of the MFR was also gradually decreased during fermentation (Figure 1A). The reduction in the pH of both MGPL and MFR were due to the increased production of organic acids (Figure 1B) and lactic acid bacteria (Figure 2).

Even though, the Thai fermented foods are known for the enrichment of beneficial microbes like LAB with glutaminase, glutamate decarboxylase

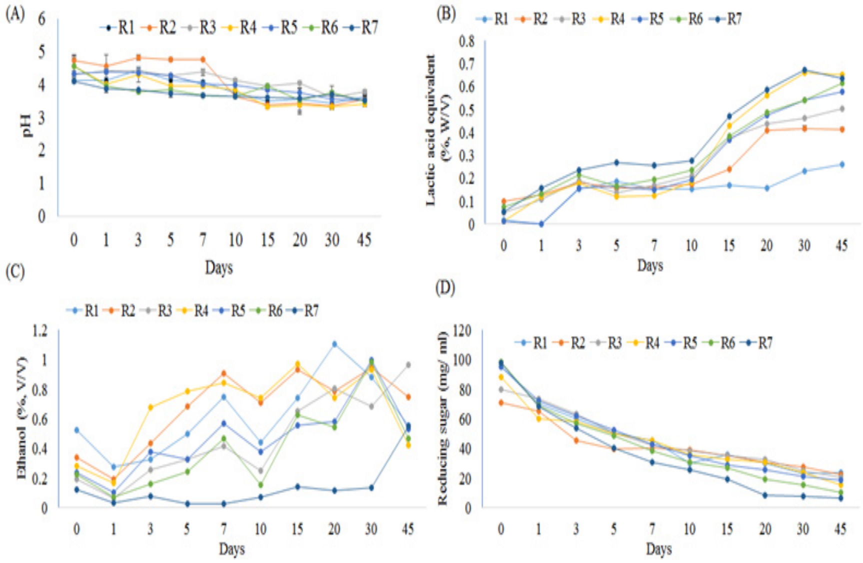

Figure 1. (A) The pH profile of MFR during fermentation. (B) The organic acids content of MFR recipes during fermentation. The values are represented as lactic acid equivalent (%). (C) The ethanol profile, and (D) the level of reducing sugar of MFR recipes during fermentation. The sample codes were detailed in table 1.

producing ability, and yeast cells with β-glucosidase production (Woraharn *et al*., 2014; Sirilun *et al.*, 2016), fermented plant beverages must be evaluated for its microbial load. Thus, the microbial load of the MGPL and MFR were determined by plated counting method with specific media for the respective microbial community. The total bacterial load of MFR was increased with respect to the duration of the fermentation (Figure 2A). A load of *Lactobacillus,* Yeast, and mold in MGPL were also increased upon incubation. Even though the total bacterial load was maximum (10.42 ± 0.32 log CFU/ml) at 10th day of fermentation, the number of *Lactobacillus*, Yeast and mold were reduced than that of the previous time point. The coliform count was not observed from the 2nd day of the process, possibly due the reduction in pH of the formula (Table 3).

The *Lactobacillus*, Yeast and mold contents of MFR were altered based on the composition of the recipes. The presence of onion extract and the concentration of the pickled starter (MGPL) influences the microbial load. The *Lactobacillus* content was observed in the R1 only after 15 days of fermentation, moreover the R2, R3 recipes were recorded for *Lactobacillus* content on the 3rd day of the process (Figure 2B). The onion can enhance the growth of Lactic acid bacteria (LAB), and suppresses the pathogenic bacteria, and also improves the nutritional content and health benefits. The role of growth stimulation of *L. acidophilus* by onion juice has been studied. The onion juice pointedly increased the growth and acidification of *L. acidophilus* in fermented milk product, and the ingredients of onion juice enhance the beneficial effect of *L. acidophilus* and its viability (Gyawali *et al.*, 2006; Li *et al.*, 2016).

In the present study also, the *Lactobacillus* load has been enhanced in the recipes that contain ROE (R5, R6, and R7). Moreover, the growth of yeast and mold have not been observed in R5 and R6. Thus, the ROE protects the recipes from contaminants, effectively. The R7 showed the yeast and mold content until the 3rd day of fermentation and R3 was accounted for the yeast and mold content at all the tested time points of the process, perhaps due to the heavy inoculation of MGPL (Figure 2C). All the recipes were microbiologically safe as per the Thai commercial product standards (TCPS 481-2547, 2004). Moreover, *M. citrifolia* is known for antibacterial, antifungal, and antiviral activity (Singh 2012). Thus, the presence of *M. citrifolia* in MFR naturally prevent or suppresses the growth of harmful microbes.

