Food Sci. Biotechnol. 25(6): 1645-1650 (2016)
DOI 10.1007/s10068-016-0253-x

Food Science
and
Biotechnology

Identification of *Zygosaccharomyces mellis* strains in stored honey and their stress tolerance

Gongliang Liu[1,2,*], Changli Tao[3], Baosheng Zhu[1], Weidong Bai[1,2], Liangliang Zhang[1], Zengpeng Wang[1], and Xingting Liang[1]

[1]*College of Light Industry and Food Science, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong 510225, China*
[2]*Key Laboratory of Traditional Cantonese Food Processing and Safety Control, Guangzhou, Guangdong 510225, China*
[3]*School of Life Science and Biopharmacology, Guangdong Pharmaceutical University, Guangzhou, Guangdong 510006, China*

Received May 15, 2016
Revised September 17, 2016
Accepted September 22, 2016
Published online December 31, 2016

*Corresponding Author
Tel: +86-020-89003179
Fax: +86-020-89013037
E-mail: gongliangliu@126.com

pISSN 1226-7708
eISSN 2092-6456

Abstract To screen yeast with high sugar tolerance and evaluate their stress tolerance, six yeast strains were selected from 17 stored honey samples. The species were identified through 26S rRNA sequencing. Their stress tolerance was determined via the Durham fermentation method and ethanol production ability was determined via flask fermentation. The results demonstrated that all the six strains were *Zygosaccharomyces mellis.* Their sugar, ethanol, and acid tolerance ranges were 500–700 g/L, 10–12% (v/v), and pH 2.5–4.5, respectively. The SO_2 tolerance was 250 mg/L. Among the six strains, 6-7431 had the best stress tolerance with sugar tolerance of 700 g/L, ethanol tolerance of 12% (v/v), and acid tolerance of pH 2.5. Furthermore, the strain of 6-7431 had the highest percentage of ethanol production at the same initial sugar content as the other strains. Therefore, the selected six yeast strains would be promising fermentation yeasts for wine-making, ethanol production, or other fermentation purposes.

Keywords: *Zygosaccharomyces mellis*, single colony isolation, phylogenetic tree analysis, stress tolerance

Introduction

High gravity fermentation (HGF) of ethanol is also known as high concentration ethanol fermentation. It refers to the fermentation process that has over 15% (v/v) ethanol production with the initial sugar content of over 270 g/L in zymotic fluid (1). Compared with traditional ethanol fermentation techniques, HGF improves equipment efficiency, increases the ethanol production, and reduces cost of production. Therefore, it has broad application prospects. In the initial stage of HGF, the stress generated by high concentration of the substrate (such as glucose) is toxic to the cells and inhibits yeast growth or fermentation (2). Moreover, the sugar tolerance of yeast directly influences its capability of ethanol production. Therefore, the key factors for increasing fermentation efficiency and reducing the production cost include improving the high sugar tolerance (HST) of the yeast and reducing the inhibition effect of high osmotic pressure on cells. The choice of HST yeast is important for effective HGF. The performance of HST varies because of different expression pathways and intensities of the yeast osmoregulation system. In addition, HST differs among yeasts from different sources (3). For example, Qiu *et al*. (4) screened HST yeast from Chinese and Italian honey, which were identified as *Zygosaccharomyces rouxii* via 18s RNA. Qi *et al*. (5) screened 99 HST yeast strains in honey with high sugar content obtained from natural hives using selective enrichment culture. Zhao *et al*. (6) screened the yeast that are tolerant to sugar content of 650 g/L using ultraviolet (UV) mutagenesis and domestication. Shen and Zhang (7) harvested *Aspergillus niger*-GS-30 with high sugar and temperature tolerance using genome shuffling enzyme control.

