Population Dynamics and Microbial Biodiversity Profiling of Spontaneous Fermentation of *Carica Papaya* Leaves

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Abstract – An aenarobic spontaneous fermentation of *Carica* papaya leaves has been conducted for 100 days. Ten sampling days has been collected in order to identify the microorganism present during the spontaneous fermentation. The maxium no. colonies (in cfu/ml) estimated at each medium; Plate Counting Agar PCA, Man Ragosa Agar MRS, Dichloran Bengal Chloramphenicol (DRBC) agar, Man Rogosa Sharpe (MRS) agar and MacConkey MC. PCA, MRS and MC clarifies bacteria from yeast as yeast is preferable to grow on DRBC media. There are six genera of bacteria present in the ecosystem consist of Klebsiella, Cronobacteria, Pantoe, Pseudomonas, Bacillus and Enterobacter. A few types of Gram negative bacteria presented which are Pseudomonas, Salmonella and also Escherichia coli. Four genera of yeast and mould presented in the ecosystem which consist of Saccharomyces cerevisiae, Candida glabrata, Zygosaccharomyces and Aspergillus oryzae. Based on this research, acidic condition of the fermented juice optimize the growth of both bacteria and yeast. Yet, the growth of pathogens after day 45 should be avoided in order to prevent from harmful effect.

I. INTRODUCTION

There are various genotypes of papaya around the world such as 'Khack Dum' cultivated in Thailand, 'Maradol' from Cuba, 'Sekaki' and 'Eksotika' cultivated in Malaysia (1).Originally comes from southern part of Mexico, *Carica* papaya or also known as paw paw, papayer, tinti, pepol, kavunagaci, kepaya etc. Papaya is describes as a fast growing plant, 7 to 8 m tall with copious latex, unbranched tree with about 20 cm diameter of trunk (2). *Carica* papaya plant has been in food and medicine related studies as the species reflect various benefits for the innovation of food and supplementation not only for human but for animals as well (3). Papaya is a perennial plant which grow well in tropics and it has been utilised fully including the fruits, leaves and by-products. The plant does not tolerate with cold weather as the best range temperature is between 21 to 33˚C. The tropical fruit is extensively consumed due to its agreeable flavour along with the beneficial pharmacological properties (4).

In order to utilize the papaya plants or even a part of the plant, research need to done to identify the microbial diversity profiling of respected parts of the plant. Previous research proves that the extraction of papaya dried leaves carried antioxidant properties. The by-products of the papaya such as the enzyme, Papain is said to act as a meat tenderizer meanwhile the latex from the plant is traditionally used to heal wounds. As the aiming of this study is to identify the dominant microorganisms present during the fermentation process of the papaya leaves extraction. Microbial biodiversity profiling consist of the population dynamic and also the identification of microorganism in the fermentation ecosystem. The knowledge of the profile of the microbial diversity presented is important to monitor and control the condition of the fermentation process to ensure the quality and safety of the product.

Fermentation is a promising technology for food processing which increase the shelf life of the food or the product. Based on the market demand, food technology is aiming for long lasting food with a condition of controlling microbiological growth of the food. A sensitive controlled condition helps to reduce toxic compounds of the fermented food such as cyanogen and aflatoxins. In regulation and safety assessment guidance of food fermentation, there is a concept of 'history of safe use' which reflect a significant consumption of the food over the years and in a genetically diverse population which adequate toxicological and allergenicity data provided to prove that the food is not harmful to be consumed (5). Papaya leaves can act as nutritional agent as well as medicinal agent as it contains carbohydrates, minerals, vitamins, lipids and also proteins (6). These components play an important role in blood coagulation, heart and nervous system (7). Other than that, fermentation helps in food preservation through formation of inhibitory metabolites such as organic acid, ethanol, becteriocins where often involved with the decrease of water activity (8). Besides, fermentation also involve with

the organoleptic quality (9) as well as improving the nutritional value of the food (10).

Therefore, this study was carried out to enumerate the population dynamic and also identify the identity of the microorganism existed during the spontaneous fermentation of *Carica* papaya leaf.