The concentration of organic acid was estimated kinetically during the preparation of MGPL and MFR. The results were suggested that the organic acids level have been increased gradually in both MGPL (Table 3) and MFR (Figure 1B) during fermentation. The organic acid contents of MGPL, in terms of lactic acid equivalent, was detected during ten days of natural fermentation (Table 3). The acidity reduces the pH of the pickling thereby it facilitates the growth of lactic acid bacteria and prevent the growth of pathogenic bacteria. Likewise, the organic acid content of the MFRs also represented. Apparently, the

Table 3. The pH, total coliforms, *Lactobacillus*, yeast, and mold content, acid content, methanol, ethanol, and reducing sugar level of MGPL at different time points. The values were represented as mean ± SD. ND = detected.

Days	pH	Total bacterial count (Log CFU/ml)	Total coliform (MPN/100 ml)	Lactobacillus (Log CFU/ml)	Yeast and mold (Log CFU/ml)	Lactic acid equivalent (%; w/v)	Methanol (ppm)	Ethanol (%;V/ V)	Reducing sugar (ppm)
0	6.5 ± 0.2	7.05 ± 0.08	17 ± 3	7.95 ± 0.03	5.90 ± 0.08	0.06 ± 0.002	ND	ND	370 ± 8.2
1	5.3 ± 0.2	7.91 ± 0.07	4 ± 1	7.82 ± 0.01	8.16 ± 0.24	0.08 ± 0.002	ND	0.019 ± 0.002	365 ± 6.9
2	4.6 ± 0.1	7.79 ± 0.15	0	7.47 ± 0.33	7.50 ± 0.24	0.12 ± 0.008	ND	0.059 ± 0.001	250 ± 5.3
3	3.8 ± 0.2	8.01 ± 0.01	0	8.00 ± 0.00	6.16 ± 0.12	0.15 ± 0.02	ND	0.110 ± 0.02	230 ± 4.9
4	3.6 ± 0.2	7.96 ± 0.04	0	7.95 ± 0.04	6.24 ± 0.04	0.155 ± 0.012	6.78 ± 0.58	0.209 ± 0.05	80 ± 2.1
5	3.5 ± 0.2	9.28 ± 0.21	0	9.36 ± 0.10	7.27 ± 0.06	0.158 ± 0.02	18.30 ± 2.2	0.273 ± 0.02	115 ± 2.9
7	3.7 ± 0.2	7.50 ± 0.00	0	9.47 ± 0.23	6.33 ± 0.12	0.164 ± 0.02	60.04 ± 5.3	0.548 ± 0.03	70 ± 3.2
10	3.8 ± 0.1	10.42 ± 0.32	0	7.33 ± 0.12	6.22 ± 0.15	0.17 ± 0.03	92.70 ± 6.1	0.634 ± 0.02	20 ± 1.9

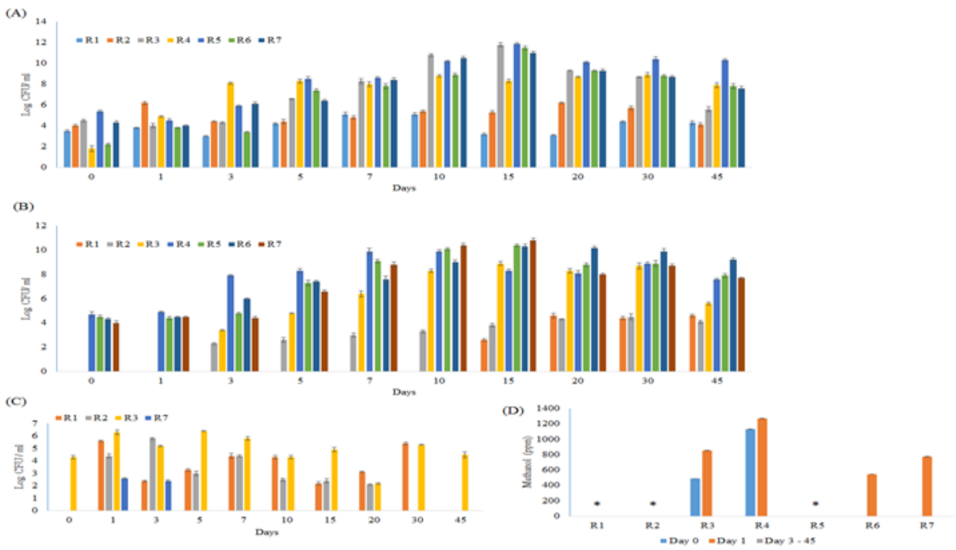

Figure 2. The microbial load in MFR recipes during the fermentation process. (A) Total bacterial count, (B) *Lactobacillus*, and (C) yeast and mold content of MFR. The methanol content of MFR recipes (D). * Means not detected. The sample codes were detailed in table 1.

