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Research review paper

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.

Contents

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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*, *Candia*, *Cryptococcus*, *Debaryomyces*, *Dipodascus*, *Filobasidium*, *Galactomyces*, *Geotrichum*, *Hanseniaspora*, *Issatchenkia*, *Kluyveromyces*, *Kodamaea*, *Lodderomyces Metschnikowia*, *Pichia, Pseudozyma*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Williopsis*, *Yarrowia*, *Zygowilliopsis*, *Mrakia*, *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.

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 saccharied 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

Table 1
Some extracellular enzymes and their applications

Extracellular enzymes	Applications	References
Amylases	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	Chi et al., 2003; Gupta et al., 2003
Cellulases	Improvement of cellulosic fibres, stone washing, detergent additives, production of single-cell protein and biofuels, waste treatment	Zhang and Chi (2007)
Lipases	Catalysis of a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis, production of biodiesel	Hasan et al. (2006)
Phytase	The component of commercial poultry, swine, and fish diets, animal and human nutrition, environmental protection	Haefner et al. (2005)
Alkaline protease	Detergents additive, leather processing, silver recovery, medical purposes, food processing, feeds, chemical industry, waste treatment, digestion of protein	Ni et al. (2008a)
Acid protease	A rennet substitute in the cheese industry, or as a catalyst in brewing industry, hydrolysis of protein in fermentation industry, removal of protein	Li et al. (2008a)
Inulinases	Production fuel ethanol, high fructose syrup and inulooligosaccharides	Pandey et al., 1999, Chi et al., 2009
Killer toxin	Medicine, biocontrol of fungal disease in plants and animal	Wang et al. (2008)

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 amylolytic 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 extracellular amylolytic enzymes include *Arxula adeninivorans*, *Lipomyces*, *Saccharomycopsis*, *Schwanniomyces*, *Candida japonica* and *Filobasidium capsuligenum* and extracellular amylolytic 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 *Saccharomycopsis fibuligera* 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. fibuligera* 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 potato starch granules and 20 U/ml of the crude enzyme. Only glucose is released from starch molecules during the hydrolysis, indicating that the crude enzyme can hydrolyze both α-1,4 and α-1,6 linkages of starch molecule in the potato starch and the enzyme is glucoamylase (Li et al., 2007b).

2.2. Properties of the amylase from the marine-derived A. pullulans N13d

It was found that the molecular weight of the purified amylase from the marine yeast *A. pullulans* N13d is 98 kDa and the purified enzyme consists of two subunits whose molecular weights are 65 and 33 kDa, respectively (Li et al., 2007c). Among the amylase-producing yeasts, molecular weights of amylase from *Cryptococcus*, *Lipomyces kononenkoae* CBS 5608, *S. cerevisiae* and *Schwanneoimyces alluvius* UCD 5483 are 66.0, 76.0, 54.1 and 61.9 kDa, respectively (Wanderley et al., 2004; Prieto et al., 1995; De Moraes et al., 1999; Wilson and Ingledew, 1982). Two forms of glucoamylases produced by the yeast *F. capsuligenum* are glycoproteins of identical molecular weight (60 kDa) (De and Verachtert, 1985). These results indicate that the molecular weight of the amylase from the marine yeast is higher than that from other yeasts.

The optima temperature of the purified enzyme is 60 °C and the enzyme is stable up to temperature of 60 °C (Li et al., 2007c). However, glucoamylase I produced by the yeast *F. capsuligenum* has a higher optimum temperature (55 °C) than glucoamylase II (50 °C) and is also more resistant to thermal inactivation (De and Verachtert, 1985). This reveals that the amylase from the marine yeast strain has a higher optimum temperature and is also more resistant to thermal inactivation 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 Ca^{2+}, Ba^{2+}, Na^{+}, Cu^{2+}, Mg^{2+} and Co^{2+} (at the concentrations of 1.0 mM) and stabilized by $CaCl_2$, suggesting that Ca^{2+} not only has an activating effect on the purified enzyme, but also can stabilize the amylase (Li et al., 2007c). On the other hand, Hg^{2+} 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 $CaCl_2$ and inhibited by Hg^{2+}, Ag^{+}, Cu^{2+} and Mg^{2+} (Iefuji et al., 1996) while that from *L. kononenkoae* CBS 5608 is inhibited by Ag^{+} and Cu^{2+} (Prieto et al., 1995). Na^{+}, Mg^{2+} and Ca^{2+} do not have any influence on the enzyme activity from *Cryptococcus* S-2, but the enzyme is stabilized by 1.0 mM $CaCl_2$. 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 Ca^{2+}.

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. kononenkoae* 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 sweet potato 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 sweet 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

glucoamylases is a feature of several fungal glucoamylases and has also been demonstrated for the purified glucoamylases from the yeasts *Candida tsukubaensis*, *S. fibuligera*, *S. alluvius*, *Schwanniomyces castellii* and *F. capsuligenum*. This means that the glucoamylase from the marine yeast strain is identical to that from the terrestrial yeasts in some respects. 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. fibuligera* and *C. antarctica* have 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 glucoamylase (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 glucoamylases from *C. rolfsii* AHU 9627 loose 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 glucoamylase 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; Dariush 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.

