

ISOLATION AND CHARACTERIZATION OF A *PICHIA ANOMALA* STRAIN: A PROMISING CANDIDATE FOR BIOETHANOL PRODUCTION

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ABSTRACT

A yeast strain designated as Y-1 was isolated and characterized from wine yeast ("Jiuqu"). Based on the morphological and biochemical results, along with the rDNA internal transcribed spacer region (ITS), Y-1 was identified to be a *Pichia anomala* strain. Y-1 is an ethanol-tolerant strain, enduring ethanol concentrations of up to 14 %. Y-1 growth medium conditions were optimized, results showing good growth in medium with pH ranges from 3.5-6.5, temperature ranges from 25-30 °C, and inoculums range of 8 %-12 %, while optimum growth conditions were reached at a temperature of 30 °C, pH 5.0, and inoculums of 10 %. Furthermore, when the alkaline hydrolyzed Shatian pummelo peel solutions were inoculated with 10 % Y-1 and fermented at 30 °C for 6 d, 4.7 % pure ethanol (w/w) was produced, as evidenced by gas chromatography analysis. Our present study shows potential for the Y-1 strain to be a promising candidate for bioethanol production.

Key words: Bioethanol; gas chromatography analysis; Jiuqu; *Pichia anomala*

INTRODUCTION

With the challenges facing the world's energy security, growing attention has been devoted to the production of renewable and clean fuel alternatives to fossil fuels. One of the most prevalent renewable fuel sources is ethanol (29, 30). In many parts of the world, demand for ethanol as an alternative fuel source has steadily increased due to dwindling fossil fuel resources and increases in gasoline prices, as well as efforts in decreasing the overall amount of greenhouse gases emitted into the atmosphere (11, 32). Accordingly, ethanol fuel production from biomass at an industrial level shows great potential for satisfying future world energy demands (13).

The production of bioethanol is a biological process in which sugars such as glucose, fructose, and sucrose are converted into cellular energy by microbial fermentation and thereby produce ethanol and carbon dioxide as metabolic waste products. The microorganisms employed in the fermentation of sugars into ethanol are principally bacteria and yeasts (16). Yeasts have proven to be more robust than bacteria by being more tolerant to ethanol (11, 23). Yeast strains are highly desired with good enological properties, such as high fermentation activity, high yields of ethanol, tolerance to ethanol, high temperature, and growth at a high osmotic pressure (2, 7, 26).

"Jiuqu," also called wine yeast or distiller's yeast, is produced with a technique that uses microbiological enzymes.

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This yeast has been selected by humans for more than 8000 years under conditions that favored the evolution towards several specialized features, such as fast growth in high-sugar fruit juices, high yield of and resistance to ethanol (14). The normally isolated strains from “Jiuqu” are yeasts, *Rhizopus*, and small quantities of *Aspergillus*(19). When compared to other industrial or laboratory strains, wine yeast strains usually exhibit a higher tolerance to ethanol and therefore produce high yields of ethanol. For this reason, there is a high demand for ethanol strains from “Jiuqu.”

The main objective of this study was to isolate, identify and characterize a yeast strain from a local “Jiuqu.” The effects of temperature, pH and inoculums of the isolated yeast strain on the culture process were also studied. In addition, ethanol production from citrus peel waste inoculated with the isolated yeast was observed.

MATERIALS AND METHODS

Plant materials

“Jiuqu” described herein was collected from the local winery. Fruits of Shatian pummelo (*C. grandis* Osbeck) were purchased from the supermarket of Xiangtan University, Xiangtan, China. Prior to hydrolysis, the peel of the Shatian pummelo fruit was subjected to a sterilization process in order to remove limonene, a monoterpene that inhibits yeast and other microorganism (30). After sterilization peels were dried at 40 °C and ground into powder for further use.

