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ORIGINAL PAPER

Production of a novel and cold-active killer toxin by *Mrakia frigida* 2E00797 isolated from sea sediment in Antarctica

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Abstract The psychrotolerant yeast *Mrakia frigida* 2E00797 isolated from sea sediment in Antarctica was found to be able to produce killer toxin against the pathogenic yeast (*Metschnikowia bicuspidata* WCY) in crab. When the psychrotolerant yeast was grown in the medium with pH 4.5 and 3.0% (wt/vol) NaCl and at 15°C, it could produce the highest amount of killer toxin against the pathogenic yeast *M. bicuspidata* WCY. The crude killer toxin activity against the pathogenic yeast *M. bicuspidata* WCY was the highest when it grew at 15°C in the assay medium with 3.0% (wt/vol) NaCl and pH 4.5. At temperatures higher than 25°C, the killing activity produced by *M. frigida* 2E00797 was completely lost and after the crude killer toxin was pre-incubated at temperatures higher than 40°C for 4 h, the killing activity was also completely lost. The killer toxin produced by *M. frigida* 2E00797 could kill only *M. bicuspidata* WCY, *Candida tropicalis* and *Candida albicans* among all the fungal species and bacterial species tested in this study.

Keywords Killer toxin · Psychrotolerant yeast · Pathogenic yeast · Cold-active enzymes · Antarctica

Introduction

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, Aureobasidium* and *Zygosaccharomyces*, indicating that the killer phenomenon is indeed widespread among yeasts (Wang et al. 2008). In environment, secretion of killer toxins (KTs) represents an efficient tool to eliminate competitors to compete for limiting nutritional resources. Some KTs, such as K1 and K28 produced by *Saccharomyces cerevisiae,* are characterized by a very narrow spectrum of activity limited to susceptible strains of the same species (Magliani et al. 1997). Some others, such as chromosomally encoded KTs produced by killer strains belonging to *Pichia* sp. and *Williopsis* sp. have their wide spectrum of activity, involving human, animal and plant pathogenic microorganisms (Magliani et al. 2008). In our opinion, some KTs that have killing activity limited to some pathogenic yeasts may have many potential applications in natural environments and pharmaceutical industries.

In recent years, many species of psychrophilic yeasts have been obtained from different cold environments, such as Antarctica, the Alps, forest substrates and the Himalayan mountains (Shivaji et al. 2008; Margesin and Fell 2008; Pathan et al. 2010; Thomas-Hall et al. 2010). Many of them were found to belong to *Mrakia* spp. (Diaz and Fell 2000). These psychrophilic yeasts that have been obtained are able to produce α-amylase, protease, lipase, β-galactosidase,

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cellulase, xylanase, phytase, ice-binding glycoprotein and pectinase as well as exopolysaccharides (Huston 2008; Song et al. 2010). Typically, the specific activity of these cold enzymes is higher than that of their mesophilic counterparts at temperatures of approximately 0–30°C. At higher temperatures, denaturation of the cold enzymes occurs. However, as mentioned above, all the killer yeasts obtained so far are mesophilic yeasts and little has been known about killer yeasts and their killer toxins from cold environments. We think that the psychrophilic or psychrotolerant killer yeasts may play important roles in low-temperature ecosystems and killer toxins produced by them may have unique characteristics. Therefore, in the current study, the psychrophilic or psychrotolerant killer yeasts isolated from sea sediment in Antarctica were screened and the killer toxin production and action by the yeast strain 2E00797, which was one of the psychrotolerant killer yeasts, were optimized. This is the first to report that the psychrotolerant killer yeast isolated from sea sediment in Antarctica has strong killing activity.

Materials and methods

Microorganisms

Metschnikowia bicuspidata WCY, which has been confirmed to be a pathogenic yeast in *Portunus trituberculatus* (Wang et al. 2007a) was supplied by Mr. W. J. Xu at the Fishery Institute of Zhejiang Province, China. *Candida tropicalis*, *Cryptococcus albidus*, *Candida albicans*, *Yarrowia lipolytica, Saccharomyces cerevisiae* and *Trichosporon montevideense* 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 possible susceptible yeast strains. *Aspergillus niger*, *Penicillium* spp., *Bacillus subtilis* and *Escherichia coli* were also used as the representatives of terrestrial microorganisms. *Mrakia frigida* 2E00797 isolated and identified in this study and deposited at the Marine Culture Collection of China (www.mccc.org.cn) was used as the killer toxin producers.