Most yeast in honey cannot survive because of high sugar stress. However, ethanol production was detected in some samples of stored natural honey. This indicated that certain types of yeast undergo proper natural domestication and convert carbohydrates to ethanol under high sugar stress. Therefore, the viable yeast that was screened from stored honey possessed high HST. Recently, HST yeasts have been widely investigated. However, all the reported yeast strains have HST below 400 g/L at the sugar content ranging from 300 to 650 g/L (3-7). Because the yeast strains with HST of above 650 g/L have not been well documented, this study aims to screen HST yeast and identifying their strains. Furthermore, the selected yeast stress tolerance, including sugar, ethanol, acid, and SO_2 tolerance were evaluated to provide important information for ethanol HGF and HST mechanism.

Materials and Methods

Chemicals and materials Litchi honey was purchased from Fengmai Apiculture Co., Ltd. (Guangzhou, China). Lungan honey and honey of various flowers were purchased from Guiling Apiculture Technology Co., Ltd. (Guangdong, China). Wine active dry yeast BV818 and SY were supplied by Angel Yeast Co., Ltd. (Hubei, China). Wort, wort and agar, Wallerstein Laboratory nutrient agar (WL), yeast extract peptone dextrose (YPD), and potato dextrose agar (PDA) mediums were purchased from Ruiwei Biotechnical Co., Ltd. (Guangdong, China). E.Z.N.A.^{TM} yeast DNA kit was purchased from Omega Bio-Tek (Norcross, GA, USA). Tris-acetate-EDTA (TAE buffer) and ethidium bromide (EB) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primers of 5'-GCATATCAATAAGCGGAGGAA AAG-3' (NL-1) and 5'-GGTCCGTGTTTCAAGACGG-3' (NL-4) were synthesized by IGE Biotechnology Ltd. (Guangzhou, China). Taq DNA polymerase was purchased from Takara (Tokyo, Japan).

Enrichment culture The sanitized liquid wort medium was used as enrichment culture. One milliliter of each honey sample was collected and transferred to a 250 mL flask containing 10 mL of wort medium. The culture solution was incubated in a shaking table at 150 rpm with constant temperature of 28°C for 3 days (8). After incubation, decimal serial dilutions of the order of 10^1–10^6 of 1 mL culture suspension were performed using sterilized distilled water. An aliquot of 100 μL 10^4, 10^5, and 10^6 dilutions were inoculated into the prepared PDA solid medium plate. Then, each diluent was spread on the medium surface by mere tilting or gentle twirling of the plate followed by surface drying (5–6 min) and incubated at 28°C for 48 h (9).

Single colony isolation and growth curve The single yeast isolate obtained from the PDA medium was further identified by streak inoculation method and incubated at 28°C for 48 h (10). After continuous subculture, the purified yeast strains were confirmed by colony morphology characteristics and microscopic examination. To preserve the purified strains, they were inoculated into wort and agar slants supplemented with 700 g/L sugar and stored at 70°C (11). The isolated strains were further morphologically, physiologically, and biochemically characterized on the WL nutrients medium (12). In addition, the growth curve of the selected strains was measured by the turbidimetric method. The purified strains were inoculated into a wort medium with 700 g/L sugar content and incubated at 28°C for 108 h. The absorbance values (OD) were measured at 560 nm every 3 h to determine the biomass of yeast. The wine active dry yeast BV818 and SY were used as control strains.

rRNA gene amplification and analysis Total genome DNA of the six yeast strains was extracted using the E.Z.N.A^{TM} Yeast DNA extraction kit by following the instructions of the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, 26S rRNA D1/D2 region of each isolate was amplified by polymerase chain reaction (PCR) (Eppendorf, Hamburg, Germany) using NL-1 and NL-4 as forward and reverse primers, respectively. The PCR was performed in a final volume of 25 μL containing 1.5 μL of extracted DNA, 10 μmol/L of each primer, 0.5 μL of Taq DNA polymerase (2.5 U/μL), 2.5 μL of 10×Taq buffer, 2.0 μL of Mg^{2+} (25 mmol/L), 2.0 μL of dNTPs (10 mmol/L), and 15.5 μL of sterilized distilled water. The thermal cycling parameters were set as follows: an initial denaturation at 94°C for 1 min followed by 30 cycles at 94°C for 30 s, 52°C for 1 min, and 72°C for 1.5 min and completed with a final extension at 72°C for 5 min (13). PCR products (2 μL) were further analyzed on 10 g/L agarose gel containing 0.7 μg/L of EB. Then, electrophoresis was performed at 165 V for 25 min and the results were obtained using the ChemiDoc MP system (Bio-Rad Laboratories). The results were visualized under UV light and compared with standard molecular weight DNA markers (100–2,000 bp, Takara, Otsu, Japan) (14).

