Chinese Journal of Oceanology and Limnology
Vol. 27 No. 4, P. 753-761, 2009
DOI: 10.1007/s00343-009-9198-8

The selection of alkaline protease-producing yeasts from marine environments and evaluation of their bioactive peptide production*

LI Jing (李静), CHI Zhenming (池振明)**, WANG Xianghong (王祥红),
PENG Ying (彭莹), CHI Zhe (池哲)
UNESCO Chinese Center of Marine Biotechnology, Ocean University of China, Qingdao 266003, China

Received Sept. 4, 2008; revision accepted Mar. 11, 2009

Abstract A total of 400 yeast strains from seawater, sediments, saltern mud, marine fish guts, and marine algae were obtained. The protease activity of the yeast cultures was estimated, after which four strains (HN3.11, N11b, YF04C and HN4.9) capable of secreting extracellular alkaline protease were isolated. The isolated strains were identified as *Aureobasidium pullulans*, *Yarrowia lipolytica, Issatchenkia orientalis* and *Cryptococcus* cf. *aureus*. The optimal pH of the protease activity produced by strains HN3.11, YF04C, and HN4.9 was 9.0, while that of the protease produced by strain N11b was 10.0. The optimal temperature for protease activity was 45°C for strains HN3.11, N11b, and YF04C, and 50°C for strain HN4.9. After digestion of shrimp (*Penaeus vannamei*) protein and spirulina (*Arthospira platensis*) protein with the four crude alkaline proteases, the filtrate from spirulina (*Arthrospira platensis*) powder digested by the crude alkaline protease of strain HN3.11 was found to have the highest antioxidant activity (61.4%) and the highest angiotensin I converting enzyme (ACE)-inhibitory activities (68.4%). The other filtrates had much lower antioxidant activity and ACE-inhibitory activities.

Keyword: marine yeasts; alkaline protease; yeast diversity; phylogenetic analysis; bioactive peptide

1 INTRODUCTION

The ocean covers 71% of the surface of the earth and represents an important bioresource of microorganisms, including yeast (Chi et al., 2006). However, little is known about the diversity and production of the bioactive substances produced by marine yeasts. Yeast has long been used in food and other industries. In addition, many hydrolytic enzymes such as amylase, lipase, protease and phytase are produced commercially using yeasts. Among these enzymes, proteases have been shown to have many applications in detergents, leather processing, silver recovery, food processing, feed production, as well as in the chemical industry, the health care industry and the treatment of waste (Kumar et al., 1999; Anwar et al., 1998). Proteases also contribute to the development of high value-added applications or products via the enzyme-aided digestion of proteins from different sources (Kumar et al., 1999). Previously (Chi et al., 2007), we found that the marine yeast *Aureobasidium pullulans* 10 could produce a high yield of alkaline protease. Additionally, the hydrolysate of shrimp protein produced using the purified alkaline protease from the marine yeast strain had high ACE-inhibitory activities while the hydrolysate of spirulina (*Arthrospira platensis*) powder produced by the purified alkaline protease from the marine yeast strain had high antioxidant activity (Ma et al., 2007). These findings suggests that alkaline protease from marine yeasts has the potential for application in the enzyme-aided digestion of proteins from different sources.

Terrestrial yeasts reported to produce alkaline proteases include *Candida lipolytica* (Tobe et al., 1976), *Yarrowia lipolytica* (Ogrydziak, 1993) and *Aureobasidium pullulans* (Donaghy et al., 1993). The extracellular alkaline protease produced by *Y. lipolytica* can reach several grams per liter under optimized conditions (Barth et al., 1996). However,

* Supported by the High Technology Research and Development Program of China (863 Program) (No. 2006AA09Z403) and the National Science and Technology Infrastructure Program of China (No. 2005DKA21209)

** Corresponding author: chi@ouc.edu.cn

very few studies have been conducted to evaluate alkaline protease-producing marine yeasts (Chi et al., 2006).

After we screened over 400 marine yeast strains from different marine environments, we found that some strains could secrete a large amount of alkaline protease into medium prepared using sea water. This study was conducted to analyze the diversity of alkaline protease-producing marine yeasts. We also evaluated the antioxidant activity and the ACE-inhibitory activity of the hydrolysis products of proteins treated using the crude alkaline protease produced by the marine yeasts.

2 MATERIALS AND METHODS

2.1 Sampling

Samples of seawater and sediments were collected from the Southern Sea of China, Indian Ocean and the Pacific Ocean during Antarctic exploration in 2004. In addition, hypersaline sea water, sediments from salterns, different species of marine animals and algae were collected from the coast of Qingdao, China.

