Enzymatic Properties of Phytase from *Escherichia coli* DH5α

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Abstract—A Escherichia coli strain producing phytase named DH5α was chosen from four *E. coli* stains, the enzyme activity was 17.88 U/mL after fermented at 37°C for 2 days. The enzymatic properties of crude enzyme solution were studied. The optimal temperature was 55°C; optimal pH was 2.5 and 7.0, enzyme had certain resistance to heat and different pH conditions, 75% activity could be maintained when treated at 65°C for 30min, and 60% activity still remained when treated in the pH range of 2 to 9 for 30 min and 60 min. Resistance to KH_2PO_4 of phytase was poor, low concentrations of KH_2PO_4 (15mg/L) could inhibit the enzyme activity to below 40%; all kinds of metal ions had no significant activation or inhibition effect on enzyme, the enzyme had resistance to pepsin but no resistance to trypsin, after treated by trypsin for 30min, only 66.69% of the activity retained, while treated by pepsin , the activity could still maintain more than 90%.

Keywords-Escherichia coli; Phytase; Enzymatic properties; Molybdenum blue method; Enzyme activity

I. INTRODUCTION

Phytase is a class of hydrolases which can hydrolyze phytic acid into phosphoric acid inositol, it exists widely in plant and animal tissues and microbial cells and mainly exists in seeds of the plant. Because of the different kinds of plants, the activity of phytase varies greatly[1-5]. Microbes are the most convenient and the most economical sources of phytase[6]. Bacteria, yeasts, fungi and many multicellular fungi can produce phytase but their properties are different[7]. According to the difference of the optimum pH values, Phytase can be divided into acidic phytase, neutral phytase and alkaline phytase. However, due to the microbial phytase has high activity and can be produced easily, the research and development on it are the most extensive and deepest[8-9]. At present, phytase is widely used in food and feed industry, it can make the utilization ratio of phosphorus which exists n plant feedstuffs increase by 60% and the amount of phosphorus in faeces reduce by 40%, so that it can reduce the amount of phosphorus in the feed and the pollution to the environment. Besides, it can also damage the affinity of phytic acid, mineral elements and protein which contain in plant feedstuffs or food, improve the bioavailability of mineral elements and the digestible rate of protein, so as to improve the nutritional value of food and livestock production efficiency [10-12].

In this study, the enzymatic properties of phytase produced by *E. coli* DH5α were examined. The objective of this study was to grasp the characteristics of phytase better and lay a good fundation for the application of phytase.

II. MATERIALS AND METHODS

A. materials and reagents

Escherichia coli DH5α, AB1157, GM2929, KK1, preserved in microbial genetics and breeding laboratory of Xuzhou Engineering Institute; Sodium phytate: High purity reagent, singma; Ammonium molybdate tetrahydrate: AR, Chemical reagent factory in hefei university of technology; Ammonium sulfate: AR, Tianjin Municipality kemi'ou Chemical Reagent Co. Ltd.; trichloroacetic acid (TDA): AR, Hedong district of tianjin red crag reagent factory; Other reagents are of analytic grade.

B. Culture medium

1) LB medium($g•L^{-1}$): beef extract 5, peptone 10, NaCl 5.

2) Fermentation medium($g•L^{-1}$): glucose 15, peptone 3 , $(NH_4)_2SO_4$ 2, $MgSO_4•7H_2O$ 2, KCl 0.5, $FeSO_4$ 0.03, $MnSO_4•7H_2O$ 0.03, pH 7.0.

C. Culture conditions

1) Seed culture: Two rings of strain growing well in test tubes was inoculated into the 250 mL conical flask containing 100 mL of LB medium and cultured on a reciprocal shaker(120 rpm) for 1 d at 37°C.

2) Fermentation culture: The seed culture was then inoculated to the 250 mL conical flask containing 50 mL fermentation medium, and fermented on a reciprocal shaker(120 rpm) for 2d at 37°C.

D. The preparation of crude enzyme

Cultures were centrifuged at 8000 r. min^{-1} for 10 minutes at 4°C and the supernatant was used for the estimation of phytase activity.

