

## CHAPTER III

### SCREENING AND ISOLATION OF LACTIC ACID BACTERIA FOR ANTI-YEAST ACTIVITY AGAINST *Rhodotorula muciliginosa*

#### 3.1 INTRODUCTION

Yeasts and molds cause major problems and economic losses in food industry as it can cause spoilage due to wasted product or inferior quality (Pitt & Hocking, 1997). Fruit juices, cut fruits, vegetables, and fermented plant beverages are often spoiled by yeast such as *Candida* spp., *C. neoformans*, *Endomycopsis* spp., *Hansenula* spp., *Rhodotorula* spp., *Saccharomyces* spp., *Schizosacchacharomyces* spp., and *Pichia* spp. (Prachyakij et al., 2007). One the yeast of the major concerns is *Rhodotorula muciliginosa* which is reported to be a problem in food spoilage and has been recognised as an emerging yeast pathogen in humans (Wirth & Goldani, 2012a).

*R. muciliginosa* is known to produce pink to red colonies that can be identified either using API 20C AUX kit or using 5.8S ITS. Identification of yeast taxonomic identification by API kit for phenotypical characteristics is widely used. The advent of molecular biology has provided many new tools for fungal taxonomists. PCR and sequencing of relevant genes provide rapid and accurate identification of a large number of yeast pathogens. The most popular targets for PCR-based assays are rDNAs. The ribosomal DNA region consists of genes encoding for small (18S), 5.8S and large (26S) subunits of ribosomal DNA separated by the internal transcribed spacers named ITS1 and ITS4 (Pincus et al., 2007).

Recently, consumers are demanding for food products without chemical preservatives. This have made researchers to look for alternatives that can be used to preserve food and at the same time safe for human consumption as well as have a prolonged shelf life. Many researches on LAB have been reported that compounds produced by lactic acid bacteria have antimicrobial and antifungal activity (Gardiner et al., 2000; Corcoran et al., 2004; Al-haj et al., 2010; Muhialdin & Hassan, 2011) which can be used for bio-preservation in foods.

Bio-preservation is defined as 'extended shelf life and enhanced safety of foods through addition of microflora and their antimicrobial products' (Ross et al., 2002). Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) and are known to produce compounds with antimicrobial activity such as organic acids, hydrogen peroxides and bacteriocin.

The purpose of this study was to isolate novel LABs from various sources. About 38 LABs isolates were isolated and were screened for their anti-yeast activity towards *R. mucilaginosa* that readily spoiled fresh cuts cucumber and carrot. The LAB isolates that demonstrated strong anti-yeast activity by dual agar overlay method were identified by phenotypic and genotypic methods.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of spoilage yeast *R. mucilaginosa*

Samples of vegetables and fruits (Table 1) were collected from Nilai, Negeri Sembilan. Initially, 100 ml of peptone water (0.1% w/v) was added to 10 g samples and was mixed using stomacher. The sample was serially diluted with peptone water (0.1% w/v) to achieve  $10^6$  cell/ml of yeast concentration. Approximately 20  $\mu$ l of yeast  $10^6$  cell/ml was spread on potato dextrose agar (PDA, Oxoid) and then incubated at 30°C for 48 to 72 h. Single red to pink colonies were further purified and checked using a light microscope (Olympus). Pure cultures were maintained on a PDA slant at 4°C and sub-cultured at intervals (Prachyakij et al., 2007).

Table 1: Samples for yeast screening and isolation

| Sample     | Codes | Sources                  |
|------------|-------|--------------------------|
| Carrot     | CRT   | Sundry Shop, Nilai       |
| Cucumber   | CBR   | Sundry Shop, Nilai       |
| Tomato     | TMT   | Giant Hypermarket, Nilai |
| Onion      | ONN   | Sundry Shop, Nilai       |
| Lettuce    | LTC   | Giant Hypermarket, Nilai |
| Papaya     | PPY   | Sundry Shop, Nilai       |
| Watermelon | WML   | Sundry Shop, Nilai       |



### 3.2.2 Phenotypic identification of spoilage yeast

Isolates from cucumber and carrot were identified using API 20C AUX kit (BioMérieux) following the manufacturer's instruction. Isolates of yeast were pre-cultured in potato dextrose broth (PDB, 2% w/v, Difco) and were incubated at 30°C for 24 h. Yeast cell concentration was determined using Nanophotometer at 600 nm wavelength, and was adjusted to  $10^6$  cells/ml with sterile peptone water (0.1% w/v) and were used as inoculums in API kit. These inoculums consist of 20 cupules containing dehydrated substrates which enable the performance 19 assimilation tests and 1 control cupule. One morphology test cupule was used for identifying the presence of hyphae or pseudo-hyphae and this test constitutes the 21<sup>st</sup> test of the strip.

The cupules were inoculated with semi solid minimal medium and the yeast will grow only if they are capable of utilizing each substrate as the sole source of carbon. The strips were incubated at 30°C aerobically for 48 h as recommended by the manufacturer. After 48 h of incubation, each cupule was compared with zero<sup>th</sup> cupule which was used as negative control cupule. Cupules that was more turbid than the control indicates a positive reaction. Identification was obtained with the numerical profile. On the result sheet provided, the test was separated into 7 groups of 3. By adding the numbers corresponding to positive reactions within each group, a 7 digit number was obtained which constitutes the numerical profile and were compared and identified by using API WEB<sup>TM</sup> (<https://apiweb.biomerieux.com>).



### 3.2.3 Genotypic identification of spoilage yeast

#### 3.2.3.1 Total genomic DNA extraction

Total genomic DNA of all yeast samples was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instruction. Yeast sample was obtained from well-grown agar plates. The samples were suspended in 480 µl at 50mM EDTA. Next, 120 µl lyticase enzyme was added with the mixture before being incubated at 37°C for 60 min. The mixtures were then centrifuged for 2 min at 13,000 rpm and 600 µl nuclei lysis solution was added to the pellet obtained.

