Production, characterization and gene cloning of the extracellular enzymes from the marine-derived yeasts and their potential applications

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Abstract

In this review article, the extracellular enzymes production, their properties and cloning of the genes encoding the enzymes from marine yeasts are overviewed. Several yeast strains which could produce different kinds of extracellular enzymes were selected from the culture collection of marine yeasts available in this laboratory. The strains selected belong to different genera such as Yarrowia, Aureobasidium, Pichia, Metschnikowia and Cryptococcus. The extracellular enzymes include cellulase, alkaline protease, aspartic protease, amylase, inulinase, lipase and phytase, as well as killer toxin. The conditions and media for the enzyme production by the marine yeasts have been optimized and the enzymes have been purified and characterized. Some genes encoding the extracellular enzymes from the marine yeast strains have been cloned, sequenced and expressed. It was found that some properties of the enzymes from the marine yeasts are unique compared to those of the homologous enzymes from terrestrial yeasts and the genes encoding the enzymes in marine yeasts are different from those in terrestrial yeasts. Therefore, it is of very importance to further study the enzymes and their genes from the marine yeasts. This is the first review on the extracellular enzymes and their genes from the marine yeasts.

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1. Introduction

The oceans cover 71% of the surface of the earth, and there are abundant biotic resources, including yeasts, yeast gene and enzymes resources. The yeast genera we isolated from marine environments include Aureobasidium, Candida, Cryptococcus, Debaryomyces, Dipodascus, Filobasidium, Galactomyces, Geotrichum, Hanseniaspora, Issatchenkia, Kluyveromyces, Kodamaea, Lodderomyces, Metschnikowia, Pichia, Pseudoyzma, Rhodosporidium, Rhodotorula, Saccharomyces, Williopsis, Yarrowia, Zygowilliopsis, Mtkia, Guehomyces, Zygoascus, Clavispora and unidentified genera (http://www.mccc.org.cn). Therefore, it is clear that a greater investment in the development of marine yeasts as a resource for enzymes and genes is needed. Marine yeasts are the yeasts that survive longer in seawater than in fresh water (Kurtzman and Fell, 2000). We think that marine yeasts are also the yeasts that are isolated from marine environments and that can grow better in the medium prepared with seawater than in the medium prepared with fresh water. If marine yeasts could be used as producers of enzymes in industries, the seawater which is the most abundant water resource in the world could be used as fermentation medium, so that a large amount of fresh water resource could be greatly saved. In our recent studies, it has been found that different species of marine yeasts could produce different kinds of extracellular enzymes, including amylase, alkaline protease, acid protease, phytase, lipase, inulinase and killer toxins. As shown in Table 1, amylase, alkaline protease, acid protease, phytase, lipase, inulinase and killer toxins from terrestrial microorganisms have many potential applications in food, pharmaceutical, maricultural and fermentation industries. Therefore, it is very important to find new yeasts from marine environments which can produce the enzymes with unique properties compared to those of the same enzymes from terrestrial yeasts and the genes encoding the enzymes different from those of terrestrial yeasts. In this chapter, the extracellular enzymes production, their properties and cloning of the genes encoding the enzymes from marine yeasts are overviewed.

<table>
<thead>
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<th>Table 1</th>
<th>Some extracellular enzymes and their applications</th>
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</thead>
<tbody>
<tr>
<td><strong>Extracellular enzymes</strong></td>
<td><strong>Applications</strong></td>
</tr>
<tr>
<td>Amylases</td>
<td>Starch liquefaction and saccharification, textile desizing, detergent additives, ethanol production, analysis in medical and clinical chemistry, production of high fructose syrup, production of yeast cells and other microorganisms</td>
</tr>
<tr>
<td>Cellulases</td>
<td>Improvement of cellulosic fibres, stone washing, detergent additives, production of single-cell protein and biofuels, waste treatment</td>
</tr>
<tr>
<td>Lipases</td>
<td>Catalysis of a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis, production of biodiesel</td>
</tr>
<tr>
<td>Phytase</td>
<td>The component of commercial poultry, swine, and fish diets, animal and human nutrition, environmental protection</td>
</tr>
<tr>
<td>Alkaline protease</td>
<td>Detergents additive, leather processing, silver recovery, medical purposes, food processing, feeds, chemical industry, waste treatment, digestion of protein</td>
</tr>
<tr>
<td>Acid protease</td>
<td>A rennet substitute in the cheese industry, or as a catalyst in brewing industry, hydrolysis of protein in fermentation industry, removal of protein</td>
</tr>
<tr>
<td>Inulinases</td>
<td>Production fuel ethanol, high fructose syrup and inulooligosaccharides</td>
</tr>
<tr>
<td>Killer toxin</td>
<td>Medicine, biocontrol of fungal disease in plants and animal</td>
</tr>
</tbody>
</table>

2. Amylase

Amylases hydrolyze starch molecule into glucose, maltose and dextrin. They can be classified into α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3). They have many applications in bread and baking industry, starch liquefaction and saccharification, textile desizing, paper industry, detergent industry, analysis in medical and clinical chemistry, food and pharmaceutical industries (Chi et al., 2003; Gupta et al., 2003). Starch is the best substrate for production of yeast cells and their fermentation products on a large scale due to its low price and easily available raw material in most regions of the world (Chi et al., 2003; Gupta et al., 2003). In starch granules, the molecules are densely packed in a polycrystalline state with inter and intramolecular bonds and are hence insoluble in cold water and often resistant to chemicals and enzymes. In the course of conventional enzymatic saccharification by amylases, a slurry containing 15% starch is gelatinized at a temperature of 105 °C and high pressure so as to open the crystalline structure of starch for the enzyme action. This increases the viscosity of the slurry so that more energy is needed for mixing and pumping in the bioreactor. The gelatinized starch is then liquefied with high temperature α-amylase at the same time, followed by the saccharification with glucoamylase at a much lower temperature of 50–60 °C. In the case of ethanol fermentation by using Saccharomyces cerevisiae, temperature of the saccharified slurry is further decreased to around 30 °C. The whole process requires a high-energy input, thus increasing the production cost of starch-based products. In view of energy costs, effective utilization of natural resources and viscosity problems, direct hydrolysis of starch below gelatinization temperature is desirable (Fu et al., 2005). Therefore, in recent years, the process for enzymatic saccharification of raw starch without heating has become well recognized, mainly from the viewpoints of energy savings and effective utilization of the biomass thereby reducing the cost of starch processing. This has generated a world wide interest in the discovery of several raw starch digesting amylases which do not require the gelatinization and can...
directly hydrolyze the raw starch in a single step and that too at moderate temperature much below the gelatinization temperature. Most of the raw starch digesting amylases is obtained from cultures of Aspergillus and Rhizopus. However, most of yeasts from environments are safe (GRAS, generally regarded as safe), interest in amyloolytic yeasts has increased in recent years as their potential value for conversion of starchy biomass to single-cell protein and ethanol has been recognized (Chi et al., 2003; Gupta et al., 2003). To date, it has been noticed that the terrestrial yeasts which can produce extra-cellular amyloolytic enzymes include Arxula adeninivorans, Lipomyces, Saccharomyces, Schwanniomyces, Candida japonica and Filobasidiu capsuligenum and extracellular amyloolytic enzymes produced by them have been well characterized (Gupta et al., 2003; De and Verachtert, 1985). Even the genes encoding α-amylase and glucoamylase from Aspergillus sp. and Saccharomyces fibuliger have been cloned and expressed in cells of S. cerevisiae (Gupta et al., 2003).

Recently, some amylases from terrestrial yeasts also have been found to have the ability to digest raw starch. For example, amylase (AMY-CS2) produced by the yeast Cryptococcus sp. S-2 is also able to digest various raw starches including wheat starch, corn starch, rice starch, sweet potato starch and potato starch (Iefuji et al., 1996). The glucoamylases from S. fibuliger and Candida antarctica have been found to be adsorbed on raw starch and have the capability to digest it (Iefuji et al., 1996). However, very few studies exist on the amylase-producing marine yeasts (Chi et al., 2006).

2.1. Amylase production by the marine-derived Aureobasidium pullulans N13d

The marine yeast strain A. pullulans N13d, producing an extracellular amylase, was isolated from the deep sea sediments of the Pacific Ocean (Li et al., 2007a). Under these conditions, 58.5 units of amylase activity per mg protein are produced within 56 h of fermentation. It was noticed that the crude glucoamylase actively hydrolyzes potato starch granules, but poorly digests raw corn starch and sweet potato starch, resulting in conversion of 68.5 and 22% of them into glucose within 6 h of incubation in the presence of 40 g/l of starch (Li et al., 2007c). It was reported that glucoamylase activity per mg protein are produced within 56 h of fermentation than that from the terrestrial yeasts.

The optimal pH of the purified amylase is 4.5 and the purified enzyme is stable from pH 4.0 to pH 8.0 (Li et al., 2007c). When pH value is higher than 9.0 or lower than 3.0, the enzyme activity decreases significantly. These results suggest that the enzyme is not very sensitive to change of pH. Usually, the pH optima of amylase from terrestrial yeasts are 4.2–6.0 (De and Verachtert, 1985; Nagasaka et al., 1998; Iefuji et al., 1996; Rene and Verachtert, 1987). This demonstrates that the amylase from most of yeasts including the marine yeast strain works best at acidic environment.

The enzyme is activated by Ca2+, Ba2+, Na+, Cu2+, Mg2+ and Co2+ (at the concentrations of 1.0 mM) and stabilized by CaCl2, suggesting that Ca2+ not only has an activating effect on the purified enzyme, but also can stabilize the amylase (Li et al., 2007c). On the other hand, Hg2+ and Ag+ (at the concentrations of 1.0 mM) inhibit the enzyme. The inhibition by mercuric ions may indicate the importance of indole amino acid residues in the enzyme function (Ramirez-Zavala et al., 2004). When the concentrations of the ions tested are higher than 1.0 mM, the enzyme is inhibited by all the ions. The α-amylase from Cryptococcus S-2 is also stabilized by CaCl2 and inhibited by Hg2+, Ag+, Cu2+ and Mg2+ (Iefuji et al., 1996) while that from L. kononenkoe CBS 5608 is inhibited by Ag+ and Cu2+ (Prieto et al., 1995). Na+, Mg2+ and Ca2+ do not have any influence on the enzyme activity from Cryptococcus S-2, but the enzyme is stabilized by 1.0 mM CaCl2. This means that some properties of the purified amylase from the marine yeast strain are different from those of the amylase from other yeasts, but all the amylases from yeasts including the marine yeast must be stabilized by Ca2+.

The enzyme is inhibited by EDTA, EGTA and SDS, but is not inhibited by iodoacetic acid and PMSF (Li et al., 2007c). This suggests that Cys residues and Ser residues are not essential for the enzyme active sites (Urek and Pazarlioglu, 2004). However, the enzyme is inactivated by SDS. Iefuji et al. (1996) reported that DTT is the inhibitor of amylase from L. kononenkoe CBS 5608. These results show that some characteristics of the amylase from the marine yeast are indeed different from those from terrestrial yeasts.

It has been well documented that raw starch adsorption of amylase is essential for raw starch digestion (Nidhi et al., 2005). Therefore, raw starch adsorption of the purified amylase produced by the marine yeast was carried out. It was found that the purified amylase from the marine yeast strain is strongly adsorbed on raw starch (35±1.2%) and potato starch (35±1.5%), but weakly adsorbed on corn starch (3±0.2%) (Li et al., 2007c). It was reported that glucoamylase from C. antarctica is strongly adsorbed onto raw starches (De Mot and Verachtert, 1987) while amylase (AMY-CS2) produced by the yeast Cryptococcus sp. S-2 has the adsorption rate of 57±4.9% (raw corn starch) (Iefuji et al., 1996). Nagasaka et al. (1998) reported that 1.0 mg sample of glucoamylase is adsorbed 100% onto 2 g raw corn starch, and 75% onto 0.2 g. This means that the purified amylase from the marine yeast strain has lower the adsorption rate on raw starch than that from other yeasts.

Most raw starch digesting enzymes reported to date hardly digest potato starch because of the larger size of these granules (Nidhi et al., 2005). On the other hand, next to corn, potato is the most important source of starch. Therefore, enzymes that are capable of digesting raw potato starch are economically attractive for they can increase the range of starch sources for direct hydrolysis (Nidhi et al., 2005). It was found that the purified amylase from the marine yeast only could actively digest raw potato starch although it could be adsorbed on raw potato starch, potato starch and corn starch (Li et al., 2007c). It is very interesting to note that only glucose is released from raw potato starch, gelatinized potato starch and gelatinized soluble starch by action of the purified amylase (Li et al., 2007c). These results again strongly suggest that the amylase produced by the marine yeast is glucoamylase with cleavage activity on both α-1,4- and α-1,6-glycoside linkages in starch molecules used in this study. It has been reported (Dariush et al., 2006) that the debranching activity of the
3. Cellulase

Cellulose is the most abundant organic material on the earth, consisting of glucose units linked together by 1,4-β-glycosidic bonds. Therefore, it has become of considerable economic interest to develop some processes for the effective treatment and utilization of cellulose wastes as inexpensive carbon sources (Wen et al., 2005; Ikeda et al., 2006).

Cellulases are enzymes that degrade crystalline cellulose to glucose. Three types of cellulases, endoglucanases (EC 3.2.1.4, endo-1,4-β-D-glucanase), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), are considered to be needed to degrade crystalline cellulose to glucose in vivo, and they act synergistically (Kim et al., 2003). Cellulases have diverse applications in environmental, food and agricultural industries. Cellulases have also been found to be adsorbed on raw starch and have the capability to digest it (Rene De Mot et al., 1987). Among the five forms of glucamylase (G1–G5) produced by C. rolfsii AHU 9627, only G1, G2 and G3 have a considerable ability to digest cereal starch, but a poor performance with root starch. For example, the purified glucamylases from C. rolfsii AHU 9627 lose the ability to hydrolyze raw potato starch (Nagasaka et al., 1998). This suggests that the raw starch digesting amylases from yeasts greatly vary in their ability to bind to starch granules and digest them.

