Microbiological Properties and Molecular Profiling Of Microbial Ecosystems during Spontaneous Fermentation of *Carica Papaya* Leaves

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Abstract **- Spontaneous fermentation has been identified that it can improve the quality of foods characteristics. It defined that this fermentation undergoes naturally without any starter culture. The microflora at the leaves in the fermentation will give the result on the unknown microorganisms present. This study is conducted to investigate the microbiological properties that present in the fermentation process. This fermentation is conducted for 100 days and this study is isolated, identified and characterized the microorganisms associated with the fermentation Carica papaya leaf on different selective media. Their biochemical characteristics have been investigated by using phenotypic characterization which is gram staining and catalase production. For further methods to know the community of the microbial, molecular profiling method has been used. The microorganisms that present in this study were genus *Lactobacillus, Bacillus, Pichia, Candida, Entrobacter, Klebsiella, Cronobacter, Saccharomyces* and *Lactococcus. Lactobacillus plantarum* is one of the Lactic Acid Bacteria and is the most frequently isolated bacteria in this study. The *Bacillus* genus also was detected throughout the fermentation process starting from the Day 60 until the end of the fermentation process. At the beginning of the fermentation process, Bacillus genus does not detected. This study indicates that *Lactobacillus spp*. is the predominant microorganisms that present in this fermentation of the *Carica* papaya leaves.**

Keywords**:** Carica papaya, Spontaneous fermentation, phenotyping, PCR, sequencing

I. INTRODUCTION

Carica papaya Linn or commonly known as papaya is tropical fruit because of it usually has been cultivated in the tropical region that often seen in orange-red, yellow orange and yellow green colour of the skin. This *Carica papaya* Linn is from the *Caricaceae*'s family [1]. *Carica papaya* is the genus in the family of Caricacea. This plants are belong to the group of plants that known as Laticiferous plants. *Carica papaya* Linn can be grows best and better in a well-drained, rich organic matter soil which is pH 5.5-6.7 and well aerated [2]. Plants are commonly used for medicinal purpose because they are generally cheap and the best sources of the pharmacologically substances that actives. They are also having good resistance to activities of bacterial [3]. Carica papaya is plant that can grow fastly, woody, short-lived, and large herbaceous plant that having vessels of latex in all fruits with an upright of the unbranched stem that has covered all over the leaf scars [4].

Fermentation is a reaction of biological where there is an organism has been changes the sugar which is a carbohydrate into acids or alcohol by process that known as glycolysis without the presence of the oxygen gas. This process is known as anaerobic fermentation [5]. Fermentation process usually has been used widely in the Food and also Pharmaceutical industries [6].

Fermentation process has been used in order to ferment the *Carica papaya* leaves that has contain microflora so that the microorganisms can grow in the broth. The unknown microorganisms will grow in the fermented broth as along the time of fermentation has been started. The mixed culture fermentation will exist as the fermentation has been started in which the inoculum have consists of two or more microorganism exist in the fermentation. The mixed cultures can be consists of mixture of the microorganism between fungi and bacteria, yeasts and fungi, or can also consists microorganism with the same group.

Every species of the microorganisms have their own characteristics and they are unique. There are some microorganisms that have enzyme catalase as their protector enzyme to protect the cells from oxidative damage by the species of reactive oxygen and some microorganism does not have the enzyme catalase. This fermentation of *Carica papaya* leaf is a process that is differs with others products of papaya leaves. It is expected that this fermentation can make the product become more bioavailable. Thus, it can render quicker therapeutic effects especially in dengue fever treatment.

Therefore, this study was carried out to identify and classification the morphology and characterization of unknown microorganism that present in the fermented *Carica papaya* leaf broth and to determine ability of unknown microorganisms to produce catalase enzyme.

II. MATERIALS AND METHODS

A. Fermentation

C.Papaya leaves were collected from farm in Banting. The fresh leaves were making sure that there is no physical dirt on it by washing the leaves. The leaves were shredding into small pieces by using kitchen blender. A little amount of distilled water was put into the kitchen blender in order to make sure the leaves shredded well. Ten percent (w/v) from the raw material has been loaded into 4L benchtop bioreactor. Distilled water has been added to make up 4L. At the 10% w/v, sugar has been added. Sugar act as initial substrate. The fermentation was carried out the anaerobically which is the leaves has been fermented for 90 days at room temperature with no present of oxygen. There is no addition of preservative or starter culture.