3. Cellulase

Cellulose is the most abundant organic material on the earth, consisting of glucose units linked together by β-1, 4-glycosidic bonds. Therefore, it has become of considerable economic interest to develop some processes for the effective treatment and utilization of cellulosic 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-glucanases), 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 found a number of industrial applications in the textile industries. For example, they are used to improve the appearance of cellulosic fibres and used as a replacement for pumice stones in the “stone washing” process. In addition, cellulases are also potentially useful for converting cellulosic plant polymeric biomass into single-cell protein and biofuels (Deshpande et al., 1992).

Cellulases produced by fungi such as *Aspergillus*, *Rhizopus* and *Trichoderma* species have been extensively studied by several researchers (Wen et al., 2005; Ikeda et al., 2006). From the importance of fungal endoglucanases in the textile and detergent industries, many fungal endoglucanases from members of the subdivision Deuteromycotina, such as *Aspergillus* sp., *Fusarium* sp., *Humicola* sp., *Penicillium* sp., and *Trichoderma* sp., have been purified and characterized (Chi et al., 2006; Murashima et al., 2002). However, little is known about the cellulases from the marine-derived yeasts.

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 (endoglucanase) 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 FPAase 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-β-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-β-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

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

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Raw starch adsorption	Digestion of raw starch	References
Marine-derived *A. pullulans* N13d	98 kDa (two subunits: 65 and 33 kDa)	4.5, 60 °C	Ca^{2+}, Ba^{2+}, Na^{+}, Cu^{2+}, Mg^{2+} and Co^{2+}	Hg^{2+} and Ag^{+}, EDTA, EGTA and SDS	Sweet potato starch, and potato starch	Potato starch	Li et al., 2007a,b
Terrestrial Yeasts	66.0–61.9 kDa	4.2–6.0, 50–55 °C	Ca^{2+}	Hg^{2+}, Ag^{+}, Cu^{2+} and Mg^{2+}, DTT	Raw corn starch	Raw cereal starch	Chi et al., 2003; Gupta et al., 2003

these results, the CMCase seems to have considerable thermosensitivity. 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^+, Mg^{2+}, Ca^{2+}, K^+, Fe^{2+} and Cu^{2+} (at the concentrations of 1.0 mM). However, Fe^{3+}, Ba^{2+}, Zn^{2+}, Mn^{2+} 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 concentrations 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 IF031125 and *R. oryzae*, is inhibited by Cu^{2+} (Akiba et al., 1995; Murashima et al., 2002). However, it is very strange that in our research, Cu^{2+} could activate the activity of the purified CMCase. These results indicate that some biochemical properties of the CMCase from the marine yeast are not the same as those from the terrestrial fungi.

The enzyme is inhibited by PMSF, SDS, DTT and iodoacetic acid and the presence of the chelating agents, EDTA and EGTA inhibits the enzyme activity, demonstrating that the purified enzyme is metalloenzyme (Zhang and Chi, 2007; George and Diwan, 1983). The enzyme activity is inhibited by PMSF, indicating that Ser residues are essential for the enzyme active sites (George and Diwan, 1983). Iodoacetic acid was also found to have a negative effect on the enzyme activity, suggesting that Cys residues are important for active sites of the enzyme. It was also found that the CMCase is inactivated by SDS. It has been reported that the CMCase from *Bacillus amyloliquefaciens* is also strongly inhibited by EDTA (Lee et al., 2008).

K_m and V_{max} values of the purified enzyme for CMC are 4.73 mg/ml and 0.566 μmol/min/mg, respectively (Zhang and Chi, 2007). It has been reported that the apparent K_m value of the CMCase from *Favolus arcularius* against CMC is 0.28 mg/ml (Enokibara et al., 1992). These results reveal that the CMCase from the marine yeast displays high affinity for CMC.

Only oligosaccharides, but no monosaccharides are released from CMC after hydrolysis for 1 h with the purified CMCase, indicating the purified CMCase indeed has endo-1, 4-β-D-glucanase activity (Zhang and Chi, 2007). Both RCE1 and RCE2 from *R. oryzae* also hydrolyze the insoluble cellooligosaccharide (G33) to G2, G3, and G4. G6 is hydrolyzed to G2 and G4. G5 is hydrolyzed to G2 and G3. G4 is weakly hydrolyzed to G2 by both enzymes. Neither of the enzymes was found to act on G2 or G3 (Murashima et al., 2002). This means that like CMCases from *R. oryzae*, the CMCase from the marine yeast cannot act on cellobiose, either.

3.3. 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 sequence of the *CMC1* 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 *CMC1* gene encodes 498 amino acid residues of a protein without signal peptide. The protein deduced from the *CMC1* gene has the conserved motifs: ENFITC, LGLNCIF, FFNYFHT and NGEFGFVY. The proteins deduced from the CMC 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 suggested 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 encoding 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 cellulases from the marine yeast and terrestrial fungi are summarized in Table 3.

