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The effect of salt concentration and fermentation time on pH value, total acidity and microbial characteristic of pickled ginger (*Zingiber officinale* Rosc.)

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Abstract

Spontaneous fermentation of ginger are very popular in China and Japan and the product are known as pickled ginger, in Indonesia its popularity is still limited in certain society, but more increasing with the present of more japanese cuisine restaurants especially in metropolitan cities. Hence a study on the effect of salt concentration and fermentation time on pH value, total acidity number and microbial characteristic of pickled ginger (*Zingiber officinale* Rosc.) was conducted. Completely Randomized Design was used in this study with two factors namely salt (NaCl) concentrations (2.5, 5.0 and 7.5 % w/w) and fermentation time : 5 and 10 days, and this experiment was replicated three times, while the parameters observed were pH value, total acidity , Lactic Acid Bacteria total counts and characterisation and identification of lactic acid bacteria of selected pickled ginger sample using index de Garmo was carried out by 16SrRNA gene sequencing analysis. The selected sample was the one prepared using 2.5% w/w salt and 5 days fermentation at 26°C with pH value of 3.40, total acidity 0.92% and lactic Acid bacteria total counts of 7.56 x 10^6 CFU/ml. Based on 16S rRNA gene sequencing analysis and similarity by Neighbor Joining (NJ) method with phylogenic tree construction, the lactic acid bacteria of selected pickled ginger identified were *Lactobacillus plantarum* strain BM4 and *Lactobacillus plantarum* LOCK 0991 with similarity of 86% .

Introduction

Ginger (*Zingiber officinale* Rosc) rhizome is one of most important spices used widely in Indonesian cuisine as well as in other countries and also well known as one of traditional herbal medicine ingredients. The volatile aromatic components give a spicy, pungent and pleasant smell and therefore ginger rhizome either in fresh, dried or powder form are used as spices and food additives in a number of food and beverages. Gingerols were found as the most abundant active components which mainly consist of gingerol [5-hydroxy-1-(4-hydroxy-3-methoxy phenyl) decan-3 (Purnomo *et al*., 2010; Singh *et al.,* 2014; Zadeh and Kor, 2014).

Traditional or spontaneous fermentation of vegetables and fruits have been practised for centuries and some of these fermented products as reported by Aryanta (2000); Tamang (2011); Law *et al.* (2011) and Sulistiani *et al.* (2014). are sauerkraut (European countries and United States of America), kimchi (South Korea), jiang gua (pickled cucumber – Taiwan), sayur asin (fermented mustard cabbage leaves – Indonesia), tempeh, natto (fermented soya beans – Indonesia, Japan), tuak,arak or toddy (fermented fresh palm sap – Indonesia, Malaysia), tape ketan, khoumak (fermented glutinous rice – Indonesia, Thailand) , yan jiang , gari (fermented sliced young ginger rhizome – Taiwan, Japan).

Yeh *et al.* (2014) noted that in some other countries ginger rhizome are not only used as spices and condiment in cooking or to flavor foods and beverages, but it has also been used as raw material of fermented sliced or pickled ginger (Chang *et al.*, 2011) and ginger candy (Nath *et al*., 2013). Japanese pickled young ginger rhizome are usually consumed with sushi and known as gari,and the thin sliced gari are bright pink with an impressive flavor and taste (Chou, 2003), while pickled young ginger Yan jiang in Taiwan according to Chang *et al*. (2011) were prepared by adding salt and sugar in the amount of 30 – 60 $g.kg^{-1}$ and fermented for 3 – 5 days at 6° – 10°C. While Setyaningsih (1993) noted that in Indonesia pickled ginger are prepared using sliced ginger (*Zingiber officinale* var. Officinarum) fermented in brine solution of 10% cooking salt and 2% cane sugar in the ratio 1 : 2 at ambient temperature (25° – 30°C) for 10 days. As information on quality traits of pickled ginger in Indonesia are very limited, therefore the aims of this study were to investigate the pH value, total acidity and microbial characteristic as part of quality traits of pickled ginger prepared using

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different salt concentration and fermentation time.