2.2 Screening and isolation of protease-producing marine yeasts

Two grams of sediments, 2 ml of seawater, the homogenized guts of marine animals or homogenized marine algae were suspended in 20.0 ml of YPD medium containing 2.0% glucose, 2.0% polypeptone and 1.0% yeast extract that was prepared with seawater and supplemented with 0.05% chloramphenicol. The samples were then incubated at room temperature for 5 days on the ship, after which they were plated on YPD agar plates containing 0.05% (w/v) chloramphenicol and incubated at 20°C–25°C for 5 days. Colonies grown on the YPD agar plates were then transferred to protease-screening plates containing 2.0% (w/v) casein and incubated for an additional 5 days at 25°C. Strains that produced a large clear zone were selected for subsequent evaluation.

2.3 Protease production and determination of protease activity

One loop of the cells of each yeast strain was transferred from the slants to 50 ml of YPD medium in a 250 ml flask and aerobically cultivated for 24 h. The cell culture (5 ml, OD_{600nm}=20.0) was then transferred to 50 ml of the production medium, which contained 2.0% glucose, 2.0% $NaNO_3$, and 50 ml of sea water, and then incubated with shaking at 160 r/min and 25.0°C for two days. The cell culture was then centrifuged at 8 000 r/min and 4°C for 10 min, after which the supernatant obtained was used as the crude protease solution.

Next, 0.5 ml of this supernatant was mixed with 1.0 ml of 0.5% casein solution in glycine-NaOH buffer (0.05 mol/L, pH 9.0 or 10.0) preincubated at 45°C or 50°C for 30 min. The mixture was then incubated at 45°C or 50°C for 30 min, after which 2.0 ml of 10% TCA (trichloroacetic acid) solution were added to the mixture to stop the reaction. The reaction mixture was then centrifuged at 12 000 r/min (4°C) for 15 min. The tyrosine content in the supernatant was determined colorimetrically at 650 nm using Folin-phenol reagent (Lowry et al., 1951). The same mixture to which TCA solution was added before the reaction was used as the blank. The enzyme activity was defined as the amount of the enzyme that liberated 1 μg of tyrosine per min under the conditions used in this study. The specific protease activity was units per mg of protein. Protein concentration was measured using the Bradford method, with bovine serum albumin as the standard (Bradford, 1976).

2.4 Phenotypic characteristics and phylogenetic analysis

The morphological and physiological characteristics were examined using the methods described by Yarrow (1998) to identify the yeast. The total genomic DNA of the yeast strains was isolated and purified using the methods described by Sambrook et al. (1989). The primers used to amplify the D1/D2 26S rDNA of the yeast were as follows: forward primer NL-1 (5′-GCATATCAATAAGCG GAGGAAAAG-3′), reverse primer NL-4 (5′-GGTC CGTGT TTCAAGACGG-3′) (Sugita et al., 2003).

The sequences obtained above were aligned using a BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) for comparison with currently available sequences. The results revealed 13 sequences with over 98% similarity belonging to 13 different genera present in the NCBI database (http://www.ncbi.nlm.nih.gov). Multiple alignments using these sequences were then conducted with ClustalX 1.83 and phylogenetic trees were constructed using PHYLIP software package version 3.75c (Felsenstein, 1995). Distance matrices were generated by the DNADIST program based on Kimura's two-parameter model (Kimura, 1980). Neighbor-joining analysis of the data sets was conducted using the neighbor program of the PHYLIP package. *Ricciocarpus natans* was

used as an outgroup during construction of the consensus tree of the isolates based on the D1/D2 26S rDNA sequences.

2.5 Effects of pH and temperature on protease activity

The effects of pH on the alkaline protease activity were determined by incubating the crude protease solution at pHs ranging from 6.0 and 10.0 using the standard assay conditions described above. The buffers used were 0.02 mol/L Na_2HPO_4-Citric acid buffer (pH 6.0–8.0) and 0.05 mol/L Glycine-NaOH buffer (pH 9.0–10.0). The optimal temperature for the activity of the crude protease was determined by incubating the solution at 35°C, 40°C, 45°C, 50°C and 55°C in the same buffer described above.

2.6 Preparation of protein sample

One g of the spirulina (*Arthrospira platensis*) powder was suspended in 2.0 ml of Na_2HPO_4-Citric acid buffer (ice-cold) to make a thick suspension. Next, 2.0 g of fresh shrimp (*Trachypenaeus curvirostris*) muscle was sliced into small pieces, which were subsequently suspended in 2.0 ml of the ice-cold buffer described above. The spirulina suspension and sliced shrimp muscle were then homogenized in a DY89-I Type Electric Glass Homogenizer (Xinzhi, Zhejiang, China) for 1 h on ice. Next, the cell debris was removed by centrifugation (12 000 r/min, 4°C, 30 min), after which approximately 2.0 ml of the supernatants were subjected to ultrafiltration to obtain proteins with a weight of greater than 10 kD using a Labscale™ TFF System (Millipore, USA). The obtained proteins were then used to conduct a bioactive assay of the marine alkaline protease.

The protein concentration of the samples was measured using the method described by Bradford with bovine serum albumin as the standard (Bradford, 1976). The protein concentration in the samples was then adjusted to 5.0 mg/ml.

