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BIOENERGY AND BIOFUELS

Display of phytase on the cell surface of *Saccharomyces cerevisiae* to degrade phytate phosphorus and improve bioethanol production

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Abstract Currently, development of biofuels as an alternative fuel has gained much attention due to resource and environmental challenges. Bioethanol is one of most important and dominant biofuels, and production using corn or cassava as raw materials has become a prominent technology. However, phytate contained in the raw material not only decreases the efficiency of ethanol production, but also leads to an increase in the discharge of phosphorus, thus impacting on the environment. In this study, to decrease phytate and its phosphorus content in an ethanol fermentation process, *Saccharomyces cerevisiae* was engineered through a surface-displaying system utilizing the C-terminal half of the yeast α-agglutinin protein. The recombinant yeast strain, PHY, was constructed by successfully displaying phytase on the surface of cells, and enzyme activity reached 6.4 U/g wet biomass weight. Ethanol productions using various strains were compared, and the results demonstrated that the specific growth rate and average fermentation rate of the PHY strain were higher 20 and 18 %, respectively, compared to the control strain *S. cerevisiae* CICIMY0086, in a 5-L bioreactor process by simultaneous saccharification and fermentation. More importantly, the phytate phosphorus concentration decreased by 89.8 % and free phosphorus concentration increased by 142.9 % in dry vinasse compared to the control in a 5-L bioreactor. In summary, we constructed a recombinant *S. cerevisiae* strain displaying phytase on the cell surface, which could improve ethanol production performance and effectively reduce the discharge of phosphorus. The strain reported here represents a useful novel engineering platform for developing an environment-friendly system for bioethanol production from a corn substrate.

Keywords Bioethanol · *Saccharomyces cerevisiae* · Phytase · Surface-displaying expression

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Introduction

About 2.9 TW liquid transportation fuels are consumed globally, and these fuels are currently mainly derived from petroleum (Nielsen et al. 2013). However, conventional petroleum-based chemical industries are now facing great socio-political challenges due to the increasing concerns on climate change and limited fossil resources (Hahn-Hägerdahl et al. 2006; Lee et al. 2015). With the availability and abundance of biomass resources, as well as environmental pollution caused by fossil

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fuels, development of biofuels as an alternative fuel has gained a lot of attention in recent times (Alper and Stephanopoulos 2009; Chen et al. 2013). Biofuels only account for 2.7 % of the total transportation energy because of the barriers that exist in both the technical and economic aspects of biotechnological production (Alper and Stephanopoulos 2009). Several disadvantages still remain in commercial-scale cellulosic ethanol production, more specifically, substrate recalcitrance, enzyme cost, and mass transfer constraints (Alper and Stephanopoulos 2009; Nielsen et al. 2013). Currently, by far, the dominant biofuel is ethanol, which is being produced at 75 billion liters annually, with the majority being produced in the USA (50 billion liters) with corn as the major feedstock (Gray et al. 2006; Nielsen et al. 2013).

Nowadays, dry grind is still the predominant process for bioethanol production from grains (mainly corn). In this process, distillers dried grains with solubles (DDGS) as a major coproduct is produced through several major steps, including dry milling, liquefaction, saccharification, fermentation, distillation, and coproduct recovery (Bothast and Schlicher 2005). In recent years, the quantity of DDGS has increased significantly with the increasing production of fuel ethanol. Owing to its rich source of various nutrients for animal feed such as protein, amino acids, and phosphorus, there are many reports on the chemical composition of DDGS and their variability (Liu 2011). However, different DDGS sources lead to high variations in the concentrations of minerals, which not only limits its value but also impacts its end use as animal feed (Liu and Han 2011). For example, recent studies showed that high phosphorus content in DDGS has made more phosphorus excretion in livestock wastes, which inevitably create environmental concerns (Liu and Han 2011; Spiehs and Varel 2009). Moreover, bioavailability of phosphorus in DDGS is another important factor that affects its value and application in animal feed. There are three types of phosphorus in DDGS and grains, inorganic phosphorus (also known as phosphate phosphorus or free phosphorus), phytate phosphorus, and other phosphorus (the sum of all phosphorus-containing compounds in a sample other than phytate phosphorus and inorganic phosphorus), in which the form of phosphorus determines its bioavailability. Specially, phytate is found in most cereal seeds and the main storage form of phosphorus in grains (Lott et al. 2000). Phytate phosphorus in DDGS leads to increased phosphorus discharge into the environment because of its lower bioavailability than inorganic phosphorus by monogastric animals (He et al. 2009; Lott et al. 2000). On the other hand, previous studies have shown that phytate can interact with various dietary components including starch and divalent cations, which therefore decreases their availability to humans and animals (Liu 2011). Liu and Han (2011) investigated the levels of different types of phosphorus in DDGS and found that phytate phosphorus had 2.54-fold of increase over corn during fermentation. Therefore, increasing bioavailability of phytate phosphorus in DDGS is a critical issue with both animal nutrient and environmental concerns.