4. Lipase

Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis. Therefore, lipases, especially microbial lipases have many industrial applications (Hasan et al., 2006). Following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume. Numerous species of bacteria, yeasts and molds were found to produce lipases. Among the terrestrial yeasts, *Candida rugosa*, *C. tropicalis*, *C. antarctica*, *C. cylindracea*, *C. parapsilosis*, *C. deformans*, *C. curvata*, *C. valida*, *Yarrowia lipolytica*, *Rhodotorula glutinis*, *R. pilimornae*, *Pichia bispora, P. maxicana*, *P. sivicola*, *P. xylosa*, *P. burtonii*, *S. crataegenesis*, *Torulaspora globosa* and *Trichosporon asteroides* have been found to be able to produce lipase (Vakhlu and Kour, 2006). Extracellular lipases from several yeasts have been purified and characterized and the genes encoding lipase in *Candida*, *Geotrichum*, *Trichosporon* and *Y. lipolytica* have been cloned and overexpressed (Vakhlu and Kour, 2006). It has been found that most of lipases are serine hydrolyases according to their biochemical properties. Although lipases from *C. rugosa* and *C. antarctica* have been extensively used in hydrolysis and synthesis of a wide range of esters of commercial interest, very few studies exist on the lipase produced by yeasts isolated from sea salterns (Chi et al., 2006).

Table 3
Some properties of cellulases from the marine yeast and terrestrial fungi

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Cellulase activity	Size of CMCase gene	References
The marine-derived *A. pullulans* 98	67.0 kDa	5.6, 40 °C	Na^+, Mg^{2+}, Ca^{2+}, K^+, Fe^{2+} and Cu^{2+}	Fe^{3+}, Ba^{2+}, Zn^{2+}, Mn^{2+} and Ag^+, PMSF, SDS, DTT, iodoacetic acid, EDTA and EGTA	CMCase and FPAase	1497 bp	Zhang and Chi, (2007)
Terrestrial fungi	34–61 kDa	5.0–6.0, 50–70 °C	Unknown	Cu^{2+}, EDTA	CMCase and FPAase	711–1493 bp	Wen et al., 2005; Ikeda et al., 2006

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, *Lodder-myces elongisporus* YF12c, *Candida quercitrusa* JHSb, *Candia rugosa* wl8, *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 are the new producers of lipase (Wang et al., 2007a).

The optimal pHs and temperatures of lipases produced by them are between 6.0 and 8.5 and between 35 and 40 °C, respectively (Wang et al., 2007a). Usually, the optimal pH and temperature for lipase from terrestrial yeasts are between 5.0 and 8.0 and between 30 and 50 °C (Vakhlu and Kour, 2006). For example, the optimal pH and temperature for activity of the crude lipase produced by *C. rugosa* are 30 °C and pH 7.2, respectively (Lotti et al., 1998). This means that the optimal pH and temperature of the crude lipase from the marine yeasts are in agreement with those from the terrestrial yeasts.

It was found that majority of lipases from the marine-derived yeast strains are cell-bound and only lipase from *A. pullulans* HN2.3 is extracellular (Wang et al., 2007a). However, it has been confirmed that most of lipases from terrestrial yeasts are extracellular, but lipase from terrestrial yeast *C. parapsilosis* CBS 604 and some lipase from *Y. lipolytica* are also cell-bound (Vakhlu and Kour, 2006).

Some lipases from the marine-derived yeast strains can actively hydrolyze different oils, indicating that they may have potential applications in industry. Although lipase from terrestrial yeast *C. rugosa* has been extensively used in industrial and clinical purpose (Vakhlu and Kour, 2006), lipase activity produced by the marine-derived yeast *C. rugosa* wl8 is lower than that produced by the marine yeasts *C. intermedia* YA01a and *L. elongisporus* YF12c (Wang et al., 2007a). This suggests that the marine-derived yeast *C. rugosa* wl8 is different from the terrestrial yeast *C. rugosa* in their ability to produce lipase.

In China, a large amount of peanut oil and lard is discharged from the restaurants and homes each day and causes heavy pollution in fresh water and seawater. Therefore, the lipase with high hydrolytic activity towards peanut oil and lard may have highly potential applications in degradation of oil in fresh water and seawater and reuse of the wasted peanut oil for biodiesel production.

As mentioned above, the marine-derived *A. pullulans* HN2.3 is found to be able to produce extracellular lipase. Therefore, the optimal media and cultural conditions for the lipase production by *A. pullulans* HN2.3 were determined. The crude lipase produced by this marine yeast works best at pH 8.5 and 35 °C (Liu et al., 2008a). The optimal medium for the crude lipase production is 3.0% (w/v) olive oil, 0.4% (w/v) glucose, 0.6% (w/v) ammonium sulfate, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4.7H_2O$, pH 7.0 while the optimal cultivation conditions for the crude lipase production are pH 7.0, 25 °C and 170 rpm (Liu et al., 2008a). We found that lipase production is dependent on the time when olive oil is added to the medium. When olive oil is added to 6 h old culture with 0.4% (w/v) glucose, the highest lipase activity is achieved. Under the optimal conditions, over 8.02±0.24 U/ml of lipase is produced within 96 h when the cell growth enters mid-stationary phase at shake flask level. The results may imply that a large amount of lipase is produced only when the yeast strain is actively growing. The results obtained also demonstrate that the lipase yields are significantly increased when the time-dependent induction strategy is used. This is the first report on the enhancement of lipase production by yeast using the time-dependent induction strategy.