Material and Methods

Sample preparation

The sample preparation was following the method as described by Setyaningsih (1993) with slightly modification. Ginger rhizome of 4 – 5 months old were obtained from ginger farmer in Batu, Jawa Timur were washed thoroughly using tap water to clean from all kind of dirts before peeled and sliced with thickness of about 0.5 mm. The sliced ginger rhizome (200 g) were then packed in a glass jar and added with brine solution which prepared using cooking salt (2.0%, 5.0% and 7.5% weight per volume) and cane sugar (2% weight per volume). The ratio of sliced ginger and brine solution were 1 : 2, and this mixture were then fermented at room temperature (25^{o} – 30^{o}C) for 5 and 10 days. The pH value, total acidty and total lactic acid bacteria counts were determined after 5 and 10 days after fermentation. While the microbial characteristic of pickled ginger were measured from the best sample after selected using index effectivity according de Garmo (1994).

pH measurement

The measurement of pH values of fermenting liquor were performed following the method as desribed in AOAC (2000) using pH meter (S426237).

Total acidity measurment

The total acidity was determined by titrating 10 ml of fermenting liquor in 50 ml Erlenmeyer flask using 0.1N NaOH and 1% phenolphthalein as the indicator , the total acidity are expressed as lactic acid (AOAC, 2000)

Lactic acid bacteria total counts

Total Plate Count of lactic acid bacteria were conducted following the method as described by Hadioetomo (1993), 10 ml of fermented ginger liqour were diluted in 90ml phosphate buffer and 1ml were then pipetted onto MRS (de Man, Rogosa, and Sharpe)-agar and incubated at 37^{o}C for 2 days before counting the colony formed.

Isolation and identification of lactic acid bacteria

The isolation and identification of lactic acid bacteria of pickled ginger liquor were carried out following the method as described by Yelnetty (2014). The isolated lactic acid bacteria from selected pickled ginger sample using effectivity index de Garmo (1994) was identified by analysis of the 16SrRNA sequence.

Isolation of lactic acid bacteria.

5 ml of pure bacteria culture were put into sterile evendrof (1 : 5) and centrifuged for 5 minutes at 13,000 rpm. The obtained sediments were added with 410 µl $T_{10}E_1$ buffer (10 mM tris HCl : 1mM ethylene diamine tetra acetic acid , pH 8), then the sediments were homogenized and 50 µl lysozyme was added and resuspended before incubation in water bath at 37ºC for 1 hour with agitation every 15 minutes. Proteinase enzyme (20 µl) was added to the culture and incubated in water bath at 37^{o}C for 1 hour with agitation every 15 minutes. Furthermore, 50 µl of Sodium Dodesil Sulfat (SDS) 10% was added and incubated at 65^{o}C for 1 hour with agitation every 30 minutes. After incubation 167 ml NaCl 5M was added and reincubated at 65^{o}C for 1 hour with agitation during incubation. The cell culture after incubation were added with ± 400 µl cold chloroform and incubated again at ambient temperature for 30 minutes with agitation every 10 minutes. Then the cell culture were centrifuged at 13,000 rpm for 10 minutes and supernatant were transfered into a new eppendorf and isopropanol (2D-propanol) as much as 10 times were added and agitated ± 50 times before incubated at 20^{o}C for 12 hours. After incubation it was centrifuged at 13,000rpm for 5 minutes and the supernatant were discarded and 100 µl of cold ethanol 70% was added continued with another centrifugation before ethanol were discarded and the sediments were aerated dried and after drying 80µl $T_{10}E_1$ Buffer 20 buffer was added to the dried sediments.

DNA purification, DNA electrophoresis, Polymerase Chain Reaction (PCR) amplification and sequencing purification uses the method described by Leonard (1998).