2.7 Protein digestion by marine alkaline protease

One hundred microliters of the protein sample were mixed with 1 ml of the crude protease (50 U/ml) and 0.8 ml of 0.05 mol/L glycine-NaOH (pH 9.0 or 10.0). The mixtures were then incubated at 45°C or 50°C for 4 h, after which they were heated in boiling water for 10 min to terminate the digesting reactions. The reaction mixture was then centrifuged (12 000 r/min, 4°C, 20 min), after which the resulting supernatants were filtered through an AmiconYM3 (MW cut-off 3 kD) membrane to remove proteins with a molecular weight of more than 3 kD. The filtrate was then used as the peptide sample.

2.8 Assay of antioxidant activity and ACE-inhibitory activity

The antioxidant activity of the peptide sample was determined according to the method described by Re et al. (1999). After adding 1.0 ml of diluted $ABTS^+$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid)] solution (A_{734nm} = 0.700 ± 0.020) to 10 μl of the peptide sample, the absorbance was measured at 30°C exactly 1 min after initial mixing and then again at 6 min. The absorbance of 1.0 ml of the diluted $ABTS^+$ solution in 10 μl of distilled water at 734 nm was regarded as no inhibition (control) and the percentage inhibition of the absorbance at 734 nm of the diluted $ABTS^+$ solution containing the peptide sample was calculated.

The angiotensin I converting enzyme (ACE) inhibitory activities of the peptide sample were determined according to the method described by Li et al. (2005). The ACE inhibitory activity of the peptide sample was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = \frac{B - A}{B - C} \times 100$$

where B is the absorbance of the control (buffer added instead of test sample), C is the absorbance of the reaction blank (HCl was added before ACE), and A is the absorbance in the presence of sample.

3 RESULTS

3.1 Screening and isolation of marine yeast strains with protease activities

Screening of over 400 yeast strains from seawater, sediments, the guts of marine fish and marine algae revealed four strains (HN3.11, N11b, YF04C, HN4.9) of marine yeasts that could secrete extracellular alkaline protease when grown in YPD medium

Table 1 Alkaline protease specific activity in the four marine yeasts from different sources

Strain	HN3.11	N11b	YFO4C	HN4.9
Source	Sea water, Qingdao, China	Sea water, Antarctica	Sea water, Changdao, China	Sediment Qingdao, China
Specific activity (U/mg protein)	127.0	43.9	34.0	26.7

(Table 1). Strains HN3.11, N11b, YF04C and HN4.9 were isolated from sea water at Qingdao in China, Antarctica, Changdao and sediment in Qingdao, respectively.

3.2 Phenotypic characteristics and phylogenetic analysis

The colonies produced by yeast strain HN4.9 were pink and butyrous and its vegetative cells reproduced by budding (Figs.1,2). There were no elaborate pseudohyphae and ascospores (data not shown). The colonies of yeast strain YF04C were light cream colored, with cells from ovoid to elongate in shape that occurred singly or in pairs. There were elaborate pseudohyphae but not ascospores (data not shown). The colonies formed by yeast strain HN3.11 were white or pink and its vegetative cells reproduced by budding. The colonies were initially white, after which they became pink to greenish, and finally black (data not shown). The colonies of yeast strain N11b were white to cream, butyrous to membranous and its vegetative cells reproduced by budding. There were elaborate pseudohyphae and/or septate hyphae and persistent asci containing up to four rough oval, round and hat Saturn- or walnut-shaped ascospores (data not shown).

Tables 2 and 3 show the fermentation spectra and carbon source assimilation spectra of the marine yeasts found to be capable of secreting extracellular alkaline protease in this study. Yeast strains HN3.11, N11b and HN4.9 could not ferment any of the tested sugars; however, strain YF04C was capable of fermenting glucose. HN3.11 could assimilate all of the sugars shown in Table 3 except for lactose. N11b could assimilate glucose, sucrose, raffinose and L-arabose, but could not assimilate lactose, xylose, maltose, cellobiose, trehalose, soluble starch, galactose, melibiose, or D- arabose. YF04C could only assimilate glucose. HN4.9 was able to assimilate all of the tested sugars. Based on these results and currently available data regarding yeast (Kurtzman et al., 2000; Takashima et al. 2003) strains HN3.11, N11b, YF04C and HN4.9 were identified as *Aureobasidium pullulans*, *Yarrowia lipolytica*, *Issatchenkia orientalis* and *Cryptococcus aureus*, respectively.

