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Isolation and evaluation of stress tolerance in acetic acid-tolerant yeasts

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Abstract

Bioethanol production from lignocellulosic materials has attracted a significant amount of interest. However, a pretreatment process of the lignocellulosic materials is needed before fermentation. This process releases microbial inhibitors, particularly acetic acid which is toxic to fermenting microorganisms and reduces ethanol yields. The aim of this study was to isolate acetic acid-tolerant yeasts obtained from natural samples. Forty-three yeast strains were isolated. The obtained isolates were then examined for their acetic acid tolerance ability by spotting a ten-fold serial dilution on YPD agar supplemented with 0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 1.2% (v/v) acetic acid using *Saccharomyces cerevisiae* BY4743 as a comparator. The results showed that eight isolated yeasts could tolerate acetic acid up to 0.8% (v/v). However, one isolated yeast sample was able to grow in 1.0% (v/v) acetic acid and this was MY2/P1. Eight isolates were then selected for testing their ability to tolerate high temperatures and high concentrations of glucose and ethanol. It was found that L/A1 and S/PA1 could grow well at 42°C. Moreover, MY1/P3, S/PA1 and L/A1 were found to be able to tolerate glucose at 45% (w/v) and L/A1 and S/PA1 were found to tolerate ethanol at 12.5% (v/v). From this research, MY2/P1, L/A1, S/PA1 and MY1/P3 were found to possess the best properties in terms of acetic acid tolerance and the ability to grow under conditions involving high concentrations of glucose and ethanol. These strains were determined

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through the sequencing of the D1/D2 region of the large subunit rRNA gene (LSU) to represent to *Pichia manshurica* MY2/P1, *P. kudriavzevii* L/A1, *P. kudriavzevii* S/PA1 and *Starmerella bacillaris* MY1/P3, respectively.

Keywords: acetic acid-tolerant yeasts, osmotolerance, thermotolerance, ethanol tolerance

1. Introduction

Because there are increasing pressures for people and nations to reduce carbon footprints, and because there is a steadily increasing demand for costly and polluting petroleum-based fuels, bioethanol obtained from lignocellulose biomass, which is known to be abundant, cost effective, and renewable, has drawn a significant amount of attention for use as an alternative source of biofuel [1-3]. Lignocellulosic materials consist of cellulose, hemicellulose, and lignin [4]. Regardless of the hydrolysis methods, glucose and xylose are the most abundant sugars in cellulosic hydrolysates, which consist of 60–70% glucose and 30–40% xylose [4]. Xylose is the most abundant sugar in hemicellulose but it cannot be utilized by wild type *Saccharomyces cerevisiae*, which has long been used in industrial ethanol production [5]. Microorganisms that can convert all types of sugars (glucose, mannose, galactose, xylose and arabinose) that are present in cellulose and hemicellulose hydrolysates to ethanol, are a prerequisite for rendering the lignocellulosic ethanol processes as an economically competitive one [6, 7]. Ideal microorganisms should not only display broad substrate utilization, high ethanol yield and productivity, but they should also have other relevant abilities such as ethanol tolerance, temperature tolerance and tolerance of the inhibitors present in hydrolysate [8]. Among the various inhibitors present, weak acid is considered to be a major source of stress responsible for inhibiting cell growth and viability, as well as for limiting fermentation productivity and ethanol yield [9, 10]. High tolerance to acetic acid is a favorable phenotype for microorganisms used in industrial biotechnology, in particular with regard to using renewable feedstock such as lignocellulosic biomass [11].

Acetic acid is a weak acid generated from the deacetylation of hemicellulose during pretreatment [12, 13]. Concentrations of acetic acid present in actual biomass hydrolysates range from 7.5 to 15 g/l [14]. A variety of previous research studies have investigated the effect of acetic acid on glucose fermentation in *S. cerevisiae*. Reference [15] studied the effect of acetic acid on intracellular pH and found that the intracellular pH value inside yeast cells decreased when the amount of undissociated acetic acid was increased. A decrease in the intracellular pH value was associated with a reduction in the ethanol production rate. Reference [16] found that biomass, specific growth rate and ethanol production were reduced when concentration of acetic acid was increased.