E. Determination of phytase activity

Phytase activity was determined using the method described by Harland (1980) with slight modifications[13]. Definition of activity unit of enzyme: Under the condition of 37 °C temperature, pH 5.5, 1 μmol inorganic phosphorus is released from the 5.0 mmol/L sodium phytate solution per minute is a unit of phytase activity(U).The enzyme activity was calculated by the following equation (1):

$$\text{Phytase activity} = \frac{C \times F}{V \times 30} \quad (1)$$

where C is the phosphorus concentration (mol/L);F is total diluted multiples of the sample solution before the reaction; V is sample volume (mL); 30 is the reaction time.

F. Enzymic properties of crude enzyme of E. coli DH5α

1) The optimal pH of enzyme reaction: The enzyme activity of crude enzyme was determined as described above. The buffer used for pH measurement was 0.1 mol/L acetic acid sodium-acetate buffer ,pH was adjusted to 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The highest enzyme activity was set as 100%, the ratio of enzyme activity under other conditions to the highest enzyme activity was relative enzyme activity.

2) pH stability of enzyme: Enzyme solution was treated at different pH values (2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 6.0, 7.0) for 30 min and 60 min at 37°C, then adjusted back to the optimal pH value. Enzyme activity was determined under routine conditions to investigate the pH stability of the enzyme at 37°C.

3) The optimal temperature of enzyme reaction: The enzyme activity according to temperature was measured at different temperatures(30°C, 35°C, 45°C, 55°C, 65°C, 75°C, 85°C). Enzyme activity was determined at 37°C.

4) Thermal stability of enzyme: Crude enzyme was treated at different temperatures（35°C, 45°C, 55°C, 65°C, 75°C) for 30 min and 60 min, and then residual enzyme activity was determined at 37°C.

5) Effect of metal ions on phytase activity: 0.1% AgCl, $AlCl_3$, $CoCl_2$, $CuSO_4$, $FeSO_4$, NH_4Cl, KCl, $GeCl_4$, $MnCl_2$ was separately added to the buffer, then enzyme activity was determined under routine conditions.

6) Effect of KH_2PO_4 on phytase activity: Effect of KH_2PO_4 whose concentration was 1, 5, 7, 10, 15, 20, 30, 40, 50, 60 mg/L on phytase was investigated. Enzyme activity was determined under routine conditions.

7) Effects of pepsin and trypsin on phytase activity: 1mg/L pepsin and trypsin was respectively added to crude enzyme liquid, treated for 10 min, 30 min, 60 min under the optimum temperature. Enzyme activity was determined under routine conditions.

III. RESULTS AND DISCUSSION

A. KH_2PO_4 standard curve

Phosphorus concentration was set as Abscissa, absorbance at 700nm was set as ordinate, then standard curve was drawn, as shown in Fig. 1. linear regression equation is y=0.0026x-0.0024, correlation coefficient R^2=0.9979, showing a good linear relationship.

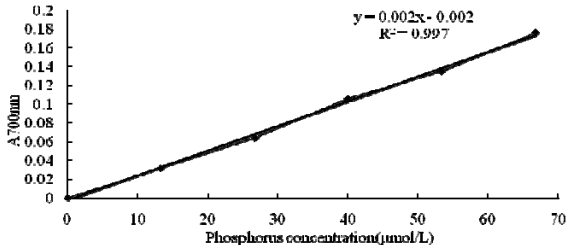

Figure 1. Standard curve of KH_2PO_4

B. Selection of the best strain and medium

Phytase activity of four strains of *E. coli* was determined every 24h, the results were shown in Table 1. As seen from table 1, in the two different culture medium, enzyme activity of phytase produced by DH5α were the highest of the 4 strains, so DH5α was the best strain to produce phytase. Meanwhile, it can be seen from the results of the experiment, phytase-producing activity of DH5α was higher when cultured in fermentation medium, up to 17.88 U/mL, so the optimum medium for phytase production was fermentation medium.

TABLE 1 SELECTION OF THE BEST STRAIN

Kinds of Culture medium	Time (h)	Enzyme activity (U/mL)			
		DH5α	AB1157	GM2929	KK1
LB medium	24	6.45±0.12c	11.76±0.11a	10.64±0.21b	4.06±0.16d
	48	12.55±0.21a	9.77±0.22c	11.89±0.05b	2.58±0.13d
	72	8.18±0.12a	6.25±0.31c	7.92±0.02b	0.98±0.09d
Fermentation medium	24	11.63±0.15a	5.19±0.26c	1.00±0.12d	9.19±0.11b
	48	17.88±0.22a	5.61±0.42d	8.13±0.25b	6.41±0.05c
	72	5.68±0.35a	5.13±0.10b	4.84±0.24c	4.49±0.12d

Data expressed as mean ± SD from triplicate experiments. different superscripts within the same row are significantly different ($P<0.05$)

C. Determination of incubation time

Phytase activity that DH5α produced was determined every 12 hours, the results were shown in Fig. 2. As seen in Fig. 2 , phytase activity of DH5α reached the peak when cultured for 48h, when phytase activity was up to 17.92 U/mL. After that, the phytase acitibity decreased. so the incubation time was 48h.

