Isolation and Molecular Identification of Potential Probiotic Yeast Strains Found in Malaysian Kefir Drinks Samples

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***Abstract*— Kefir drink is a product from the fermentation process of milk using symbiotic mixture of bacteria and yeast consortium. *Saccharomyces* and *lactobacillus* are the major genera found in the kefir drink. The present work involves the isolation and identification of potential probiotic yeast strains in the kefir drink samples from Malaysia. The molecular identification was done by PCR using ITS1 and ITS4 amplified regions. Nine different yeast strains were isolated, and the strains were successfully identified based on the sequence analysis. *Saccharomyces* and *Kodamaea* were found to be the major population in the kefir drink samples. Lastly, kefir milk is one of the excellent sources of probiotic yeast strains and could be used as a new yeast probiotic formulation or in food supplements. Moreover, the amplification of ITS region can be used as a useful method to identified yeast strains.**

***Index Terms*—kefir, yeast, probiotics, molecular identification, PCR**

I. Introduction

Kefir drink is a product that undergoes a fermentation process using milk as a medium. The taste is quite acidic and it has a creamy-like texture [1]. It is normally produced from a traditional kefir grains or kefir starter cultures by fermentation process. Kefir comprises a microbial symbiotic mixture of bacteria and yeast that attached to a polysaccharide matrix [2]. Although the bacterial population is dominant in kefir, the presence of yeast plays an important role to develop the flavour as well as chemical composition of the kefir product. Moreover, the yeast strains are important for the microbial balance by providing the essential nutrients for a probiotic bacteria population such as vitamins and amino acid and produce some compounds that contribute to the kefir drink taste [3].

Based on a previous report, majority of the bacterial and yeast strains that can be found in the kefir drink are *Lactobacillus* and *Saccharomyces* [4] that are recognised as probiotic microorganisms. The term probiotics originates from the Greek word 'probios', which stands 'for life', but the latest definition by FAO/WHO describes them as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [5].

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In the recent years, the use of a probiotic product containing beneficial strain is not only for gastrointestinal disease treatment but also for the overall human health in medicine and dentistry. A considerable amount of literature has been published on the therapeutic potential and health benefit of probiotics such as the prevention of diarrheal disease [6], colon cancer [7], and upper GI tract disease [8], and to improve the immune system against intestinal infections [9].

In the nutraceutical industry, most of the probiotic products contain lactic acid bacterial strains. Yeast strains have been also reported to have the probiotic ability. However, a very few reports regarding probiotic from yeast is available compared to that from bacterial strains although it has a good potential of probiotic ability that can benefit the human health. Compared to the probiotic bacterial strains, products containing yeast are not as widely available but are fast developing and becoming more common. Therefore, the purpose of this research is to isolate, classify and sellect the predominant probiotic yeast from the Malaysian kefir drink using molecular identification technique through PCR method for potential probiotic attrubutes.

II. Material and Methods

A. Isolation of Yeast from Kefir

The yeast samples used for this work were isolated from a kefir drink bought from a supplier in Gambang, Pahang, Malaysia. The samples were kept chilled at 4 °C in the sterile bottle until further use. The isolation process was done using standard serial dilution technique and spread over Yeast Extract Peptone Dextrose (YEPD) agar for 48 h at 30 °C. After incubation, yeast colonies were selected and streaked again on YEPD agar plates for purification. The grown single colonies were stored at 4 °C for further study.

B. DNA Isolation

The isolated yeasts strains were cultured on Yeast Extract Peptone Dextrose (YEPD) broth for 24 h at 30 °C. The yeast strains were treated with 10 mg/mL of proteinase K and 2.5 mg/mL of lyticase followed by genomic DNA extraction using GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, UK) using the protocol provided. The PCR products were run

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on 1% (w/v) agarose gel, stained with GelRed®, and visualised under ultraviolet light transilluminator.

C. PCR Analysis

Polymerase Chain Reaction (PCR) was carried out using universal primers named ITS1 and ITS4. The reaction was carried out in 0.2 mL PCR tubes in 25 µL reaction mixture containing purified genomic DNA, PCR buffer, 1 unit of Taq polymerase, forward and reverse primers, and dNTPs. The reaction was carried out for 30 cycles under the following condition: 2 min of initial denaturation at 98 °C followed by the PCR cycle consisting of denaturation at 98 °C for 15 s, annealing temperature at 60 °C for 30 s, and extension at 72 °C for 30 s. The PCR cycles were followed by an additional final extension at 72 °C for 10 min. The PCR products were run on 1% (w/v) agarose gel, stained with GelRed®, and visualised under ultraviolet light transilluminator.

D. Gene Sequencing and Identification

The PCR products were gel-purified and cloned into pJET1.2 according to the manufacturer's protocol (Thermo Fisher Scientific). The plasmids of nine positive clones were purified and sequenced with 1st BASE universal primer LpJET1.2F using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) by 1st Base Sequencing services (Selangor, Malaysia). The bioinformatics analysis of the sequencing results was performed using the basic local alignment search tool (BLAST). The identification at the taxonomic level of species were allowed between 99% and 100%.

