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Marine killer yeasts active against a yeast strain pathogenic to crab *Portunus trituberculatus*

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ABSTRACT: Some marine yeasts have recently been recognised as pathogenic agents in crab mariculture, but may be inhibited or killed by 'killer' yeast strains. We screened multiple yeast strains from seawater, sediments, mud of salterns, guts of marine fish, and marine algae for killer activity against the yeast *Metchnikowia bicuspidata* WCY (pathogenic to crab *Portunus trituberculatus*), and found 17 strains which could secrete toxin onto the medium and kill the pathogenic yeast. Of these, 5 strains had significantly higher killing activity than the others; routine identification and molecular methods showed that these were *Williopsis saturnus* WC91-2, *Pichia guilliermondii* GZ1, *Pichia anomala* YF07b, *Debaryomyces hansenii* hcx-1 and *Aureobasidium pullulans* HN2.3. We found that the optimal conditions for killer toxin production and action of killer toxin produced by the marine killer yeasts were not all in agreement with those of marine environments and for crab cultivation. We found that the killer toxins produced by the killer yeast strains could kill other yeasts in addition to the pathogenic yeast, and NaCl concentration in the medium could change killing activity spectra. All the crude killer toxins produced could hydrolyze laminarin and the hydrolysis end products were monosaccharides.

KEY WORDS: Marine-derived yeasts · Killer yeasts · Killer toxin · Pathogenic yeasts · Crab

INTRODUCTION

Many diseases in marine animals are caused by marine bacteria and marine viruses. However, in recent years studies have shown that some marine yeasts are also pathogenic to some marine animals. Like bacterial and virus diseases, yeast disease has caused large economic losses in the maricultural industry in some regions of China (Xu et al. 2003, Xu 2005). For example, an explosive epidemic of a yeast disease called 'milky disease' has occurred in cultured *Portunus trituberculatus* since 2001 in Zhoushan, Zhejiang Province, China, leading to high mortality of this crab and great economic loss in this area. Results of conventional and molecular identification show that the pathogenic agent for milky disease is the yeast *Metschnikowia bicuspidata* (Xu et al. 2003). The purified yeast strain obtained from diseased parts of marine animals also produced the same symptoms in the muscle, heart and hepatopancreas in a challenge test (Xu et al. 2003). It was also found that nystatin, benzalkonium bromide and extracts of goldthread root and garlic were active against the pathogenic yeast. However, the compounds with minimum inhibitory concentration (MIC) were toxic to the crab and it would be impossible to apply antibiotics in the open sea.

The yeast *Torulopsis mogii* is a pathogen to some shrimp in China (Sun & Sun 1998). *Metschnikowia bicuspidata* var. *bicuspidata*, a pathogenic yeast of aquatic invertebrates, was capable of infecting aquaculture-reared and disease-free *Artemia* (Moore & Strom 2003). A new species of marine yeast, *Kluyveromycs penaeid,* was isolated from the heart tissue of subadult shrimp *Peneus chinensis* during tissue culture. The yeast grew well in seawater supplemented with 2% shrimp extract, but did not grow in yeast peptone dextrose (YPD) and malt extract medium in which most yeast cells grow well (Tong & Miao 1999). Much research has shown that killer yeasts can be applied to

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control the growth of pathogenic yeasts in humans, animals and plants. Killer toxins produced by some yeast strains are low molecular mass proteins or glycoprotein toxins which kill sensitive cells of the same or related yeast genera without direct cell–cell contact (Magliani et al. 1997). The killer strains themselves are immune to their own toxin, but remain susceptible to the toxins secreted by other killer yeasts. The killer phenotype is very common in occurrence and can be found both in natural yeast isolates and in laboratory yeast strain collections. To date, toxin-producing killer yeasts have been identified in the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Ustilago*, *Torulopsis*, *Williopsis* and *Zygosaccharomyces*, indicating that the killer phenomenon is indeed widespread among yeasts (Magliani et al. 1997). Killer determinants are cytoplasmically inherited encapsulated dsRNA viruses, linear dsDNA plasmids or nuclear genes (Schmitt & Breinig 2002). Analysing the mechanisms of killer toxins can also provide important information for combating yeast infections caused by certain human pathogenic strains of the yeasts *Candida albicans* and/or *Sporothrix schenkii* (Comitini et al. 2004). However, little is known about marine killer yeasts and the killer toxins they produce. The ecological distribution, killer-susceptible interaction, killing activity and spectrum of killing activity of the terrestrial yeast killer phenomenon (toxin production and susceptibility) have been extensively studied in yeast communities, particularly in decaying stems and fruits (Magliani et al. 1997). However, diversity of killer yeasts in different marine environments is still unclear. Therefore, the main objective of the present study was to investigate marine-derived yeasts. We specifically examined those that are active against the pathogenic yeast which can cause milky disease in *Portunus trituberculatus*, in order to develop an effective tool against infection of the crab by the pathogenic yeast in the future.