Media

Growth medium was YPD medium (prepared with seawater) containing 20 g/L glucose, 20 g/L peptone, 10 g/L 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 25–35 g/L 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 glycerol adjusted to pH 4.5, 5.0, 6.0 and 7.0 with 0.05 M citrate–phosphate buffer. *E. coli* and *B. subtilis* were grown in the broth medium containing beef extract 0.3 g, peptone 1.0 g and NaCl 0.5 g, 100 ml distilled water, pH 7.4–7.6 at 37°C for 18 h. *A. niger* and *Penicillium* sp. were cultivated in PDA medium (200.0 g/L potato extract and 20.0 g/L glucose) by shaking at 30°C for 18 h.

Sampling

Different samples of sea sediments (ZS1: S69°22′22″, salt 3.00% and temperature 1.5°C; ZS2: S69°22′18″, salt 3.00% and temperature 1.5°C; ZS3: S69°22′13″, salt 2.53% and temperature 1.5°C; ZS4: S69°22′13″, salt 2.80% and temperature 1.5°C) in Antarctica were collected and frozen at −80°C during the Antarctic exploration on 9 March 2007.

Isolation of psychrotolerant yeasts

After the samples reached the laboratory, 2.0 g of the sediments was suspended in 20.0 mL of YPD medium prepared with seawater and supplemented with 0.5 g/L chloramphenicol and cultivated at 15°C for 5 days. After suitable dilution of the cell cultures, the dilution was plated on the YPD plates with 0.5 g/L chloramphenicol and the plates were incubated at 15°C for 5 days. Different yeast colonies from the plates were transferred to the YPD slants. Each yeast strain obtained was tested for its ability to produce killer toxin as described below.

Screening of killer toxin-producing psychrotolerant yeasts

Each yeast strain obtained was grown in YPD liquid medium for 24 h at 15°C and the cells in 2.0 mL of the culture were collected and washed 3 times with sterilized saline water by centrifugation. Cells of the pathogenic yeast *M. bicuspidata* WCY were suspended in sterile saline water; 0.2 mL of the suspension (10^7 cells/mL) was spread on the assay medium. For examination of the killing activity, the washed cells were inoculated onto the above-mentioned assay medium on which the cells of the pathogenic yeast *M. bicuspidata* WCY had been spread. After 3–4 days of incubation at 15°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 clear zone to the diameter of the colony. Finally, the yeast strain 2E00797 was found to have the highest ratio. Therefore, the yeast strain was used in the subsequent studies.

DNA extraction and PCR

The total genomic DNA of the yeast strain 2E00797 was isolated and purified using the methods as described by Sambrook et al. (1989). The common primers for amplification of D1/D2 26S rDNA sequence in the yeast used were: the forward primer NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and the reverse primer NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (Sugita et al. 2003). The reaction system (25 μL) was composed of 10× buffer 2.5 μl, dNTP 0.8 mM, $MgCl_2$ 1.5 mM, NL-1 or NL-4 0.5 mM, *Taq* DNA polymerase 1.25 U, template DNA 1.0 μl and H_2O 16.6 μl. The conditions for the PCR amplification were as follows: initial denaturation at 94°C for 10 min, denaturation at 94°C for 1 min, annealing temperature at 53°C for 1 min, extension at 72°C for 2 min, final extension at 7°C for 10 min. PCR was run for 32 cycles and PCR cycler was GeneAmp PCR System 2400 made by Perkin–Elmer. PCR products were separated by agarose gel electrophoresis and recovered using UNIQ column DNA gel recovery kits (BIOASIA, Shanghai). The recovered PCR products were ligated into pGEM-T easy vector (Promega, USA) and transformed into competent cells of *E. coli* JM109. The transformants were selected on plates with ampicillin. The plasmids in the transformant cells were extracted using the methods as described by Sambrook et al. (1989). In order to confirm that the PCR products had been ligated into the vector, the purified plasmids were used as templates for amplification of D1/D2 26S rDNA in the yeast strain, respectively. The reaction system and the conditions for PCR amplification of D1/D2 26S rDNA were the same as described above. The D1/D2 26S rDNA fragments inserted on the vector were sequenced by Shanghai Sangon Company.