4.2. 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 extracelluar, 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 there 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 confimed 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; Kakugawa et al., 2001; Oishi et al., 1999). It also has 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 thermosensitivity. 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). However, 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 Hg^{2+}, Fe^{2+} and Zn^{2+} (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 Hg^{2+}, but are enhanced by Ca^{2+} and Mg^{2+}. At alkaline pH, extracellular phospholipase B from *Kluyveromyces lactis* requires Ca^{2+}, Fe^{3+} or Al^{3+} 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
Some properties of lipases from the marine and terrestrial yeasts

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Hydrolysis	Size of lipase gene	References
The marine-derived *A. pullulans* HN2.3	63.5 kDa	8.5, 35 °C	No metal ions tested	Hg^{2+}, Fe^{2+} and Zn^{2+},PMSF, iodoacetic acid	Prefers peanut oil and lard	1245 bp (one intron: 55 bp)	Liu et al., 2008a,b
Terrestrial yeasts	33–65 kDa	5.0–8.0, 50–70 °C	Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+}	Ag^{+}, Hg^{2+}, PMSF	All the lipid	1347–1494 bp (no intron)	Hasan et al. (2006)

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. lipolytic* 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 distantly related to other lipases. Ni et al. (2008a,b, 2009) also found that the alkaline protease sequence deduced from *ALP1* gene cloned from *A. pllulans* 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*LIP1*gene 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 hexakiphosphate), 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. castellii* (Pandey et al., 2001), *Schwanniomyces occidentalis* (Nakamura et al., 1999), *Pichia anomala* (Vohara and Satyanarayana, 2004), *A. adeninivorans* (Sano et al., 1999) and *Hansenula polymorph* (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, *Issatchenkia 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 (*Hexagrammes 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 bioresources in marine environments. Phytase-producing marine yeasts may be more suitable as maricultural feed additives than the added phytase from terrestrial microorganisms.

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 mU/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. ficuum* 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. adeninivorans* 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 phytate 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 in optimizing 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% (Segueilha et al., 1992) and the structure of the deglycosylated protein is tetrameric, 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 (Segueilha 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 (Segueilha 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 Mn^{2+}, Ca^{2+}, K^{+}, Li^{+}, Na^{+}, Ba^{2+}, Mg^{2+} and Co^{2+} (at a concentrations of 0.1 and 5.0 mM), but it is inhibited by Cu^{2+}, Hg^{2+}, Fe^{2+}, Fe^{3+}, Ag^{+}, and Zn^{2+} (at a concentration of 0.1 and 5.0 mM) (Li et al., 2008c). However, the phytase from *A. adeninivorans* is only slightly sensitive to low concentrations of various ions (Mg^{2+}, Ca^{2+}, and Zn^{2+}) (Sano et al., 1999) while the phytase from one strain of *A. niger* is inhibited by Cu^{2+}, Zn^{2+}, Hg^{2+}, Sn^{2+}, and Cd^{2+} ions and activated by Ca^{2+}, Mg^{2+}, and Mn^{2+} 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, iodoacetic acid (at a concentration of 1.0 mM), and phenylgloxal 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 (Kerovuo 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. castellii* CBS 2923 phytase and the *APHO1* gene encoding extracellular acid phosphatase from *A. adeninivorans* 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*-glycosylations and other processings 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. adeninivorans* 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. castellii* 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 (RHG*X*R*X* 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 (RHG*X*R*X* P) and Arg residue of RHG*X*R*X* 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. castellii* 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. castellii*. 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. castellii* 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 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, apartic acid protease and metellaprotease. 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 (Kurmar and Tagaki, 1999; Anwar and Saleemuddin, 1998). Proteases also contribute to the development of high-added applications or products by using the enzyme-aided digestion of proteins from different sources (Kurmar 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 (Kurmar and Tagaki, 1999). Terrestrial yeasts reported to produce alkaline proteases include *Candida lipolytica*, *Y. lipolytica* and

Table 5
Some properties of phytases from the marine yeast and terrestrial fungi

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Hydrolysis	Size of phytase gene	References
The marine-derived *K. ohmeri* BG3	92.9 kDa	5.0, 60 °C	Mn^{2+}, Ca^{2+}, K^{+}, Li^{+}, Na^{+}, Ba^{2+}, Mg^{2+} and Co^{2+}	Cu^{2+}, Hg^{2+}, Fe^{2+}, Fe^{3+}, Ag^{+}, Zn^{2+}, PMSF, iodoacetic acid, phenylgloxal hydrate	Phytate is partially hydrolyzed	1389 bp	Li et al. (2008a,b)
Terrestrial fungi	33–65 kDa	2.5–6.0, 50–70 °C	Unknown	Mg^{2+}, Ca^{2+}, and Zn^{2+} and phenylgloxal hydrate	Phytate is fully hydrolyzed	1383–1449 bp	Haefner et al. (2005)