MATERIALS AND METHODS

Yeast strains. *Metschnikowia bicuspidata* WCY, which has been confirmed as a pathogenic yeast in *Portunus trituberculatus* (Xu et al. 2003, Xu 2005) was supplied by W. J. Xu at the Fishery Institute of Zhejiang Province, China. *Cryptococcus aureus*, *Lodderomyces elongisporus*, *Candida albicans*, *Yarrowia lipolytica* and *Rhodotorula mucilaginosa* isolated from different marine environments, identified in our laboratory and deposited at the Marine Culture Collection of China (www.mccc.org.cn) were used as susceptible yeast strains. *Saccharomyces* sp. W0, which is a high ethanol-producing yeast (Chi & Arneborg 2000), was also used as a representative of terrestrial yeasts. *Williopsis saturnus* WC91-2, *Pichia guilliermondii* GZ1, *Pichia anomala* YF07b, *Debaryomyces hansenii* hcx-1 and *Aureobasidium pullulans* HN2.3, isolated and identified in our laboratory and deposited at the Marine Culture Collection of China (www.mccc.org.cn) were used as killer toxin producers.

Media. Growth medium was YPD medium (prepared with seawater) containing 20 g l^{-1} glucose, 20 g l^{-1} peptone, 10 g l^{-1} yeast extract. The assay medium (prepared with seawater) for killer toxins and their actions was YPD medium with 1.5 mg of methylene blue per 100 ml, and 25 to 35 g l^{-1} agar adjusted to pH 4.5, 5.0, 6.0 and 7.0 with 0.05 M citrate-phosphate buffer (Santos et al. 2000). Killer toxin production medium (prepared with seawater) was YPD medium with different concentrations of NaCl and 50 g l^{-1} glycerol adjusted to pH 4.5, 5.0, 6.0 and 7.0 with 0.05 M citrate-phosphate buffer.

Sampling. Different samples of seawater and sediments in the South China Sea (100 m depth, 20°C, pH 8.1 and 2.89% salinity, summer of 2004), Indian Ocean (50 m depth, 25°C, pH 8.1 and 2.89% salinity, summer of 2005) and the Pacific Ocean (200 m depth, 20°C, pH 8.1 and 2.89% salinity, winter of 2004) were collected during an Antarctic expedition in 2004. Hypersaline seawater (1 m depth, 26°C, pH 8.1 and 15% salinity, Spring of 2004), sediments of the salterns (1 m depth, 26°C, pH 8.1 and 15% salinity, autumn of 2004), different species of marine animals and algae along the coast of Qingdao, China (10 m depth, 15°C, pH 8.1 and 2.89% salinity, autumn of 2005) were also collected.

Isolation of marine yeasts. We suspended 2 ml of the seawater or 2 g of sediments or 2 ml of homogenized guts of marine animals or homogenised marine algae in 20 ml of YPD medium prepared with seawater and supplemented with 0.5 g l^{-1} chloramphenicol immediately after sampling and cultivated at natural temperature on the ship for 5 d. After suitable dilution of the cell cultures, the dilute was plated on YPD plates with 0.5 g l^{-1} chloramphenicol and incubated at 20 to 25°C for 5 d. Different colonies from the plates were transferred to the YPD slants.

Screening of marine killer yeasts. Each yeast strain from the slants was grown in YPD liquid medium for 24 h at 28°C and the cells were collected in 2 ml of culture and washed 3 times with sterilized saline water by centrifugation. Cells of the pathogenic yeast strain and susceptible yeasts grown in YPD medium were suspended in sterile saline water; 0.2 ml of the suspension (10^7 cells ml^{-1}) were spread on the assay medium. For examination of the killing activity, the washed cells were inoculated onto the above mentioned assay medium on which cells of the pathogenic yeast strain

and susceptible yeast strains had been spread. After 2 to 3 d of incubation at 23°C, a clear killing zone was observed around the colonies of killer yeasts. The killing activity was calculated based on the ratio of the diameter of the inhibition zone to the diameter of the colony.

DNA extraction, PCR and DNA sequencing. DNA extraction and PCR techniques for amplification of ITS and 26S rDNA in the yeasts were performed according to the methods described by Chi et al. (2007). The common primers for amplification of D1/D2 26S rDNA sequence in yeasts were used, the forward primer NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and the reverse primer NL-4 (5′-GGTCCGTGTTT CAAGACGG-3′) (Sugita et al. 2003). The common primers for amplification of ITS in yeasts were used; the forward primer P11 (5′-TCCGTAGGTGAACCT-GCGG-3′) and the reverse primer P21 (5′-TCCTC-CGCTTATTGATATGC-3′) (Josefa et al. 2004). The ITS and D1/D2 26S rDNA fragments inserted into the vector (pMD-19T) were sequenced by Shanghai Sangon.