Yeast isolation

One gram of “Jiuqu” was added into 100 ml of sterilized physiological saline. The suspension was serially diluted with sterilized distilled water. A 0.1 ml aliquot of the diluted suspension was spread on the YPD medium containing 1 % yeast extract, 2 % peptone, 2 % dextrose and 2 % agar. The plates were incubated at 28 °C for 2~3 d. The predominant representative colonies were selected based on colony

morphology differences under a microscope. Selected colonies were sub-cultured on new plates and purified by repeated streaking. The isolated colonies were maintained on YPD agar slants at 4 °C for further identification.

Phenotypic identification of the isolated yeast

The purified yeast colonies were subjected to standard tests and classification schemes as described by Kreger-van Rij (1984) (17).

Molecular characterization

The molecular method for yeast identification was based on the amplification and sequence analysis of the ribosomal DNA internal transcribed spacer region (ITS) (5). The primers used to amplify the rDNA internal transcribed spacer region were ITS1 (5'-CGG GATCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-CGGGATCCTCCGCTTATTGAT ATGC-3'). The amplification reaction was done in 20 µl (final volume) containing 20 pmol of each primer, 300 ng of genomic DNA template, 0.2 mM dNTP, 1.5 mM MgCl₂ and 1 U Taq polymerase. The reactions were run for 40 cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The PCR products were cloned into the pMD18-T vector and sequenced. Sequences of the PCR product were compared with the ITS region deposited in the GenBank (<http://www.ncbi.nlm.nih.gov>) and the percentage of similarity among the fragments was calculated using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Ethanol tolerance

The yeast isolates were inoculated in 10 ml of liquid YPD (10 g/l yeast extract, 10 g/l peptone, 20 g/l glucose) supplemented with 80, 100, 120, 140 and 160 ml/l ethanol and incubated at 28 °C for one week. The increase in cell number was determined by the gas amount collected in the Durham's tubes.

Determination of growth parameters of the isolated yeast

Temperature: Overnight cultures of the yeast strain were centrifuged at 5000 rpm for 10 min and cells were washed twice with sterilized normal saline. Then, cells were suspended in the same solution to give a concentration of about 10^7 cfu/ml. An aliquot of 10 μ l suspension was inoculated into the YPD liquid medium and incubated at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C for 72 h. The increase in cell number was determined by measuring the optical density (OD) of cultures at 600 nm.

pH: Activated yeast was inoculated into YPD liquid medium with varying pHs. The pH of the broth was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 by sodium hydroxide or sulfuric acid. All pH measurements were done on an Orion 710A pH meter equipped with a glass electrode calibrated for H⁺ ion concentration. After growth at 30 °C for 24 h, the OD value of each broth was determined at 600 nm by a spectrophotometer.

Inoculum: Overnight cultures of the yeast strain were centrifuged at 5000 rpm for 10 min and cells were washed twice with sterilized physiological saline (NaCl 8.5 g/l). Then, cells were suspended in the same solution to give a concentration of 0.01 g/ml, and a specified volume of the strain suspension was added into the YPD liquid medium to reach 6 %, 8 %, 10 %, 12 %, 14 % inoculation quantity. After growth at 30 °C for 24 h, the OD value of each broth was determined at 600 nm by a spectrophotometer.

The orthogonal experiment: Based on the single factor experiments, a L₉ (3³) orthogonal experimental design was used to investigate the effect of A (temperature: 25 °C, 30 °C, 55 °C), B (pH: 4.0, 4.5, 5.0) and C (inoculum: 8 %, 10 %, 12 %) on the culture process.

Fermentation condition and analytical techniques

Ten grams of dry peel powder of Shatian pummelo was suspended in 150 ml of 10 % NaOH. After soaking in 10 % NaOH for 20 min, a proper amount of 6 M H₂SO₄ solution was added to adjust the pH to 5.0. Then the solution was filtered

through a piece of gauze and 150 ml filtrate was obtained. The sterilized solution was inoculated with 10 % Y-1. After fermenting at 30 °C for 6 d, enough CaO was added to the fermentation broth to remove the water. The resulting solutions were subjected to distillation and were analyzed by a Capillary Gas Chromatogram. The column was FFAP (0.32 mm \times 30 m, 0.25 μ m). The column temperature was increased from 50 °C to 120 °C, and the rate was 10 °C/min. The FID temperature was set at 240 °C, and the carrier gas was nitrogen whose flow rate was 5 ml/min. The internal standard was n-butanol.