II. MATERIALS AND METHODS

A. Materials and Chemicals

Carica papaya leaf was collected from Banting, Selangor and sterile saline-peptone water was purchased from Sigma Aldrich. There were four types of media used in the experiment which include Plate Count Agar (PCA), Dichloran Bengal Chloramphenicol (DRBC) agar, Man Rogosa Sharpe (MRS) agar and MacConkey agar also purchased from Sigma Aldrich. There were also Brain Heart Infusion (BHI) broth, MRS broth, Lysogeny broth (LB) and MacConkey broth from Sigma Aldrich for the conservation of pure culture. L. cysteine of 0.05 % w/v as reducing agent for the media, 30% glycerol, ethanol, isopropanol, Promega DNA extraction kit, unrefined sugar, distilled water, candles and aluminium foil.

B. Equipment and tools

20 L benchtop fermenter was used for the fermentation. Other equipment used were autoclave, laminar hood, incubator, centrifuge, pH probe, conical flask, magnetic stirrer, pipette, micro pipette, pipette tip, microcentrifuge tubes, Bunsen burner, lighter, hockey stick, petri dish plates, inoculum loop, colony counter, kitchen blender, scissor, parafilm and candle jar.

C. Fermentation

The main process of the research study is the fermentation process where the leaf obtained was kept in the refrigerator to keep its original characteristics. The first step was prepared by cleaning the dirt on the leaf surface and later cut into small pieces. Then, with a little addition of water, the leaves was blended by using kitchen blender. Some amount of distilled water were added into the blender to ensure the leaves shredded well. The extraction of the papaya leaf with the residues was poured into 30 L benchtop fermenter (INOFRS) at 10 % w/v. For initial substrate, 10 % w/v of unrefined sugar was added into the fermenter. Before closing the lead, distilled water was added into the fermenter until working volume is achieved. The fermentation process was in anaerobic condition at room temperature for 100 days away from direct exposure towards sunlight. Within 100 days of the fermentation process, about ten samples taken and examined including day 0, 2, 5, 10, 15, 30, 40, 60, 75, 90 and day 100. The samples pH was measured by pH probe (Mettler-Toledo).

D. Preparation of media

Plate Count Agar (PCA), Dichloran Bengal Chloramphenicol (DRBC) agar, Man Rogosa Sharpe (MRS) agar and MacConkey agar were prepared by weighing approximate amount of respective powder and dissolved it in 500 mL of distilled water in conical flask. The conical flask were plugged with cotton wool wrapped with aluminium foil. The media were boiled to allow homogenization before undergo sterilizing in the autoclave at 121 ˚C for 15 minutes. Later, each media were allowed to cool for a few minutes before being poured into 20 pieces of petri dishes inside the laminar hood and allowed to solidify

E. Microbiology analysis

On each sampling, 50 mL of samples were taken from the fermenter into a centrifuge tube. After determined the pH value of the sample, 0.1 mL from the sample were homogenized with 0.9 mL of sterile peptone water in the micro-centrifuge tube for dilution $10.^{-1}$. 0.1 mL from the dilution 10^{-1} was pipetted and transferred into the second micro-centrifuge tube with the addition of 0.9 mL peptone water for dilution 10^{-2}. The next dilution continue by transferring 0.1 mL from the dilution 10^{-2} into the third micro-centrifuge until dilution 10^{-6} is achieved. 0.1 mL of each dilution were pipetted into two petri dishes of every agar and spread with hockey stick. MRS and PCA were prepared for the growth of bacteria, MacConkey agar for pathogens while DRBC for yeast and mould. Later, all agar that has been inoculated were sealed and incubated anaerobically in the candle jar at 30˚C for 24 hours except for MacConkey. MacConkey plates were incubated anaerobically in the candle jar at 37˚C for 24 hours as well. After 1 day incubation, the growth of the microorganism inside the petri dishes were counted. The growth of the bacteria and yeast were purified by randomly picked a three single colonies from every types of petri dishes. The inoculation for the purification was prepared by using inoculation loop. The chosen colonies were streaked into a new petri dish of the same type. Second purification was carried out by inoculating the first purification plates and streaked into three new petri dishes of the same type. The second purification was then inoculated into respective for enrichment purpose. The preservation step was carried out in glycerol and stored at -30˚C.