Moreover, it was found that acetic acid not only affected ethanol fermentation in *S. cerevisiae*, but also influenced xylose-fermenting yeasts. Reference [17] found that 0.5% (v/v) (pH 4.1) acetic acid had an effect on the specific xylose-fermenting yeasts: *Candida shehatae*, *Scheffersomyces* (*Pichia*) *stipitis* CBS5773, fusant F101 and fusant F198, by reducing fermentation and growth. No growth of all tested yeasts was observed at 1.0% (v/v) (pH 3.7). These study revealed that acetic acid sensitivity was found to be more prevalent in xylose-fermenting yeast than *S. cerevisiae*.

The goal of this study was to screen and isolate acetic acid-tolerant yeasts. Moreover, the obtained acetic acid-tolerant yeasts were further tested to determine their tolerance to other stresses that affected ethanol fermentation such as heat, glucose and ethanol at elevated levels.

2. Materials and Experiment

2.1 Isolation of yeast from natural samples

Natural samples of soil, rotten fruit and decayed wood were collected from three provinces (Chiang Mai, Chiang Rai and Lampang). 1 g of each sample was added to 9 ml of Yeast extract-Malt extract (YM) broth. After thorough mixing, a further ten-fold serial dilution was achieved to obtain 10^{-1}-10^{-6} solutions of dilution. 100 µl of appropriate dilution was spread on acidified YM agar (YM agar plus 0.7% v/v HCl), which was a selective medium using for yeast isolation. Then the plates were incubated at 30ºC for 48 h. After confirming that colonies presented on the plates were yeast by microscope, they were purified by the streak plate technique on YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) and collected for testing for acetic acid tolerance.

2.2 Screening of acetic acid-tolerant yeasts

Single colonies of each isolated yeast strain grown in YPD medium at 30°C, 160 rpm for 24 h, were collected. After washing the cells with deionized water, the suspended cells (1×10^7 cells/ml) were 10-fold sequentially diluted and then spotted 5 µl of each dilution onto YPD agar supplemented with acetic acid values at 0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 1.2% v/v. These plates were incubated at 30°C for 48 h. *S. cerevisiae* BY4743 was used as a comparator. Yeast strains that could tolerate acetic acid at high levels were selected for further examination.

2.3 Testing of osmo-, ethanol- and thermo-tolerances

Cells of acetic acid-tolerant yeast were examined via the same method as was employed for the screening of acid-tolerant yeasts. Concentrations of glucose and ethanol used for testing were (25%, 30%, 35%, 40% and 45% w/v) and (5.0%, 7.5%, 10.0% and 12.5% v/v), respectively by using YPD medium as a control. To test thermo-tolerance, cells were streaked on YPD agar and then incubated at 30°C, 37°C, 40°C and 42°C for 48 h.

2.4 Identification of acid-tolerant yeasts by molecular method

Identification of the isolated strains was performed by 26S rDNA gene sequence. Genomic DNA was extracted using the existing protocol [18] with some modifications. The 26S rDNA D1/D2 domain was amplified by PCR using forward primer NL1 (5′-GCATATCAATAA GCGGAGGAAAAG-3′) and reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG-3′). The PCR products were purified using silica-based spin columns (QIAquick, QIAGEN) and the PCR products were then sequenced. The DNA sequences were submitted to the National Center for Biotechnology Information (NCBI) in order to identify them using the Basic Local Alignment Search Tool (BLAST). The evolutionary history was inferred using the neighbor-joining method [19]. The evolutionary distances were computed using the Kimura 2-parameter method [20]. Evolutionary analyses were conducted in MEGA7 [21].

3. Results and Discussion

3.1 Isolation of yeast from natural samples

The colonies of microorganisms screened from natural samples were confirmed as yeast strains using a microscope. After confirmation, forty-three yeast strains were obtained.

3.2 Screening of acetic acid-tolerant yeasts

There were 43, 42, 28, 13, 8 and 1 isolates that could grow on YPD agar supplemented with acetic acid at 0.0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0% (v/v), respectively. At a concentration of 1.2% (v/v), no growth of any isolated yeast strain was observed. The best acetic acid tolerant-yeast was MY2/P1, which was able to grow on 1.0% (v/v) acetic acid, while *S. cerevisiae* could tolerate up to 0.4% (v/v), but could not grow at 0.6% (v/v). This result was consistent with that of the research findings of [22]. It was reported that the concentration of acetic acid of at least 0.6% w/v (100 mM) could inhibit *S. cerevisiae* growth. Eight isolates that could tolerate acetic acid at up to 0.8% were MY1/P3, MY2/P1, MY2/P2, S/PA1, L/A1, MY3/4, YG1/1 and YG3/2 (Table 1). The cell morphology observed under microscope of the eight isolates that could tolerate acetic acid up to 0.8% (v/v) was shown in Fig. 1. Moreover, weak-acid resistance was examined in *Zygosaccharomyces bailii* comparing with *S. cerevisiae* [23]. The minimum inhibitory concentration of acetic acid in various strains of *Z. bailii* and *S. cerevisiae* BY4741 & NCYC 3253 was recorded at 400-500 mM (2.3%–2.8% v/v) and 120-145 mM (0.69%-0.83% v/v), respectively. Therefore, MY2/P1 tolerated acetic acid is less effectively than *Z. bailii,* but more effectively than *S. cerevisia.*

