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Survival of Isolated Probiotic Yeast Strains from Kefir Towards Bile and Acidic Environments

Azhar, M.A[1] & Munaim, M.S.[2]

[1,2]*Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang As-Sultan Abdullah, Lebuh Persiaran Tun Khalil Yaakob, 26300 Kuantan, Pahang, Malaysia.*

Abstract

Probiotic yeast is one of the most important probiotics in kefir drinks and has been shown to have positive effects on gut health. The abundance of probiotic yeast strains in kefir drinks provides an opportunity to identify potential probiotic yeast as dietary supplements or functional food. Currently, yeast products are not as widely available as bacterial strains, but this is expected to change in the near future. Probiotics must resist acid and bile and adhere to the intestinal surface to thrive and colonise the gastrointestinal tract. Therefore, this study aims to investigate the ability of locally isolated yeast strains to resist acid and bile stress. An in vitro experiment was conducted using sample strains isolated from kefir beverages such as *Kodamaea ohmeri*, *Kazachstania unispora*, *Saccharomyces cerevisiae*, and *Saccharomyces boulardii*. Acid tolerance was determined by varying the pH of YEPD broth from 2 to 7, and bile tolerance was determined using YEPD broth with bile salt concentrations of 0%, 0.3%, 0.5%, and 1%. The study findings show that most isolated yeast strains survive acidic and bile environments. They were able to survive pH 3 for 24 hours (about log 6 CFU/mL). Only *Saccharomyces boulardii* and *Kodamea ohmeri* could survive for 24 hours in 1% bile acid (log 6.41 to log 7.34 CFU/mL), whereas all strains survive in 0.3% bile salt (log 3.42 to 8.95 CFU/mL). This study shows that all isolated yeasts have remarkable potential probiotic properties.

Keywords: Probiotics; yeast; acid tolerance; bile tolerance; *Saccharomyces*

Full length article **Corresponding Author*, e-mail: akmalazhar@ump.edu.my

1. Introduction

Fermented foods have long been an important part of the human diet. Fermentation is one of the most common ways of food processing, as it prevents food spoilage and extends its shelf life [1]. Various fermented foods are traditionally produced worldwide, including wine, beer, yoghurt, kimchi, milk, cereals, soybeans, fruit, and fish [2]. The microorganisms and by-products produced during fermentation contribute to the unique taste and texture of fermented foods. The beneficial microorganisms are known as probiotics. FAO-WHO defines probiotics as "living microorganisms that provide health benefits to the host when administered in sufficient quantity" [3]. In fact, probiotics have functional capacities with anticancer, antigenotoxic, antioxidant, anti-inflammatory, osteoporotic, antihypertensive, and antidiabetic potentials [4]. In gastroenterological therapy, most probiotic products contain strains of lactic acid bacteria. However, it has been reported that only certain yeast strains can act as probiotics, such as *Kluyveromyces marxianus*, *Saccharomyces boulardii*, *Pichia kudriavzevii*, and *Debaryomyces hansenii* [5]. Yeast cells are 10 times larger than bacterial cells due to their larger size, giving them an advantage in colonising mucosal surfaces. They are resistant to antibiotics, which makes them suitable candidates for treatment in combination with antibiotics. There is also no evidence of antibiotic resistance gene transfer [6]. In addition, certain yeasts have beneficial probiotic properties such as resistance to gastric bile and acid as well as an antibacterial effect on intestinal infections. Currently, yeast products are not widely used as bacterial strains, but this trend will likely change soon.

Probiotic yeast as one of the main probiotics in kefir has shown to have positive effect on gut health. Kefir is a fermented milk drink that originated from the Caucasus region. It is made by adding kefir grains to milk, which then ferments to produce a slightly acidic, fizzy drink. Kefir is rich in probiotics, which contain yeast and bacteria that are beneficial for gut health. The high proportion of a probiotic yeast strain in kefir drink provides an opportunity to identify probiotic yeast that can be used as dietary supplements or functional food. However, to develop an oral probiotic formulation, the probiotic microorganism should remain viable during the administration and transport to the recipient. At least 10^6 CFU/g or mL of viable probiotic organisms in the small intestine is recommended for them to be useful [7].

Probiotics need to resist acid and bile and adhere to the intestinal surface to thrive and colonise the gastrointestinal tract. Therefore, this study aims to investigate the ability of locally isolated yeast strains to resist acid and bile stress.

2. Materials and methods

2.1 Samples

This study employed yeast strains obtained from a kefir drink. The strains used in this investigation include *Kodamaea ohmeri, Kazachstania unispora, Saccharomyces cerevisiae*, and *Saccharomyces boulardii*. The identified yeast strains were grown in a YEPD liquid medium at 35 °C for 24 hours at 200 rpm. YEPD cultures with 20% (v/v) glycerol were used to create long-term stocks to be stored at -80°C, while short-term stocks were grown on YEPD agar plates. The culture was incubated for 24 hours.