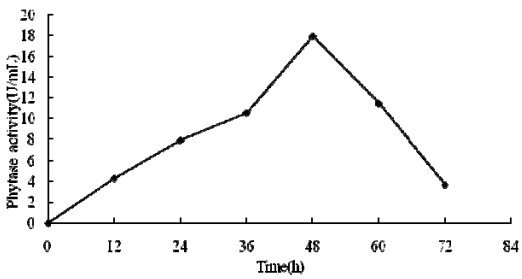

Figure 2. Effect of culture time on phytase activity

D. Optimal pH of enzyme reaction

The activity of phytase was determined at different pH values ranged from 2 to 9, the results were shown in Fig. 3. As shown in Fig. 3, phytase produced by DH5α reached the peak at pH 2.5 and its relative enzyme activity was 100%, then enzyme activity gradually decreased, but reached another peak at pH 7, when relative enzyme activity was 61%. After that, the enzyme activity gradually declined, so enzyme activity was higher both at pH 2.5 and pH 7, it had certain adaptability to acidic and neutral conditions and had the characteristics of both acidic phytase and neutral phytase. So it would have a good application prospect.

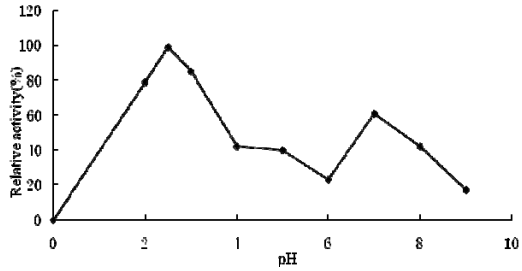

Figure 3. Effect of pH on phytase activity

E. pH stability of phytase

To measure pH stability, the remaining phytase activity was determined after incubation at various pHs for 30 min and 60 min at 37°C The results were shown in Fig.4.The enzyme was more stable in the pH range of 2.5–5.0, with more than 80% of the initial activity remained. The enzyme could always keep the activity of about 60% at different pHs, it showed that the phytase had good pH stability. The phytase could maintain the activity of more than 70% in the pH range of 2-5, which showed that it had strong resistance to acid; the phytase could maintain the activity of more than 60% in the pH range of 6~9, which showed that it also had good stability under neutral pH condition. It was concluded that the enzyme had higher resistance to pH and it had certain application prospect.

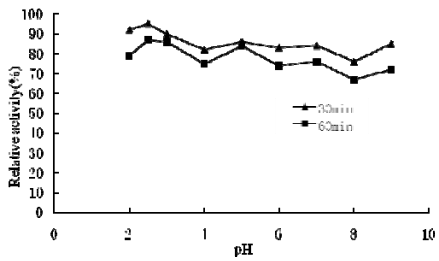

Figure 4. pH stability of phytase

F. Optimal temperature of enzyme reaction

The effect of temperature on phytase activity was detected, the results were shown in Fig.5. The relative enzyme activity of phytase increased firstly, enzyme activity was higher from 30°C to 55°C,maximum activity was exhibited at 55°C, then the activity of the enzyme decreased rapidly when temperature was higher than 55°C. When the temperature was above 80°C, the enzyme lost activity completely., the result would have a certain guide significance to the application in the future.

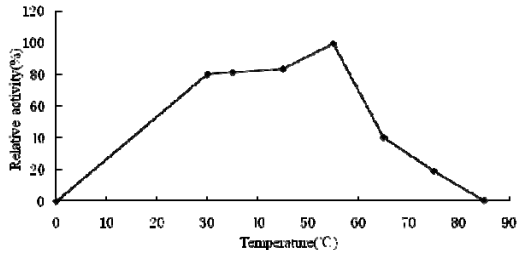

Figure 5. Effect of temperature on phytase activity

G. Thermal stability of phytase

The thermal stability of phytase was measured by incubating the enzyme at various temperatures for 30 min and 60 min, the residual enzyme activity was determined. The results were shown in Fig.6. Phytase activity could still remain around 75% after incubated for 30 min and 60 min below 65°C, then the enzyme activity reduced quickly and could just stay around 30% at 75°C. Prolonging the time did not much affect the thermal stability. It could be concluded that the enzyme had certain thermal stability.