In summary (Table 2), the results above clearly demonstrate that the glucamylase produced by the marine yeast is different from that produced by other yeasts and fungi. It may have great potential use in direct digestion of raw potato starch in food and fermentation industries. The results also suggest that marine yeasts offer the potential for the production of novel enzymes, which would not be observed from terrestrial yeasts and fungi (Gupta et al., 2003; Darilish et al., 2006). Therefore, the mechanisms of its action towards raw potato starch and the cloning of the gene encoding the enzyme in this marine yeast are undertaken in this laboratory.

Table 2
Some properties of amylases from the marine and terrestrial yeasts

<table>
<thead>
<tr>
<th>Producers</th>
<th>Molecular mass</th>
<th>Optimal pH and temperature</th>
<th>Activated by</th>
<th>Inhibited by</th>
<th>Raw starch adsorption</th>
<th>Digestion of raw starch</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine-derived</td>
<td>98 kDa (two subunits: 65 and 33 kDa)</td>
<td>4.5, 60 °C</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;, Ba&lt;sup&gt;2+&lt;/sup&gt;, Na&lt;sup&gt;+&lt;/sup&gt;, Cu&lt;sup&gt;2+&lt;/sup&gt;, Mg&lt;sup&gt;2+&lt;/sup&gt; and Co&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt; and Ag&lt;sup&gt;+&lt;/sup&gt;, EDTA, EGTA and SDS</td>
<td>Sweet potato starch, and potato starch</td>
<td>Raw corn starch</td>
<td></td>
</tr>
<tr>
<td>Terrestrial Yeasts</td>
<td>66.0–61.9 kDa</td>
<td>4.2–6.0, 50–55 °C</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;, Ag&lt;sup&gt;+&lt;/sup&gt;, Cu&lt;sup&gt;2+&lt;/sup&gt; and Mg&lt;sup&gt;2+&lt;/sup&gt;, DTT</td>
<td>Raw corn starch</td>
<td>Raw cereal starch</td>
<td>Li et al., 2007a,b; Chi et al., 2003; Gupta et al., 2003</td>
</tr>
</tbody>
</table>

3.1. Cellulase production by the marine-derived A. pullulans 98

A. pullulans is popularly known as black yeast and widely distributed in the phyllosphere of many crop plants, on various tropical fruits, in fresh water, estuarine, marine sediments, hypersaline habitats, seawater and deep sea (Akiba et al., 1995; Nagahama, 2006; Gunde-Cimerman et al., 2000). It has been observed that most of the cultures of A. pullulans have usually failed to show any cellulolytic activity (Dennis and Buhagiar, 1973; Federici, 1982; Leathers, 1986; Buzzini and Martini, 2002). However, Kudanga and Mwenje (2005) reported that some isolates of A. pullulans of tropical origin produce CMCase (endo-glucanase) and alpha-cellulase (exoglucanase) activity.

A total of 19 strains of marine yeasts which could produce cellulase were screened from the marine yeasts deposited in our laboratory, and strain 98 which could produce high yield of cellulase was selected for the subsequent investigation (Zhang and Chi, 2007). The marine yeast strain 98 was isolated from surface seawater of sea saltern at Yellow sea, China. This strain was identified to be A. pullulans by 18 S rDNA, 26 S rDNA and ITS gene sequence analysis and routine yeast identification methods. Maximum production of enzyme (CMCase 4.51 U/mg FPase 4.75 U/mg protein) is obtained in a medium containing 2.0 g of soluble starch, 0.5 g of peptone, 2.0 g of yeast extract, 100 ml of sea water, initial pH 7.0 after aerobic cultivation at 28 °C for 24 h. The TLC results indicate that the crude enzyme can hydrolyze CMC and filter paper, producing a large amount of monosaccharide and a trace amount of disaccharide.

3.2. Properties of the CMCase from the marine derived A. pullulans 98

The molecular mass of the purified CMCase from A. pullulans 98 is 67.0 kDa (Zhang and Chi, 2007). Usually, the molecular masses of endo-1,4-glucanase purified from terrestrial fungi and yeasts are 34–61 kDa (Akiba et al., 1995; Murashima et al., 2002; Hatano et al., 1994). These results indicate that the molecular mass of the CMCase from the marine yeast is higher than that from fungi and other yeasts. Because A. pullulans is widely distributed in different marine environments (Nagahama, 2006; Gunde-Cimerman et al., 2000; Li et al., 2007b) and can produce different kinds of extracellular enzymes, including cellulase (Chi et al., 2007; Ma et al., 2007; Wang et al., 2007a; Li et al., 2007b; Zhang and Chi, 2007), it may play an important role in degradation of polymers including cellulose in marine environments.

The optimal pH of the purified CMCase is 5.6 and the enzyme is stable in the range of pH 5.0 to pH 6.0 (Zhang and Chi, 2007). Our recent studies also showed that the optimal pH of the crude CMCase produced by the same yeast strain is 5.6 (Zhang and Chi, 2007). Usually, the purified endo-1,4-glucanase from fungi A. niger strain IF031125, Aspergillus aculeatus and R. oryzae has the optimal pH 5.0–6.0 and is stable in the pH range of 2.0–10.0 (Minamiguchi et al., 1995; Murashima et al., 2002). This demonstrates that the CMCase from most of microorganisms including the marine-derived A. pullulans 98 works best at acidic environment.

The optimal temperature of the purified enzyme is 40 °C and the enzyme is stable up to 30 °C (Zhang and Chi, 2007). From
these results, the CMCase seems to have considerable thermostabi-
licity. It has been reported that the optimum temperature of the
extracellular endoglucanases from fungi are in the range of 50 to 70 °C
(Murashima et al., 2002; Minamiguchi et al., 1995; Murashima et al.,
2002). These results indicate that the optimal temperature of the
CMCase from the marine yeast is much lower than that from fungi.
These characteristics of the CMCase may be of interest in textile and
detergent industries because it can be easily inactivated at room
temperature after its action.

The enzyme is activated by Na⁺, Mg²⁺, Ca²⁺, K⁺, Fe²⁺ and Cu²⁺ (at the
concentrations of 1.0 mM). However, Fe³⁺, Ba²⁺, Zn²⁺, Mn²⁺ and Ag⁺ (at
the concentrations of 1.0 mM) inhibit the enzyme, suggesting that they
are able to alter the enzyme conformation (Barth and Gaillardin,
1997; Zhang and Chi, 2007). It was found that when the concentra-
tions of the ions tested are higher than 1.0 mM, the enzyme is
inhibited by all the ions. It has been reported the purified endo-β-1,4-
glucanase from A. niger strain IF03125 and R. oryzae, is inhibited by
Cu²⁺ (Akiba et al., 1995; Murashima et al., 2002). However, it is very
strange that in our research, Cu²⁺ could activate the activity of the
enzyme. It was found that the CMCase from the marine yeast cannot
act on cellobiose, either.

CMCase from A. pullulans, like CMCases from terrestrial fungi, was found to act on G2 or G3 (Murashima et al., 2002). This means that
G2 and G4 are weakly hydrolyzed to G2 by both enzymes. Neither of the enzymes
hydrolyzed to G2 and G4. G5 is hydrolyzed to G2 and G3. G4 is
hydrolyzed to G2 and G4. G3 is hydrolyzed to G2 and G3. G2 is
hydrolyzed to glucose. G1 is not hydrolyzed to any monosaccharides.

Cloning and characterization of the CMCase gene from the
marine-derived A. pullulans 98

The CMC1 gene cloned from A. pullulans 98 has an open reading
frame of 1497 bp long encoding a CMCase. It is found that the se-
quence of the CMCI gene has high similarity to that of the CMCase
genes from Aspergillus terreus (62.12%) and Aspergillus fumigatus
(60.92%). It has been noticed that the CMCI gene encodes 498 amino
acids of a protein without signal peptide. The protein deduced
from the CMCI gene has the conserved motifs: ENFITC, LGLNCIF,
GFFNYFHT and NGEFGFVY. The proteins deduced from the CMCI genes in
A. terreus, Neosartorya fischeri, A. fumigatus, Gibberella zeae also have
such conserved motifs. Participation of carboxyl groups in the catalytic
function has been reported in a variety of glycosyl hydrolases and the
carboxylate seems to be an integral part of catalytic site of these
enzymes where acid/base catalysis is involved. The results strongly
Suggest that during the cellulose hydrolysis by cellulase, the two
carboxyl groups Glu and Asp are necessary in their catalysis. These
two carboxyl residues are also totally conserved in the CMCase from
the marine yeast and are surrounded by a highly homologous region
of the conserved motifs.

The gene encoding endo-1,4-β-glucanase in Pichia stipitis CBS 6054
has 1443 bp and the protein also has 481 amino acids (accession
number: XP_001387349) without signal peptide. The gene encoding
the hypothetical protein (cellulase, glycosyl hydrolase family 5) in
Cryptococcus neoformans var. neoformans B-3501A has 1493 bp and
the protein has 491 amino acids (accession number: XP_774830)
without the amino acids of signal peptide. However, the cDNA of
FICMCase from A. aculeatus no. F-50 has a 711 bp open reading frame
coding a protein consisting of 237 amino acid residues, including a
putative signal sequence for secretion (Minamiguchi et al., 1995).
This means that the size of the CMCase gene and its signal peptide from
the marine derived A. pullulans 98 are similar to those from other yeasts,
but different from those of A. aculeatus.

Some properties of cellulosases from the marine yeast and terrestrial
fungi are summarized in Table 3.

Table 3

<table>
<thead>
<tr>
<th>Producers</th>
<th>Molecular mass</th>
<th>Optimal pH and temperature</th>
<th>Activated by</th>
<th>Inhibited by</th>
<th>Cellulase activity</th>
<th>Size of CMCCase gene</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>The marine-derived</td>
<td>A. pullulans 98</td>
<td>670 kDa</td>
<td>5.6, 40 °C</td>
<td>Na⁺, Mg²⁺, Ca²⁺, K⁺, Fe²⁺ and Cu²⁺</td>
<td>CMCase and FPase</td>
<td>1497 bp</td>
<td>Zhang and Chi, (2007)</td>
</tr>
<tr>
<td>Terrestrial fungi</td>
<td>34–61 kDa</td>
<td>5.0–6.0, 50–70 °C</td>
<td>Unknown</td>
<td>Fe³⁺, Ba²⁺, Zn²⁺, Mn²⁺ and Ag⁺, PMS, SDS, DT, iodoacetic acid, EDTA and EGTA Cu²⁺, EDTA</td>
<td>CMCase and FPase</td>
<td>711–1493 bp</td>
<td>Wen et al., 2005; Ikeda et al., 2006</td>
</tr>
</tbody>
</table>
4.1. Lipase production by marine yeasts

A total 427 yeast strains from seawater, sediments, mud of salterns, guts of the marine fish and marine algae were obtained. After lipase activity of the yeast cultures was measured, we found that nine yeast strains grown in the medium with olive oil could produce lipase (Wang et al., 2007a). The results of routine identification and molecular methods show that they belong to Candida intermedia YA01a, Pichia guilliermondii N12c, Candida parapsilosis 3eA2, Loddermyces elongisporus YF12c, Candida quercitrusa JHSb, Candida rugosa N8b, Y. lipolytica N9a, Rhodotorula mucilaginosa L10-2 and A. pullulans HN2.3, respectively. Therefore, it can be seen that the yeast strains C. intermedia YA01a, L. elongisporus YF12c, P. guilliermondii N12c, C. quercitrusa JHSb, R. mucilaginosa L10-2 and A. pullulans HN2.3 obtained the new producers of lipase (Wang et al., 2007a).

The optimal pHs and temperatures of lipases produced by A. pullulans HN2.3 are extracellular (Wang et al., 2007a). However, it has been confirmed that majority of yeast lipases are extracellular, but lipase from terrestrial yeasts are cell-bound and only lipase from terrestrial yeast works best at pH 8.5 and 35 °C (Liu et al., 2008a). The optimal pH and temperature for lipase from the marine-derived yeast A. pullulans HN2.3 is 3.0% (w/v) olive oil, whilst others are required for activity. With strain HN2.3, only three isoforms with apparent molecular mass of 33 and 65 kDa (Vakhlu et al., 2006).

Some properties of the lipase from the marine-derived A. pullulans HN2.3

The molecular mass of the purified lipase from the marine-derived A. pullulans HN2.3 is estimated to be 63.5 kDa. It has been reported that majority of yeast lipases are extracellular, monomeric glycoproteins with molecular mass ranging between 33 and 65 kDa (Vakhlu and Kour, 2006). However, the results from SDS–PAGE of the purified lipase from C. rugosa DMS 2031 revealed three distinct bands, indicating that they are three isoforms with apparent molecular mass of 64, 62 and 60 kDa, respectively (Benjamin and Pandey, 1998) (Table 4).

The optimal pH of the purified lipase is 8.5 and the enzyme is stable from pH 4.0 to pH 8.5 (Liu et al., 2008a). It has been confirmed that the optimum pH range of yeast lipases is generally between pH 5 and 8, with a few exceptions of lower pH optima of 2.0 (Vakhlu and Kour, 2006; Kugawa et al., 2001; Oishi et al., 1999). It has also been shown that most of the yeast lipases are generally stable between pH 4.0 and 8.0. However, it seems that optimal pH (8.5) for the purified lipase from the yeast A. pullulans HN2.3 is a little higher than that from other yeasts. This may be related to the sea saltern environment where the yeast strain is isolated (Table 4).