3.4 Phylogenetic analysis of the sequences of the D1/D2 26SrDNA

According to Kurtzman et al. (2000), traditional and routine identification methods that depend on phenotype generally lead to uncertain and inaccurate interpretations of species interactions. Accordingly, sequence analysis of phylogeny for microbial taxonomy is a more accurate method of determining inter- and intra-specific relationships. Therefore, the D1/D2 26S rDNA of the yeast strains were determined and aligned using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees were constructed by using PHILIP software package version 3.75c (Felsenstein, 1995). A search for similarities between the D1/D2 26S rDNA sequences of the isolates and those in the NCBI database revealed that many phylogenetically related yeast

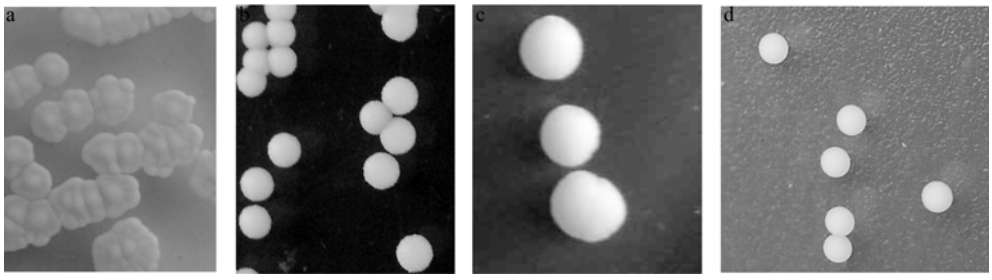

Fig.1 Photographs of colonies of the four protease-producing marine yeasts

a. HN3.11; b. N11b; c. YF04C; d. HN4.9. Medium: YPD medium; temperature: 28°C; incubation time: 3 days

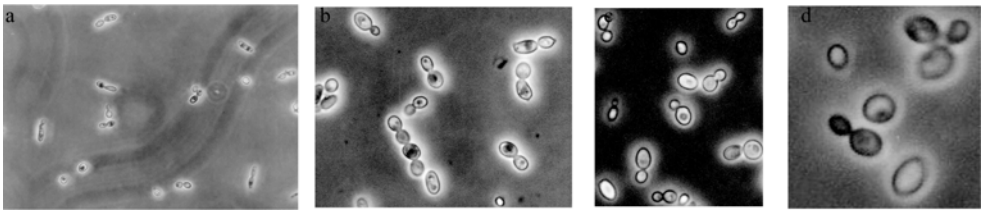

Fig.2 Micrographs of vegetable cells of the four protease-producing marine yeasts

a. HN3.11; b. N11b; c. YF04c; d. HN4.9. Medium: YPD medium; temperature: 28°C; incubation time: 24h

Table 2 Fermentation of different carbohydrates by the marine yeast strains

Strains	HN3.11	N11b	YF04C	HN4.9
Glucose	–	–	+	–
Sucrose	–	–	–	–
Maltose	–	–	–	–
Lactose	–	–	–	–
Galactose	–	–	–	–
Raffinose	–	–	–	–
Melibiose	–	–	–	–

Fermentation was conducted at 25°C and media containing 2.0% sugar at native pH. +: positive; –: negative

Table 3 Assimilation of different carbohydrates by marine yeast strains

Strains	HN3.11	N11b	YF04C	HN4.9
Glucose	+	+	+	+
Sucrose	+	+	–	+
Lactose	–	–	–	+
Xylose	+	–	–	+
Maltose	+	–	–	+
Cellobiose	+	–	–	+
Trehalose	+	–	–	+
Starch	+	–	–	+
Galactose	+	–	–	+
Raffinose	+	+	–	+
Melibiose	+	–	–	+
L-arabinose	+	+	–	+
D-arabinose	+	–	–	+

Assimilation was evaluated at 25°C using media that contained 0.5% sugar at native pH. +: positive; –: negative

species were similar to the strains obtained in this study. The phylogenetic relationships of the D1/D2 26S rDNA sequences of the yeast strains isolated in this study are shown in Fig.3. The topology of the phylogram shown in Fig. 3 revealed a 99% bootstrap value between the D1/D2 26S rDNA sequence of strain HN4.9 and that of *Cryptococcus* cf. *aureus* NRRL Y-27710. Additionally, the D1/D2 26S rDNA sequences of strains HN3.11, N11b and YF04C were found to be 100% homologous with those of *Aureobasidium pullulans* HN2.3, *Yarrowia lipolytica* ATCC 18942 and *Issatchenkia orientalis* CCTCC M206098, respectively. Based on the phenotypic characteristics and phylogenetic analyses, strain HN4.9 was identified *Cryptococcus* cf. *aureus*, whereas strains HN3.11, N11b and YF04C were identified as *Aureobasidium pullulans, Yarrowia lipolytica* and *Issatchenkia orientalis*, respectively.