Phylogenetic analysis and identification of the yeast

The sequences obtained were aligned using BLAST analysis (http://www.ncbi.nlm.nih.-gov/BLAST). For comparison with currently available sequences, 7 sequences were retrieved with over 98% similarity belonging to 7 different genera from NCBI (http://www.ncbi.nlm.nih.gov) and multiple alignment was performed by using Bioedit 7.0. The routine identification of the yeast was performed using the methods as described Kurtzmam and Fell (1998).

Production of killer toxin

The yeast strain 2E00797 was cultivated for 4 days at different temperatures from 10 to 25°C in 500-mL Erlenmeyer flasks with 150 mL of the production medium with different pH (3.5, 4.5, 5.0, 6.0 and 7.0) and NaCl concentrations [0, 1.0, 2.0, 3.0 and 4.0%, (wt/vol)]. The cultures were incubated in a rotary bed shaker (130 rpm). After centrifugation (5000×*g*, 10 min, 4°C), the supernatant obtained was used as the crude killer toxin (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 from 0 to 8% (wt/vol) and different pH values from 4.0 to 7.0] seeded with yeast *M. bicuspidata* WCY. Finally, 200 μL of the crude killer toxin was added to each cup and incubated at different temperatures from 10 to 30°C and pH values for 72 h and the diameter of the clear zone was used as a measure of the yeast killer toxin activity (Santos et al. 2000).

Effects of pre-incubation of the killer toxin on its activity

The crude killer toxin was pre-incubated at 4, 30, 37, 40 and 45°C for 4 h. Then, the killer toxin activity of the treated killer toxin was determined at 15°C, pH 4.5 and 3.0% (wt/vol) NaCl as described above.

Assay of killing activity spectra of the crude killer toxin

The pathogenic yeast *M. bicuspidata* WCY and possible susceptible yeasts grown in YPD medium, the bacterial strains grown in the broth medium and the molds grown in PDA medium were seeded on the assay medium plate, the broth medium plate and PDA medium plate, respectively. 200 μL of the crude killer toxin was added to the sterile cups which had been put on the plates and incubated at 15°C for 96 h and the diameter of the clear zone formed was measured.

Results and discussion

Screening of killer yeasts against the pathogenic yeast *M. bicuspidata* WCY

A total of 9 yeast strains were obtained from the sea sediments in Antarctica (data not shown). We found that only 3 yeast strains (2E00797, 2E00798 and 2E00800) belonging to various species of yeasts isolated from sea sediments could secrete killer toxins onto the medium and kill the pathogenic yeast *M. bicuspidata* WCY (data not shown). The results indicate that of the three strains, the yeast strain 2E00797 had the highest killing activity against the pathogenic yeast *M. bicuspidata* WCY (the ratio of the diameter of clear zone to diameter of colony was = 2.0)

(Fig. 1). Therefore, the yeast strain was used in the subsequent investigations.

Characterization of the yeast strain 2E00797

The colony of the yeast strain 2E00797 was white to cream and yellowish-white. The vegetative cells of this strain were reproduced by budding, there were abundant pseudohyphae and it had teliospores (data not shown). The yeast strain could ferment glucose, galactose, sucrose, maltose and raffinose, but could not ferment lactose, melibiose, trehalose and cellobiose. However, it could assimilate glucose, galactose, sucrose, maltose, cellobiose, melibiose, raffinose, L-arabinose, D-arabinose and L-rhamnose (data not shown). The morphologies of colony and cells and physiological and biochemical characteristics of the yeast strain 2E00797 were identical with those of type species of *Mrakia frigida* (Kurtzman and Fell 1998). Phylogenetic analysis of 7 D1/D2 26S rDNA sequences with over 97% similarity belonging to 7 different genera from NCBI server shows that the similarities between D1/D2 26S rDNA sequences of the yeast strain 2E00797 and the type species of *M. frigida* were 99% (Fig. 2). Therefore, the yeast strain 2E00797 was finally identified as *M. frigida*. After the yeast cells were grown at different temperatures from 10 to 30°C, it was found that they could grow the best at 15°C, but grew poorly over 20°C and stopped growth at 25°C (data not shown). It is generally accepted that cold-adapted organisms are classified to two overlapping groups: psychrophiles and psychrotrophs (or psychrotolerants). The minimum, optimal and maximum growth temperature values are generally taken to be in the range of <0, 15 and 20°C for psychrophiles and >0, >20 and >30°C for psychrotrophs (Morita 1975). Therefore, the yeast strain 2E00797 was regarded as a psychrotolerant yeast. Kurtzman and Fell (2000) reported that *M. frigida* was commonly isolated from Antarctic soil, Greenland soil and other cold environments. The psychrophilic yeast *M. frigida* AG25 isolated from remote geographical locations (European Alps, north Siberia) has been found to be able to produce extracellular cold-active pectate lyase (Margesin et al. 2005). This means that *M. frigida* indeed is widely distributed in different cold environments. So, this is the first to report that the psychrotolerant yeast *M. frigida* 2E00797 isolated from sea sediments in Antarctic could produce killer toxin against the pathogenic yeast *M. bicuspidata* WCY in the crab.