Subsequently, the mixture was mixed gently before incubating for 5 min at 80°C. Three µl of RNase solution was added to the mixture and was incubated at 37°C for 15 to 60 min. 200 µl of protein precipitation solution was added to the mixture, vortexed continuously and were then incubated in ice for 5 min. The mixture was then followed by centrifugation at 13,000 rpm for 3 min. The supernatant obtained was transferred to a clean tube containing 600 µl of room temperature (25°C) isopropanol and mixed properly. The mixture was centrifuged for another 2 min at 13,000 rpm, and the supernatant was decanted. After that, 600 µl of 70% ethanol was added, mixed and centrifuged again for 2 min at 13,000 rpm. The ethanol was aspirated and the pellet was air-dried for 10 to 15 min. The DNA pellet was rehydrated in 100 µl of rehydration solution for 1 h at 65°C or overnight at 4°C.

3.2.3.2 PCR amplification

Amplification was carried out using master mix (BIORON). The amount of mixture used was showed in Table 2. Polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) DNA yeast samples were identified by sequencing the internal transcribed spacer (ITS) regions. To determine the identity of yeast, the amplified ITS PCR products obtained from total genomic DNA using primer set ITS1-F (5'- CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al., 1990) were sequenced commercially.

Table 2: Polymerase chain reaction mixture using BIORON master mix

| PCR Reaction Mixture     | Amount (µl) | Final Concentration |
|--------------------------|-------------|---------------------|
| DNA template             | 2.0         | -                   |
| Complete 10X PCR-Buffer  | 5.0         | 1×                  |
| dNTP mix                 | 1.0         | 800 µM              |
| Forward primer (ITS 1)   | 2.5         | 0.5 µM              |
| Reverse primer (ITS 4)   | 2.5         | 0.5 µM              |
| DFS-Taq DNA polymerase   | 0.5         | 0.1 unit            |
| Sterile distilled water  | 36.5        | -                   |
| Total volume of reaction | 50.0        | -                   |

The amplification of PCR products was done by using T100 Thermal Cycler (Bio-Rad, Singapore). The PCR condition was stated in Table 3. The amplified DNA was kept at -20°C until further use.

**Table 3:** PCR cycling condition for the thermocycler

| Reaction Parameters  | Temperature | Time  |
|----------------------|-------------|-------|
| Initial Denaturation | 95°C        | 5 min |
| (30 Cycles)          |             |       |
| Denaturation         | 95°C        | 1 min |
| Annealing            | 60°C        | 1 min |
| Extension            | 72°C        | 5 min |
| Final Extension      | 72°C        | 5 min |
| Hold                 | 4°C         | -     |

**3.2.3.3 Gel Electrophoresis**

Gel electrophoresis was used to analyse the PCR product by separating the amplified DNA on agarose gel according to their size. The 1% (w/v) agarose gel was prepared by dissolving 0.4 g of agarose powder in 40 ml 1 × TAE buffer. Five µl of 1 kb ladder (Norgen High Ranger) was loaded into the well of agarose gel and used as a standard. Aliquots of 7 µl of amplified product were then loaded into the well together with 3 µl of loading dye (Norgen). The gel was run in TAE buffer at 100 V constant voltages for 35 min. The separated DNA was stained with gel red (Biotium) and was visualized using computer based gel documentation system (Uvitec Cambridge).



#### 3.2.3.4 PCR products purification

PCR products were purified using high pure PCR product purification kit (Roche Diagnostic GmbH, Germany) by following the manufacturer's instruction. 5 volumes of binding buffer and 1 volume of PCR product was briefly mixed well and transferred into high pure filter tube. The DNA was bind to the filter by centrifugation at 13,400 rpm (Eppendorf mini spin, USA) for 60 sec at 5°C. The flow-through was then discarded. An additional 500 µl of wash buffer was added and centrifuged at 13,400 rpm for 1 min. The flow-through was also discarded. Additional centrifugation at 13,400 rpm for 1 min was performed after 200 µl of wash buffer was added. Finally, DNA was eluted into a clean 1.5 ml micro-centrifuge tube by adding 50 µl of elution buffer (10 mM Tris-HCl) and centrifuged at 13,400 rpm for 1 min.

#### 3.2.3.5 PCR product sequencing

The DNA fragments of 5.8S-ITS were sent for sequencing to 1<sup>st</sup> base laboratory in Shah Alam, Selangor after PCR amplification using the ITS1 and ITS4 primers. The sequences obtained were assembled using the Chromas Lite software, version 2.1.1. The assembled sequences were aligned and compared with the GenBank databases of the National Center for Biotechnology Information (NCBI). Searches in GenBank with Basic Local Alignment Search Tool (BLAST) program were performed to determine the closest known relative of DNA fragment of 5.8S-ITS (Altschul et al., 1997).

### 3.2.4 Isolation of LAB

LABs were isolated from various sources. Appropriate dilutions were prepared using sterile peptone water (0.1% w/v). Then, 100  $\mu$ L were spread on modified de Man Rogosa and Sharpe agar (MRS, Oxoid) with (0.8%  $\text{CaCO}_3$ ) followed by incubation of the plates under anaerobic conditions at 37°C for 48 h (De Man et al., 1960; Wanchai et al., 2007). Colonies showing clear zones around them were streaked on MRS agar and incubated at 37°C for 48 h to obtain pure colonies. The colonies obtained were tested for catalase activity by placing a drop of 4% hydrogen peroxide solution on the cells. Bubbles formation indicated the presence of catalase in the cells. Gram staining was done and the morphology of the bacteria was observed using a Nikon microscope (Nikon Eclipse 80i).

