J. Ocean Univ. China (Oceanic and Coastal Sea Research)
DOI 10.1007/s11802-010-1765-2
ISSN 1672-5182, 2010 9 (4): 359-364
http://www.ouc.edu.cn/xbywb/
E-mail:xbywb@ouc.edu.cn

Optimum Production and Characterization of an Acid Protease from Marine Yeast *Metschnikowia reukaufii* W6b

LI Jing, PENG Ying, WANG Xianghong, and CHI Zhenming*

UNESCO Chinese Center of Marine Biotechnology, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

(Received April 29, 2010; revised August 6, 2010; accepted August 27, 2010)

Abstract The marine yeast strain W6b isolated from sediment of the South China Sea was found to produce a cell-bound acid protease. The crude acid protease produced by this marine yeast showed the highest activity at pH 3.5 and 40 ℃. The optimal pH and temperature for the crude acid protease were in agreement with those for acid protease produced by the terrestrial yeasts. The optimal medium of the acid protease production was seawater containing 1.0% glucose, 1.5% casein, and 0.5% yeast extract, and the optimal cultivation conditions of the acid protease production were pH 4.0, a temperature of 25 ℃ and a shaking speed of 140 $rmin^{-1}$. Under the optimal conditions, 72.5 UmL^{-1} of acid protease activity could be obtained in cell suspension within 48 h of fermentation at shake flask level. The acid protease production was induced by high-molecular-weight nitrogen sources and repressed by low-molecular-weight nitrogen sources. Skimmed-milk-clotting test showed that the crude acid protease from the cell suspension of the yeast W6b had high skimmed milk coagulability. The acid protease produced by *M. reukaufii* W6b may have highly potential applications in cheese, food and fermentation industries.

Key words acid protease; marine yeasts; *Metschnikowia reukaufii*; milk coagulability

1 Introduction

Proteases are the most important group of enzymes produced commercially so far and are used in the detergent, protein, food, brewing, photographic, collagenous fiber leather and dairy industries (Kalisz, 1988). All proteinases catalyze the hydrolysis of peptide bonds (CO-NH) in proteins but can differ markedly in specificity and mechanism of action (Barrett and Rawlings, 1991). Acid proteases, commonly known as aspartic proteases including pepsin, chymosin, renin, cathepsin D and related microbial enzymes such as endothiapepsin and penicillopepsin, with a pH optimum in the acidic range (pH3-4), have been reported in a variety of microorganisms as both 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 (Davies, 1990).

The enzymes from fungi and yeasts have been studied most extensively, and several of them have been purified and cloned (Kocabiyik and Ozel, 2007). For example, acid protease from *Rhodotorula glutinis* K-24 (Kamada *et al.*, 1972) has been purified and characterized in some detail. *Saccharomyces carlsbergensis* has been reported to secrete four different proteases, each of which has a double pH optimum: one acidic between pH 2 and 4 and the other near neutrality between pH 6 and 8 (Maddox and Hough, 1970). Abdelal *et al.* (1977) reported that *Yarrowia lipolytica* 37–1 produced extracellular acid protease (s) active at pH 3.2 when grown with casein at pH 3.2. A psychrotrophic, dimorphic yeast *Candida humicola*, isolated from Antarctic soil, has been reported to secrete an acidic protease with a pH optimum (pH 1.0) which is much lower than that of the acidic protease from other yeasts (Ray *et al.*, 1992). *Candida olea* (Nelson and Young, 1987) was also reported to secrete aspartic protease.

Some acid proteases 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 environments. For example, it may be used to remove proteins in shrimp shell to obtain chitin and chitosan.

The oceans cover 71% of our planet, and there are

* Corresponding author. Tel: 0086-532-82032266
E-mail: zhenming@sdu.edu.cn

abundant biotic resources including marine yeasts (Chi *et al.*, 2006). In recent years, marine yeasts and their bioactive substances have received increasing attention. However, few studies exist on the acid protease and its production from marine yeasts (Chi *et al.*, 2006). After we screened 427 marine yeast strains from different marine environments, one marine yeast strain W6b, isolated from sediment of the South China Sea, was found to produce a cell-bound acid protease. To extend knowledge of the acid protease from the strain, W6b, the *SAP6* gene encoding the acid protease has been cloned and expressed in *E. coli* (Li *et al.*, 2009); thereafter, the recombinant acid protease rSAP6 has been purified and characterized (Li *et al.*, 2008). The main purpose of the present study was to optimize the conditions for acid protease production by the marine yeast strain W6b. We also tested the milk-clotting ability of the crude acid protease produced by this marine yeast. To our knowledge, this is the first report that *Metschnikowia reukaufii* from the marine environment could produce cell-bound acid protease.