F. 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

G. PCR amplification.

V3 region of the bacterial 16S rRNA gene were targeted for the PCR amplification where two universal primers which consisted of forward primer; 27f (5-AGAGTTTGATCCTGGCTCAG-3) and reverse primer; 1492r (5-GGTTACCTTGTTACGACTT-3). The universal primers were used to amplify the rRNA of the bacteria. There are 5 µL of DNA template and 0.25 µL of REDiant 2x MasterMix (1st BASE) inside each of the mixture of the PCR of 50 µL of total volume. In the 0.25 µL of REDiant 2x MasterMix (1st BASE), there is a mixture of reaction buffer, 0.06 U/µL of Taq DNA polymerase, 3 mM MgCl2 and 400 µL of each dNTPs and nuclease-free water. A conventional thermocycler (Eppendorf Mastercycler) was used to carry out 35 cycles of denaturation at 95˚C for 30 s, followed by the annealing at 55.5 ˚C for 30 s and elongation at 72˚C for 1.5 minute. The initial denaturation was carried out for 5 minute at 95 ˚C while the final extension was carried out for 10 minute at 72 ˚C. The DNA fragment purity were then analysed by running 2% w/v of agarose gel with 1 x TAE buffer.

For the fungal isolate, 5.8S –Internally transcribe Spacer (5.8S-ITS) rDNA region was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Then, Eppendorf

Mastercycler in 35 cycles of denaturation was used for thermocycling. It was conducted at 95˚C for 2min and followed by annealing the temperature at 56˚C in 2min. Then elongate to 72˚C for 2min. The initial denaturation were carried out 5minutes at 95˚C while final extension take 10min at 72 ˚C. Other PCR condition and purification were similar to bacterial isolates.

H. Molecular sequencing and microbial identification

The sequences obtain from the PCR amplification were analysed by using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) website. Nucleotide BLAST was chosen to run the sequence. The sequences gained from the PCR was uploaded through Enter Query Sequence. 16S ribosomal RNA sequences (Bacteria and Archaea) was selected from the Database. The most homology sequence were selected and analysed by the system thus, the identification of the microbial was obtained.

I. Cluster analysis of microbial profiles.

Phylogenetic tree of both bacteria and yeast were obtained by clustering all microbial profiles with the use of Mega7 software.

III. RESULTS AND DISCUSSION

A Microbial growth of *Carica* papaya spontaneous fermentation

Each graph has been constructed based on colony counting of the microbial growth on every media plate consist of Plate Count Agar (PCA), Man Rogosa Sharpe (MRS) agar, Dichloran Bengal Chloramphenicol (DRBC) agar and MacConkey agar. 10 sampling points were taken during 100 days of fermentation of *Carica* papaya leaves. The microbial growth presented includes bacteria and also yeast. Each type of medium only allowed specific growth of microorganisms; i.e. DRBC for yeast and mould, MRS for *lactobacillus sp* and MC agar grows Gram negative bacteria. Colony forming units (Cfu/ml) of bacterial culture was estimated and log Cfu/ml versus sampling days correlation was constructed. Whenever there was more than 200 colonies presented on the media plate, the data will be presented as too numerous to count or TNC.

Based on Figure 1, PCA shows a peak growth of bacteria present starting from day 0 until day 5 as well as Gram negative bacteria on MC media. Yet, the graph shows a decrement of bacterial growth during day 5 and day 30. However, a new growth arise later during day 45 and maintain for a few days until day 90. After that, the graph decreases again at day 100. Basically, PCA media is a standard media that allows the growth of bacteria. The graph shows quite a similarities of microbial growth among PCA, MRS and MC media.

Based on Figure 2, the growth of the bacteria increase rapidly at the early stage of fermentation, day 2 until day 5. Later, the growth starts to decrease per day until day 75. After day 75, the growth of bacteria start to increase again. Same goes with the result of microbial growth on DRBC and MC media, the growth start to increase again after day 90.

As for DRBC media, based on Figure 3, the growth of yeast can be seen grow slowly from day 0 to day 2, different than the others microbial growth on MRS and PCA plate. Yeast and mould grow slowly and reach it peak at day 30. Later, the growth of yeast and mould slowly decrease until day 90 and increase again at day 100. Yeast and mould show a different pattern of growth than the others which relate with the pH of the fermentation solution. It can be seen that yeast and mould grow at optimum level in acidic solution. pH of the fermentation solution at day 30 is pH 3.33.

As for MacConkey media, the growth of Gram negative bacteria or pathogens can be seen increase slightly at day 2 and then decrease at day 5 on Figure 4. The highest growth of the pathogens is at day 15. Yet, after that, the growth of the pathogens decrease gradually until day 45. However, the growth of pathogens increase again at day 60. The presence of these pathogens should be avoided as it is not welcomed and unwanted. There may be some contamination occurred during the lab works especially during the sampling of day 60. Their presence should be overcome as they may cause unwanted effect towards the consumer of the fermented *Carica* papaya leaves.