RESULTS

Isolation and identification of the isolated yeast

A yeast strain, designated as Y-1, was successfully isolated from the local "Jiuqu." The colony morphology of this strain was ivory-white in color, smooth with a finely serrate margin (Figure 1). It could form one ascospore (Figure 2), and no pseudo-hypha was observed during cultivation. In the fermentation sugar source test, it was capable of utilizing almost all the test sugars (glucose, maltose, sucrose, galactose and raffinose) except lactose (Table 1). In the assimilation carbon source test, it could make use of the entire tested carbon source except methanol (Table 1). Y-1 could assimilate both ammonium sulfate and potassium nitrate. In addition, Y-1 could grow well on the substrate without vitamins and with high osmotic pressure. It also had the potential ability to produce a compound similar to starch. According to these morphological properties, Y-1 was preliminary identified to be a *Pichia anomala* (= *Hansenula anomala*) strain (5, 17).

To confirm the taxonomic identity of Y-1, the 18s rDNA ITS region was cloned and sequenced. After PCR amplification using the ITS1 and ITS4 primer combinations, a fragment about 600 bp in size was obtained (Figure 3). Sequencing results revealed that it was 617 bp in length (Figure 4). BLAST results showed that it shared complete identity with the corresponding sequences of *Pichia anomala* (EU343844,

AB469881, AB467307, and EU380207) deposited in GenBank. Based on these results, Y-1 was identified to be a *Pichia anomala* strain.

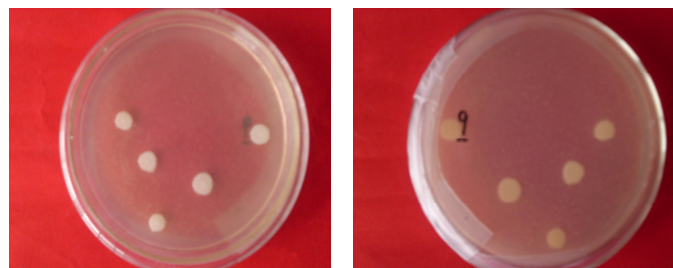


Figure 1. The pictures of purified yeast strains in the medium
A. The face of medium **B.** The inverse of medium

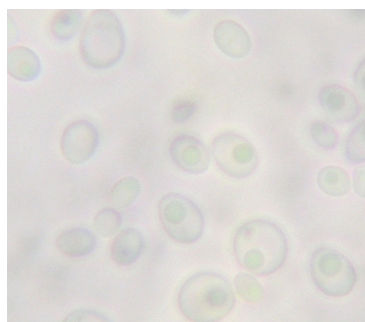


Figure 2. The ascospore morphological characters of Y-1

Table 1. Biochemical characteristics of Y-1

Characteristic	results
Sugar fermentation	
1.Glucose	+
2.Maltose	+
3.Sucrose	+
4.Lactose	-
5.Galactose	+
6.Raffinose	+
Carbon sources assimilation	
7.Lactose	+
8.Xylose	+
9.Arabinose	+
10.Citric Acid	+
11.Amidulin	+
12.Methanol	-
13.Glycerin	+
Assimilative capacity of ammonium sulfate	+
Assimilative capacity of potassium nitrate	+
The growth on the substrate without vitamin	+
Producing similar starch compound	v
The growth on the substrate with high osmotic pressure	+

Note “+” mean positive; “-” negative, “v” mean variable.