3.5 Effects of different temperatures and pH on the activity of the crude proteases

The results shown in Fig.4 demonstrate that the optimal pH of the crude proteases produced by *Aureobasidium pullulans* HN3.11, *Issatchenkia orientalis* YF04C and *Cryptococcus* cf. *aureus* HN4.9 was 9.0. The optimal pH for the crude protease produced by *Yarrowia lipolytica* N11b was between 9.0 and 10.0, which is similar to that of the terrestrial yeast *Yarrowia lipolytica* CX161-1B (Ogrydziak and Scharf, 1982). As shown in Fig.5, the optimal temperature of the crude proteases produced by *Aureobasidium pullulans* HN3.11, *Yarrowia lipolytica* N11b, and *Issatchenkia orientalis* YF04C was 45ºC, while the optimal temperature of the crude protease produced by *Cryptococcus* cf. *aureus* HN4.9 was 50°C. Additionally, the results presented in Fig.4 and Fig.5 clearly demonstrate that *Aureobasidium pullulans* HN3.11 could produce much more alkaline protease than any other yeast strains used in this study.

3.6 Antioxidant activity and ACE-inhibitory activity

After determination of the antioxidant activity of the filtrates, it was found that all of the filtrates had antioxidant activity. The antioxidant activity of the filtrate from the digest of spirulina (*Arthrospira platensis*) protein obtained using crude alkaline protease produced by *Aureobasidium pullulans* HN3.11 was 61.4% (Fig.6). However, the antioxidant activity of filtrates produced by the crude alkaline proteases of the other yeast strains was much lower than that of the protease produced by *Aureobasidium pullulans* HN3.11.

After determination of the ACE-inhibitory activity of the filtrates, the ACE-inhibitory activity of the filtrate from the digest of spirulina (*Arthrospira platensis*) protein using crude alkaline protease produced by *Aureobasidium pullulans* HN3.11 was 68.4% (Fig.7). Additionally, as shown in Fig.7 and 8, the filtrates from the digest of spirulina (*Arthospira platensis*) protein produced by the crude alkaline proteases had a greater ACE-inhibitory activity and antioxidant activity than those from the digest of shrimp (*Penaeus Vannamei*).

4 DISCOSSION AND CONCLUSION

Recently, we demonstrated that the diversity of marine yeasts is very rich. However, to the best of our knowledge, marine yeasts have not yet been utilized for extracellular enzyme production (Chi et al., 2006). In this study, four marine-derived strains of yeast capable of secreting extracellular alkaline protease were isolated and identified. Interestingly, most of the protease-producing marine yeasts identified in this study were isolated from marine environments (Table 1).