Recently, Mikulski et al. (2014) investigated the effect of phytase hydrolysis of phytate complexes on starch availability during the alcoholic fermentation process using high-gravity maize mashes. Their observations showed that addition of phytase resulted in a better availability of starch for enzymatic hydrolysis and higher fermentation yield (Mikulski et al. 2014). To reduce phytates in DDGS and corn gluten feed, Noureddini and Dang (2009) evaluated the effect of phytase on the dephosphorylation of phytates in light steep water (LSW) and whole stillage (WS) and the results showed that approximately 90 % phytate phosphorus of LSW and 66 % phytate phosphorus of WS were released, respectively. The maximum amounts of released phosphorus of 4.52 mg/g LSW and 0.86 mg/g WS were obtained, respectively. It is worthwhile to note that addition of exogenous phytase can decrease phytates and corresponding phosphorus content; however, this process increases the economic cost because of high cost of phytase.

In this study, to efficiently and cost-effectively decrease phytate phosphorus content in an ethanol fermentation process, *Saccharomyces cerevisiae* was engineered for heterologous expression of phytase on the cell surface and its application was evaluated extensively.

Materials and methods

Strains and media

Escherichia coli JM109 was used for gene cloning and routinely cultured at 37 °C in Luria-Bertani medium (1 % peptone, 0.5 % yeast extract, and 1 % sodium chloride). *S. cerevisiae* CICIMY0086, an industrial ethanol producer, was used as an expression host. All yeast strains were grown in yeast extract peptone dextrose (YPD) medium (1 % yeast extract, 2 % peptone, 2 % glucose) or synthetic complete (SC) medium (0.67 % yeast nitrogen base without amino acid, 2 % glucose) supplemented with the appropriate concentrations of antibiotic geneticin (G418). Incubation conditions were standardized on a rotary shaker at 30 °C with 200 rpm.

Construction of the recombinant plasmids

The basic plasmid for cell surface display, pMGK-*AG*, with the strong constitutive expression (*PGK1*) promoter (Fig. 1a), was constructed as described below. Using *S. cerevisiae* genomic DNA as template, the *PGK1* promoter and terminator were amplified by PCR with primers PPF, PPR, TPF, and TPR, respectively (Table 1). The two fragments were digested with *Sal*I and then inserted into the pMD18-T simple vector

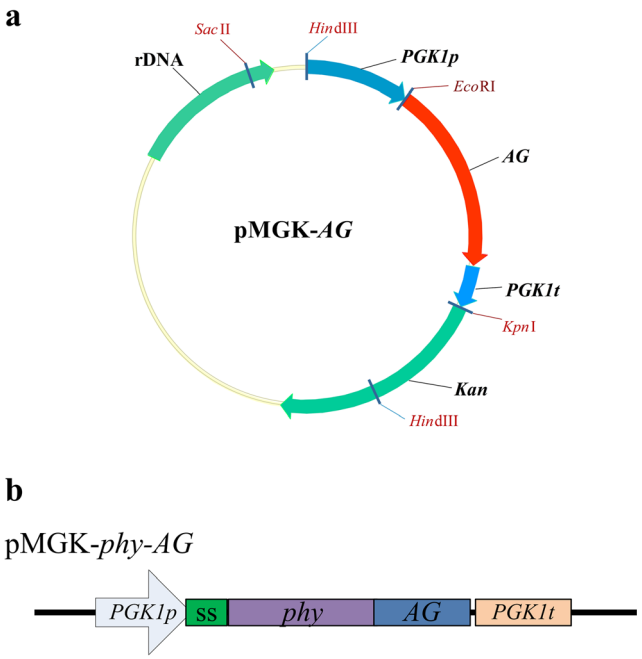

Fig. 1 Construction of the recombinant plasmid for cell surface display of phytase. **a** Expression plasmid (pMGK-AG) for display on the *S. cerevisiae* cell surface. **b** Structure of relevant part of cell surface display plasmid of pMGK-*phy-AG*. PGK promoter and PGK terminator were used. *AG* C-terminal half of the yeast α-agglutinin gene, *phy* phytase gene, *ss* α secretion signal

(TaKaRa, Dalian, China) to yield recombinant plasmid pMG. The G418 resistance gene was amplified from the plasmid pPIC9K using primers KNF and KNR (Table 1) and cloned into the *Not*I site of pMG to yield pMG1. A ribosomal DNA (rDNA) fragment, which was used as homologous integration site for phytase expression, was amplified from *S. cerevisiae* using primers rRF and rRR (Table 1) and inserted into the *Nde*I site of pMG1 to yield plasmid pMGK. An anchor sequence of the C-terminal half of the yeast α-agglutinin gene (*AGα1*) (Van der Vaart et al. 1997), designated *AG1*, was amplified by PCR using primers AGF and AGR (Table 1). This fragment was digested with *Sal*I and inserted into the vector pMGK, resulting in plasmid pMGK-*AG* (Fig. 1a). The *phy* gene, encoding codon-optimized phytase (Accession number: JQ976672.1), was cloned by PCR amplification using PhyF1 and PhyR primers (Table 1) and using pKK-*phy* plasmid (pKK223-3 cloning vector, accession number: M77749.1) as template. After blunting by *pfu* polymerase, the 1236-bp *phy* gene fragment was inserted into the pPIC9K vector at the *Sna*BI site, resulting in plasmid pPIC9k-*phy*. Then, the fragment containing α-factor signal sequence fused with the *phy* gene was obtained by PCR using plasmid pPIC9K-*phy* as template and P1 and phyR as primers (Table 1). The amplified fragment was treated by nuclease *S1* and then inserted into the plasmid pMGK-*AG* at the *Eco*RI site, which was treated also by nuclease *S1*. The resulting plasmid was pMGK-*phy-AG* (Fig. 1b). All primers used in this study are listed in Table 1.