DNA purification

The DNA of isolated bacteria were purified using phenol-chloroform method i.e. 100 µl mixed with $T_{10}E_1$ buffer solution and phenol-chloroform (1 : 1) then homogenised and centrifuged at 13,000 rpm for 10 minutes. The supernatant replaced to a new eppendorf tube and added with 1 x volume of Na-acetate 3M and 2 x volume absolute ethanol then incbated at 20^{o}C for 5 minutes. The sediment obtained were added with 100 µl ethanol 70% before recentrifuged at 13,000 rpm for 5 minutes. Furthermore the sediments obtained were air dried and then added with 20 µl $T_{10}E_1$ buffer solution before determined using DNA electrophoresis method.

DNA electrophoresis

DNA result of isolation were observed by electrophoresis using 0.8% agarose at 100 volt for 45 minutes, then observed under UV light and continued the identification by Polymerase Chain Reaction (PCR) method.

Polymerase Chain Reaction (PCR) amplification.

Polymerase Chain Reaction (PCR) identification process was conducted using 10 µl solution (Total 50 µl PCR reactant) consist of DNA as temp[late, megamix blue kit for amplication and primer 27F (5'-AGA GTT TGA TCC TGG CTC CG-3') and reverse primer 1492 R (5'-TAC GGH TAC CTT GTT ACG ACT T-3'). Early denaturation was carried out at 96°C for 4 minutes, followed by DNA duplication at 94°C for 1 minute, aneling at 51.5°C for 1.30 seconds and extension at 68°C for 8 minutes continued with last extension at 68°C for 10 minutes.

Electrophoresis using polyacrilamide gel 80%.

Polyacrilamide gel which consist of 12.5 ml aquabidest, 5.3 ml polyacrilamide 30%, 2 ml TBE (Tris Borat – EDTA) and 20 µl TEMED. 5 µl of electrophoresis PCR result were homogenised using 1µl loading dye and filled into gel well with retention of 70 volt for 2.5 hours with TBE buffer solution media one time only.

Sequencing purification

Sequencing purification was conducted using 10 µl mixture in PCR tube added with 1µl natrium acetate 3 M and 25 µl cold ethanol absolute (-20°C) and mixed thoroughly using vortex for 10 minutes. Samples were then kept at ambient temperature to settle down before centrifuged at 15,000 rpm for 20 minutes and 4°C. Supernatant were discarded, then 10 µl ethanol 70% were added with slow agitation before recentrifuged at 15.000 rpm for 15 minutes, 4°C. Supernatant were discarded and residue of ethanol in sample were evaporated using vacuum evaporator for 10 minutes and then 10 µl formamide added before mixed with vortex and rinsed. As much as 10 µl purified PCR result were filled in PCR tube or tray and put into sequencer genetic analyzer. The DNA sequence were read at TH analyzer and copied to the bioedit program and conducted blasting at DDBJ/NCBI to observe the sequence similarity. Based on that result then phylogenic tree were constructed using MEGA 5.2 with Neighbor Joining (NJ) method.

Phylogenic tree reconstruction

Reconstruction were carried out by comparing 16S rRNA gen sequence from selected pickled ginger sample isolate with 16SrRNA gen sequence from standard strain obtained from gen database DNA bank *(http://www.ncbi.nml.nih.gov)* using MEGA 5.2. program.

Statistical analysis

Data on the above parameters was taken in triplicate and analyzed statistically by using Randomized Complete Block Design (RCBD) while means were separated by Least Significant Difference (LSD) test at 5% level of significance as described by Steel and Torrie (1997).

Results and Discussion.

Determination of pH value, total acidity number and LAB total counts of pickled ginger.

The pH value, total acidity and Lactic Acid Bacteria (LAB) total counts of pickled ginger prepared using different salt concentration and fermentation time are presented in Table 1. The lowest pH value (3.33) was observed in pickled ginger prepared using 2.5% salt for 10 days, while highest pH value (4.77) was observed in pickled ginger prepared using 7.5% salt for 5 days. The total acidity and total LAB count were decreasing if salt concentrations were increased and longer fermentation time.