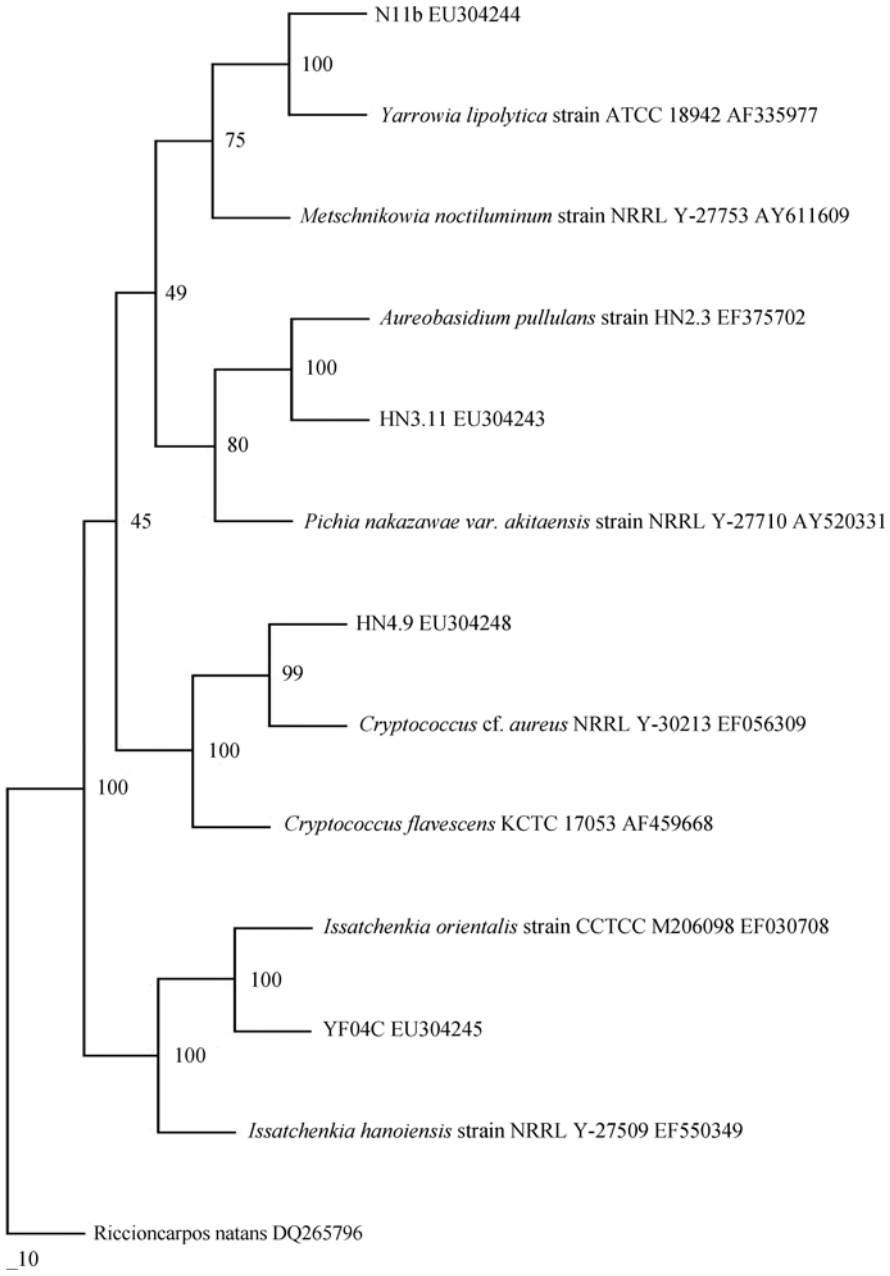

Fig.3 Consensus tree of the isolates based on D1/D2 26S rDNA sequences obtained in this study and 13 previously published sequences obtained from GenBank

The outgroup was *Ricciocarpus natans*. The numbers on the tree branches indicate the percentages of bootstrap samplings derived from 1 000 samples that supported the internal branches by 45% or higher. All strains shown are type strains

The results of routine identification and molecular methods show that the marine yeast strain HN3.11 was similar to *Aureobasidium pullulans*, that strain N11b was similar to *Yarrowia lipolytica,* strain YF04C to *Issatchenkia orientalis* and strain HN4.9 to *Cryptococcus* cf. *aureus*. Terrestrial yeasts that have been reported to produce alkaline proteases include *Candida lipolytica* (Tobe et al., 1976), *Yarrowia lipolytica* (Ogrydziak, 1993) and *Aureobasidium pullulans* (Donaghy et al., 1993). Among the extracellular enzymes of *Y. lipolytica*, alkaline protease could reach several grams per liter under optimized conditions (Barth et al., 1996). Recently, several strains of marine *Yarrowia lipolytica* were found to have a high ability to degrade oil pollutants in marine environments (Jain et al., 2004). Previously (Chi et al., 2007), we found that the marine yeast *Aureobasidium pullulans* 10 could

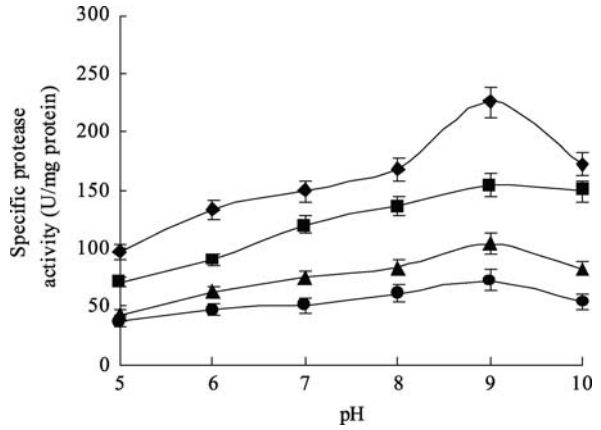

Fig.4 The optimal pHs of the crude proteases produced by the four marine yeasts

Data are given as means ± SD, *n*=3. Strain HN3.11 (♦); Strain N11b (■); Strain YF04C (▲); Strain HN4.9 (●)

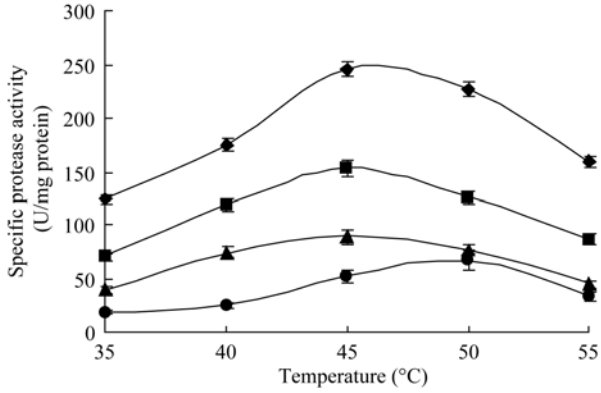

Fig.5 The optimal temperatures of the crude proteases produced by the four marine yeasts

Data are given as the means ± SD, *n*=3. (×). Strain HN3.11 (♦); Strain N11b (■); Strain YF04C (▲); Strain HN4.9 (●)