B. Preparation of media

Man Rogosa Sharpe (MRS), MacConkey agar, Dichloran Bengal Chloramphenicol and plate count agar. Media were prepared by weighing approximate amount of the powder and dissolved it in 500 mL of distilled water in conical flasks or Duran Wide Mouth Bottles. All media that has been used was added with L. cysteine (0.05%). The flasks were plugged with cotton wool wrapped with aluminium foil or if used the Duran bottles, the caps of the bottles were not fully closed. Then, the media were homogenized by boiling during the mixing process before sterilizing in the autoclave at 121°C for 15minutes. Finally, the sterile media were allowed to cool to about 50°C before being poured into sterile petri-dishes and allowed to set.

A. Microbiology analysis.

i) Enumeration of microbial populations

The broth samples were collected for 0.1 ml at day 0, 2, 4, 8, 15, 33, 45, 60, 75, 90 and 100 fermentation. The samples were homogenized with 0.9 ml of sterile peptone water. The samples were serially diluted into one to six dilution factor. 0.1 ml of each dilution was speared inoculated onto several plates of different selective agar media. The chosen selective agar media were Man Rogosa Sharpe (MRS) agar for lactic acid bacteria, MacConkey agar for Enterobacteriaceae (e.g. Escherichia coli, Salmonella and Yersinia pestis), Dichloran Bengal Chloramphenicol (DRBC) agar for mould and yeast and plate count agar (PCA) for aerobic bacteria (e.g. Bacillus subtilis, Lactobacillus casei. Staphylococcus aureus). All the agar that has been used was added with L. cysteine (0.05%). L. cysteine acts as reducing agent. All the sample plates were incubated anaerobically in a candle jar at temperature of 37°C for MacConkey. And at temperature of 30°C for DRBC, PCA and MRS. All the plates incubated for about 24 hours (1 day). After the incubation, the bacteria and also yeast colonies that have grown were counted and a log colony forming unit (CFU) per ml of the broth sample were expressed. Further purification was done by randomly selected at least three single colonies for each types of agar for each sampling time. The taken colonies were re-streaking in each agar type for the. Each sample was purified two times for further purification.

ii) Preservation of isolates

The pure isolates were inoculated onto freshly prepared MRS for bacteria grown on MRS and LB broth for the rest agar. The isolates were incubated in an incubator at 37°C for 24 hours. The pure isolates were preserved in liquid broth (MRS for bacteria grown on MRS and LB broth for the rest agar) that containing 30% glycerol and then stored in the refrigerator at - 30°C.

B. Phenotypic characterization of microbial species.

i) Gram staining and cell morphology.

Inoculation loop was sterile by flame it under Bunsen burner. By using the sterile inoculation loop, one drop of distilled water was added to the slide. A bacteria colony was selected by using sterile inoculation loop and the colony was smeared on to the slide. The slide was air dried and heat fix by passing the slide through a flame for a few times. Five to six drops of Crystal Violet was added to the culture slide and was stand for about one minute. Then, the culture slide was washed briefly with distilled and the water excess was shaken off. Five to six drops of Gram's Iodine solution was added to the culture slide and was stand for about one minute. The culture slide was washed briefly with distilled water and the excess water was shaken off. The culture slide was decolorized using acetone-alcohol solution until the purple colour has stopped running. The culture slide was washed immediately with distilled water. After that, five to six drops of Safranin solution was added and was stand for about 30 seconds. The slide was washed briefly with distilled water and the excess water was shaken off. The slide was blotted with bibulous paper. Cover slip was put on the slide. The culture slide was examined under microscope with 4X objective, 10X objective, 40X objective and 100X objective. At 100x objective, one drop of oil immersion was added to the cover slip.

ii) Catalase production.

A small amount of bacteria colony was transferred to a sterile slide by using sterile inoculation loop. A drop of 3% H_2O_2 was added to the slide and was mixed. The mixed culture was observed whether there was a present of bubble or not.

