

# 毕赤酵母中植酸酶和内切葡聚糖酶共表达菌株的构建及其高效表达

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**摘要:** 植酸酶和内切葡萄糖苷酶广泛应用于动物饲料添加剂中, 能更有效地帮助饲料的利用, 同时为动物的生命活动提供必需的重要物质。首先构建重组质粒 pPICZ $\alpha$ -EG, 经过线性化后, 电转化到含有植酸酶 phyA 基因的毕赤酵母感受态细胞中, 构成含有植酸酶基因和内切葡萄糖苷酶基因的重重组体菌株 GS115-phyA-EG。经甲醇诱导后其活性分别达到出发菌株 GS115-phyA 和 GS115-EG 的 39.4% 和 56.2%。酶学性质的分析显示, 最适反应温度和最适反应的 pH 值分别为 55°C 和 5.5, 植酸酶和内切葡萄糖苷酶在温度为 45°C~55°C, pH 值为 4.5~5.5 时, 酶活相对稳定, 均能达到最高酶活的 80% 以上。结果表明这种在同一个系统种同时表达 2 种酶的方法能更好地节约时间和成本, 使其在饲料工业的应用上有更广阔的前景。

**关键词:** 植酸酶, 内切葡聚糖酶, 毕赤酵母, 混合表达

## Construction of a *Pichia pastoris* recombinant strain capable of over-expressing phytase and endoglucanase

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**Abstract:** Both phytase and endoglucanase are additives in feed for mono-gastric animal known for their effects. Recombinant vector pPICZ $\alpha$ -EG was constructed and transformed to GS115-phyA, a *Pichia pastoris* strain that had integrated with phytase gene, generating GS115-phyA-EG. Both phytase and endoglucanase activities in the supernatant were determined after methanol induction of GS115-phyA-EG. Phytase and endoglucanase activity reached 39.4% and 56.2% activity compared to GS115-phyA and GS115-EG, respectively. Properties of the mixed enzyme suggest that the optimal temperature and pH value be 55°C and 5.5 respectively. Both phytase and endoglucanase showed greater than 80% activity across temperature ranges 45°C to 55°C and pH ranges 4.5 to 5.5. Expressing more than one enzyme in one system could save time and money during induced expression, and the mixed enzyme might apply for treating forge before feeding with poultry.

**Keywords:** phytase, endoglucanase, *Pichia pastoris*, mix-expression

### Introduction

Phytases (myo-inositol hexakisphosphate phosphohydrolase;

EC 3.1.3.8) belong to the family of histidine acid phosphatases and are ubiquitous in plants and fungi. They catalyze phosphomonoester cleavage of phytic

acid (myo-inositol hexakisphosphate), which is the major storage form of phosphorus in plants [1]. Supplementing with inorganic phosphate to feed can solve the problem that non-ruminants such as pigs and poultry, virtually lack phytase activity in their digestive tract. However, this supplementation imposes ecological problems. Since the application of the phytic acid has been available in animal feeds, phytase been a good choice to take place of inorganic phosphate, which has solved ecological problems to some extent [2]. Cellulase is a term for a group of enzymes able to transform cellulose to glucose. This group mainly consists of endo-1,4- $\beta$ -glucanase (EC 3.2.1.4), 1,4- $\beta$ -D-cellobiohydrolase (EC 3.2.1.9) and 1,4- $\beta$ -glucosidase (EC 3.2.1.21). These three enzymes cooperate to translate cellulose to glucose completely. The major sources of these enzymes are microorganisms, including bacteria and fungi, especially those that utilize cellulose as their energy source [3-4]. Endoglucanases, especially from fungi, have drawn the most interest in the previous cellulase study [5]. Recently, bacterial cellulases have taken on satisfactory application performance and economic value, because they show relative stability in neutral and basic conditions [6].

Both phytases and endoglucanases are additives in forage for mono-gastric animal, which acknowledged for their effects [7]. However, most of enzymes may be digested by proteinases *in vivo*, which reduced their effectiveness as additives. Therefore, treating forage with mixed enzymes *in vitro* would be more effective for feeding domestic animals. Expressing more than one enzyme in a suitable expression system simultaneously became available for obtaining mixed enzymes.