III. Results and Discussions

A. DNA Isolation

In the present study, all the isolated yeast strains were treated with lyticase and proteinase K before proceeding with the genomic extraction protocol. Proteinase K is often used during the extraction of DNA to digest protein and remove contamination from the preparation of nucleic acid. It acts as a serine protease that cleaves the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups [10]. The major component of the yeast cell wall contains mannoprotein, chitin, β-(1→6)-D-glucan, and β-(1→3)-D-glucan. One of the obstacles in the yeast genomic DNA recovery is the disruption of the cell wall. Therefore, lyticase was used to lyse β-(1→3)-D-glucan in the yeast cell wall structure [11]. The extracted chromosomal DNA (Fig. 1) from the isolated yeasts were purified and dissolved in deionised water and stored at −20 °C.

The genomic DNA from the potential probiotic yeast strains were successfully isolated and visualised on the agarose gel electrophoresis. A single band obtained indicates that the isolated genomic DNA was not degraded and stayed intact — the genes were isolated in good quality. Moreover, the band located above the 10kb ladder proves that it was chromosomal genomic based on the larger sized observed [12].

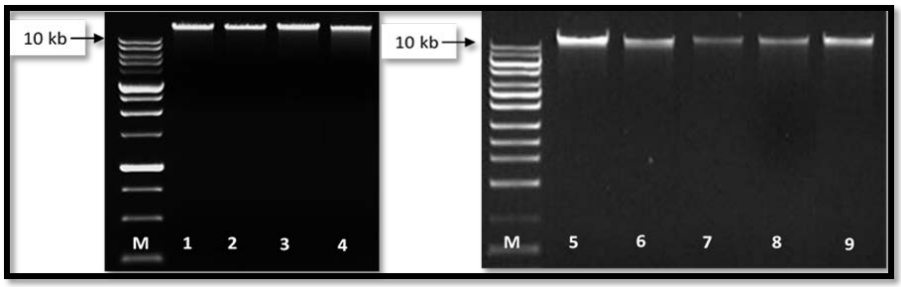

Figure 1. Electrophoresis separation of genomic DNA fragments from the isolated yeast strains. Lane M: 1 kb ladder (Promega); Lanes 1–9: isolated yeast genomic DNA.

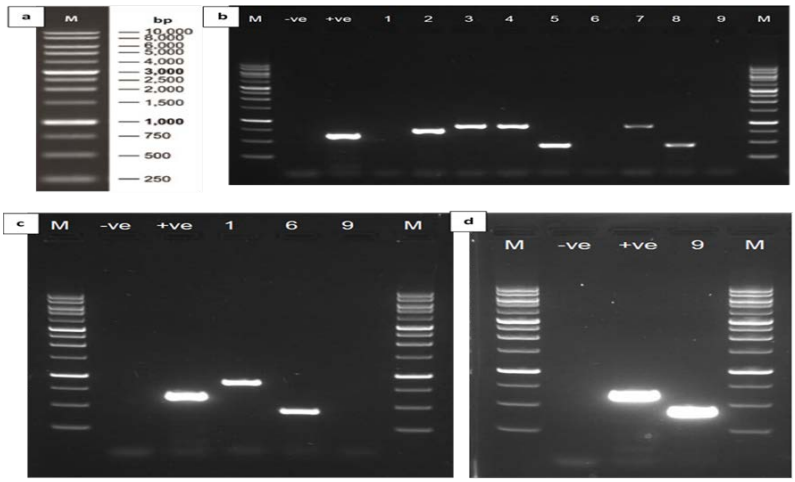

Figure 2. PCR amplification of ITS region for all isolated yeast strains. Lane M: 1 kb ladder; Lanes 1–9: fragment of ITS region for all isolated yeast.

B. PCR Analysis

The genes of the isolated yeast strains were successfully amplified using the universal primers internal transcribed spacers (ITS), ITS1 and ITS4. The resulting amplicons are shown in Fig. 2. The DNA ladder as a control size for all gel separation is shown in Fig 2a while Fig 2b–d shows nine amplicons from different samples. Single bands of PCR amplification were obtained between 300 and 800 bp. These results are consistent with the data obtained from a previous study where the normal ITS amplicon size for yeast size ranged from 300 to 900 bp depending on the type of species [13].

Internal transcribed spacer (ITS) refers to the spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome. It contains two noncoding spacer regions between 18s and 28s including 5.8s rRNA gene [14]. In yeast and fungal species, the universal primers ITS1 and ITS4 are commonly used to amplify the region. In fungal and yeast species, the PCR amplified ITS region will be highly important to differentiate the strains species [15]. The polymorphism distance between ITS1-ITS4 region among the species was in the range of 400 and 800 bp and can be detected in the PCR products (Fig. 2).

From the result, lanes 1, 3, 4, and 7 have the longest amplified sequence around 800 bp. On the other hand, lanes 5, 6, 8, and 9 show the shortest sequence around 400 bp. The similarity in sequence length indicates that they might be in the same genus or species with small variation of strain.