Sequencing and phylogenetic tree analysis The PCR products were purified by AxyPrep PCR purification kit (Axygen, Union City, CA, USA) and sequenced by the Facility of IGE Biotechnology Ltd. The results were manually confirmed by Chromas using double-barreled data as reference. BLAST searches of sequences (26S rRNA D1/D2 region) were performed at the GENBANK collection data library in order to compare the homology of corresponding sequences in six extracted strains and the comparable yeast strains (*Z. mellis* ML343, *Z. mellis* ABT401, *Z. rouxii* CBS4837, *Z. saoae* M21, *Z. lentus* CBS8574, and *Z. kombuchaensis* CBS 8849) (15). In general, a strain is assigned to a species in the database if its sequence identity to that species exceeds 99%. Based on the results from the homology sequence search, MUSCLE alignment of 26S rRNA D1/D2 region sequence in the comparable yeast strains and six extracted strains were performed to explore their genetic relationship and taxonomic status. The phylogenetic tree was developed using Kimura 2-parameter distance measure and neighbor-joining method (MEGA, version 6.0) (16). Then, confidence values of the phylogenetic tree were further analyzed by interior-branch and bootstrapping tests with 1,000 replicates.

Sugar, ethanol, acid, and SO_2 tolerance of the selected yeast strains Sugar, ethanol, acid, and SO_2 tolerance of the six selected strains were evaluated by Durham's fermentation method based on the gas production. In the study for sugar tolerance, the Litchi honey used as a culture medium was autoclaved to eliminate any microorganisms. Then, each of the six yeasts was inoculated with a ratio of 10% (v/v) into the honey medium with the sugar concentrations adjusted to 400, 500, 600, 650, 700, and 775 g/L (17). The samples were incubated at 28°C for 108 h and the production of gas was recorded. For the evaluation of ethanol tolerance, each of the six yeasts was inoculated with a ratio of 10% (v/v) into the wort medium comprising 6% (v/v), 8% (v/v), 10% (v/v), and 12% (v/v) of ethanol (18). The samples were incubated at 28°C for 108 h and the production of gas was recorded.

In the study for acid tolerance, each of the six yeasts was inoculated into a wort medium with pH of 2.5, 3.0, 3.5, 4.0, and 4.5 at the inoculation ratio of 10% (v/v) (2). The samples were incubated at 28°C for 108 h and the production of gas was recorded. In the study for SO_2 tolerance, each of the six yeasts was inoculated into a wort medium with 250 mg/L of SO_2 at the inoculation ratio of 10% (v/v) (19,20). The samples were incubated at 28°C for 108 h and the production of gas was recorded at different time intervals (12, 24, 36, 48, 60, 72, 84, 96, and 108 h). The wine active dry yeast BV818 and SY were used as control in all tolerance evaluation studies.

Ethanol production under high sugar concentration Five percentages of each selected yeast were inoculated into the honey medium with sugar contents of 550, 600, 650, and 700 g/L and incubated at 28°C for 10 days for scale-up culture. The ethanol production was quantified by alcohol meter, whereas the total sugar content was determined using Fehling reagents (21). The wine active dry yeast BV818 and SY were used as control strains.

Statistical analysis All data are presented as mean±standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test using the Statistical Package for Social Studies (SPSS) (IBM Corp., Armonk, NY, USA). A result of $p<0.05$ was regarded as statistically significant.

