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Phytase Production by a Marine Yeast *Kodamea ohmeri* BG3

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Abstract The marine yeast strain *Kodamea ohmeri* BG3 isolated from the gut of a marine fish (*Hexagrammes otakii*) was found to secrete a large amount of phytase into the medium. The crude phytase produced by this marine yeast showed the highest activity at pH 5.0 and 65 °C. The optimal medium for phytase production contained 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 phytase production were 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 was produced within 72 h of fermentation at the shake flask level. This is a very high level of phytase activity produced by yeasts. We think that the medium and process for phytase production by the marine yeast strain were very simple, and such marine yeast from the gut of natural marine fish may have a potential application in the maricultural industry and marine environmental protection. The results demonstrate that phytate was actively degraded by the crude phytase within a short period.

Keywords Marine yeasts · Phytase · *Kodamea ohmeri* · Marine fish · Oat

Introduction

Phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyzes the release of phosphate from phytate (myco-inositol hexakiphosphate), which is the principle type of phosphorus present in cereal grains, legumes, and oilseeds [1].

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, contribute significantly toward environmental protection, and lead to improved availability of minerals, trace elements, amino acids, and energy [2].

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In the recent decades, global maricultural industry, including shrimp, bivalves, seaweeds, and sea cucumber farming, has been developing rapidly because of the high demand of seafood. Non-eco-friendly management practices that are very common in intensive modern farming systems such as discharges of waste manures and wastewater from treatment plants are found to be associated with phosphorus pollution in the marine environment [3]. Therefore, phytase also can be incorporated into the maricultural feed because of the lack of phytase in the guts of marine farming animals [3].

Microorganisms are the best sources for commercial production of phytases because of their easy cultivation and high yields of the enzyme. At present, all phytase preparations authorized in the EU as feed additives are produced by recombinant strains of filamentous fungi, and the expressed phytase genes are of fungal origin and originate in two cases from the genus *Aspergillus* [4]. In the last decade, phytate-degrading enzymes of terrestrial yeasts such as *Schwanniomyces castellii* [1], *Schwanniomyces occidentalis* [5], *Pichia anomala* [6], *Arxula adeninivorans* [7], *Hansenula polymorph* [8], and *Rhodotorula gracilis* [9] also have received increasing attention as they can be easily incorporated into feed diets and are rich in nutrients. However, little is known about phytase-producing marine yeasts.

In our recent studies [3], marine yeast strains from different marine habitats were isolated, and phytase-producing marine yeasts were screened. Of the 327 yeast strains isolated, ten strains showed comparatively higher phytase activity. We found that *Kodamea ohmeri* BG3, one of the yeast strains, could produce more phytase than any other marine yeast strains tested. To our knowledge, phytase-producing marine yeasts are still untouched bioresources in marine environments. We also think that phytase-producing marine yeasts as maricultural feed additives are more suitable than the added phytase from terrestrial microorganisms. Therefore, the present study aimed at the optimization of medium and cultivation conditions for phytase production by *K. ohmeri* BG3 isolated from the gut of the marine fish (*Hexagrammes otakii*) and exploration of its potential applications in maricultural industry.

Materials and Methods

Marine Yeast Strains

The marine yeast strains W2B, BG3, N12C, YF04C, MA6, YF08, NY4E, YF12C, MB2, and WZ1, which were isolated from the gut of a sea cucumber (*Holothuria scabra*) in China, gut of marine fish *H. otakii*, seawater at the Pacific Ocean, gut of marine fish *H. otakii*, seawater at the Indian Ocean, gut of marine fish *Synecogobius hasts*, seawater from salterns, a sea cucumber (*H. scabra*) in Srilanka, seawater in China South Sea, and gut of an unknown fish, respectively, were maintained on a YPD medium containing 10.0 g/l yeast extract, 20.0 g/l polypeptone, and 20.0 g/l glucose at 4 °C.