C. DNA extraction and purification

Total of the DNA from the broth culture and the bacterial and yeast DNA from the pure culture were extracted by using the Promega DNA extraction kit which it has followed the manufacturer's protocol. The extracts of the DNA were stored at -30°C

D. PCR amplification.

Two universal primers were used for amplification of the V3 region of the 16S rRNA gene which were forward primer; 27f (5-AGAGTTTGATCCTGGCTCAG-3) and reverse primer; 1492r (5-GGTTACCTTGTTACGACTT-3). These universal primers were used to amplification the rRNA of bacteria. Each of the mixture of the PCR of 50 µl total volume consist of 5 µl of DNA template, 0.25 µM of each primer (forward and reverse), 25 µl of REDiant 2× MasterMix (1st BASE) which comprised of reaction buffer, 0.06 U/µl of *Taq* DNA polymerase, 3 mM $MgCl_2$ and 400 µM of each dNTPs and nuclease-free water.

Each of the reaction was carried out using a conventional thermocycler which was Eppendoff Mastercycler at 35 cycles of denaturation at 95 °C for 30s, followed by the annealing at 55.5 °C for 30 s and elongation at 72 °C for 1.5 min. the initial denaturation and also the final extension were carried out at 95 °C for 5 min and 10 min at 72 °C respectively. The DNA fragment purity were analysed by running the 2% (w/v) of agarose gel with 1 x TAE buffer. For fungal isolates, 5.8S-Internally Transcribed Spacer (5.8S-ITS) rDNA region was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Other PCR conditions were similar to bacterial isolates analysis.

E. Molecular sequencing and microbial identification

The sequences that have obtained from PCR amplification were analysed using BLASTN website. Firstly, the Basic Local Alignment Search Tool page was opened from the National Center for Biotechnology Information website by clicking on BLAST in the Popular Resources menu. Nucleotide Blast program were chosen to run the sequence. File of the sequence was uploaded under the menu of Enter Query Sequence. 16S ribosomal RNA sequences (Bacteria and Archaea) was selected

from the Database pull-down menu under Choose Search Set. Highly similar sequences (megablast) were selected under the menu Program Selection. The button BLAST was clicked to submit the research of the sequences. Finally, the identification of the microbial was obtained after the sequences were analysed.

F. Cluster analysis of microbial profiles.

The microbial profiles were clustered by using the Mega7 software to grouping the microbial that have same group which are more similar to each other than the microbial in other group. Phylogenetic tree of microbial were obtained from the cluster analysis.

III. RESULTS AND DISCUSSION

Phenotypic characterization of microbial species: Gram staining, cell morphology and catalase production

In this research, spontaneous fermentation was used and this fermentation has used biochemical test and molecular methods in order to identify the microorganisms. Spontaneous fermentation of the *Carica papaya* leaves is the process where the natural bacteria has already present on the surface of the raw materials and it is the starting point to start the fermentation process or fermentation that has been microflora without any starter culture being added in the process. The preliminary criterion for identification is the morphological characteristics of individual microorganisms. In the several properties of the morphological, the gram staining reactions and the colonial characteristics were employed in order to identify the identification of the isolates. In this research, four types of selective media were chosen to isolate the microbial that present in the fermentation which was Man Rogosa Sharpe (MRS) agar, Dichloran Bengal Chloramphenicol (DRBC) agar, Plate Count Agar (PCA) and MacConkey agar. Types of microorganisms that can growth from the fermentation were different because of the different type of selective media have been used. The Gram's stain is very useful in this identification of microbial because it helps to eliminate thousands of the possible organisms that present during the fermentation process [7]. Biochemical test was used in the identification of microbial because each of the microbial family or species can produce certain unique metabolites [7].

From Table 3.0 until Table 3.3, all isolates were obtained with each of them having their own characteristics and morphology. All microorganisms were growth for each type of selective media. All microorganisms that were growth on each type of selective media showing that for each day of the fermentation gave different count of microbial.

