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Characterization of newly isolated thermotolerant yeasts and evaluation of their potential for use in *Cayratia trifolia* wine production

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Abstract:

Thermotolerant ethanologenic yeasts have attracted the interest of many scientists due to the current challenges caused by increasing global temperature, the benefits associated with processing at high temperatures, and the potential to reduce cooling costs. The objectives of this study are to characterize the selected thermotolerant yeasts and to evaluate their use in *Cayratia trifolia* fermentation at high temperatures. A total of 151 yeast strains isolated from 53 samples of *Cayratia trifolia* were studied for their morphology, physiology, biochemistry, and their phylogenetic relationship. Based on the results of tests for thermotolerance ability (37-45^{0}C) and ethanol tolerance capacity (9-12% v/v), 57 of the 151 yeast isolates were selected to be tested for use in wine fermentation from three-leaf cayratia at 37^{0}C. Thirty isolates that were found to have high fermentation ability and that produced an ethanol concentration of between 6.0 and 9.9% (v/v) were selected for identification using amplified 26S rDNA sequences. The yeasts were identified as follows: *Candida glabrata* (BL2.1, CT1.1, CT1.3, CT2.3, HG2.1), *Candida tropicalis* (KG1.1, KG3.2, CM3.3, HG3.3, TG1.1, TG3.1), *Candida nivariensis* (DT1.2, CM3.2, ST2.1, BT1.2), *Pichia kudriavzevii* (KG2.1, KG5.1, AG2.1, AG2.3, AG4.2, DT3.2, LA1.3, CM4.4, BT2.1, BT3.3, TV4.2, CT4.2, VL1.1), *Clavispora lusitaniae* (TG4.2), and *Saccharomyces cerevisiae* (HG1.3). The phylogenetic tree constructed using MEGA 6 with bootstrap analysis performed by repeating the data 1,000 times revealed that the selected yeast strains were closely related. The newly isolated strain of *S. cerevisiae* HG1.3 producing the highest ethanol concentration of 9.9% (v/v) in *Cayratia trifolia* wine fermentation at 37^{0}C was selected for further study.

Keywords: ***Cayratia trifolia*, ethanol fermentation, ethanol tolerance, *Saccharomyces cerevisiae*, thermotolerant yeast.**

Classification number: **3.5**

Introduction

Cayratia trifolia (L.) Domin is a rich source of biologically active compounds with antioxidant properties that can reduce tumor growth [1, 2]. It is used as a medicinal ingredient and in alcoholic wines. Currently, fermentation products are being researched for quality, yield and scale, for their application in industrial production to meet consumer demand. Wine, which is an indispensable drink that contributes greatly to supporting human health, is made from a variety of ingredients other than grapes. Temperature is a factor that significantly affects the fermentation capacity of yeast. In summer, the temperature in the South of Vietnam increases dramatically, particularly with global warming [3]. Thus, the use of thermotolerant yeast strains is essential for dealing with climate change. Furthermore, high temperature fermentation has several advantages, such as a reduction in the cost of cooling fermentation vats, higher saccharification yields, continuous removal of ethanol, and decreased risk of bacterial contamination [4-7]. Therefore, the use of thermotolerant yeast strains in ethanol production contributes to lowering manufacturing expenses.

The aims of this study are to isolate thermotolerant yeasts and evaluate their fermentation capacity for the production of three-leaf cayratia (*Cayratia trifolia* L.) wine.

Materials and methods

Culture and materials

Fifty-three samples of *Cayratia trifolia* were collected from 13 provinces in the Mekong Delta region. This was carried out in three phases:

I: the *C. trifolia* berries were collected from the four provinces of Kien Giang, An Giang, Dong Thap, and Long An.

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II: then, berries was collected from the four provinces of Can Tho, Hau Giang, Vinh Long, and Tien Giang.

III: finally, berries was collected from the five provinces (Ca Mau, Bac Lieu, Soc Trang, Ben Tre, and Tra Vinh).

The berries were brought fresh to the Laboratory of Food Microbiology at the Biotechnology Research and Development Institute, Can Tho University and were processed immediately.

The microbiological medium used was YPD broth (g/l, D-glucose 20, peptone 5, yeast extract 5) with 20 g/l of agar added to make a YPD agar medium.

