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Isolation and identification of yeasts from local fruits in Thua Thien Hue province, Vietnam

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Abstract. In this study, a total of 30 yeast isolates were recovered from local fruits in Thua Thien Hue province. Genetic characterization based on the ITS sequences identified isolates belonging to 3 species including *Saccharomyces cerevisiae, Lachancea fermentati*, and *Clavispora fructus*, with high sequence homology (over 99%) compared to published sequences in the GenBank. All identified *S. cerevisiae* isolates could grow well at 30°C and ferment several sugar including fructose, galactose, sucrose, mannose, maltose, and raffinose with different performances, but were inhibited at temperature higher than 35°C. The strains also grew well in the medium containing 5% ethanol (v/v) and 200 g/L glucose, but their growth ability was decreased gradually with an increase in ethanol and glucose concentrations. Interestingly, D14 strain was able to grow in the medium supplemented with 12% of ethanol, and 500 g/L of glucose at 45°C, while D7 strain could utilize both mannitol and glycerol at a low level. Our results also indicated that some strains have relatively high sedimentation efficiency, which are favorable conditions for beer fermentation and biomass recovery. The isolated yeast strains with good tolerance properties may provide a potential source of valuable raw materials for applications in beverage production and food processing.

Keywords: Yeast, fermentation, tolerance, high temperature, *Saccharomyces cerevisiae*

1 Introduction

Yeasts play an essential role in various fermentation processes such as baking and brewing, while the ethanol released by yeast, carbon dioxide is of utmost need for the rising of flour dough, maturation, and creation of flavor [1]. Fermentation is a relatively complex process in which numerous adverse conditions may damage yeast cells, for example, osmotic pressure, ethanol concentration, and high temperature [2]. Therefore, tolerance to high temperatures is one of the most desirable characteristics of yeasts, which is of interest in the fermentation industry. In particular, this property is beneficial for reducing cooling costs, increasing conversion rates of sugar to ethanol, and reducing contamination by other strains, resulting in an increase in fermentation productivity [3]. Thermotolerant yeasts isolated from nature have gained considerable attention since they can grow and ferment efficiently in uncomfortable conditions [4–6]. Particularly, these properties are usually not found in *S. cerevisiae* which has been widely used for industrial production at optimal temperatures of around 25-35°C. For example, some isolated yeasts can grow and ferment at temperatures above 40°C, such as *Kluyveromyces marxianus* [7], *Pichia kudriavzevii* [8], and *Candida tropicalis* [9].

New *S. cerevisiae* strains from different sources are expected to have different phenotypic and genotypic profiles in comparison with

traditional strains used in the industry [10]. The wild yeast *S. eubayanus* has been used in the industrial production of lager beer [11]. Similarly, the *S. uvarum* strain isolated from apple chicha also exhibited a good potential for the production of commercial cider without any apparent flavour defects [12]. Many other yeast species are also emerging as candidates for industrial production of food and beverages [13].

Vietnam has a long history of agriculture and diverse ecosystems along whole regions grant the existence of more than 13,000 plant species, belonging to 3,500 genera and 500 families, 60% of which are indigenous origin that constitute a potential source of yeast strains [14]. Among them, fruits harbour a complicated community of yeast species associated with spontaneous ethanol ic fermentation [15]. Thua Thien Hue province, located in the North Central Coast region of Vietnam, has a transitional climate between two regions of the North and South of Vietnam, scorching in the dry season and humid cold in the rainy season. Therefore, this is one of the most abundant places in fruit biodiversity.

The aim of this study is to isolate some *S. cerevisiae* strains that can tolerate high temperature, ethanol content, and exhibit flocculation from local fruits in Thua Thien Hue province.

2 Materials and Methods

2.1 Materials

Fruit samples (mango, orange, pineapple, and mangosteen) without growth stimulants and pesticides were collected from the farms in Thua Thien Hue province, Vietnam. These samples were preserved in sterile plastic bags and left to naturally ferment at room temperature (25-30°C) for 2-5 days.

Medium for yeast inoculation: YEPD broth composes of 1% yeast extract, 1% peptone, and 2% dextrose.