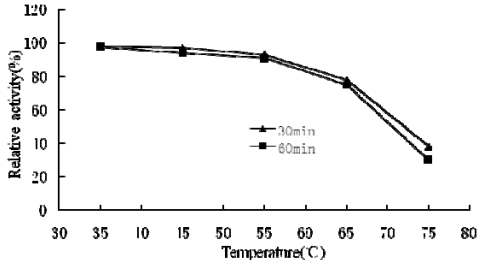

Figure 6. Thermal stability of phytase

H. Effect of metal ion on phytase activity

Effect of metal ion on phytase activity was measured, the results were shown in Table 2. No significant inhibition was observed in the presence of all kinds of metal ions listed in Table 2. So the enzyme had certain resistance to metal ions. In the presence of the metal ions, all of the relative phytase activity measured could keep high. As a consequence, phytase was not sensitive to metal ions. The results provided reference for its future use in the feed industry.

TABLE 2 EffECT OF METAL ION ON PHYTASE ACTIVITY

Metal ions	Relative enzyme activity/%
None	100.00±0.17a
Fe^{2+}	99.41±0.71ab
K^{+}	98.39±0.65bc
Al^{3+}	99.95±0.58a
Cu^{2+}	98.88±0.48ab
Co^{2+}	99.39±0.61abc
NH_4^{+}	99.25±0.78bc
Ag^{+}	99.09±0.25bc
Mn^{2+}	98.51±0.39cd
Ge^{4+}	97.82±0.41d

Data expressed as mean ± SD from triplicate experiments. different superscripts within the same column are significantly different (P＜0.05)

I. *Effect of KH_2PO_4 on phytase activity*

Different concentrations of KH_2PO_4 solution (1, 5, 7, 10, 15, 20, 30, 40, 50, 60mg/L) was added to the crude enzyme, enzyme activity was determined under routine conditions. The results were shown in Fig.7. As seen from the Figure, KH_2PO_4 solution with lower concentrations had significant inhibitory effect on phytase activity, when the concentration was over 15 mg/L, the remaing activity of the enzyme was less than 40%; When the concentration reached 60 mg/L, the enzyme almost lost activity. So in the process of application, the effect of element phosphorus on phytase activity should be avoided and the conditions during the use of the procedure should be controlled.

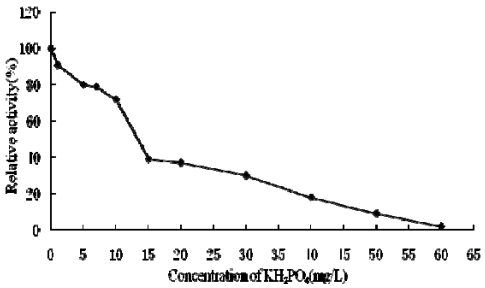

Figure 7. Effect of KH_2PO_4 concentration on phytase activity

J. *Effect of pepsin and trypsin on phytase activity*

1 mg/L of pepsin and trypsin solution was added to the crude enzyme for 10 min, 30 min, 60 min, then the phytase activity was determined. The results were shown in Table 3. The results showed that pepsin had no obvious inhibition or activation effect on the enzyme. On the contrary, trypsin had obvious inhibition effect on the enzyme. Thus it could be inferred that when the enzyme was added in animal feed, it would be more stable in the animal stomach than in the intestines.