Research method

Isolation of yeast strains:

Five grams of each *Cayratia trifolia* sample was added to 100 ml of YPD broth and incubated at 30^0C, 150 rpm for 24-48 hours. Yeast colonies were selected, streaked on YPD agar, and incubated at 30^0C. Purified yeast cultures were stored in YPD agar slants at 4^0C.

Examination of morphological, physiological, and biochemical characteristics:

Morphological characteristics: the shapes and dimension of colonies and cells were observed under a microscope and recorded.

Glucose, sucrose and maltose fermentation ability: after 24 hours' incubation, yeast suspensions were inoculated into Durham tubes containing a 2% (w/v) sucrose or maltose solution and incubated at 30^0C. The accumulated CO_2 in the inner Durham tubes was measured after 48 hours.

Urea anabolism: yeast isolates were inoculated into tubes containing 3 ml of Stuart's Urea broth and the change in the color of the medium was recorded after incubating at 30^0C for 48 hours.

Gelatin liquefaction: yeast isolates were inoculated into tubes containing 3 ml of gelatin medium and then incubated at 30^0C for 48 hours. The tubes were immediately cooled and the gelatin liquefaction recorded.

Testing the thermo- and ethanol-tolerant capacity of yeast isolates:

Thermo-tolerance: yeast isolates were streaked onto YPD agar and then incubated at 30, 35, 37, 39, 41, 43, 45 and 47^oC for 48 hours. The formations of the colonies that appeared on the medium were recorded.

Ethanol tolerance: yeast isolates were streaked onto YPD agar supplemented with 0, 3, 6, 9, 12 and 15% (v/v) of ethanol and then incubated at 37^oC for 48 hours. The formations of the colonies that appeared on the medium were recorded.

Screening for the ethanol fermentation capacity of yeast isolates:

This test was carried in Durham tubes containing a 2% (w/v) glucose solution and three-leaf cayratia juice (pH 4 and 22^0Brix) incubated at 30^0C. The accumulated CO_2 in the inner Durham tubes was measured at 6-hour intervals for 48 hours.

Testing ethanol fermentation from three-leaf cayratia juice:

The selected yeast isolates were inoculated into YPD broth and incubated for 48 hours. Then, 1 ml of yeast cell suspension (10^8 cells/ml) was inoculated into 99 ml of three-leaf cayratia juice (pH=4 and 22^0Brix) and incubated at 37^0C. The pH, ^{0}Brix and ethanol concentration were determined.

Identification of selected yeast isolates:

The DNA of selected yeast isolates was extracted and used for nucleotide sequencing. The divergent D1/D2 (500 bp) domain of the LSU rRNA gene was amplified with the specific primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG) [8]. Nucleotide sequences were aligned and compared with the database on the National Center for Biotechnology Information website. The identification was conducted at the Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan.

Analytical method and statistical analysis:

The pH was measured with a digital pH meter (Sartorius PB-20). The total dissolved solids of the saccharified liquid (^{0}Brix) was measured using a manual refractometer (FG102/112, Euromex-Holland). The alcohol content was determined using the distillation method [9]. The experimental data were statistically analyzed using Statgraphics Centurion XV software from Manugistics Inc., USA.

Results and discussion

Morphological, physiological, and biochemical characteristics of yeast isolates

One hundred and fifty-one yeast strains were isolated and purified from 53 *C. trifolia* berry samples. The yeast strains were cultured on YPD agar medium for 36 hours at 30^0C and were investigated for their colony and cell morphology. Based on cell morphology and physiological and biochemical characteristics, the 151 yeast isolates were divided into 7 groups (Table 1).

Table 1. Summary of yeast cell shape.