2 Materials and Methods

2.1 Yeast Strain

The yeast strain W6b was isolated from sediment in the South China Sea. This yeast strain was then maintained in YPD medium (prepared with seawater) containing 2.0% glucose, 2.0% yeast extract and 1.0% polypeptone at 4 ℃.

2.2 Acid Protease Production

One loop of the cells of the purified yeast strain was transferred to 50 mL of YPD medium in 250 mL flask and aerobically cultivated for about 24 h. In order to determine the optimal medium and cultivation conditions of the acid protease production by the yeast strain, on the basis of YPD medium (prepared with seawater), different amounts of cell culture (2.5×10^7–2.5×10^8) obtained above were transferred to 50.0 mL of the medium in 250 mL flask which contained different carbon (0.3%–2.0%) and nitrogen sources (0.5%–2.5%) and yeast extract (0.2%–1.5%), and the yeast strain was grown at different initial pHs (2.5–8.0) and temperatures (22–35 ℃), and grown by shaking at different shaking speeds (120–200 $rmin^{-1}$) for 2 d.

2.3 Determination of Acid Protease Activity

The crude acid protease activity was determined according to the methods described by Larson and Whitaker (1970). The substrate contained 1.0% bovine hemoglobin (Sigma, USA) dissolved in citrate buffer (20 $mmolL^{-1}$, pH 3.4). The supernatant of cell suspension (0.4 mL) and the substrate (0.4 mL) were combined and incubated for 1 h at 40°C. The reaction was stopped by addition of 0.4 mL of 15.0% trichloroacetic acid and cooled in the ice for 20 min and the precipitate was removed by centrifugation (12000 ×*g*) for 20 min and the absorbance of the supernatant was read at 280 nm. The reaction mixture to which 0.4 mL of 15.0% trichloroacetic acid was added before the substrate (contained 1.0% bovine hemoglobin) was used as the control. One acid protease unit (U) was defined as the amount of protease causing an increase in absorbance of 0.1 at 280 nm after 1 h. All the assays were done in duplicate.

2.4 Determination of Cell Growth

Cell dry weight was used to represent cell growth. The yeast cells from 5.0 mL of culture were harvested and washed three times with distilled water by centrifugation at (5000 × *g*) for 5 min. Then, the cells in the tube were dried at 100 ℃ until the cell dry weight was constant (Chi *et al.*, 2007).

2.5 Effects of Temperature and pH on Acid Protease Activity

The optimal temperature of enzyme activity was determined at temperatures of 30, 35, 40, 45, 50 and 55℃. The effect of pH on the enzyme activity was determined by incubating the cell suspension at different pHs between 2.0 and 8.0. The buffers used were of the 20 mM McIlvaine buffer category.

2.6 Skim Milk Clotting Test

The milk-clotting ability was assayed by the method of Berridge (Berridge, 1952). A total of 10 g of skimmed milk powder was suspended in 100 mL of 0.05 mol L^{-1} $CaCl_2$ by stirring with a glass rod. The pH of the milk substrate was adjusted to 5.8 with 0.1 mol L^{-1} NaOH or HCl. A volume of 0.5 mL of the cell suspension of the marine yeast *M. reukaufii* W6b was added to 4.0 mL of the pre-incubated skimmed milk for complete coagulation at 50 ℃. A volume of 0.5 mL of water and 0.5 mL of the cell suspension boiled in 100 ℃ water for 10 min were used as negative controls.