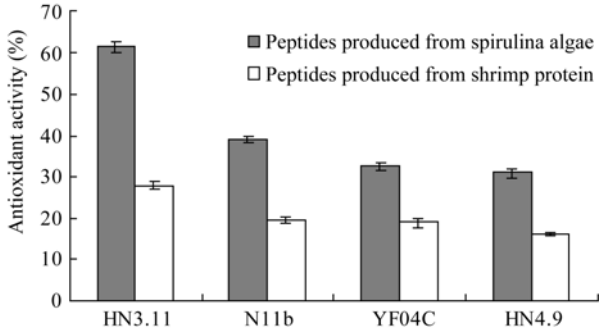

Fig.6 Antioxidant activity of peptides produced by the crude alkaline protease from different marine yeasts

All data shown are the means±SD, *n*=3

produce a high yield of alkaline protease. However, terrestrial *Cryptococcus aureus* and *Issatchenkia orientalis* have not been reported to produce protease. In another study (Sheng et al., 2007), we found that the marine yeast *Cryptococcus aureus* G7a grown in medium prepared with sea water

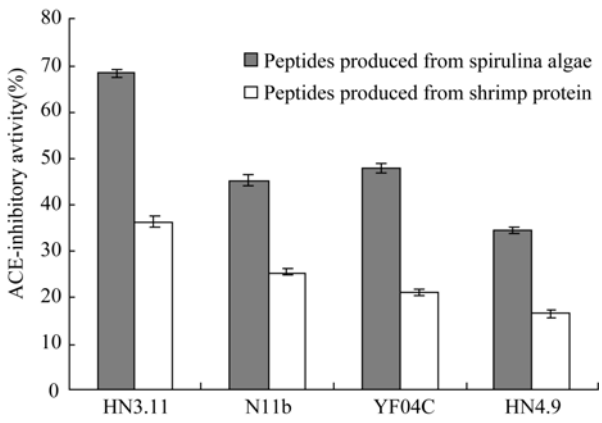

Fig.7 ACE-inhibitory activity of peptides produced by the crude alkaline protease produced by different marine yeasts

All data shown are the means±SD, *n*=3

secreted a large amount of inulinase. *Cryptococcus aureus* has only recently been described (Takashima et al., 2003). Prillinger et al. (1999) reported that *Issatchenkia orientalis* is widely distributed in different cheeses. Additionally, Costas et al. (2004) found that the yeast *Issatchenkia orientalis* CECT 10688 could secrete proteins with lipolytic activity in submerged culture. Overall, these findings indicate that *Cryptococcus* cf. *aureus* HN4-9 and *Issatchenkia orientalis* YF04C obtained in this study are novel alkaline protease producers.

As shown in Fig.4, the optimal pH of the crude alkaline protease produced by the marine yeast strains HN3.11, YF04C and HN4.9 was 9.0. The optimal pH of the crude protease produced by *Yarrowia lipolytica* N11b was between 9.0 and 10.0, which is similar to that of the terrestrial yeast, *Yarrowia lipolytica* CX161-1B (Ogrydziak and Scharf, 1982). Kumar et al. (1999) reported that the optimum pH range of alkaline proteases was generally between pH 9 and 11, with a few exceptions in which the pH was greater than 11.5. Therefore, the results obtained in this study were in agreement with those reported by other researchers (Kumar et al., 1999).

As shown in Fig.5, the optimal temperature of the crude proteases produced by HN3.11, N11b and YF04C was 45°C. Additionally, the optimal temperature of the crude protease produced by HN4.9 was 50°C, which was higher than that of other marine yeasts tested in this study and that of *Aureobasidium pullulans* 10 (Chi et al., 2007). It has been reported that the optimum temperatures of

alkaline proteases produced by bacteria range from 50°C to 70°C (Kumar et al., 1999). An alkalophilic *Bacillus* sp. B189A has even been shown to secrete an alkaline protease with a higher optimum temperature of 85°C (Kumar et al., 1999). These results suggest that the optimum temperatures of alkaline protease from marine yeasts are lower than those of bacteria.

Assay of the antioxidant activity and ACE-inhibitory activity of the yeasts isolated in this study (Fig.6 and Fig.7) showed that the filtrate from spirulina (*Arthospira platensis*) powder digested by the crude alkaline protease produced by strain HN3.11 had the greatest antioxidant activity (61.4%) and the highest angiotensin I converting enzyme (ACE)-inhibitory activity (68.4%). In another study (Ma et al., 2007), the ACE inhibitory activity of the resulting peptides produced by the shrimp (*Arthospira platensis*) protein digested with the purified alkaline protease produced by *Aureobasidium pullulans* 10 was found to be 85.3% and the antioxidant activity of those produced from the spirulina protein digested by the same protease was 54.6%. It is well known that short peptides produced by enzymatic hydrolysis and fermentation from different sources of protein have ACE inhibitory activity, antioxidant activity, opioid agonistic and antagonistic activity and immunomodulatory effects (Silva et al., 2005). Therefore, the alkaline protease from the marine yeast *Aureobasidium pullulans* HN3.11 has the potential for use in the production of bioactive peptides from spirulina (*Arthospira platensis*) powder. There are large amounts of spirulina (*Arthospira platensis*) powder produced by many biotech companies in China each year. The results of the present study indicate that the protein resources for bioactive peptide production are very high in China.

