

Breeding of wastewater treatment yeasts that accumulate high concentrations of phosphorus

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Abstract Inorganic phosphate is an essential nutrient. In general, microorganisms take up phosphorus when the extracellular phosphorus concentration is low, but not when it is high. In *Saccharomyces cerevisiae*, the major phosphate transporters, such as Pho84p, and acid phosphatases (APases), such as Pho5p, are regulated in parallel by the phosphate signal transduction pathway (*PHO* pathway). We found that *PHO* mutants expressing *PHO84* and *PHO5*, even under high-P conditions, could take up phosphorus at twice the rate of the wild-type strain. The regulatory pathway for phosphorus accumulation in two wastewater treatment yeasts, *Hansenula fabianii* J640 and *Hansenula anomala* J224-1, was found to be similar to that in *S. cerevisiae*. We screened for mutants of these yeasts that constitutively expressed APase. Such mutants formed blue colonies on high phosphorus concentration agar plates containing 5-bromo-4-chloro-3-indolylphosphate (X-phosphate). We found four mutants of *H. fabianii* J640 and one mutant of *H. anomala* J224-1 that accumulated from 2.2 to 3.5 times more phosphorus than the parent strains. The growth rates and abilities to remove dissolved total nitrogen and dissolved organic carbon of the mutants were similar to those of the parent strains. In addition, the mutants removed

95% of dissolved total phosphorus from *shochu* wastewater, while the parent strain removed only 50%.

Keywords Phosphorus · Wastewater treatment · Yeast · Polyphosphate accumulation · *PHO* pathway

Introduction

Phosphorus is an essential nutrient for all organisms. It is widely used in the biosynthesis of cellular components, such as nucleic acids, phospholipids, and proteins. Therefore, phosphorus is added to plant fertilizers and animal feeds. However, phosphorus accumulation in the environment due to runoff from land treated with fertilizers and to the discharge of industrial and domestic waste, is a global problem which causes eutrophication of lakes, bays, and other surface waters. Considerable attention is therefore being paid to the removal of phosphorus from wastewaters (Kornberg et al. 1999). At the same time, it is anticipated that rock deposits high in phosphorus will be exhausted in the coming decades (Abelson 1999). Therefore, developing methods for removing and recycling phosphorus from wastewater would address both of these problems.

Two methods are currently used to remove and recycle phosphorus: chemical precipitation and biological removal. Phosphorus can be removed from sewage by precipitation using aluminum, calcium (to form hydroxyapatite compounds) and magnesium (to form magnesium ammonium phosphate (MAP) compounds, also called struvite; Durrant et al. 1999). Biological phosphorus removal (BPR) processes have been developed over the past 20 to 30 years. Conventional BPR processes are characterized by cycling between anaerobic and aerobic conditions (the A/O process; Seldark 1991) and are useful for sewage treatment.

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However, wastewater is a highly variable feedstock for these processes, so many treatment methods are required to deal with the large variation in wastewater characteristics. For example, wastewater with high concentrations of organics, such as *shochu* wastewater (filtered wastewater from *shochu* distilleries), and beer, beverage, and food industry wastewater, not only contains high concentrations of total phosphorus (T-P), but also have high chemical oxygen demand and high total nitrogen (T-N). It is therefore difficult to treat these various wastewaters using only the activated sludge method.

The upflow anaerobic sludge blanket (UASB) system is widely used for pretreatment of activated sludge (Seghezzi et al. 1998). Although the UASB system converts organic matter into methane effectively (Yamada et al. 2006), it is an anaerobic treatment and therefore cannot remove phosphorus. For this reason, a physicochemical phosphorus post-treatment removal process, such as MAP precipitation, is also required. Together UASB pretreatment and physicochemical post-treatment are expensive. Thus, it would be useful if these processes could be replaced with treatment with yeast strains that accumulate high concentrations of phosphorus. Such yeasts would not only treat organic matter, but also remove and allow recovery of phosphorus. Previously, the National Research Institute of Brewing (NRIB) of Japan developed a “wastewater treatment method using yeasts” (Yoshizawa 1978). This system removes large amounts of organic compounds, requires little space, and discharges little waste sludge but it was not designed for the removal of phosphorus. New phosphorus-accumulating strains are thus needed.