### 3.2.5 Anti-yeast activity of lactic acid bacteria

Isolates of yeast were grown in potato dextrose broth (PDB, 2% w/v, Difco) and incubated at 30°C for 24 h. Yeast cell counts were determined using a Nanophotometer, and adjusted to  $10^6$  cells/ml with sterile peptone water (0.1% w/v) to be used as an inoculum for testing. Inhibition activities of the isolates were determined using overlay method as described by Magnusson and Schnurer (2001). Afterward, LAB was inoculated on MRS agar plates and incubated anaerobically at 37°C for 24 h. The plates were then overlaid with 10 ml of potato dextrose soft agar (0.05% PDB and 1% Oxoid Agar Technical No. 3) containing  $10^6$  cells/ml of *R. mucilaginosa*. After 48 h of aerobic incubation at 30°C, the diameter of clear zone was measured. Analyses were done in duplicate.

### 3.2.6 Cell free supernatant preparation

The isolates were inoculated into MRS broth and incubated for 48 h at 30°C with 150 rpm (Orbital shaker). The cell free supernatant was prepared by centrifuging the broth (10,000 rpm for 20 min), to remove the pellet. The supernatant of each isolates was then filtrated using sterile filtered (0.45 µm-pore-size filter, Millipore).

### 3.2.7 Phenotypic identification of LAB

Three isolates (YG1, DF22 and C16) that showed strong yeast inhibition activity in all the tests were identified using API 50 CH kit (API system, BioMérieux, France). The three isolates were catalase negative and Gram positive. Overnight cultures of the isolates were grown on MRS plates at 30°C and were suspended in API 50 CHL medium (API system, BioMérieux, France). The suspension was transferred into each of the 50 wells of the API CH strips. All wells were overlaid with sterile oil to make it anaerobic. The strips were incubated at 30°C as recommended by the manufacturer. Changes in colours either to yellow, blue or green were observed after 48 h. The results were compared and identified by using API WEB™ (<https://apiweb.biomerieux.com>).



### 3.2.8 Genotypic identification of LAB

#### 3.2.8.1 DNA extraction

Genomic DNA of LAB isolates was extracted using (Genomic DNA Extraction Mini Kit, Yeastern Biotech CO., LTD, Taiwan) the method described by Magnusson et al. (2003) with some modifications. A 1500  $\mu\text{l}$  of overnight culture was harvested by centrifuging at  $14,000 \times g$  for 1 min using (Eppendorf centrifuge 5804 R) and the supernatant was discarded. The cell pellet was re-suspended in 200  $\mu\text{l}$  of lysozyme and then incubated at room temperature for 10 min with shaking of the tubes every 2-3 min. 200  $\mu\text{l}$  of GB buffer was then added to the sample, and mixed by vortexing for 5 sec. The LAB samples were incubated for 20 min until the sample lysate was clear. During incubation, the sample tubes were inverted every 5 min. After the sample lysate was clear, elution buffer (200  $\mu\text{l}$  per sample) incubated at  $60^{\circ}\text{C}$  (for DNA elution) was added. Following that, a volume of 200  $\mu\text{l}$  of the ethanol was added to each sample and mixed immediately by vortexing for 10 sec. The GD column was placed on a 2 ml collection tube. Then all the mixture from the previous step was applied to the GD column, and then centrifuged at  $14000 \times g$  for 2 min. The flow-through was discarded and the GD column placed in a new 2 ml collection tube. 400  $\mu\text{l}$  of W1 buffer (ethanol added) into the GD column, and then centrifuged at  $14000 \times g$  for 30 sec. the flow-through was discarded and the GD column placed back in the 2 ml collection tube, the wash step was repeated with 600  $\mu\text{l}$  of wash buffer (ethanol added) with centrifuge at  $14000 \times g$  for 30 sec. The flow-through was discarded and the GD column placed back in the 2 ml collection tube, then centrifuged at  $14000 \times g$  for 3 min to dry the column matrix of any ethanol excess. The dried GD column was

transferred into a clean 1.5 ml micro-centrifuge tube, and then 100  $\mu$ l of the preheated elution buffer was added in the centre of the column matrix. It was left to stand for 3-5 min until elution buffer was absorbed by the matrix. The purified DNA was eluted by centrifuging at  $14000 \times g$  for 30 sec. Finally, the DNA sample was kept at  $-20^{\circ}\text{C}$  for long term storage.

### 3.2.8.2 PCR amplification

The extracted DNAs were used directly in PCR reactions to amplify the 16S rDNA gene from LAB. The 16S rDNA region was amplified by using primers (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3). The PCR reaction mixture contained 5  $\mu$ l of  $1\times$  reaction buffer ( $\text{NgCL}_2$ ) (Yeastren Biotech CO., LTD. Taiwan), 1  $\mu$ l of 0.2 mM dNTPs mix (Yeastren Biotech CO., LTD. Taiwan), 1  $\mu$ l of 10  $\mu$ M 16S forward and 1  $\mu$ l of 10  $\mu$ M 16S reverse primers, 1  $\mu$ l of template DNA, and 0.5  $\mu$ l of (1.25 U/  $\mu$ l) YEAtaq DNA polymerase (Yeastren Biotech CO., LTD. Taiwan), negative control without DNA template was included in parallel. Each sample was topped up with 40.5  $\mu$ l ddH<sub>2</sub>O until 50  $\mu$ l. The PCR were as follows: (LID 105°C), initial at 95°C for 3 min, denaturation at 95°C for 30s, annealing at 57°C for 15 sec and extension at 73°C for 1 min, with 34 cycles for each step. Final elongation was at 73°C for 5 min; and then held at 4°C. From each amplification mixture 10 $\mu$ l were mixed with 2  $\mu$ l of 6 $\times$  DNA loading dye (Fermentans), and then subjected to electrophoresis in 1.0% (w/v) agarose gels (Conda S.A, Spain) in  $1\times$  TAE buffer (Bio Basic Canada INC, Canada) for 40 min and 80 V. 6  $\mu$ l of 1Kb DNA ladder (250 to 10000 bp) from (1<sup>st</sup> Base, Malaysia) was used as standard. After electrophoresis the gel was stained in ethidium bromide



(Merck, Germany) for 30 min and then washed with dH<sub>2</sub>O for another 30 min. The gel was visualized and photographed with UV transilluminator (BIORAD).