2.2 Methods

Yeast isolation

Yeasts were isolated in accordance with a previous study of Nguyen [16] with some modifications. Briefly, ground 1 g of fermented fruit (non-peels) in 0.5 mL of sterile NaCl 0.9%, followed by centrifugation at 3,000 rpm for 1 min and removal of the supernatant. The precipitate was resuspended in YEPD broth and serially diluted. Finally, 50 µL of the solution was spread on YEPDA plates (YEPD supplemented with 1.5% agar, 50 µg/mL ampicillin, and 50 µg/mL chloramphenicol). The plates were then incubated at 35°C for 24-48 h. Yeast was preliminarily identified by morphological method according to the yeast classification guideline by Kurtzman [17]. Observing the yeast-like colonies was implemented by a microscope (Olympus).

DNA extraction and yeast identification

Single yeast-like colonies on YEPDA were inoculated in 10 mL YEPD broth with shaking incubation (160 rpm) at 35°C for 20 h. Cells were collected by centrifugation at 10,000 rpm for 1 min and supernatant discard. Then cells were resuspended in 200 µL of buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA pH 8.0) [18]. Complete solidification of these solutions occurred by deep refrigeration at -70°C for 5 min and thereupon rapidly soaked in boiled water at 95°C for 10 min. The freeze-thaw procedure was repeated once more and vigorously vortexed for 30 sec. After that, 200 µL of phenol-chloroform solution was added, and centrifuged at 13,000 rpm for 5 min. The upper clear supernatant was then transferred to a new tube containing 400 µL of ethanol 95%. DNA was

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precipitated at -20°C for 30 min before centrifuging at 13,000 rpm for 10 min at 4°C. After removal of the supernatant, the pellet was washed with 0.5 mL of ethanol 70% twice, and dried at room temperature for ethanol evaporation. Finally, pellet was dissolved in DNase-free water (TOP-Bio, Czech Republic) and stored at -20°C for further experiments.

One µL of genomic DNA (100 ng/µL) was used for amplification of the internal transcribed spacers regions ITS1 and ITS2 using forward primer ITS1 (5′-CAAGGTTTCCGTAGGTGAAC-3′) and reverse primer ITS4 (5′-CGGGTACTCCTACCTGATTT-3′) as described in Fig. 1 based on ITS1/2 sequences in the *Saccharomyces* Genome Database (SGD). The PCR program was pre-denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were checked by electrophoresis in 1.2% agarose gel, and purified by PCR purification kit (Favorgen, Taiwan). Purified PCR products were directly sequenced by the Sanger sequencing method at GenLab (Hanoi, Vietnam) using primer pair ITS1 and ITS4 as mentioned above. Yeasts were identified based on the ITS1/2 sequencing analysis. Sequences were compared pairwise using the Basic Logarithmic Alignment Search Tool (BLAST) algorithm in the National Centre for Biotechnology Information (NCBI) database (minimum of 97% sequence similarity and 95% coverage).