3 Results and Discussion

3.1 Screening the Acid Protease-Producing Yeasts

After 427 yeast strains from seawater, sediments, guts of marine animals and marine algae were screened, only one strain W6b isolated from sediment in the South China Sea was found to produce acid protease. The activity of the acid protease in the cell suspension was 15.7 UmL^{-1} ± 1.1 UmL^{-1} while low acid protease activity in the supernatant was detected (data not shown).It was indicated that the acid protease produced by this marine yeast W6b was cell-bound enzyme. The marine yeast strain W6b was identified as a strain of *Metschnikowia reukaufii* based on the phenotypic characteristics and phylogenetic analyses (data not shown). Some other hydrolytic enzymes were also found to be cell-bound enzymes. For example, lipase from *Yarrowia lipolytica* (Pignede *et al.*, 2000.) and *Candida parapsilosis* CBS604 (Neugnot *et al.*, 2002) was the cell-bound enzyme. Three aspartic proteases *i.e.* Yapsin,

Yapsin2 and Yapsin3 (Cawley and Loh, 1998; Olsen *et al.*, 1999) were reported to be cell-bound enzymes from *Saccharomyces cerevisiae*. To our knowledge, this is the first report about cell-bound acid protease from the genus *Metschnikowia.*

3.2 Effects of Different Initial pHs and Temperature on Acid Protease Production and Cell Growth

Effects of different initial pH in the medium prepared with seawater on the acid protease production by the marine yeast W6b were tested. Fig.1a showed that the optimum initial pH of the medium for the acid protease production was 4.0 while the cells of the marine yeast strain grew well between pH 3.5-7.0. The results also showed that when initial pH was higher than 5.0, the acid protease activity decreased sharply, indicating that the acid protease produced by this marine yeast strain was very sensitive to the change in initial pH (Fig.1a). It was reported that a large amount of alkaline protease can be secreted under neutral condition and three acid proteases were secreted under acid condition by *Y. lipolytica* (Gerold and Gaillardin, 1997). *S. lipolytica* could secret at least three acid proteases when grown at pH 3.4 in medium containing glycerol and Difco peptone. However, when its cells grew under the condition of pH 7.0, neutral protease was secreted (Ogrydziak and Mortlmer, 1977). This suggested that the effect of initial pH on the acid protease production of the marine yeast W6b was consistent with the case of the terrestrial yeasts, although *M. reukaufii* W6b was isolated in a slight alkline marine environment with pH of sea sediment being around 8.0. However, it is completely unknown if *M. reukaufii* W6b can yield such protease in natural sea sediment.

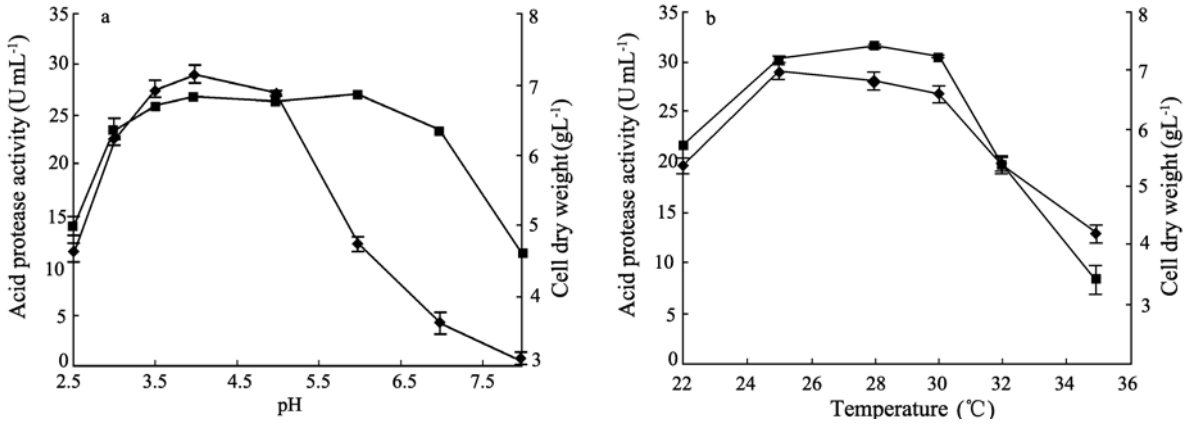

Fig.1 Effects of initial pHs and temperature on acid protease production (◆) and cell growth (■). Data are given as means ± SD, n=3.