### 3.2.8.3 PCR product purification

The PCR products purification was processed using PCR clean up kit from (Yeastern Biotech, Taiwan). 40 µl of the PCR product was added into micro-centrifuge tube with 200 µl of DF buffer and mixed by vortex. The sample was applied into DF column with collection tube, and then centrifuged at  $6000 \times g$  for 30 sec. The flow-through was discarded and the DF column placed back in the collection tube. The centrifugation was repeated at  $14000 \times g$  for 2 min. The dried DF column was transferred to a new micro-centrifuge tube. 15 µl of the elution buffer was added in the centre of the DF column matrix, stand for 2 min until the elution buffer absorbed by the matrix. Purified DNA was eluted at  $14000 \times g$  for 2 min.

### 3.2.8.4 PCR product sequencing

The partial 16S-S DNA and 16S rRNA gene sequences of all the isolates were determined by 1<sup>st</sup> Base at Shah Alam, Malaysia. The sequences obtained were assembled using the Chromas Lite software, version 2.1.1. The assembled sequences were aligned and compared with the GenBank databases of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). Searches in GenBank with Basic Local Alignment Search Tool (BLAST) program were performed to determine the closest known relative of DNA fragment of 16S rDNA.

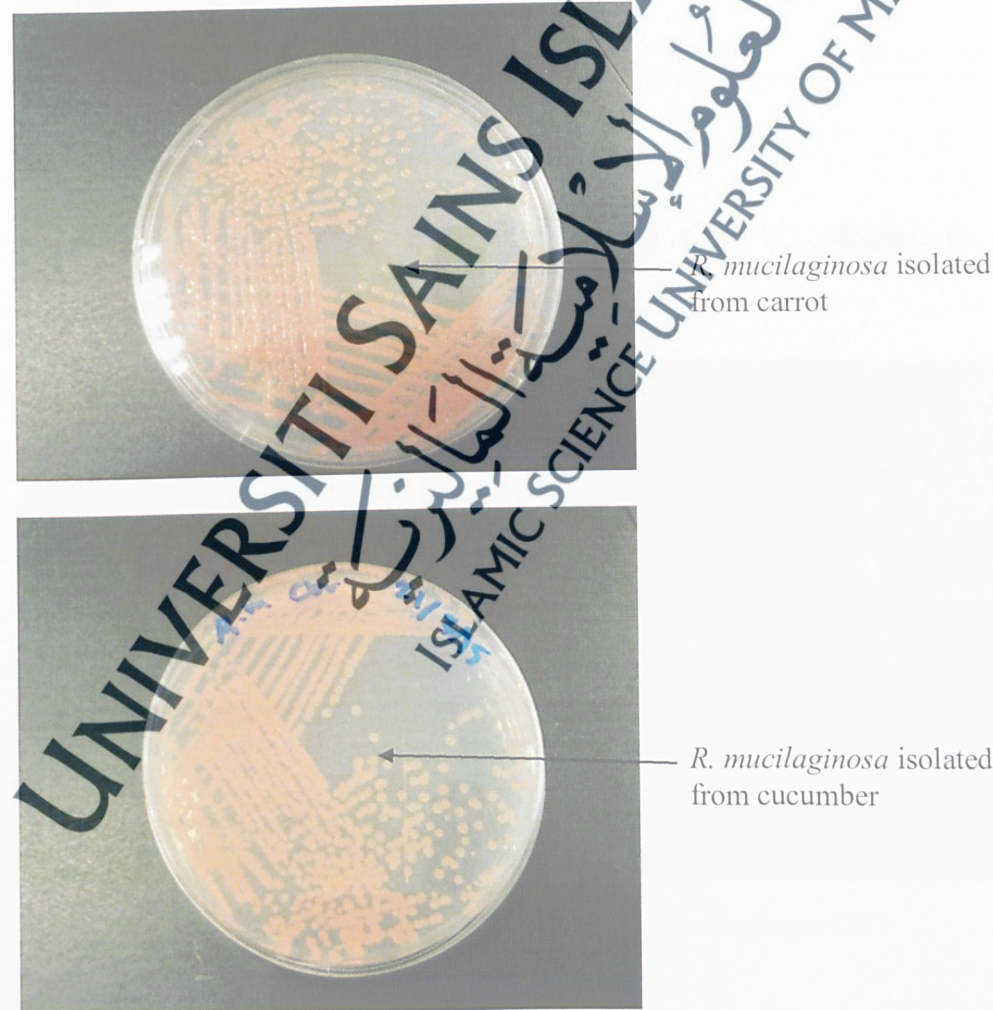


3.3 RESULTS

3.3.1 Screening and identification of isolated yeast

From 7 samples of fresh cut vegetables and fruits only 2 samples were contaminated by red yeast after 48 h of incubation on PDA. Both of the yeast isolates from carrot and cucumber (Figure 1) were later identified as *R. mucilaginosa* (Figure 2) as shown in Table 4 and 5. Figure 3 shows the DNA band of PCR product.