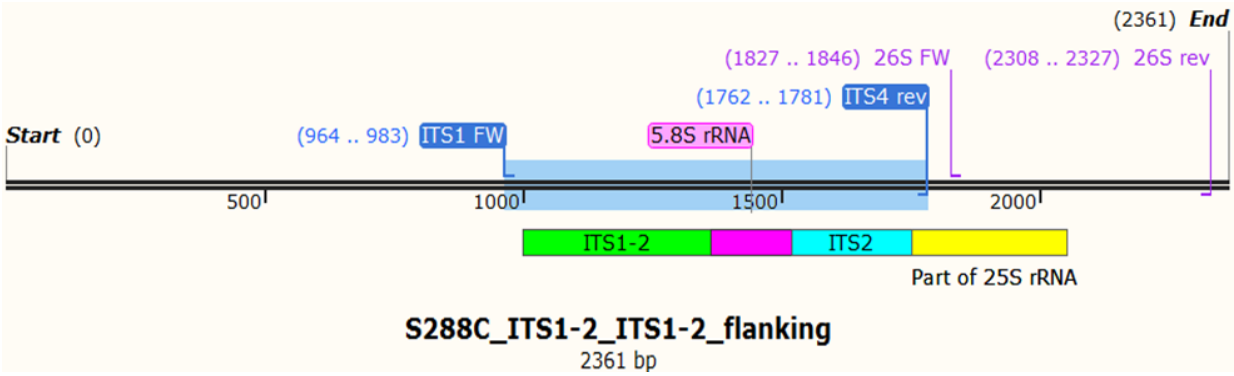

Fig. 1. Internal transcribed spacers (ITS) regions ITS1 and ITS2

Examining high temperature and ethanol tolerance of yeast isolates

To evaluate the growth ability under stress conditions as ethanol tolerance of each yeast strain, ethanol was supplemented in YPD broth (2% glucose, 1% yeast extract, 1% bacto peptone [w/v]) to reach the final ethanol concentration (0, 2, 5, 8, and 12% [v/v]). Growth curve was defined by optical density at wavelength 600 nm (OD_{600}) after incubating for 24 h at 30°C.

Analogously, verified yeast strains were cultivated in YPD broth at different temperatures, including 20, 25, 30, 35, and 45°C, for 24 h. The growth diagram of strain was developed based on the survival capacity of cells in proportion to every level of temperature.

Fermentation by different carbon sources

Glucose fermentation of selected strains was investigated in a YPD broth medium containing glucose level of 20, 100, 200, 400, and 500 g/L at 30°C for 24 h.

These strains were inoculated in the medium of dependent carbon ingredients such as glycerol, maltose, galactose, fructose, raffinose, mannose, sucrose, and mannitol with each sugar

concentration of 100 g/L, thereby assessing the assimilation of hydrocarbons by measuring at OD_{600}.

Flocculation test

The flocculation of yeast cells was determined according to Bester et al. [19]. Yeast cells were cultured in YD broth (glucose 2%, yeast extract 1% [w/v]) for 48 h, then cells were collected by centrifugation at 13,000 rpm. The sedimentation and cell interactions were disrupted by deionized water with EDTA (pH 8, final concentration 50 mM), and the cell suspension OD_{600} was determined (as A). Then, the cells were washed twice with distilled water and suspended in 30 mM $CaCl_2$. After being allowed to stand for 60 seconds, the OD_{600} of the upper layer of the cell suspension was measured (as B). The flocculation proportion (%) was calculated using the formula: 100×(A-B)/A. The flocs or free cells were imaged by a transmitted light microscope (DMR microscope, Leica, Germany).

3 Results and discussion

3.1 Morphological and phenotypical characteristics

One hundred and twenty yeast-like colonies were isolated from the collected fruits on YEPDA plates. The isolated yeast strains exhibited identical morphology, such as whitish or cream color and round shape, but there were differences in the surface and margin of colony morphology. This result is consistent with the description by Kurtzman et al. [17], who reported that yeasts exhibited a range of colors from white-cream to tan, besides varying in texture, surface, elevation, and margin.

Based on the morphology, color, and dimension of colonies, 30 typical isolates were selected and divided into 6 groups, as described in Table 1. Colony morphology with rough, smooth, or flat outside was captured by a camera ProgRes® CT3 CMOS (Germany) (Fig. 2), while oval or ellipse of yeast cell shape was observed by a microscope (Olympus) with 100x magnification (Fig. 3).

Table 1. Characteristics of yeast colonies in each group

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Smooth and wet on surface	Big and milk white	Rough	Smooth	Rough and edge on surface	Big and quite rough
isolates: C1, C5, D7, D8, D9, M20, N21	C4, D11, D12, D13, X30	D14, M15, M16, M17, M18, M19	C2, D10	N22, N23, N24, N25, X28,	C3, D6, X26, X27, X29