Phylogenetic analysis of the yeasts. Maximum parsimony analyses were performed using MEGA 4.0 (Tamura et al. 2007). Bootstrap analysis was performed on 1000 random resamplings. Reference sequences were retrieved from GenBank under the accession numbers indicated in the tree.

Identification of the yeasts. Routine identification of the yeasts was performed using the methods described by Kurtzman & Fell (1998).

Production of killer toxin. The killer yeasts were cultivated for 3 d at different temperatures ranging from 20 to 35°C in 500 ml-Erlenmeyer flasks with 150 ml of the production medium with different pH and NaCl concentrations. Cultures were incubated in a rotary bed shaker (130 rpm). After centrifugation (5000 × *g*, 10 min, 4°C), the supernatant was thoroughly mixed with glycerol (the final glycerol concentration was 15 g 100 ml^{-1}) and the mixture was concentrated to a volume of 15 ml by ultrafiltration with a 5-kDa-cutoff™ membrane with a Labscale TFF System (Millipore). These partially purified concentrated supernatants were used as the killer toxin concentrates (Santos et al. 2000).

Measurement of killer toxin activity. We assayed killer toxin activity with a diffusion test, using 6-mm-diameter sterile Oxford-cups (6 × 10 mm) which were put on the assay medium (with different NaCl concentrations) seeded with yeast strain WCY. Finally, 200 µl of the killer toxin concentrate was added to each cup and incubated at different temperatures and pH for 72 h and the diameter of the inhibition zone was used as a measure of the yeast killer toxin activity (Santos et al. 2000).

Laminarin hydrolysis. Reaction mixtures containing 20 µl of 26.8 U ml^{-1} of the concentrated crude killer toxin and 2.0 g l^{-1} laminarin in 0.1 ml of buffer (0.05 M, pH 4.5) was incubated at 35°C for 10 h. After that, the toxin in the mixture was immediately inactivated by heating at 100°C for 15 min (Gong et al. 2007). The end products of laminarin hydrolysis were determined by thin layer chromatography (Gong et al. 2007). The reaction mixtures in which the crude killer toxin was inactivated prior to addition by heating at 100°C for 15 min were used as controls.

RESULTS

Screening of marine killer yeasts against the pathogenic yeast strain WCY

We found that 17 yeast strains isolated from seawater, sediments, mud of salterns, guts of the marine fish and marine algae could secrete killer toxins onto the medium and kill the pathogenic yeast *Metschnikowia bicuspidata* WCY (data not shown). However, 5 killer yeast strains (WC91-2, GZ1, YF07b, hcx-1 and HN2.3), belonging to various species of yeasts, had higher killing activity (ratio of diameter of inhibition zone to diameter of colony was ≥1.5) (Fig. 1) against the pathogenic yeast than the others and these were used for further analyses. Of these strains, WC91-2 from the rhizosphere of *Barringtonia asiatica* and YP07b from the gut of sea squirts had the highest killing activities against the pathogenic yeast (Table 1).

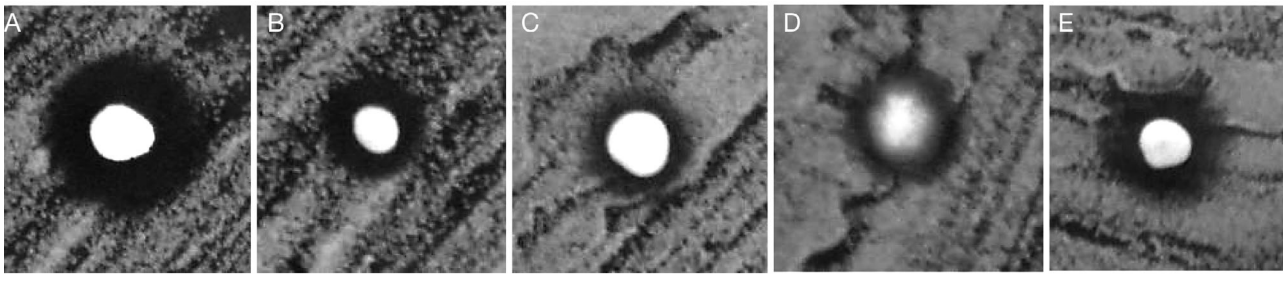

Fig. 1. Clear zones formed on assay medium seeded with pathogenic yeast WCY. Strains: (A) WC91–2; (B) GZ1; (C) hcx–1; (D) HN2.3; (E) YF07b