Table 1 Ability of isolated yeast strains to tolerate acetic acid

No.	Strains	Acetic acid concentration (% v/v)						
		0.0	0.2	0.4	0.6	0.8	1.0	1.2
control	*S.cerevisiae*	++++++	+++++	++	-	-	-	-
1	F/B1	++++++	++++++	++++++	++	-	-	-
2	F/C1	++++++	++++	-	-	-	-	-
3	F/C2	++++++	++++	-	-	-	-	-
4	F/C3	++++++	+++++	++	-	-	-	-
5	L/A1	++++++	++++++	++++++	+++	+	-	-
6	L/A3	++++++	++++++	++	-	-	-	-
7	L/PA1	++++++	++++++	+	-	-	-	-
8	L/PA2	++++++	++++++	+	-	-	-	-
9	MY1/P1	++++++	++++++	+++++	+	-	-	-
10	MY1/P2	++++++	++++++	+++++	+	-	-	-
11	MY1/P3	++++++	++++++	++++++	++++	+++	-	-
12	MY2/P1	++++++	++++++	++++++	+++	+++	+	-
13	MY2/P2	++++++	++++++	++++++	++++	++++	-	-
14	S/C1	++++++	++++++	++++++	-	-	-	-
15	S/C2	++++++	++++++	++	-	-	-	-
16	S/C3	++++++	++++++	+	-	-	-	-
17	S/C4	++++++	++++++	++++	+	-	-	-
18	S/C5	++++++	++++++	++	-	-	-	-
19	S/C6	++++++	++++++	+++++	-	-	-	-
20	S/M1	++++++	++++++	-	-	-	-	-
21	S/M2	++++++	+	-	-	-	-	-
22	S/O1	++++++	++++++	++	-	-	-	-
23	S/PA1	++++++	++++++	++++++	++	+	-	-
24	SM/S1	++++++	++++++	-	-	-	-	-
25	SM/S2	++++++	++++	++	-	-	-	-
26	SM/S3	++++++	++++++	++++	-	-	-	-
27	SM/S4	++++++	++++++	++++	-	-	-	-
28	SM/S5	++++++	+++++	-	-	-	-	-
29	F5	++++++	-	-	-	-	-	-
30	MY3(4)	++++++	++++++	++++++	+++	+	-	-
31	MY3(5)	++++++	+	-	-	-	-	-
32	WF2(1)	++++++	+++++	-	-	-	-	-
33	WF2(2)	++++++	++++	-	-	-	-	-
34	YG1/1	++++++	+++++	+++	+	+	-	-
35	YG1/2	++++++	+++++	++	-	-	-	-
36	YG2/1	++++++	++++++	+	-	-	-	-
37	YG3/1	++++++	+++	-	-	-	-	-
38	YG3/2	++++++	+++++	+++	++	++	-	-
39	YG4/1	++++++	-	-	-	-	-	-
40	YG5/1	++++++	+++++	-	-	-	-	-
41	YG5/2	++++++	++++++	+++	-	-	-	-
42	YG6/1	++++++	++++++	+++++	+	-	-	-
43	YG6/2	++++++	++++++	+++++	-	-	-	-

3.3 Testing of osmo-, ethanol- and thermo-tolerance

Effective yeast strains should display osmo-, ethanol- and thermo-tolerance properties. Therefore, eight isolates, which could tolerate acetic acid up to 0.8% (v/v), were selected to evaluate their tolerance levels.