presence of ROE enhances the growth of LAB and facilitates the improvement of organic acid content in the recipes R5, R6, and R7. The maximum level of organic acids, in terms of lactic acid equivalent, was recorded in the recipes, R7 due to the rich inoculum and ROE (Figure 1B). The presence of several acids in noni has been reported previously (Singh 2012), and the microbial actions increase the acid content of fermented plant beverages by sugar fermentation.

The level of methanol and ethanol content was assessed in MGPL for ten days and found that the alcohol content was gradually increased during fermentation. The presence of methanol and ethanol were detected at fourth day and the first day of the process, respectively. To the maximum 92.7042 ± 6.1 ppm of methanol and 0.6348 ± 0.02% v/v of ethanol was recorded after ten days of MGPL preparation (Table 3). Yeast can change the sugar to alcohol by the aerobic reaction, during 4-7th days of fermentation. As per the Thai community product standards (TCPS) norms, the content of methanol (≤ 240 ppm) and ethanol (≤ 3% v/v) in MGPL was acceptable in fermented products (Chaiyasut *et al*., 2016). There was no perpetual trend of ethanol concentration during MFR fermentation. The frequent fluctuations were observed in ethanol content whereas gradual upsurge has been recorded in R7. After 45 days of MFR fermentation, the ethanol content was under or near the permissible level in fermented plant juice. There is no methanol content detected in the R1, R2, and R5 during fermentation, whereas, recipes R3, R4, R6 and R7 accounted with methanol during the initial period of processing (Figure 1C, Figure 2D). The methanol formation during *M. citrifolia* fermentation due to the presence of pectinmethylesterase enzyme in the fruit, which facilitates the formation of methanol from pectin of plant raw materials. Naturally, the reducing sugar level has been gradually reduced in MFR by microbial utilization (Figure 1D).

The fermented noni or *M. citrifolia* juices were used in many parts of Asian countries. The production of probiotic-based *M. citrifolia* juice with lactic acid bacteria or *Bifidobacteria* is typical and the formulations were tested for probiotic nature. A study suggested that *B. longum* and *L. plantarum* are the best starter culture for producing probiotic seedless noni fruit juice (Wang *et al*., 2009). The stability of fermented *M. citrifolia* juice has not been studied in detail except a report by Nandhasri *et al*., 2011 about the stability of concentrated Thai *M. citrifolia* fruit juices. The nutritional value of the different part of *M. citrifolia* has been reported by several report (Singh 2012). But there is no proper recommended daily dose of fermented *M. citrifolia* juice except an oral dose of 2 g per day (Assi *et al*., 2015), due to the nutritional changes in fermented *M. citrifolia* juice depends on the type of fermentation and nature of starter culture. To the best of our knowledge, the current study is the initial report about the fermentation of *M. citrifolia* fruit with naturally fermented Thai-MGPL juice as starter culture deals with the microbial load, organic acids, alcoholic content and other physical properties.

Conclusion

The naturally fermented mustard green pickles (MGPL) have been developed and assessed the physicochemical and microbiological nature. The fermented *M. citrifolia* Linn. fruit was prepared using MGPL as starter culture and revealed that the fermented mustard green pickled liquid (MGPL) harbor the adequate amount of *Lactobacillus* sp. (10^8 CFU/mL), which can be further used as a safe starter for fermented plant beverages. Moreover, the microbiological and physiochemical analysis suggested that fermented *M. citrifolia* Linn with 10% of MGPL and ROE produces the safe consumable fermented juice. Also, the results indicated that onion extract enhances the growth of LAB during *M. citrifolia* fermentation. The current study was an attempt to use traditional mustard green pickle formulation as a starter for controlled fermentation but some of the aspects are not explained like bioactivities of MGPL fermented *M. citrifolia* juice. Thus, additional studies are recommended on traditional formulations and its applications in modern food technology, especially in the production of fermented plant beverages.

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