Yeast transformation

The plasmid pMGK-*phy-AG* was cleaved with *Sac*II. After purification, the resultant linear DNA fragment was introduced into the ethanol-producing strain *S. cerevisiae* CICIMY0086 (deposited in CICIMCU, http://cicim-cu.jiangnan.edu.cn/) by the lithium acetate method (Ito et al. 1983). For the initial selection of yeast transformants, G418 was added to a final concentration 0.25 mg/mL. Transformants with the G418 resistance phenotype were isolated. Then, a higher concentration of G418, 0.5 mg/mL, which provides a stronger selective pressure leading to higher copy numbers was used (Wang et al. 2004).

Table 1 Sequence of the oligonucleotide primers used in this study

Primer name	Primer sequence (5′—3′)	Restriction enzyme
PPF	ATTTTAGATTCCTGACTTCAACTC	
PPR	GCG*GTCGACGGATCC*TGTTTTATATTTGTTGTAAAAAGTAG	*Sal*I, B*am*HI
TPF	CCG*GTCGAC*TTCTTTGGAATTATTGGAAGGTA	*Sal*I
TPR	GCG*GCGGCCGC*GAACGCAGAATTTTCGAGTTAT	*Not*I
KNF	AG*GCGGCCGC*GCCCAGTAGTAGGTTGAGG	*Not*I
KNR	AG*GCGGCCGC*TTGAAGTCGGACAGTGAGT	*Not*I
P1	CCGGAATTCCGATGAGATTTCCTTCAA	*Eco*RI
PhyF1	CAGAGTGAGCCTGAGTTGAAA	
PhyR	CCGGAATTCTTACTACAAGGAACAAGCTGG	*Eco*RI
AGF	CCG*GTCGAC*AGCGCCAAAAGCTCTTTTATC	*Sal*I
AGR	CCG*GTCGAC*TTTGATTATGTTCTTTCTAT	*Sal*I
rRF	CCG*CATATG*CTCTATCCCCAGCACGA	*Nde*I
rRR	CCC*CATATG*GAGAAACGGCTACCACATC	*Nde*I

Measurement of enzyme activities

Supernatant and cell-associated samples were obtained by centrifuging crude samples at 10,000 rpm for 8 min. Cell pellets were washed twice with buffer (50 mM acetic acid/sodium acetate buffer, pH 3.0) in order to determine the intact cell pellet enzyme activity and isolate cell wall fractions. The cells were disrupted using a sonic dismembrator (VC750, Sonics, Newtown, USA) with 30 % of the total working energy for 5 min at 0 °C, and the cell wall fractions were recovered by centrifugation of the homogenate at 10,000 rpm for 5 min and washed with the same buffer.

Phytase activity was determined according to a previously reported method (Bennett and Yang 2012) with slight modifications. Briefly, 200 μL of crude enzyme extract was combined with 800-μL substrate solution consisting of 625 mM sodium phytate in 0.1 M Tris-HCl buffer (pH 7.0) and incubated at 37 °C for 30 min. The reaction was terminated with the addition of 1-mL 5 % (*w*/*v*) trichloroacetic acid solution.

Enzyme blanks as control were also prepared according to the above reference. The optical density was detected at 700 nm with a spectrophotometer (UNIC UV2000, Shanghai, China) and compared with a standard made from K_2HPO_4 solution increasing in 0.25 mg/mL increments from 0 to 1.0 mg/mL. One phytase unit of activity is defined as the amount of the enzyme, which liberates 1.0 μmol/min of inorganic phosphorus from 5 mmol/L sodium phytate under the optimal conditions. The specific phytase activity is presented as enzyme activity per gram wet cell mass (U/g).

Ethanol fermentation

Simultaneous saccharification and fermentation (SSF) was carried out in a flask and a 5-L bioreactor (Bailun Biotechnology, Shanghai, China) as described previously with a slight modification (Guo et al. 2010). The fermentation medium was prepared by dispensing the corn powder in deionized water at a ratio of 1:3 (*w*/*v*) of corn powder to water. An aliquot of 10 U of thermostable α-amylase was added to 1-g corn powder and incubated for 2 h at 95 °C and pH 6.0. This was followed by glucoamylase and urea addition at concentrations of 130 U/g and 0.05 % (*w*/*w*), respectively. Yeast cells were precultured in 500-mL Erlenmeyer flasks at 30 °C in a seed medium (2 % glucose, 0.85 % yeast extract, 0.13 % NH_4Cl, 0.01 % $MgSO_4$, 0.006 % $CaCl_2$) until the optical density (OD) value reached 10.0 (approximately 18 h). This stationary phase culture was used to inoculate the flask or bioreactor with approximately 1.0×10^8 cells/mL. During the fermentation process, the temperature was maintained at 30 °C with no air supplied. The agitation rate was maintained at 100 rpm, and the pH was maintained at a constant 5.0 by addition of 2 M NaOH. All fermentation experiments were performed in triplicate.