*Pichia pastoris* is a methylotrophic yeast, well-known for its ability to express high levels of heterologous proteins [8-9]. We have constructed a recombinant *Pichia pastoris* strain, capable of over-expressing phytase and endoglucanase simultaneously. The properties of phytase and endoglucanase expressed in this system have also been discussed.

## 1 Materials and methods

### 1.1 Experimental materials

*Escherichia coli* DH5 $\alpha$  was used as host for DNA manipulation and transformation. DH5 $\alpha$  which contains endoglucanase gene was constructed in our laboratory [10]. Plasmid pPICZ $\alpha$  was used for endoglucanase expressing vector construction. *Pichia pastoris* GS115 and

GS115-*phyA* which contains *phyA* gene, were used as host for electroporation and expression. These strains were constructed and stored in our laboratory [11]. *Pfu* DNA polymerase (TIAGEN Beijing, China) and the restriction enzymes (TaKaRa Dalian, China) used in this study were commercially available; and the primer pair was synthesized by Invitrogen (Shanghai, China).

### 1.2 Recombinant vector construction for endoglucanase expression

Amplification of endoglucanase gene was carried out by *Pfu* DNA polymerase, and a pair of primers were designed and synthesized. The forward and reverse primers were 5'-CCGGAATTCGCAGAGACAAAAACGCCAGT-3' and 5'-GAATGCGGCCGCCTAATTTGGTTCTGTTC-3'; the underline indicated *EcoR* I and *Not* I site. The PCR products were digested by *EcoR* I and *Not* I restriction enzymes then ligated into pPICZ $\alpha$  at the corresponding restriction sites, to generate pPICZ $\alpha$ -END. The recombinant was identified by restriction analysis and DNA sequencing.

### 1.3 Transformation of *P. pastoris* GS115 and GS115-*phyA* with pPICZ $\alpha$ -END

An amount of 10  $\mu$ g pPICZ $\alpha$ -END plasmid was linearized with *Sac* I, and then electroporated into *P. pastoris* GS115 and GS115-*phyA* containing *phyA* gene. The transformants were plated on YPD-Zeocin agar plates (1% yeast extract, 2% peptone, 2% glucose and 100  $\mu$ g/mL zeocin), and incubated at 30°C for 2-4 days until colonies obtained. GS115-*phyA* which integrated with pPIC9k-*phyA* plasmid didn't grow in YPD-Zeocin agar plates. Only the colonies inserted with pPICZ $\alpha$ -END were able to grow on this culture. The recombinant *P. pastoris* GS115 strains were named GS115-END and GS115-*phyA*-END, respectively (Fig. 1).

### 1.4 Expression of GS115-END and GS115-*phyA*-END in shaking flask

The colonies were selected randomly and inoculated into 3 mL BMGY medium (2% peptone, 1% yeast extract, 100 mmol/L potassium phosphate (pH 6.0), 1.34% YNB, 1% glycerol and  $4 \times 10^{-5}$ % biotin) in 50 mL flasks. After shaken at 30°C for 18-22 h, the cells were collected by centrifugation (4 000 r/min, 10 min), then resuspended in 5 mL BMMY medium (BMMY with 0.5% methanol instead of 1% glycerol in BMGY). The culture was shaken for 48 h at 30°C before measuring the enzymatic activity in supernatant. 100% methanol was supplemented every 24 h to a final concentration of 1% for maintaining induction throughout the induction phase. GS115-*phyA*,

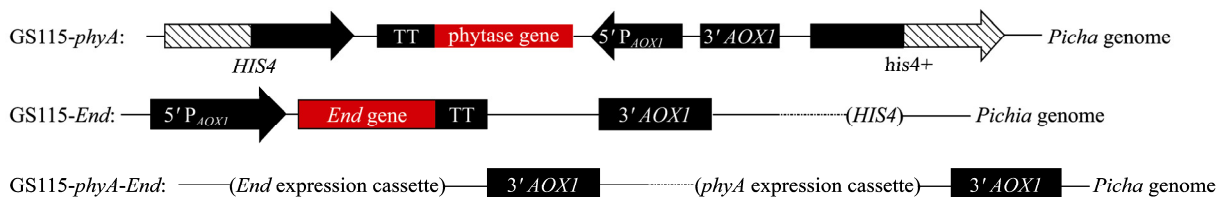


Fig. 1 Schematic diagram of construction of GS115-*phyA-End*.