Figure 1: Isolates of *R. mucilaginosa*



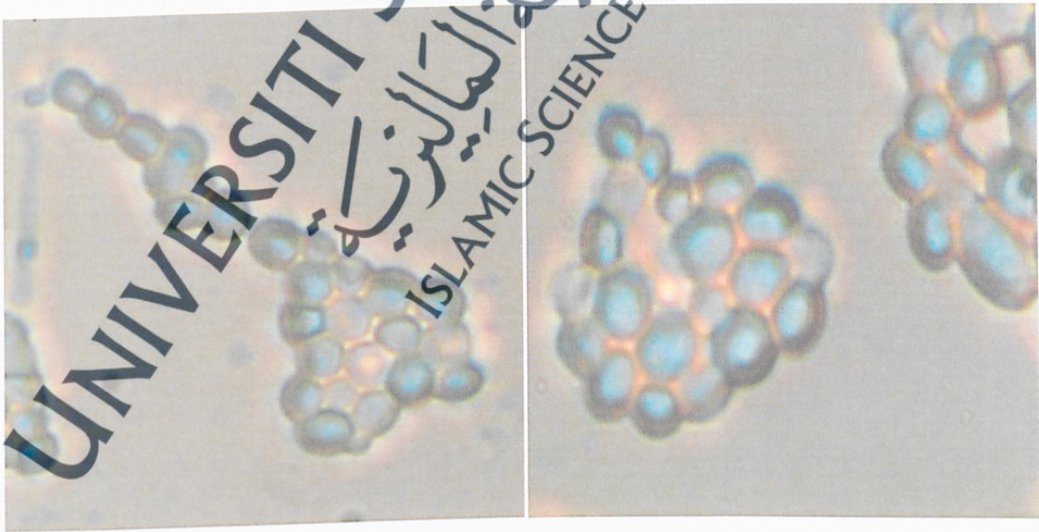
**Table 4:** Identification of spoilage yeast phenotypically using API 20C AUX kit

| Yeast Source | Phenotypic Identification |                    |
|--------------|---------------------------|--------------------|
|              | Description               | Similarities Index |
| Carrot       | <i>R. mucilaginosa</i>    | 91.3%              |
| Cucumber     | <i>R. mucilaginosa</i>    | 92.7%              |

**Table 5:** Identification of spoilage yeast using 5.8S-ITS

| Yeast Source | 5.8S-ITS Identification |         |              |               |
|--------------|-------------------------|---------|--------------|---------------|
|              | Description             | E value | Similarities | Accession No. |
| Carrot       | <i>R. mucilaginosa</i>  | 0.0     | 99.0%        | HO702343.1    |
| Cucumber     | <i>R. mucilaginosa</i>  | 0.0     | 99.0%        | GU220714.1    |