For Man Rogosa Sharpe (MRS) agar, the Table 3.0 shows that the microorganisms gave the same cell shape which was bacilli with the positive gram staining except for the Day 15 (isolate 3) which it gave negative result for the gram staining. From the Table 3.0, it shows that for the catalase test mostly the microorganisms have gave positive results for the catalase test and some of them gave negative results. For microorganism that growth on the MRS agar, they should gave positive results in the catalase test and gram staining because MRS agar is the selective media that essential for the growth of the lactic acid bacteria. The negative results that obtained may cause by the contamination that occurred during the experiment. For Dichloran Bengal Chloramphenicol (DRBC) agar, the Table 3.1 shows that the mostly the microorganisms gave the same cell shape which was oval and there was some gave bacilli shape with the positive gram staining and positive catalase test. For the Day 0, there was no growth on the DRBC agar.

For the Plate Count Agar (PCA), the Table 3.2 shows that mostly the microorganisms gave the same cell shape which was bacilli and there was some gave cocci shape with the positive gram staining and positive catalase test. For MacConkey agar, all microorganisms gave negative results for gram staining with the same bacilli shape and positive catalase test. There were no growths for Day 33 until Day 90 for MacConkey agar.

The positive results of the Gram's stain for all isolates were indicated that the organisms were retained the crystal violet dye and the negative results were indicated that organisms does not retained the crystal violet dye. The crystal violet dyes were retained because the organisms consists thick peptidoglycan layer in the cell wall of the organisms and organisms that does not retained crystal violet dyes due to they consist of thin peptidoglycan cell wall [8]. The positive results were obtained because of the presence of bubbles when the drop of hydrogen peroxide was mixed with single colony on glass slide. This means that the microorganisms consist of catalase enzyme in their cells.

Table 3.0: Biochemical characteristics of bacteria on MRS

Day	MRS Agar					
	Gram staining and shape			Catalase test		
	1	2	3	1	2	3
0	(+) Bacilli	(+) Bacilli	(+) Bacilli	-	-	-
2	(+) Bacilli	(+) Bacilli	(+) Bacilli	-	-	-
4	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	-	-
8	(+) Bacilli	(+) Bacilli	(+) Bacilli	-	-	-
15	(+) Bacilli	(+) Bacilli	(-) Bacilli	-	-	-
33	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
45	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
60	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
75	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
90	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
100	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+

Table 3.1: Biochemical characteristics of bacteria on DRBC

Day	DRBC Agar					
	Gram staining and shape			Catalase test		
	1	2	3	1	2	3
0	No growth	No growth	No growth	x	x	x
2	(+) Oval	(+) Oval	(+) Oval	+	+	+
4	(+) Oval	(+) Oval	(+) Oval	+	+	+
8	(+) Oval	(+) Oval	(+) Oval	+	+	+
15	(+) Oval	(+) Oval	(+) Oval	+	+	+
33	(+) Oval	(+) Oval	(+) Oval	+	+	+

45	(+) Oval	(+) Oval	(+) Oval	+	+	+
60	(+) Oval	(+) Oval	(+) Oval	+	+	+
75	(+) Oval	(+) Oval	(+) Oval	+	+	+
90	(+) Oval	(+) Oval	(+) Oval	+	+	+
100	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+

Table 3.2: Biochemical characteristics of bacteria on PCA

Day	PCA					
	Gram staining and shape			Catalase test		
	1	2	3	1	2	3
0	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
2	(+) Bacilli	(+) Bacilli	(+) Cocci	+	+	+
4	(+) Cocci	(+) Cocci	(+) Cocci	+	+	+
8	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
15	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
33	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
45	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
60	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
75	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
90	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+
100	(+) Bacilli	(+) Bacilli	(+) Bacilli	+	+	+