Phytase Production

One loop of the cells of the yeast strains was transferred to 50 ml of YPD medium prepared with distilled water in a 250-ml flask and aerobically cultivated for 24 h. The cell culture (1.0 ml, optical density [$OD_{600\ nm}$]=10.0) was centrifuged at 5,000×*g* and 4 °C for 5 min and washed three times with sterile saline water. The washed cells were transferred to 50 ml of the production medium, which contained 10.0 g/l oat, 30.0 g/l glucose, 15.0 g/l ammonium sulfate, and 20.0 g/l NaCl, pH 5.0, and grown by shaking at 170 rpm and 28 °C for 3 days. To

determine the optimal medium and cultivation conditions for phytase production by the yeast strain, different phytate-containing substances (corn meal, wheat flour, oat meal, soybean meal, pea meal, horsebean meal, and phytic acid), carbon sources (glucose, sucrose, fructose, maltose, and galactose), and nitrogen sources (ammonium sulfate, ammonium citrate, peptone, beef extract, casein, and tryptone) with different concentrations were added to the medium prepared with distilled water or seawater, and the yeast strain was grown at different pH (3–9) and temperature (15–35 °C).

Phytase Assay

One milliliter of the cell culture was centrifuged at 5,000×*g* for 10 min. The supernatant obtained was used as the crude extracellular phytase preparation. The phytase activity was assayed as follows: 0.8 ml of sodium phytate solution (5.0 mM sodium phytate in 0.2 M sodium acetate pH 5.0) was preincubated at 65 °C for 5 min, and 0.2 ml of the crude extracellular phytase preparation was added and mixed well. The mixture was incubated at 65 °C for 30 min. The reaction was stopped by the addition of 1.0 ml of 50.0 g/l trichloroacetic acid. Inorganic phosphate liberated was quantitatively determined by using the ammonium molybdate method [10] spectrophotometrically at 700 nm. One unit of phytase activity was defined as the amount of enzyme causing the release of 1.0 μM of inorganic phosphate per minute under the assay conditions.

Effects of Different pH and Temperature on Phytase Production

To examine effects of different temperature on phytase activity, the phytase activity was determined at 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, and 85 °C, respectively, as described above. The effect of pH on the enzyme activity was determined by incubating the mixture between pH 3.0 and 9.0 using the standard assay condition. The buffers used were 0.2 M acetate buffer (pH 3.0–6.0) and 0.2 M $Na_2B_40_7$ 10 H_2O–H_3BO_3 buffer (pH 7.0–9.0).

The Phytate-containing Substances and Phosphate Determination

Corn meal, wheat flour, soybean meal, oats meal, pea meal, and horsebean meal were purchased from a local supermarket in Qingdao, China. Phytic acid was bought from Sigma in the USA. The amount of inorganic phosphate in the medium plus different phytate-containing substances was measured by using the ammonium molybdate method [10] spectrophotometrically at 700 nm.

Phytate Hydrolysis

Sodium phytate solution (0.8 ml; 5.0 mM sodium phytate in 0.2 M sodium acetate, pH 5.0) was preincubated at 65 °C for 5 min, and 0.2 ml of the extracellular phytase preparation (557.9 mU/ml) was added and mixed well. The mixture was incubated at 65 °C for 3 h and sampled every hour for determination of degradation.

The end products of phytate hydrolysis after 3 h at 65°C were withdrawn and identified to ascertain the extent of hydrolysis by ascending thin-layer chromatography (Silica gel 60, MERCK, Germany) with the solvent system of 160 ml of chloroform, 60 ml of acetone, 52 ml of methanol, 48 ml of glacial acetic acid, and 28 ml of water. The spots were located by exposing the plates to iodine vapor.

Results

The Marine Yeast Strains with Phytase Activity

A total of 327 yeast strains from seawater, sediments, guts of the marine fish, and marine algae were obtained, but only ten strains among them could secrete a large amount of phytase into the medium (Table 1). Strains W2B, BG3, N12C, YF04C, MA6, YF08, NY4E, YF12C, MB2, and WZ1 were isolated from the gut of *H. scabra* in China, gut of *H. otakii*, seawater at the Pacific Ocean, gut of *H. otakii*, seawater at the Indian Ocean, gut of *S. hasts*, seawater from salterns, gut of *H. scabra* in Srilanka, seawater in the southern sea of China, and gut of an unknown marine fish around the coastal line of Qingdao, respectively. It should be stressed that all the marine animals were healthy and obtained from natural marine environments. These results showed that most of the marine yeasts producing phytase were obtained from the guts of the natural marine animals, suggesting that they could exist in such environments. It also can be noticed from Table 1 that the marine yeast strain BG3 could produce more extracellular phytase than any other marine yeast strains tested in this study. In our previous studies [3], it was found that the marine yeast strain BG3 was closely related to *K. ohmeri* after identification by using the routine methods and molecular methods. Therefore, this marine yeast strain was used in the subsequent studies.