**Figure 2:** (a) Morphology of *R. mucilaginosa* CBR (b) Morphology of *R. mucilaginosa* CRT

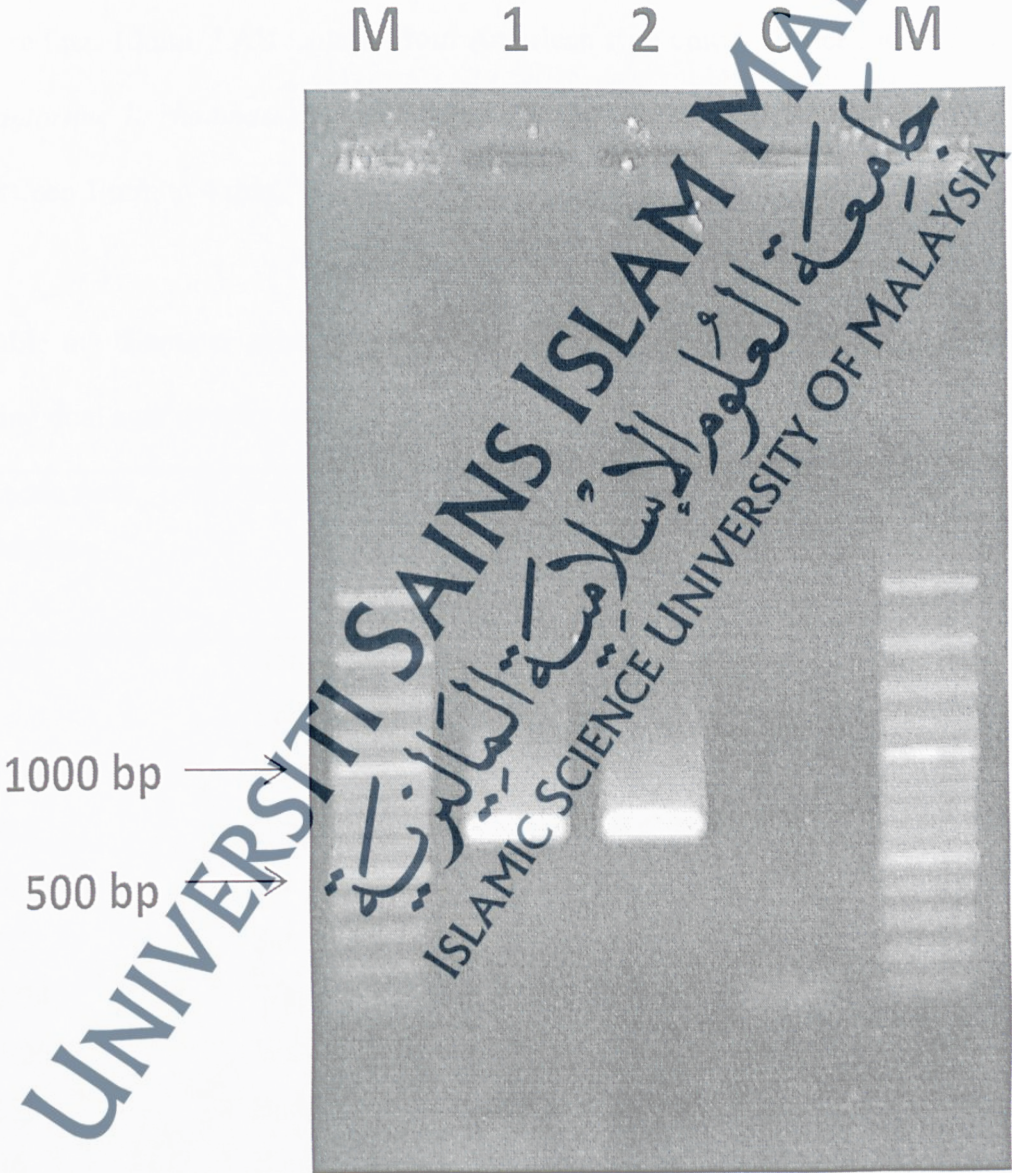


(a) *R. mucilaginosa* CBR

(b) *R. mucilaginosa* CRT



**Figure 3:** PCR products using primers ITS1-F and ITS4. M, Fermentas GeneRuler 100 bp DNA ladder Plus; Lanes 1, PCR products from sample Cucumber (CBR); Lanes 2, PCR products from sample Carrot (CRT); Lanes C, PCR products from negative control (without DNA template); Gel electrophoresis was performed on 1% agarose gel at 100V for 30 min





3.3.2 Screening for anti yeast activity of LABs isolates against *R. mucilaginosa* using dual agar overlay method

From 38 LAB isolates, only 35 LAB isolates inhibit *R. mucilaginosa* in dual layer agar overlay method as shown in Table 6 and Figure 4. However only 3 LABs from homemade yogurt YG1, dragon fruit DF22 and fermented cocoa C16 shows inhibition more than 10mm. LAB isolates from American type culture collection (ATCC) of *L. plantarum*, *L. rhamnases*, *L. paracasei* and *L. acidophilus* only gave weak inhibition between 1 mm to 4 mm.

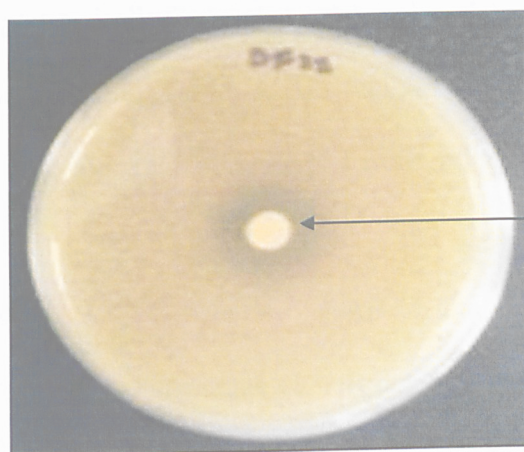
**Table 6:** Diameter zone of inhibition of lactic acid bacteria against spoilage yeast using dual agar overlay method\*

| Lactic Acid Bacteria | Sources         | Diameter of Inhibition Zone (mm) |                        |
|----------------------|-----------------|----------------------------------|------------------------|
|                      |                 | <i>R. mucilaginosa</i>           | <i>R. mucilaginosa</i> |
|                      |                 | CRT                              | CBR                    |
| YG1                  | Homemade Yogurt | 13 ± 0.0                         | 18 ± 0.0               |
| C16                  | Fermented Cocoa | 13 ± 0.0                         | 14 ± 0.0               |
| F22                  | Dragon Fruit    | 13 ± 0.0                         | 13 ± 0.0               |
| DF20                 | Dragon Fruit    | -                                | 5 ± 0.0                |
| S23                  | Star Fruit      | 1 ± 0.0                          | 2 ± 0.0                |
| W24                  | Watermelon      | 3 ± 0.0                          | 2 ± 0.0                |
| W25                  | Watermelon      | 3 ± 0.0                          | 5 ± 0.0                |
| B3                   | Banana          | 2 ± 0.0                          | 1 ± 0.0                |
| B6                   | Banana          | 1 ± 0.0                          | 1 ± 0.0                |
| BM8                  | Buffalo milk    | -                                | -                      |
| CM                   | Cow milk        | -                                | 1 ± 0.0                |
| B2                   | Banana          | -                                | 1 ± 0.0                |
| B3                   | Banana          | -                                | -                      |

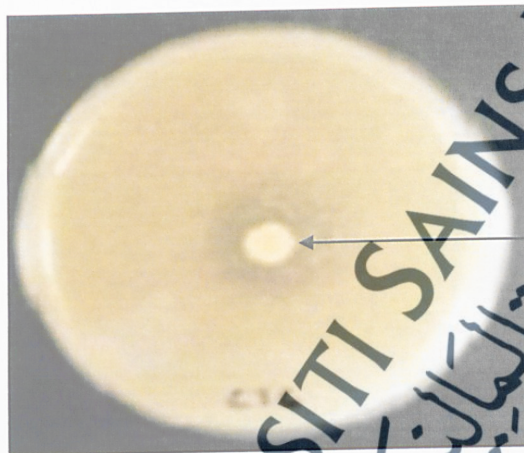
|                       |                 |         |         |
|-----------------------|-----------------|---------|---------|
| B19                   | Banana          | -       | 1 ± 0.0 |
| <i>L. casei</i>       | ATCC 393        | 2 ± 0.0 | 2 ± 0.0 |
| <i>L. plantarum</i>   | ATCC 8014       | 3 ± 0.0 | 4 ± 0.0 |
| <i>L. acidophilus</i> | ATCC 4356       | 4 ± 0.0 | 3 ± 0.0 |
| <i>L. rhamnases</i>   | ATCC 7469       | -       | 1 ± 0.0 |
| D1                    | Fermented Dates | 5 ± 0.0 | 4 ± 0.0 |
| M1                    | Mango           | 4 ± 0.0 | 4 ± 0.0 |
| P1                    | Pickles         | 4 ± 0.0 | 3 ± 0.0 |
| G1                    | Grape           | 3 ± 0.0 | 5 ± 0.0 |
| BM1                   | Buffalo Milk    | 2 ± 0.0 | 1 ± 0.0 |
| BM3                   | Buffalo Milk    | 2 ± 0.0 | 3 ± 0.0 |
| BM9                   | Buffalo Milk    | 2 ± 0.0 | 4 ± 0.0 |
| BM7                   | Buffalo Milk    | -       | 1 ± 0.0 |
| BM4                   | Buffalo Milk    | -       | 1 ± 0.0 |
| G7                    | Grape           | -       | -       |
| DF8                   | Dragon Fruit    | -       | 1 ± 0.0 |
| DF10                  | Dragon Fruit    | 3 ± 0.0 | 1 ± 0.0 |
| B9                    | Banana          | 1 ± 0.0 | -       |
| B12                   | Banana          | 4 ± 0.0 | 3 ± 0.0 |
| S11                   | Star Fruit      | 2 ± 0.0 | 2 ± 0.0 |
| S18                   | Star Fruit      | -       | 1 ± 0.0 |
| P13                   | Papaya          | 3 ± 0.0 | 1 ± 0.0 |
| DR14                  | Durian          | 4 ± 0.0 | 2 ± 0.0 |
| GN15                  | Ginger          | 3 ± 0.0 | 5 ± 0.0 |
| PR17                  | Pear            | 1 ± 0.0 | 4 ± 0.0 |