GS115-*END* and GS115-*phyA-END* were expressed. Each strain was inoculated into 25 mL BMGY medium in 250 mL flasks. The cells were collected until the  $OD_{600}$  reached 2–6, then resuspended with 100–200 mL BMMY medium (the final cell density  $OD_{600}=1$ ) in 1 000 mL flasks [12]. GS115-*phyA* and GS115-*END* were also cultured and expressed together for mix-expression of phytase and endoglucanase to compare with the capability of GS115-*phyA-END*. After expressed for 4 d at 30°C, the supernatants were used for determination of enzymatic activity and enzymatic characters.

### 1.5 Enzymatic activity assays

The activities of phytase and endoglucanase were determined in the culture supernatants. The phytase activity was estimated by determining the free-phosphorus concentration and following the method described by Han *et al* [13]. The crude enzyme was diluted with acetic acid buffer (pH 4.6), and 2 mL diluted enzyme was transferred to a 10 mL tube. Then, 4 mL sodium phytate (diluted with acetic acid buffer) was added to start the reaction. The reaction was stopped by adding 4 mL color/termination solution after incubating at 37°C for 30 min. Blanks were enzyme with color/termination solution before adding sodium phytate.

The endoglucanase activity was determined using classical method described by Mandels *et al* [14]. In this method CMC-Na was used as substrate; the amount of reduced sugars which released from the assay was detected by the DNS method. 200  $\mu$ L supernatant was diluted to 1 mL PBS buffer (pH 6.0). 100  $\mu$ L diluted enzyme and 1 mL 1% CMC-Na were mixed in a 10 mL tube and incubated at 50°C for 30 min. The reaction was terminated adding 2.5 mL DNS, then boiled for 5 min. Blanks were measurement of the endoglucanase activity before adding CMC-Na.

### 1.6 Enzyme characterization assays

The optimal temperature for reaction was determined at various temperatures from 30°C to 85°C in corresponding buffer. To determine thermostability, the enzyme was pre-incubated at 40°C to 85°C in

buffer for 30 min, then perform the standard activity assay. The enzyme and substrate were diluted to pH range from 3.0 to 10.0 using various buffers to estimate the optimal pH. Also the enzyme was pre-incubated at 50°C with different buffers which has the pH range from 3.0 to 10 to determine the pH stability. The standard DNS activity assay was then performed.

### 1.7 Genome isolation and amplification of genomic DNA

Chromosome DNA isolation and genome PCR were performed in order to check the integrity of phytase and endoglucanase gene in *Pichia* genome DNA. *Pichia* genomic isolation was performed following the method described by Holm *et al* [15]. Genome PCR was completed through a standard PCR amending template with *Pichia* genome DNA. The primers used for amplifying phytase [16] and endoglucanase genes were added respectively to the reaction.

### 1.8 The genetic stability of the recombinant strain

The recombinant colonies were selected and inoculated into 25 mL BMGY medium and incubated at 30°C in a shaking incubator until  $A_{600}$  of the culture medium reaches 2–6. The cells were centrifuged at 4 000 r/min for 10 min then resuspended in 100 mL BMMY medium. During the 108 h incubation period, methanol was added every 24 h to reach a final volume fraction of 1%. Then the enzyme activity was determined and 1ml cells were used for the next inducible expression. And this experiment was performed for 10 generations of the recombinant strain.