Effects of Different Phytate-containing Substances on Phytase Production

The results in Fig. 1 show that in the absence of phytate in the medium, the marine yeast strain did not produce any phytase. This means that the phytase production by this yeast strain was inducible. However, phytate does not seem to have an inducible effect on phytase synthesis by terrestrial yeasts used by Sano et al. [7]. To know the effects of phytate-containing substances and pure phytic acid on phytase production and look for a cheap substance for promotion of phytase production by it, effects of different phytate-containing substances and phytic acid on phytase production by the marine yeast were examined. It can be clearly observed from the data in Fig. 1 that oat was the most suitable for phytase production by the marine yeast strain. Our results also show that the optimal concentration of oat for phytase production was 10.0 g/l in the medium (data not shown). It

Table 1 Phytase-producing marine yeast strains, phytase activity, and their sources.

Strains	Phytase activity (mU/ml)	Habitats
W2B	61±0.011	Gut of sea cucumber (*Holothuria scabra*) in China
BG3	62±0.006	Gut of fish (*Hexagrammes otakii*)
N12C	47±0.026	Seawater at Pacific Ocean
YF04C	30±0.004	Gut of fish (*Hexagrammes otakii*)
MA6,	35±0.006	Seawater at Indian Ocean
YF08,	31±0.034	Gut of marine fish (*Synecogobius hasts*)
NY4E,	44±0.006	Seawater from salterns
YF12C,	49±0.008	Sea cucumber (*Holothuria scabra*) In Sri Lanka
MB2	34±0.023	Seawater in Southern sea of China
WZ1	19±0.01	Gut of unknown fish

The yeast cells were aerobically grown in the minimal synthetic medium containing vitamins, mineral salts, 10.0 g/l corn meal, and 10.0 g/l ammonium sulfate for 5 days at 28 °C, and phytase activity in the supernatant of the culture was determined as described in "Materials and Methods." Data are given as means±SD, $n=3$.

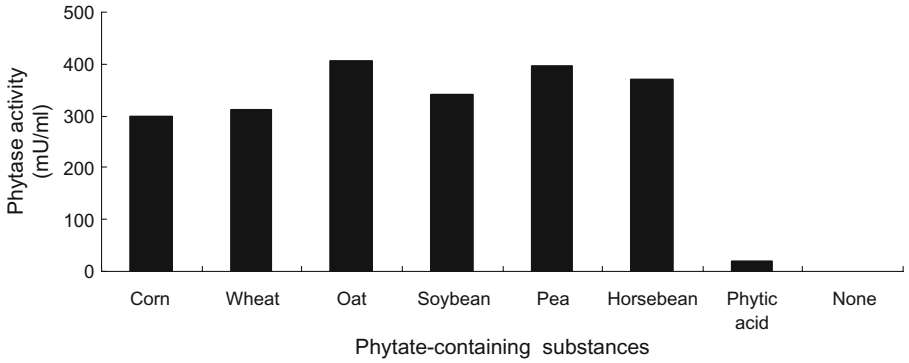

Fig. 1 Effects of different phytate-containing substances and phytic acid on phytase production. Data are given as means±SD, $n=3$. The growth medium: glucose 30.0/l, ammonium sulfate 15.0 g/l, and NaCl 20.0 g/l. Initial pH=5.0, temperature=28 °C, shaking speed=170 rpm, incubation time=3 days

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. For example, in a survey of phytase-producing microorganisms, *A. ficuum* produced the highest amount of phytase (113 nkat/ml in shake flask in 5 days) when the inorganic phosphorus content was in the range of 0.0001–0.005%, the optimum being 0.4 mg/100 ml with 8.0% cornstarch [11]. In a continuous culture, phytase production by a strain of *S. castellii* decreased when the phytic acid or phosphate content increased [1]. Therefore, the phosphorus content in the phytate-containing substances was determined. The results in Table 2 indicate that the medium plus oat contained 0.001724% inorganic phosphate. It has been reported that oat contains 0.27 g of phytate phosphorus per 100 g of dry weight [4]. All these may have contribution to the enhancement of phytase production by the marine yeast strain.