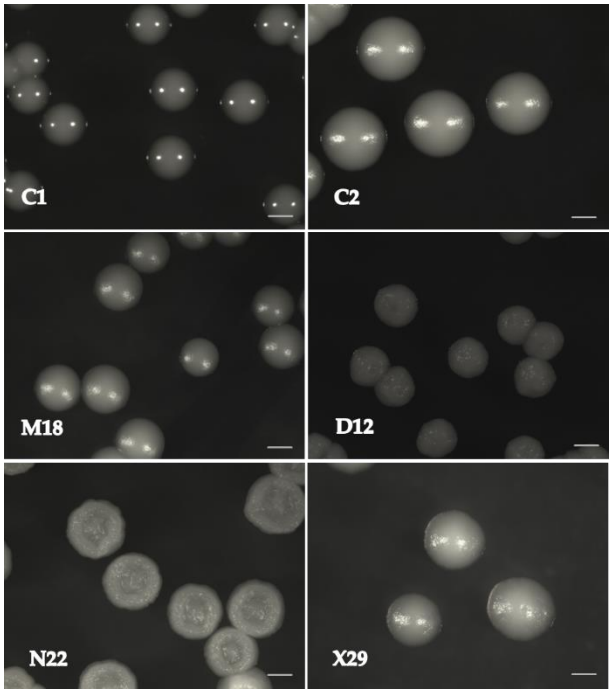

Fig. 2. The colony morphologies of isolated yeast strains *(C1, C2, D12, M18, N22, and X29).* Bar, 1 mm

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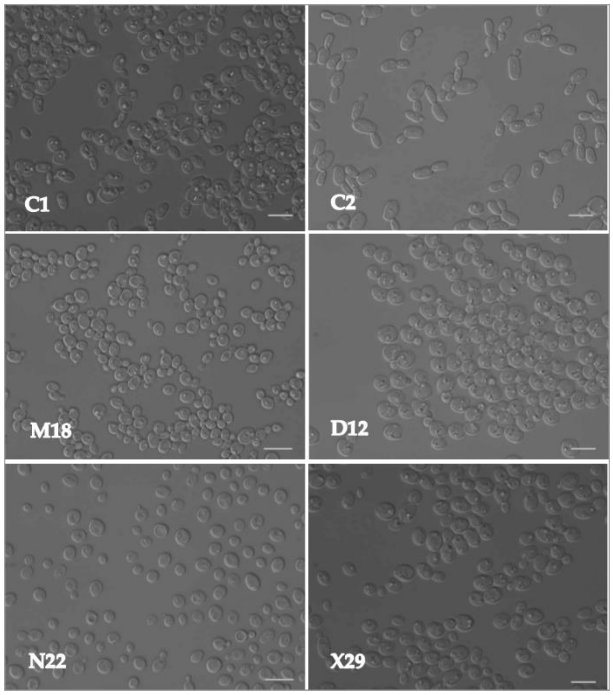

Fig. 3. The cell shapes of isolated yeast strains *(C1, C2, D12, M18, N22, and X29).* Bar, 10 µm

3.2 Identification of yeast strains

Sequencing fragments belonging to the 5.8S-ITS of 30 yeast isolates were amplified by PCR as described above and checked by electrophoresis on 1.2% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen) in 1x TAE buffer (Fig. 4). The results indicated that PCR products of 400-600 bp in size depending on each strain were amplified successfully, except D11 (that has two bands, it may be contaminated or multiply non-specific gene). Based on the preliminary data about morphology, and the PCR results, 12 isolates named C1, C2, C3, C5, D6, D7, D10, D14, M19, N22, X29, and X30 were further characterized by DNA sequencing.

Comparing the ITS1/ITS2 sequences of these isolates with published sequences on GenBank, seven isolates namely C1, C5, D7, D14, M19, N22, and X30 belong to *S. cerevisiae* with nucleotide sequence similarity of 99%. C2 and D10 isolates were identified as *L. fermentati*, with 100% sequence homology. C3, D6, and X29 isolates showed 99% identity to *C. fructus* (Table 2).

Table 2. GenBank accession numbers for the ITS nucleotide sequences of isolated yeast strains

Sample ID	GenBank ID	Sequence homology		
		Identity	GenBank ID	Strain description
C1	OQ711757	99%	MK439496	*S. cerevisiae* FC3469
C2	OQ711765	100%	GU237044	*L. fermentati*
C3	OQ711770	99%	EU484318	*C. fructus* PEIPFFB10
C5	OQ711758	99%	MK439496	*S. cerevisiae* FC3469
D6	OQ711771	99%	MT974663	*C. fructus* 2MG-A0603-50
D7	OQ711759	99%	MZ098648	*S. cerevisiae* ICMP 349
D10	OQ711766	100%	OK050766	*L. fermentati* YHKB279
D14	OQ711760	99%	MZ098648	*S. cerevisiae* ICMP 349
M19	OQ711761	99%	MZ098648	*S. cerevisiae* ICMP 349
N22	OQ711762	99%	MZ098648	*S. cerevisiae* ICMP 349
X29	OQ711772	99%	AJ539377	C. *fructus* CECT 11884T
X30	OQ711763	99%	MZ098648	*S. cerevisiae* ICMP 349

