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RESEARCH PAPER

Evaluating the microbial growth kinetics and artificial gastric digestion survival of a novel *Pichia kudriavzevii* FOL-04

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Abstract

Present study aims to explore *Pichia kudriavzevii* FOL-04 (**FOL-04**)'s: i) survival against artificial gastric juice (**AGJ**) and artificial bile juice (**ABJ**), ii) growth kinetics in shake flask (**SF**) and fed-batch trials (**FBT**). Survival of FOL-04 as measured by relative cell density (**RCD**) against AGJ and ABJ was screened at four different pH-levels (control, 3, 2, 1.5) and ox-bile concentrations (control, 0.2%, 1%, 2%), respectively. Growth kinetics was calculated by periodic measurement of OD_{600} in SF (225 rpm, 30°C) or in FBT using exponential feeding regimen where pH, dissolved-oxygen and temperature were controlled at 5.5, 21%, and 30°C, respectively. The doubling-time, maximum specific growth rate, and final cell densities achieved for SF and FBT were 81.7min, 1.67, 11.79 and 170.4 min, 4.75, 37.95, respectively. RCDs calculated were similar for pH=3 and control vs both were significantly higher($p<0.05$) than pH=1.5 and 2 with the latter two pH-levels were not significantly different($p>0.05$). RCDs were similar across control, 0.2%, and 1% ox-bile levels($p>0.05$). However, 2% ox-bile yielded significantly lower RCD ($p<0.05$) compared to all except 1%. FOL-04 is a potential probiotic candidate showing robustness against AGJ and ABJ and remarkable biomass increase was achieved when grown under FBT which could pave the way for developing a yeast-based probiotic using this strain.

Introduction

Yeast is a eukaryotic microorganism that is widely used for the fermentation of alcohol or acids to produce different fermented products such as baking dough, beer, wine, and vinegar. The first use of yeast for fermentation by humans' dates before 8000 BC (Liti, 2015). Some yeast strains have probiotic characteristics that help to prevent certain intestinal disorders. McFarland and Bernasconi (1993) stated that *Saccharomyces boulardii* treatment could inhibit *Clostridioides difficile*, a toxin secreting bacterium that causes nosocomial diarrhea in adults.

Strains of *Pichia kudriavzevii* are widespread in the environment and are often seen in sporadic fermentations (Douglass et al., 2018). The *P. kudriavzevii* strains are also used to manufacture various traditionally fermented foods (Kurtzman, 2011; Smukowski Heil et al., 2018). This species is not under the pathogens list and possesses GRAS status (Bourdichon et al., 2012) due to its safe use over centuries to make fermented foods such as cassava and cacao in Africa, fermented milk in Tibet and Sudan, and maize beverages in Colombia (Bourdichon et al., 2012). It is also utilized as a starter culture for Chinese vinegar and sourdough (De Vuyst et al., 2016; Li et al., 2014). *P. kudriavzevii*'s potential utilization includes probiotic applications (Chelliah et al., 2016) owing to its high stress-tolerance thus displays a growing role in the biotechnology industry such as production of bioethanol (Mukherjee et al., 2017; Radecka et al., 2015), succinic acid, and glycerol (Wang et al., 2001; Xiao et al., 2014).

Shalgam is a non-alcoholic traditional beverage manufactured in Turkey. It is produced by lactic acid fermentation of turnip and red carrot (Karaoglan et al., 2019). Studies showed that predominant microbiota of Shalgam is composed of lactic acid bacteria (LAB) and yeast organisms where *Lactobacillus plantarum* is the most abundant LAB in Shalgam. There are other LAB in Shalgam such as *L. paracasei, L. brevis, and L. fermentum* (Tanguler & Erten, 2012). Also, several health promoting compounds exist in Shalgam, such as anthocyanins and phenolic compounds (Konczak & Zhang, 2004).

Although majority of research on probiotics focused on lactic acid bacteria, there is a growing trend on finding new yeast strains carrying probiotic effects (Hatoum et al., 2012; Kumura et al., 2004; Moslehi-Jenabian et al., 2010; Perricone et al., 2014). As a general approach, most probiotics are evaluated according to their capability to survive and subsequently colonize in the gastrointestinal system. In fact, survival against gastrointestinal conditions and the ability to attach to the intestinal mucosa is crucial to reach the target organ in certain numbers to confer health benefits to the hosts (Ouwehand et al., 2002). In the gastrointestinal tract, firstly, probiotics must pass through the harsh acidic conditions of the stomach that exerts a strong barrier for entry to the gut (Greppi et al., 2017).