References

Anwar A, Saleemuddin M. 1998. Alkaline protease: a review. *Bioresource Technol.*, **64**(3): 175-183.

Barth G, Garlardin C. 1996. Yarrowia lipolytica. *In*: Wolf K ed. Nonconventional yeasts in biotechnology. Springer-Verlag. Press, Berlin, Germany. p. 313-388.

Bradford M M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**(1-2): 248-254.

Chi Z M, Liu Z Q. 2006. Marine yeasts and their applications in mariculture. *J. Ocean University of China*, **5**(3): 243-247.

Chi Z M, Ma C L, Wang P, Li H F. 2007. Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. *Bioresource Technol.*, **98**(3): 534-538.

Costas M, Deive F J, Longo M A, 2004. Lipolytic activity in submerged cultures of *Issatchenkia orientalis*. *Process Biochem.*, **39**(12): 2 109-2 114.

Donaghy J A, McKay A M. 1993. Production and properties of alkaline protease by *Aureobasidium pullulans*. *J. Appl. Microbiol.*, **74**(6): 662-666.

Felsenstein J. 1995. PHYLIP (Phylogenetic Inference Package), Version 3.75. Distributed by author, Department of Genetics, University of Washington, Seattle, WA.

Jain M R, Zinjarde S S, Deobagkar D D, Deobagkar D N. 2004. 2,4,6-Trinitrotoluene transformation by a tropical marine yeast, *Yarrowia lipolytica* NCIM 3589. *Mar. Pollut. Bull.*, **49**(9-10): 783-788.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies on nucleotide sequences. *J. Mol. Evo.*, **16**(2): 87-90.

Kumar C G, Takagi H. 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnol. Adv*., **17**(7): 561-594.

Kurtzman C P, Fell J W. 2000. The yeasts. A taxonomic Study. Elsevier Press, Amsterdam, Lausanne, New York, Oxford, Shannon, Singapore, Tokyo. 1 055p.

Li G H, Liu H, Shi Y H, Le G W. 2005. Direct spectrophotometric measurement of angiotensin I-converting enzyme inhibitory activity for screening bioactive peptides. *J. Pharmaceut. Biomed.*, **37**(2): 219-224.

Lowry O H, Rosebrough N J, Farr A L, Randall R J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, **193**(1): 265-275.

Ma C L, Ni X M, Chi Z M, Ma L Y, Gao L M. 2007. Purification and characterization of an alkaline protease from the marine yeast *Aureobasidium pullulans* for bioactive peptide production from different sources. *Mar. Biotechnol.*, **9**(3): 343-351.

Ogrydziak D M, Scharf S. 1982. Alkaline extracellular protease produced by *Saccharomycopsis lipolytica* CX161-1B. *J. Gen. Microbiol.*, **128**(6): 1 225-1 234.

Ogrydziak D M. 1993. Yeast extracellular proteases. *Crit. Rev. Biotechnol.*, **13**(1): 1-55.

Prillinger H, Molnár O, Eliskases-Lechner F, Lopandic K. 1999. Phenotypic and genotypic identification of yeasts from cheese. *Anton. Leeuw.*, **75**(4): 267-283.

Re R, Pellergini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio. Med.*, **26**(9-10): 1 231-1 237.

Sambrook J, Fritsch E F, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press. Jin D Y, Li M F. Beijing, Science Press, 1992: 367-370. (in Chinese)

Sheng J, Chi Z M, Li J, Gao L M, Gong F. 2007. Inulinase production by the marine yeast Cryptococcus aureus G7a and inulin hydrolysis by the crude inulinase. *Process Biochem.*, **42**(5): 805-811.

Silva S V, Malcata F X. 2005. Caseins as source of bioactive peptides. *Intern. Dairy J*., **15**: 1-15.

Sugita T, Takashima M, Kodama M, Tsuboi R, Nishikawa A. 2003. Description of a new yeast species, *Malassezia japonica*, and its detection in patients with atopic dermatitis and healthy subjects. *J. Clin. Microbiol.*, **41**(10): 4 695-4 699.

Takashima M, Sugita T, Shinoda T, Nakase T. 2003. Three new combinations from the *Cryptococcus laurentii* complex: *Cryptococcus aureus*, *Cryptococcus carnescens* and *Cryptococcus peneaus*. *Int. J. Syst. Evol. Mic.*, **53**(4): 1 187-1 194.

Tobe S, Takami T, Ikeda S, Horikoshi K. 1976. Production of some enzymatic properties of alkaline protease of *Candida lipolytica*. *Agric. Biol. Chem.*, **40**(3): 1 087-1 092.

Yarrow D. 1998. Methods for the isolation, maintenance and identification of yeasts. *In*: Kurtzman C P and Fell J W ed. The Yeasts, A Taxonomic Study. Elsevier Press, Amsterdam. p. 77-100.