Results and Discussions

Morphological identification and purification The single colony that could grow normally on a wort agar medium with typical morphology was isolated via enrichment culture, plate streaking, and multiple subculture. Six selected yeast strains were named as 7-7,142, 7-7,352, 6-7,431, 7-7,551, 7-7,651, and 7-7,751. Among these, 7-7,142 was obtained from Litchi honey and 6-7,431 was obtained from Longan honey, whereas the other four strains were obtained from honey of various flowers. The six isolated strain colonies mainly appeared yellowish and green in non-transparent creamy status with a spherical-shaped cylindrical hill and glossy surface. The cell morphology presented a globose or ovoid shape and the reproductive mode was budding. The colonies of all six strains were opalescent with similar color in the colony margin and center. The surface of the colony was glossy with moist and homogenous texture. Strains of 7-7,551, 7-7,651, and 7-7751 had larger sized cylindrical colonies than the strains of 7-7,142, 7-7,352, and 6-7,431. Moreover, the cell morphologies of 7-7,352 and 6-7,431 were globose, whereas the other four strains were in ovoid shape. Therefore, the six selected strains were yeasts (22).

26S rRNA D1/D2 gene analysis The six yeast strains were identified on the basis of colony characteristics and morphology, and they were confirmed using molecular techniques. As shown in Fig. 1, the DNA strand amplified from PCR presented from 500 to 750 bp, which was similar to the reported 600 bp of 26S rRNA D1/D2 region in the yeast ribosomal large subunit (23). Thus, it indicated that the 26S rRNA D1/D2 region gene fragment was successfully obtained from PCR amplification.

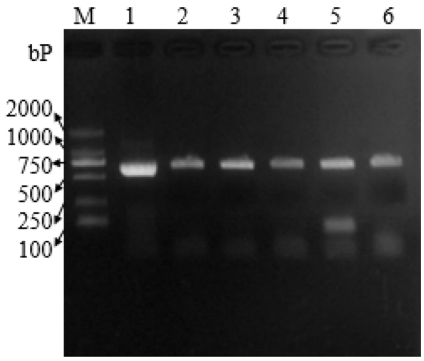

Fig. 1. The electrophoresis of 26S rRNA D1/D2 PCR products. *M, DNA marker; Channel 1, 7-7142 strain; Channel 2, 7-7352 strain; Channel 3, 6-7431 strain; Channel 4, 7-7551 strain; Channel 5, 7-7651 strain; Channel 6, 7-7751 strain

26S rRNA D1/D2 sequence analysis and phylogenetic tree analysis The phylogenetic tree of the six selected strains and related yeast strains (*Z. mellis* ML343, *Z. mellis* ABT401, *Z. rouxii* CBS4837, *Z. saoae* M21, *Z. lentus* CBS8574, and *Z. kombuchaensis* CBS 8849) were constructed on the basis of their 26S rRNA D1/D2 region sequence. Figure 2 demonstrated that the strains of 7-7352, 6-7431, 7-7551, 7-7651, and 7-7751 were grouped with *Z. mellis* ML343 with 99% similarity, whereas 7-7142 was in the same clade cluster with *Z. mellis* ABT401 with 99% similarity. The consistency of all the six yeast strains were all above 99%, which met the standard that the difference of strains in the same species is not more than 1% (24). Thus, the six isolated yeast strains were cataloged as *Z. mellis.*

Stress tolerance of *Z. mellis*

***The time parameters of growth curve*:** In this study, 17 stored honey samples (18 months) with active ethanol fermentation were used to screen the HST yeast strains. During storage, certain types of yeasts exhibited great HST performance with natural domestication (6). Generally, the growth curves of the strains are estimated by OD values, reflecting the biomass of the yeast. The biomass of the six selected yeast strains increased gradually with the time of culture. The strains maintained the lag phase for 20–50 h, which was less than that maintained by the control yeasts of BV818 (56 h) and SY (56 h). In particular, the three strains of 7-7352, 6-7431, and 7-7751 had the lag phase time of 20, 22, and 22 h, respectively, which was approximately 36% of that of BV818 and SY. It indicated that the six selected yeast strains could adapt to the high sugar environment in a short time and perform glycolysis and enzyme activities related to fermentation and metabolism (6). The strain of 7-7352 maintained a logarithmic phase for 30 h, followed by the strains of 6-7431, 7-7651,