The activity of the purified lipase is the highest at 35 °C and the enzyme is stable up to 20 °C (Liu et al., 2008a). Therefore, the lipase from the marine-derived yeast seems to have considerable thermostability. It has been reported that the optimum temperatures of most of lipases from yeasts range from 30 to 50 °C. For example, the optimum temperatures of all the three forms of lipases purified from the supernatant of C. rugosa DMS 2031 range from 35 to 40 °C (Benjamin and Pandey, 1998).

The optimal temperature for lipase from Kurtzmanomyces sp. I-11 is 75 °C (Oishi et al., 1999). This means that the optimal temperature of lipase produced by the marine-derived yeast is constant with that of lipases produced by other yeasts (Table 4).

The purified lipase from the marine-derived yeast is greatly inhibited by Hg2+, Fe3+ and Zn2+ (at the concentrations of 1.0 mM) (Liu et al., 2008a). The inhibition by mercuric ions may indicate the importance of thiol-containing amino acid residues in the enzyme function (Barth and Gaillardin, 1997). When the concentration of all the cations was 5.0 mM, the purified lipase activity was further inhibited greatly. Benjamin and Pandey (1998) reported that all the three forms of lipases produced by C. rugosa DM 2031 are inhibited strongly by Ag+ and Hg2+, but are enhanced by Ca2+ and Mg2+. At alkaline pH, extracellular phospholipase B from Klyveromyces lactis requires Ca2+, Fe3+ or Al3+ for activity (Oishi et al., 1999). With the terrestrial strains cited here, some ions are reported to inhibit activity whilst others are required for activity. With strain HN2.3, only three

### Table 4

<table>
<thead>
<tr>
<th>Producers</th>
<th>Molecular mass</th>
<th>Optimal pH and temperature</th>
<th>Activated by</th>
<th>Inhibited by</th>
<th>Hydrolysis</th>
<th>Size of lipase gene</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>The marine-derived</td>
<td>63.5 kDa</td>
<td>8.5, 35 °C</td>
<td>No metal ions tested</td>
<td>Hg2+, Fe3+ and Zn2+, PMSF,</td>
<td>All the lipid</td>
<td>1245 bp (one intron: 55 bp)</td>
<td>Liu et al., 2008a</td>
</tr>
<tr>
<td>A. pullulans HN2.3</td>
<td>Terrestrial yeasts: 33–65 kDa</td>
<td>5.0–8.0, 50–70 °C</td>
<td>Ca2+, Mg2+, Fe3+ or Al3+</td>
<td>isoiodoacetic acid, Ag+, Hg2+, PMSF</td>
<td>urrected by prefer peanut oil and lard</td>
<td>1347–1494 bp (no intron)</td>
<td>Hasan et al. (2006)</td>
</tr>
</tbody>
</table>

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metal ions are identified to inhibit the enzyme activity, whereas all the other ions tested have no affect on its activity. Based on this, the yeast strain HN2.3 lipase may be described as a different class of lipase (Table 4).

The enzyme is strongly inhibited by PMSF, not inhibited by EDTA, but weakly inhibited by iodoacetic acid, indicating that Ser residues are essential for the enzyme active sites (Liu et al., 2008a; George and Diwan, 1983). Indeed, many results have shown that microbial lipases are initially classified as serine hydrolyases based on the inhibition of their enzyme activity by chemical modification (Vakhlu and Kour, 2006) (Table 4).

It was also found that the purified lipase from the marine-derived yeast strain has the highest hydrolytic activity towards peanut oil although it can hydrolyze all the oils tested in this study, suggesting that the purified lipase has highly potential application in digestion of lipids (Liu et al., 2008a) (Table 4).

4.3. Characterization of the lipase gene from A. pullulans HN2.3

The extracellular lipase structural gene (LIP1 gene) isolated from cDNA of A. pullulans HN2-3 has an open reading frame of 1245 bp long encoding a lipase. The coding region of the gene is separated by only one intron (55 bp) (Liu et al., 2008b). However, the coding regions of the LIP1 gene from Geotrichum candidum and Y. lipolytica contain no introns (Vakhlu and Kour, 2006). These results indicate that the LIP1 gene cloned from A. pullulans HN2-3 is different from that of other yeast cells (Table 4).

The LIP1 gene encodes 414 amino acid residues of a protein with a putative signal peptide of 26 amino acids (Liu et al., 2008b). The lipase genes from A. adeninivorans and Y. lipolytica harbors an ORF of 1347 bp encoding a 420 amino acid protein of some 50 kDa and 1494 bp coding for 498-aa lipase, respectively (Boer et al., 2005; Barth and Gaillardin, 1997). This means that the size of the LIP1 gene from the marine yeast is similar to that from other yeasts.

The protein deduced from the cloned LIP1 gene has three potential N-linked glycosylation sites of the protein:-N-R-T-(41), -N-C-T-(163) and -N-P-T-(222), respectively (Liu et al., 2008b). However, each deduced lipase sequence from lipase genes of Candida albicans has 4 conserved putative N-glycosylation sites (Hube et al., 2000). These results also indicate that the lipase produced by the marine yeast strain is different from that of other yeast cells. The amino acid sequence deduced from cDNA/LIP1 gene was found to be closely related to that of A. fumigatus (XP_750543) and N. fischeri (XP_001257768) lipase and more distinctly related to other lipases. Ni et al. (2008a,b, 2009) also found that the alkaline protease sequence deduced from ALP1 gene cloned from A. pullulans 10 is closely related to alkaline protease from A. fumigatus. Like Aspergillus spp. terrestrial and marine-derived A. pullulans can produce cellulase, xylanase, lipase, amylase and protease (Chi et al., 2006; Wang et al., 2007a,b,c; Li et al., 2007a; Ma et al., 2007). Therefore, we think that A. pullulans and Aspergillus spp. may have the close phylogenetical relationship.

The protein sequence deduced from the cloned LIP1 gene contains the lipase consensus sequence (G-X-S-X-G) (Liu et al., 2008b). It has been known that lipases from fungi also have the consensus sequence Gly-His-Ser-Leu-Gly while lipases from yeasts have the consensus sequence Gly-Glu-Ser-Ala-Gly. As lipase is a serine hydrolyase, the trimer Ser-Asp-His or Ser-Glu-His is essential for its activity. In addition to this, some lipases need an extra Ala residue for their high catalytic activity. It is considered that the Ser residue in lipases acts as a nucleophile, taking part in lipid hydrolysis while the two Gly residues near to the Ser residue do not play an important role in the catalysis. However, it is thought that the two Gly residues increase the flexibility of the peptide and decrease spacial hindrance so that its substrate can bind more efficiently to the catalytic center of lipase and catalysis can be improved (Wu, 2001; Liu, 2008b).

The LIP1 gene cloned from the marine yeast can be expressed in E. coli and the molecular weight of the expressed fusion protein is about 47 kDa, which is the similar size (46.2 kDa) as estimated from the deduced amino acid sequence of cDNA/LIP1gene cloned from the same yeast. The optimal pH and temperature of the crude recombinant lipase are 8.0 and 35 °C, respectively and the crude recombinant lipase has the highest hydrolytic activity towards peanut oil. This means that the recombinant lipase has the similar catalytic activity and characteristics to the native one and may also have highly potential applications in biotechnology.

5. Phytase

Phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyses the release of phosphate from phytate (myo-inositol hexakisphosphate), which is the principle type of phosphorus present in cereal grains, legumes and oilseeds (Pandey et al., 2001). Therefore, phytase can be incorporated into commercial poultry, swine, and fish diets and has a wide range of applications in animal and human nutrition as it can reduce phosphorus excretion of monogastric animals by replacing inorganic phosphates in the animal diet, contributes significantly toward environmental protection and leads to improved availability of minerals, trace elements, amino acids and energy (Haefner et al., 2005).

In the last decade, phytate-degrading enzymes of terrestrial yeasts such as S. castelli (Pandey et al., 2001), Schwanomyces occidentalis (Nakamura et al., 1999), Pichia anomala (Vohara and Satyanarayana, 2004), A. adeninivorans (Sano et al., 1995) and Hansenula polymorpha (Mayer et al., 1999) and Rhodotorula gracilis (Bindu et al., 1998) also have received increasing attention as they can be easily incorporated into feed diets and are rich in nutrients. However, little is known about the phytases from marine yeasts. If the marine yeasts, especially the marine yeasts that contain high content of protein, can secrete high level of phytase, they can be added to maricultural feed for release of phosphorus from phytate and as the protein source for marine animals.

5.1. Phytase production by marine yeasts

In our recent studies (Hirimuthugoda et al., 2006), we found that 10 strains isolated from marine environments showed comparatively higher phytase activity. They include Hanseniaspora uvarum WZ1, Y. lipolytica W2B, Candida sp. N12C, Issatchenka orientalis YF04C, Candida sp. MA6, Y. lipolytica YF08, Candida sp. NY4E, Candida sp. YF12C, Candida sp. MB2 and Kodamaea ohmeri BG3. They were isolated from the gut of the marine fish (Scomberomorus niphonius), the gut of sea cucumber (Holothuria scabra), seawater at Pacific Ocean, the gut of the marine fish (Hexagrammus otakii), seawater at Indian Ocean, the gut of the marine fish (Synecogobius basts), seawater from salterns, the gut of sea cucumber, seawater in South China Sea and the gut of the marine fish (H. otakii), respectively. This means that phytase-producing yeasts are widely distributed in different marine environments. We found that K. ohmeri BG3, one of the yeasts, could produce more phytase than any other marine yeast strains tested. To our knowledge, phytase producing marine yeasts are still unexploited. After the medium and cultural conditions for phytase production by K. ohmeri BG3 were optimized, we found that the optimal medium for its phytase production contains oat 10.0 g/l, ammonium sulfate 15.0 g/l, glucose 30 g/l, and NaCl 20.0 g/l, while the optimal cultivation conditions for its phytase production are pH 5.0, a temperature of 28 °C, and a shaking speed of 170 rpm. Under the optimal conditions, over 557.9 ml/ml of phytase activity is produced within 72 h of fermentation at the shake flask level. This is a very high level of
phytase activity produced by yeasts (Li et al., in press-a). We think that the medium and process for phytase production by the marine yeast strain are very simple.

It is interesting to note that the marine yeast produces the highest level of phytase in the medium containing 1.0% oat. It has been well documented that high phosphate conditions are to repress the synthesis of acid phosphatases and phytases, while limiting phosphate conditions result in their expression (Wodzinski and Ullah, 1996). For example, in a survey of phytase-producing microorganisms, A. ficium produces the highest amount of phytase (113 nkat/ml in shake flask in 5 days) when the inorganic phosphorus content is in the range of 0.0001–0.005%, the optimum being 0.4 mg/100 ml with 8.0% corn starch (Wodzinski and Ullah, 1996). Therefore, the phosphorus content in the phytate-containing substances mentioned above was determined. The results indicate that the medium plus oat contains only 0.02 g inorganic phosphate per liter. It has been reported that oat contains 0.27 g of phytate phosphorus per 100 g of dry weight (Haefner et al., 2005). All these may have contribution to the enhancement of the phytase production by the marine yeast strain and such marine yeast from the gut of natural marine fish may have a potential application in the maricultural industry and marine environmental protection as the marine yeast may produce phytase in the marine environments and the phytase produced by it could remove phosphorus from the phytate-containing materials.

Glucose is found to be the best carbon source for its phytase production (Li et al., 2008a). However, the lowest phytase is produced in the medium containing galactose. In contrast, Sano et al. (1999) found that when glucose is replaced by galactose, A. adenicinivorans secretes high levels of phytase into the culture medium. This means that the glucose effect on the phytase production by the marine yeast strain is completely different from that by the terrestrial yeast. The results may imply that there is no glucose repression on phytase synthesis in the cells of K. ohmeri BG3. However, glucose or glucose syrups were also used as main carbon sources for the phytase production by H. polymorpha during fermentation (Mayer et al., 1999).

It was observed that 20.0 g/l of added NaCl is the most suitable for phytase production by the marine yeast and the marine yeast strain can produce a high level of phytase in the production medium prepared with seawater. This may be related to the marine environment where the marine yeast strain is obtained (Li et al., in press-a). Therefore, this yeast strain will be suitably applied in marine environments as it can produce high level of phytase in seawater.

It was found that phytate can be converted into different sizes of hydrolysis products by the action of the crude phytase produced by the marine yeast strain within 3 h. However, it is still unknown how phytase is dephosphorylated by the phytase (Li et al., in press-a).

Plackett–Burman design (Plackett and Burman, 1946) has been successfully applied to optimization of medium components and cultivation conditions for phytase production by yeasts and other microorganisms. For example, Vohra and Satyanarayana (2002) carried out statistical optimization of the media components by response surface methodology (RSM) to enhance the phytase production by P. anomala. Therefore, the statistical experimental designs were also applied for the optimization of the phytase production by the marine yeast K. ohmeri BG3 in the cost-effective oats medium (Li et al., 2008b). Finally, it was found that the optimum variables that supported maximum phytase activity were found to be oats 1.0%, ammonium sulfate 2.3%, glucose 2.0%, NaCl 2.0% and initial pH 6.3. An overall 9-fold enhancement in phytase activity (62.0–575.5 U/ml) is attained due to the optimization. This means that Plackett–Burman and RSM designs are effective for the phytase production by the marine yeast K. ohmeri BG3 in the submerged fermentation. These results again demonstrate that the marine yeast strain can produce high yield of extracellular phytase in the simple medium and this may have wide uses in phytase production. In China, the price of oat meal is almost the same as that of corn meal. So, we think that oat can be cheaply used as one component of the production medium for the phytase production by the marine yeast.