In general, the uptake of phosphorus by organisms is regulated by the extracellular phosphorus concentration. In *Saccharomyces cerevisiae*, phosphorus is taken up by the action of the *PHO* genes (Oshima 1997; Auesukaree et al. 2004), which are regulated by several phosphate-response genes. Two important *PHO* genes, *PHO84* and *PHO5*, are regulated by the same transcriptional activators Pho2p and Pho4p. Pho84p is a high affinity phosphate transporter located on the plasma membrane, and Pho5p is a repressible acid phosphatase which liberates phosphate from extracellular phosphoric compounds. When yeast strains encounter phosphate starvation conditions, the

PHO regulatory pathway is up-regulated to express the *PHO81* and *PHO5* and the yeast take up extracellular phosphorus (Urech et al. 1978; Ogawa et al. 1995; Kaffman et al. 1998). On the other hand, when phosphate concentrations are high, the *PHO* regulatory pathway is down-regulated (Oshima 1997).

Thus, *PHO* mutants in which Pho4p activates the expression of the phosphate transporter, Pho84p, and the acid phosphatase, Pho5p, even in high phosphate conditions, would be expected to effectively remove phosphorus from the culture solution. Furthermore, the *PHO* mutants that constitutively express *PHO84* and have enhanced phosphorus removal ability would also be expected to constitutively express *PHO5*, because *PHO84* and *PHO5* are regulated by the same transcriptional activators. The mutants that express acid phosphatase activity in high phosphate conditions would be easier to select by plate assay than the mutants that express phosphate transporter, because phosphatase activity can be identified by a color reaction on the plates. Thus, the goal was to find mutants that constitutively express phosphate transporter and acid phosphatase.

We thought this strategy would be useful to enhance phosphorus removal ability of wastewater treatment yeasts. We therefore attempted to select wastewater treatment yeast strains whose acid phosphatase activities are regulated by phosphorus concentration as they are in *S. cerevisiae*. Two yeast strains, *Hansenula fabianii* J640 (Kato et al. 1997) and *Hansenula anomala* J224-1 (Saito et al. 1990) were selected as candidates for creating mutants that efficiently remove phosphorus. Both strains were originally isolated in our laboratory as wastewater treatment yeasts. Here, we describe single mutants of each of these stains that accumulate high concentrations of phosphorus, and show that they would be useful for wastewater treatment.

Materials and methods

Strains

All the *S. cerevisiae* strains used (Table 1) were kindly provided by Dr. N. Ogawa at Stanford University. Waste-

Table 1 *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Reference
<i>S. cerevisiae</i> NBW7	<i>MATa ade2 his3 leu2 trp1 ura3 pho3-1</i>	Ogawa and Oshima (1990)
<i>S. cerevisiae</i> NOF1 ^a	<i>MATa ade2 his3 leu2 trp1 ura3 pho3-1 PHO81^c-1</i>	Ogawa et al. (2000)
<i>S. cerevisiae</i> NBD82-1 ^a	<i>MATa ade2 his3 leu2 trp1 ura3 pho3-1 PHO4^c-1</i>	Ogawa and Oshima (1990)
<i>S. cerevisiae</i> NBD4-1 ^a	<i>MATa ade2 his3 leu2 trp1 ura3 pho3-1 Δpho4::HIS3</i>	Ogawa and Oshima (1990)

^a These strains were derived from strain NBW7.

water treatment yeast strains *H. fabianii* J640 (Kato et al. 1997), *H. anomala* J224-1 (Saito et al. 1990), *Cryptococcus* sp. S-2 (Iefuji et al. 1994a), and *Geotrichum* sp. M111 (Iefuji et al. 1994b) were isolated in our laboratory.

H. anomala J224-1 was isolated as a non-flocculating mutant from the flocculating yeast strain, *H. anomala* J224 (Saito et al. 1990).