To date, relatively few artificial gastric juice and artificial bile juice survival tests, which are the fundamental challenge conditions that probiotics come across when ingested (Sun & Griffiths, 2000; Yetiman et al., 2022; Klaenhammer & Kleeman, 1981; Song et. al., 2003), were explored for *P. kudriavzevii* strains. Also, bioprocess studies targeting *P. kudriavzevii* microbial biomass production characteristics are limited if any. To fill those gaps in the literature i) survival of a novel *P. kudriavzevii* strain FOL-04, isolated from traditional Shalgam, was evaluated under artificial gastric and bile juice conditions, ii) shake flask and fed-batch bioreactor experiments were conducted to explore and compare growth kinetics of *P. kudriavzevii* FOL-04 under two bioprocess conditions. This is the first report describing artificial gastric and bile juice tolerance in addition to microbial growth kinetics of a new *P. kudriavzevii* strain isolated from Shalgam produced in the Southern Anatolia region.

Materials and Methods

Isolation of *P. kudriavzevii* FOL-04

A traditionally fermented lactic acid beverage called Shalgam was purchased from a local store in Southern Anatolia region. After performing serial dilutions of liquid Shalgam sample, Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Merck, Germany) was used to grow yeast strains. The FOL-04 was streak plated and purified for further DNA isolation, PCR, Sanger sequencing, survival against artificial gastric and bile juice, and microbial growth kinetic determinations.

DNA Isolation and PCR

DNA of the yeast sample was isolated with MACHEREY-NAGEL's NucleoSpin® Microbial DNA Mini kit according to yeast DNA extraction protocol. The following primers were used to amplify the 5.8S-ITS rRNA region and D1/D2 domains of the 26S rRNA region:
ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'),
ITS4 (5'-TCCTCCGCTTATTGATATGC-3'),
NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3'),
NL4 (5'-GGTCCGTGTTTCAAGACGG-3'), respectively.

Reagents of PCR amplification (EasyTaq® DNA Polymerase, 10X EasyTaq ® Buffer, 2.5 mM dNTPs, 6X DNA loading dye) was obtained from Transgen Biotech, otherwise specified. A 50 µl of total PCR master mix was prepared by adding 1 µL of templated DNA, forward and reverse primers at 0.2 µM final concentration, EasyTaq® buffer at 1X final concentration, dNTPs at 0.2 µM final concentration, 0.5 µL of EasyTaq® DNA polymerase, and 37.5 µL of nuclease-free water. Thermal cycling started with the first denaturation at 95°C for 5 minutes. Then, it continued with denaturation at 94°C for 30 seconds, annealing at 55°C for ITS1/4, 52.5°C for NL1/4 for 30 seconds. After annealing, samples were extended at 72°C for 2 minutes. Denaturation, annealing, and extension phases were repeated 36 times. Samples were incubated at 72°C for 10 minutes for the last extension, followed by running samples on agarose gel, and the NL amplified DNA fragment was sent for Sanger sequencing. The sequencing results were analyzed at the BLAST at blast.ncbi.nlm.nih.gov (Altschul et al., 1990).

Cultivation in Shake Flasks

Yeast extract-peptone-dextrose (YPD) media, containing 1% yeast extract, 2% peptone, and 2% dextrose (g/L), was used in shake flask cultivations. Firstly, 5 mL of pre-inoculated for 16 hours with the *P. kudriavzevii* YPD media was mixed with 65 mL of YPD media inside the Erlenmeyer flask. Then, the Erlenmeyer flask was incubated at 30°C at 225 rpm in a shaker incubator for 24 hours. Samples were taken every hour to measure optical density at 600 nm wavelength. Doubling time (minutes) was calculated during the exponential phase as follows (Roth, 2006)

$$t_d = \frac{duration \cdot log(2)}{log(finalOD) \cdot log(initialOD)}$$

Where the t_d is doubling time. Maximum specific growth rate (μ_{max}) was calculated by measuring the slope of the steepest linear portion of the growth curve.