\*Results are mean values of duplicate determinations ± standard deviation

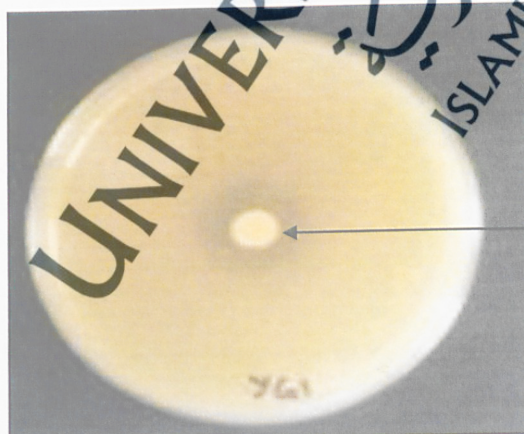
**Figure 4:** Inhibition zone of 3 isolates of LAB isolated from dragon fruit DF22, homemade yogurt YG1 and fermented cocoa C16 against *R. mucilaginosa* CBR using dual layer agar overlay method



*L. casei* DF22



*L. plantarum* C16



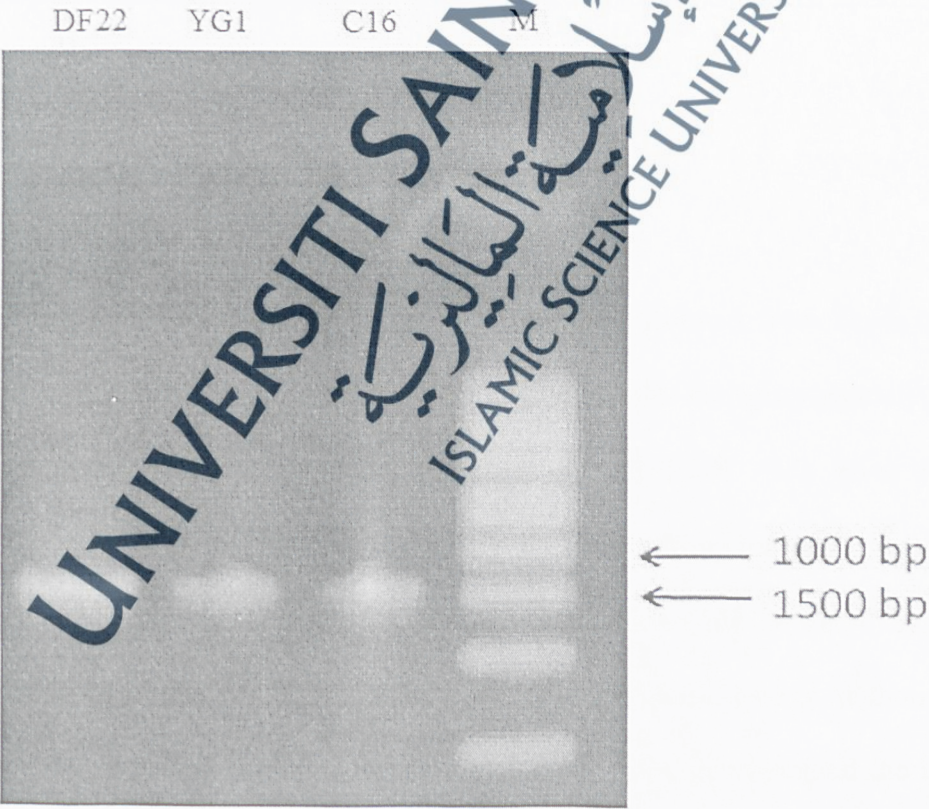
*L. casei* YG1



3.3.3 Genotypic and phenotypic identification of LAB isolates.

16S rDNAs sequence were determined for the three selected isolates as shown in Figure 5. The sequences derived from the isolates were used to search databases for highest similarity rank to determine the species identity of the isolates as shown in Table 7 and Table 8. The cell morphology of three isolates was captured under microscope indicated the cell were rod shaped (Appendix A).