Effects of Different Carbon Sources on Phytase Production

It has been reported that different carbon sources have significant influences on phytase production by terrestrial yeasts [2]. Indeed, as shown in Fig. 2, phytase production by the marine yeast strain was also influenced greatly by different carbon sources in the medium. It can be seen clearly from Fig. 2 that glucose was the best carbon source for phytase production and fructose was the second better carbon source for it. However, the lowest

Table 2 Inorganic phosphate content in different phytate-containing substances.

Phytate-containing substances	Inorganic phosphate content (%)
Corn meal	0.000018±0.0000012
Wheat flour	0.000817±0.00002
Oat meal	0.001724±0.00005
Soybean meal	0.004374±0.00013
Pea meal	0.003185±0.00011
Horsebean meal	0.005702±0.00021

The amount of inorganic phosphate was determined in the sterilized medium containing phytate-containing substances 10.0 g/l, glucose 30.0 g/l, ammonium sulfate 15.0 g/l, NaCl 20.0 g/l, pH 5.0.

Data are given as means±SD, $n=3$.

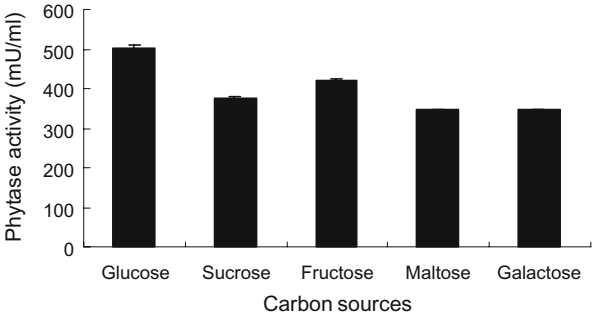

Fig. 2 Effects of different carbon sources on phytase production. Data are given as means±SD, n=3. The growth medium: oat 10.0 g/l, ammonium sulfate 15.0 g/l and NaCl 20.0 g/l. Initial pH=5.0, temperature=28 °C, shaking speed=170 rpm, incubation time=3 days

phytase was produced in the medium containing galactose. We also found that the optimal concentration of glucose for the phytase production was 30.0 g/l. However, beyond this concentration, enzyme yield declined gradually (data not shown). However, Sano et al. [7] found that when glucose was replaced by galactose, *A. adeninivorans* secreted high levels of phytase into the culture medium. This means that the glucose effect on phytase production by the marine yeast strain was completely different from that by the terrestrial yeast. The results may imply that there was no glucose repression on phytase synthesis in the cells of *K. ohmeri* BG3 used in this study. However, glucose or glucose syrups were also used as main carbon sources for phytase production by *H. polymorpha* during fermentation [8].

Effects of Different Nitrogen Sources on Phytase Production

Little has been known about the effects of different nitrogen sources on phytase production by yeasts so far [2, 7]. To study effects of different nitrogen sources on phytase production by the marine yeast strain, different nitrogen sources were supplemented in the production medium containing 30.0 g/l glucose and 10.0 g/l oat. Among all the nitrogen sources tested, ammonium sulfate (15.0 g/l) increased phytase production (534.8 mU/ml), and beef extract was the second better nitrogen source for it (250 mU/ml), as shown in Fig. 3. To determine the optimum concentration of ammonium sulfate for phytase production, different concentrations (1.0–25.0 g/l) of ammonium sulfate were used in the production medium. A concentration of 15.0 g/l of ammonium sulfate was found to be optimal for phytase production by the marine yeast strain (data not shown). Sano et al. [7] also found that phytate-assimilating yeasts could secrete a large amount of phytase into the phosphate-depleted minimal phytate medium in which ammonium sulfate was the sole nitrogen source.