The effects of different temperatures on the acid protease production and cell growth by strain W6b were studied. Fig.1b shows that the maximum acid protease production (29.3 UmL^{-1}) was observed at 25 ℃ and thereafter a sharp decrease in enzyme activity was seen at higher temperatures. A similar decrease in cell growth is shown in Fig.1b. *Candida parapsilosis* CHUV E-18 was reported to secret two acid proteases when grown at 30℃. Both enzymes had an apparent pH optimium close to PH 3.5 (Fusek *et al.*, 1993). *Saccharomycopsis lipolytica* CX161-1B was reported to produce at least three extracellular acid proteases during exponential growth in medium containing glycerol, Difco Proteose Peptone at pH 3.4 and 23℃ (Yamada and Ogrydziak, 1983). Few researches were concerned with the optimization of culture temperatures for the acid protease secretion by yeasts. Possibly, a lower optimal temperature for the acid protease production by the yeast strain used in this study may be related to the marine environment where it was isolated.

3.3 Effects of Different Carbon Sources on Acid Protease Production and Cell Growth

Many researches showed that different carbon sources have significant influences on the production of extracellular enzymes and the growth of different strains (Chi and Zhao, 2003). As shown in Fig.2, the acid protease production by the marine yeast strain W6b was influenced by different carbon sources in the medium. The highest amount of acid protease was produced in the medium containing glucose. However, the different carbon sources had no notable influences on cell growth of the marine

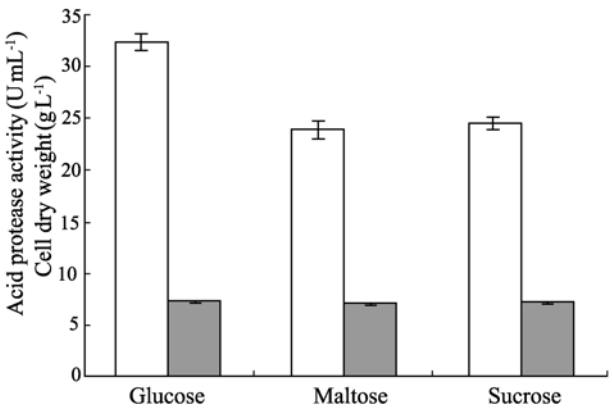

Fig.2 Effects of different carbon sources on acid protease production (□) and cell growth (■). Data are given as means ± SD, n=3.

yeast strain (Fig.2). Acid protease activity decreased sharply whereas the growth of the yeast strain W6b increased steadily when the concentrations of glucose in the medium were higher than 1.0% (Fig.3). When the concentration of glucose was increased from 0.3 to 1.0%, acid protease activity was increased from 26.9 to 53.2 $U mL^{-1}$. Therefore, the optimal concentration of glucose for the acid protease production was found to be 1.0 % (w/v). However, it was reported that a change in the concentration of glucose (*e.g.*, from 0.1 to 1%) did not alter the level of acid protease from *Candida humicola* in the medium (Ray *et al.*, 1992). The concentration of glucose had an obvious influence on the acid protease production of marine yeast strain W6b in our study.

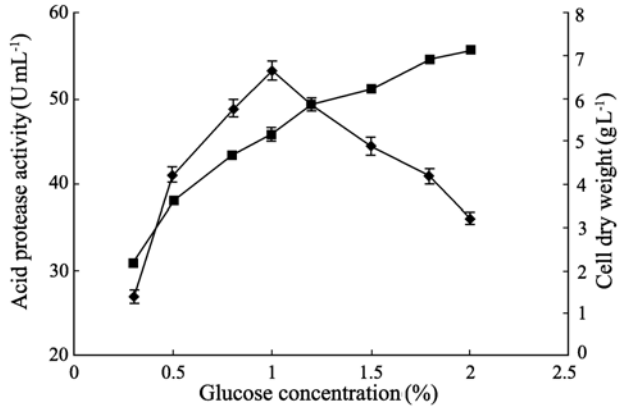

Fig.3 Effects of different glucose concentrations on acid protease production (◆) and cell growth (■). Data are given as means ± SD, n=3.