Table 1. Marine killer yeast strains, killing activity and source. Ratio = diameter of inhibition to diameter of colony. Values are mean ± SD, n = 3

Strain	Inhibition (mm)	Colony (mm)	Ratio	Source
hcx-1	4.5 ± 0.1	3.0 ± 0.1	1.5 ± 0.1	Gut of *Hexagrammos otakii*
WC91-2	9.0 ± 0.2	3.0 ± 0.15	3.0 ± 0.15	Rhizosphere of *Barringtonia asiatica*
GZ1	5.0 ± 0.15	2.0 ± 0.1	2.5 ± 0.1	Gut of *Cleisthenes herzensteini*
HN2.3	3.0 ± 0.1	2.0 ± 0.2	1.5 ± 0.15	Sea mud in salterns in Qingdao
YF07b	6.0 ± 0.2	2.0 ± 0.1	3.0 ± 0.1	Gut of sea squirts

Characterisation of the marine killer yeast strains

We found that the colony and cell morphology of the 5 killer yeast strains were very diverse (data not shown). Based on the fermentation and carbon source assimilation spectra (Table 2) and the type strains of these marine yeasts (Kurtzman & Fell 1998), we found that strains WC91-2, GZ1, YF07b, hcx-1 and HN2.3 were closely related to *Williopsis saturnus*, *Pichia guilliermondii*, *Pichia anomala*, *Debaryomyces hansenii* and *Aureobasidium pullulans*, respectively. ITS and D1/D2 26S rDNA sequences of the 5 yeast strains were determined and aligned and the identification based on these sequences and the similarity to the references are shown in Table 3. Phylogenetic trees were constructed as described in 'Materials and methods' above. The search for similarities between ITS and D1/D2 26S rDNA of the isolates and those in the NCBI database showed that many phylogenetically related yeast species were similar to the marine yeast strains obtained in this study (Figs. 2 & 3). The topology of the phylograms in Figs. 2 & 3 confirms that strain WC91-2 was closely related to *W. saturnus*, and strain GZ1 has a close relationship to *P. guilliermondii*. The strain YF07b was assigned to *P. anomala*. D1/D2 26S rDNA sequences and ITS of yeast strains hcx-1 and HN2.3 were identical to those of *D. hansenii* and *A. pullulans*, respectively.

Table 2. Fermentation and assimilation of different sugars by marine killer yeasts. Fermentation carried out at 25°C on media with 2% sugars at natural pH. Assimilation performed at 25°C on media with 0.5% sugars at natural pH. + = positive result; – = negative result; w = weak

	Marine killer yeast strain				
	WC91-2	GZ1	YF07b	hcx-1	HN2.3
Fermentation					
Glucose	+	+	+	+	–
Maltose	–	–	+	–	–
Galactose	+	+	+	+/w	–
Sucrose	+	+	+	–	–
Lactose	–	–	–	–	–
Raffinose	+	+/w	+	–	–
Melibiose	+	–	–	–	–
Assimilation					
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Galactose	+	+	+	+	+
Sucrose	+	+	+	+	+
Lactose	+	–	+	–	–
Raffinose	+	+	+	+	+
Melibiose	–	+	–	+	+
Amidulin	+	–	–	+	+
Trehalose	–	+	+	+	+
Cellobiose	+	+	+	+	+
D-arabinose	–	+	+	+	+
Xylose	–	+	+	+	+
L-arabinose	+	+	+	+	+

Table 3. Identification based on 26S rDNA and ITS sequences of 5 marine killer yeast strains. Similarity = similarity to reference sequences

Strain	Identification	Similarity (%)
WC91-2	*Williopsis saturnus*	99
GZ1	*Pichia guilliermondii*	99
YF07b	*Pichia anomala*	100
hcx-1	*Debaryomyces hansenii*	99
HN2.3	*Aureobasidium pullulans*	99

Effects of NaCl, pH and temperature on killer toxin production

Cultivation temperature and pH and NaCl concentrations in growth medium have obvious influences on killer toxin production by yeasts (Llorente et al. 1997, Marquina et al. 2001). Indeed, we found that the optimal pH for killer toxin production was 4.5 by *Williopsis saturnus* WC91-2 and *Pichia anomala* YF07b, 5.0 for