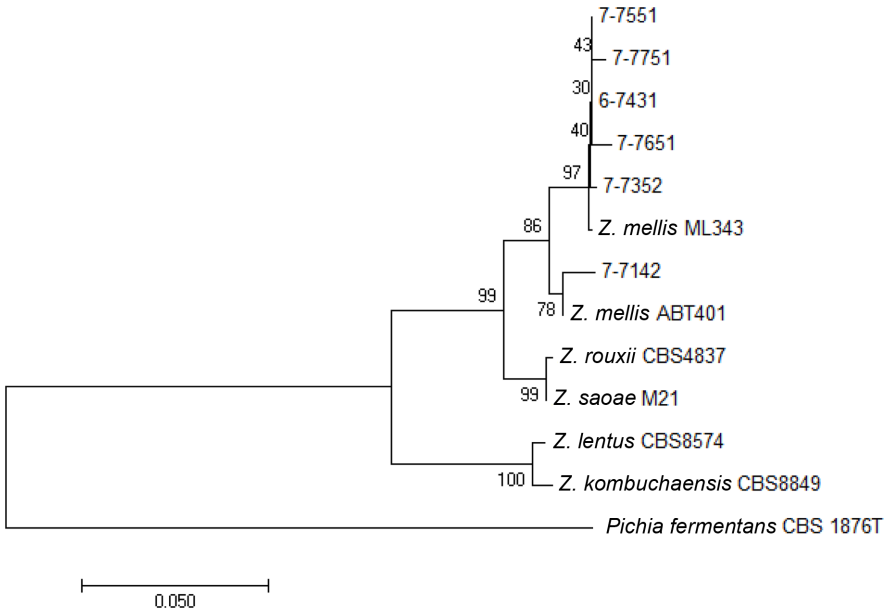

Fig. 2. Phylogenetic tree analysis based on 26S rRNA D1/D2 region sequence

and 7-7751, which maintained the logarithmic phase for 20 h. However, the strains of BV818 and SY only maintained the logarithmic phase for 8 h, which may have significantly less biomass than the strains of 7-7352, 6-7431, 7-7651, and 7-7751. Moreover, compared with the strain of *Pichia-guillier-mondii* (obtained from *Sapium sebiferum* honey), which is tolerant to 300 g/L sugar content as reported by Shi *et al*. (25) the selected strain in this study had significantly higher sugar tolerance of 700 g/L.

***HST of Z. mellis*:** The production of gas indicated the growing status of the *Z. mellis* strains. The produced gas was trapped inside the Durham tube and a visible air bubble appeared in the tube. The result demonstrated that the gas produced in the strains of the 7-7551 and 7-7352 samples filled the entire Durham tube at the sugar content of 400–650 g/L (Table 1). However, as the sugar concentration increased to 700 g/L, less gas (filling less than one-third of the Durham tube) was produced in the strains of 7-7551 and 7-7352, indicating the test strains were not able to grow normally. Thus, the highest sugar content that the strains of 7-7551 and 7-7352 could tolerate was 650 g/L. The strains of 7-7751 and the control yeasts of BV818 and SY produced sufficient gas to fill the entire Durham tube at the sugar content of 400–500 g/L, whereas its grow stopped at 600 g/L. Thus, the strains of 7-7751, BV818, and SY can tolerate sugar up to 500 g/L. However, the strains of 7-7651 and 6-7431 had a sugar tolerance at the sugar content of 700 g/L, which was higher than the current reported mutated strains with sugar tolerance of 650 g/L and commercial Angel dry yeast with sugar tolerance of 600 g/L (6,26). The HST of the selected strains was owing to their response ability to high external solute concentrations. It has been described as system-level coordination between the extracellular environment and the genetic make-up inside the cell with the interconnected modules of sensing, signal transduction, and effector processes. This osmotolerant mechanism has been found in strains of *Z. rouxii* and *Saccharomyces cerevisiae* (27).