Table 3.3: Biochemical characteristics of bacteria on MacConkey

Day	MacConkey Agar					
	Gram staining and shape			Catalase test		
	1	2	3	1	2	3
0	(-) Bacilli	(-) Bacilli	(-) Bacilli	+	+	+
2	(-) Bacilli	(-) Bacilli	(-) Bacilli	+	+	+
4	No growth	No growth	No growth	x	x	x
8	(-) Bacilli	(-) Bacilli	(-) Bacilli	+	+	+
15	(-) Bacilli	(-) Bacilli	(-) Bacilli	+	+	+
33	No growth	No growth	No growth	x	x	x
45	No growth	No growth	No growth	x	x	x
60	No growth	No growth	No growth	x	x	x
75	No growth	No growth	No growth	x	x	x
90	No growth	No growth	No growth	x	x	x
100	(-) Bacilli	(-) Bacilli	(-) Bacilli	+	+	+

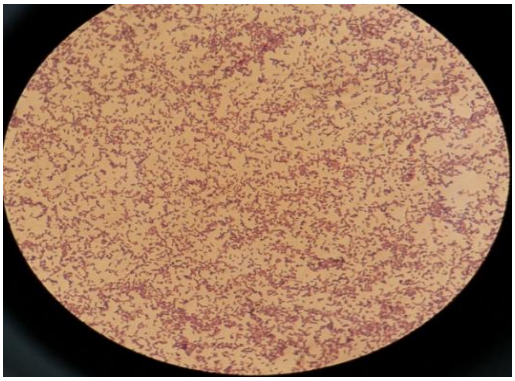

Figure 3.0: Photomicrograph of Gram positive bacilli

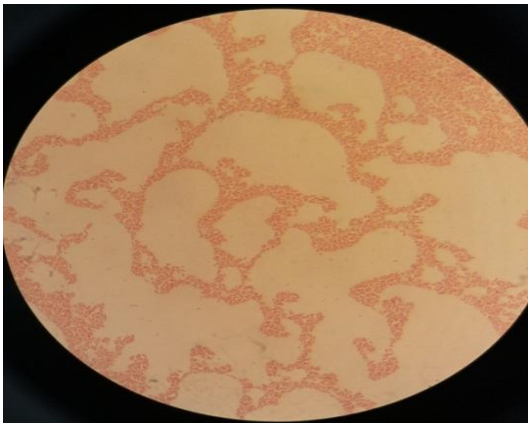

Figure 3.1: Photomicrograph of Gram negative bacilli

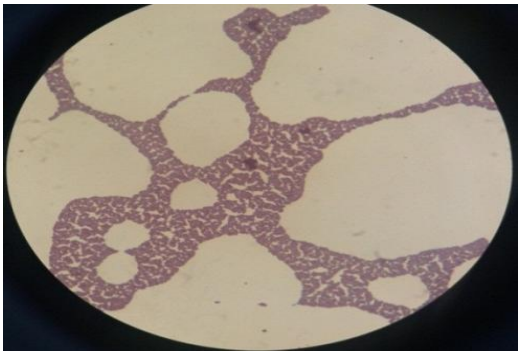

Figure 3.2: Photomicrograph of Gram positive cocci

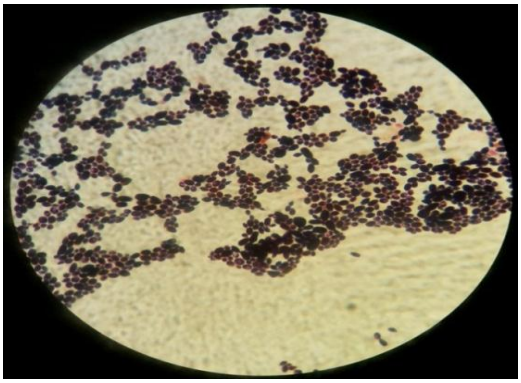

Figure 3.3: Photomicrograph of Gram positive oval (bacilli)

Molecular sequencing, microbial identification and cluster analysis of microbial profiles.

The colonies that were chosen from the selective media were colonies from MRS agar, PCA, DRBC agar and MacConkey agar. These colonies were clustered by amplification of PCR and they were identified by 16S RNA partial sequencing of gene or species-specific PCR by using the two primers which were forward primer; 27f and reverse primer; 1492r [9]. The duplicates and also the selection of the positive and negative controls were used in this research to evaluate the reproducibility and also the specificity of the identification [9]. The Figure 3.4 and Figure 3.5, shows the list of name of the species that has been grouped by using Mega7 software. There are many types of species that has been present in the raw materials of the fermentation process. The result of the fermentation process is depending on the dominate species that produce in the fermentation process [10].