The higher salt concentration (2.5% - 7.0%) did not decreasing the pH value of sample although at longer fermentation time, however it affected either total acidity number as well as total LAB count at 5 and 10 days of fermentation. Chia *et al.* (1982) reported that high salt (NaCl) content up to 5% (w/v) added during fermentation of sugars by yeasts showed that yeasts growth was decreased and observed that the effect on semisolid culture were similar to liquid culture. Furthermore Tabatabaei-Yazdi *et al*. (2013) noted that salt concentration added affected the growth, activity as well as the role of microorganisms involved during fermentation process. The higher pH values after 5 and 10 days of fermentation with 5% and 7.5% salt concentration reduced the total LAB as well hence the acidity numbers were also decreased. Therefore the best pickled ginger obtained was the one using 2.5% salt and 5 days of fermentation although same concentration of salt and 10 days of fermentation showed a similar results from the point of view of pH value, total acidity number and total LAB counts.

LAB identification based on electrophoresis, Polymerase Chain Reaction and phylogenic tree reconstruction

DNA of LAB isolated from the best pickled ginger sample and PCR amplification were

Table 1. Physicochemical and microbial quality traits of pickled ginger prepared using different salt concentration and fermentation time*

Salt concentration	Fermentation	pH	Total acidity	TPC LAB
%	time, d			CFU/ml x 10^6
2.5	5	3.47 ± 0.06^{ab}	0.92 ± 0.01^{d}	7.56 ± 1.15^{c}
2.5	10	3.33 ± 0.12^{a}	0.93 ± 0.01^{d}	7.49 ± 0.30^{c}
5.0	5	4.13 ± 0.12^{abc}	0.53 ± 0.01^{bc}	6.50 ± 0.21^{bc}
5.0	10	4.13 ± 0.06^{ab}	0.52 ± 0.01^{ab}	6.83 ± 0.23^{bc}
7.5	5	4.77 ± 0.21^{c}	0.52 ± 0.01^{abc}	5.68 ± 0.80^{ab}
7.5	10	4.40 ± 0.17^{bc}	0.50 ± 0.01^{a}	4.46 ± 0.41^{a}

*) means with standard deviation followed by same superscript in one column was significant diference (P < 0.05) and means were measurement from three different samples.

determined using electrophoresis and the result are presented in Figure 1. The identification were then continued using Polymerase Chain Reaction (PCR) 16SrRNA program and Neghbour Joining (NJ) method to construct phylogenic tree in order to find out percentage of similiraty between the sequences obtained with 16SrDNA from the nucleotide databases from the Genbank *(http://www.ncbi.nml.nih.gov/BLAST)* using the Basic Local Alignment Search Tool (BLAST) program.

The size of PCR product of DNA isolated from best pickled ginger sample as measured using electrophoresis were about 1500bp as showed in Figure 1. and the sequencing analysis result of LAB isolate from pickled ginger prepared using 2.5% salt and 5 days fermentation are as follow:

GCATAGCGTCACCGTTGTGTCTCATGTTACGGTGGCGGGTACACGGA
CTGCGCGGATCCTAAGTGCACCGAAACTTAGTGCTGAGTGCCCATCTT
TGGTCCCGCTAGCGATGTGCCCCTCGTGTCCGCAGCCATTCTGAGAA
TCGGGACCCGACAGCTGAGACAGCCGTAGTCGAATCTTTAGACATATT
GAACTGTAGGTTGTATAAACCACCCGGCGCCCGAATCTGGAGCTTGC
CGATGGCGAAGCAGCGTTAGCTGAGGAACCTCCAACTCATCGACGAC
TGACGTCCTCGGCGTAGTAAAGACCCGGGTCTTTACTACGCCGAGGA
CGTTACCGTCCATCCGACTTGGGAGGTTTCCTCAGCTAACGCTGCTTC
GCCATCGGCAAGCTCCAGATTCGGGCCAGGTCTGTGGTTAATCAAGA
GCTCGTCTTACAATTAGAGAACTTCCTGGCTGTCAGGTCATGTTCCCG
CTCTCGTGATGGGTAGTCCCGCACCCTCGTCCATTTGGGCACTGTAC
CCGGACCGAAGGGTCAACCATGCCTTAGACCTGGGTCCCCGTCGGTT
CACCAGTCACTCCAGAAACATATTTCTTCTGGACCTAAA