Analytical methods

Yeast growth in YPD medium was monitored by measuring the OD at OD_{600} using a spectrophotometer (UNIC UV2000, Shanghai, China). During the ethanol fermentation process, direct detection of OD was difficult and inaccurate due to the presence of insoluble solids in the broth. Therefore, cell counts were used to determine the biomass concentration in the fermentation broth by the direct microscopic method using a hemocytometer (Thomas and Ingledew 1990). The specific growth rate of yeast was measured as described previously (Nissen et al. 1997).

The concentration of glucose and ethanol in filtered samples withdrawn from the batch cultivations was determined by HPLC (Agilent Technologies 1260 infinities, Santa Clara, USA) using an HPX87-H (Bio-Rad, Hercules, USA) column eluted with 0.05 M H_2SO_4 at 65 °C. The quantity of mineral elements, Zn^{2+}, Mg^{2+}, and Ca^{2+}, was determined with a Perkin-Elmer Optima 3200 inductively coupled plasma-optical emission spectrometer(ICP-OES, Waltham, USA) in an aqueous solution following nitric acid digestion of the samples, as previously described (Liu and Han 2011). The concentration of starch was determined according to a previously described method (Guo et al. 2010). Total sugar concentration was determined by the phenol sulfuric acid method, as described previously (Demoraes et al. 1995). Fermentation efficiency *Y* was calculated using the formula below:

Y = ethanol concentration / initial total sugar × 0.511 × 100 %.

Statistical analysis

A Student's one-tailed *t* test for paired samples was performed with the Microsoft Office 2013 Excel software (Microsoft, Redmond, USA). The mean values, standard deviations, and *P* values were calculated in all quantitative analysis. Statistical significance was accepted at $P < 0.05$ level.

Results

Expression of pMGK-*phy-AG1* fusion proteins on cell surface

To display phytase on the yeast cell surface, a recombinant plasmid of the pMGK-*phy-AG1* was constructed via an *AG1* anchor protein (Fig. 1). After linearization and purification, the resultant DNA fragments were introduced into the ethanol-producing strain *S. cerevisiae* CICIMY0086 by the lithium acetate method (Ito et al. 1983). Transformants with the $G418^R$ phenotype were isolated. Correct insertion of the gene into the rDNA locus was verified by colony PCR. The phytase activity of 20 transformant colonies was investigated. A recombinant denoted PHY (deposited in

China Center for Type Culture Collection, collection number: CCTCC M 2015190) expressing the enzyme on the cell surface was used in subsequent experiments. It showed an apparently high phytase activity of 6.4 U/g wet cell mass compared with the wild-type strain *S. cerevisiae* CICIMY0086, which produced no detectable phytase activity (Table 2). Furthermore, the cell wall showed more enzyme activity (9.2 U/g) when compared to supernatant of cell lysate which showed very low activity (0.7 U/mL) (Table 2), which indicated that the phytase was successfully expressed in the cell surface. Recombinant enzyme was characterized, and we found that phytase presented the highest phytase activity at pH 4.5 and 55 °C (data not shown).

Enzyme activity and its effect on the strain growth

Figure 2 shows the time course of phytase activity on the cell surface of *S. cerevisiae* harboring pMGK-*phy-AG1* cultivated in YPD medium at 30 °C. Phytase activity reached a maximum value of 6.5 U/g wet cell weight at 24 h while no detectable enzyme activity was observed in the culture medium. Interestingly, phytase activity of the cell-surface-displayed enzyme was not significantly changed by lyophilization (data not shown). All yeast strains reached stationary phase following cultivation for 16–20 h at 30 °C (Fig. 2). The enzymes were produced and accumulated on the cell surface during both growth and stationary phases. Phytase activity gradually increased after cell growth reached stationary phase. The amount of cells in the stationary phase was slightly reduced by cell surface expression of enzymes. The accessibility of phytase to protease was assayed by trypsin treatment on recombinant PHY cells cultivated for 24 h, and it was found that phytase activity decreased to 52 % compared to untreated cells (data not shown), which indicated that a large proportion of the phytase was anchored on the cell surface.