Cultivation in Fed-batch Bioreactors

For optimizing *P. kudriavzevii* FOL-04's growth conditions and increase its biomass yield, fed-batch cultivations were performed. Bioreactor cultivations were performed at 700 mL YPD media (50 mL pre-inoculated and 650 ml sterile YPD media). The composition of YPD media was adjusted to 1% yeast

extract, 2% peptone, and 1% dextrose (g/L) to shorten the batch phase. The dissolved oxygen level was set at 25% and controlled with airflow and stirrer cascade. pH was set to 5.5, and the temperature was set to 30°C to achieve the highest biomass (Ndubuisi et al., 2020) during both batch and fed-batch cultivations. Exponential feeding regimen was tested on the growth kinetics of *P. kudriavzevii* FOL-04. A 50% dextrose feed solution was used during fed-batch phase, which started upon arrival of batch fermentation which was evident with no more base consumption in alignment with pH curve flattening. Fed-batch cultivations lasted for around 22 hours, and samples were taken every 2 hours to measure the biomass yield of the *P. kudriavzevii* FOL-04 by measuring its optical density at 600 nm. Doubling time (minutes) was calculated during the exponential phase as follows (Roth, 2006):

$$t_d = \frac{duration \cdot log(2)}{log(finalOD) \cdot log(initialOD)}$$

Where the t_d is doubling time. Maximum specific growth rate (μ_{max}) was calculated by measuring the slope of the steepest linear portion of the growth curve.

Artificial Gastric Juice

To investigate the influence of pH on survival of *P. kudriavzevii* FOL-04, a modified method from Sun & Griffiths (2000) and Yetiman et al. (2022) was applied to prepare artificial gastric juice. First, YPD media was prepared according to manufacturer's instructions and following pH conditions were adjusted using HCl solution: control (no acid supplementation), pH = 1.5, pH = 2, and pH = 3 with each treatment being prepared in six replicates. Each treatment media was autoclaved at 121°C for 15 minutes at 2 atmospheres. Each sterile treatment media was inoculated with fresh culture of *P. kudriavzevii* FOL-04 cells. Each treatment tube was incubated at 30°C under 225 rpm shaking conditions for two days. Then, the optical density of each treatment tube was measured with Shimadzu UVmini-1240 spectrophotometer at 600 nm wavelength. The experiments were performed in six replicates and results were presented as relative cell density ratio (OD_{600} t_{final}/OD_{600} t_0). Statistical analysis of relative cell density ratios were performed with the analysis of variance (ANOVA) and Tukey tests in R programming language (R Core Team, 2020).

Artificial Bile Juice

Artificial bile juice (ABJ) was prepared according to a modified method of Klaenhammer & Kleeman (1981), Song et. al. (2003), and Yetiman et al. (2022). Simply, YPD broth supplemented with following concentrations of ox bile extract (Sigma, Germany): control (no bile), 0.2%, 1%, and 2% by w/v (Yetiman et al., 2022) was autoclaved at 121°C for 15 minutes at 2 atmospheres. Each sterile treatment media was inoculated with fresh culture of *P. kudriavzevii* FOL-04 cells. Each treatment tube was incubated at 30°C under 225 rpm shaking conditions for two days. Then, the optical density of each treatment tube was measured with Shimadzu UVmini-1240 spectrophotometer at 600 nm wavelength. The experiments were performed in six replicates and results were presented as relative cell density ratio (OD_{600} t_{final}/OD_{600} t_0). Statistical analysis of relative cell density ratios were performed with the analysis of variance (ANOVA) and Tukey tests in R programming language (R Core Team, 2020).

Results

PCR Fingerprinting and Sanger Sequencing

After running PCR samples on the agarose gel, both ITS and NL primers amplified PCR products yielded clear bands on pulse field gel electrophoresis. However, non-specific binding was seen on the sample amplified with ITS primer (Figure 1). The sample that was amplified with NL primers had only one clear band on the gel image which shows specific amplicon has been achieved. Therefore, the NL primers amplified DNA fragment of yeast isolate was sent to Sanger sequencing for strain level identification (Sanger et al., 1977).

Figure 1. Gel image of PCR fingerprinting assay of *P. kudriavzevii* FOL-04. There is a non-specifically bonded fragment on ITS.