***Ethanol tolerance of Z. mellis*:** All the six strains were tolerant to 10% (v/v) ethanol because they produced sufficient gas to fill the entire Durham tube (Table 2). However, compared with the commercial

Table 1. Gas production by the six strains under different sugar content

Sugar content (g/L)	Strains							
	7-7142	7-7352	6-7431	7-7551	7-7651	7-7751	BV818	SY
400	+++[1)]	+++	+++	+++	+++	+++	+++	+++
500	+++	+++	+++	+++	+++	+++	+++	+++
600	+[2)]	+++	+++	+++	+++	0	0	0
650	0	+++	+++	+++	+++	0	0	0
700	0	-[3)]	+++	-	+++	0	0	0
775	0	0[4)]	0	0	0	0	0	0

[1)]+++, the Durham tube was completely fill with gas
[2)]+, one-third of the Durham tube was fill with gas
[3)]-, less than one-third of the Durham tube was fill with gas
[4)]0, no gas was produced

Table 2. Gas production by the six strains under different ethanol content

Ethanol content (%, v/v)	Strains							
	7-7142	7-7352	6-7431	7-7551	7-7651	7-7751	BV818	SY
6	+++[1)]	+++	+++	+++	+++	+++	+++	+++
8	+++	+++	+++	+++	+++	+++	+++	+++
10	+++	+++	+++	+++	+++	+++	+++	+++
12	++[2)]	+[3)]	+++	+++	+++	-[4)]	+++	+++

[1)]+++, the Durham tube was completely filled with gas
[2)]++, two-third of the Durham tube was filled with gas
[3)]+, one-third of the Durham tube was filled with gas
[4)]-, less than one-third of the Durham tube was filled with gas

Table 3. Ethanol production of the six strains at different initial sugar contents

Initial sugar content (g/L)	Ethanol production (%, v/v)							
	7-7142	7-7352	6-7431	7-7551	7-7651	7-7751	B[2)]	S[3)]
550	3.17±0.06*[1)]	1.53±0.06*	4.27±0.15*	3.07±0.06*	2.57±0.06*	3.17±0.15*	-[4)]	-
600	2.93±0.06*	1.17±0.06*	3.80±0.10*	2.27±0.06*	2.13±0.06*	2.97±0.06*	-	-
650	2.50±0.10*	0.73±0.06*	3.33±0.06*	2.10±0.10*	1.77±0.06*	2.37±0.06*	-	-
700	1.53±0.06*	0.40±0*	2.63±0.06*	1.47±0.06*	1.03±0.06*	1.10±0.09*	-	-

[1)]Data are expressed as mean±SD (n=3) using Microsoft Excel 2013. All one-way ANOVAs for difference were statistically significant with *p<0.05.
[2)]B, BV818 strain
[3)]S, SY strain
[4)]-, No ethanol production

yeasts of BV818 and SY, only strains of 6-7431 and 7-7651 had similar ethanol tolerance as ethanol percentage increased to 12% (v/v) (Table 2). The ethanol percentage (12%, v/v) that they could tolerate was significantly higher than 10% (v/v) for the reported UV-mutated strain of Y316-23 (28).

***Acid tolerance of Z. mellis*:** In general, acid environment can inhibit the interference of hazardous microorganisms and also promote the fermentation process of yeast (29). Moreover, it can improve the aroma of fermented food such as wine. Therefore, a yeast strain with good fermentation ability should have high tolerance of low pH ranging from 4 to 6. However, most of the yeasts were unable to grow in the environment with pH below 3 (30). In this study, the six selected strains could produce sufficient gas to fill the entire Durham tube in the growing environment with pH range of 2.5–4.5, indicating that they all had good adaptability of low pH. Thus, they would be useful for improving the conversion rate and utilization of fermenting materials as well as increasing ethanol production.

***SO_2 tolerance of Z. mellis*:** In fruit wine industry, the addition of the correct amount of SO_2 will effectively inhibit microorganism growth; SO_2 has antioxidant and color protection functions (31). Thus, the tolerance of yeasts to SO_2 is an important index in wine making. In China, the maximum level of SO_2 in wine was limited to 250 mg/L (32). Thus, the SO_2 concentration of 250 mg/L was used in this study to test the SO_2 tolerance of the six selected strains. After the fermentation time reached 48 h, the six strains started to produce gas and maintained a good growing status until 108 h. In practice, the concentration of SO_2 used in fruit wine industry was much less than 250 mg/L. In that case, the fermentation characteristic of strains would be better. Moreover, the concentration of organic compounds, including fusel oil and methanol as by-product, will be determined in future studies. Thus, such strains would be promising candidates for fruit wine brewing.