5.2. Properties of the phytase from the marine-derived K. ohmeri BG3

The molecular mass of the purified phytase from the supernatant of cell culture of the marine yeast K. ohmeri BG3 was estimated to be 92.9 kDa (Li et al., 2008c). It has been reported that the majority of yeast extracellular phytase has a molecular mass greater than 95 kDa. However, the phytase produced by S. castellii has a molecular mass of 490 kDa with a glycosylation rate of approximately 31% (Seguelilha et al., 1992) and the structure of the deglycosylated protein is tetramer, with one large subunit (MW 125 kDa) and three identical small subunits (MW 70 kDa).

The optimal temperature of the purified phytase is 60 °C and the enzyme is stable up to 60 °C (Li et al., 2008c). From these results, the phytase seems to have considerable thermostability. Thermostability is considered an important and useful criterion for industrial application of phytase. For example, thermostability is a prerequisite for the successful application of enzymes in marine animal feeds that are exposed to 60 °C to 90 °C during the pelleting process. Phytase, in general, shows high activity in the temperature range of 50 °C to 70 °C while the optimum temperature is mostly between 45 °C and 60 °C (Vats and Banerjee, 2004). For example, the phytase produced by S. castellii exhibits an uncommon preference for high temperatures, with optimum activity at 77 °C and thermostability up to 74 °C (Seguelilha et al., 1992; Pandey et al., 2001). This means that the thermostability of phytase from the marine yeast is lower than that from S. castellii.

The optimal pH of the purified phytase is 5.0 and the enzyme is stable from pH 3.0 to pH 9.0 (Li et al., 2008c). Generally, the phytases from the bacterial source have optimum pH in the neutral to alkaline range, while in fungi the optimum pH range is 2.5 to 6.0 and the stability of phytase decreases dramatically above pH 7.5 and below pH 3.0, with a few exceptions of lower pH optima of 2.0 (Han et al., 1999; Pandey et al., 2001; Vats and Banerjee, 2004). For example, the optimum pH of the phytase produced by S. castellii is 4.4 (Seguelilha et al., 1992). However, to date little has been known regarding pH stability of the phytases secreted by terrestrial yeasts. Our results indicate that the phytase from the marine yeast has greater pH stability in the alkaline range than that from other fungi, which may be related to the marine environment where the yeast strain was isolated.

The purified phytase is stimulated by Mn2+, Ca2+, K+, Li+, Na+, Ba2+, Mg2+ and Co2+ (at a concentrations of 0.1 and 5.0 mM), but it is inhibited by Cu2+, Hg2+, Fe2+, Fe3+, Ag+, and Zn2+ (at a concentration of 0.1 and 5.0 mM) (Li et al., 2008c). However, the phytase from A. adenicinivorans is only slightly sensitive to low concentrations of various ions (Mg2+, Ca2+, and Zn2+) (Sano et al., 1999) while the phytase from one strain of A. niger is inhibited by Cu2+, Zn2+, Hg2+, Sn2+, and Cd2+ ions and activated by Ca2+, Mg2+, and Mn2+ ions (Dvorakova et al., 1997). This suggests that some biochemical characteristics of the phytase from the marine yeast are different from those from terrestrial yeasts and fungi.

The purified phytase is inhibited by PMSF, iodoacetate acid (at a concentration of 1.0 mM), and phenylglyoxal hydrate (at a concentration of 5.0 mM) which is a specific inhibitor of histidine acid phosphatases (Li et al., 2008c). However, the purified phytase is not inhibited by EDTA and 1,10-phenanthroline (at concentrations of 1.0 mM and 5.0 mM), demonstrating that the purified enzyme is not a metalloenzyme (Ramirez-Zavala et al., 2004). The phytase from Klebsiella oxytoca MO–3 is not inhibited by EDTA or N-ethylmaleimide (Jareonkitmongkol et al., 1997), either. In contrast, it has been reported that a purified phytase from B. subtilis strain VTT E-68013 is readily inhibited by EDTA (Kerovu et al., 1998). However, to date little has been known about the effects of the enzyme inhibitors on phytase activity produced by terrestrial yeasts.
5.3. Characterization of the phytase gene from K. ohmeri BG3

So far, the gene encoding phytase has been cloned from D. castelli, S. occidentalis, P. stipitis, L. elongisporus, P. guilliermondii, K. lactis and S. cerevisiae. The phytase A gene (phyA), phytase B gene (phyB) from Aspergillus niger (ficuum) NRRL 3135, the ORF encoding the D. castelli CBS 2923 phytase and the APHO1 gene encoding extracellular acid phosphatase from A. adreninivorans have been overexpressed (Kaur et al., 2007).

The gene encoding phytase in K. ohmeri BG3 was also cloned and expressed in E. coli in our laboratory. The gene has an open reading frame of 1389 bp long and the coding region of the gene has no intron. It encodes 462 amino acid residues of a protein with a calculated molecular mass of 51.9 kDa and has a putative signal peptide of 15 amino acids (Li et al., 2009). However, we found that the molecular mass of the purified phytase from the marine yeast strain was estimated to be 92.9 kDa. As discussed below, there are many N-glycosylation sites in the enzyme. Therefore, the phytase may undergo many N-glycosylation and other processing during secretion in the cells of wild type so that molecular mass of the produced phytase is much higher than calculated molecular mass of the phytase. The APHO1 gene encoding extracellular acid phosphatase from A. adreninivorans gene harbours an ORF of 1449 bp encoding a protein of 483 amino acids with a calculated molecular mass of 52.4 kDa. The 461-amino-acid sequence deduced from the ORF encoding the D. castelli CBS 2923 phytase corresponds to a 51.2 kDa protein (Ragon et al., 2008), no signal sequence cleavage site is detected. Therefore, the calculated molecular mass of other phytases is similar to that of the phytase from the marine yeast.

The protein sequence deduced from the extracellular phytase structural gene from the marine yeast contains the consensus motifs (RHGXRX P and HD) which are conserved among histidine acid phosphatases. In histidine acid phosphatases, the dipeptidic region containing His-Asp (HD) residues are shown to be important for catalysis and are thought to be involved in donation of a proton to the substrate leaving group during the formation of phosphohistidine. The substrate is bound to the consensus motif (RHGXRX P) and Arg residue of RHGX RX P is necessary for the enzyme activity. The positively charged Arg residue can accommodate the negatively charged phytate as substrate by static action, forming ES complex. After that, the substrate is hydrolyzed under the catalysis of the consensus motif HD (Li et al., 2009).

The deduced protein sequence from the extracellular phytase structural gene of the marine yeast contains six conserved putative N-glycosylation sites. However, the amino acid sequence deduced from the ORF encoding the D. castelli CBS 2923 phytase has nine potential N-glycosylation sites.

According to the phylogenetic tree of the phytase, the phytase from K. ohmeri BG3 is closely related to C. albicans (XP_713452) and P. stipitis (XP_001385108) phytase protein and more distantly related to other phytases, respectively. This suggests that the deduced amino acid sequence from cDNA PHY1 gene of K. ohmeri BG3 also has a distance relationship to APHO1 and phytase from D. castelli. All of these results demonstrate that PHY1 produced by the marine yeast K. ohmeri BG3 is greatly different from the phytases from other yeasts (Li et al., 2009).

The gene encoding phytase in K. ohmeri BG3 can be expressed in E. coli and the recombinant phytase has molecular mass of about 51.0 kDa. Optimal pH and temperature of the crude recombinant lipase are 5.0 and 65 °C, respectively and the crude recombinant phytase has hydrolytic activity towards phytate. This means that the optimal temperature and stability of the crude recombinant phytase from E. coli cells is similar to those of the native phytase from K. ohmeri BG3.

The recombinant phytase obtained in this study can actively hydrolyze phytate, but it cannot fully hydrolyze the 6 phosphates binding of the myo-inositol hexakisphosphate. It has been reported that a novel phytase from the yeast D. castelli is especially able to fully hydrolyse the 6 phosphates binding of the myo-inositol hexakisphosphate (Ragon et al., 2008). It has been shown that lower phosphoric esters of myo-inositol (mono, bis, tris, and tetrakisphosphates) play a crucial role in transmembrane signaling processes and in calcium mobilization from intracellular store in animal cells as well as in plant tissues (Haefner et al., 2005). The use of phytase has been shown to be very effective in producing different inositol phosphate species (Vats and Banerjee, 2004). Therefore, the phytase that cannot fully hydrolyze the 6 phosphates binding of the myo-inositol hexakisphosphate may have potential applications in producing different inositol phosphate species.

Table 5 summarizes some properties of phytases from the marine yeast and terrestrial fungi.

6. Protease

A protease is any enzyme that conducts proteolysis. Proteases are currently classified into six groups: serine proteases, threonine proteases, cysteine proteases, aspartic acid protease and metalloprotease. Proteases have many applications in different biotechnological fields. Bacteria, filamentous fungi and yeasts are found to be able to produce different kinds of protease.

6.1. Alkaline protease

Alkaline proteases have been shown to have many applications in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, chemical industry as well as waste treatment (Kummar and Tagaki, 1999; Anwar and Saleemmuddin, 1998). Proteases also contribute to the development of high-added applications or products by using the enzyme-aided digestion of proteins from different sources (Kummar and Tagaki, 1999). In recent years, many results have shown that alkaline proteinase in the intestine of marine animals can help digest protein in the feed and the activity of alkaline protease in the intestine regulates the use of components in the compound diet and shows the stage of development in marine animals. Therefore, alkaline protease in the guts of marine animals also has received much attention in recent years (Chong et al., 2002; Fu et al., 2005). So far, it has been found that microorganisms are the most suitable resources for industrial production of protease as protease-producing microorganisms are easily cultivated in a large scale, protease yields from microorganisms are very high and different proteases produced by microorganisms have different biochemical and physical characteristics and physiological functions (Kummar and Tagaki, 1999). Terrestrial yeasts reported to produce alkaline proteases include Candida lipolytica, Y. lipolytica and
A. pullulans (Tobe et al., 1976; Ogrydziak, 1993; Donaghy and McKay, 1997). However, very few studies exist on the alkaline protease-producing marine yeasts (Chi et al., 2006).

6.1.1. Alkaline protease production by marine yeasts

A yeast strain, A. pullulans strain 10, which could produce the high yield of protease was isolated from sediment of sea salttern of Yellow sea, China. Maximum production of enzyme (623.1 U/mlg protein; 7.2 U/ml) is obtained in a medium containing 2.5 g soluble starch and 2.0 g NaN3, 100 ml seawater, initial pH 6.0, after fermentation at 24.5 °C for 30 h. The crude protease produced by A. pullulans strain 10 has the highest activity at pH 9.0 and 45 °C (Chi et al., 2007). These results suggest that the enzyme is alkaline protease (Anwar and Saleemuddin, 1998).

It is interesting to note that soluble starch and corn starch are the best carbon sources for the protease production and sodium nitrate is stimulatory for the alkaline protease production by the yeast strain (Chi et al., 2007). However, increased yields of alkaline proteases were reported by several other workers who used different sugars such as lactose, maltose, sucrose and fructose (Malathis and Chakraborty, 2001; Tsuchiya et al., 1991; Phadatare et al., 1993).

It has been well-known that bioactive peptides from different sources of proteins have opioid agonistic and antagonistic activity, angiotensin-converting enzyme (ACE) inhibitory activity, immuno-modulatory effects, antimicrobial activity, and antioxidant activity (Silva and Malcata, 2005). Although some bacteria can produce more protease than yeasts, the protease-producing yeasts are generally regarded as safe (GRAS). Therefore, in order to use protease from marine yeasts for production of bioactive peptides, over 400 yeast strains from seawater and sediments were obtained in this laboratory, but only 5 strains named HN2-3, N13d, N13C, Mb5 and HN3-2 among them can form clear zone around their colonies on the double plates with 2.0% casein (Ni et al., 2008a). However, only strains HN2-3 and N13d can produce a large amount of protease in the cultures. At the same time, it was observed that peptides in the hydrolysate produced by the proteases from strains HN2-3 and N13d have higher angiotensin I-converting-enzyme (ACE)-inhibitory activity. The two strains strains were identified to be A. pullulans according to the results of routine yeast identification and molecular methods although the colony morphology of strain HN 2-3 is significantly different from that of strain N13d (Ni et al., 2008a).

After determination of ACE inhibitory activity and antioxidant activity of the crude bioactive peptides, it was found that although all the crude bioactive peptides have ACE inhibitory activity and antioxidant activity, ACE inhibitory activity of the crude bioactive peptides from shrimp (T. curvirostris) muscle under the catalysis of the purified protease from strain HN 2-3 is the highest (88.3%) while antioxidant activity of the crude bioactive peptides from spirulina (A. platensis) powder is the highest (82.8%) under the catalysis of the purified protease from strain N13d. Therefore, the alkaline protease from the two marine yeasts A. pullulans has potential uses in production of bioactive peptides from shrimp muscle and spirulina (Ni et al., 2008a).

Sodium caseinates prepared from bovine, sheep, goat, pig, buffalo, or human milk were hydrolyzed by a partially purified proteinase of Lactobacillus helveticus PR4 and peptides in each hydrolysate have ACE-inhibitory activity, antimicrobial activities (Minervini et al., 2003). The proteases produced by the marine yeasts also can be used for production of bioactive peptides from marine proteins.

The Chinese maricultural industry produces a large amount of the shrimp (T. curvirostris) each year and the spirulina powder produced by many biotechnological companies in China is widely available in markets. So, the protein resources for the bioactive peptide production are very rich in China. He et al. (2006) also found that antioxidant activities of the hydrolysate and ultrafiltrate from the shrimp (Acetes chinensis) treated with the crude protease from Bacillus sp. SM80111 are 42.38% and 67.95%, respectively. The hydrolysate and ultrafiltrate also have good ACE inhibitory activity.