Sanger sequencing results were analyzed with the Basic Local Alignment Tool (BLAST) (Altschul et al., 1990). BLAST results show that the isolated *P. kudriavzevii* FOL-04 has the highest homology against *P. kudriavzevii* feni92 (KM234470.1) and *P. kudriavzevii* cs280 (KM234470.1) with 98.93% and 58% identity score and query cover score achieved, respectively. BLAST results are represented in a phylogenetic tree to show the phylogenetic distance of species against the *P. kudriavzevii* FOL-04 (Figure 2) (Paradis & Schliep, 2019).

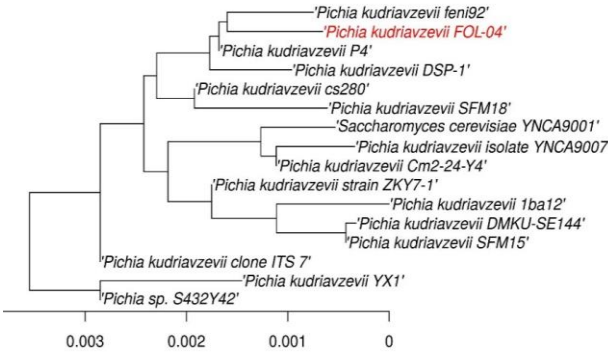

Figure 2. The phylogenetic distance between species and *P. kudriavzevii* FOL-04 (red) based on D1/D2 domains of the 26S rRNA region.

Biomass in Shake Flasks

The biomass yield of *P. kudriavzevii* was observed by measuring optical density values at 600 nm. Results showed that the lag phase he *P. kudriavzevii* strain was around 6 hours and the exponential phase took around 8 hours (Figure 3). During the shake flask cultivation, μ_{max} achieved was 1.67/hour, and the doubling time was calculated as 81.7 minutes.

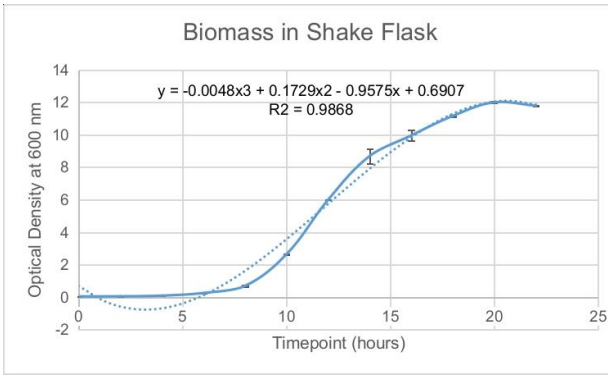

Figure 3. Time vs optical density at 600 nm results of shake flask cultivations.

Biomass in Fed-batch Bioreactor Cultivation

The biomass yield of the *P. kudriavzevii* FOL-04 was measured with the same method that was applied for the shake flask cultivations. Results showed that the lag phase of the *P. kudriavzevii* strain was shortened to 4 hours. The exponential phase took up to 8 hours. Moreover, biomass yield increased after the deceleration phase perhaps due to feeding with 50% dextrose (Figure 4).

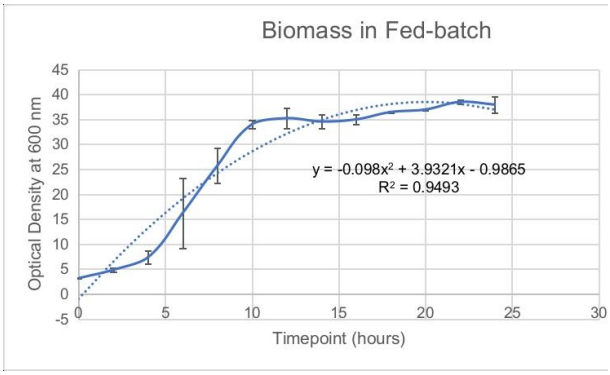

Figure 4. Time vs optical density at 600 nm results of fed-batch bioreactor cultivations.