Fig. 1 The clear zone formed by the yeast strain 2E00797 grown in the plate seeded with the pathogenic yeast *M. bicuspidata* WCY at 15°C for 4 days

Effects of different temperatures, pHs and NaCl concentrations on killer toxin production

As shown above, the yeast strain 2E00797 used in this study was isolated from sea sediments in Antarctic and was a psychrotolerant yeast. Therefore, it is very important to

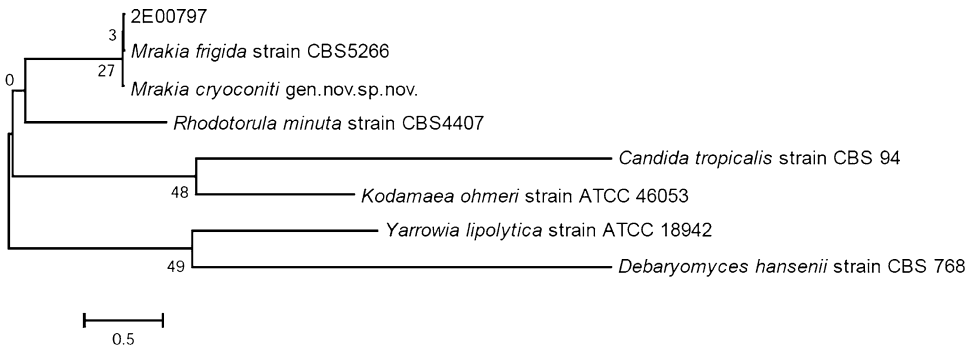

Fig. 2 Phylogenetic tree of the yeast strain 2E00797 and 7 closest relatives based on a maximum parsimony analysis of D1/D2 rDNA sequences. Bootstrap values (1000 pseudoreplications) of ≥36%. Strain numbers and sequence accession numbers are given. All strains shown are type strains and their accession numbers were: 2E00797 EU680778, *Mrakia frigida* CBS5266 AF189849, *Rhodotorula minuta* CBS4407 AF189949, *Yarrowia lipolytica* ATCC 18942 AF335977, *Saccharomyces cerevisiae*, *Debaryomyces hansenii* CBS 768 EU816352, *Candida tropicalis* CBS 94 AY497695, *Kodamaea ohmeri* ATCC 46053 AF335976

Table 1 Effects of different cultivation temperatures, pH values and NaCl concentrations on killer toxin production

Temperature (°C)	Killing activity[a]	pH values	Killing activity[a]	NaCl (%) (wt/vol)	Killing activity[a]
10	11.0 ± 0.3	3.5	11.0 ± 0.3	0.0	14.0 ± 0.3
15	12.0 ± 0.4	4.5	12.0 ± 0.4	1.0	16.0 ± 0.4
23	10.0 ± 0.2	5.0	11.0 ± 0.2	2.0	16.5 ± 0.2
25	0	6.0	9.0 ± 0.1	3.0	20.0 ± 0.6
		7.0	9.0 ± 0.1	4.0	18.5 ± 0.3

[a] Killing activity: diameter of the clear zone (mm); Data are given as mean ± SD, $n = 3$