6.2. Properties of the alkaline protease from the marine-derived A. pullulans strain 10

The molecular mass of the purified alkaline protease from the marine yeast A. pullulans strain 10 was estimated to be 32.0 kDa (Ma et al., 2007). It has been reported that the molecular masses of alkaline proteases from microorganisms range from 15 to 30 kDa with few reports of higher molecular masses of 31.6 kDa, 33 kDa; 36 kDa, and 45 kDa (Kumar and Takagi, 1999). For example, alkaline extracellular protease from Y. lipolytica is also a 32-kDa protease of the subtilisin family (Barth and Gaillardin, 1997) (Table 6).

The optimal pH of the purified alkaline protease is 9.0 and the enzyme is stable from pH 4.0 to pH 12.0 (Ma et al., 2007). Therefore, the enzyme can work best in the alkaline environments and this characteristic of the enzyme has many applications in maricultural and other industries as mentioned above. It has been indicated that the optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima of 11.5 (Kumar and Takagi, 1999). They also have high isoelectric points and are generally stable between pH 6 and 12 (Kumar and Takagi, 1999). The results about the alkaline protease from the marine yeast A. pullulans strain 10 are in agreement with those reported by other researchers (Kumar and Takagi, 1999) (Table 6).

The optimal temperature of the purified alkaline protease is 45 °C and the enzyme is stable up to 20 °C (Ma et al., 2007), indicating that the protease seems to have considerable thermostability. It has been reported that the optimum temperatures of alkaline proteases from bacteria range from 50 to 70 °C (Kumar and Takagi, 1999). For example, the alkaline protease

### Table 6

<table>
<thead>
<tr>
<th>Producers</th>
<th>Molecular mass</th>
<th>Optimal pH and temperature</th>
<th>Activated by</th>
<th>Inhibited by</th>
<th>Hydrolysis</th>
<th>Size of alkaline protease genes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>The marine-derived A. pullulans HN2-3</td>
<td>33.0 kDa</td>
<td>9.0, 52 °C</td>
<td>Zn²⁺, Mg²⁺, Na⁺</td>
<td>PMSF-1-10-phenanthroline, EDTA and iodoacetic acid</td>
<td>Production of bioactive peptides from marine proteins</td>
<td>1248 bp (two introns: 54 and 52 bp)</td>
<td>Ni et al. (2008a,b)</td>
</tr>
<tr>
<td>The marine-derived A. pullulans strain 10</td>
<td>32.0 kDa</td>
<td>9.0, 45 °C</td>
<td>Cu²⁺ and Mn²⁺</td>
<td>Hg²⁺, Fe²⁺, Fe³⁺, Zn²⁺, and Co²⁺, PMSF, 1-10-phenanthroline, EDTA and iodoacetic acid</td>
<td>Production of bioactive peptides from marine proteins</td>
<td>1248 bp (two introns: 54 and 50 bp)</td>
<td>Ma et al. (2007)</td>
</tr>
<tr>
<td>Terrestrial yeasts</td>
<td>32 kDa</td>
<td>9–11, 50–70 °C</td>
<td>Unknown</td>
<td>PMSF</td>
<td>Unknown</td>
<td>1364 bp</td>
<td>Madażek et al. (2004)</td>
</tr>
</tbody>
</table>
from an alcalophilic Bacillus sp. B189 shows an exceptionally high optimum temperature of 85 °C (Kurmar and Tagaki, 1999). This means that the optimum temperatures for the alkaline protease from the bacterium are much higher than those from the marine yeast.

The enzyme is activated by Cu2+ (at a concentration of 1.0 mM) and Mn2+ and inhibited by Mg2+, Fe2+, Fe3+, Zn2+, and Co2+ (at a concentration of 1.0 mM) and higher concentrations of the ions can further inhibit the enzyme activity (Ma et al., 2007). In contrast, Cu2+ (at a concentration of 5.0 mM) inhibits the enzyme activity. However, alkaline proteases from bacteria require a divalent cation such as Ca2+, Mg2+, and Mn2+ or a combination of these cations for maximum activity (Kurmar and Tagaki, 1999). The inhibition by mercuric ions may indicate the importance of thiol-containing amino acid residues in the function of the alkaline protease (Barth and Gaillardin, 1997). It may indicate the importance of thiol-containing amino acid residues in the function of the alkaline protease (Barth and Gaillardin, 1997). It also was reported that the mature extracellular alkaline protease from Y. lipolytica is formed after N-glycosylation, signal peptide cleavage and other modifications of the propeptide (Matoba et al., 1988). We also found that there are three potential N-linked glycosylation sites of the protein, among them, -N-G-T- at 68 amino acid is the most possible N-glycosylation site (Ni et al., 2008b). The protein has the conserved serine active site and histidine active site of serine proteases in the subtilase family from 356 to 366 amino acids and from 188 to 198 amino acids, respectively. Therefore, the results demonstrated that ALP1 obtained from A. pullulans strain 10 belongs to one member of serine proteases in the subtilisin family (Ni et al., 2008b) so that the enzyme activity is strongly inhibited by phenylmethylsulfonyl fluoride (Ma et al., 2007).

It has been reported that expression vector pNA1317 and its recipient yeast strain Y. lipolytica Po1h have many advantages (Mdzak et al., 2004). For example, the expression of the target gene on this plasmid does not need to be induced as it has a strong recombinant growth-phase-dependent promoter, hp4d. Therefore, the cDNAALP1 gene cloned from A. pullulans strain 10 was ligated into pNA1317 and expressed in the yeast strain Po1h. The results demonstrated that cDNAALP1 gene can be expressed in Y. lipolytica Po1h and the enzyme secreted into the medium has alkaline protease activity (Ni et al., 2008b).

6.2.2. Cell surface display of the alkaline protease and bioactive peptide production

It has been mentioned above that the alkaline protease produced by A. pullulans HN2-3 is more suitable for production of bioactive peptides from shrimp (T. curvirostris) muscle and spirulina (A. platensis) powder than that produced by other marine yeasts (Ni et al., 2008a). Therefore, the alkaline protease structural genes (cDNAALP2 gene and ALP2 gene) were amplified from cDNA and genomic DNA of the marine yeast strain, respectively (Ni et al., 2009). The gene has an open reading frame of 1248 bp encoding a 415 amino-acid proteins. The ALP2 gene contains two introns which had 54 bp and 52 bp, respectively. However, ALP1 gene from A. pullulans 10 contains two introns with 54 and 50 bp, respectively (Ni et al., 2008b). It also was found that the sequence of the ALP2 gene has very high similarity (85.01%) to that of ALP1 gene isolated from A. pullulans strain 10 (Table 6).

In recent years, yeast surface display techniques have received increasing attention as they have many applications in biotechnological and industrial fields, such as cell adhesion, molecular recognition, immobilized biocatalysis, bioconversion, bioremediation, change of cell function, signal transduction, biosensor, live vaccine development, and ultra-high-throughput screening for the identification of novel biocatalysts (Becker et al., 2004; Ueda and Tanaka 2000; Won et al., 2006; Yue et al., 2008; Zhu et al., 2006). Amylasles, cellulases, xylanases, hemolysin, and other proteins have been successfully immobilized on yeast cells and their potential applications are evaluated (Yue et al., 2008; Zhu et al., 2006; Ueda and Tanaka 2000). For example, the yeast cell displaying amylases and cellulases can be used to produce ethanol from starch and cellulose, respectively (Ueda and Tanaka, 2000). The yeast cell displaying hemolysin can be used to develop live vaccines in marine animals (Zhu et al., 2006). However, alkaline protease has not been displayed

The calculated molecular mass of the protein deduced from cDNAALP1 gene is 42.9 kDa, and the protein contains 415 amino acids compared with 42.2 and 46.9 kDa for A. fumigatus and Y. lipolytica alkaline protease, respectively (Ni et al., 2008b). However, the estimated mass of the purified alkaline protease from the supernatant of the culture of A. pullulans strain 10 is only 32 kDa (Ma et al., 2007). This may imply that the alkaline protease encoded by cDNAALP1 gene underwent various processings during the secretion in A. pullulans strain 10. It also was reported that the mature extracellular alkaline protease from Y. lipolytica is formed after N-glycosylation, signal peptide cleavage and other modifications of the propeptide (Matoba et al., 1988). We also found that there are three potential N-linked glycosylation sites of the protein, among them, -N-G-T- at 68 amino acid is the most possible N-glycosylation site (Ni et al., 2008b). The protein has the conserved serine active site and histidine active site of serine proteases in the subtilase family from 356 to 366 amino acids and from 188 to 198 amino acids, respectively. Therefore, the results demonstrated that ALP1 obtained from A. pullulans strain 10 belongs to one member of serine proteases in the subtilisin family (Ni et al., 2008b) so that the enzyme activity is strongly inhibited by phenylmethylsulfonyl fluoride (Ma et al., 2007).

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on yeast cells for production of bioactive peptides so far. As mentioned earlier, the plasmid pINA1317 and its host Y. lipolytica have many advantages over other expression plasmids and hosts. A surface display vector for protein display on the yeast Y. lipolytica has been constructed in our laboratory (Yue et al., 2008). When the cDNAALP2 gene obtained above is cloned into the multiple cloning sites of the surface display vector pINA1317-YlCWP110 and expressed in cells of Y. lipolytica, the protease displaying cells can form clear zone on the double plate containing milk protein and have high protease activity (Ni et al., 2009). It can also be noticed that 100% of the observed cells displays the alkaline protease. In our another study (Yue et al., 2008), it was also found that 100% of the Y. lipolytica cells displays enhanced green fluorescent protein or hemolysin using the same GPI-anchor-fusion expression system and the Y. lipolytica cells displaying hemolysin exhibit hemolytic activity toward erythrocytes from flounder.

The proteins were extracted from shrimp, spirulina, and single cells of marine yeast strain G7a (Gao et al., 2007b). The cell-free extracts, milk, and casein solution were filtrated. The filtrates with proteins more than 10 kDa were digested by the yeast cells displaying the alkaline protease. The supernatants obtained were filtrated again and the filtrates with short peptides less than 3 kDa were collected. After determination of ACE inhibitory activity and antioxidant activity of the filtrates (Ni et al., 2009), it was found that although all the filtrates have ACE inhibitory activity and antioxidant activity, ACE inhibitory activity of the filtrate from the digest of the single-cell protein of the marine yeast strain G7a is the highest (80.82%), while antioxidant activity of the filtrate from the digest of the spirulina (A. platensis) powder is the highest (73.97%). Therefore, the results in this study demonstrate that the yeast cells displaying alkaline protease can be used for bioactive peptide production. These results confirm that the recombinant vector and the yeast cells displaying the alkaline protease have the promising uses in biotechnology, food industry, and pharmaceutical industry. It was found that the marine yeast C. aureus G7a used in this study contains a high level of protein (53.0 g of crude protein per 100 g of cell dry weight) when it grows on Jerusalem artichoke extract (Gao et al., 2007b). So, the protein resources for bioactive peptide production are very rich.

It is very interesting to note that the activity of the alkaline protease with 6×His tag are much higher than that of the alkaline protease without 6×His tag (Ni et al., 2009). For example, most of the transformants displaying the alkaline protease with 6×His tag have the specific alkaline protease activity of over 600 U/g of cell dry weight while most of the transformants displaying the alkaline protease without 6×His tag have the specific alkaline protease activity of less than 400 U/g of cell dry weight. The colonies carrying the alkaline protease with 6×His tag also can form bigger clear zones on the double plates with 2.0% casein than those carrying the alkaline protease without 6×His tag. Thus, the transformants displaying the alkaline protease with 6×His tag are more suitable for the production of bioactive peptides. Strauss and Gotz (1996) also reported that the activity of surface-immobilized lipase varied with the spacer length. The activity increases from 0.8 to 83 units per milligram lipase as the spacer length varies from 10 to 92 amino acids. They thought that the length of the cell-wall-spanning region of the carrier protein must exceed a critical length to allow efficient folding of the passenger protein. Therefore, 6×His tag as the spacer of the displayed alkaline protease on the yeast cells may also play positive role in the efficient folding of the alkaline protease on the yeast cells, resulting in the increased alkaline protease activity. However, it is still unknown if the longer His tag could further increase alkaline protease activity.

6.3. Acid protease

Acid proteases, commonly known as aspartic proteases with a pH optimum in the acidic range (pH 3–4) have been reported in a variety of microorganisms as intracellular and extracellular enzymes (Kocabiyik and Ozel, 2007). They have two reactive aspartyl residues (Asp32 and Asp215, according to pepsin numbering) in the active site within the characteristic sequences (hydrophobic generally Phe) Asp32-Thr-Gly-Ser in the N-terminal domain, and a corresponding (hydrophobic)-Asp215-Thr-Gly-Ser/Thr in the C-terminal domain (De Viragh et al., 1993). These proteases are recognized by their specific inhibition by pepstatin. The enzymes from fungi and yeast have been studied the most extensively, and several of them have been purified and cloned (Kocabiyik and Ozel, 2007). For example, the acid protease from R. glutinis K-24 (Kamada et al., 1972) has been purified and characterized in some detail. Other yeasts which can produce acid protease include Saccharomyces carlsbergensis (Maddox and Hough, 1970), Y. lipolytica 37-1 (Tobe et al., 1976), Candida olea (Nelson and Young, 1987), Candida humicola (Ray et al., 1992) and S. fibuligera (Abdelh et al., 1977). Among them, some are of particular interest for their successful commercial applications, e.g. as a rennet substitute in the cheese industry, or as a catalyst in brewing industry (Kocabiyik and Ozel, 2007). Acid protease also plays an important role in fermentation industry because it hydrolyzes protein in the fermentation mash to liberate amino acids or peptides under the acidic condition (Kitano et al., 2002). This may imply that acid protease can play an important role in degradation of proteinous materials in acid environment. For example, it may be used to remove proteins in shrimp shell to obtain chitin and chitosan. However, many acid proteases are also involved in infection of plant pathogen and human pathogen. For example, C. albicans, the common human fungal pathogen, possesses at least eight genes encoding enzymes of this type (Hube et al., 1994).