Media

YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% dextrose) and YPAD medium (1% yeast extract, 2% peptone, 2% dextrose, 0.04% adenine) were used for pre-cultivation. SD medium (2% glucose, 0.227% L-asparagines monohydrate, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.033% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1% vitamin mixture, 0.1% trace elements) was used for the acid phosphatase activity test. KH_2PO_4 was used to generate various phosphorus concentrations, and KCl was used to maintain a constant potassium concentration (Yoshida et al. 1989). Adenine and required amino acids were added to auxotrophic mutants. High P-YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 0.4% KH_2PO_4) was used for the phosphorus removal test and for screening mutants stained blue by the acid phosphatase hydrolysis of 5-bromo-4-chloro-indolyphosphate (X-phosphate; 50 mg/l in high P-YPD medium; Morohoshi et al. 2002). For auxotrophic mutants, adenine and required amino acids were added (high P-YPAD medium). Sweet potato *shochu* wastewater or barley *shochu* wastewater filtered through a miracloth (Wako) and diluted 1:1 with Milli-Q water was used for the wastewater treatment tests.

Phosphorus removal test

Yeast strains were pre-cultivated with shaking in 5 ml of YPAD medium for 24 h. The absorbance at 660 nm (OD_{660}) was measured in order to determine cell density. The cells were harvested, washed with sterilized H_2O twice, then used to inoculate 50 ml of high P-YPAD medium (0.2 OD_{660}) in a 200-ml flask. The cells were grown at 30°C, 120 rpm; 5 ml aliquots were harvested at 0, 12, 24, and 36 h, the OD_{660} was determined, the cells were centrifuged at 3,500 rpm for 10 min, and the dissolved total phosphorus (DTP) in the supernatant was determined using standard methods (APHA et al. 1998).

Acid phosphatase activity test

Acid phosphatase activities at low and high phosphorus concentrations in the medium were tested using a plate assay (Dorn 1965). Yeast strains were cultivated in low and high phosphorus concentration SD medium for 1 or 2 days.

Then, 100 ml of stain solution (0.1 M acetic acid buffer (pH 4.0), 50 mg α -naphthyl calcium phosphate, 500 mg Fast Blue B Salt) and 100 ml of 3% heated agar solution were mixed and stratified on cultivation plates. After 0.5 to 1 h, yeast strains secreting acid phosphatase were stained red.

Mutation method

Yeast strains were cultivated in 5 ml of YM medium with shaking for 24 h. Culture (250 μl), 4.6 ml of 0.2 M phosphoric acid buffer (pH 8.0), and 150 μl of ethyl-methanesulfonate (EMS: mutagenesis agent) were mixed in test tubes and the test tubes were shaken gently for 1 h at 30°C. Hydrosulfite sodium solution (6%) was added to stop EMS-induced mutation after 10 min (Lindegren et al. 1965). Following dilution with 67 mM phosphoric acid buffer by 50 times, the diluted culture was spread on high P-YPD plates containing X-phosphate. Following overnight cultivation, about 1,000 colonies were apparent on the plates.

DAPI fluorescence

Polyphosphate granules in the cell were detected by 4',6'-diamidino-2-phenylindole (DAPI) fluorescence (Allan and Miller 1980; Morohoshi et al. 2002). Yeast strains were cultivated with shaking in 5 ml of YPD medium for 24 h. One milliliter of the culture was harvested and washed with sterilized H_2O twice. The cell pellets were resuspended in 100 μl of 0.05% DAPI solution. When excited with UV light, nuclei fluoresces blue, and polyphosphate fluoresces yellow. The samples were observed by fluorescent microscopy.

Elution of phosphorus from yeast cells

Yeast strains were cultivated with shaking in 5 ml of YPD medium for 24 h. The cells ($\text{OD}_{660} \times \text{culture fluid (ml)} = 12$) were harvested and washed with sterilized H_2O twice. The cells pellets were resuspended in 50 ml of 0.1 mM Tris-HCl buffer (pH 8.0) and incubated at 70°C for 1 h (Kuroda et al. 2002; Takiguchi et al. 2007). After incubation, the samples were centrifuged for 3 min at 15,000 rpm. The concentration of inorganic phosphate ($\text{PO}_4\text{-P}$), poly-phosphate (Poly-P), and total phosphate (T-P) eluted in the supernatants was determined. $\text{PO}_4\text{-P}$ and T-P were assayed by standard methods, and Poly-P was determined by hydrolysis in 1 N HCl at 100°C for 7 min (Kuroda et al. 2002).