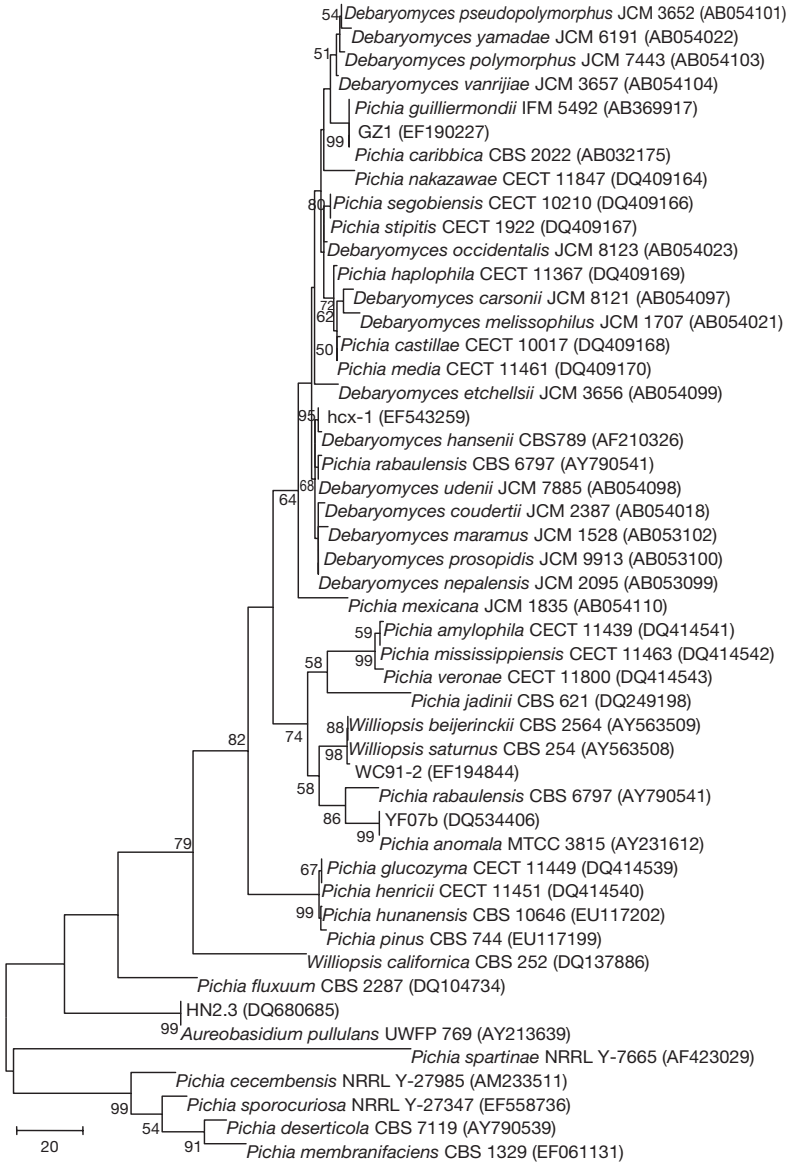

Fig. 2. Phylogenetic tree of 5 marine killer yeasts and 43 closest relatives based on a maximum parsimony analysis of ITS sequences. Bootstrap values (1000 pseudoreplications) of ≥50%. Strain numbers and sequence accession numbers given. All strains shown are type strains

Aureobasidium pullulans HN2.3, and 6.0 for *P. guilliermondii* GZ1 and *Debaryomyces hansenii* hcx-1 (Table 4). Thus, most of the killer yeasts produced the highest amount of killer toxin under acidic conditions. The optimal NaCl concentration for killer toxin production by strains *W. saturnus* WC91-2 and *P. anomala* YF07b was 20 g l^{-1} NaCl, and for *A. pullulans* HN2.3 was 40 g l^{-1} (Table 4). However, the highest yield of killer toxin produced by strains *P. guilliermondii* GZ1 and *D. hansenii* hcx-1 was achieved in the medium lacking NaCl. The optimal temperature for killer toxin production by all the yeast strains was around 20°C (Table 4).

Optimal conditions for action of killer toxins produced by the yeasts

The yeast strains used in this study could produce the largest amount of killer toxins under different conditions (Table 4). Therefore, the supernatant of the culture was mixed with glycerol and the mixture was concentrated. Killer toxin activity in these partially purified concentrated supernatants was measured under different conditions. Killer toxin activity from all the killer yeast strains was the highest when the pathogenic yeast *Metschnikowia bicuspidata* WCY grew at 15°C in the assay medium with pH 4.5 (Table 4). However, killer toxin activity from the yeast strains *Pichia anomala* YF07b and *Debaryomyces hansenii* hcx-1 toxins was the highest when the pathogenic yeast *M. bisuspidata* WCY grew in the assay medium with 30.0 g l^{-1} NaCl whereas the killer toxin activity from *Williopsis saturnus* WC91-2, *P. guilliermondii* GZ1 and *Aareobasidium pullulans* HN2.3 toxins was the highest when the pathogenic yeast strain *M. bicuspidata* WCY grew in the assay medium without added NaCl.