2.2 In vitro screening of probiotic potential for acidic tolerance

pH resistance was determined by cultivating the yeast in YEPD broths with varying pH of 2–7 at 35 °C for 24 hours at 200 rpm [8]. In this study, pH 7 is used as the control. The resistance was determined by estimating the viable cell count using a colony-forming unit (CFU) on YEPD agar for 24 hours at 35 °C.

2.3 In vitro screening of probiotic potential for bile salt tolerance

Resistance to bile in intestinal juices was tested and modified, as reported by Narayanan et al. (2012). Briefly, YEPD broths with bile salt concentrations of 0%, 0.3% (w/v), 0.5% (w/v), and 1.0% (w/v) were used. The fluid was inoculated in the yeast culture and incubated for 24 hours at 35 °C at 200 rpm. The bile resistance was determined by estimating the viable cell count using a colony-forming unit (CFU) on YEPD agar for 24 hours at 35 °C.

2.4 Cell viability measurement

CFU was used to express the viable cell count in the probiotic yeast (CFU/mL) samples. CFU was determined using the spread plate technique to make a serial dilution with YEPD agar. It was determined after 24 hours of incubation at a specific temperature by counting the colony visible on the agar after an incubation period. The amount of CFU on the plate is multiplied by the dilution factor to get the amount of CFU in the original sample.

2.5 Statistical evaluation

All analysis was performed using Excel 2010. The data are shown as the mean ± standard deviation (SD).

3. Results and Discussions

3.1 Acidic tolerance

In the previous studies, potential probiotic yeast strains were isolated from kefir beverage and molecularly identified as *Kodamaea ohmeri, Kazachstania unispora, Saccharomyces cerevisiae*, and *Saccharomyces boulardii* [10]. The pure cultures of all isolates were obtained and grown in YEPD media prior to analysis. In this study, the probiotic ability of the isolated strains was evaluated by assessing their resistance to acidic and bile environments. Table 1 shows that all yeasts tested are sensitive to an extremely acidic environment (pH 2). There is no viable cell at pH 2, indicating that most isolated yeast strains die and cannot grow. In contrast, the survival rate of viable cells was moderate at pH 3 and 4, whereas the survival rate was high at pH 5 and 6, as well as in the YEPD control medium (pH 7.0). The findings indicate that the cells can proliferate without difficulty and survive in the human intestinal environment.

Table 1. Acidic tolerance of isolated yeast culture at 35 °C for 24 hours

	Viable cell count (log CFU/mL)					
Strains	**pH 2**	**pH 3**	**pH 4**	**pH 5**	**pH 6**	**Control**
Saccharomyces boulardii	TFTC	6.91 ± 0.10	7.93 ± 0.05	8.99 ± 0.05	8.98 ± 0.11	9.01 ± 0.06
Kazachstania unispora	TFTC	6.97 ± 0.07	7.97 ± 0.11	9.00 ± 0.12	9.00 ± 0.12	9.06 ± 0.14
Saccharomyces cerevisiae	< 1 ± 0	6.10 ± 0.15	8.02 ± 0.10	9.10 ± 0.13	8.99 ± 0.09	9.00 ± 0.08
Kodamaea ohmeri	TFTC	6.78 ± 0.14	7.90 ± 0.15	8.99 ± 0.05	8.80 ± 0.14	8.95 ± 0.10

(TFTC): Too few to count. Values are the mean of three samples ± SD.

For a microbe to become an effective probiotic, it must have a high level of viability as it travels through the stomach's acidic environment to reach the target site. However, some probiotic strains are less tolerant to the acidic environment and may not survive or achieve the desired effect, resulting in reduced efficacy or adverse effect. Normally, probiotics are very sensitive to acidic conditions. Therefore, pH is one of the key elements in measuring the performance of a probiotic product. Due to acid secretion in the stomach, the stomach environment is very acidic, so this becomes the main barrier once probiotics are consumed [11]. The stomach pH varies, but its natural pH is between 1.3 and 3.5 before meal consumption [12]. In this experiment, most isolated strains can survive 24 hours of incubation at pH 3.0. Thus, they fulfill the preliminary in vitro selection criteria for potential probiotics. Survivability at pH 3 is deemed as having moderate tolerance to an acidic medium. That is why pH 3 is suitable for the selection of acid-resistant yeast strains. There are potential benefits that are associated with the tolerance of probiotics in an acidic environment. Probiotics that survive and thrive in harsh conditions may be better at fighting harmful bacteria and improving digestive function. In addition, probiotics that have a high tolerance to the acidic environment are less likely to cause side effects when consumed in larger amounts. This leads to a healthier population and better healthcare.