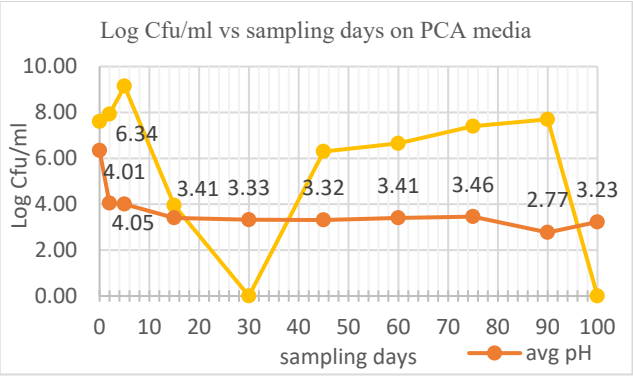

Figure 1: Microbial growth on PCA media

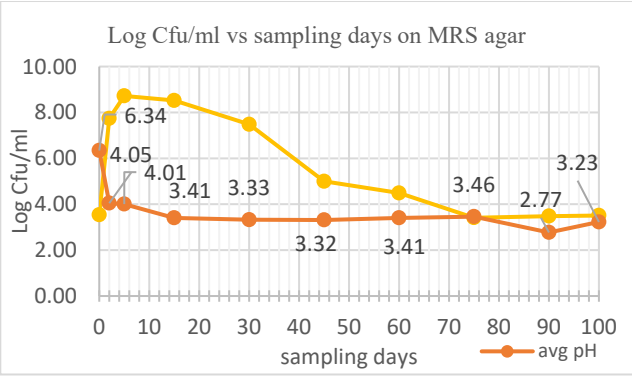

Figure 2: Microbial growth on MRS media

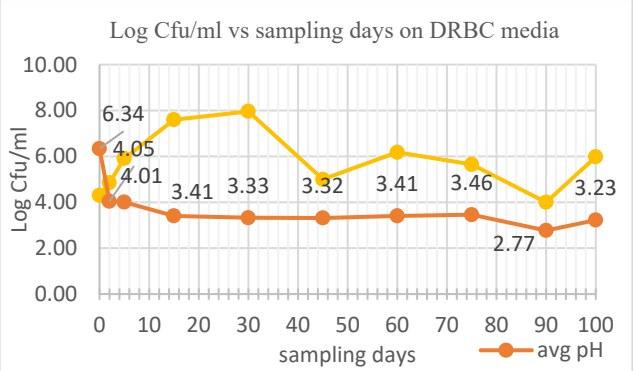

Figure 3: Microbial growth on DRBC media

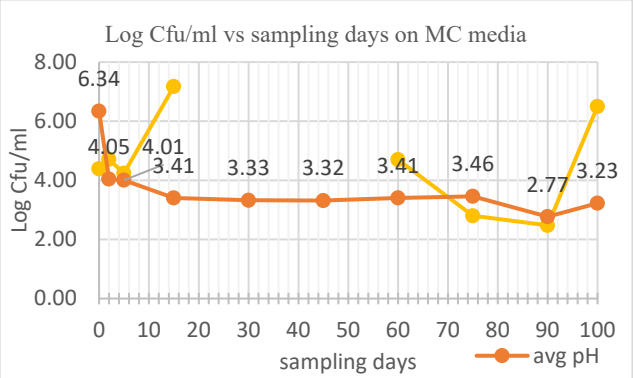

Figure 4: Microbial growth on MC media

B Phylogenetic tree of the microbial growth using MEGA software 7.0.26 ver

Phylogenetic tree of populations of bacteria and yeast has been determined by utilizing the ENA sequences determined by BLASTn search at NCBI database. Both phylogenetic tree has been constructed using MEGA software 7.0.26 ver. Based on Figure 5, there are six genera of bacteria present in the ecosystem consist of Klebsiella, Cronobacteria, Pantoe, Pseudomonas, Bacillus and Enterobacter. Other than that, there are also presence of Escherichia coli strain, Salmonella enterica strain, Erwinia soli strain, Erwinia tasmaniensis strain, Moraxella osloensis strain, Lactobacillus planetarium strain, Enterococcus faecalis strain, Weissell cibaria gene and predicted Aotus nancymaae valosin containing protein lysine methyltransferase. Based on the populations determined, there are a few types of Gram negative bacteria which are Pseudomonas, Salmonella and also Escherichia coli which may arise after 45th day of fermentation. Table 1 shows the identity percentage of the sequence detected for bacteria.