Group	Cell conformation	Name of yeast isolate*			No. of isolate
		Group I	*Group II*	*Group III*	
1	Small spherical		CT3.2, CT4.5, HG1.1 HG1.3, HG4.3, VL3.3, TG1.1, TG3.1	CM1.1, CM1.2, ST1.3	11
2	Large spherical		CT3.3, CT4.1, HG4.4 VL1.2, VL3.1		5
3	Small oval	KG1.1, KG1.3, KG2.1, AG1.3, AG2.1, AG4.1, DT1.3, DT3.1, LA2.1	CT1.1, CT1.2, CT1.3 CT2.3, CT3.1, CT4.2, HG1.2, HG2.1, VL4.2 TG2.2, TG4.3, TG4.4	CM1.3, CM2.1, CM4.1, CM4.3, BL1.1, BL2.1, BL2.3, BL3.1, BL4.3, ST2.1, ST2.3, ST3.1, ST4.3, BT1.2, BT3.1, TV2.1, TV2.2, TV3.2	39
4	Large oval	KG1.2, KG2.2, KG2.3, KG3.1, KG4.2, KG5.1, KG5.2, AG1.1, AG2.4, AG3.2, DT1.1, DT1.2, DT2.1 DT2.3, DT3.2, LA1.1, LA1.2, LA1.3, LA3.1, LA3.2, LA3.3	CT2.1, CT2.2, CT4.4 HG3.1, HG3.3 VL2.2 VL4.4, TG1.2, TG2.3 TG4.2	CM2.2, CM3.1, CM4.2, BL1.2, BL2.2, ST1.2, BT1.3, BT2.1, BT3.2, BT3.3, TV1.2, TV4.4	43
5	Short ellipse	DT4.2, DT4.3, LA4.1	CT4.3, HG3.2, HG4.1, VL2.1, VL4.3, TG2.1, TG4.1	BL4.2, ST1.1, ST2.2, BT4.2	14
6	Elongated ellipse	KG3.2, AG1.2, AG2.3, AG3.1, AG4.2, DT2.2, LA2.2, LA2.3, LA3.4	HG2.2, HG4.2, HG4.5, VL1.1, VL1.3, VL3.2, VL4.1, TG3.2	CM4.4, CM3.2, CM3.3 TV4.2, BL3.2, ST3.3, BT1.1, TV2.3, TV3.1, TV4.1	27
7	Apiculate ellipse	KG4.1, AG2.2, DT4.1, LA4.2		BL4.1, ST3.2, ST4.1, ST4.2, BT2.2, BT4.1, TV1.1, TV4.3	12
Total					**151**

*Notes: Group I: yeast isolates from three-leaf cayratia were collected from Kien Giang, An Giang, Dong Thap, and Long An; Group II: yeast isolates from three-leaf cayratia were collected from Can Tho, Hau Giang, Vinh Long, and Tien Giang; Group III: yeast isolates from three-leaf cayratia were collected from Ca Mau, Bac Lieu, Soc Trang, Ben Tre, and Tra Vinh.

Colony morphology of yeast isolates: the colonies of yeast isolates measured 1-4 mm in diameter and 0.1 mm in height. Some colonies had smooth surfaces while others had rough surfaces. The margins of colonies were also diverse and included entire, undulate, serrated, filiform and lobate. The colonies of yeast were creamy white or white in color.

Cell morphology of yeast isolates: cell shape of yeast isolates were diverse but can be categorized into 4 main forms: spherical, ovoid, elliptical and cylindrical. There were also differences in the dimensions of yeast isolates but generally cell length was approximately 3-11 µm and cell width was approximately 2-5 µm.

Budding and endospore formation: yeast isolates in group 1, 2, 3, 4, 5 and 6 grew by multilateral budding, while isolates in group 7 grew by bipolar budding. All the yeast isolates had the ability to sporulate when nutritionally deficient except those in group 7. Although the endospore dimensions of yeast isolates were not homogeneous, each cell had four ascospores. Yeast tends to form four ascospores after meiosis in their sexual reproduction [10].

Glucose, sucrose and maltose fermentation ability: of 151 yeast isolates, 138 were capable of using glucose and 101 of using sucrose as a carbon source for fermentation after 24 hours. Most strains in groups 1, 2, 4, 5 and 6 were capable of fermenting sucrose while none of the strains in group 3 could ferment this sugar. Of 151 yeast isolates, 104 were able to ferment maltose. The sugar fermentation capacity of the yeast strains was assessed by measuring the among of CO_2 generated during fermentation [11]. Thus, testing the ability to consume sugar was one of criteria for classification the yeast and was also used to select appropriate yeast strains for fermenting different substrates.

Urea assimilation: of 151 yeast isolates, 26 were able to use urea as a source of nitrogen. None of the yeast isolates in groups 1 and 2 were capable of urea resolution. Yeasts belonging to Ascogenous species were able to resolve urea, while those of the Basidiomycetous species had this capability [12].