Ethanol production of *Z. mellis* The ethanol production of the six selected strains was determined under the growing environment of high sugar content. The initial sugar content was set at 550, 600, 650, and 700 g/L. As shown in Table 3, the strain of 6-7431 had ethanol production of 4.27±0.15 (%, v/v) at 550 g/L, 3.80±0.10 (%, v/v) at 600 g/L, 3.33±0.06 (%, v/v) at 650 g/L, and 2.63±0.06 (%, v/v) at 700 g/L, which was significantly higher than that of the other five strains at the same initial sugar content. It was followed by strains of 7-7142, 7-7551, and 7-7751 wherein ethanol production by each strain was above 3.0 (%, v/v) at 550 g/L. With the increasing initial sugar content, the ethanol production of the six strains gradually decreased. The six strains produced different amounts of ethanol at the same initial sugar content of 700 g/L. However, the commercial yeasts of BV818 and SY were unable to produce ethanol at the initial sugar content of above 500 g/L. Therefore, the results will be useful for the selection of yeast strains for optimizing fermentation techniques required for ethanol production or other fermentation purposes.

Disclosure The authors declare no conflict of interest.

References

1. Vásquez Castillo JA, Laguado JA, López J, Gil NJ. New sources and methods to isolate vinasse-tolerant wild yeasts efficient in ethanol production. Ann. Microbiol. 66: 187-195 (2016)
2. Meijnen JP, Randazzo P, Foulquié MR, Brink J, Vandecruys P, Stojiljkovic M, Dumortier F, Zalar P, Boekhout T, Gunde-Cimerman N, Kokošar J, Štajdohar M, Curk T, Petroviè U, Thevelein JM. Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. Biotechnol. Biofuel. 9: 5 (2016)
3. Kłosowski G, Mikulski D, Macko D, Miklaszewska B, Kotarska K, Czupryñski B. Influence of various yeast strains and selected starchy raw materials on production of higher alcohols during the alcoholic fermentation process. Eur. Food. Res. Technol. 240: 233-242 (2015)
4. Qiu JX, Qu JY, Lin DX, He TF, Peng Y. The strain identification of Chinese honey and Italian honey. J. Bee 1: 10-11 (2015)
5. Qi XH, Wang X, Lin J, Zhu JF, Luo Y, Sun WJ. Screening and identification of osmophilic yeast and studies on its capacity of producing polyols. Food Ferment. Ind. 40: 16-21 (2014)
6. Zhao S, Li P, Wang Q. Breeding of high-sugar-tolerant yeast and application. J. Anhui Agr. Sci. 38: 4801-4803 (2010)
7. Shen YP, Zhang ZJ. Selection of high temperature and high sugar producing bacteria by GS. J. Hunan Univ. Sci. Eng. 35: 43-45 (2014)
8. Tikka C, Osuru HP, Atluri N, Raghavulu PCV, Kumar N, Mannur IS, Prasad UV, Aluru S, Varma K, Bhaskar M. Isolation and characterization of ethanol tolerant yeast strains. Bioinformation 9: 431-425 (2013)
9. Xu XL, Feng GL, Liu HW, Li XF, Zhao GL, Xiao XL. Isolation, identification and control of osmophilic spoilage yeasts in sweetened condensed milk. Afr. J. Microbiol. Res. 8: 1032-1039 (2014)
10. Osho A. Ethanol and sugar tolerance of wine yeasts isolated from fermenting cashew apple juice. Afr. J Biotechnol. 4: 660-662 (2005)
11. Gupta N, Dubey A, Tewair L. High efficiency alcohol tolerant *Saccharomyces* isolates of Phoenix dactylifera for bioconversion of sugarcane juice into bioethanol. J. Sci. Ind. Res. India 68: 401-405 (2009)