Based on Figure 6, there are four genera which consist of Saccharomyces cerevisiae, Candida glabrata, Zygosaccharomyces and Aspergillus oryzae. Aspergillus is a filamentous fungi while the other genera are yeast. There is also an uncultured Aspergillus isolate and Maytenus fournieri a gene from flowering plant present in the ecosystem. Table 2 shows the identity percentage of the sequence detected for yeast and fungi.

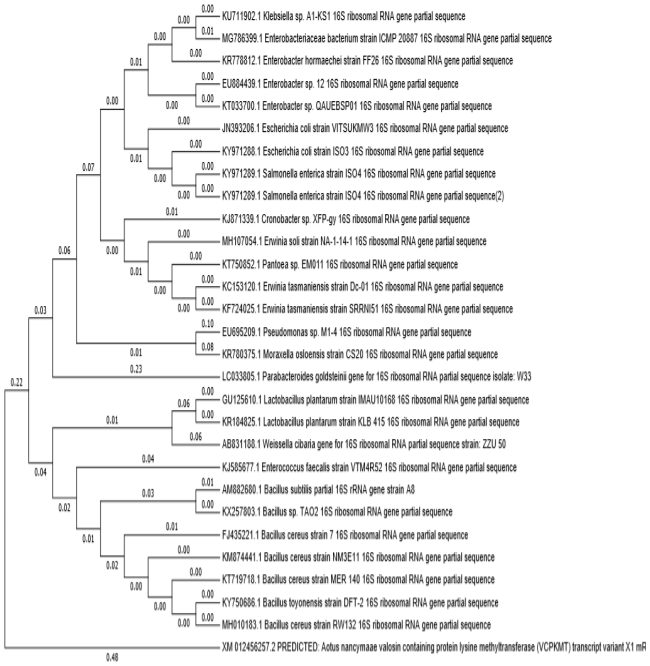

Figure 5: Phylogenetic tree of pure isolation of bacteria

Table 1: List of bacteria identified

ACCESSION NO	STRAINS	IDENT %
MH107054.1	Erwinia soli strain NA-1-14-1 16S ribosomal RNA gene partial sequence	93
KF724025.1	Erwinia tasmaniensis strain SRRNI51 16S ribosomal RNA gene partial sequence	95
LC033805.1	Parabacteroides goldsteinii gene for 16S ribosomal RNA partial sequence isolate: W33	93
MH010183.1	Bacillus cereus strain RW132 16S ribosomal RNA gene partial sequence	99
KM874440.1	Bacillus cereus strain NM3E11 16S ribosomal RNA gene partial sequence	80
FJ435221.1	Bacillus cereus strain 7 16S ribosomal RNA gene partial sequence	90
MG786399.1	Enterobacteriaceae bacterium strain ICMP 20887 16S ribosomal RNA	74

	gene partial sequence	
KU711902.1	Klebsiella sp. A1-KS1 16S ribosomal RNA gene partial sequence	95
KX257803.1	Bacillus sp. TAO2 16S ribosomal RNA gene partial sequence	97
KY750686.1	Bacillus toyonensis strain DFT-2 16S ribosomal RNA gene partial sequence	95
KY971288.1	Escherichia coli strain ISO3 16S ribosomal RNA gene partial sequence	94
JN393206.1	Escherichia coli strain VITSUKMW3 16S ribosomal RNA gene partial sequence	94
EU884439.1	Enterobacter sp. 12 16S ribosomal RNA gene partial sequence	96
KT719718.1	Bacillus cereus strain MER 140 16S ribosomal RNA gene partial sequence	89
AB831188.1	Weissella cibaria gene for 16S ribosomal RNA partial sequence strain:ZZU_50	90
KR778812.1	Enterobacter hormaechei strain FF26_16S ribosomal RNA gene partial sequence	97
KR184825.1	Lactobacillus plantarum strain KLB 415 16S ribosomal RNA gene partial sequence	96
GU125610.1	Lactobacillus plantarum strain IMAU10168 16S ribosomal RNA gene partial sequence	96
KC153120.1	Erwinia tasmaniensis strain Dc-01 16S ribosomal RNA gene partial sequence	96
XM_012456257.2	PREDICTED: Aotus nancymaae	91
	valosin containing protein ysine methyltransferase (VCPKMT) transcript variant X1 mRNA	
AM882680.1	Bacillus subtilis partial 16S rRNA gene strain A8	87
KY971289.1	Salmonella enterica strain ISO4 16S ribosomal RNA gene partial sequence	95
KR780375.1	Moraxella osloensis strain CS20 16S ribosomal RNA gene partial sequence	97
KT750852.1	Pantoea sp EM011 16S ribosomal RNA gene partial sequence	96
KC410779.1	Pantoea sp. EM011 16S ribosomal RNA gene partial sequence	84
KY971289.1	Salmonella enterica strain ISO4 16S ribosomal RNA gene partial sequence (2)	95
EU695209.1	Pseudomonas sp. M1-4 16S ribosomal RNA gene partial sequence	96
KT033700.1	Enterobacter sp. QAUEBSP01 16S ribosomal RNA gene partial sequence	97
KJ871339.1	Cronobacter sp. XFP-gy 16S ribosomal RNA gene partial sequence	97