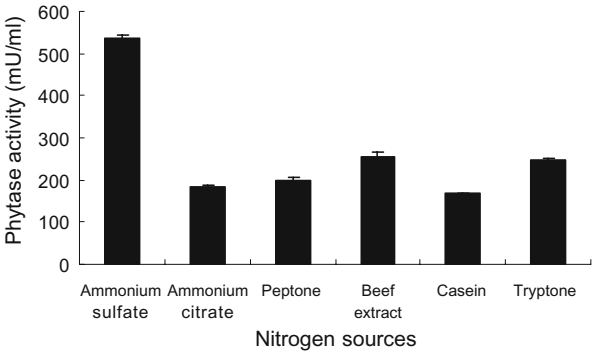

Fig. 3 Effects of different nitrogen sources on phytase production. Data are given as means±SD, n=3. The growth medium: oat 10.0 g/l, glucose 30.0 g/l, and NaCl 20.0 g/l. Initial pH=5.0, temperature=28 °C, shaking speed=170 rpm, incubation time=3 days

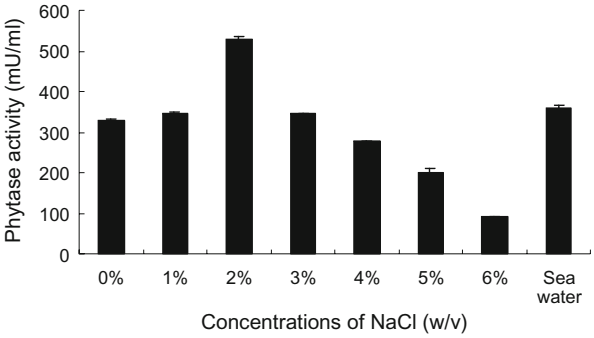

Fig. 4 Effects of different concentrations of NaCl on phytase production. Data are given as means±SD, $n=3$. The growth medium: oat 10.0 g/l, glucose 30.0 g/l, ammonium sulfate 15.0 g/l, and NaCl 20.0 g/l. Initial pH=5.0, temperature=28 °C, shaking speed=170 rpm, incubation time=3 days

Effects of Different Concentrations of NaCl on Phytase Production

Many results have shown that many enzyme activities in halotolerant yeasts and moderately halotolerant bacteria were enhanced in the presence of NaCl [12–13]. For example, the activity of NADP–glutamate dehydrogenase activity is fivefold higher when the halotolerant yeast *Debaryomyces hansenii* is grown in the presence of 1 M NaCl [13]. *M. varians* subsp. *halophilus* produced the highest amylase activity when grown in 2.0 M NaCl [12]. Because the yeast strain used in this study was isolated from the marine environment, it is very important to examine effects of different concentrations of NaCl in the production medium and seawater on phytase production by the marine yeast. As indicated in Fig. 4, when concentrations of added NaCl were increased from 0 to 20.0 g/l, phytase activity was increased from 330.4 to 530.3 mU/ml, whereas when the concentrations of NaCl was further increased from 20.0 to 60.0 g/l, phytase activity was decreased gradually. These results indicate that 20.0 g/l of added NaCl was the most suitable for phytase production by the marine yeast. The results in Fig. 4 also indicate that the marine yeast strain could produce a high level of phytase in the production medium prepared with seawater.

Effects of Different pH and Temperature on Phytase Production

Effects of different initial pH in the production medium on phytase production by the marine yeast strain were tested. The results in Fig. 5 reveal that the optimal initial pH in the medium for phytase production was 5.0. The results also show that when initial pH was higher or lower than 5.0, phytase activity decreased sharply, indicating that phytase production by the marine yeast strain was very sensitive to the change in initial pH.