6.3.1. Acid protease production by the marine-derived Metschnikowia reukaufi W6b

A total of 427 yeast strains from seawater, sediments, the guts of marine fish, and marine algae were obtained, only one strain (W6b) among them is found to be able to produce acid protease when they are grown in the medium with 20.0 g/l glucose (Li et al., 2008a). This yeast strain was isolated from sediment of South China Sea. The crude acid protease produced by strain W6b has the optimal temperature of 40 °C and the optimal pH of 3.5 and most of the acid protease produced by strain W6b is found to be the cell-bound enzyme. The activity of the crude acid protease produced by strain W6b is significantly inhibited by pepstatin A, suggesting that the acid protease belongs to aspartic protease. After identification using the routine and molecular methods (the accession number of 26S rDNA is EU439452), it was found to be closely related to M. reukaufi. Although as mentioned above many terrestrial yeasts can produce acid protease, this is the first report that M. reukaufi isolated from the marine environment can produce acid protease. Gonzalez-Lopez et al. (2002) found that the type of protease synthesized in the yeast Y. lipolytica is strictly dictated by ambient pH. At acidic pH, induction of the AXP1 gene leads to secretion of an acid protease (Axp), whereas at neutral pH, an alkaline protease (Aep) is produced as the XPR2 gene becomes induced. Therefore, it is unusual that M. reukaufi W6b isolated from marine environment can produce acid protease as pH of sea sediment is around 8.0. However, it is completely unknown if M. reukaufi W6b can yield such protease in natural sea sediment.

6.3.2. Characterization of the acid protease gene from M. reukaufi W6b

The gene named SAP6 (accession number EU186020) was cloned from cDNA of M. reukaufi W6b and has 1527 bp long encoding the acid protease (Li et al., 2008a). It was found that the gene has no intron (Li et al., 2008a). It has been reported that most of the genes encoding acid protease from yeasts do not contain introns (Hirata et al., 1988). The ORF encodes 508 amino acid residues with an estimated molecular mass of 53.5 kDa and the pl of the deduced protein is 4.2 (Li et al., in press-a). In contrast, the secreted aspartic proteinase from Glomerella
SAP6, C-terminal anchor domain of the gene encoding GPI-CWP in *M. reukaufi* hydropathy profile of yeast cells (Ueda and Tanaka, 2000; Yue et al., 2008). Therefore, the results above indeed have shown that most of the acid protease in the marine yeast *M. reukaufi* M6b is much higher than that of the acid proteases produced by other yeasts.

The deduced acid protease from the cloned gene has a typical conserved region (from 55 to 390 amino acids) which is the characteristics of eukaryotic aspartyl protease and the conserved eukaryotic and viral aspartyl proteases active site “VLDTCGSSDLRM” from 67 to 88 amino acids and “ALLDSGTTITQF” from 278 to 289 amino acids, respectively. Therefore, the results demonstrate that the acid protease belongs to one member of aspartic proteases in the eukaryotic aspartyl protease (EC 3.4.23) family (Li et al., in press-a). The amino acid sequence deduced from the SAP6 gene is also closely related to that of *S. cerevisiae* GPI-anchored aspartic protease I, *S. cerevisiae* GPI-anchored aspartic protease and *S. cerevisiae* GPI-anchored aspartic protease 2. This suggest that the SAP6 is GPI-anchored aspartic protease in the marine yeast *M. reukaufi* M6b (Li et al., in press-a). It has been reported that such protease is covalently linked to cell membrane by GPI-anchor (Ueda and Tanaka, 2000). The results above indeed have shown that most of the acid protease in *M. reukaufi* M6b is cell bound (Li et al., 2008a). The Kyte and Doolittle hydrophathy profiles of the Cwp1 from *S. cerevisiae* and the SAP6 from *M. reukaufi* M6b demonstrated that both two proteins have characteristic of glycosylphosphatidylinositol cell-wall proteins (GPI-CWPs) (Li et al., 2008a). Like the gene encoding the Cwp1 in *S. cerevisiae*, C-terminal anchor domain of the gene encoding GPI-CWP in *M. reukaufi* M6b may be used to construct surface display vector in yeast cells (Ueda and Tanaka, 2000; Yue et al., 2008). Therefore, the SAP6 gene cloned from *M. reukaufi* M6b may have many applications in biotechnology.

The SAP6 gene obtained from the marine yeast strain *M. reukaufi* W6b can be expressed in *E. coli* cells and the recombinant SAP6 produced by *E. coli* cells becomes the mature form with acid protease activity. The molecular mass of the recombiant SAP6 is 54 kDa, which is the similar size range (53.5 kDa) as estimated from the deduced amino acid sequence of the SAP6 gene (Li et al., 2008a,b). It has been reported that the molecular mass of a secreted aspartic protease from the terrestrial yeasts is around 36.0 kDa (Clark et al., 1997; Ray et al., 1992; Abdehl et al., 1977). This means that the molecular mass of the SAP6 is higher than that of acid proteases produced by other yeasts. The genes encoding the acid protease from *S. lignicolum* and *S. fibuligera* also have been expressed in *S. cerevisiae* (Shimuta et al., 2000; Yamashita et al., 1986).

The milk clotting activity is the common characteristics of all the aspartic protease (Kumar et al., 2005). After the milk clotting activity of the crude acid protease from the culture of the yeast strain *M. reukaufi* W6b and the recombinant SAP6 was determined. It was found that the crude acid protease from the culture of the yeast strain *M. reukaufi* W6b and the recombinant SAP6 indeed have the skimmed milk coagulability. The results demonstrate that the cDNASAP6 gene amplified from the marine yeast strain *M. reukaufi* W6b indeed encodes the aspartic protease (Li et al., in press-b).

### Table 7

<table>
<thead>
<tr>
<th>Producers</th>
<th>Molecular mass</th>
<th>Optimal pH and temperature</th>
<th>Activated by</th>
<th>Inhibited by</th>
<th>Location</th>
<th>Size of acid protease gene</th>
<th>Skimmed milk coagulability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine-derived</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. reukaufi</em> W6b</td>
<td>54.0 kDa</td>
<td>3.4, 40 °C</td>
<td>Mn²⁺</td>
<td>Cu⁺⁺, Ag⁺⁺, Zn⁺⁺ and Mg⁺⁺, pepstatin</td>
<td>Cell-bound</td>
<td>1527 bp</td>
<td>Yes</td>
<td>Li et al. (2008a,b)</td>
</tr>
<tr>
<td>Terrestrial yeasts</td>
<td>36 kDa</td>
<td>2.5–3.9, 50 °C</td>
<td>Ca²⁺</td>
<td>Cu⁺⁺ and Ag⁺, pepstatin</td>
<td>Extracellular</td>
<td>1,170–1212 bp</td>
<td>Yes</td>
<td>Kocabiyik and Ozel (2007)</td>
</tr>
</tbody>
</table>

### 6.3.3. Properties of the recombinant acid protease

The activity of the purified rSAP6 from the engineered *E. coli* carrying the SAP6 gene was found to be the highest at 40 °C and the enzyme is stable up to 40 °C (Li et al., in press-b). However, Ray et al. (1992) reported that when the acid protease produced by *C. humicolata* isolated from Antarctic soil is exposed for 2 h at 15, 22 or 37 °C, its activity retains 100%. When exposed to 45 °C for 2 h, its activity only looses 25% while it is incubated at 56 °C for 10 min, its activity is lost totally. This means that thermostability of the rSAP6 obtained in this study is higher at higher temperature than that of the acid protease produced by *C. humicolata*.

The maximum activity of the purified rSAP6 from the engineered *E. coli* carrying the SAP6 gene is observed at pH 3.4 and the enzyme is stable just between pH 2.6 and 5.0 (Li et al., in press-a). In general, the optimal pH for acid proteases is in the pH range of 2.5–3.9. For example, the optimal pH for three acid proteases produced by *S. lipolytica* CX161-1B are 3.5, 4.2 and 3.1, respectively (Yamada and Orzydzik, 1983) and their pH stability is in the pH range of 2.0–5.0. The optimal pH for the acid protease produced by *C. albicans* is 3.2 (Remold et al., 1968). In contrast, the optimal pH for the extracellular acid protease produced by *R. glutinis* is in the pH range of 2.0–2.5 (Kamada et al., 1972). This means that optimal pH for rSAP6 obtained from the engineered *E. coli* carrying the SAP6 gene is identical to that for acid proteases produced by other yeasts.

The results show that only Mn²⁺ (5.0 mmol/l) tested has an activating effect on the rSAP6 from the engineered *E. coli* carrying the SAP6 gene and Cu⁺⁺ and Ag⁺ act as inhibitors in decreasing the enzyme activity while Zn⁺⁺ and Mg⁺⁺ can weakly inhibit rSAP6 activity (Li et al., in press-a). In addition, Cu⁺⁺ has no obvious influence on the rSAP6 activity. When the concentrations of the ions are higher than 1.0 mM, the enzyme is inhibited by all the ions tested. However, Ca⁺⁺ has an activating effect on the acid protease produced by *S. cerevisiae* (Anahit et al., 1993). Alessandro and Federico (1980) reported that Cu⁺⁺ and Ag⁺ can inhibit the acid protease produced by *C. albicans*. Like acid protease from other yeasts, the rSAP6 enzyme activity is also strongly inhibited by pepstatin. This means that some properties of the rSAP6 obtained from the engineered *E. coli* carrying the SAP6 gene are different from those of acid proteases produced by other yeasts. Therefore, it seems that the SAP6 produced by the marine-derived yeast is a novel acid protease which may have potential applications in biotechnology and food industries.

Table 7 summarizes some properties of acid proteases from the marine yeast and terrestrial yeasts.

### 7. Inulinase

Among the hydrolytic enzymes, inulinase has been receiving much attention as it can be widely applied to production of fuel ethanol, high fructose syrup and inulooligosaccharides from inulin. Inulin is a linear β-[2, 1]-linked fructose polymer that occurs as a reserve carbohydrate in Jerusalem artichoke, dahlia tubers, chicory and yacon root (Chi et al., 2009; Rocha et al., 2006; Gazetta et al., 2005). Ethanol is the most employed liquid biofuel either as a fuel or as a gasoline
enhancer (Sanchez and Cardona, 2008). Fructose is widely used in many foods, pharmaceuticals and beverages instead of sucrose as fructose is a GRAS sweetener, sweeter than sucrose (up to 1.5 times), with lower cost, and has functional properties that enhance flavor, color, and product stability and fructose metabolism bypasses the known metabolic pathway of glucose and therefore does not require insulin (Kaur and Gupta, 2002). Inulooligosaccharides have very similar structure and functionalities to fructooligosaccharides whose beneficial effects on humans and animals have been well characterized as functional sweeteners (Sheng et al., 2007). Inulin also can be converted into fructose by chemical approach. However, the chemical approach is currently associated with some drawbacks as the process gives rise to coloring of the inulin hydrolysate and by-product formation in the form of difructose anhydrides (Pandey et al., 1999; Gill et al., 2006). Fructose can also be produced from starch by enzymatic methods involving amylase, amyloglucosidase and glucose isomerase (Gill et al., 2006). The best procedure involves the use of microbial inulinase, which after one step enzymatic hydrolysis of inulin, yields 95% pure fructose. Inulinase is produced by many microorganisms, such as Kluyveromyces, Aspergillus, Staphylococcus, Xanthomonas and Pseudomonas. Yeasts such as Kluyveromyces fragilis, Kluyveromyces marxianus, Candida kefyr, Debaryomyces cantarelli and fungi, Penicillium and Aspergillus species are the common inulinase producers (Pandey et al., 1999). However, it has been shown that some yeast strains can produce higher exoinulinase activity than filamentous fungi. Among the yeasts which can produce inulinas, two species of K. fragilis and K. marxianus have high potential for producing commercially acceptable yields of the enzyme (Rocha et al., 2006). After we screened over 300 marine yeast strains from different marine environments, we found that some marine yeast strains could secrete a large amount of inulinase into the medium prepared with seawater or artificial seawater (Gao et al., 2007a).

7.1. Inulinase production during submerged fermentation by Cryptococcus aureus G7a and P. guilliermondii strain 1

The marine yeast C. aureus G7a isolated from sediment of South China Sea was found to secrete a large amount of inulinase into the medium (Sheng et al., 2007). The crude inulinase produced by this marine yeast shows the highest activity at pH 5.0 and 50 °C. The optimal medium for its inulinase production is the artificial seawater containing inulin 4.0% (w/v), K2HPO4 0.3% (w/v), yeast extract 0.5% (w/v), KCl 0.5% (w/v), CaCl2 0.12% (w/v), NaCl 4.0% (w/v) and MgCl26H2O 0.6% (w/v), while the optimal cultivation conditions for inulinase production by this yeast strain are pH 5.0, a temperature of 28 °C and a shaking speed of 170 rpm. Under the optimal conditions, over 850 U/ml of inulinase activity is produced within 42 h of fermentation at shake flask level.

Because the yeast strain used in this study was isolated from marine environment, it is very important to examine effects of different concentrations of NaCl in the artificial seawater on inulinase production and cell growth by the marine yeast. It is worthy to observe that 4.0% (w/v) of added NaCl and 0.6% (w/v) of added MgCl26H2O is the most suitable for the inulinase production (84.1 U/ml) by the marine yeast (Sheng et al., 2007). However, it is still completely unknown why the inulinase production is enhanced in the presence of 4.0% (w/v) NaCl and 0.6% MgCl2 6H2O in the artificial seawater. However, cell growth is best in the presence of 3.0% NaCl and 0.6% MgCl2 6H2O in the artificial seawater, respectively.