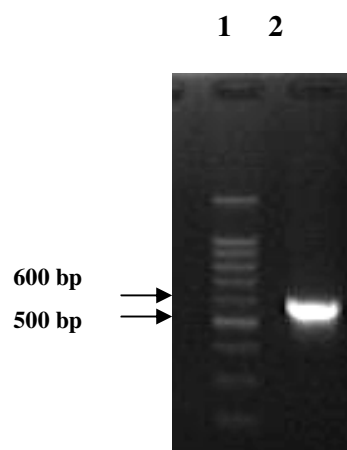


Figure 3. PCR product of the ITS region of Y-1. Lane 1: 100 bp DNA ladder, lane 2: the PCR product.

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TCCGTAGGTGAACCTGCGGAGGATCATTATAGTATTCTATTGCCAGCGCTTAATTGCGCGCGATAAACCTTACACAC
ITS1
ATTGCTAGTTTTTTTTGAACITTTGCTTTGGGTGGTGAGCCTGGCTTACTGCCCAAAGGCTTAAACACATTTTTTTAATG
TTAAACCTTTAACCAATAGTCATGAAAATTTTTAACAAAAATTTAAATCTTCAAACTTTCAACAACGGATCTCTGG
TTCTCGCAACGATGAAGAADGCAGCGAAATCGGATACGTATTGTGAATTGCAGATTTTCGTGAATCATCGAATCTTTGA
ACGCACAITGCACCCTCTGGTATTCCAGAGGGTATGCCTGTTTGAGCGCTATTCTCTCTCAAACCTTCGGGTTTGTA
TTGAGTGATACTCTGTCAAGGGTTAACTTGAATATTGACTTAGCAAGAGTGTACTAATAAGCAGTCTTTCTGAAATAA
TGTATTAGGTTCTTCCAACCTGTTATATCAGCTAGGCAGGTTTAGAAGTATTTTAGGCTCGGCTTAACAACAATAAAT
AAAAGTTTGAECTCAAATCAGGTAGGACTADCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
ITS4
    
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Figure 4. Sequencing results of the ITS region of Y-1. Primer sequences were underlined

Ethanol tolerance

Table 2 shows the effect of ethanol on the growth rate of Y-1. As revealed by Table 2, Y-1 could grow well in the presence of 8 % and 10 % ethanol. The growth of Y-1 was remarkably inhibited with the increase of exogenously added ethanol. Only 3/4 gas of the Durham's tube was obtained in the presence of 12 % ethanol, and even half the amount of gas was present in the tube in the presence of 14 % ethanol. The growth of Y-1 was severely inhibited in the presence of 16 % and 18 % ethanol, since no gas was released. Consequently, it could be inferred that Y-1 was able to endure 14 % ethanol.

Table 2. Ethanol endurance of Y-1

	Ethanol Degree (v/v)					
	8 %	10 %	12 %	14 %	16 %	18 %
Y-1	++++	++++	+++	++	-	-

++++: the Durham's fermentation tube was full of gas; +++: the Durham's fermentation tube was filled with 3/4 gas; ++: the Durham's fermentation tube was filled with 1/2 gas; -: no gas was observed

Optimization of culture conditions

Temperature: As revealed by Figure 5A, the OD value increased as temperatures increased from 25 °C to 30 °C, and then declined when the temperatures were above 30 °C. A sharp decrease of OD values was detected when the temperature increased from 35 °C to 45 °C. The OD value almost reached zero at 40 °C and 45 °C, suggesting that 40 °C might be a lethal temperature for Y-1.

pH: The effects of pH on the yeast cell growth are given in Figure 5B. The OD value increased by a fraction when the pH ranged from 3.0 to 4.5, while 4.5 was obviously the optimal pH for Y-1. The medium pH fluctuation between 5.0 and 6.0 did not significantly affect the growth rate of Y-1. When the pH ranged from 6.5 to 7.5, it underwent a remarkable decrease in OD, reducing from 0.997 to 0.415.

Inoculum: The effect of inoculum on the fermentation process was shown in Figure 5C. The biomass increased

steadily when the inoculums varied from 6 % to 10 %. There was a moderate decrease when the inoculums varied from 10 % to 12 %. In contrast, a notable decrease was observed between inoculums of 12 % and 14 %.