Killing activity spectra of the killer toxins

The killer toxins produced by *Williopsis saturnus* WC91-2 and *Pichia anomala* YF07b could kill all the yeast strains tested except *Rhodotorula mucilaginosa* in the absence of NaCl (Table 5). The results were identical to those in Table 4. However, the killer toxin produced by *P. anomala* YF07b and *W. saturnus* WC91-2 lost the ability to kill *Candida albicans*, while the killer toxin produced by *P. anomala* YF07b lacked the ability to kill *Cryptococcus aureus* in the presence of 30.0 g l^{-1} NaCl. The killer toxins produced by *P. guilliermondii* GZ1 only actively killed the pathogenic yeast in the absence of NaCl (Table 5). However, killer toxins produced by *D. hansenii* hcx-1 killed *Saccharomyces* sp. W0 in addition to the pathogenic yeast in the presence of NaCl. These results clearly suggest that changes in concentration of NaCl affects the killing activity spectra of the toxins produced by the yeast strains used in this study.

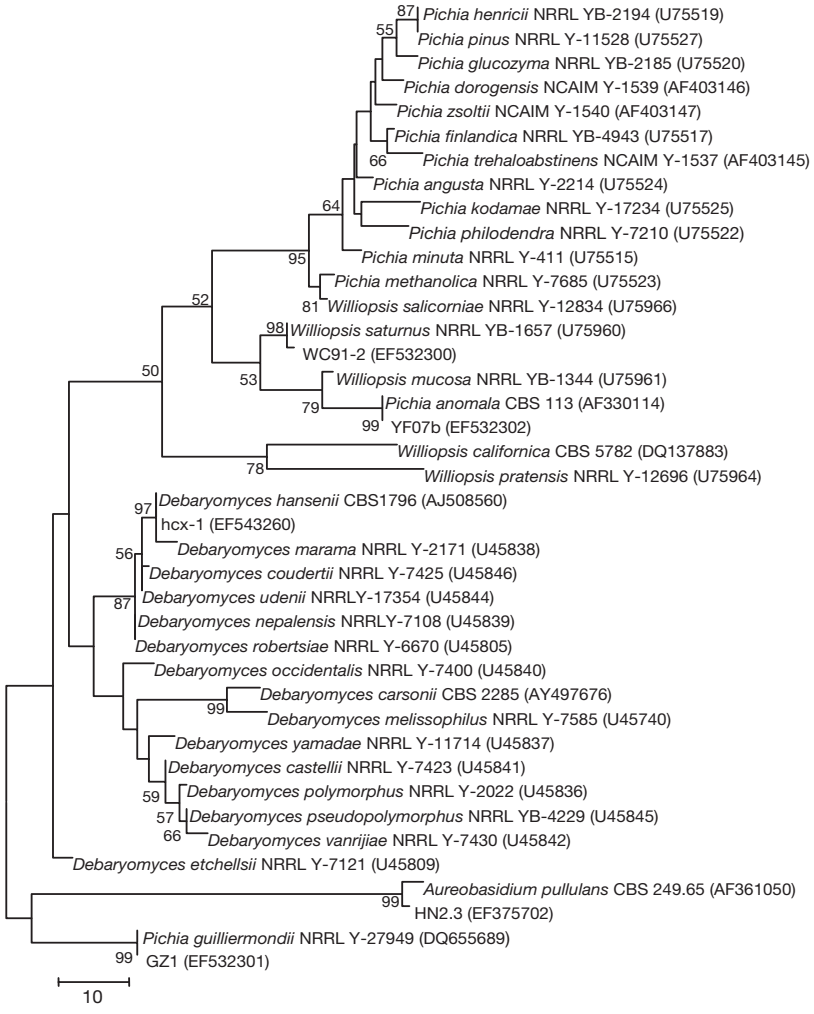

Fig. 3. Phylogenetic tree of 5 marine killer yeasts and 35 closest relatives based on a maximum parsimony analysis of D1/D2 rDNA sequences. Bootstrap values (1000 pseudoreplications) of ≥50 %. Strain numbers and sequence accession numbers are given. All strains shown are type strains

Laminarin hydrolysis

Laminarin (β-1,3-glucan) exists in the cell wall of most yeast cells (Izgu et al. 2006). All killer toxins produced by the yeast strains could actively hydrolyze laminarin and end products of laminarin hydrolysis were monosaccharides (Fig. 4). The results imply that the killer toxins had very high exo-β-1,3-glucanase activity. Such killer toxins are of medical and ecological importance because they are not toxic to animal cells, which lack a cell wall containing β-1,3-glucan.