The marine-derived yeast P. guilliermondii strain 1, isolated from the surface of a marine alga, was also found to secrete a large amount of inulinase into the medium (Gong et al., 2007). The crude inulinase produced by this marine yeast works optimally at pH 6.0 and 60 °C. The optimal medium for inulinase production by the marine yeast strain 1 is the seawater containing 4.0% (w/v) inulin and 0.5% (w/v) yeast extract, while the optimal cultivation conditions for its inulinase production are pH 8.0, 28 °C and 170 rpm. Under the optimal conditions, over 60 U/ml of inulinase activity is produced within 48 h of fermentation in shake flasks. A large amount of monosaccharides and a trace amount of oligosaccharides are detected after the hydrolysis, indicating that the crude inulinase has a high exoinulinase activity.

These results indicate that 2.0% (w/v) of added NaCl is the most suitable for the inulinase production by the marine yeast strain 1 (Gong et al., 2007). Especially, when the marine yeast strain is grown in the medium prepared with seawater, the inulinase activity reaches the highest (615 U/ml). However, as mentioned earlier, it is still completely unknown why the inulinase production by the marine yeast is enhanced in the presence of 2.0% (w/v) NaCl or seawater (Gong et al., 2007).

One inulinase overproducer mutant (M-30) that produces 115 U/ml of inulinase activity was obtained from the marine yeast P. guilliermondii strain 1 (Gao et al., in press). Under the optimized conditions, 1277 U/ml of inulinase activity is reached in the liquid culture of the mutant M-30. Under the same conditions, its parent strain only produces 481.0 U/ml of inulinase activity. This is the highest inulinase activity produced by the yeast strains reported so far. We also found that inulin can be actively converted into monosaccharides by the crude inulinase (Yu et al., 2009).

So far, it has been shown that the inulinase activity produced the terrestrial yeasts is less than 60 U/ml (Singh et al., 2006; Zhang et al., 2003, 2005; Singh and Bhermi, 2008). The fungus A. niger shows good growth on a medium containing 40% (w/v) of dandelion tap root extract composed of 50 g tap roots blended with 200 ml water and 2% yeast extract medium and produces 55 U/ml of inulinase activity in 96 h at 30 °C and 150 rpm (Kango, 2008). This demonstrates that the inulinase activity produced by the marine yeasts, especially the mutant M-30 reaches very high level in the liquid culture within the short fermentation period. Therefore, the inulinas produced by the marine yeast strains and their mutants are potentially useful in food industry.

7.2. Inulinase production during solid state fermentation by C. aureus G7a and P. guilliermondii

The solid state fermentation (SSF) offers numerous advantages for the production of bulk chemicals and enzymes. Therefore, the optimization of process parameters for high inulinase production by the marine yeast strain C. aureus G7a in solid-state fermentation (SSF) was carried out using response surface methodology (RSM) based on Central Composite Designs (CCD) (Sheng et al., in press). Finally, the optimal parameters obtained with RSM are the initial moisture 61.5%, inoculum 2.75%, the amount ratio of wheat bran to rice husk 0.42, temperature 29 °C, pH 5.5. Under the optimized conditions, 420.9 U/g of dry substrate of inulinase activity is reached in the solid state fermentation culture of strain G7a within 120 h whereas the predicted maximum inulinase activity of 436.2 U/g of dry weight is derived from RSM regression.

Response surface methodology (RSM) was also used to optimize the medium compositions and cultivation conditions for the inulinase production by the inulinase overproducer (the mutant M-30) in solid-state fermentation (Guo et al., in press). The initial moisture, inoculum, the amount ratio of wheat bran to rice bran, temperature, pH for the maximum inulinase production by the mutant M-30 were found to be 60.5%, 2.5%, 0.42, 30 °C and 6.5, respectively. Under the optimized conditions, 455.9 U/g of dry substrate of inulinase activity is reached in the solid state fermentation culture of the mutant M-30 whereas the predicted maximum inulinase activity of 459.2 U/g is derived from RSM regression. Under the same conditions, its parent strain only produces 291.0 U/g of inulinase activity. This is the highest inulinase activity in the culture of solid state fermentation produced by the yeast strains reported so far. For example, under the optimized conditions, inulinase activity of 391.9 U/g of dry fermented bagasse from K. marxianus NRRL Y-7571 is produced by SSF (Mazutti, 2008).
et al., 2006). Pandey et al. (1999) reported that the extracellular inulinase concentration reaches a peak (122.88 U/g of dry fermented substrate) with K. marxianus by SSF under the optimized conditions. After the optimization of solid state medium for production of inulinase by Kluyveromyces sp. S120 using surface response methodology, the average inulinase activity (409.8 U/g initial dry substrate) is obtained (Chen et al., 2007).

The results above demonstrate that like in terrestrial yeasts, inulinase activity produced by the marine yeasts can be greatly enhanced using surface response methodology.

7.3. Properties of the inulinases from the two marine yeasts

The molecular mass of the purified inulianses from the marine yeast C. aureus G7a and P. guilliermondii strain 1 was estimated to be 60.0 and 50.0 kDa, respectively (Sheng et al., 2008; Gong et al., 2008). It has been reported that the extracellular inulinase from the terrestrial yeast, K. fragilis has 250 kDa of molecular weight whereas the apparent molecular weight of exoinulinae from K. marxianus CBS 6556 is 72 kDa (SDS–PAGE) (Pandey et al., 1999; Rouwenhorst et al., 1990). The purified extracellular inulinase from the yeast K. marxianus var. bulgaricus was found to be 57 kDa by SDS–PAGE (Kushi et al., 2000). It has been reported that most of the inulinses from terrestrial fungi have more than 50.0 kDa of molecular weight (Pandey et al., 1999). This means that the molecular mass of the purified inulinase from P. guilliermondii strain 1 is smaller than that of the inulinses from terrestrial fungi and the marine-derived C. aureus G7a.

The optimal pH of the purified inulinase from the marine yeast C. aureus G7a is 5.0 and the enzyme is stable in the range of pH 4.0 to 6.5 (Sheng et al., 2008). In contrast, the maximum inulinase activity produced by P. guilliermondii strain 1 is observed at pH 6.0 and the enzyme is stable in the range of pH 6.0 to pH 7.0 (Gong et al., 2008). Generally, the inulinase from terrestrial fungi and yeasts is stable in the range of pH 4.0–8.0 whereas pH optima of the inulinases are in the range of 4.5–6.0 (Pandey et al., 1999; Zhang et al., 2005). This indicates that the inulinase from the marine yeasts have similar pH optima to that from the terrestrial yeast.

The optimal temperature of the purified inulinase from the marine yeast C. aureus G7a is 50 °C and the enzyme is very stable up to 65 °C (Sheng et al., 2008). From these results, the inulinase seems to have considerable thermostability. However, the inulinase activity produced by P. guilliermondii strain 1 is the highest at 60 °C and the enzyme is very stable up to 60 °C (Gong et al., 2008). Inulinase from terrestrial microorganisms, in general, shows the highest activity below 50 °C whereas optimum temperature is mostly between 30 and 55 °C (Pandey et al., 1999; Kushi et al., 2000; Zhang et al., 2005). This means that the optimal temperature and thermostability of inulinase from C. aureus G7a are also almost the same as those from the terrestrial K. marxianus, K. marxianus var. bulgaricus, and K. fragilis, which have been confirmed to be the common inulinase producers. However, the optimal temperature of the inulinase from P. guilliermondii strain 1 is higher than that from other yeasts (Gong et al., 2008).

The purified inulianses produced by the marine yeasts C. aureus G7a and P. guilliermondii strain 1 are activated by Ca2+, K+, Na+, Fe2+ and Cu2+, respectively. However, Mg2+, Hg2+, and Ag+ (at concentrations of 1.0 mM) acts as inhibitors in decreasing the activity of the two purified inulianses, suggesting that they are able to alter the enzyme conformation (Sheng et al., 2008; Gong et al., 2008; Sharon et al., 1998). However, when the concentrations of the ions are higher than 1.0 mM, the enzyme activity is inhibited by all the ions tested. The inhibition by mercuric ions may indicate the importance of thiol-containing amino acid residues in the enzyme function (Barth and Gaillardin, 1997). These results indicate that the inulianses from the two marine yeasts have some common properties. However, the inulinase from Penicillium sp is inhibited by Ag+2 and Cu2+ (Pandey et al., 1995) whereas the inulianses from Aspergillus sp. are inhibited by Fe3+, Mn2+, and Mg2+ (Pandey et al., 1999). Fe2+ increases the activity of the recombinant exoinulinase from K. marxianus expressed in P. pastoris by 41.97%, but Mg2+, Cu2+, Zn2+, Ca2+, and Fe3+ inhibits the activity of the recombinant exoinulinase from K. marxianus expressed in P. pastoris dramatically (Zhang et al., 2005). It was found that Mg2+ does not affect the activity of the purified extracellular inulinase from the yeast K. marxianus var. bulgaricus, but Ca2+ inhibits the enzyme by approximately 27%, Ba2+, Zn2+, and Na+ inhibits 50% whereas ferric chloride completely inhibits the enzyme (Kushi et al., 2000). This may imply that some physical and biochemical properties of the inulianses from the marine yeast strains are different from those from terrestrial yeasts and the unique characteristics may have many potential applications.

The purified inulianses from both the marine yeast C. aureus G7a and P. guilliermondii strain 1 are strongly inhibited by PMSF, iodoacetic acid, EDTA, and 1,10-phenanthroline (Sheng et al., 2008; Gong et al., 2008). This means that the purified inulianses are metalloenzyme and Ser and Cys residues of the enzyme are essential for the enzymes active sites. This again demonstrates that the inulianses from the two marine yeasts have some common properties.

The KM and Vmax of the purified inulinase from the marine yeast C. aureus G7a for inulin are 20.06 mg/ml and 0.0085 mg/min, respectively (Sheng et al., 2008) while apparent KM and Vmax values of the purified inulinase from P. guilliermondii strain 1 for inulin are 21.1 mg/ml and 0.1 mg/min, respectively (Gong et al., 2008). However, the inulinase from terrestrial yeast K. marxianus has a KM value of 11.9 and 3.92 mM for sucrose and inulin, respectively (Pandey et al., 1999), whereas the apparent KM value of the purified extracellular inulinase from the yeast K. marxianus var. bulgaricus for inulin is 86.9 mg/ml (Kushi et al., 2000). These results reveal that the inulianses from the marine yeast displays very high affinity for inulin.

It is very interesting to note that only monosaccharides (glucose and fructose) are released from inulin by the action of the purified inulianses from both the two marine yeasts C. aureus G7a and P. guilliermondii strain 1 (Sheng et al., 2008; Gong et al., 2008). These results strongly suggest that the inulianses produced by the marine yeasts have high exoinulinase activity. However, analysis of the hydrolysis products of inulin by the crude inulinase produced by the marine yeast C. aureus G7a shows that a large amount of monosaccharides and oligosaccharides with different molecular sizes are released from inulin after inulin hydrolysis by the crude inulinase (Sheng et al., 2007). The monosaccharides and oligosaccharides are also detected after inulin hydrolysis for more than 2 h by the purified exoinulinase produced by K. marxianus var. bulgaricus (Kushi et al., 2000). Therefore, the inulianses from the two marine yeasts may have great potential use in the direct digestion of inulin in food and fermentation industries for production of bioethanol and ultra-high fructose.

7.4. Characterization of the inulinae gene from the two marine yeast strains

The extracellular inulinase structural gene cloned from C. aureus G7a has an open reading frame of 1557 bp long encoding an inulinase while the gene cloned from P. guilliermondii strain 1 has an open reading frame of 1542 bp long encoding an inulinase. There is no intron in the coding region of both the two genes (Zhang et al., 2009). The inulinae genes from A. niger AF10, K. marxianus, K. marxianus var. marxianus ATCC 12424 and K. cicerisporus CBS54857 are 1551 bp (encoding 516 amino acids), 1670 pb, 1665 bp and 1665 bp, respectively (Zhang et al., 2005; Bergkamp et al., 1993; Laloux et al., 1991; Wen et al., 2003). This means that the size of the INUI gene cloned from the marine yeast P. guilliermondii strain 1 is similar to that of the inulinae gene from A. niger AF10 and smaller than that of inulinae genes from other yeasts.

The predicted protein from the INUI gene cloned from P. guilliermondii strain 1 consists of 514 amino acids with a calculated molecular mass of 58.04 kDa while the inulinase gene from C. aureus
G7a encodes 518 amino acid residues of a protein (Zhang et al., 2009). However, the molecular mass of the purified inulinase from the same \textit{P. guilliermondii} strain 1 was estimated to be 50.0 kDa (Gong et al., 2008) while the molecular mass of the purified inulinase from \textit{C. aureus} G7a was estimated to be 60.0 kDa (Sheng et al., 2008). This means that the size of the calculated molecular mass of the inulinase deduced from the \textit{INU1} gene cloned is close to that of the molecular mass of the purified inulinase from \textit{P. guilliermondii} strain 1 as mentioned above. It has been reported that the extracellular inulinase from the terrestrial yeast, \textit{K. fragilis} has 250 kDa of molecular mass while the apparent molecular mass of exoinulinase from \textit{K. marxianus} CBS 6556 is 72 kDa (by SDS–PAGE) (Pandey et al., 1999; Rouwenhorst et al., 1990). Meanwhile, the purified extracellular inulinase from the yeast \textit{K. marxianus} var. \textit{bulgaricus} was found to be 57 kDa by SDS–PAGE (Kushi et al., 2000). This means that the extracellular inulinase from the terrestrial yeast has a higher little molecular mass than that from the marine yeast \textit{P. guilliermondii} strain 1.