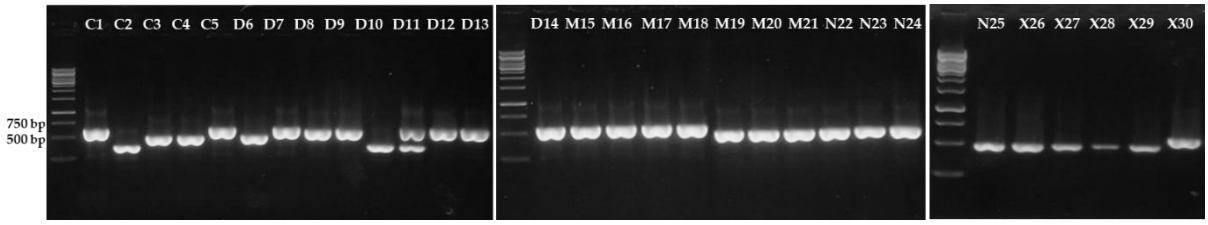

Fig. 4. Electrophoresis of PCR products of 30 yeast strains on 1.2% agarose gel

3.3 Growth characterization of isolated *S. cerevisiae* under different stress conditions

High temperature tolerance

According to literatures, the optimal temperature for *S. cerevisiae* growth is usually ranged from 25°C to 30°C [20,21]. At high temperatures, yeast cells face to stress which induces increasing membrane fluidity, changing in protein structures and functions that lead to growth inhibition or cell death [22,23]. In this study, *S. cerevisiae* isolates grew well at temperatures ranging from 20 to 30°C (Fig. 5A). But the growth rate of all isolates significantly declined when temperature reached 35°C or could not grow at 45°C, except D14 and X30 strains that were able to grow up to 45°C. Similar results were also observed in the study of Techaparin [24] who found some *S. cerevisiae* isolated from Mekong region exhibited moderate growth at 45°C, but others could not. Nasir A [25] also reported that *S. cerevisiae* strains isolated from fruit sources (pineapple and orange) were highly thermotolerant as growing well up to 40°C.

As stated in the study of Sree [20], yeast can grow at temperatures as high as 40°C, being a thermotolerant yeast. Therefore, D14 and X30 isolates are thermotolerant yeasts and may be suitable for industrial **Fig. 5.** applications.

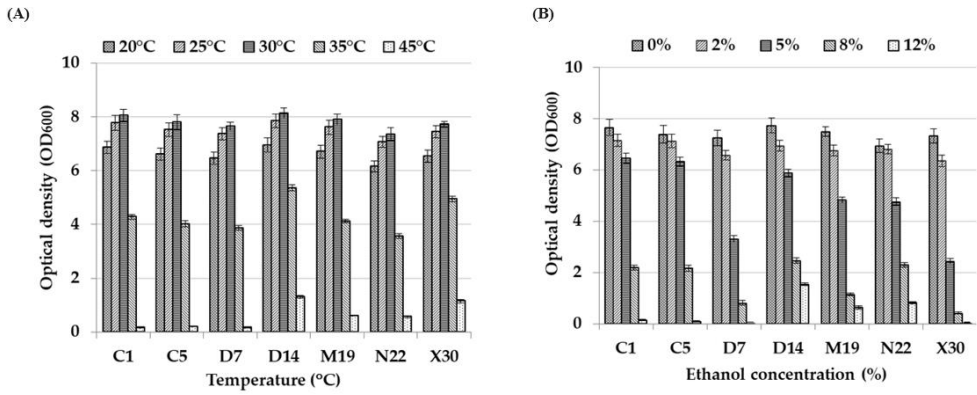

Fig. 5. The growth of yeast strains under stress conditions in YPD medium after 24 h
(A) Different temperatures; (B) Levels of ethanol