The phylogenic tree construction using MEGA5.2 application with neighbour joining method and statistically test using bootstrap with 500 replication. Boostrap method have the function of randomizing same bacteria characteristic into a new set of data with same amount of bacteria. Number below each

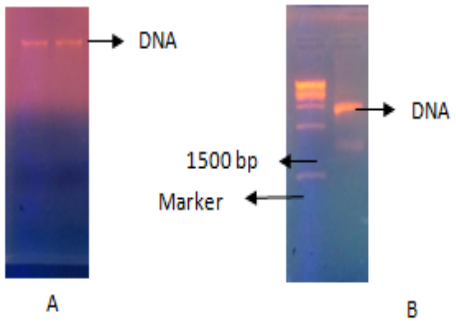

Figure 1. DNA spot detected using electrophoresis running by 5 µl loading dye 1 µl (A), and PCR 16S rRNA product which was running in the amount of 3 µl, loading dye1µl, and marker 1 kb vivantis 6 µl (B).

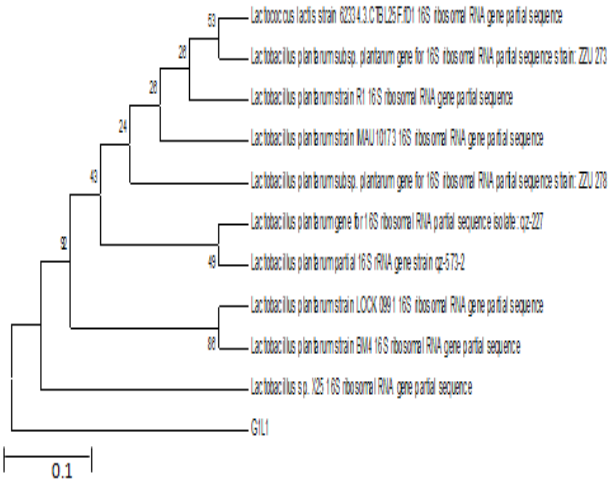

Figure 2. Phylogenic tree based on 16S rRNA sequencing which showed similarity between LAB of sample prepared using 2.5%(w/w) salt and 5 days fermentation and comparison bacteria group obtained from NCBI.

phylogenic tree is bootstrap value as level similarity of bacteria, and the sequencing reconstruction result could be find out by observing the similarity level of LAB sample with LAB standard at phylogenic tree as shown in Figure 2. The result showed that LAB found in pickled ginger sample prepared using 2.5% salt and 5 days of fermentation was *Lactobacillus*

plantarum with similarity 92%. According to phylogenic tree construction *Lactobacillus plantarum* strain BM4 and *Lactobacillus plantarum* LOCK 0091 have the similarity of 86%. According to Goodfellow dan O'Donnel (1993) a strain could be classified as one species if this strain has index imilarity of ≥ 70%. While Chang *et al.* (2011) and Swain *et al.* (2014) also observed *Lactobacillus plantarum* as one of LAB found after the third day of pickled ginger added with plum produced in Taiwan. The other LAB species reported were *Lactobacillus sakei, Lactococcus lactis* subsp. *Lactis* and *Weissella Cibaria*. Saeedi *et al.* (2015) noted that *Lactobacillus plantarum* is the major LAB identified at early stage and *Pediococcus pentosaceus* at later stage of winter salad fermentation.

Conclusion

Results showed that the selected pickled ginger is the one prepared using 2.5% w/w salt and fermented for 5 days at 26^{o}C with pH value of 3.40, total acidity 0.92%, lactic acid bacteria total counts 7.56 x 10^{6} CFU/ml. The 16S rRNA gene analysis and phylogenic tree construction results showed that *Lactobacillus plantarum* strain BM4 and *Lactobacillus plantarum* LOCK 0091 have the similarity of 86% are lactic acid bacteria species identified in this sample.

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