Table 2 Distribution of phytase activity in wild-type (CICIMY0086) and recombinant (PHY) *S. cerevisiae* strains

Strain	Enzyme activity[a]		
	Cell pellet[b]	Cell wall[b]	Culture supernatant
CICIMY0086	ND	ND	ND
PHY	6.4 ± 0.2 (U/g)	9.2 ± 0.3 (U/g)	ND

Data ± the standard deviation

CICIMY0086 wild type, *PHY* recombinant surface-expressing the phytase enzyme, *ND* not detectable

[a] Triplicate experiments

[b] Indicates the enzyme activity per gram wet cell mass. Cells were in exponential phase (OD600 nm = 2)

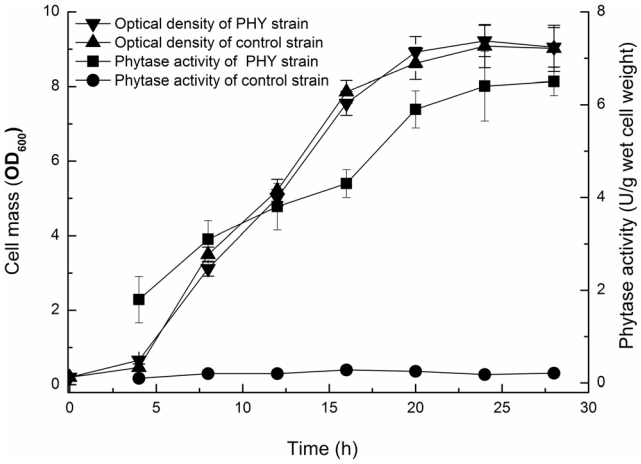

Fig. 2 Time course of phytase activity on the cell surface and cell growth (optical density at 600 nm). *S. cerevisiae* PHY harboring the plasmid pMGK-*phy-AG* and control strain were grown at 30 °C in YPD medium, respectively. The data points represent the average of three independent experiments (mean ± standard deviations)

In addition, Fig. 2 shows that the growth rate and final biomass of the recombinant strain PHY were similar to the control strain, which indicated that anchoring phytase on the cell surface did not have a significant impact or burden on the strain growth pattern. Therefore, the recombinant strain was further investigated for the ethanol production.

Optimization of ethanol production by expressing phytase on the cell surface

In shake flask fermentations, the recombinant strain PHY had a higher growth rate compared to the control strain during the early phase of growth with the final cell biomass of the PHY strain being 8.2 % higher than that of the control strain (Fig. 3). However, when the control strain was inoculated into the fermentation medium supplemented with 200 U/L exogenous phytase, the growth rate and final biomass were similar with those of the recombinant strain (Fig. 3a). The results demonstrated that either anchoring phytase on the yeast cell surface or exogenous addition of phytase into the fermentation medium could lead to an improvement in cell growth. Furthermore, the recombinant strain PHY produced 112.7 g/L ethanol, which was 5.5 % higher than the wild strain ($p < 0.05$, Fig. 3b). When extra phytase was added to the fermentation medium, more ethanol was also produced by the control strain compared with the control strain without phytase in the medium (Fig. 3b). On the other hand, the recombinant strain PHY utilized similar amounts of sugars and had a similar sugar consumption rate compared to the control strain in the fermentation medium regardless of whether containing extra phytase (Fig. 3b). The release of reduced sugars from corn dextrin was high due to high glucoamylase activity, but *S. cerevisiae* cell biomass was not high enough to use this

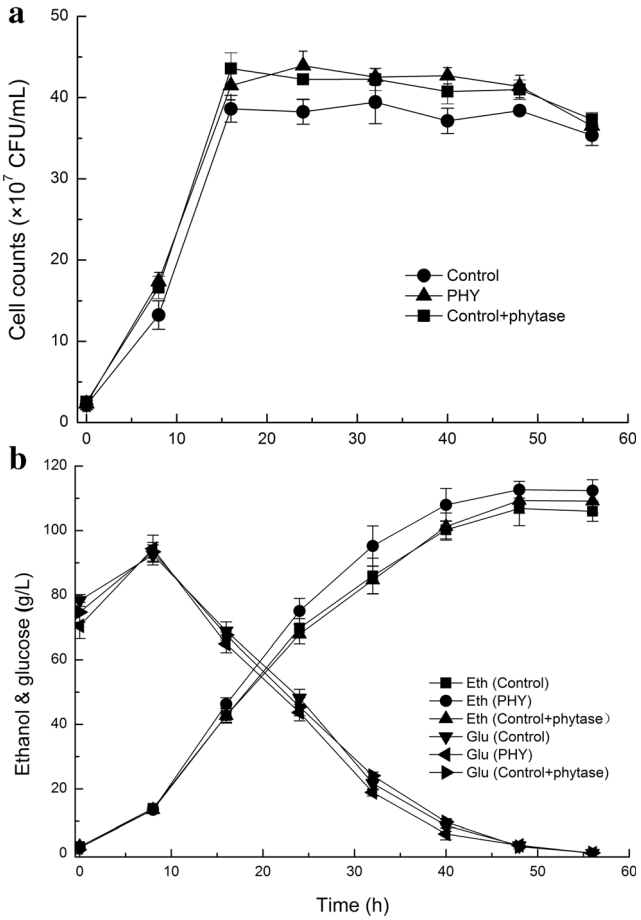

Fig. 3 Time course of ethanol production by fermentation of raw corn starch. **a** Time course of biomass of *S. cerevisiae* CICIMY008 (either or not adding phytase in the fermentation medium), recombinant yeast PHY, and *S. cerevisiae* CICIMY008. **b** Glucose and ethanol concentrations of *S. cerevisiae* CICIMY008, recombinant yeast PHY, and the control strain CICIMY008 supplemented with phytase in the fermentation medium. Data are averages from three independent experiments (mean ± standard deviations)

abundant substrate during the initial phase of growth; glucose concentration increased constantly in the first 8 h (Fig. 3b). Furthermore, the fermentation efficiency of the PHY strain reached 89.96 %, which was 4.82 % higher than the control strain which had 85.82 % fermentation efficiency ($p < 0.05$). When phytase was added into the medium, fermentation efficiency of the control strain was similar to the recombinant strain. Therefore, surface-displaying phytase in *S. cerevisiae* could directly enhance the fermentation efficiency.