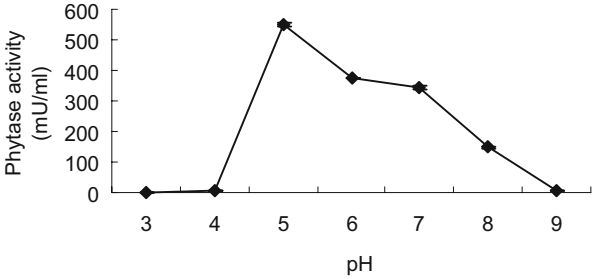

Fig. 5 Effects of different initial pH on phytase production. Data are given as means±SD, $n=3$. The growth medium: oat 10.0 g/l, glucose 30.0 g/l, ammonium sulfate 15.0 g/l, and NaCl 20.0 g/l. Temperature=28 °C, shaking speed=170 rpm, incubation time=3 days

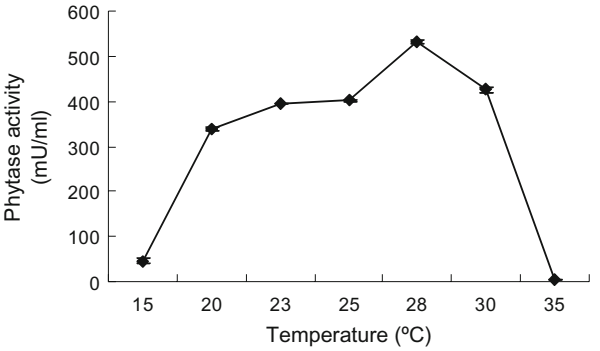

Fig. 6 Effects of different temperature on phytase production. Data are given as means±SD, n=3. The growth medium: oat 10.0 g/l, glucose 30.0 g/l, ammonium sulfate 15.0 g/l, and NaCl 20.0 g/l. Initial pH=5.0, shaking speed=170 rpm, incubation time=3 days

The effect of temperature (15–35 °C) on phytase production was studied. Our experiments show that the maximum phytase production (532.2 mU/ml) was observed at 28 °C, and thereafter, a significant decrease in enzyme activity was seen at higher temperatures (Fig. 6).

Time Course of Phytase Production at Shake Flask Level

All the data above have shown that the optimal medium for phytase production contained oat 10.0 g/l, glucose 30.0 g/l, ammonium sulfate 15.0 g/l, and NaCl 20.0 g/l, while the optimal cultivation conditions for phytase production were pH 5.0, a temperature of 28 °C, and a shaking speed of 170 rpm. We also found that 1.0 ml of 24-h-old yeast culture ($OD_{600\ nm}$=10.0) per 50 ml of the production medium was the best inoculation size to produce the maximum phytase activity (data not shown). Therefore, the time course of phytase production during fermentation was checked under the conditions. The results in Fig. 7 indicate that 557.9 mU/ml of phytase activity could be reached within 72 h of the fermentation. These results demonstrate that the marine yeast strain could produce high yield of extracellular phytase in the simple medium, and this may have wide uses in phytase production.

Phytate Hydrolysis

It can be noted from Fig. 8 that most of phytate in the reaction mixture could be converted into different sizes of hydrolysis products by the action of the crude phytase within 3 h. This means that the crude phytase could actively hydrolyze phytate within a short period.

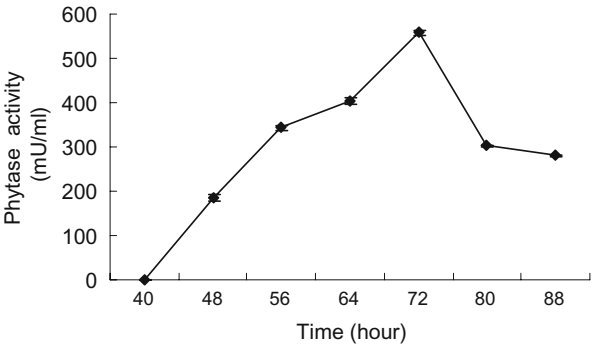

Fig. 7 Time course of phytase production by the marine yeast strain BG3. All the data are given as means±SD, n=3. The growth medium: oat 1.0%, glucose 3.0%, ammonium sulfate 1.5%, and NaCl 2.0%. Initial pH=5.0, temperature=28 °C, shaking speed=170 rpm, incubation time=88 h