With regard to testing thermo-tolerance, *S. cerevisiae* BY4743, *Kluyveromyces marxianus* DMKU3-1042 and *K. marxianus* BUNL-21 were used as comparators. The results showed that all isolates, except MY1/P3, could grow well at 30°C and 37°C. At 40°C, three isolates, MY1/P3 MY2/P2 and MY3(4) were not able to grow at these temperatures. Only two isolates, S/PA1 and L/A1, still grew well at 42°C, and at the same levels as *K. marxianus* DMKU3-1042 and *K. marxianus* BUNL-21, while *S. cerevisiae* BY4743 could grow well up until 37°C (Fig.2).

In terms of the osmo-tolerance test, the results showed that there were three isolates, MY1/P3, S/PA1 and L/A1, that could tolerate glucose at the highest concentration value of 45% (w/v), which was the same as *S. cerevisiae* (Fig. 3). MY1/P3, S/PA1 and L/A1 could tolerate glucose at the same level as osmo-tolerant yeast, namely *Zygosaccharomyces rouxii* CBS732 [24]

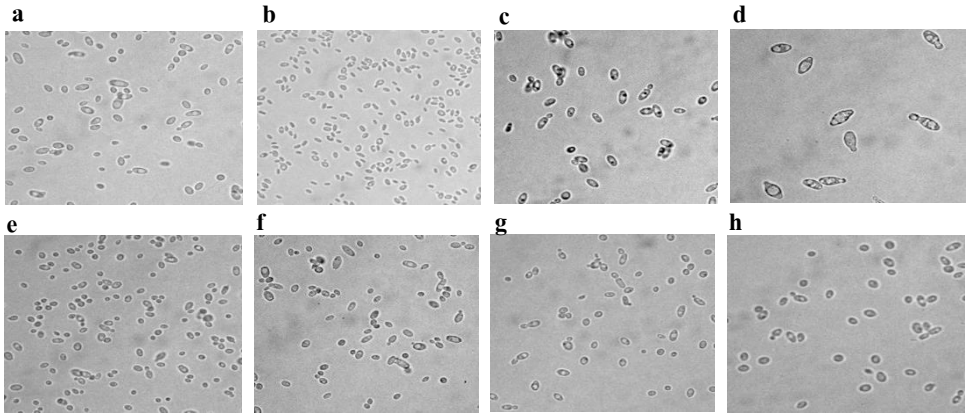

Figure 1 Cell morphology of the isolated yeast strains under a microscope at 400X that could tolerate acetic acid at values up to 0.8% (v/v). L/A1 (**a**); MY1/P3 (**b**); MY2/P1 (**c**); MY2/P2 (**d**); S/PA1 (**e**); MY3(4) (**f**); YG1/1 (**g**) and YG3/2 (**h**).

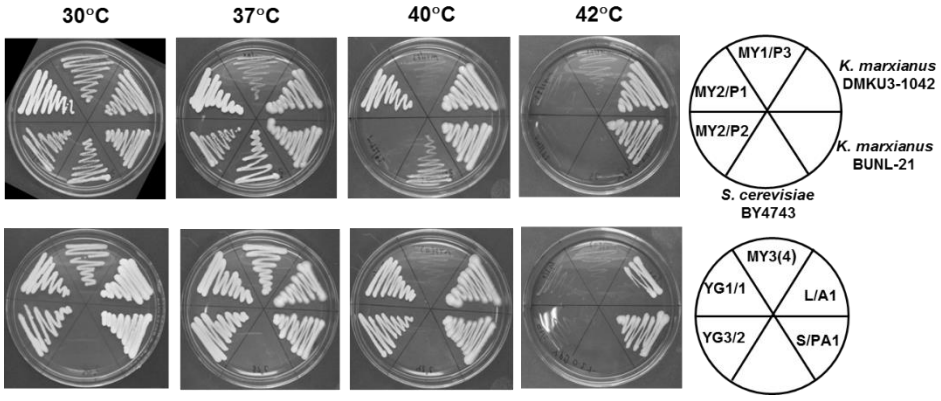

Figure 2 Thermo-tolerance of isolated yeast strains grown on YPD agar incubated at 30°C, 37°C, 40°C and 42°C for 48 h, using *S. cerevisiae* BY4743, *K. marxianus* DMKU3-1042 and *K. marxianus* BUNL-21 as comparators.