Decrease of phytate phosphorus by surface-displaying recombinant phytase

Here, to evaluate the effect of surface-displaying recombinant phytase on the phytate phosphorus content in DDGS, the fate of phytate phosphorus was investigated during the ethanol fermentation process by an engineered *S. cerevisiae* strain. For the recombinant strain PHY, the phytate phosphorus content declined during the whole process. Upon completion of the ethanol fermentation process, the phytate phosphorus content decreased to 0.06 % in the DDGS (Fig. 4a). However, when the control strain was used in a batch fermentation, the phytate phosphorus content in the DDGS showed a small fluctuation during the whole process with an exception at the early phase, in which the phytate phosphorus content increased slightly. Compared to the control strain, the phytate phosphorus content decreased by 91 % for the recombinant strain under the same fermentation conditions (Fig. 4a), which is significantly beneficial for DDGS being used as animal feedstock. Moreover, when extra phytase was added into medium containing the control strain, the change in phytate phosphorus content was similar to that of the recombinant strain PHY (Fig. 4a, b).

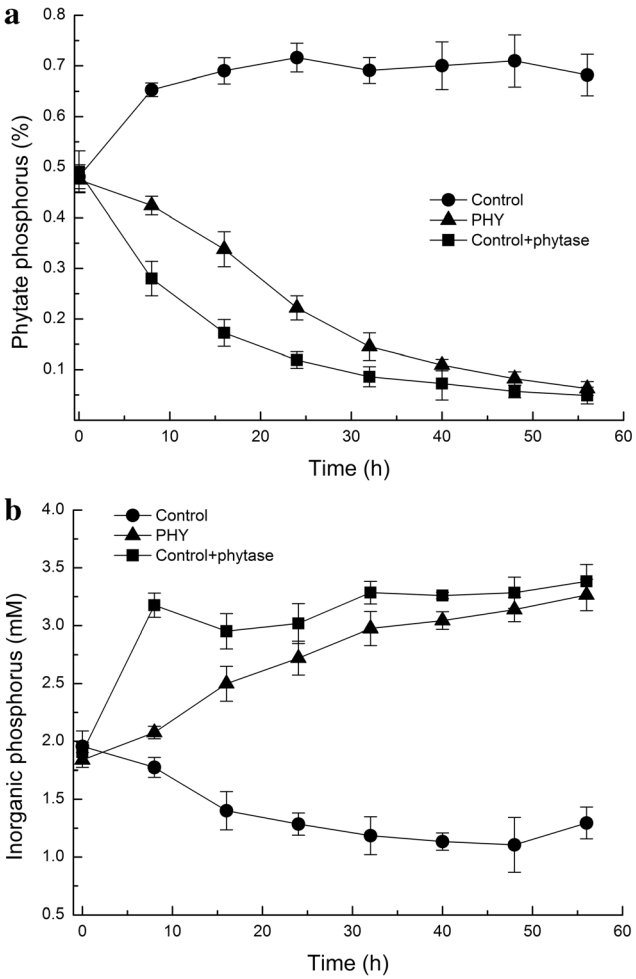

Fig. 4 Changes in phytate phosphorus (**a**, % means of the phytate phosphorus weight per 100 g dry grain) and inorganic phosphorus (**b**) concentration of inorganic phosphorus produced by the control strain *S. cerevisiae* CICIMY008, recombinant yeast PHY, and the control strain *S. cerevisiae* CICIMY008 with added phytase in the fermentation medium. The data points represent the average of three independent experiments (mean ± standard deviations)

Furthermore, the release of free phosphorus (inorganic phosphorus) during the fermentation process was investigated. In the initial phase, the control strain grew quickly which led to a significant decline in free phosphorus, as the manufacture of cell components and DNA synthesis requires large quantities of phosphorus. This was then followed by a slow decrease in the rate of free phosphorus utilization which continued until the final concentration was 1.29 mM (Fig. 4b). Due to degradation of phytate phosphorus by the surface-expressing phytase during the whole process, a higher free phosphorus content was found for the recombinant strain PHY compared to the control strain. Although the phytase activity was lower, constitutive expression and stable anchoring on the cell surface facilitated the persistent digestion of phytate. Figure 4b shows a gradual increase in free phosphorus concentration compared to the control. After 8 h, the free phosphorus content in the medium for PHY and the control strain reached to 2.07 and 3.26 mM, respectively (Fig. 4b). This means that there was a 152.7 % increase in the free phosphorus concentration at the end of process for the recombinant strain PHY ($p < 0.01$). When extra phytase was added to the fermentation medium containing the control strain, phytate was more efficiently digested and the free phosphorus content increased significantly. However, the final free phosphorus content reached a similar concentration compared to the recombinant strain PHY (Fig. 4b).