3.4 Effects of Different Nitrogen Sources on Acid Protease Production and Cell Growth

The effects of various nitrogen sources on the acid protease production by the yeast strain were investigated. The results in Fig.4 show that the yeast grew well with most nitrogen sources tested except with urea. Among all the nitrogen sources tested, casein (2%) increased the acid protease production (64.4 $U mL^{-1}$) most markedly, and bovine hemoglobin and bovine serum albumin were the second and third best nitrogen source (61.7 $U mL^{-1}$ and 60.9 $U mL^{-1}$). Other nitrogen sources including casamino acid, $(NH_4)_2SO_4$, NH_4Cl and urea decreased the enzyme production apparently while they could promoted the cell growth. It can be seen clearly from Fig.4 that acid protease production by marine yeast W6b was induced by higher-molecular-weight nitrogen sources, while repressed by low-molecular-weight nitrogen sources. Similar observations have been reported for *Candida albicans* (Renold *et al.*, 1968) and *Candida humicola* (Ray *et al.*, 1992).

In order to determine the optimum concentration of casein for the acid protease production, different concentrations (0.5%–2.5%) of casein were tested in the medium. A concentration of 1.5% of casein was found to be optimal for the acid protease production. A concentration of 0.5% of yeast extract was also found to be optimal for acid protease production (data not shown). The vitamins and trace elements in the yeast extract may enhance acid protease production by this marine yeast strain.

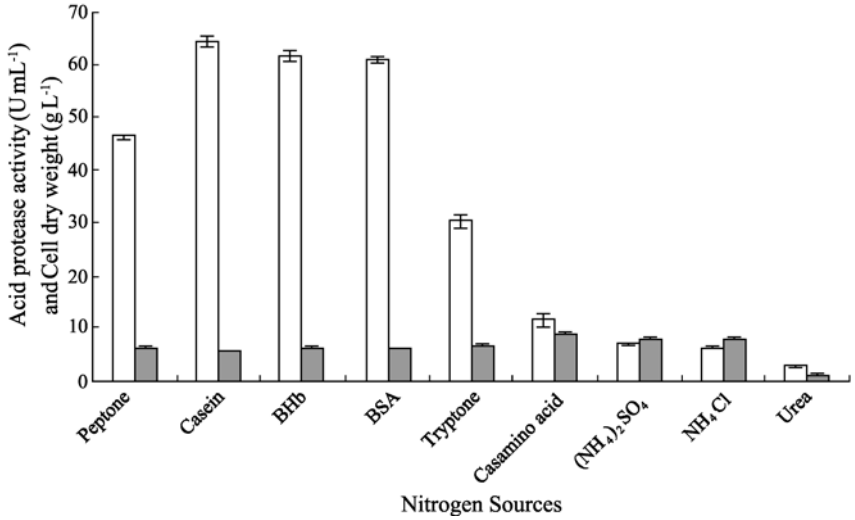

Fig.4 Effects of different nitrogen sources on acid protease production (◆) and cell growth (■). Data are given as means ± SD, n=3.

3.5 Time Courses of Acid Protease Production and Cell Growth at Shake Flask Level

All the data above have shown that the optimal medium for the acid protease production was the seawater containing glucose 1.0%, casein 1.5%, and yeast extract 0.5%, while the optimal cultivation conditions for the acid protease production were pH 4.0, at a temperature of 25 ℃ and a shaking speed of 140 r min^{-1}. And 7.5×10^7 cells $(50 mL)^{-1}$ was the best inoculation size (data not shown). Therefore, the time course of acid protease production and cell growth of the yeast strain during the cultivation was checked under these conditions. The results in Fig.5 indicate that the production of acid protease was paralleled with the cell growth. The results showed that maximum acid protease activity (72.5 $U mL^{-1}$) could

be reached in the cell suspension when cell growth was at the middle stationary phase. It is also observed in Fig.5 that acid protease activity in the supernatant could be detected after about 32 h of the cultivation and kept increasing thereafter while the cell- bound acid protease activity began to decrease after 48 h of the cultivation.