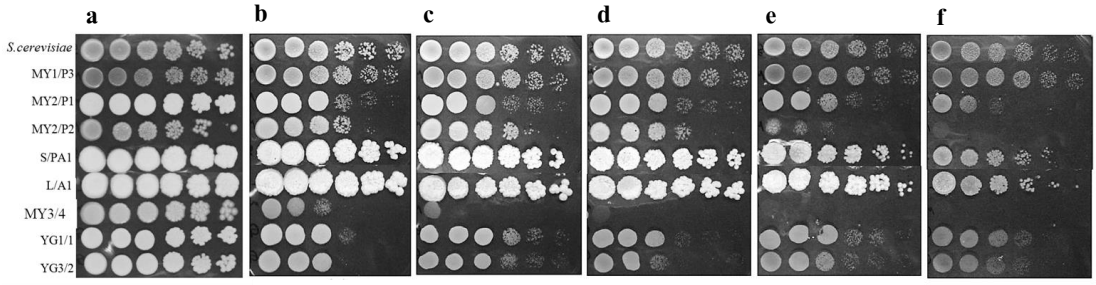

Figure 3 Osmo-tolerance of isolated yeast strains grown on YPD supplement with glucose at 0%(**a**); 25%(**b**); 30%(**c**); 35%(**d**); 40%(**e**) and 45%(**f**) using *S. cerevisiae* BY4743 as a comparator.

In terms of the ethanol tolerance test, the results showed that at elevated concentrations, the ability of growth of all isolates was reduced. All strains were normally grow at 7.5% (v/v) ethanol, except MY3(4). Only two isolates, S/PA1 and L/A1, tolerated ethanol more effectively up until 12.5% (v/v) than *S. cerevisiae*, which could grow well up until 7.5% (v/v) and its growth could be observed even at 12.5% (v/v) (Fig. 4). Reference [24] showed that various strains of *S. cerevisia*e presented different degrees of ability of ethanol tolerance from 4-12% (v/v).

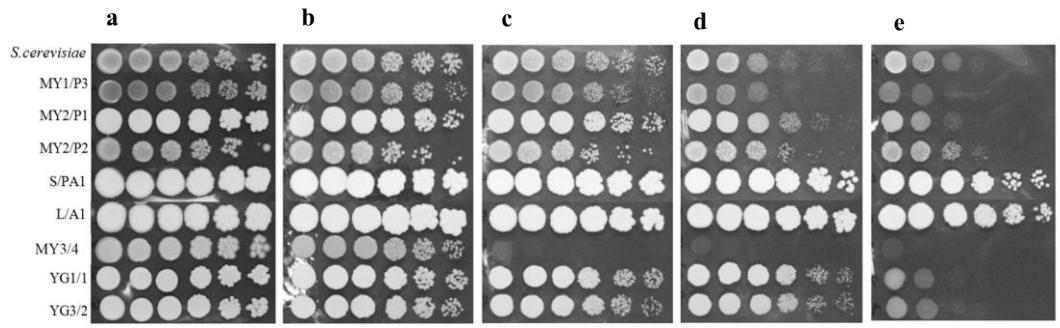

Figure 4 Ethanol tolerance of isolated yeast strains grown on YPD supplement with ethanol at 0% (**a**); 5.0% (**b**); 7.5% (**c**); 10.0% (**d**), 12.5% (**e**) using *S. cerevisiae* BY4743 as a comparator.

3.4 Identification of acid-tolerant yeasts by molecular method

Four isolates, MY2/P1, L/A1, S/PA1 and MY1/P3, that displayed high potential as candidates for ethanol producing strains due to their ability to tolerate other stresses, such as high concentrations of acetic acid or glucose or ethanol and high temperatures, were identified as *Pichia manshurica*, *P. kudriavzevii* and *Starmerella bacillaris*, respectively with 100% identity (Fig. 5). The phylogenetic tree was based on sequences of the D1/D2 domain in the large-subunit rDNA gene. It demonstrated that MY2/P1 was located in the same position as *P. manshurica*. L/A1 and S/PA1 were located in the same position as *P. kudriavzevii*, while MY1/P3 was located in the same position as *S. bacillaris* (Fig. 5).