C. Gene Sequencing and Identification

All the purified PCR fragments were ligated into pJET1.2 vector (Thermo Fisher) and then transformed into *E.* coli strain JM109. pJET1.2 vector is the cloning system generated with Pfu DNA polymerase, Taq DNA polymerase, or another thermostable DNA polymerase with blunt or sticky ends. A successful cloning of insert into the pJET1.2 vector was identified by growing the colonies on LB–ampicillin agar plates. The positive recombinant plasmids were sequenced using universal primer LpJET1.2F by 1st Base Laboratories (M) Sdn. Bhd., Malaysia.

The determined nucleotide sequences of the amplified fragments were subjected to search against all known sequences in database (BLAST search). The yeast species similarity was determined based on the highest bit score and identified percentage from the hit BLAST from the National Centre for Biotechnology Information (NCBI) as shown in Fig. 3.

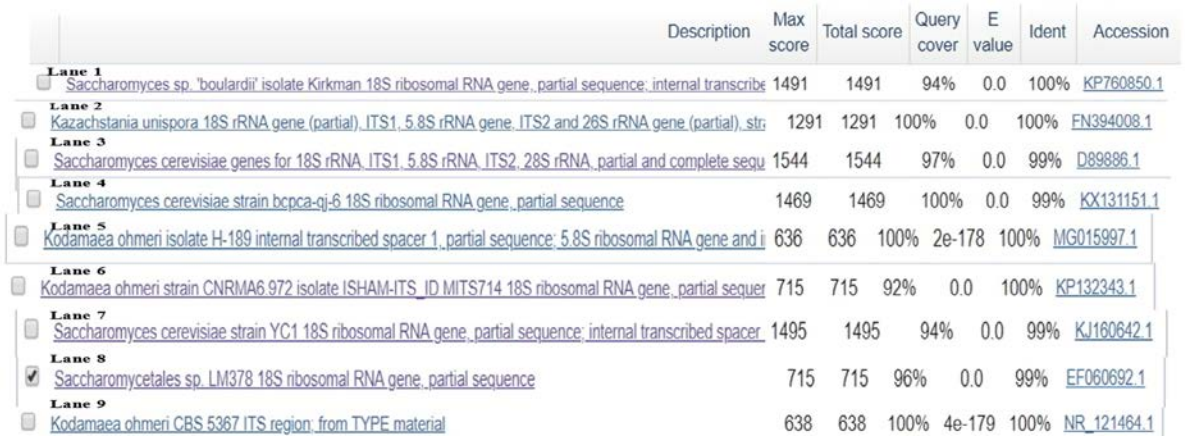

Figure 3. Sequence alignment using BLAST. The highest identity was shown between 99% and 100%.

TABLE I. GENETIC IDENTIFICATION OF THE SPECIES

PCR Lane	PCR product length (bp)	Yeast species	Identity (%)	Accession no.
1	853	Saccharomyces boulardii	100	KP760850.1
2	699	Kazachstania unispora	100	FN394008.1
3	861	Saccharomyces cerevisiae	99	D89886.1
4	804	Saccharomyces cerevisiae	99	KX131151.1
5	366	Kodamaea ohmeri	100	MG015997.1
6	420	Kodamaea ohmeri	100	KP132343.1
7	862	Saccharomyces cerevisiae	99	KJ160642.1
8	405	Saccharomycetales	99	EF060692.1
9	345	Kodamaea ohmeri	100	NR_121464.1

Table I shows the species of probiotic yeast found in the samples of kefir drink from Malaysia along with the accession number obtained from the database. Based on the 16s-rDNA identification, the indigenous yeast isolated were classified into three major cluster which are Kodamaea, Kazachstania and Saccharomyces group respectively. *Saccharomyces* and *Kodamaea* were found to be the major population in all the isolated yeast strains.

These results are consistent with a previous research [16] where the most common yeast species that can be found in the microbial mixture in the kefir is *Saccharomyces cerevisiae*. Another study done from Brazil reported that *Saccharomyces, Candida and Kluyveromices* are the abundance genera that can be found in the kefir drink in the region [17].

However, the yeast species diversity observed in kefir samples may be influenced by the milk's characteristics

and the variety of microbial mixture in the samples where it may be different based on the origin of the kefir samples. In this case, the local Malaysian kefir drink samples can be found up to three different types of yeast genera.

IV. Conclusion

In the present study, nine of yeast strains were isolated from the complex microbial consortium of kefir drink. The ITS region of all the isolated strains were successfully amplified by using PCR method and identified by using sequencing analysis. Genus *Saccharomyces* and *Kodamaea* were found to be the major population of yeast strains in the kefir drink samples.

Since, indigenous yeast strains from genera Saccharomyces and Kodamaea has been isolated from the local Malaysia kefir drink that regularly consumed by the Malaysian people. Therefore, the potential of probiotic role seem to be quite important for the yeast strains found in the Malaysian kefir drink. The yeast strains found could be used as a new source of probiotic formulation such as in tablet or capsule form or can be incorporate in the supplement or functional food. However, further study to observe the probiotic traits should be carried out to investigate potential of isolated yeast strains.

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