During the fed-batch cultivation, μ_{max} achieved was 4.75/hour and doubling time calculated was 170.4 min. The first 12 hours of bioreactor cultivation, similar biomass results were achieved. After that, the difference in biomass of the *P. kudriavzevii* strain got bigger in favor of the yeast fed with the exponential feeding regimen. Figure 5 shows that pH was oscillating at 5.4 at the beginning of the batch process. At that time base pump works to increase the pH to 5.5. When the pH exceeds the 5.5 base pump stops to control pH. Base pump restarts to work around 13 h time point to adjust the pH of the vessel. Pre-set dissolved oxygen levels throughout the fed-batch process was adjusted by stirrer and airflow intake through cascade system (Figure 6).

Survival Against AGJ and ABJ

Results of the AGJ tolerance test show that the *P. kudriavzevii* FOL-04 is able to grow in a pH 3 level acidic environment similar to the control environment at pH 6.5. However, the survival of the *P. kudriavzevii* FOL-04 decreases significantly when pH is at or lower than 2. Moreover, Tukey's test results show that there is not a

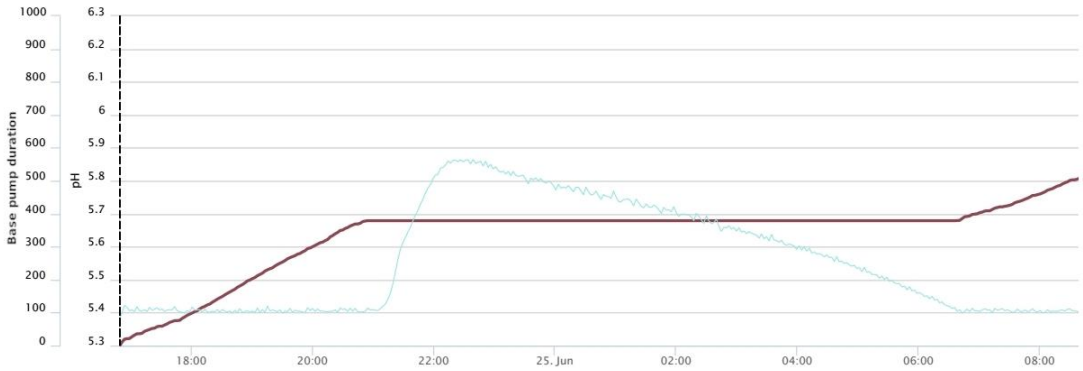

Figure 5. Time vs. pH level (light blue) and base pump duration (purple) during the fed-batch cultivation. X axis shows the time in hours while bioreactors are operating.

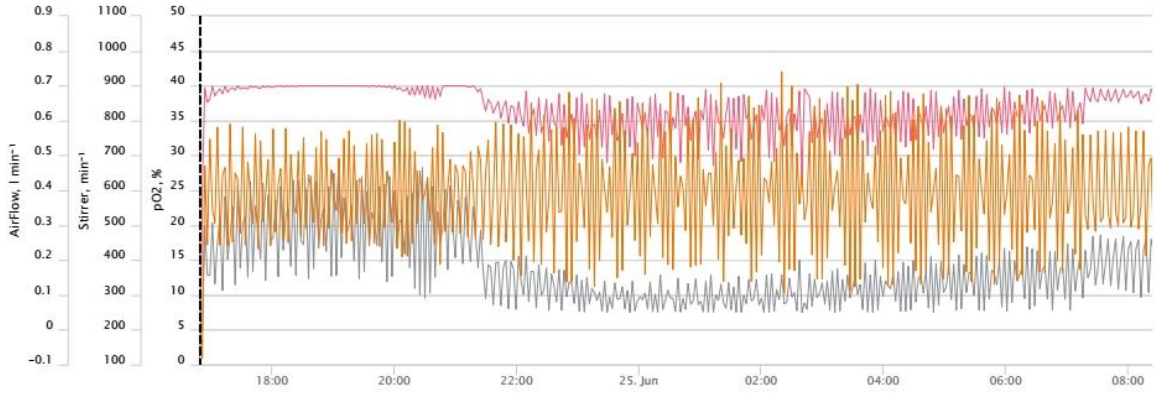

Figure 6. Time vs dissolved oxygen (pO_2) (orange), airflow rate (grey), and stirrer rate (red) during the fed-batch cultivation.

significant difference (p = 0.05) in survival of the *P. kudriavzevii* FOL-04 at pH 2 and pH 1.5 (Table 1, Figure 7).