From this study, *P. kudriavzevii* L/A1 and S/PA1 could tolerate multi-stresses that were acetic acid, glucose, ethanol and temperature at 0.8% (v/v), 45% (w/v), 12.5% (w/v) and 42°C, respectively. Several studies have revealed the application of *P. kudriavzevii* to ethanol production from various sources such as lignocellulosic waste, cassava starch, sugarcane juice and orange peels [25-33]. Moreover, *P. kudriavzevii* had been characterized as being robust and a multi-stress-tolerant form of yeast, resisting low pH values, elevated temperatures and high salt contents [27]. For example, *P. kudriavzevii* KVMP10 was found to be highly thermo-tolerant [31] (grown at 42°C as was *P. kudriavzevii* L/A1 and S/PA1 in this study). *P. kudriavzevii* D1C could produce ethanol at 40°C and revealed multi-stress tolerance towards 5-hydroxymethyl furfural, ethanol (20 %, v/v), high gravity and H_2O_2 (0.3 M). However, the growth of strain D1C was totally arrested at 0.76% (v/v) (8 g/l) acetic acid [29]. The multistress-tolerant *P. kudriavzevii* SI could tolerate acetic acid values at up to 18 g/l [33]. All the evidences support that *P. kudriavzevii* had a significant potential for applications in the ethanol production processes.

P. manshurica MY2/P1 could tolerate acetic acid at up to 1.0% (v/v) and could grow at 40°C. There have not been any reports on the acid tolerance or for other applications of the ethanol fermentation of this strain.

S. bacillaris MY1/P3 showed a tolerance to 45% (w/v) glucose. This result was in accordance with those of certain previous findings that found that *S. bacillaris* was a sugar-tolerant form of yeast [34-36]. However, no evidence to support acetic acid tolerance was found in this strain. On the other hand, this yeast was found to be suitable for the evaluation of some oenological properties in wine, but not for ethanol production [37-39].

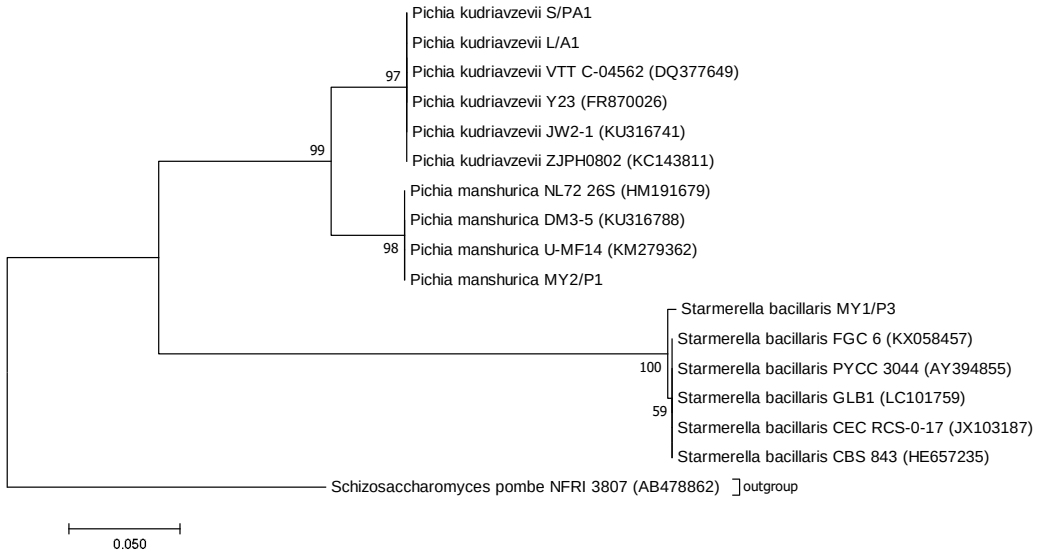

Figure 5 A phylogenetic tree is presented based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of the isolated strains with respect to the type strain of each species. The phylogenetic tree was constructed as described in the Materials and Methods section. The marker bar denotes relative branch length. Bootstrap values are expressed as percentages of 1,000 replications and are given at branch points. The numbers in parentheses are GenBank accession numbers. *Schizosaccharomyces pombe* NFRI 3807 was an out-group in the analysis. The bar represents 0.05 distances.

4. Conclusions

Pichia manshurica MY2/P1 was the most effective acid-tolerant yeast, since it was able to grow in acetic acid at up to 1.0%. Moreover, *Starmerella bacillaris* MY1/P3, *P. kudriavzevii* S/PA1 and *P. kudriavzevii* L/A1 displayed the most noteworthy properties for multistress-tolerance. From this study, *P. kudriavzevii* S/PA1 and L/A1 were of the greatest interest because they were tolerant of acetic acid, glucose, ethanol and temperature at values of 0.8% (v/v), 45% (w/v), 12.5% (w/v) and 42°C, respectively.

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