A. pullulans (Tobe et al., 1976; Ogrydziak, 1993; Donaghy and McKay, 1993). Especially, among the extracellular enzymes of *Y. lipolytica*, alkaline protease can reach several grams per liter under optimized conditions (Barth and Gaillardin, 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 saltern of Yellow sea, China. Maximum production of enzyme (623.1 U/mg protein; 7.2 U/ml) is obtained in a medium containing 2.5 g soluble starch and 2.0 g $NaNO_3$, 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, 1991; 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, immunomodulatory 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 marine yeast 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 and antibacterial activities (Minervini et al., 2003). ACE-inhibitory peptides were also produced in fermented milks started by *Lactobacillus delbrueckii* subsp. bulgaricus SS1 and *L. lactis* subsp. *cremoris* FT4 (Gobbetti et al., 2000). A glutamic-acid-specific endopeptidase from *Bacillus subtilis* ATCC 6051 was applied in the recovery of bioactive peptides from fusion proteins (Okamoto et al., 1997). The prawn of *Penaeus japonicus* was hydrolyzed by various proteases, and antioxidant activity of the hydrolysates was examined. Among the digests, pepsin digest shows the most potent antioxidant activity (Suetsuna, 2000). Therefore, we found that the alkaline 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 chinersis*) treated with the crude protease from *Bacillus* sp. SM98011 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 (Kurmar and Tagaki, 1999). They also have high isoelectric points and are generally stable between pH 6 and 12 (Kurmar and Tagaki, 1999). The results about the alkaline protease from the marine yeast *A. pullulans* strain 10 are in agreement with those reported by other researchers (Kurmar and Tagaki, 1999) (Table 6).

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

Table 6
Some properties of alkaline proteases from the marine yeasts and terrestrial yeasts

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Hydrolysis	Size of alkaline protease genes	References
The marine-derived *A. pullulans* HN2–3	33.0 kDa	9.0, 52 °C	Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}	PMSF 1-10-phenanthroline, EDTA and iodoacetic acid	Production of bioactive peptides from marine proteins	1248 bp (two introns: 54 and 52 bp)	Ni et al. (2008a,b)
The marine-derived *A. pullulans* strain 10	32.0 kDa	9.0, 45 °C	Cu^{2+} and Mn^{2+}	Hg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Co^{2+}, PMSF, 1-10-phenanthroline, EDTA and iodoacetic acid	Production of bioactive peptides from marine proteins	1248 bp (two introns: 54 and 50 bp)	Ma et al. (2007)
Terrestrial yeasts	32 kDa	9–11, 50–70 °C	Unknown	PMSF	Unknown	1364 bp	Madzak et al. (2004)

from an alkalophilic *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 Cu^{2+} (at a concentration of 1.0 mM) and Mn^{2+} and inhibited by Hg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Co^{2+} (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, Cu^{2+} (at a concentration of 5.0 mM) inhibits the enzyme activity. However, alkaline proteases from bacteria require a divalent cation such as Ca^{2+}, Mg^{2+}, and Mn^{2+} 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 was found that Cu^{2+} (at a concentration of 1.0 mM) has an activating effect on the alkaline protease activity produced by *A. pullulans* strain 10. This demonstrates that some biochemical properties of the alkaline protease produced by *A. pullulans* strain 10 are greatly different from those of other alkaline proteases.

The purified alkaline protease is strongly inhibited by phenylmethylsulfonyl fluoride, but weakly inhibited by EDTA, 1-10-phenanthroline, and iodoacetic acid (Ma et al., 2007), demonstrating that the purified enzyme is metalloenzyme (Ma et al., 2007; Ramirez-Zavala et al., 2004). The results also show that Ser residues are essential for the enzyme active sites (George and Diwan, 1983) and Cys residues are important for active sites of the enzyme. However, it has been reported that thiol inhibitors have little effect on the alkaline proteases of *Bacillus* spp., although they do affect the alkaline enzymes produced by *Streptomyces* sp. (Kurmar and Tagaki, 1999). This again demonstrates that some biochemical properties of the alkaline protease produced by *A. pullulans* strain 10 are greatly different from those of other alkaline proteases (Table 6).

The K_m and V_{max} values of the purified alkaline protease from *A. pullulan* strain 10 for casein are 0.25 mg/ml and 0.0286 mmol/min/mg of protein, respectively (Ma et al., 2007). These results reveal that the alkaline protease from the marine yeast displays a high affinity for casein.

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 (*Trachypenaeus curvirostris*) muscle under the catalysis of the purified protease from *A. pullulans* strain 10 is the highest (85.3%) while antioxidant activity of the crude bioactive peptides from spirulina (*Arthospira platensis*) powder under the catalysis of the purified protease from *A. pullulans* strain 10 is the highest (54.6%). It was also found that all the crude bioactive peptides have no antimicrobial activity (Ma et al., 2007). Therefore, the alkaline protease from the marine yeast *A. pullulans* strain 10 also has potential uses in production of bioactive peptides from shrimp (*T. curvirostris*) muscle and spirulina (*A. platensis*) powder.