## 2 Results

### 2.1 Expression of endoglucanase and phytase enzyme

The recombinant vector pPICZ $\alpha$ -*END* was constructed and identified by restriction analysis and DNA sequencing. Then, pPICZ $\alpha$ -*END* plasmid was linearized with *Sac* I, and electroporated into *P. pastoris* GS115 and GS115-*phyA*. Ten colonies from each strain were randomly selected and methanol inductions continued for 48 h. Crude enzyme in supernatant was

measured through standard activity assay. Only two colonies of GS115-*phyA-END* had activity of both phytase and endoglucanase; the rest colonies had either phytase activity or endoglucanase activity (Table 1).

The 4th and 8th colonies showed both activity of phytase and endoglucanase, however, they lost both enzymatic activities to some extent. The phytase activity of these two colonies was only about half of it in other strains. Meanwhile, endoglucanase activity reached to approximately 80%. The 6th and 4th in GS115-*END* and GS115-*phyA-END* were selected for the following experiments.

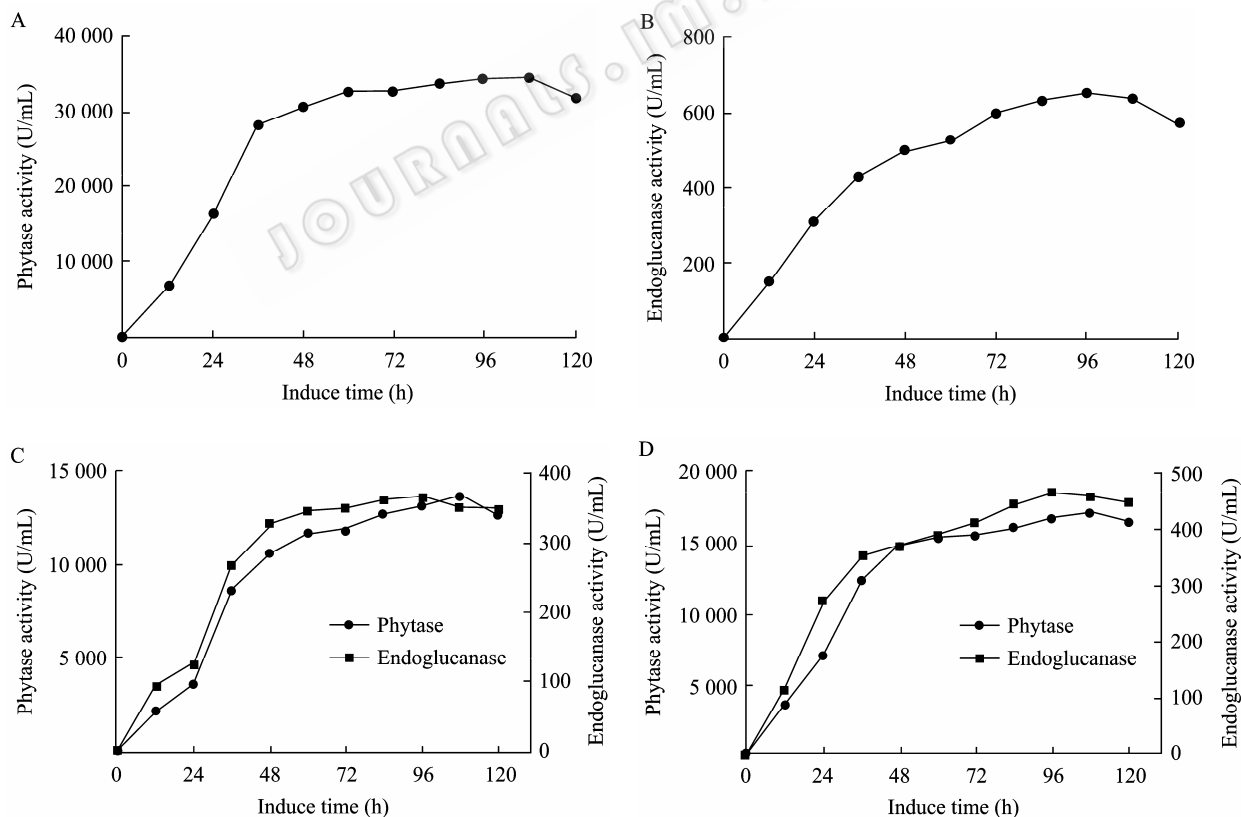
The GS115-*phyA*, GS115-*END* and GS115-*phyA-END* were performed expression in scale, the supernatants at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h were sampled for enzymatic activity determination (Fig. 2).