Gelatin liquefaction: of the 151 yeast isolates, 32 had the capacity to liquify gelatin using gelatinase. This capacity of yeasts was also often associated with protease activity, but only some yeast species were capable of producing protease [11].

The ethanol- and thermo-tolerant capacities of the yeast isolates

Thermotolerant ability: all yeast isolates could grow well in the temperature range 30-35^{0}C. Ten of the 151 yeast strains showed high heat resistance by growing at 45^{0}C. However, the number of yeast colonies generally decreased when the incubation temperature was increased. Among 48 yeast isolates with a high fermentation capacity, 10 isolates (BT2.1, TG2.3, VL1.1, HG4.3, LA1.1, DT3.2, AG4.2, AG3.1, AG2.3, AG2.1) could grow at temperature of 45^{0}C and 38 isolates were able to grow at 43^{0}C after 48 hours of incubation. Based on the results of the thermotolerant screening test, 141 yeast isolates that could grow at 37-45^{0}C were selected for further testing of their ethanol tolerant ability.

Ethanol tolerant ability: when the ethanol concentration in the culture medium was increased, the number of yeast colonies that developed in the medium gradually decreased. This can be explained for causing affect to the yeast growth. Of the 141 isolates, 27 could tolerate an ethanol concentration of up to 12% (v/v), and 64 could tolerate a 9% (v/v) ethanol concentration after 48 hours of incubation.

Screening of ethanol fermentation ability of yeast isolates

The results reveal that 57 out of 64 yeast isolates were able to ferment tubes containing 2% (w/v) glucose solution and three-leaf cayratia juice after 48 hours. Yeast strains including KG2.2, KG3.1, DT1.2, CM3.2 and BT1.2 showed highest fermentation abilities which created maximum CO_2 amount

in Durham tubes within a 6-hour fermentation. The two yeast isolates KG4.1 and AG3.2 had no fermentation capacity. A total of 57 isolated strains that could grow at 37-45^{0}C and tolerate 9-12% (v/v) ethanol were evaluated for their ability to ferment three-leaf cayratia at 37^{0}C.

Ethanol fermentation by selected yeast isolates at high temperatures

The ethanol fermentation ability of 30 out of the 57 selected yeast isolates is presented in Table 2. These isolated yeast strains showed the best fermentation activity and an ethanol content of at least 6.0% (v/v). The highest ethanol concentration was produced by strain HG1.3, which reached at 9.9% (v/v). Isolates HG1.3, CM3.2 and AG2.1 produced the highest ethanol concentration in each group at 9.9, 8.95 and 8.0% (v/v), respectively. The obtained ethanol concentrations of these novel thermotolerant yeasts were better than many thermotolerant yeasts isolated from drainage samples containing hot spring water. Isolates collected from hot spring water could generate maximum ethanol concentrations of approximately 7.0-7.2% (w/v) at 30^{0}C with a nutritional substrate containing 15% (w/v) glucose [13].

At 37^{0}C, there was a clear difference in ethanol concentration produced by 39 tested yeast isolates. The growth of yeast cells also went up when the temperature was increased to a level within the tolerance threshold of the yeast, but the amount of ethanol produced was reduced. Enzymes which control microbial activity and fermentation are sensitive to high temperatures which can denature their tertiary structure and deactivate them [14]. The five yeast isolates BT3.3, BT2.1, HG2.1, HG3.3, VL1.1 and TG4.2 showed lower fermentation ability, whereby ethanol concentrations reached only around 6.0% (v/v).

Table 2. Ethanol producing capacity of 30 selected yeast isolates at 37^{o}C.