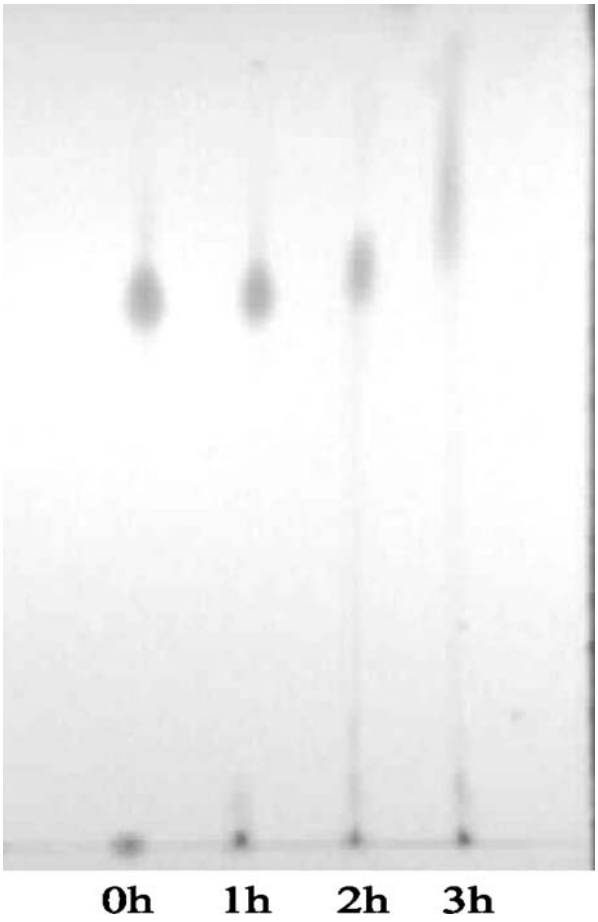

Fig. 8 Thin-layer chromatogram of the hydrolysis products of phytate with the crude phtase produced by the marine yeast strain used in this study. *Lane 1*: phytate+the crude phytase within 0 h); *lane 2*: the hydrolysis products for 1 h; *lane 3*: the hydrolysis products for 2 h; *lane 4*: the hydrolysis products for 3 h. The end products of phytate hydrolysis were analyzed by using a TLC plate (Silica gel 60, MERCK, Germany) with the solvent system 160 ml of chloroform, 60 ml of acetone, 52 ml of methanol, 48 ml of glacial acetic acid, and 28 ml of water. The spots were located by exposing the plates to iodine vapor

Discussion

To reduce phosphorus excretion from marine fish and improve availability of minerals, trace elements, amino acids, and energy to marine fish, phytase is incorporated into commercial fish diets to enhance phosphorus release from phytate in the fish diets [2]. If marine yeasts in the gut of marine animals can produce a large amount of extracellular phytase, such marine yeasts can play an important role in degradation of phytate in the guts of marine animals. The results in Table 1 showed that most of the marine yeasts producing phytase were obtained from the guts of the natural marine fish, suggesting that they could exist in such environments. Among the marine yeasts, *K. ohmeri* BG3 isolated from the gut of the marine fish *H. otakii* was found to produce more extracellular phytase than any other marine yeast strains tested in this study. Therefore, the medium and cultivating conditions for phytase production by this marine yeast were optimized in this study. We found that the optimal medium for phytase production contained oat 10.0 g/l, ammonium sulfate 15.0 g/l, glucose 30 g/l, and NaCl 20.0 g/l (Figs. 1, 2, 3, 4, and 7). The results in Table 2 indicate that the production medium plus oat only contained a trace amount of phosphorus, and these may have contribution to enhancement of phytase production by the marine yeast strain as phosphorus in the medium will repress phytase production. In China, the price of