The inulinase protein sequence deduced from both the cloned inulinase gene in \textit{P. guilliermondii} strain 1 and that in \textit{C. aureus} G7a contains the consensus motifs R-D-P-K-V-F-W-H and W-M-N-D-P-N-R which are conserved among the inulinases from other microorganisms (Zhang et al., 2009, 2005; Nagem et al., 2004; Kim et al., 2008). It has been confirmed that the consensus motif (WMNXPNGL) of inulinases acts as a nucleophile and the consensus motif (RDPKVF) of inulinases has an essential role in catalytic activity (Nagem et al., 2004). The amino acid sequence from 20 to 350 amino acids at N-terminal of the inulinase from both two marine yeast strains also contains the conserved domain of glycosyl hydrolases family 32 (Zhang et al., 2009).

The inulinase gene from \textit{P. guilliermondii} strain 1 can be expressed in \textit{P. pastoris} X-33 and the expressed fusion protein has the molecular mass of about 60 kDa (Zhang et al., 2009). However, the molecular mass of the purified inulinase from the culture supernatant of \textit{P. guilliermondii} strain 1 was estimated to be 50.0 kDa (Gong et al., 2008), indicating that the molecular mass of the purified inulinase is smaller than that of the recombinant inulinase from \textit{P. pastoris} X-33. This is due to that the recombinant inulinase produced by \textit{P. pastoris} X-33 is 6×His tag fusion protein. Another reason is that the protein modification difference between in \textit{P. guilliermondii} strain 1 and in \textit{P. pastoris} X-33 may occur as there were ten potential N-linked glycosylation sites of the protein deduced from the \textit{INU1} gene cloned from \textit{P. guilliermondii} strain 1.

It was found that like the crude and purified inulinases produced by \textit{P. guilliermondii} strain 1, the crude recombinant inulinase produced by \textit{P. pastoris} X-33 carrying the \textit{INU1} gene has exo-inulinase activity (Zhang et al., 2009). This means that the action mode of the recombinant inulinase produced by \textit{P. pastoris} X-33 carrying the \textit{INU1} gene was little different from that of the purified inulinase and crude inulinase from \textit{P. guilliermondii} strain 1. Such exo-inulinase has many potential applications in the production of either ultra-high fructose syrups or bioethanol as a large amount of reducing sugar can be released from inulin during the enzymatic hydrolysis (Pandey et al., 1999).

In order to make comparison of different inulinases, some properties of inulinases from marine yeasts and terrestrial yeasts are given in Table 8.

### 8. Killer toxin

It has been well known that many diseases in marine animals can be caused by some species of marine bacteria and marine viruses. However, in recent years, many evidences have shown that some marine yeasts are also pathogenic to some marine animals. Like bacterial and virus disease, the yeast disease has caused big economic losses in mariculture industry in some regions of China (Xu, 2005; Xu et al., 2003). For example, an explosive epidemic disease which is called milky disease has happened in cultured \textit{Portunus trituberculatus} since 2001 in Zhoushan, Zhejiang Province, China, leading to high mortality of this crab and great economic loss in this area. The pathogenic agent for the milky disease was found to be \textit{Metschnikowia bicuspidata} according to the results of preliminary identification (Wang et al., 2007b). The purified yeast strain from the diseased parts of the marine animal can develop the same symptom in the muscle, heart and hepatopancreas of the infected marine animals in the challenging test (Wang et al., 2007b). It was found that nystatin, benzalkonium bromide and extract of goldthread root and garlic are active against the pathogenic yeast. However, the compounds with minimum inhibitory concentration (MIC) are toxic to the crab and it is impossible to apply the expensive antibiotics to the open sea (Xu et al., 2003). It was found that the yeast \textit{Torulopsis mogii} is the pathogen to some shrimp in China (Sun and Sun, 1998). The yeast \textit{M. bicuspidate var. bicuspidate}, a pathogenic yeast of aquatic invertebrates is capable of infecting aquaculture-reared, disease-free Artemia (Moore and Strom, 2003). A new species of the marine yeast \textit{Klyveromyces petroel} was isolated from the heart tissue of subadult shrimp \textit{Penaeus chinensis} during tissue culture. The yeast grows well in seawater supplemented with 2.0% shrimp extract, but does not grow in YPD and malt extract medium in which most of yeast cells grow well (Tong and Miao, 1999). The research has shown that killer yeasts can be applied to control growth of pathogenic yeasts in human, animal and plant. Killer toxin produced by some yeast strains is a low molecular mass protein or glycoprotein toxin which kills sensitive cells of the same or related yeast genera without direct cell–cell contact (Magliani et al., 2004; Schmitt and Breining, 2002). The killer strains themselves are immune to their own toxin, but remain susceptible to the toxins secreted by other killer yeasts. The results have shown that some killer toxins that interfere with the synthesis of β-1,3-d-glucan can hydrolyze laminarin. Therefore, they are regarded as glucanase (Wang et al., 2008). The killer phenotype is very common in occurrence and can be found both in natural yeast isolates and in laboratory yeast strain collections. Up to now, toxin-producing killer yeasts have been identified in genera \textit{Candida}, \textit{Cryptococcus}, \textit{Debaromyces}, \textit{Hanseniaspora}, \textit{Hansenula}, \textit{Klyveromyces}, \textit{Metschnikowia}, \textit{Pichia}, \textit{Saccharomyces}, \textit{Ustilago}, \textit{Torulopsis}, \textit{Williopsis} and \textit{Zygosaccharomyces}, indicating that the killer phenomenon is indeed widespread among yeasts (Magliani et al., 2004). Killer determinants are either

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**Table 8**

Some properties of inulinases from the marine yeasts and terrestrial yeasts

<table>
<thead>
<tr>
<th>Producers</th>
<th>Molecular mass</th>
<th>Optimal pH and temperature</th>
<th>Activated by</th>
<th>Inhibited by</th>
<th>Location</th>
<th>Size of inulinase gene</th>
<th>Type of inulinase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>The marine-derived \textit{C. aureus} G7a</td>
<td>60.0 kDa</td>
<td>5.0, 50 °C</td>
<td>Ca2+, K+, Na+, Fe3+ and Cu2+</td>
<td>Mg2+, Hg2+, Ag+, PMSF, iodoacetic acid, EDTA, and 1,10-phenanthroline</td>
<td>Extracellular</td>
<td>1537 bp</td>
<td>Exoinulinase</td>
<td>(Sheng et al., 2008; Zhang et al., 2009)</td>
</tr>
<tr>
<td>The marine-derived \textit{P. guilliermondii} strain 1</td>
<td>50.0 kDa</td>
<td>6.0, 60 °C</td>
<td>Ca2+, K+, Na+, Fe3+ and Cu2+</td>
<td>Mg2+, Hg2+, Ag+, PMSF, iodoacetic acid, EDTA, and 1,10-phenanthroline</td>
<td>Extracellular</td>
<td>1542 bp</td>
<td>Exoinulinase</td>
<td>(Gong et al., 2008; Zhang et al., 2009)</td>
</tr>
<tr>
<td>Terrestrial yeasts</td>
<td>57–250 kDa</td>
<td>4.5–6.0, 30–55 °C</td>
<td>Fe3+</td>
<td>Ra2+, Na+, Mg2+, Cu2+, Zn2+, Ca2+, and Fe3+</td>
<td>Extracellular</td>
<td>1551–1670 pb</td>
<td>Exoinulinase</td>
<td>Chi et al. (2009)</td>
</tr>
</tbody>
</table>
cytoplasmically inherited encapsulated dsRNA viruses, linear dsDNA plasmids or nuclear genes (Schmitt and Breinig, 2002). The analysis in mechanisms of killer toxin can also provide important information for combating yeast infections caused by certain human pathogenic strains of the yeasts C. albicans and/or Sporothrix schenckii (Comitini et al., 2004). However, it still is little known about the killer toxin produced by marine yeasts.

In order to fight against the pathogenic yeast in crab, killing activity of different marine yeast cultures was determined. We found that 17 strains of the marine yeasts from seawater, sediments, mud of salterns, guts of the marine fish and marine algae could secrete killer toxin into the medium and kill the pathogenic yeast M. bicuspidata WCY in crab. However, only five strains (WC91-2, GZ1, YF07b, hcx-1 and HN2.3) among them have higher killing activity against the pathogenic yeast than others. The results of routine identification and molecular methods show that the five yeast strains belong to Williopsis saturnus WC91-2, P. guilliermondii GZ1, P. anomala YF07b, Debaryomyces Hansenii hcx-1 and A. pullulans HN2.3, respectively. We found that not all the optimal conditions for the killer toxin production and action of killer toxin produced by the marine killer yeasts are in agreement with those of marine environments and for crab cultivation. It was found that NaCl concentration in the medium could change killing activity spectra. All the crude killer toxins produced by them could hydrolyze laminarin and the hydrolysis end products are monosaccharides (Wang et al., 2008), indicating that the killer toxins produced by the marine yeasts have exo-β-1,3-glucanase activity.

The molecular mass of the purified killer toxin from the marine killer yeast YF07b was estimated to be 47.0 kDa and it is a monomer protein (Wang et al., 2007c). It has also been reported that the extracellular killer toxin from the terrestrial yeasts, P. anomala NCYC432 and P. anomala NCYC434 K5 type, has 47 kDa and 49 kDa of molecular mass, respectively, whereas the killer toxin from P. anomala WC65 has 83.3 kDa of molecular mass (Izgu et al., 2005; Izgu et al., 2006). Usually, most of the killer toxins from Pichia spp. are monomer. However, a novel type of killer toxin (salt-mediated killer toxin) produced by the halotolerant yeast P. farinosa, is a heterodimer (14.214 kDa) (Kashiwagi et al., 1997).

The optimal temperature of the purified killer toxin from the marine killer yeast YF07b is 40 °C and the enzyme is very stable up to 60 °C. From these results, the toxin seems to have considerable thermostability (Wang et al., 2007c). The toxins from P. anomala show high stability up to 37 °C (Izgu et al., 2005; Izgu et al., 2006). At pH 4, optimal killer activity of the killer toxin produced by Pichia membranifaciens CYC 1106 was observed at temperatures up to 20.6 °C (Santos et al., 2000). This means that the killer toxin from the marine killer yeast strain YF07b has higher thermostability than that from other Pichia spp.

The maximum activity of the purified killer toxin from the marine killer yeast YF07b is observed at pH 4.5 and the killer toxin is stable in the range of pH 3.0–5.0 (Wang et al., 2007c). Naturally, the killer toxin shows its maximum killer activity at pH 2.5–4.0, its toxicity steeply decreases with increasing pH (Izgu et al., 2005, 2006; Santos et al., 2000). This results show that the killer toxin from the marine killer yeast strain YF07b has similar pH stability to that from most terrestrial yeasts. However, the most stable killer toxins are those of Hansenula mrakii (stable at pH 2–11) and H. saturnus (stable at pH 3–11) (Kashiwagi et al., 1997).

The toxin from the marine killer yeast strain YF07b is activated by Ca²⁺, K⁺, Na⁺, Mg²⁺, and Co²⁺ (accession number: EF029071) of the deduced amino acids has 99% identity for laminarin than that from P. anomala (accession number: AJ222862) of P. anomala strain K.

Therefore, it will be further investigated in this laboratory whether the killer toxin produced by the marine killer yeast YF07b can kill the pathogenic yeast in crab and in marine environment.

Some properties of killer toxins from marine yeasts and terrestrial yeasts are summarized in Table 9.

9. Conclusions and future prospects

The results above strongly show that production of the enzymes by different marine yeasts can be greatly enhanced in the presence of Na⁺ and seawater. Some enzyme activity, such as amylase, protease, inulinase, phytase, CMCase and killer toxin also can be significantly activated in the presence Na⁺, Mg²⁺ and Mn²⁺ which are the main ions in the seawater (Tables 2–9). From the results in Tables 2–9, it can be clearly seen that the genes encoding the extracellular enzymes in the marine yeasts tested have big differences from those in terrestrial

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Table 9

<table>
<thead>
<tr>
<th>Producers</th>
<th>Molecular mass</th>
<th>Optimal pH and temperature</th>
<th>Activated by</th>
<th>Inhibited by</th>
<th>Location</th>
<th>Size of killer toxin gene</th>
<th>Enzyme activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>The marine-derived P. anomala YF07b</td>
<td>47.0 kDa</td>
<td>4.5, 40 °C</td>
<td>Ca²⁺, K⁺, Na⁺, Mg²⁺, Co²⁺</td>
<td>Fe²⁺, Fe³⁺, Hg²⁺, Cu²⁺, Zn²⁺, Ag⁺</td>
<td>Extracellular</td>
<td>1589 bp</td>
<td>β-1,3-Glucanase</td>
<td>Wang et al., 2007</td>
</tr>
<tr>
<td>Terrestrial yeasts</td>
<td>47–83.3 kDa</td>
<td>2.5–4.0, 20.6–37 °C</td>
<td>Na⁺, K⁺</td>
<td>Unknown</td>
<td>Extracellular</td>
<td>1589 pb</td>
<td>β-1,3-Glucanase</td>
<td>Izgu et al., 2005</td>
</tr>
</tbody>
</table>
yeasts and fungi. The inulinases, lipase and phytase activities from the marine yeasts are found to be much higher those from the terrestrial yeasts and fungi and the enzymes can be used to actively hydrolyze their substrates for production of different useful products. Therefore, the enzymes from the marine yeast strains have so many unique properties and many potential applications in biotechnology. So, it is very important to clone and overexpress the genes encoding the extracellular enzymes. It is also necessary to genetically modify the cloned genes so that the activity, optimal pH and temperature, stability of the recombinant enzymes can be further greatly improved.

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