Ethanol fermentation by the engineered yeast strain in a 5-L fermentation bioreactor

In order to evaluate the effect of the surface-expressing phytase on the ethanol fermentation, a scale-up experiment was done in a 5-L bioreactor. The results indicated that the recombinant strain PHY exhibited an overall improved growth and a higher final cell density when compared to the control strain (Fig. 5, Table 3). The overall specific growth of the recombinant strain reached 0.53, which is a 20.1 % increase compared to the control strain (Table 3). The expression of phytase which generated a higher amount of available phosphorus not only increased the rate of fermentation but also resulted in a higher ethanol yield. The average fermentation rate and ethanol yield (Table 3) reached 3.02 g glucose/h and 0.50 g/g glucose, respectively, which is significantly higher than the control fermentation rate with a maximum ethanol yield of 2.56 g glucose/h and 0.48 g/g glucose, respectively. At the end of the process, the final ethanol concentration reached 113.5 g/L and increased by 6.2 % compared to the control strain (Fig. 5). However, the growth yield of the recombinant strain was similar to that of the control strain, which indicated that the release of free phosphorus into the medium had a slight impact on the final cell mass. In addition, we investigated the main by-products such as acetate, pyruvic acid, and glycerol and similar concentrations of by-products

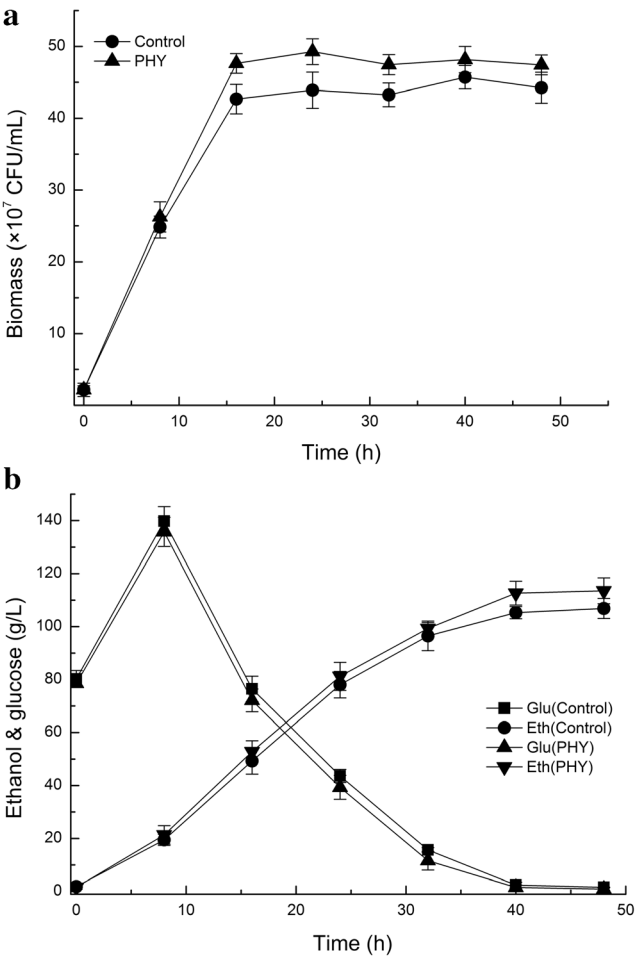

Fig. 5 Comparison of ethanol production of the recombinant *S. cerevisiae* strain PHY and the control strain under 5-L bioreactor conditions. **a** Growth pattern of the two strains in a 5-L bioreactor. **b** Ethanol production and glucose consumption from *S. cerevisiae* CICIMY008 and PHY strain. Data are averages from three independent experiments (mean ± standard deviations)

were found in broth fermented by the recombinant strain and by the control strain (data not shown). Furthermore, the phytate phosphorus content in DDGS of the recombinant strain decreased significantly by 89.8 % compared to the control strain (Table 3). The final free phosphorus content reached 3.4 mmol/L by the recombinant strain PHY. However, only 1.4 mmol/L of free phosphorus content was generated by the control strain under the same fermentation conditions (Table 3).

Discussion

Bioethanol production using starch as raw materials has become a prominent technology. However, phytate contained in the raw material not only decreases ethanol production efficiency, but also leads to a decrease in the DDGS nutrient value and an increase in the discharge of phosphorus. In recent

Table 3 Comparison of fermentation performance of the recombinant strain PHY with the control strain for ethanol production in a 5-L fermentor

Strain	Specific growth rate (1/h)	Average fermentation rate (g glucose/h)	Growth yield (g biomass/g glucose)	Ethanol yield (g ethanol/g glucose)	Phytate phosphorus content (%)[b]	Free phosphorus content (mmol/L)
CICIMY008[a]	0.44 ± 0.05	2.56 ± 0.31	0.553 ± 0.12	0.482 ± 0.04	0.59 ± 0.15	1.4 ± 0.1
PHY[a]	0.53 ± 0.07	3.02 ± 0.35	0.562 ± 0.08	0.501 ± 0.05	0.06 ± 0.01	3.4 ± 0.2
Fold	1.20	1.18	1.02	1.04	0.10	2.43