No.	Isolate	Thermo- tolerance (^{0}C)	Ethanol-tolerance % (v/v)	CO_2 in Durham tube (24 h)	Ethanol (% v/v)
Group I: Isolates of yeast from three-leaf cayratia collected from the four provinces of Kien Giang, An Giang, Dong Thap, and Long An					
1	KG1.1	41	12	30 (18 h)	7.12^{b*}
2	KG2.1	41	12	19	6.25^{def}
3	KG3.2	43	9	8	6.08^{efg}
4	KG5.1	43	12	14	6.49^{cde}
5	AG2.1	45	12	30 (18 h)	8.00^{a}
6	AG2.3	45	12	18	6.23^{def}
7	AG4.2	45	12	20.67	6.16^{defg}
8	DT1.2	43	12	30 (6 h)	6.19^{defg}
9	DT3.2	45	12	29.33	6.68^{bcd}
10	LA1.3	43	12	17.33	6.84^{bc}
Group II: Isolates of yeast from three-leaf cayratia collected from the four provinces of Can Tho, Hau Giang, Vinh Long, and Tien Giang					
11	CT1.1	43	9	30	8.05^{b}
12	CT1.3	42	9	30 (18 h)	6.4^{c}
13	CT2.3	43	12	30 (18 h)	6.4^{c}
14	CT4.2	41	9	30	8.2^{b}
15	HG1.3	43	9	30	9.9^{a}
16	HG2.1	41	12	25.7	6.1^{d}
17	HG3.3	41	12	30 (18 h)	6de
18	VL1.1	45	12	24	6de
19	TG1.1	43	9	30 (18 h)	6.4^{c}
20	TG3.1	41	9	30	6.4^{c}
21	TG4.2	41	12	23.3	6de
Group III: Isolates of yeast from three-leaf cayratia collected from the five provinces of Ca Mau, Bac Lieu, Soc Trang, Ben Tre, and Tra Vinh					
22	CM3.2	43	12	30 (6 h)	8.95^{a}
23	CM3.3	41	9	30	7.01^{b}
24	CM4.4	39	9	19.7	6.56^{cd}
25	BL2.1	43	9	30 (6 h)	6.61^{cd}
26	ST2.1	39	9	30 (6 h)	6.48^{cd}
27	BT1.2	39	9	30 (6 h)	6.79^{bc}
28	BT2.1	45	9	30 (18 h)	6.09^{e}
29	BT3.3	41	12	30 (18 h)	6.09^{e}
30	TV4.2	39	12	30	6.32^{de}

*Note: values in the table were the average values of triplication. The average values in a group with the same letter were not significantly different at the 95% confidence level.

Generally, wine fermentation of *C. trifolia* berries using thermotolerant yeast showed the same trend, whereby ethanol concentrations decreased when temperatures were increased. In this study, ethanol concentration were lower than that produced in the optimal temperature. At high temperatures, the accumulation of intracellular ethanol in yeast cells was increased, which stalled yeast growth. As a result, the fermentation ability of the yeast was affected and lower ethanol concentrations were generated [15].

Identification of selected yeast isolates

The results of aligning the 26S rDNA sequences of 30 selected yeast strains with the GenBank database (NCBI) along with an analysis of their morphology, physiology, and biochemistry indicated that all strains belonged to one of the four genera *Saccharomyces, Candida, Clavispora* and *Pichia*. The results of identification of 30 selected yeast isolates are presented in Table 3. *S. cerevisiae* was popularly ultilized for alcoholic fermenting in industrial manufacturing. *S. cerevisiae* was able to yield an ethanol concentration of between 7.4 and 7.7% (w/v) fermenting molasses at room temperature. This species is also likely to grow at high temperatures ranging from 40 to 44^{0}C [16, 17]. Thus, it was decided to use the thermotolerant yeast *S. cerevisiae* HG1.3 to make wine from the fresh berries of *C. trifolia.*

The genetic relation of selected thermotolerant yeasts was determined by constructing a phylogenetic tree based on the 26S rDNA gene using MEGA 6 software (Neighbor-Joining). The phylogenetic tree for 30 selected yeast strains is shown in Fig. 1.

Table 3. The identification results of 30 selected yeast isolates.

No.	Genera	Species	Name of yeast isolate	No. of isolate
1	*Candida*	*Candida tropicalis*	KG1.1, KG3.2, CM3.3, HG3.3, TG1.1	5
		Candida nivariensis	DT1.2, ST2.1, BT1.2	3
		Candida glabrata	BL2.1, CT1.1, CT1.3, CT2.3,	4
2	*Pichia*	*Pichia kudriavzevii*	KG5.1, AG2.3, AG4.2, CM4.4, BT2.1, BT3.3, CT4.2, VL1.1	8
3	*Clavispora*	*Clavispora lusitaniae*	TG4.2	1
4	*Saccharomyces*	*Saccharomyces cerevisiae*	HG1.3, CM3.2, AG2.1, TV4.2, DT3.2, LA1.3, KG2.1, TG3.1, HG2.1	9
	Total			**30**