DISCUSSION

Xu (2005) found that nystatin, benzalkonium bromide and extracts of goldthread root and garlic are active against the pathogenic yeast used in this study. However, the compounds with minimum inhibitory concentration (MIC) are toxic to the crab. Furthermore, the application of antibiotics to the open sea is not permissible (Xu 2005). Therefore, it is very important to find a method for combating pathogenic yeasts. Killer yeasts can be used to control the growth of harmful yeasts in plants, humans, animals, grains and fermentation medium (Sugita et al. 2003). Thus, marine killer yeasts may be an important agent against pathogenic yeasts. Among the 17 killer yeast strains

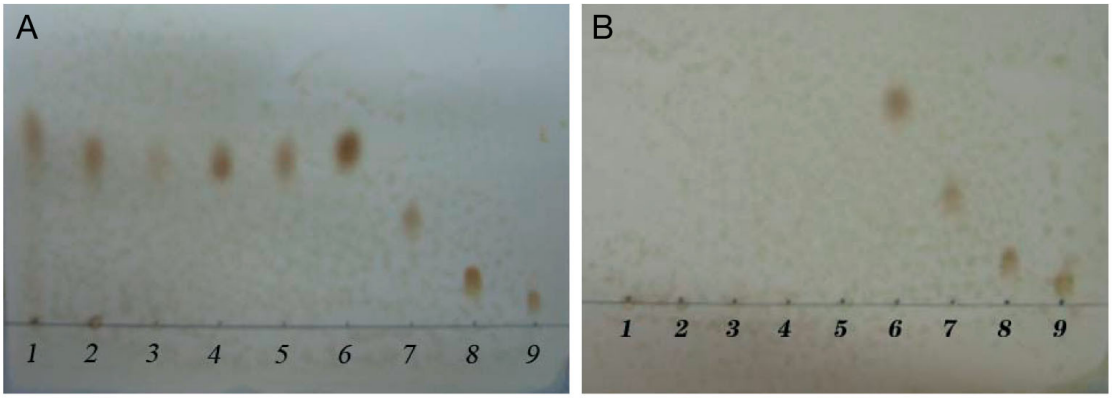

Fig. 4. Thin-layer chromatogram of hydrolysis products of laminarin with crude killer toxins. Lanes A1, A2, A3, A4 and A5: hydrolysis products (laminarin + crude killer toxins produced by strains HN2.3, GZ1, hcx-1, YF07b and WC91-2, respectively); Lanes B1, B2, B3, B4 and B5: controls (laminarin + heat inactivated crude killer toxins produced by strains HN2.3, GZ1, hcx-1, YF07b and WC91-2, respectively); Lanes A6, B6, A7, B7, A8, B8, A9 and B9: glucose, maltose, kestose (trisaccharides) and nystose (tetrasaccharides)

Table 4. Optimal NaCl concentration, pH and temperature for killer toxin production and action of killer toxins produced by the yeast strains. Values are mean ± SD, n = 3. A = optimal conditions for killer toxin production. Killer toxin production medium was YPD medium, various concentrations of NaCl, and 50.0 g l^{-1} glycerol adjusted to pH 4.5, 5.0, 6.0 and 7.0 with 0.05 M citrate-phosphate buffer. B = optimal conditions for action of killer toxins produced by yeast strains. Assay medium for killer toxin and its action was YPD medium, 1.5 mg of methylene blue per 100 ml, and 25 to 35 g l^{-1} agar

		WC91-2	GZ1	YF07b	hcx-1	HN2.3
pH	A	4.5 ± 0.3	6.0 ± 0.5	4.5 ± 0.2	6.0 ± 0.3	5 ± 0.2
	B	4.5 ± 0.4	4.5 ± 0.3	4.5 ± 0.2	4.5 ± 0.3	4.5 ± 0.1
Temperature (°C)	A	20 ± 2	25 ± 1	20 ± 3	25 ± 2	25 ± 1
	B	15 ± 2	15 ± 1	15 ± 3	15 ± 2	15 ± 2
NaCl concentrations (g l^{-1})	A	20.0 ± 2.0	0	20.0 ± 3.0	0	40.0 ± 1.0
	B	0	0	30.0 ± 3.0	30 ± 2.0	0

Table 5. Killing activity spectra of killer toxins. Ratio of inhibition zone diameter to colony diameter was: >2.5 (+++); 1 to 2.5 (++); 0 to 1 (+); 0 (–)