From Figure 3.4 and Figure 3.5, the partial 16S rDNA amplicons indicated that the *Lactobacillus* and *Bacillus* species to be the major bacterial groups during the fermentation course and followed by others partial 16S rDNA sequences that belonging to the genera *Pichia, Candida, Entrobacter, Klebsiella, Cronobacter, Saccharomyces* and *Lactococcus.* Fermented food mostly contain of three groups of lactic acid bacteria which were homolactic *(Streptococcus sp., Pediococcus sp*.), facultative (*Lactobacillus sp*.) and heterolactic (*Leuconostoc sp*.) [11]. The metabolism of the homolactic which is *Lactobacillus plantarum* has the ability to achieve a high of density of cell within a reasonable time of the fermentation and produces high amounts of lactic acid [12]. The isolates that are belonging to the *L.plantarum* group are more tolerant to acid. They were often to become dominant in the fermentation processes. This is because of their ability to transport and metabolize the different carbohydrates of plant [13].

The lactic acid bacteria that has found in this study were also has been presented in other fermented food such as fermented vegetables in the Himalaya are sinki, khalpi and gundruk. The predominant lactic acid bacteria that have been involve in the ethnic fermented vegetables are *Lactobacillus brevis, L. plantarum, Pediococcus pentosaceus, P. acidilactici,* and *Leuconostoc fallax* [14]. The results of this research may indicate that the lactic acid bacteria may play an important role during the fermentation of the Carica papaya leaves.

According to Cintia et al. 2011 [13], lactic acid bacteria are able to ferment the important nutrient which is carbohydrates such as maltose, sucrose and glucose. LAB has positive gram staining and positive catalase and this can be referred to Table 3.0 for MRS Agar. According to Arena et al. 2016 [15], among the genus of *Lactobacilli, Lactobacillus plantarum* was one of the most versatile species. This is also has been included the strains with the valuable skills of the technologies. This LAB is a recognized probiotics features. The number of the probiotic of the *Lactobacillus plantarum* strains can holds multipurpose features.

This is because they can carried out the metabolic processes and also appreciable fermentative. For examples, it can promote the maintenance of health of the consumers and also can increased the specific amount of beneficial compounds like vitamins in the product of fermented foods. This is due to their capacity to modulate the response of the host immune and to de novo produce in the human gut.

The *Bacillus* genus also was detected throughout the fermentation process. But only at the beginning of the fermentation process, Bacillus genus does not detected. The Bacillus genus of the microorganisms is typically was found in the soils. This genus can secrete wide range of enzyme degradation including proteases and amylases. This enzyme may be important in the fermentation processes.

Lactococcus lactis only be detected to be found only at Day 2 of the fermentation because this species do not survive long-term of acidification processes [13]. Starting from Day 0 until the Day 15 of the fermentation, the *Enterobacteriaceae* family which were *Entrobacter, Klebsiella,* and *Cronobacter* were isolated. This may be due to the duration of time of the fermentation which this fermentation takes longer time to complete. This can be refer to the Table 3.2 (day 2 until day 8) and Table 3.3 (day 0 until day 15). These bacteria were Gram negative and catalase positive.

From the Table 3.1, which the DRBC agar's table, the species of yeast were isolated from the fermentation of the Carica papaya leaves which included the Genus of Pichia (*Pichia kudriavzevii*) and Candida (*Candida glabrata* and Candida tropicalis). The *Saccharomyces cerevisiae* also was found but it only be found only at Day 15 of the fermentation time. Candida species have been reported to present in the fermented foods. They have given impact to human health. *Candida glabarata* has been shown to be present in the alcoholic drink in Malaysia which is in tuak which it was made from ragi [16]. These bacteria were Gram positive and positive catalse. This can be referred to Table 3.1.