Table 1. Acid tolerance of *P. kudriavzevii* FOL-04 after 42 hours of incubation. OD_{600} values of AGJ at the onset of incubation were as follows: 0.494 ±0.02 (control), 0.511 ±0.02 (pH 3), 0.470 ±0.09 (pH 2), and 0.550 ±0.02 (pH 1.5). The relative cell density ratio was calculated by dividing final cell densities achieved against initial cell turbidity measured at 600 nm wavelength. Means sharing the same superscript letters are not significantly different than each other (α = .5)

pH	Relative Cell Density Ratio ($OD_{600}\ t_{final}/OD_{600}\ t_0$)	Standard Deviation
1.5	10.4^{cx}	±4.3
2	18.8^{bx}	±10.5
3	34.7^{a}	±9.8
6.5 (Control)	42.2^{a}	±7.7

P. kudriavzevii FOL-04 can easily survive at 0.2% bile salt concentration in YPD media. However, survival of the *P. kudriavzevii* FOL-04 drops significantly when the bile concentration is higher than 1%. In addition, there is not a significant difference in survival of *P. kudriavzevii* FOL-04 observed while bile salt concentration is between 1% and 2% (Table 2, Figure 7).

Table 2. Bile salt tolerance of *P. kudriavzevii* FOL-04 after 42 hours of incubation. OD_{600} values of ABJ at the onset of incubation were as follows: 0.546 ±0.01 (control), 0.593 ±0.01 (0.2%), 0.786 ±0.02 (1%), and 1.026 ±0.06 (2%). The relative cell density ratio was calculated by dividing final cell densities achieved against initial cell turbidity measured at 600 nm wavelength. Means sharing the same superscript letters are not significantly different than each other (α = .5)

Bile Salt Concentration (%)	Relative Cell Density Ratio ($OD_{600}\ t_{final}/OD_{600}\ t_0$)	Standard Deviation
0 (Control)	28.24^{a}	±7.46
0.2	29.18^{a}	±9.51
1	24.47^{ax}	±0.92
2	18.04^{bx}	±1.54

Discussion

Pichia kudriavzevii FOL-04 showed similar growth characteristics during batch cultivation in the bioreactor with a previous study (Ndubuisi et al., 2020). Lag phases

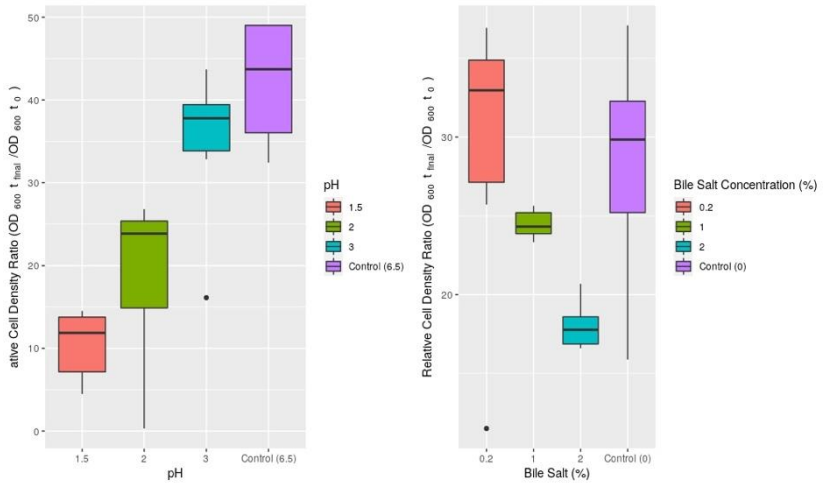

Figure 7. Relative cell density ratios of *P. kudriavzevii* FOL-04 against various pH and bile salt conditions after 42 hours. OD_{600} values of AGJ at the onset of incubation were as follows: 0.494 ±0.02 (control), 0.511 ±0.02 (pH 3), 0.470 ±0.09 (pH 2), and 0.550 ±0.02 (pH 1.5). OD_{600} values of ABJ at the start are following: 0.546 ±0.01 (control), 0.593 ±0.01 (0.2%), 0.786 ±0.02 (1%), and 1.026 ±0.06 (2%).