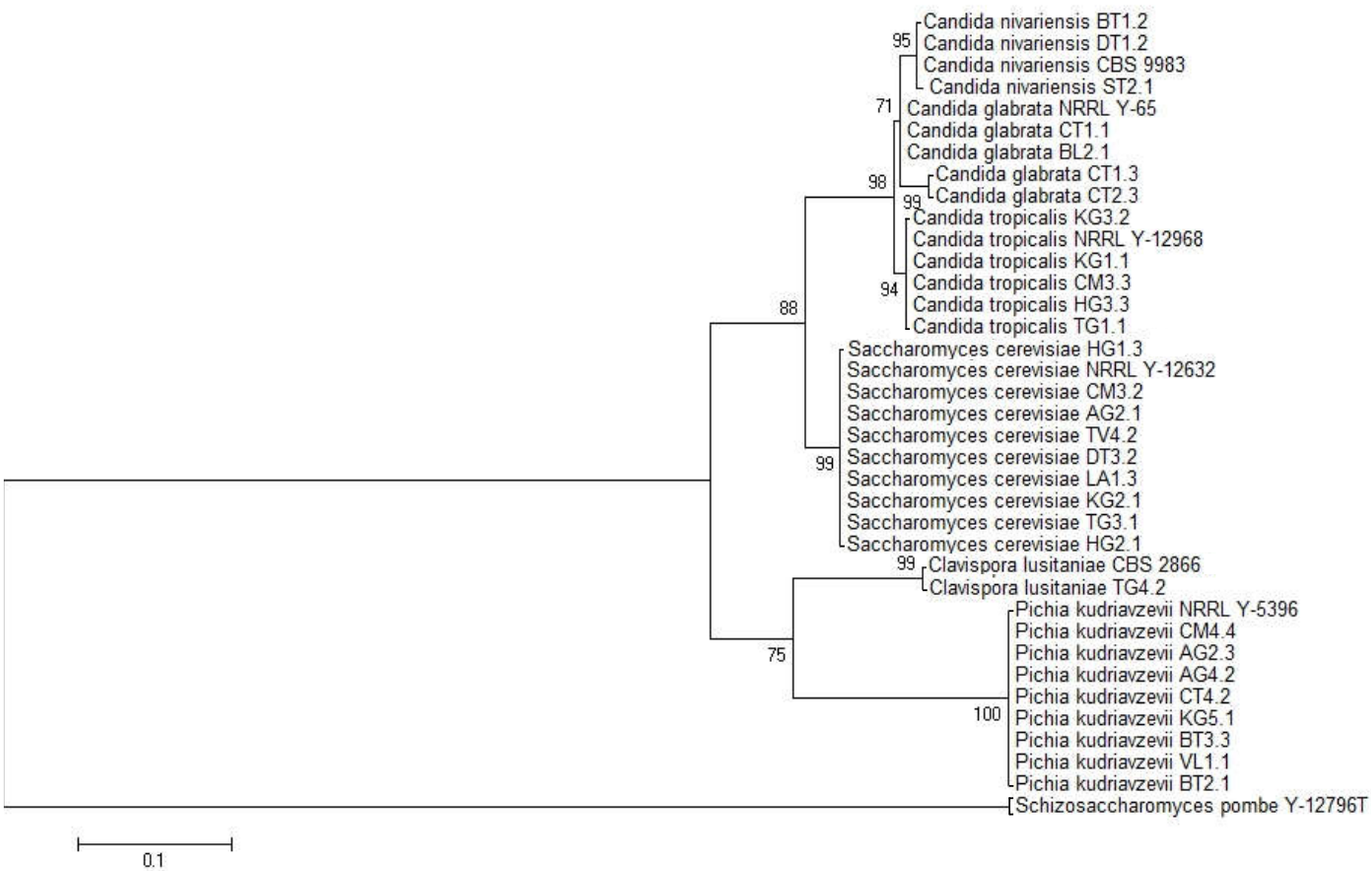

Fig. 1. Phylogenetic tree of 30 selected yeast strains.

The phylogenetic tree showed the genetic relation of the selected thermotolerant yeasts. It indicated that *Saccharomyces cerevisiae* HG1.3, CM3.2, AG2.1, TV4.2, DT3.2, LA1.3, KG2.1, TG3.1, and HG2.1 are the most closely related strains because of their high reliability (with 100% Bootstrap) and that the first distinct branch is *Candida nivariensis*.