6.2.1. Characterization of the alkaline protease genes from A. pullulans

The alkaline protease structural gene (*ALP1* gene) from *A. pullulans* strain 10 has an open reading frame of 1248 bp. The gene contains two introns which have 54 bp and 50 bp, respectively (Table 6). The promoter of *ALP1* gene is located from −62 to −112 and has two CCAAT boxes and one TATA box (Ni et al., 2008b). The terminator of the *ALP1* gene contains the sequence with a hairpin structure (AAAAAGTT TGGTTTTT), meaning that transcription of the gene can be terminated effectively. In the promoter region, there is no element that can mediate transcription of the gene in *E. coli* so that the gene cannot be expressed in *E. coli* (Liiv et al., 2001).

The protein sequence deduced from the *ALP1* gene exhibits 55.24%, 50.35% and 31.68% identity with alkaline proteases from *A. fumigatus*, *Acremonium chrysogenum* and *Y. lipolytica*, respectively (Ni et al., 2008b). The amino acid sequence deduced from the cDNA*ALP1* gene cloned from cDNA of *A. pullulans* strain 10 is also closely related to that of *A. fumigatus* alkaline protease. As mentioned above, this may imply that *A. pullulans* is closely related to *Aspergillus* spp.

The calculated molecular mass of the protein deduced from cDNA*ALP1* 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 cDNA*ALP1* 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 propreprotein (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 pINA1317 and its recipient yeast strain *Y. lipolytica* Po1h have many advantages (Madzak 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 cDNA*ALP1* gene cloned from *A. pullulans* strain 10 was ligated into pINA1317 and expressed in the yeast strain Po1h. The results demonstrated that cDNA*ALP1* 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 (cDNA*ALP2* 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 protein. 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). Amylases, 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

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 cDNA*ALP2* 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* (Abdehl 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 reukaufii 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. reukaufii*. Although as mentioned above many terrestrial yeasts can produce acid protease, this is the first report that *M. reukaufii* 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. reukaufii* 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. reukaufii* W6b can yield such protease in natural sea sediment.

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

The gene named *SAP6* (accession number EU186020) was cloned from cDNA of *M. reukaufii* 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 encods 508 amino acid residues with an estimated molecular mass of 53.5 kDa and the pI of the deduced protein is 4.2 (Li et al., in press-a). In contrast, the secreted aspartic proteinase from *Glomerella*

cingulata (GcSAP) has a molecular mass of 36.0 kDa (Clark et al., 1997). The complete nucleotide sequence of a 2753-base-pair-long DNA fragment from *S. fibuligera* is essential for acid protease production in *S. cerevisiae* and the fragment contains an open reading frame of 1170 bp that encodes a 390-amino acid polypeptide (Hirata et al., 1988). However, the acid protease-encoding gene (*PepA*) of *A. oryzae* encodes 404 amino acid residues and contains three putative introns ranging in length from 50 to 61 nucleotides (Gomi et al., 1993). This may imply that the molecular mass of the acid protease produced by the marine yeast *M. reukaufii* 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 "VLLDTGSSDLRM" 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 1, *S. cerevisiae* GPI-anchored aspartyl protease and *S. cerevisiae* GPI-anchored aspartic protease 2. This suggest that the SAP6 is GPI-anchored aspartic protease in the marine yeast *M. reukaufii* 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. reukaufii* M6b is cell bound (Li et al., 2008a). The Kyte and Doolittle hydropathy profiles of the Cwp1 from *S. cerevisiae* and the SAP6 from *M. reukaufii* 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. reukaufii* 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. reukaufii* M6b may have many applications in biotechnology.

The *SAP6* gene obtained from the marine yeast strain *M. reukaufii* 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 recombinant 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 proteinase 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 rSAP6 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. reukaufii* W6b and the recombinant SAP6 was determined. It was found that the crude acid protease from the culture of the yeast strain *M. reukaufii* W6b and the recombinant SAP6 indeed have the skimmed milk coagulability. The results demonstrate that the cDNA*SAP6* gene amplified from the marine yeast strain *M. reufaukii* W6b indeed encodes the aspartic protease (Li et al., in press-b).

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. humicola* 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. humicola.*

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 Orydziak, 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 Mn^{2+} (5.0 mmol/l) tested has an activating effect on the rSAP6 from the engineered *E. coli* carrying the *SAP6* gene and Cu^{2+} and Ag^{+} act as inhibitors in decreasing the enzyme activity while Zn^{2+} and Mg^{2+} can weakly inhibit rSAP6 activity (Li et al., in press-a). In addition, Ca^{2+} 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^{2+} has an activating effect on the acid protease produced by *S. cerevisiae* (Anahit et al., 1993). Alessandro and Federico (1980) reported that Cu^{2+} 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; Cazetta et al., 2005). Ethanol is the most employed liquid biofuel either as a fuel or as a gasoline

Table 7
Some properties of acid proteases from the marine yeast and terrestrial yeasts