The function of yeast species in this research is not known. The yeast species may be can cause spoilage to the fermentation and may contributed to the flavour development. The taxonomic structure of the bacterial comparison were performed by doing the analysis of cluster of the microbial profiles and the phylogenetic tree were constructed using UPGMA method by using Mega7 software [13]. Figure 3.4 and Figure 3.5 shows that phylogenetic tree derived from cluster analysis of bacteria and fungi communities associated with *Carica papaya* leaves spontaneous fermentation. The species have been classified by Domain, Class, Family and Genus.

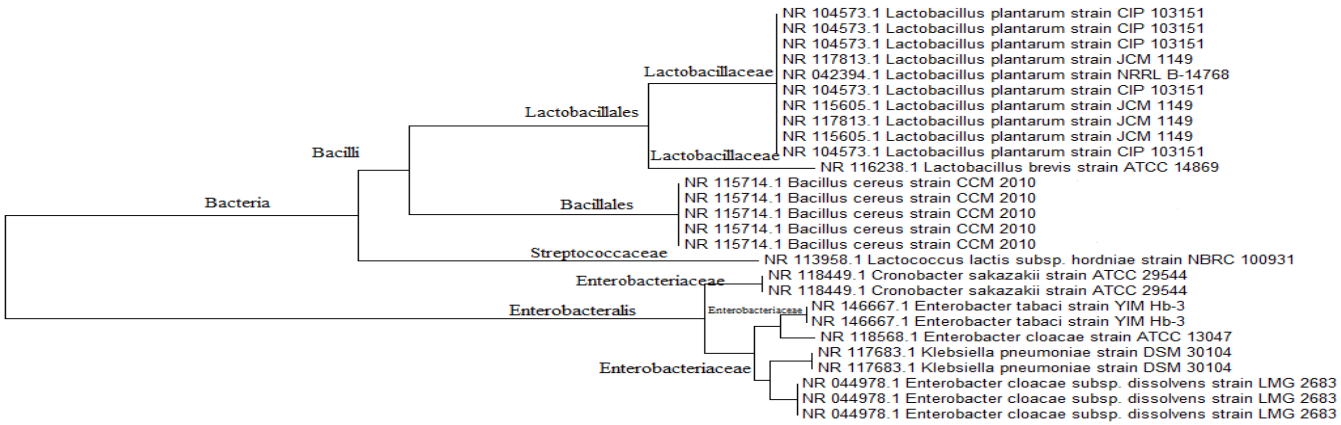

Figure 3.4: Phylogenetic tree of bacteria that growth on MRS, PCA and MacConkey agar.

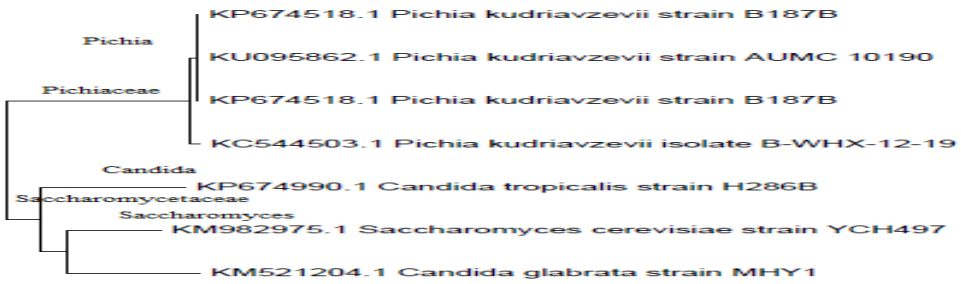

Figure 3.5: Phylogenetic tree of fungi that growth on DRBC agar

IV. CONCLUSION

In conclusion, this research is the first research on the population of microbial involved in the spontaneous fermentation of the *Carica papaya* leaves. The results shows that the among the microorganisms that present in the fermentation, lactic acid bacteria which were *Lactobacillus spp.* were the predominant microorganisms that present in the fermentation of the *Carica papaya* leaves. According to Gilliland E. et al. 1990[17], lactic acid bacteria have several nutritional benefits which were it can improved the nutritional value of food, control of some types of cancer, control the level of serum cholesterol , improved the digestion of the lactose and control the infections of intestinal. Generally, the PCR amplification and molecular sequencing methods have seemed that it have offered a reliable and also is a fast method in order to known the specific name of the microorganisms that present in the fermentation.

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