Ethanol tolerance

The effect of ethanol on the growth of *S. cerevisiae* isolates was also examined, the results shown in Fig. 5B indicated that all isolates grew well at 2% ethanol (v/v) when compared to the control (without ethanol supplementation). But the growth of yeast was significantly affected when ethanol concentration increased from 5 to 8%, of which D7 and X30 were less tolerant compared to others. Interestingly, D14 strain grew well in 12% ethanol, while most isolates could not. This finding is consistent with the previous studies, in which *S. cerevisiae* isolated from pineapple can tolerate up to 12% ethanol [25], and *S. cerevisiae*

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KKU-VN35 isolated from agricultural products exhibited tolerance to 13% ethanol [24]. The range of ethanol tolerance of *S. cerevisiae* isolated from palm wine [26] and sugar cane [27] was also determined from 7-12%. In contrast, a higher ethanol tolerance (16%) of *S. cerevisiae* was found in the study of Tsegaye [28]. According to Coulibaly [29], the higher ethanol tolerance exhibited by these strains could be due to their greater capability to consume ethanol in the presence of oxygen, as the ethanol tolerance of yeasts greatly depends on mitochondria. Ethanol resistance is an extremely complex mechanism involved in multiple physiological processes that each rely on many different genes, in addition to combining alleles and mutations sophisticatedly can lead to improve ethanol tolerance [30].

3.4 Sugar fermentation

All species of the genus *Saccharomyces* can utilize glucose as a sole carbon, and the distinct ability is up to each strain [31,32]. As illustrated in Fig. 6, all isolates were capable to use efficiently glucose with concentrations in the range of 20 to 100 g/L after 24 h of incubation. However, the increase in glucose concentration, from 200 to 400 g/L, inhibited yeast cell growth. Attractively, the D14 isolate could grow in a medium containing up to 500 g/L glucose, which means that the cell membranes of the D14 strain could endure great osmotic tension. Ortiz-Zamora [33] reported that the yeast isolated from agricultural sources (grape juice, sugarcane molasses, and cane juice) had a good adaptation to 200 g/L glucose and remarkable growth inhibition at glucose levels ranging from 25 to 40% (w/v, equivalent to 250 to 400 g/L), depending on the strain. Thatipamala [34] suggested that sugar inhibition is related to instantaneous biomass yield and typically begins at concentrations above 150 g/L glucose, and the specific growth rate was found to decrease linearly with further increase in substrate content.

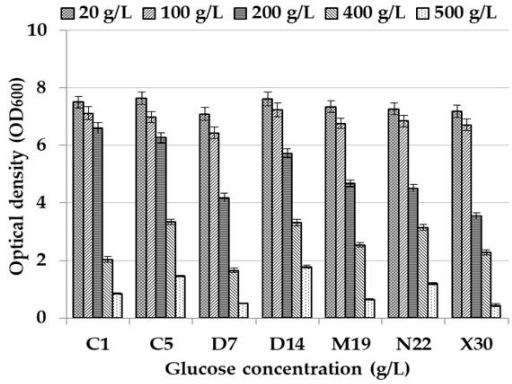

Fig. 6. The growth of yeast strains by glucose concentration in YPD medium after 24 h

The ability to facilitate different carbon sources of isolated *S. cerevisiae* was also tested. As results shown in Table 3, all isolates were able to ferment fructose, galactose, sucrose, mannose, maltose, and raffinose. In contrast, manitol was only fermented by D7 and M19, while C5, D7, and X30 could use glycerol as a sole carbon source. However, the assimilation of glycerol or mannitol at low levels in these isolates was consistent with the result of Swinnen [35] who found that *S. cerevisiae* only grows poorly on glycerol as a carbon source with complex supplements. The study of Quain and Boulton [36] evidenced that some strains of *S. cerevisiae*, but not all, can assimilate mannitol and such adaptation is likely due to the induction of key degradative enzymes or transport permeases. According to Tra Bi [37], glucose is the most preffered sugar (100%), followed by fructose, sucrose, maltose, galactose, and raffinose with 93.3, 93.3, 80.0, 66.7, and 43.3% of fermentation efficiency used by isolated *S. cerevisiae*, respectively. This property was also informed by other studies, which found that wild yeasts can use certain sugars, such as maltose, sucrose, glucose, mannose, fructose and galactose [3]. Several isolates are able to ferment many different carbon sources besides glucose, which is of great importance in industrial production.