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Location	Size of acid protease gene	Skimmed milk coagulability	References
The marine-derived *M. reukaufii* W6b	54.0 kDa	3.4, 40 °C	Mn^{2+}	Cu^{2+}, Ag^{+} ,Zn^{2+} and Mg^{2+}, pepstatin	Cell-bound	1527 bp	Yes	Li et al. (2008a,b)
Terrestrial yeasts	36 kDa	2.5–3.9, 50 °C	Ca^{2+}	Cu^{2+} and Ag^{+}, pepstatin	Extracellular	1,170–1212 bp	Yes	Kocabiyik and Ozel (2007)

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 inulinases, 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), K_2HPO_4 0.3% (w/v), yeast extract 0.5% (w/v), KCl 0.5% (w/v), $CaCl_2$ 0.12% (w/v), NaCl 4.0% (w/v) and $MgCl_26H_2O$ 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 85.0 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 $MgCl_2{\cdot}6H_2O$ 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% $MgCl_2 \cdot 6H_2O$ in the artificial seawater. However, cell growth is best in the presence of 3.0% NaCl and 0.6% $MgCl_2{\cdot}6H_2O$ 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 (61.5 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 (Guo et al., in press). Under the optimized conditions, 127.7 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 48.1 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% (v/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 inulinases 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/gram of dry substrate (gds) 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/gds is derived from RSM regression. Under the same conditions, its parent strain only produces 291.0 U/gds 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

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 exoinulinase 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 inulinases 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 inulinases 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 inulinases 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 Ca^{2+} K^{+}, Na^{+}, Fe^{2+} and Cu^{2+}, respectively. However, Mg^{2+}, Hg^{2+}, and Ag^{+} (at concentrations of 1.0 mM) acts as inhibitors in decreasing the activity of the two purified inulinases, 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 inulinases from the two marine yeasts have some common properties. However, the inulinase from *Penicillium* sp is inhibited by Ag^{2+} and Cu^{2+} (Pandey et al., 1999) whereas the inulinases from *Aspergillus* sp. are inhibited by Fe^{3+}, Mn^{2+}, and Mg^{2+} (Pandey et al., 1999). Fe^{2+} increases the activity of the recombinant exoinulinase from *K. marxianus* expressed in *P. pastoris* by 41.97%, but Mg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, and Fe^{3+} inhibits the activity of the recombinant exoinulinase from *K. marxianus* expressed in *P. pastoris* dramatically (Zhang et al., 2005). It was found that Mg^{2+} does not affect the activity of the purified extracellular inulinase from the yeast *K. marxianus* var. bulgaricus, but Ca^{2+} inhibits the enzyme by approximately 27%, Ba^{2+}, Zn^{2+}, 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 inulinases from the marine yeast strains are different from those from terrestrial yeasts and the unique characteristics may have many potential applications.

The purified inulinases 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 inulinases are metalloenzyme and Ser and Cys residues of the enzyme are essential for the enzymes active sites. This again demonstrates that the inulinases from the two marine yeasts have some common properties.

The K_m and V_{max} 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 K_m and V_{max} 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 K_m value of 11.9 and 3.92 mM for sucrose and inulin, respectively (Pandey et al., 1999), whereas the apparent K_m 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 inulinases 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 inulinases 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 inulinases 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 inulinases 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 inulinase 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 inulinase genes from *A. niger* AF10, *K. marxianus*, *K. marxianus* var. *marxianus* ATCC 12424 and *K. cicerisporus* CBS4857 are 1551 bp (encoding 516 amino acids), 1670 pb, 1667 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 *INU1* gene cloned from the marine yeast *P. guilliermondii* strain 1 is similar to that of the inulinase gene from *A. niger* AF10 and smaller than that of inulinase genes from other yeasts.

The predicted protein from the *INU1* 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 *P. guilliermondii* strain 1 was estimated to be 50.0 kDa (Gong et al., 2008) while the molecular mass of the purified inulinase from *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 *INU1* gene cloned is close to that of the molecular mass of the purified inulinase from *P. guilliermondii* strain 1 as mentioned above. It has been reported that the extracellular inulinase from the terrestrial yeast, *K. fragilis* has 250 kDa of molecular mass while the apparent molecular mass of exoinulinase from *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 *K. marxianus* var. *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 little higher molecular mass than that from the marine yeast *P. guilliermondii* strain 1.

The inulinase protein sequence deduced from both the cloned inulinase gene in *P. guilliermondii* strain 1 and that in *C. aureus* G7a contains the consensus motifs R-D-P-K-V-F-W-H and W-M-N-D-P-N-G 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 *P. guilliermondii* strain 1 can be expressed in *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 *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 *P. pastoris* X-33. This is due to that the recombinant inulinase produced by *P. pastoris* X-33 is 6×His tag fusion protein. Another reason is that the protein modification difference between in *P. guilliermondii* strain 1 and in *P. pastoris* X-33 may occur as there were ten potential N-linked glycosylation sites of the protein deduced from the *INU1* gene cloned from *P. guilliermondii* strain 1.