Sensitive strain	NaCl conc. (g l^{-1})	Killer yeast strain HN2.3	WC91-2	GZ1	YF07b	hcx-1
Cryptococcus aureus	0	–	++	–	++	–
	30.0	–	+	–	–	–
Lodderomyces elongisporus	0	–	++	–	++	–
	30.0	–	++	–	++	–
Candida albicans	0	–	++	–	+	–
	30.0	–	–	–	–	–
Metschnikowia bicuspidata	0	++	+++	+++	+++	++
	30.0	+	++	+	+++	++
Saccharomyces sp.W0	0	–	++	–	+	–
	30.0	–	+++	–	+	+
Yarrowia lipolytica	0	–	+	–	++	–
	30.0	–	++	–	+	–
Rhodotorula mucilaginosa	0	–	–	–	–	–
	30.0	–	–	–	–	–

isolated from different sources in marine environments, 5 produced relatively larger amounts of killer toxin against the pathogenic yeast *Metschnikowia bicuspidata* WCY. The 5 yeast strains were identified as *Williopsis saturnus* WC91-2, *Pichia guilliermondii* GZ1, *P. anomala* YF07b, *Debaryomyces hansenii* hcx-1 and *Aureobasidium pullulans* HN2.3, according to the results of routine identification and molecular methods, which were in complete agreement. Our results also indicated that killer yeasts can be found in various marine environments, especially the guts of marine animals. Magliani et al. (1997) stated that the killer phenotype is very common in occurrence and can be found both in natural yeast isolates and in laboratory yeast strain collections. So far, toxin-producing killer yeasts isolated from terrestrial environments have been identified in the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces, Ustilago*, *Torulopsis*, *Williopsis* and *Zygosaccharomyces* (Magliani et al. 1997). Therefore, *A. pullulans* HN2.3 found in this study is a newly identified producer of killer toxin. In recent years, *A. pullulans* has been found to be widely distributed in sea salterns and the deep sea and has many applications in biotechnological field (Chi et al. 2007, Li et al. 2007).

We found that optimal NaCl concentration and temperature for the production of killer toxins by the yeast strains were similar to the natural conditions of marine environments and cultivation conditions for crabs. The lethal activity of the killer toxin produced by marine yeast *Debaryomyces hansenii* increases with the presence of NaCl (60 g l^{-1}) in the medium used for testing killing action (Marquina et al. 2001), and the yeast strain produces a strong active killer substance which is effective against various genera of yeasts (Llorente et al. 1997). Also, *Pichia membranifaciens*' killer toxin is active against some sensitive strains such as *Candida boidinii* IGC 3430 only in the presence of NaCl, and this peculiarity has been studied to establish the role of NaCl in the killer character of this yeast (Santos et al. 2000). However, it is still completely unknown how NaCl availability in the medium affects killer toxin production by the yeasts. The optimal pH for production of killer toxins (pH 4.5 to 6.0) was not consistent with that found in marine environments (i.e. around pH 8.0). It has been previously reported that the optimal pH for killer toxin production by *Williopsis saturnus* var. *mrakii* MUCL 41968 is 3.5, while for *Debaryomyces hansenii* CYC 1021 it is 4.0 (Marquina et al. 2001).

We also found that optimal temperature for action of killer toxins produced by all the yeast strains was similar to the natural conditions of marine environments and cultivation conditions for crabs, as was the optimal NaCl concentration for action of killer toxins produced by *Pichia anomala* YF07b and *Debaryomyces hansenii* hcx-1. However, the optimal pH for action of killer toxins produced by all yeast strains and NaCl concentrations for action of killer toxins produced by *Williopsis saturnus* WC91-2, *P. guilliermondii* GZ1 and *A. pullu-*

lans HN2.3 were not consistent with that in marine environments (around pH 8.0). Therefore, whether or not the killer yeasts can effectively protect the crabs against infection by *Metschnikowia bicuspidata* WCY is currently being addressed in our laboratory using a challenge test. The killer toxins produced by *W. saturnus* WC91-2 and *P. anomala* YF07b were able to kill most of the yeast strains tested and they also had very high exo-β-1,3-glucanase activity. It has been previously reported that the *Pichia* and *Williopsis* killer systems are of great interest because they interfere with the synthesis of β-1,3-D-glucan, the major cell wall polysaccharide polymer in sensitive yeasts (Magliani et al. 1997). Also, their activity is against a wide range of unrelated microorganisms, such as yeasts, hyphomycetes and bacteria, including important opportunistic pathogens such as *Candida albicans* and mycelial and yeast forms of dimorphic fungi (Magliani et al. 1997). It is possible that multiple toxins, active against different susceptible strains, are produced by a single killer strain (Llorente et al. 1997). Therefore, killer toxins produced by these 2 killer yeasts may have some advantages over those produced by other killer yeasts. Killer toxin production and killing activity by some yeast strains, especially *W. saturnus* WC91-2 and *P. anomala* YF07b were enhanced in the presence of NaCl. It has also been reported that *P. membranaefaciens'* killer toxin is active against some sensitive strains such as *C. boidinii* IGC 3430 only in the presence of sodium chloride (Santos et al. 2000). The lethal activity of the killer toxin produced by *D. hansenii* CYC 1021 increased with the presence of NaCl in the medium (Marquina et al. 2001). This discovery is of biological and biotechnological importance. Therefore, the mechanisms of the enhancement at molecular levels are also of interest.

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