Table 2 Effects of different temperatures, pH values and NaCl concentrations on killing activity of the crude killer toxin

Temperature (°C)	Killing activity[a]	pH values	Killing activity[a]	NaCl (%) (wt/vol)	Killing activity[a]
10	13.0 ± 0.3	4.0	14.5 ± 0.3	0.0	11.5 ± 0.3
15	17.0 ± 0.4	4.5	15.0 ± 0.4	3.0	16.5 ± 0.4
20	14.0 ± 0.2	5.0	14.0 ± 0.2	6.0[b]	19.5 ± 0.2
25	0	6.0	0	8.0[b]	24.5 ± 0.6

[a] Killing activity: diameter of the clear zone (mm)

[b] When the sensitive yeast *M. bicuspidata* WCY was grown in the medium with more than 6.0% (wt/vol) NaCl, it grew very poorly. Data are given as mean ± SD, $n = 3$

know the effects of different temperatures, pH values and NaCl concentrations on killer toxin production by this yeast strain. After the yeast cells were grown at different temperatures, killing activity of the supernatant obtained was determined as described in "Materials and methods". The results in Table 1 indicate that when the yeast cells were grown at 15°C, the supernatant obtained had the highest killing activity.

After the yeast cells were grown in the medium with different pH values, killing activity of the supernatant obtained was determined as described in "Materials and methods". The results in Table 1 reveal that when the yeast cells were grown in the medium with pH 4.5, the supernatant obtained had the highest killing activity.

After the yeast cells were grown in the medium with different concentrations of NaCl, the killing activity of the supernatant obtained was determined as described in "Materials and methods". The results in Table 1 show that when the yeast cells were grown in the medium with 3.0% (wt/vol) NaCl, the supernatant obtained had the highest killing activity. Therefore, when the psychrotolerant yeast was grown in the medium with pH 4.5 and 3.0% (wt/vol) NaCl and at 15°C, it could produce the highest amount of killer toxin. In our previous studies (Wang et al. 2007a), it was found that the largest amount of killer toxin was produced by the marine-derived yeast *Pichia anomala* YF07b in the production medium with 2.0% (wt/vol) NaCl, pH 4.5 and at 20°C. This suggests that the optimal conditions for killer toxin production by the psychrotolerant yeast were similar to those for killer toxin production by the marine-derived yeast *P. anomala* YF07b. In fact, the killer toxin production by many marine yeast strains can be enhanced in the presence of NaCl (Wang et al. 2008). For example, the lethal activity of the killer toxin produced by the marine yeast *Debaryomyces hansenii* increased with the presence of NaCl in the medium used for testing the killing action (Marquina et al. 2001). This may be related to the marine environments where the killer yeasts were isolated.

Effects of different temperatures, pHs and NaCl concentrations on killing activity

So far, much research work has been focusing only on mesophilic killer yeasts and their killer toxin (Magliani et al. 2008). In contrast, little has been known about the killer toxins produced by psychrotolerant or psychrophilic yeasts, to date. Therefore, the effects of different temperatures, pH values and NaCl concentrations on the killing activity of the killer toxin produced by the yeast strain 2E00797 were examined. It can be seen from the results in Table 2 that when the crude killer toxin was incubated at 15°C, pH 4.5 and in the medium with 3.0% (wt/vol) NaCl, the killing activity was the highest. This means that the optimal conditions for killer toxin production were almost identical with those for killing activity. The results in Table 2 confirmed that the crude killer toxin produced by the yeast strain 2E00797 was cold-active because when the incubation temperature was higher than 25°C, the killing activity was completely lost. In our previous study (Wang

Table 3 Effects of temperature on stability of the crude killer toxin

Pre-incubation temperatures (°C)	4	30	37	40	45
Diameter of the clear zone (mm)	15.0 ± 0.3	12.5 ± 0.4	10.0 ± 0.2	0	0