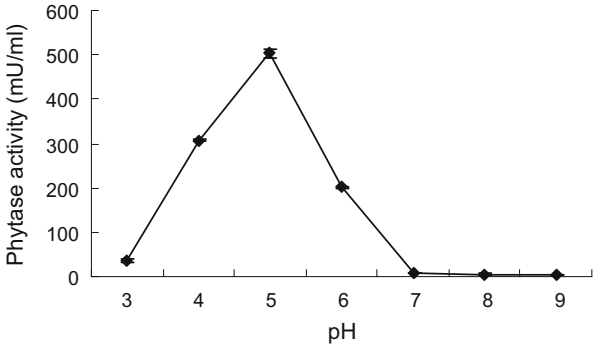

Fig. 9 Effects of different pH on the crude phytase activity. Data are given as means±SD, n=3. Different buffers with 5.0 mM sodium phytate. Temperature=65 °C, incubation time=30 min

oat meal is almost the same as that of corn meal. Therefore, we think that oat instead of phytate can be cheaply used as one component of the production medium for phytase production by the marine yeast. This is the first time to report that oat meal can be used for the production of phytase. The results in Fig. 4 show that phytase production by the marine yeast used in this study can be enhanced in the presence of 20.0 g/l NaCl and seawater. However, it is still completely unknown why phytase production was enhanced in the presence of 20.0 g/l NaCl and seawater. This may be related to the marine environment where the marine yeast strain was obtained. Therefore, this yeast strain will be suitably applied in marine environments.

The optimal cultivation conditions for phytase production by the marine yeast were pH 5.0, a temperature of 28 °C, and a shaking speed of 170 rpm (Figs. 5 and 6). Sano et al. [7] reported that the terrestrial yeasts they used could secrete high level of phytase in the medium with pH 5.5. This suggests that the optimal initial pH for phytase production by the marine yeast was consistent with that by the terrestrial yeast. However, it was observed that *A. adeninivorans* strains were capable of producing high levels of phytase while growing actively at the temperature of 44 °C [7]. This means that the optimal temperature for phytase production by the marine yeast was lower than that by the terrestrial yeast. This may also be related to the marine environment where the marine yeast strain was obtained.

Under the optimal conditions, more than 557.9 mU/ml of phytase activity was produced within 72 h of fermentation at shake flask level (Fig. 7). This is a very high level of phytase activity produced by yeasts. Although it was reported that *A. adeninivorans* CBS 7377 and *A. adeninivorans* CBS 8335 could secrete more than 1,427 and 1,921 mU/ml of phytase, respectively, in the phosphate-depleted yeast extract medium [7], the medium and process for phytase production were too complicated and were not feasible for industrial purposes.

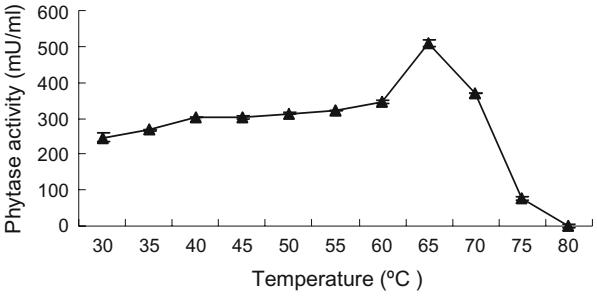

Fig. 10 Effects of different temperature on the crude phytase activity Data are given as means±SD, n=3. Acetate buffer (0.2 M, pH 5.0) with 5.0 mM sodium phytate. Incubation time=30 min

The results in Figs. 9 and 10 show that the optimal pH and temperature for the crude phytase produced by the marine yeast strain BG3 were 5.0 and 65 °C, respectively. The optimum pHs for phytases secreted by *A. adeninivorans* and *Pichia pastoris* were 4.5 and 5.5, respectively, while optimal temperatures for phytases from *A. adeninivorans* and *P. pastoris* were 75 and 60 °C [2, 7], respectively. This means that the optimal pH and temperature for the crude phytase produced by the marine yeast strain were in agreement with those for phytases produced by the terrestrial yeasts. In our previous study [14], it was found that optimal pH and temperature for the purified phytase from the marine yeast strain used in this study were also 5.0 and 65 °C, respectively.

The results in Fig. 8 demonstrate that phytate was converted into different sizes of hydrolysis products by the action of the crude phytase within 3 h. However, it is still unknown how phytate is dephosphorylated by the phytase.

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