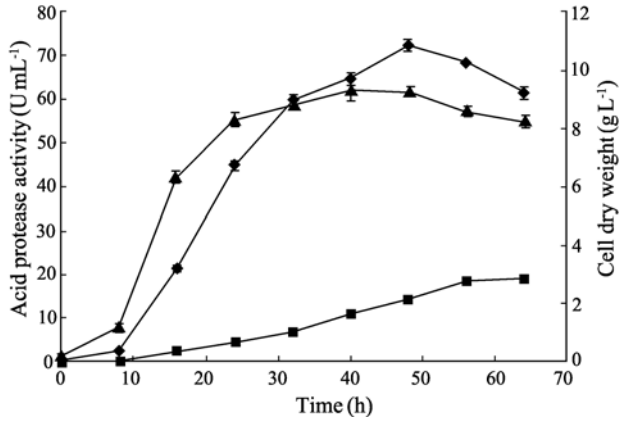

Fig.5 Time course of acid protease production and cell growth by the marine yeast. ◆: Acid protease activity in cell suspension; ■: Acid protease activity in supernatant; ▲: Cell growth data are given as means ± SD, n=3.

3.6 Effects of Temperature and pH on Activity of the Crude Acid Protease

The results in Fig.6 show that the optimal temperature and pH of the crude acid protease were 40 ℃ and 3.5, respectively. The optimum activity against hemoglobin of the acid protease from *Candida olea* 148 was at 42℃ and pH 3.3, which were in agreement with those of the acid protease from the marine yeast W6b. However, it was reported that an optimum temperature for the acid protease acted on casein from *Rhodotorula glutinis* K-24 (Kamada *et al.*, 1972) was 60℃. Generally, the optimum pH for acid protease secreted by yeasts was between 2.5 to 3.9. For example, the optimal pHs for the three extracellular acid proteases secreted by *Saccharomycopsis lipolytica* CX161-1B were 3.5, 4.2, 3.1 (Yamada and Ogrydziak, 1983) respectively, and the optimum pH for the acid proteases produced by *Candida albicans* was 3.2 (Renold *et al.*, 1968). It means that the optimum pH for the crude acid protease produced by the marine yeast strain W6b was in agreement with that of the acid protease produced by the terrestrial yeasts.

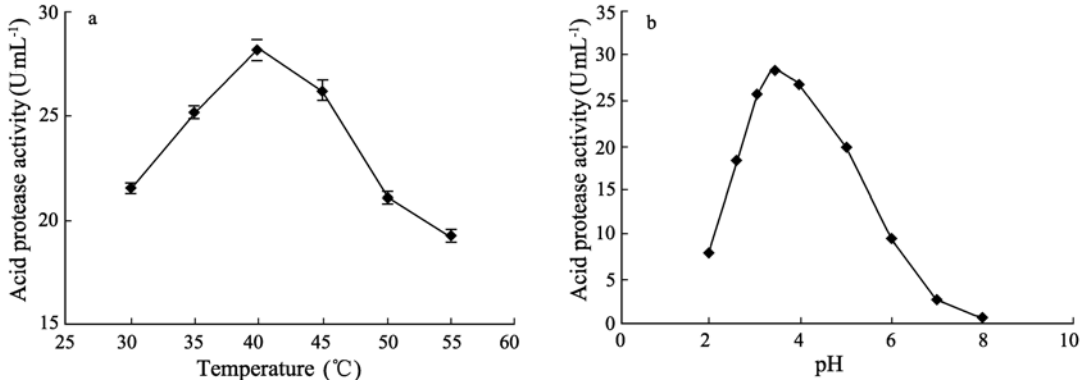

Fig.6 Effects of different temperatures (a) and pHs (b) on activity of the crude acid protease. Data are given as means ± SD, n=3.

3.7 Skimmed-Milk-Clotting Test of the Crude Cell-Bound Acid Protease

The milk-clotting activity is a common characteristic of all the aspartic proteases (Kumar *et al.*, 2005). So the milk-clotting activity of the crude acid protease produced by the marine yeast strain *M. reukaufii* W6b was determined. It can be clearly seen from the results in Fig.7 that there was high skimmed milk coagulability of the crude cell-bound acid protease from the cell suspension of the yeast W6b (Fig.7c), while no such coagulability was observed on the addition of water (Fig.7a) or the inactivated crude cell-bound acid protease (Fig.7b). This result suggested a milk-clotting activity associated with the acid protease of marine yeast W6b. In recent years, milk-clotting enzymes from fungi or bacteria to be used as calf rennet substitutes to make cheese have received increasing attention due to the reduced supply, consumer constraints on the use of rennet and increased price of calf rennet (Shieh *et al.*, 2009). Therefore, the acid protease produced by *M. reukaufii* W6b may have highly potential applications in cheese, food and fermentation industries.