The crude killer toxin was pre-incubated at different temperatures for 4 h. Then, the killing activity of the treated killer toxin was determined as described in "Materials and methods". Data are given as mean ± SD, $n = 3$

et al. 2007a), it was found that the killer toxin activity produced by the marine-derived *P. anomala* YF07b was the highest when the yeast strain WCY grew at 15°C in the assay medium with 6.0% (wt/vol) NaCl, pH 4.5. However, the killer toxin produced by the marine-derived *P. anomala* YF07b is still active at the temperatures higher than 25°C. The optimal NaCl concentration and temperature for production of killer toxin by the psychrotolerant yeast and the optimal NaCl concentration and temperature for action of the killer toxin on the pathogenic yeast *M. bicuspidata* WCY were in agreement with the natural conditions in the marine environments. However, the optimal pH for the production of killer toxin and its action was not consistent with that (around pH 8.0) in the marine environments. Therefore, it is still unknown if the killer toxin can be produced or the produced killer toxin can actively act on the pathogenic yeasts in natural marine environments. So far, most of the killer yeasts produce killer toxin at acidic pH (Wang et al. 2008).

Effects of temperature on stability of the crude killer toxin

In order to know the effects of different temperatures on the stability of the crude killer toxin, the supernatant obtained was pre-incubated at different temperatures for 4 h. Then, the killing activity of the treated crude killer toxin was measured at 15°C, pH 4.5 and 3.0% (wt/vol) as described in "Materials and methods". The results in Table 3 indicate that when the pre-incubation temperatures were increased from 4 to 37°C, killing activity was greatly decreased. After the crude killer toxin was pre-incubated at temperatures higher than 40°C for 4 h, the killing activity was completely lost. This suggests that the crude killer toxin was very sensitive to the treatment at high temperature. In fact, all the cold enzymes and proteins produced by the psychrophilic yeasts have their activity at temperatures of approximately 0–30°C. At higher temperatures, denaturation of the cold enzymes and proteins occurs (Gerday et al. 2000). In order to know why the killer toxin produced by the psychrotolerant yeast used in this study is cold active and sensitive to high temperature, the killer toxin is being purified and the gene encoding the killer toxin is being cloned and characterized in our laboratory.

Table 4 Killing activity spectra of the crude killer toxin

Possible sensitive yeast or bacterial strains	Killing activity
Metschnikowia bicuspidata WCY	+
Candida tropicalis	+
Candida albicans	+
Cryptococcus albidus	−
Yarrowia lipolytica	−
Saccharomyces cerevisiae	−
Trichosporon montevideense	−
Aspergillus niger	−
Penicillium sp.	−
Escherichia coli	−
Bacillus subtilis	−

+ Positive killing activity, − no killing activity

Killing activity spectra of the crude killer toxin

Table 4 shows that the killer toxin produced by the yeast strain 2E00797 could kill only *M. bicuspidata* WCY, *C. tropicalis* and *C. albicans* among all the fungal species and bacterial species tested in this study. It has been confirmed that *M. bicuspidata* WCY is the pathogenic yeast in crab (Wang et al. 2007a). It also has been well documented that *C. tropicalis* and *C. albicans* are potentially harmful in an immunocompromised hosts including humans or in the hosts who are under stress environmental conditions (Naglik et al. 2003). This may imply that the crude killer toxin produced by the yeast strain had some specificity to the pathogenic yeasts. The killer toxins that only kill the pathogenic yeasts may have many potential uses in natural environments and medicine. The reason for this is still completely unknown. In our previous studies (Wang et al. 2008), it was found that the killer toxins produced by *Williopsis saturnus* WC91-2 and *P. anomala* YF07b can kill many species of yeasts except *Rhodotorula mucilaginosa*. This means that the killer toxin produced by the psychrotolerant yeast strain 2E00797 killed less yeast species than that produced by *W. saturnus* WC91-2 and *P. anomala* YF07b. It has been documented that the killer toxin produced by *P. anomala* YF07b has very strong exo-β-1,3-glucanase activity against the cell wall of the sensitive yeasts and the killer toxin produced by *W. saturnus* WC91-2 can actively inhibit biosynthesis of β-1,3-glucan in the cell wall of the sensitive yeasts (Wang et al. 2007b; Peng et al. 2010). Our results have shown that the killer toxin produced by the psychrotolerant yeast strain 2E00797 had only weak endo-β-1,3-glucanase activity (data not shown). This is may be related to the

narrow killing activity spectra of the crude killer toxin produced by the psychrotolerant yeast *M. frigida* 2E00797 used in this study.

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