Supernatants at 96–108 h reached the maximal activity. GS115-*phyA-END* showed 13 726.7 U/mL phytase activity and 364.5 U/mL endoglucanase activity, 39.4% of GS115-*phyA* (34 820.6 U/mL) and

56.2% of GS115-*END* (649.0 U/mL). In the mix-expression flask, both the activities of phytase and endoglucanase are also reduced, which suggested the total expressing mass of heterologous proteins in *P. pastoris* may have a limitation.

**Table 1** Enzymatic activity screening of GS115-*END* and GS115-*phyA-END*

GS115- <i>END</i>	Endoglucanase activity (U/mL)	GS115- <i>phyA-END</i>	Enzymatic activity (U/mL)	
			Phytase	Endoglucanase
1	368.4	1	18 940	0
2	409.7	2	23 857	0
3	421.7	3	22 381	0
4	398.3	4	8 667	256.4
5	406.2	5	22 160	0
6	426.8	6	0	336.9
7	386.9	7	18 225	0
8	408.4	8	7 280	298.6
9	374.5	9	0	371.4
10	394.3	10	21 485	0



**Fig. 2** Phytase and endoglucanase activity determination of *P. pastoris* recombinant strains. (A) Phytase activity of GS115-*phyA*. (B) Endoglucanase activity of GS115-*END*. (C) Phytase and endoglucanase activities of GS115-*phyA-END*. (D) Phytase and endoglucanase activities of mix-expression of GS115-*phyA* and GS115-*END*.

## 2.2 Enzymatic characters assays

Phytase and endoglucanase mixed enzyme properties were determined using crude enzyme expressed in GS11-*phyA-END*. The optimal temperatures of phytase and endoglucanase were 55°C and 65°C, respectively. The crude enzyme exhibited above 60% both phytase and endoglucanase activities across narrow temperature (35°C–55°C) ranges (Fig. 3).

Both of their activity lost much after being treated at 70°C for 30 min (Fig. 4).

The optimal pH values of the two enzymes were 4.5 and 6.0, respectively. The pH ranges where both phytase and endoglucanase can reach above of 60% of their highest activities were between 4.0–6.0 (Fig. 5).

The phytase was stable at acid conditions (pH 3.0–5.5), while the endoglucanase can preserve most of activity at neutral and basic conditions (even when pH value reached 10.0) (Fig. 6).

## 2.3 *Pichia* genomic isolation and genome PCR

Chromosome DNA isolation and genome PCR were performed to verify the integration of *phyA* gene and endoglucanase gene in *Pichia* genome DNA. Genome DNAs of GS115-*phyA*, GS115-*END* and GS115-*phyA-END* were then used as templates for amplifying phytase and endoglucanase gene (Fig. 7).

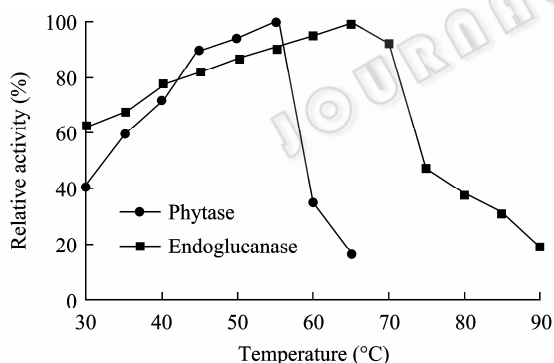


Fig. 3 Optimal temperature of phytase and endoglucanase.

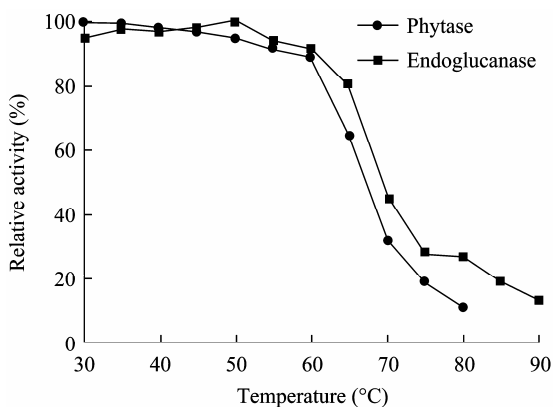


Fig. 4 Thermostability of phytase and endoglucanase.