TABLE 3 EFFECT OF PEPSIN AND TRYPSIN ON PHYTASE ACTIVITY

Time/min	Relative enzyme activity after treated by pepsin/%	Relative enzyme activity after treated by trypsin/%
0	100±0.10a	100±0.09a
10	99.23±0.45b	84.19±0.15b
20	98.09±0.35c	75.48±0.23c
30	96.25±0.46d	66.69±0.38d

Data expressed as mean ± SD from triplicate experiments. different superscripts within the same column are significantly different (P＜0.05)

IV. CONCLUSIONS

Four strains were cultured in different mediums, the phytase activity of the strains was determined, DH5α produced the highest enzyme activity in the fermentation medium. The phytase enzyme properties of crude enzyme from DH5α were studied. The optimal pH of phytase was 2.5 and 7, this enzyme had certain stability under the condition of acidic and neutral pH; the optimal temperature of enzyme was 55°C. In the aspect of heat resistance, the enzyme activity could still keep 75% after incubated at 65°C for 30 min, then the enzyme activity reduced quickly and it could just remain 30% at 75°C. Metal irons had no obvious inhibition or activation effect on phytase activity but KH_2PO_4 solution in low concentration had obvious inhibition on phytase activity. Pepsin had no obvious inhibition or activation effect on the enzyme, on the contrary, trypsin had obvious inhibition effect on this enzyme.

Based on the study, we could conclude that the enzyme had certain advantages to resist different pH values. The enzyme had higher activity under the acidic and neutral conditions, which showed that phytase from *E.Coli* DH5α had advantage compared with other phytase studied before.The phytase had good resistance to various metal ions, so it had a wide application prospect. Meanwhile, the phytase had better resistance to pepsin, so when it was added to the feed , phytic acid could be decomposed effectively and the absorption of nutrients could be improved.

In this study, the phytase was extracted from *E. coli* in nature, so some enzymatic properties were not very good, such as thermal stability, which could be improved by mutation or genetic engineering methods, so as to make it meet the requirements of industrial production.

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REFERENCES

[1] Chen W, Ye L, Guo F, et al. Enhanced activity of an alkaline phytase from *Bacillus subtilis* 168 in acidic and neutral environments by directed evolution[J]. Biochemical Engineering Journal, 2015, 98: 137-143.

[2] Gaind S, Singh S. Production, purification and characterization of neutral phytase from thermotolerant *Aspergillus flavus* ITCC 6720[J]. International Biodeterioration & Biodegradation, 2015, 99: 15-22.

[3] Bala A, Jain J, Kumari A, et al. Production of an extracellular phytase from a thermophilic mould *Humicola nigrescens* in solid state fermentation and its application in dephytinization[J]. Biocatalysis and Agricultural Biotechnology, 2014, 3(4): 259-264.

[4] Kim Y O, Lee J K, Oh B C, et al. High-level expression of a recombinant thermostable phytase in *Bacillus subtilis*[J]. Bioscience, biotechnology, and biochemistry, 1999, 63(12): 2205-2207.

[5] Kerovuo J, Lauraeus M, Nurminen P, et al. Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*[J]. Applied and Environmental Microbiology, 1998, 64(6): 2079-2085.

[6] Yoon S J, Choi Y J, Min H K, et al. Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4, and enzymatic properties of phytase enzyme[J]. Enzyme and microbial technology, 1996, 18(6): 449-454.

[7] Mullaney E J, Ullah A H J. The term phytase comprises several different classes of enzymes[J]. Biochemical and biophysical research communications, 2003, 312(1): 179-184.

[8] Huang H, Shi P, Wang Y, et al. Diversity of beta-propeller phytase genes in the intestinal contents of grass carp provides insight into the release of major phosphorus from phytate in nature[J]. Applied and environmental microbiology, 2009, 75(6): 1508-1516.

[9] Phillippy B Q, Wyatt C J. Degradation of phytate in foods by phytases in fruit and vegetable extracts[J]. Journal of food science, 2001, 66(4): 535-539.

[10] Liu N, Ru Y J, Li F D, et al. Effect of diet containing phytate and phytase on the activity and messenger ribonucleic acid expression of carbohydrase and transporter in chickens[J]. Journal of animal science, 2008, 86(12): 3432-3439.

[11] Wyss M, Pasamontes L, Friedlein A, et al. Biophysical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance[J]. Applied and Environmental Microbiology, 1999, 65(2): 359-366.

[12] Laboure A M, Gagnon J, Lescure A M. Purification and characterization of a phytase (myo-inositol-hexakisphosphate phosphohydrolase) accumulated in maize (Zea mays) seedlings during germination[J]. Biochem. J, 1993, 295: 413-419.

[13] Harland B F, Harland J. (1980). Fmermentative reduction of phytase in rye ,white , and whole wheat breads[J] . Cereal Chemistry, 1980,57(3): 226 - 229.