Wastewater treatment with yeasts on a flask scale

Yeast strains were pre-cultivated with shaking in 5 ml of YM medium for 24 h. The cells were harvested, washed with

sterilized H₂O twice, inoculated into 50 ml of sweet potato *shochu* wastewater or barley *shochu* wastewater (0.2 OD₆₆₀) in 200 ml flasks, and grown at 30°C with shaking at 120 rpm. Aliquots of the culture (1 ml) were removed after 0, 12, 24, or 36 h, the OD₆₆₀ was determined, and the samples were centrifuged at 3,500 rpm for 10 min; the pH, dissolved total phosphorus, dissolved total nitrogen (DTN), and dissolved organic carbon (DOC) levels in the supernatants were determined using standard methods (APHA et al. 1998).

Results

Phosphorus removal by the model yeast, *S. cerevisiae*

We first tested our strategy for enhancing phosphorus removal with *PHO* regulatory mutants of *S. cerevisiae*. Figure 1 shows growth curves for these cells (OD₆₆₀:

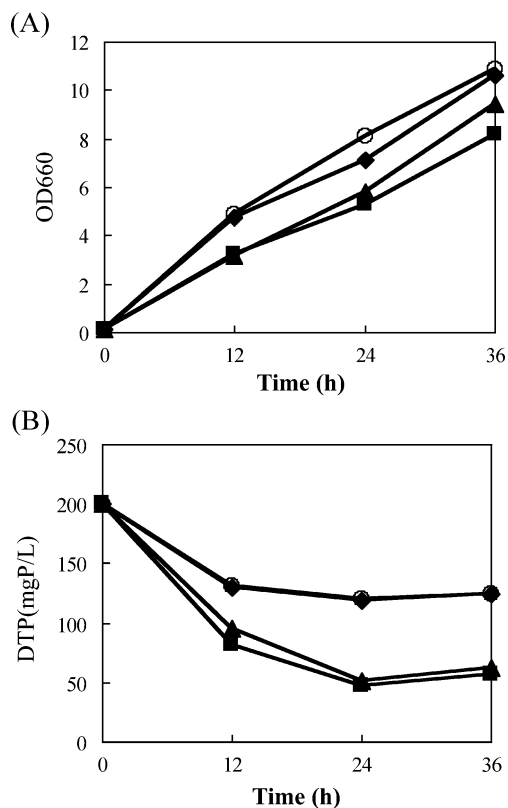


Fig. 1 Growth of the yeast strains (a) and the concentration of DTP left in the media (b) after 36 h of incubation. The wild-type, NBW7 (filled diamond), NOF1 (filled square), NBD82-1 (filled triangle), and NBD4-1 (empty circle) strains were incubated in 5 ml of YPAD medium with shaking for 24 h. The cells were harvested, washed with sterilized H₂O twice, and then inoculated into 50 ml of high P-YPAD medium (OD₆₆₀=0.2) in a 200-ml flask and incubated at 30°C with shaking at 120 rpm. The culture (5 ml) was harvested at each time point (0, 12, 24, and 36 h), the OD₆₆₀ assayed, and then the samples were centrifuged at 3,500 rpm for 10 min. DTP of the supernatants were determined using standard methods

Fig. 1a) and the concentration of residual phosphorus in high-P YPAD medium (DTP: Fig. 1b).

NOF1 and NBD82-1, whose *PHO* regulation pathways were up-regulated, grew more slowly than the two other strains (Fig. 1a). The wild-type strain NBW7 and the *PHO* down-regulated mutant NBD4-1 could remove only 35% of the soluble phosphate after 24 h, apparently because their *PHO* regulation pathways were down-regulated. However, the mutants NOF1 and NBD82-1 could remove about 75% of the DTP from the cultivation medium after 24 h.

Selection of candidates from wastewater treatment yeasts

When the phosphorus concentration of the medium was low, all but NBD4-1 were stained red, showing that they exhibit significant acid phosphatase activity (Table 2). When the phosphorus concentration of the medium was high, NOF1 and NBD82-1 showed activity, whereas NBW7 and NBD4-1 did not. *H. fabianii* J640 and *H. anomala* J224-1 stained red in low-P medium but were not stained in high-P medium, indicating that the acid phosphatase activity of these two strains is affected by phosphorus concentration. *Cryptococcus* sp. S-2 was stained red in both low-P and high-P medium, showing that its acid phosphatase is likely activated constitutively. *Geotrichum* sp. M111 was not stained in either low-P or high-P medium.