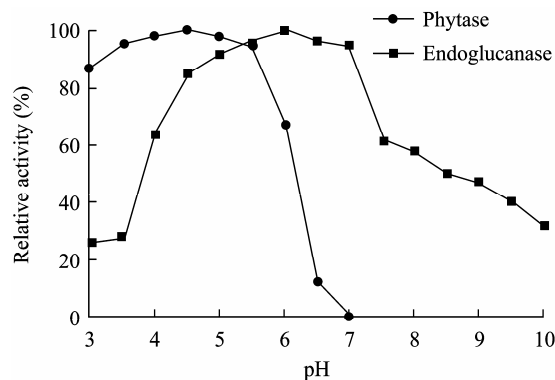


Fig. 5 Optimal pH profile of phytase and endoglucanase.

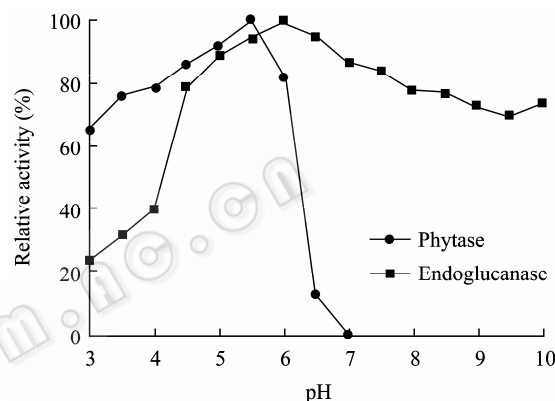


Fig. 6 pH stability of phytase and endoglucanase.

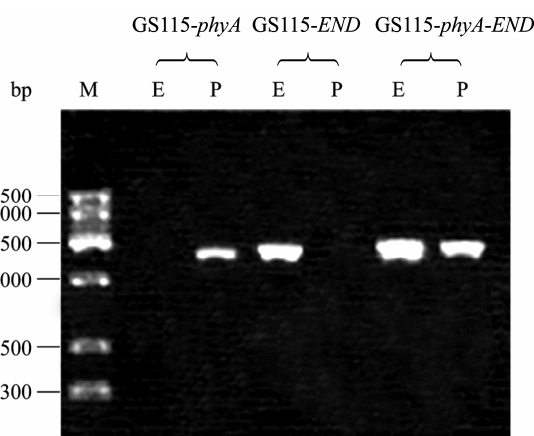


Fig. 7 Genome PCR of GS115-*phyA*, GS115-*END* and GS115-*phyA-END*. M: DNA marker VII; E and P indicate genome PCR were performed adding the primers amplifying endoglucanase and phytase gene, GS115-*phyA* genome DNA can be only used for amplifying *phyA* gene fragment, and it is contrary to GS115-*END*. However, applying GS115-*phyA-END* genome DNA as template, we only obtained *phyA* and endoglucanase gene fragments in accordance to the corresponding primers. The results indicated that both phytase and endoglucanase gene have integrated into the GS115-*phyA-END* chromosome DNA.

## 2.4 The genetic stability of the recombinant strain

The results of 10 generations of consecutive culture and the activity determination indicated that the recombinant strain GS115-*phyA-END* remained stable on the growth and the expression of phytase and endoglucanase. This result showed that the recombinant strain is genetic stable (Table 2).