Orthogonal experiment: The optimal conditions for the culture process were obtained by using orthogonal design $L_9(3^3)$ based on single factor experiments. Table 3 showed factors at different levels in nine experiments conducted and the statistical analysis. Results showed that the order of the effect of factors affecting the fermentation process was found to be: $B > A > C$. The optimum culture conditions obtained from the statistical analysis were $A_2B_3C_2$. The optimal culture conditions for Y-1 were suggested to be at a temperature of 30 °C, pH 5.0 and inoculums of 10 %. These conditions were later tested to ascertain the reliability of these results. As a result, the OD value in the experimental result was 2.56, higher than results in the orthogonal experiment. Therefore, the hypothesis of the orthogonal experiment was valid.

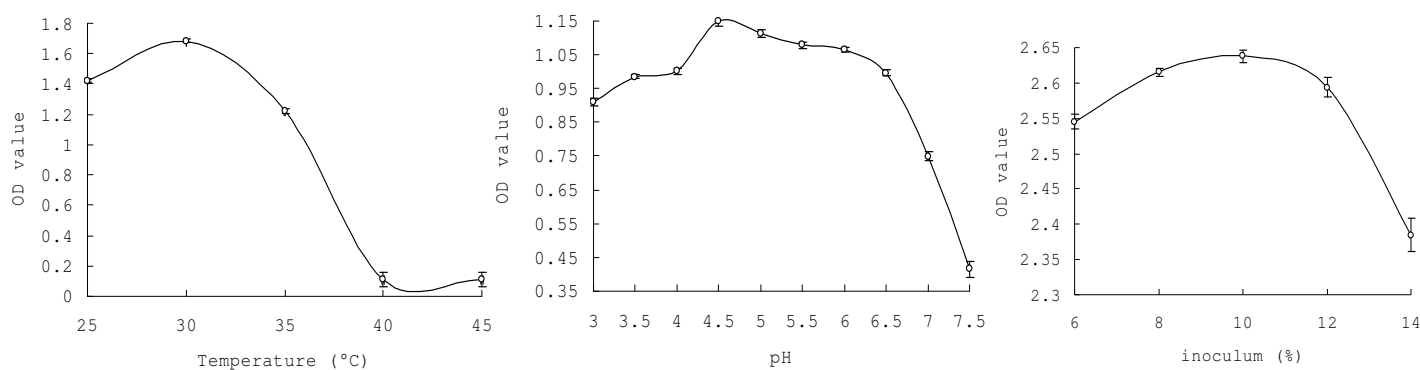
**Figure 5.** Influences of temperature (A), pH (B), inoculum(C) on the growth of Y-1

Table 3. The experimental designs and the orthogonal test results

Run	A	B	C	OD value
1	1	1	1	2.184
2	1	2	2	2.347
3	1	3	3	2.411
4	2	2	3	2.393
5	2	3	1	2.346
6	2	1	2	2.341
7	3	3	2	2.396
8	3	1	3	2.094
9	3	2	1	2.174
K ₁	6.942	6.619	6.704	
K ₂	7.080	6.914	7.084	
K ₃	6.664	7.153	6.898	
k ₁	2.314	2.206	2.235	
k ₂	2.360	2.305	2.361	
k ₃	2.221	2.384	2.299	
R	0.139	0.178	0.126	

The yield of ethanol from alkaline hydrolyzed citrus peel

When the fermentation broth was distilled, about 6ml of ethanol solution was obtained. It was then analyzed by Gas chromatography. As revealed by Figure 6A and 6B, only one peak of the collected sample was observed, which was consistent with the typical peak of the ethanol standard. The yield of ethanol was calculated to be 46.9 ± 1.25 g/l.