12. Pallmann CL, Brown JA, Olineka TL, Cocolin L, Mills D, Bisson L. Use of WL medium to profile native flora fermentations. Am. J. Enol. Viticult. 52: 198-203 (2001)
13. Park WK, Yang JW, Kim HS. Identification of novel genes responsible for salt tolerance by transposon mutagenesis in *Saccharomyces cerevisiae*. J. Ind. Microbiol. Biot. 42: 567-575 (2015)
14. Cheng C, Alamrio MP, Kao KC. Genome shuffling to generate recombinant yeasts for tolerance to inhibitors present in lignocellulosic hydrolysates. Biotechnol. Lett. 37: 2193-2220 (2015)
15. Snoek T, Nicolino MP, den Bremt SV, Mertens S, Saels V, Verplaetse A, Steensels J, Verstrepen KJ. Large-scale robot-assisted genome shuffling yields industrial Saccharomyces cerevisiae yeasts with increased ethanol tolerance. Biotechnol. Biofuel. 8: 1-19 (2015)
16. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis Version 6.0. Mol. Biol. Evol. 30: 2725-2729 (2013)
17. Gasmalla MA, Yang RJ, Nikoo M, Man S. Production of ethanol from Sudanese sugar cane molasses and evaluation of its quality. J. Food Process. Technol. (2012)
18. Lam FH, Ghaderi A, Fink GR, Stephanopoulos G. Engineering alcohol tolerance in yeast. Science 346: 71-75 (2014)
19. Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314: 1565-1568 (2006)
20. Ma C, Wei XW, Sun CH, Zhang F, Xu JR, Zhao XQ, Bai FW. Improvement of acetic acid tolerance of Saccharomyces cerevisiae using a zinc-finger-based artificial transcription factor and identification of novel genes involved in acetic acid tolerance. Appl. Microbiol. Biot. 99: 2441-2449 (2015)
21. Santos J, Sousa MJ, Cardoso H, Inácio J, Silva S, Spencer-Martins I, Leão C. Ethanol tolerance of sugar transport, and the rectification of stuck wine fermentations. Microbiology 154: 422-430 (2008)
22. Yang Y, Xu YW, Xue JX, Liu YL. Validate the identification effect of WL nutrient agar on wine yeast. J. Microbiol. 27: 75-78 (2007)
23. Giuliani A, Pirri G, Nicoletto SF. Antimicrobial peptides: An overview of a promising class of therapeutics. Cent. Eur. J. Biol. 2: 1-33(2007)
24. Kurtzman CP, Robnett CJ. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. A. Van. Leeuw. J. Microb. 73: 331-371 (1998)
25. Shi Y, Zhang LZ, Zeng ZJ, Wang ZL, Yang WY. Isolation, identification of honey yeast for sapium discolor mead and application. J. Chinese Inst. Food Sci. Tech. 13: 197-204 (2013)
26. Yu XF, Li XS, Li ZJ. Angel about temperature tolerant alcohol active dry yeast application in the China. Liquor Mak. Sci. Tech. 29: 57-59 (2002)
27. de Nadal E, Ammerer G, Posas F. Controlling gene expression in response to stress. Nat. Rev. Genet. 12: 833-845 (2011)
28. Wang B, Zhang GZ, Lu FP, Du LX. Study on the breeding of saccharonyces cerevisiae of high alcohol gravity. J. Tianjin Univ. Light Ind. 3: 33-36 (2001)
29. Chen JF, Guo WJ, Hu HJ. Separation and properties determination of an acid resistant yeast. China Brewing 32: 39-42 (2013)
30. Luo HB, Li GH. Study of screen for the acid-proof yeast fungus. J. Sichuan Univ. Sci. Eng. 17: 151-154 (2004)
31. Li Z, Han BZ, Chen JY, Li SS, Chen C. Study on fermentation characteristics of three excellent *Saccharomyces cerevisiae* strains. China Brewing 19: 10-12 (2008)
32. GB/T2760-2014, Hygienic standards for uses of food additives[S]. Standards Press of China, Beijing, China. pp. 17-18 (2015)