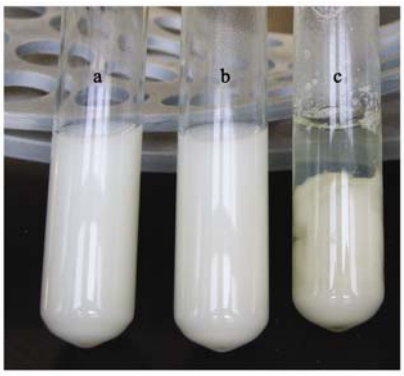

Fig.7 Milk-clotting test of the crude acid protease produced by the marine yeast strain *M. reukaufii* W6b. a. Control: 4.0 mL milk + 0.5 mL of distilled water. b. Control: 4.0 mL milk + 0.5 mL of the inactivated crude cell-bound enzyme. c. Sample: 4.0 mL milk + 0.5 mL of the crude cell-bound enzyme.

4 Conclusions

The marine yeast strain *Metschnikowia reukaufii* W6b isolated from sediment of the South China Sea was found to secrete a cell-bound acid protease in the medium with casein. The crude acid protease produced by marine yeast W6b showed the highest activity at pH 3.4 and 40℃. The optimal pH and temperature for the crude acid protease were in agreement with those for acid protease produced by the terrestrial yeasts. Our experiments also showed the optimal medium for acid protease production was seawater containing glucose 1.0%, casein 1.5%, and yeast extract 0.5%, while the optimal cultivation conditions for acid protease production were pH 4.0, a temperature of 25 ℃ and a shaking speed of 140 r min^{-1}. Under the optimal conditions, over 72.5 U mL^{-1} of cell-bound acid protease activity was produced within 48 h of the fermentation at shake flask level.

To our knowledge, this is the first report about the production of cell-bound acid protease from marine yeasts. More researches such as its physiological role in the yeast cells will be further investigated.

Acknowledgements

This research was supported by the National High Technology Research and Development Program of China (2006AA09Z403) and the National Natural Science Foundation of China (30771645).

References

Abdelal, A. T. H., Kennedy, E. H., and Ahearn, D. G., 1977. Purification and characterization of a neutral protease from *Saccharomycopsis lipolytica*. *J. Bacteriol.*, **130** (3): 1125- 1129.

Barrett, A. J., and Rawlings, N. D., 1991. *Types and Families of endopeptidases*. Trans-Biochem-Soc. Portland Press, London, **19** (3): 707-715.

Berridge, N. J., 1952. Some observation on the determination of the activity of rennet. *Analyst* (*Lond*), **77**: 57-62.

Cawley, N. X., and Loh, Y. P., 1998. In Handbook of Proteolytic Enzymes (Barrett AJ, Rawlings ND, Woessner JF, Eds.). Academic Press, London, 905-907.

Chi, Z. M., Liu, Z. Q., Gao, L. M., Gong, F., Ma, C. L., Wang, X. H., and Li, H. F., 2006. Marine yeasts and their applications in mariculture. *J. Ocean. Univ. China.*, **5** (3): 251-256.

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

Chi, Z. M., and Zhao, S. Z., 2003. Optimization of medium and cultivation conditions for pullulan production by a new pullulan-producing yeast strain. *Enzyme Microb. Technol.*, **33**: 206-211.

Davies, D. R., 1990. The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Chem.*, **19**: 189-215.

Fusek, M., Smith, E. A., Monod, M., and Foundling, S. I., 1993. *Candida parapsilosis* expresses and secretes two aspartic proteinases. *FEBS Lett.*, **327** (1): 108-112.

Gerold, B., and Gaillardin, C., 1997. Physiology and genetic of the dimorphic fungus *Yarrowia lipolytica. FEMS Microbiol. Rev.*, **19** (4): 219-237.