of both strains last about four hours in the YPD medium. Moreover, their exponential phases took around six hours. Recent studies showed that *P. kudriavzevii* is a viable candidate yeast for biodiesel (Sankh et al., 2013) and ethanol biosynthesis (Díaz-Nava et al., 2017). *P. kudriavzevii* can utilize glycerol, fructose, and glucose as a carbon source. However, *P. kudriavzevii* ITV-S42 lacks the utilization of sucrose and xylose sugars, and it ferments ethanol while sugar concentration is high (Díaz-Nava et al., 2017). A previous study on thermotolerant *P. kudriavzevii* isolated from nuruk, a traditional Korean fermentation starter, showed that *P. kudriavzevii* KCTC17763 provides optimal growth at 30°C in YPD media and synthesizes the highest amount of ethanol (Choi et al., 2017). Moreover, the *P. kudriavzevii* used in the present study performed a slightly longer exponential phase than *P. kudriavzevii* KCTC17763, MBY1358 (Choi et al., 2017), and LC375240 (Ndubuisi et al., 2020).

Bile salt and acid tolerance test results revealed that *P. kudriavzevii* FOL-04 can be a potential probiotic yeast strain. For example, *P. kudriavzevii* FOL-04 is tolerant to bile salts at similar concentrations with other *P. kudriavzevii* strains previously studied, such as M26, M28, M29, M30, M31, O6, G5, G6 (Greppi et al., 2017). These strains survived satisfactorily in pH 2 conditions although *P. kudriavzevii* FOL-04 still survived at pH 2, it showed significant loses in viability compared to pH 3 and pH 6.5. Several *P. kudriavzevii* strains are resistant to elevated temperatures and ethanol conditions, which were previously discovered (Pongcharoen et al., 2018). Chelliah et al. (2016) indicated that an *P. kudriavzevii* strain isolated from traditional Indian food had better survivability than FOL-04 in artificial gastric and bile juice. However, *P. kudriavzevii* OG32, a probiotic strain, possessed remarkably lower survivability in artificial gastric juice compared FOL-04 (Ogunremi, Agrawal, & Sanni, 2015). Chen et al. (2010) reported that *P. kudriavzevii* BY10 and BY15 strains, isolated from raw milk, showed probiotic potential in terms of survivability in ABJ and AGJ. In addition to *P. kudriavzevii* strains, *P. fermentans* BY5 sufficiently survived in ABJ and AGJ to be a potential probiotic strain (Chen et al., 2010). *P. pastoris* X-33 strain has been reported that it can survive in animal feed and gastrointestinal conditions while showing antibacterial activity against *Salmonella* Typhimurium (França et al., 2015). Similarly, *P. guilliermondii* isolated from table olives survived in low pH and high bile salt conditions also possessed antimicrobial activity against *Staphylococcus aureus* ATCC 8702, *Salmonella* Enteridis ATCC 564, and *Listeria monocytogenes* ATCC 19117 (Simões et al., 2021).

Conclusion

A new *Pichia kudriavzevii* FOL-04 was isolated from fermented plant material. We identified this strain using NL primers to amplify the D1/D2 domains of the 26S rRNA region, followed by Sanger sequencing. BLAST analysis against publicly available organisms in NCBI revealed this strain did not completely match with any other *P. kudriavzevii* strains. Artificial gastric juice and artificial bile juice trials were conducted to determine potential survivability of *P. kudriavzevii* FOL-04 against gastrointestinal conditions which revealed that FOL-04 is tolerant to low pH and high ox-bile salt conditions. *P. kudriavzevii* FOL-04 was also processed through shake flask and fed-batch fermentations to determine and compare microbial growth kinetics of this new strain. Fed-batch fermentation trials with 50% dextrose supplementation using exponential feeding regimen provided remarkably higher final biomass yield compared to shake flask experiments as measured by optical density at 600 nm wavelength. *P. kudriavzevii* FOL-04 could be a potential candidate for probiotic yeast strain with promising robustness under harsh AGJ and ABJ conditions also carrying bioprocess compatibility which should lead to further in-depth probiotic characterizations by *in vitro* and *in vivo* trials.

Author Contributions

Conceptualization: FO, Data Curation: FO, IG, Formal Analysis: FO, IG, Funding Acquisition: FO, Investigation: FO, IG, Methodology: FO, Project Administration: FO, Resources: FO, Supervision: FO, Visualization: FO, IG, Writing -original draft: IG, Writing -review and editing: FO, IG.

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