However, it is shown that all strains have a very low survival rate (< 1 ± 0 log CFU/mL) after 24 hours at an extremely low pH. Table 1 shows that the strains that survive the extreme pH of 2 are S. cerevisiae, while the remaining strains (*K. ohmeri, K. Unispora, and S. boulardii*) can only survive pH 3 and above. In general, resistance to the acidic medium depends on the species and strain, which explains the variance in acid tolerance between the isolated yeast strains. The result is in line with other studies that found different survivability of probiotic yeast strains under low pH conditions [8,13,14]. Survival under acidic conditions may be due to several reasons, such as cell wall composition and size, which are affected by the H+ ions produced by the acid [15]. The change in extracellular pH affects the yeast life cycle and viability. Therefore, the maintenance of pH homeostasis is critical for cell viability. In an acidic environment, pH homeostasis is a crucial indicator of the cell physiological state, as it regulates pH both inside and outside the cell [16].

3.2 Bile salt tolerance

In addition to gastric acid tolerance, the tolerance of microorganisms to bile salt is critical in screening probiotics for the human gut. One of the challenges in ensuring the survival of probiotic strains is the presence of bile in the upper section of the small intestine. In fact, the bile salt concentration ranges from 1% to less than 0.05% under normal physiological conditions. The ability of probiotic strains to tolerate bile salt becomes a critical factor in determining their ability to live in the small intestine and plays a functional role as probiotics.

In this study, in terms of growth pattern, the yeast isolates show varying resistance to bile salts, in which 0% indicates the yeast growth in the control medium without the addition of bile salt to the medium. Table 2 shows the tolerance to bile salt at different bile concentrations, namely 0 %, 0.3 %, 0.5 %, and 1 %. The concentrations were chosen because they correspond to the amount of bile normally found in the human gut. The ability of probiotics to tolerate 0.3−0.5% bile enhances the colonisation of probiotics in the gastrointestinal tract [17]. The 1% concentration was chosen to indicate extreme tolerance to bile salts. The findings show that most isolated yeasts can survive when grown in a medium containing 0.3% bile, indicated by a viable cell count of log 3 CFU/ml and above. However, only *Saccharomyces boulardii* and *Kodamea ohmeri* have proven to be very tolerant and survive in 0.5 % and 1.0 % bile salt environments.

Table 1. Bile tolerance of isolated yeast culture at 35 °C for 24 hours

	Viable cell count (log CFU/mL)			
Strains	**0 % (Control)**	**0.3 %**	**0.5 %**	**1 %**
Saccharomyces boulardii	9.03 ± 0.04	7.78 ± 0.05	7.42 ± 0.08	6.41 ± 0.07
Kazachstania unispora	9.02 ± 0.06	3.42 ± 0.08	TFTC	TFTC
Saccharomyces cerevisiae	9.01 ± 0.11	3.57 ± 0.06	TFTC	TFTC
Kodamaea ohmeri	8.96 ± 0.10	8.95 ± 0.15	8.94 ± 0.12	7.34 ± 0.10

(TFTC): Too few to count. Values are the mean of three samples ± SD.

Bile is highly toxic to microorganisms not adapted to intestinal conditions. When a microbial cell is exposed to bile, the lipid bilayer and cell membrane integral protein begin to dissociate due to cellular homeostasis. This leads to microbial cell leakage and, eventually, cell death. The cell membrane is the main target of bile acid due to the lipophilic steroid ring in nature, where lipid packing and proton driving force are disrupted, leading to cell death. There are other effects of bile, including alteration of sugar metabolism, induction of oxidative stress and DNA repair mechanism, and protein misfolding [18]. However, the result shows that bile does not completely inhibit the growth of isolated yeast, but some isolated strains can grow well even in the 0.5% bile medium. The high growth of the isolated yeast in 1% bile may be due to the stress adaptation mechanism. Another possible explanation is that the probiotic cell can survive and function in bile by secreting anti-bile salt enzymes known as bile salt hydrolase [19]. The enzymes cleave the primary amine from the secondary amine and thus neutralise the acidity of bile. The results are consistent with the previous studies, which show that yeast strains such as *Saccharomyces* can grow in higher bile salt concentrations [20–22].

4. Conclusions

In general, this study shows that all the isolated yeasts being tested have remarkable potential probiotic properties. The probiotics demonstrate better survival in gastric juice and bile acid environments. They were able to survive for 24 hours at pH 3 (about log 6 CFU/mL). Only *Saccharomyces boulardii* and *Kodamea ohmeri* can survive for 24 hours in 1% bile acid (log 6.41 to log 7.34 CFU/mL), whereas all strains can survive in 0.3% bile salt (log 3.42 to 8.95 CFU/mL). This study represents a preliminary investigation of probiotic yeast selection. The other parameters, such as antimicrobial activity, hydrophobicity, and intestinal adhesion, have to be studied in more detail to evaluate their potential as effective probiotics.

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