Kalisz, H. M., 1988. Microbial proteinases. *Adv. Biochem. Eng. Biotechnol.*, **36**: 1-65.

Kamada, M., Oda, K., and Murao, S., 1972. The purification of the extracellular acid protease of *Rhodotorula glutinis* K-24 and its general properties. *Agric. Biol. Chem.*, **36** (7): 1095-1101.

Kitano, H., Kataoka, K., and Furukawa, K., 2002. Specific expression and temperature-dependent expression of the acid protease-encoding gene (*PepA*) in *Aspergilhs oryzae* in solid-state culture (rice-koji). *J. Biosci. Bioeng.*, **93**: 563- 567.

Kocabiyik, S., and Ozel, H., 2007. An extracellular-pepstatin in sensitive acid protease produced by *Thermoplasma volcanium*. *Bioresour. Technol.*, **98** (1): 112-117.

Kumar, S., Sharma, N. S., Saharan, M. R., and Singh, R., 2005. Extracellular acid proteasefrom *Rhizopus oryzae*: purification and characterization. *Proc. Biochem.*, **40** (5): 1701-1705.

Larson, M. K., and Whitaker, J. R., 1970. *Endothia parasitica* protease. Parameters affecting activity of the rennin-like enzyme. *J. Dairy Sci.*, **53** (3): 253-261.

Li, J., Chi, Z. M., Liu, Z. Q., Yue, L. X., Peng, Y., and Wang, L., 2009. Cloning and characterization of a novel aspartic protease gene from marine-derived *Metschnikowia reukaufii* and its expression in *E. coli*. *Appl. Biochem. Biotechnol.*, **159** (1): 119-132.

Li, J., Chi, Z. M., and Wang, X. H., 2008. Cloning of the *SAP6* gene of *Metschnikowia reukaufii* and its heterologous expression and characterization in *Escherichia coli. Microbiol. Res.*, **165** (3): 173-182.

Maddox, I. S., and Hough, J. S., 1970. Proteolytic enzymes of *Saccharomyces carlsbergensis*. *Biochem. J.*, **117** (5): 843-852.

Nelson, G., and Young, T. W., 1987. Extracellular acid and alkaline proteases from *Candida olea*. *J. Gen. Microbiol.*, **133** (6): 1461-1469.

Neugnot, V., Moulin., G., Dubreng., E., and Bigey, F., 2002. The lipase/acyltransferase from *Candida parapsilosis*: Molecular cloning and characterization of purified recombinant enzymes. *Eur. J. Biochem.*, **269** (6): 1734-1745.

Ogrydziak, D. M., and Mortlmer, R. K., 1977. Genetics of extracellular protease production in *Saccharomycopsis lipolytica*. *Genetics*, **87** (4): 621-632.

Olsen, V., Cawley, N. X., Brandt, J., Egel-Mitani, M., and Loh, Y. P., 1999. Identification and characterization of *Saccharomyces cerevisiae* yapsin 3, a new member of the yapsin family of aspartic proteases encoded by the *YPS3* gene. *Biochem. J.*, **339**: 407-411.

Pignede, G., Wang, H., Fudalej, F., Gaillardin, C., Seman, M., and Nicaud, J. M., 2000. Characterization of an extracellular lipase encoded by Lip2 in *Yarrowia lipolytica*. *J. Bacteriol.*, **182** (10): 2802-2810.

Ray, M. K., Devi, K. U., Kumar, G. S., and Shivaji, S., 1992. Extracellular protease from the antarctic yeast *Candida humicola*. *Appl. Environ. Microbiol.*, **58** (6): 1918-1923.

Renold, H., Fasold, H., and Staib, F., 1968. Purification and characterization of a proteolytic enzyme from *Candida albicans*. *Biochim. Biophys. Acta*, **167** (2): 399-406.

Shieh, C. J., Thi, L. A. P., and Shih, L. A. P., 2009. Milk-clotting enzymes produced by culture of Bacillus subtilis natto. *Biochem. Eng. J.*, **43** (1): 85-91.

Yamada, T., and Ogrydziak, D. M., 1983. Extracellular Acid Proteases Produced by *Saccharomycopsis lipolyti*ca. *J. Bacteriol.*, **154** (1): 23-31.

(Edited by Ji Dechun)