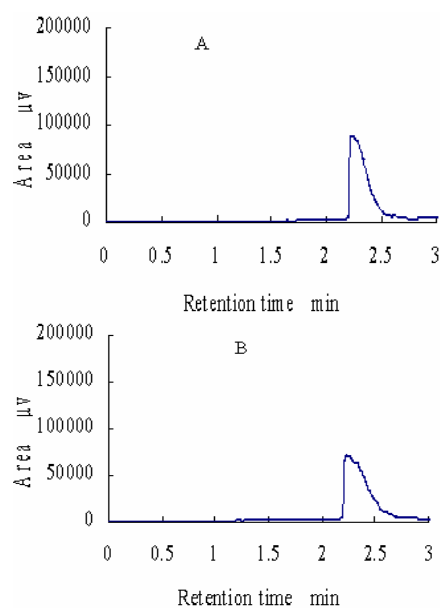


Figure 6. GC analysis of the ethanol (A: samples; B: the ethanol standard)

DISCUSSION

It is commonly believed that in a natural or spontaneous fermentation non-*Saccharomyces* yeasts such as *Candida*, *Debaryomyces*, *Hanseniopsis*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulopsis*, *Zygosaccharomyces* and many others grow and participate in early stages of fermentation (15). As the fermentation proceeds, the ethanol concentration increases. This (high ethanol) limits the growth and activity of the native non-*Saccharomyces* yeasts thus creating a condition favorable to the growth and domination of native *Saccharomyces* yeast, which then conduct the fermentation (12, 19). Therefore, bioethanol is dominantly produced by different *Saccharomyces cerevisiae* strains (10, 11, 21, 22). However, recent researches, as well as our present study, indicate that in many cases non-*Saccharomyces* strains also showed potential for ethanol production. A previous study revealed that ethanol yields produced by *Kluyveromyces marxianus* from hydrolyzed Valencia orange peel waste could be 37.1 g/l, close to that of 40.9 g/l by *Saccharomyces cerevisiae* (30). The ethanol produced by *Kluyveromyces marxianus* in shaking flask cultivation in sugar cane juice media at 37 °C reached 8.7 %

(w/v), productivity 1.45 g/l/h and yield 77.5% of theoretical yield (20). Tomás-Pejó *et al* (28) reported that the ethanol concentration from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus* could be 36.2 g/l. In our present study, the final ethanol yield from hydrolyzed Shatian pummelo peel waste was measured to be 46.9 g/l, which was near the theoretical value (33).

Ethanol endurance is an important property concerning fermentation efficiency. Ethanol is a well known toxic metabolite for yeast cells. Ethanol stress inhibits the amino acid transport system and glucose transport, and can lead to the loss of cell viability and the inhibition of cell growth (1, 18, 25). The rates of ethanol production are reduced with the accumulation of ethanol in the culture broth, especially when high concentrations of sugar substrates are used (24). Therefore, industrial yeast with high ethanol tolerance is highly desired for the improvement of ethanol concentration. It was generally accepted that *S. cerevisiae* was the most ethanol-tolerant species (9), with an average ethanol endurance of 12 % (v/v), which varies depending on the strain (27). However, our present data supports that non-*S. cerevisiae* yeasts may also be ethanol-tolerant, given that Y-1 was able to endure 14 % of ethanol.

In the conversion process of sugars to ethanol, growth of microorganisms was highly linked with the stress or environmental factors in the culture medium, which thereby affected the fermentation efficiency (3). These factors included temperature, osmotic stress, anaerobic conditions, heavy metals, growth regulators, ultraviolet radiation, metabolic repressors, pH and so on (4, 6, 8, 11, 28, 31). Consequently, a sound understanding of these factors is essential to achieve a successful fermentation and an increased ethanol yield. Our present study demonstrated that the optimum culture condition for Y-1 was a temperature of 30 °C, pH 5.0, and inoculums of 10 %, which was similar to those of some *S. cerevisiae* strains in bioethanol production, suggesting that Y-1 could also be widely used in the ethanol industry.

In conclusion, we have successfully isolated a *Pichia anomala* strain from Jiuqu, which demonstrated tolerance to high ethanol concentrations and showed potential as an ideal strain for bioethanol production.

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