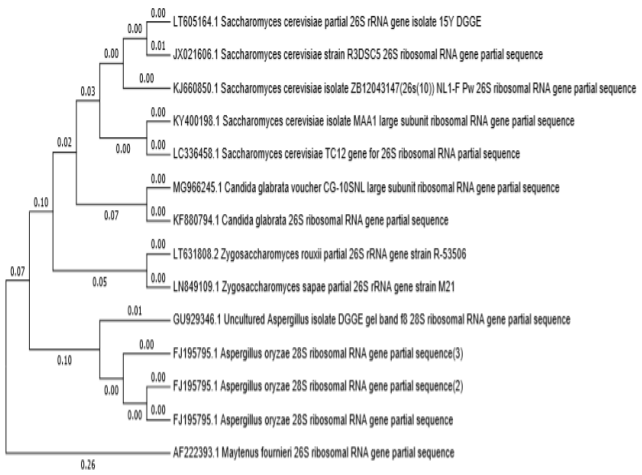

Figure 6: Phylogenetic tree of pure isolation of yeast

Table 2. List of yeast and fungi identified

ACCESSION NO.	STRAIN	IDENTITY%
LT 605164.1	Saccharomyces cerevisiae partial 26S Rrna gene isolate 15Y_DGGE	100
JX021606.1	Saccharomyces cerevisiae strain R3DSC5 26S ribosomal RNA gene partial sequence	92
KJ660850.1	Saccharomyces cerevisiae ZB12043147(26s (10)) NL1-F Pw 26S ribosomal RNA gene partial sequence	97
KY400198.1	Saccharomyces cerevisiae isolate MAA1 large subunit ribosomal RNA gene partial sequence	100
LC336458.1	Saccharomyces cerevisiae isolate MAA1 large subunit ribosomal RNA gene partial sequence	99
MG966245.1	Candida glabrata voucher CG-10SNL large subunit ribosomal RNA gene partial sequence	99
KF880794.1	Candida glabrata 26S ribosomal RNA gene partial sequence	96
LT631808.2	Zygosaccharomyces rouxii partial 26S rRNA gene strain R-53506	96
LN849109.1	Zygosaccharomyces sapae partial 26S rRNA gene strain M21	96
GU929346.1	Uncultured Aspergillus isolate DGGE gel band f8 28S ribosomal RNA gene partial sequence	83
FJ195795.1	Aspergillus oryzae 28S ribosomal RNA gene partial sequence (3)	97
FJ195795.1	Aspergillus oryzae 28S ribosomal RNA gene partial sequence (2)	99
FJ195795.1	Aspergillus oryzae 28S ribosomal RNA gene partial sequence	97
AF222393.1	Maytenus fournieri 26S ribosomal RNA gene partial sequence	97

IV. CONCLUSION

In conclusion, this research helps in identifying types of microorganism presented during the spontaneous fermentation of Carica papaya leaves. Acidic condition of the fermented juice

optimize the growth of both bacteria and yeast. The growth of good and bad bacteria fluctuate during 100 days of the fermentation. The growth of pathogens after day 45 should be avoided as their presence may cause harmful effect. Further research need to be done in order to specify the best populations remained in the fermented juice that gives benefits towards the consumers.

V. ACKNOWLEDGEMENT

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