It was found that like the crude and purified inulinases produced by *P. guilliermondii* strain 1, the crude recombinant inulinase produced by *P. pastoris* X-33 carrying the *INU1* gene has exo-inulinase activity (Zhang et al., 2009). This means that the action mode of the recombinant inulinase produced by *P. pastoris* X-33 carrying the *INU1* gene was little different from that of the purified inulinase and crude inulinase from *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 maricultural 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 *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 *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 *Torulopsis mogii* is the pathogen to some shrimp in China (Sun and Sun, 1998). The yeast *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 *Kluyveromycs penaeid* was isolated from the heart tissue of subadult shrimp *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 Breinig, 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 *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Ustilago*, *Torulopsis*, *Williopsis* and *Zygosaccharomyces*, indicating that the killer phenomenon is indeed widespread among yeasts (Magliani et al., 2004). Killer determinants are either

Table 8
Some properties of inulinases from the marine yeasts and terrestrial yeasts

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Location	Size of inulinase gene	Type of inulinase	References
The marine-derived *C. aureus* G7a	60.0 kDa	5.0, 50 °C	Ca^{2+} K^{+}, Na^{+}, Fe^{2+} and Cu^{2+}	Mg^{2+}, Hg^{2+}, Ag^{+}, PMSF, iodoacetic acid, EDTA, and 1,10-phenanthroline	Extracellular	1557 bp	Exoinulinase	(Sheng et al., 2008; Zhang et al., 2009)
The marine-derived *P. guilliermondii* strain 1	50.0 kDa	6.0, 60 °C	Ca^{2+} K^{+}, Na^{+}, Fe^{2+} and Cu^{2+}	Mg^{2+}, Hg^{2+}, Ag^{+} , PMSF, iodoacetic acid, EDTA, and 1,10-phenanthroline	Extracellular	1542 bp	Exoinulinase	(Gong et al., 2008; Zhang et al., 2009)
Terrestrial yeasts	57–250 kDa	4.5–6.0, 30–55 °C	Fe^{2+}	Ba^{2+}, Na^{+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, and Fe^{3+}	Extracellular	1551–1670 pb,	Exoinulinase	Chi et al. (2009)

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 schenkii* (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 belongs 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 Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, Na^{+}, and Co^{2+} (at the concentrations of 5.0 mM). However, Fe^{2+}, Fe^{3+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, and Ag^{+} (at the concentrations of 5.0 mM) acts as inhibitors in decreasing activity of the toxin (Wang et al., 2007c). The inhibition by mercuric ions may indicate the importance of thiol-containing amino acid residues in the killer toxin function (Barth and Gaillardin, 1997). However, all the ions at higher concentrations (more than 5.0 mM) inhibit the killer toxin activity. The killing effect of the killer toxin from *P. membranifaciens* CYC 1106 is also enhanced with increased NaCl or KCl concentrations (Santos et al., 2000). However, our results are different from those reported by Izgu et al. (Izgu et al., 2005).

The toxin from the marine killer yeast strain YF07b is strongly inhibited by PMSF, iodoacetic acid, EDTA, and 1,10-phenanthroline (Wang et al., 2007c). However, EDTA has no effects on activity of the killer toxin from *P. anomala* 434 K5 type (Izgu et al., 2005).

The K_m of the purified toxin from the marine killer yeast strain YF07b for laminarin is 1.17 g/l. The purified toxin also actively hydrolyzes laminarin and kills the whole cells of the pathogenic yeast in crab (Wang et al., 2007c). The apparent K_m of the toxin from *P. anomala* NCYC 432 and *P. anomala* NCYC 434 K5 type is 0.3 g/l and 0.25 g/l, respectively (Izgu et al., 2005; Izgu et al., 2006). This suggests that the killer toxin from the marine yeast YF07b displays lower affinity for laminarin than that from *P. anomala*.

Only monosaccharides and disaccharides are released from laminarin after hydrolysis for 1 h with the purified killer toxin from the marine killer yeast strain YF07b (Wang et al., 2007c). The results imply that the killer toxin has very high exo-β-1, 3-glucanase activity.

The gene encoding the killer toxin from the marine killer yeast YF07b is 1589 bp long which includes an ORF from 209 bp to 1492 bp (Wang et al., 2007d). The deduced protein from the gene has 427 amino acids which contains signal peptide of 17 amino acids appeared in the N-terminal domain of the kilter toxin. Therefore, the mature protein consists of 410 amino acids, its molecular mass was estimated to be 47.4 kDa and its isoelectronic point is 4.5. The sequence (accession number: EF029071) of the deduced amino acids has 99% match with that of the mature exo-β-1, 3 glucanase (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^{+}, Mg^{2+} and Mg^{2+} 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

Table 9
Some properties of killer toxins from the marine yeast and terrestrial yeasts

Producers	Molecular mass	Optimal pH and temperature	Activated by	Inhibited by	Location	Size of killer toxin gene	Enzyme activity	References
The marine-derived *P. anomala* YF07b	47.0 kDa	4.5, 40 °C	Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, Na^{+}, and Co^{2+}	Fe^{2+}, Fe^{3+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, and Ag^{+} PMSF, iodoacetic acid, EDTA, and 1,10-phenanthroline	Extracellular	1589 bp	β-1,3-Glucanase	Wang et al., 2007
Terrestrial yeasts	47–83.3 kDa	2.5–4.0, 20.6–37 °C	Na^{+} ,K+	Unknown	Extracellular	1589 pb	β-1,3-Glucanase	Izgu et al., 2005

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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