[a] Data represent the average of three samples(mean ± standard deviations)

[b] Data indicate the phytate phosphorus weight per 100 g dry grain

years, surface-displaying *S. cerevisiae* utilizing the α-agglutinin adhesion receptor has been used as whole-cell biocatalysts for bioethanol production from various substrates (Guo et al. 2010; Tanaka et al. 2012; Wen et al. 2010). Here, a surface-expressed phytase was developed and found to stimulate corn powder fermentation by supplying more available free phosphorus via decomposition of phytate. Moreover, the process using a recombinant strain could lead to decomposition of phytate and increase free phosphorus content during ethanol production.

The recombinant phytase showed that the optimal pH is consistent with the ethanol fermentation process, in which pH 4–5 is beneficial for ethanol production (Lin and Tanaka 2006; Vallet et al. 1996; Zhang et al. 1997). However, the optimal temperature of the recombinant phytase differs for ethanol production (Vallet et al. 1996; Zhang et al. 1997), which may, to some extent, affect its application in ethanol fermentation. On the other hand, the recombinant enzyme has high stability at 30–40 °C (data not shown), which will be suitable for the fermentation process (Vallet et al. 1996). Furthermore, protease accessibility assays and the intact cell pellet enzyme activity detection indicated that a large proportion of the enzyme was displayed on the cell surface.

The effects of surface-displaying phytase on the ethanol production were evaluated. We found that surface-displaying phytase in *S. cerevisiae* could not only significantly improve cell growth properties but also directly enhance the ethanol production efficiency compared to the control strain (Fig. 3). Moreover, the fermentation efficiency of the control strain generated the similar growth pattern and ethanol production efficiency when fermentation medium contained 200 U/L exogenous phytase (Fig. 3). Furthermore, in a 5-L bioreactor process by SSF, the specific growth rate and average fermentation rate of the recombinant strain were higher by 20 and 18 % respectively, compared to the parent strain (Table 3). In previous reports, Khullar et al (2011) found that exogenous addition of phytase could slightly improve final ethanol concentration in the E-Mill dry grind corn process for bioethanol production. Also, phytase treatment resulted in a higher protein content and a lower residual starch in DDGS (Khullar et al. 2011). In our investigation, displaying phytase on the *S. cerevisiae* cell surface led to a similar impact on ethanol production. It is noted that although fermentation performance could be increased by adding extra phytase, the cost of production would rise and the fermentation process would be made more complicated.

Phosphorus is an essential nutrient for livestock, but utilization efficiency is below 40 %, contributing to environmental issues (Kebreab et al. 2012). Therefore, enhancement of phosphorus availability in DDGS, which is a major coproduct available for ethanol production and widely used in feedstock, would increase its value and application (Liu 2011). In our study, a significant difference in the degradation profile of phytate phosphorus by the various strains was observed. The recombinant phytase could be constitutively expressed and stably anchor on the cell surface, which facilitated the engineered strain PHY to degrade the phytate persistently. The final phytate phosphorus concentration decreased by 89.8 % and free phosphorus concentration increased by 152.7 % in dry vinasse compared to the control under the shake flask conditions (Fig. 4).

Also, it is known that free phosphorus is one of the critical components of yeast cells and more easily utilized compared to the other forms of phosphorus (Vallet et al. 1996). Moreover, phytate has the capacity to strongly chelate divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}, which decreases the digestion and absorption of minerals by single-stomached animals when DDGS is used for animal feed (Fredlund et al. 2006; Selle et al. 2000). Higher concentrations of Zn^{2+} and Ca^{2+} were obtained from the recombinant strain fermentation broth compared with the control strain ($p < 0.05$, data not shown). It is well understood that the degradation of phytate by surface expression of phytase or the addition of extra phytase could lead to an increase in free phosphorus content and release of metal ions. Displaying phytase in a SSF process may assist in increasing coproduct values as well as lead to increased ethanol concentrations. A previous study indicated that the addition of ionic nutrients could improve phenotype performance of *S. cerevisiae*, such as the acetic acid tolerance (Ismail et al. 2014). Ethanol production by a surface-displaying phytase *S. cerevisiae* strain PHY increased the release of inositol and metal ions. This could not only improve the nutrient composition but also

decompose complexes of phytate-starch and phytate-protein, which are beneficial to yeast cell growth, ethanol production, and viability of *S. cerevisiae* (Alfenore et al. 2002; Furukawa et al. 2004). Therefore, the expression of surface-displaying phytase could stimulate corn fermentation by supplying more available phosphorus and more significantly decrease phytate and its phosphorus form in DDGS.

In summary, we constructed a recombinant *S. cerevisiae* strain displaying phytase on the cell surface, which could effectively reduce the phytate content of DDGS, improve the utilization value of vinasse, and reduce the discharge of phosphorus. The strain reported here represents a useful novel engineering platform for developing an environment-friendly system for bioethanol production from a corn substrate.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

Research involving human participants and/or animals Not applicable.

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