**Table 2 Generations and enzymatic activity of GS115-*phyA-END***

GS115- <i>phyA-END</i> (generations)	Enzymatic activity (U/mL)	
	Phytase	Endoglucanase
1	20 345	287.4
2	23 175	355.1
3	24 079	306.8
4	19 273	367.5
5	18 521	269.2
6	20 947	379.4
7	18 903	347.6
8	22 548	298.3
9	18 943	383.5
10	24 051	398.6

## 3 Discussion

*Pichia pastoris* is methylotrophic yeast. It is well-known for its ability to express high levels of heterologous proteins. Many heterologous proteins have been reported over-expression through this system [8-9,17]. *Aspergillus Niger* N25 phytase gene (*phyA*) has been expressed in *Pichia pastoris* in our previous research, and the phytase activity reached above 30 000 U/mL which is more than 50 fold compared with the original strain [16]. Many factors, such as integration form, integration sites and multicopy inserts, can affect the heterologous proteins expressing level in *Pichia pastoris* [18]. Multiple copy insertion in *Pichia* has been reported to improve the expressing level in some cases, and some expressing vectors, such as pPIC9k and pAO815, have been designed for multicopy insertion *in vivo* and *in vitro* respectively [19-20]. Integration form has also been demonstrated as an important factor in affecting the expressing level. Heterologous fragments can be inserted into *Pichia pastoris* chromosome DNA at *AOX1* and *HIS4* region according to the linearization sites of expressing vector [8].

Both phytase and endoglucanase are additives in forage for mono-gastric animal, which are often pelleted, and then added to animal feeds. However,

many natural enzymes can't withstand the temperatures during the pelleting process [21]. In the other hand, most of enzymes may be digested by proteinase *in vivo*, which reduced much of their effect as additives [7]. Treating forage with mixed enzymes *in vitro* was considered more effective for feeding domestic animal, and more than one enzyme expressed in *Pichia pastoris* can be suitably developed for obtaining mixed enzyme expediently.

In this study, endoglucanase gene has integrated into GS115-*phyA* genome DNA containing phytase gene. Activity screening was performed to obtain recombinant *Pichia* containing both phytase and endoglucanase gene. This recombinant strain was named GS115-*phyA-END*. Some strains showed either phytase or endoglucanase activity, which demonstrated several integration form happened, such as insertion and replacement. In our previous study, pPIC9k-*phyA* was linearize with *BspE* I then transformed into GS115. Therefore the integration site located at *HIS4* region [10]. In this study, pPICZ $\alpha$ -*END* was inserted into *AOX1* region of *Pichia* chromosome DNA. Genome PCR of GS115-*phyA*, GS115-*END* and GS115-*phyA-END* was performed to verify the insertion, and the results indicated that both phytase and endoglucanase gene had inserted into the GS115-*phyA-END* genome DNA. Phytase and endoglucanase activities expressed in GS115-*phyA-END* were 13 726.7 U/mL and 364.5 U/mL, 39.4% of GS115-*phyA* (34 820.6 U/mL) and 56.2% of GS115-*END* (649.0 U/mL), respectively. GS115-*phyA* and GS115-*EG* were cultured and expressed in a flask for controlling the two enzymes expression level of GS115-*phyA-END*. The results showed that both the two enzymatic activities also reduced above the half of the highest activities. The total expression mass of heterologous proteins in *Pichia* may have a limitation, especially with a constant capacity. However, inserting two genes into one strain provided convenience for strain store, expression, and so on. Some colonies which resistant to zeocin, showed low endoglucanase activity (about 80 U/mL). The cause may be the genetic stability of transformant [22], which will be discussed in the future study.

The enzymatic properties of phytase and endoglucanase expressed in GS115-*phyA-END* were determined in this study. Both of the enzymes were demonstrated same as expressed alone. The crude enzyme exhibited more than 60% both phytase and endoglucanase activities between temperature range

35°C to 55°C, and the optimal temperature of the mixed enzyme was estimated to be 55°C. The pH values where both phytase and endoglucanase can reach above 60% of their highest activities were located at pH 4.0–6.0 range, and pH 5.5 was considered as the optimal pH. Expressing more than one relative enzyme in one system offered convenience for obtaining these enzymes simultaneously, which can save time and money for induced expression. In the other hand, since it was reported forage additives would reduce by digestion of proteinase *in vivo*, treating forage with mixed enzyme before feeding with animal was considered as more effective strategy. These enzymatic properties could be convenient for application of the mixed enzyme to industry.

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