**Figure 5:** DNA band of LAB on the 1% (w/v) agarose gel using primers 27F and 1492R. M, Fermentas GeneRuler 100 bp DNA ladder Plus; Lane DF22, Lane YG1 and Lane C16 PCR products from sample DF22, YG1 and C16 respectively;



**Table 7:** Similarity index of LAB isolates determined phenotypically by API 50 CHL

| LAB Isolates | Description                      | Similarities Index (%) |
|--------------|----------------------------------|------------------------|
| YG1          | <i>Lactobacillus paracasei</i>   | 99.9                   |
| DF22         | <i>Lactobacillus paracasei</i>   | 99.3                   |
| C16          | <i>Lactobacillus plantarum</i> 1 | 97.0                   |

**Table 8:** Similarity index of LAB isolates determined genotypically by 16S rDNA

| LAB Isolates | Description                    | 16S rDNA |              |               |
|--------------|--------------------------------|----------|--------------|---------------|
|              |                                | E value  | Similarities | Accession No. |
| YG1          | <i>Lactobacillus casei</i>     | 0.0      | 99.0%        | KF500575.1    |
| DF22         | <i>Lactobacillus casei</i>     | 0.0      | 99.0%        | KF673517.1    |
| C16          | <i>Lactobacillus plantarum</i> | 0.0      | 98.0%        | HM130542.1    |

3.4 DISCUSSION

In this study, spoilage yeasts were successfully isolated from fresh cut vegetables (cucumber and carrot) and were both phenotypically and genotypically characterized as *R. mucilaginosu*. From the results (Table 4), API 20C AUX gives the similarities index 91.3, and 92.7% for yeast isolates from carrot and cucumber respectively. However, approximately 99.0% of similarities index with E-value 0.0 (Table 5) were obtained for both yeast isolates from 5.8S-ITS identification method. Arias et al. (2002) stated that partial sequence of the 26S rRNA gene yielded the best results in terms of correct identification, followed by classical techniques and 5.8S-ITS analysis while for API 20C AUX, slightly less accuracy of identification was observed.



*Rhodotorula* spp. contaminates vegetables especially in fresh cut carrot and cucumber after 24 h at room temperature. Fleet (1992) and Fleet (2011) stated that most yeast exhibit optimal growth in the range between 20-30°C. Similar study, *Rhodotorula* spp. contaminated variety of fruits such as strawberries, cantaloupe, honeydew and melon varieties (Tournas et al. 2006). Prachyakij et al. (2007) also reported that *Rhodotorula* sp. contaminates fermented plant beverages. Pitt and Hocking (1997) reported that *R. mucilaginosa* are significant as spoilage agents and commonly found in fruit and vegetables as spoilage organisms.

Furthermore LAB has been associated in bio-preservative (Vaughan et al., 2001; Marilley & Casey 2004; Tamminen et al., 2004; Mittra et al., 2005). LABs are considered as bio-inhibitor agents of high potential against fungi (Crowly et al., 2013b). *R. mucilaginosa* CBR and *R. mucilaginosa* CRT were strongly inhibited by 3 LAB isolates identified by API 50 CH kit as *L. paracasei* DF22, *L. paracasei* YG1 and *L. plantarum* C16. All the isolates showed inhibition zones that were greater than 13 mm (Table 6). Rouse et al. (2008) also reported that *P. pentosaceus* R47, *W. cibaria* R16 and *W. confusa* I5 have the ability to inhibit *R. mucilaginosa* between 6mm to 10 mm in dual layer agar overlay method however only *L. plantarum* CM8 isolates has inhibition greater than 10 mm.

PCR based technique, 16S rDNA were determined for the three isolates selected in this study. The sequences derived from the three isolates were used to search databases from the highest similarity rank to determine the species identify of the three isolates in GenBank. Each isolate was identified based on their sequence homology of 16S rDNA. The sequences of the isolates, YG1, DF22, and C16 showed



higher homology to the sequence of *Lactobacillus* species; 99.0% to *Lactobacillus casei*, 99.0% to *Lactobacillus casei*, and 98.0% to *L. plantarum*, respectively (Table 8). It suggests that the three isolates belong to the genus *Lactobacillus*. While for API identification, isolates YG1 and DF22 were identified as *L. paracasei* with similarities index 99.9% and 99.3%, respectively (Table 7). API identification is based on phenotypic and biochemical characters indicated that these characteristics may not be enough to definitely assign a strain to a particular species. Using API 50 CHL system for phenotypic characterization is sufficient for a rough characterization but not for unequivocal identification purposes (Montel et al., 1991). It is useful to characterize the isolates based on substrate utilization such as carbohydrates. However, for identification of the isolate the genotypic characterization using 16S rDNA is preferred (Maryam et al., 2013). Since PCR based techniques 16S rDNA is more accurate, the identification of the LAB were selected as *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16.

Muhialdin and Hassan (2011) reported that LABs produced antimicrobial agents including organic acids, proteinaceous compound, bacteriocin and hydrogen peroxide. Several researchers have reported that *L. plantarum* has the ability to inhibit fungi (Strom et al., 2002; Sjogren et al., 2003). Laitila et al. (2002) and Lavermicocca et al. (2000) suggested that the inhibitory activity of *L. plantarum* could be the results of many organic acids such as lactic, acetic and phenyllactic acids. Since more than one compound is responsible for the antimicrobial activity, the specific compound or combination of compounds need to be further studied for their potential use as food bio-preservation.

### 3.5 CONCLUSION

Lactic acid bacteria were successfully isolated from various sources. Among 38 isolates, only 35 isolates showed inhibitory activity against *R. mucilaginosa*. The inhibitory activity was observed from bacteria cell and cell free supernatant. The isolates YG1 identified as *L. casei*, DF22 as *L. casei*, and C16 as *L. plantarum* have strong inhibitory activity against *R. mucilaginosa* using dual agar overlay method. Hence, LAB isolates from fermented cocoa, homemade yogurt, and dragon fruit were proven to have inhibition activity against spoilage yeast, *R. mucilaginosa* and might be a potential to be use as food bio-preservatives.