Table 3. The carbohydrate assimilation of isolated strains

Carbon source	C1	C5	D7	D14	M19	N22	X30
Fuctose	+	+++	++	+	+++	+	++
Maltose	++	++	+	+++	++	++	+++
Raffinose	++	+++	+++	++	++	+	+
Mannose	+	++	++	+	++	++	++
Galactose	+	+	+	++	+	++	++
Sucrose	++	+++	+++	++	+++	++	+
Manitol	-	-	+	-	+	-	-
Glycerol	-	+	+	-	-	-	+

Note: (+), (++), (+++) express the assimilation of carbon source from low to high; (-) no assimilation.

3.5 Flocculation

Yeast flocculation is a crucially known phenomenon in the brewing industry that may enhance the survival of yeast cells in an environment with limited nutrient conditions [38]. In the phase of pre-final fermentation, single cells begin to gather up dense clusters and settle at the bottom of the reaction tank. The flocculation of yeast can facilitate the filtration process and biomass recovery of byproducts, concurrently reduce the toxicity and increase the sweetness of beer [38,39].

To determine the flocculation ability of isolated *S. cerevisiae*, 5 mL of overnight cultures were vigorously vortexed for 1 min and transferred to glass tubes for sedimentation analysis. As results shown in Fig. 7A, cells of isolates C5, D14, and N22 markedly formed macroscopic flocs (clusters of cells), which sedimented efficiently after 10 min; the sedimentation of X30 was less efficient, while C1, D7 and M49 isolates exhibited poor flocculation. Based on the formula described in the method section, the sedimentation rates of C5, D14, N22 and X30 isolates were 68.3, 61.2, 76.6, and 43.3%, respectively, as shown in Fig. 7B. Similar findings were also observed in previous studies [26,40], who found the flocculation efficiency of some *S. cerevisiae* varied between 58 and 93.1%. Since flocculation ability varies among strains and could be the result of differences in the expression of flocculin genes, further studies need to be carried out to understand the molecular mechanism that controls this event.

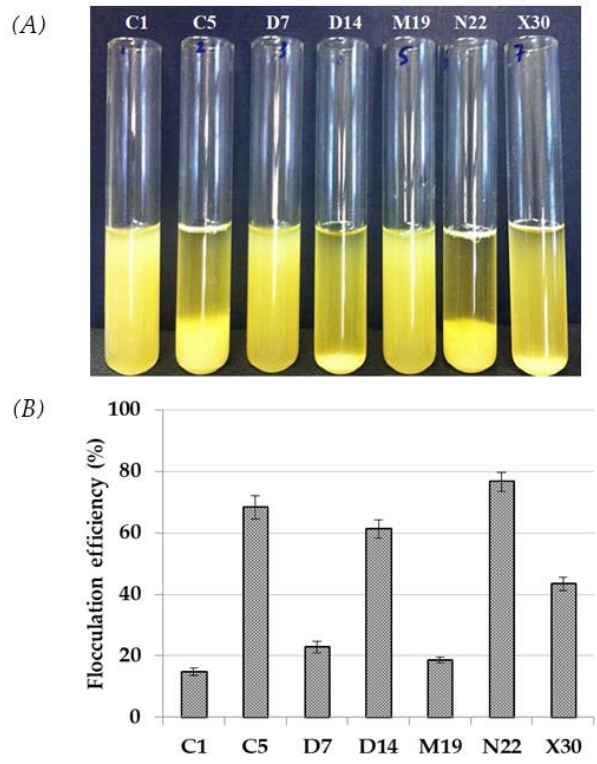

Fig. 7. The flocculation of isolated yeast strains
(A) Image of flocculation; (B) Flocculation efficiency diagram

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4 Conclusion

In this study, we obtained 30 different yeast isolates which belonged to different species: *S. cerevisiae*, *L. fermentati*, and *C. fructus*. Among them, seven *S. cerevisiae* isolates grew well in medium containing 20-100 g/L glucose and 2-5% (v/v) ethanol at 30°C, and used a wide range of other sugars in addition to glucose. Remarkably, D14 isolate was able to grow in harsh conditions (up to 45°C, 12% ethanol, 500 g/L glucose, and highly flocculation), that make it a potential candidate for industrial applications.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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