Conclusions

The diversity of yeast isolates purified from *C. trifolia* berry samples was examined, and a number of ethanol- and thermo-tolerant ethanologenic yeasts were found. The feasibility of fermentation products from *C. trifolia* by the selected yeast isolates at high temperature was confirmed. This study indicated the promising applications of such isolates for the controlled *C. trifolia* wine fermentation at high temperature.

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REFERENCES

[1] A.K. Gupta, M. Shamar (2007), "Review on Indian medical plant", *Council of Medical Research,* **7**, pp.879-882.

[2] P.C. Perumal, S. Sowmya, P. Pratibha, B. Vidya, P. Anusooriya, Starlin, S. Ravi, and V.K. Gopalakrishnan (2015), "Isolation, structural characterization and in silico drug-like properties prediction of a natural compound from the ethanolic extract of *Cayratia trifolia* (L.)", *Pharmacognosy Reviews*, **7(1)**, pp.121-125.

[3] H.X. Phong, N.T.C. Giang, S. Nitiyon, M. Yamada, P. Thanonkeo, and N.T.P. Dung (2016), "Ethanol production from molasses at high temperature by thermotolerant yeasts isolated from cocoa", *Can Tho University Journal of Science*, **3**, pp.32-37.

[4] I.M. Banat, P. Nigam, D. Singh, R. Marchant, A.P. McHale (1998), "Review: ethanol production at elevated temperatures and alcohol concentrations: part I - yeasts in general", *World Journal of Microbiology and Biotechnology*, **14**, pp.809-821.

[5] M. Roehr (2001), *The biotechnology of ethanol: classical and future applications*, 1st edition, Wiley-VHC, Weinheim.

[6] S. Limtong, C. Sringiew, W. Yongmanitchai (2007), "Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*", *Bioresource Technology*, **98**, pp.3367-3374.

[7] B.A. Abdel-Banat, H. Hoshida, A. Ano, S. Nonklang, R. Akada (2010), "High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast", *Applied Microbiology and Biotechnology*, **85**, pp.861-867.

[8] K. O'Donnell (1993), "Fusarium and its near relatives", *The Fungal holomorph: mitotic, meiotic and pleomorphic speciation in Fungal systematics*, Reynolds D.R. & Taylor J.W. (Eds), pp.225-233, CAB International, Wallingford.

[9] W.Z., Bruce, C.F. Kenneth, H.G. Barry, S.N. Fred (1995), *Wine analysis and production*, pp.447-449, Chapman and Hall, New York.

[10] A.M. Neiman (2005), "Ascospore formation in the yeast *Saccharomyces cerevisiae*", *Microbiology and Molecular Biology Reviews*, **69(4)**, pp.565-584.

[11] P.C. Kurtzman, J.W. Fell, T. Boekhout, V. Robert (2011), "Methods for isolation, phenotypic characterization and maintenance of yeast", *The Yeast, a taxonomic study*, **1**, 5th ed., Elsevier, Amsterdam.

[12] J.A. Barnett, R.W. Payne, D. Yarrow (1983), *Yeast: characteristics and identification*, Cambridge University Press, Cambridge.

[13] R. Ueno, N. Urano, S. Kimura (2002), "Effect of temperature and cell density on fermentation by a thermotolerant aquatic yeast strain isolated from a hot spring environment", *Fisheries Science*, **68**, pp.571-578.

[14] M. Phisalaphong, N. Srirattana, W. Tanthapanichakoon (2006), "Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation", *J. Biochem. Eng.*, **28**, pp.36-43.

[15] T. D'Amore, C.J. Panchal, I. Russell, G.G. Stewart (1990), "A study of ethanol tolerance in yeast", *Critical Reviews in Biotechnology*, **9(4)**, pp.287-304.

[16] W.R. Abdel-Fattah, M. Fadil, P. Nigam, I.M. Banat (2000), "Isolation of thermotolerant ethanologenic yeasts and use of selected strains in industrial scale fermentation in an Egyptian distillery", *Biotechnology and Bioengineering*, **68(5)**, pp.531-535.

[17] K.N. Sree, M. Sridhar, K. Suresh, I.M. Banat, L. Venkateswar-Rao (2000), "Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production", *Bioresource Technology*, **72(1)**, pp.43-46.