Based on these results, we selected *H. fabianii* J640 and *H. anomala* J224-1 as candidates for improving their phosphorus removal capability.

Screening of constitutive acid phosphatase expression mutants

We first selected constitutive acid phosphatase expression mutants, which formed blue colonies on high P-YPD plates

Table 2 Acid phosphatase activity of the yeast strains in media with low and high phosphorus concentration (6 and 163 mg/l total phosphorus, respectively)

Strain	Acid phosphate activity	
	Low-P medium	High-P medium
<i>S. cerevisiae</i> NBW7	+	–
<i>S. cerevisiae</i> NOF1	+	+
<i>S. cerevisiae</i> NBD82-1	+	+
<i>S. cerevisiae</i> NBD4-1	–	–
<i>H. fabianii</i> J640	+	–
<i>H. anomala</i> J224-1	+	–
<i>Cryptococcus</i> sp. S-2	+	+
<i>Geotrichum</i> sp. M111	–	–

+ Detected acid phosphatase activity, – not detected acid phosphatase activity

Table 3 The growth and amount of phosphate removal per unit OD₆₆₀ of the yeast strains

Strain	OD ₆₆₀	Residual P (mg P/l)	Removal P per OD ₆₆₀ (mg P/l-OD ₆₆₀)
<i>H. fabianii</i> J640 (wild strain)	32.1	62.7	4.3
<i>H. fabianii</i> J640 PFW1	17.7	59.2	8.4
<i>H. fabianii</i> J640 PFW2	17.2	46.2	9.0
<i>H. fabianii</i> J640 PFW3	12.0	60.5	11.7
<i>H. fabianii</i> J640 PFW4	23.9	23.4	7.4
<i>H. anomala</i> J224-1 (wild strain)	24.3	69.4	4.8
<i>H. anomala</i> J224-1 PAW1	17.1	58.4	8.4

Each strain was incubated in 5 ml of YM medium with shaking for 48 h. The cells were harvested, inoculated into 50 ml of high P-YPAD medium (OD₆₆₀=0.2) in a 200-ml flask, and grown at 30°C with shaking at 120 rpm for 24 h. The growth was measured by OD₆₆₀, and residual phosphorus was measured in the cultivation media. Removal of phosphorus per OD₆₆₀ was calculated removal phosphorus, phosphorus concentration of initial media make allowance after cultivation media, divided by OD₆₆₀ ((initial P – residual P) / OD₆₆₀).

containing X-phosphate. The mutagenized samples of *H. fabianii* J640 and *H. anomala* J224-1 were spread on about 600 plates, and each plate yielded about 1,000 colonies (total number of colonies: 0.6 million). Sixty-six candidates from *H. fabianii* J640 and 322 candidates from *H. anomala* J224-1 were isolated as blue colonies, indicating that these candidates were constitutive acid phosphatase expression mutants.

Selection of mutants capable of removing high levels of phosphorus

The mutants constitutively expressing acid phosphatase, and the parental strains, were each cultivated in 5 ml of high-P YPD medium for 24 h at 30°C with shaking, and the residual phosphorus concentration in the media was assayed. Ten mutants were isolated from *H. fabianii* J640 and 22 mutants were isolated from *H. anomala* J224-1 which could remove more phosphorus than the parent strains.

These selected mutants and their parent strains were pre-cultivated in YM medium and then cultured in 50 ml of high P-YPAD medium (0.2 OD₆₆₀) for 24 h, after which the

OD₆₆₀ and residual phosphorus concentrations in the media were assayed. Four mutants from *H. fabianii* J640 (PFW1, PFW2, PFW3, and PFW4) and one mutant from *H. anomala* J224-1 (PAW1) were isolated. These mutants remove phosphorus concentrations 1.7 to 2.7 times higher than the parent strains (Table 3).

Accumulation of phosphorus and polyphosphate

Following staining with DAPI, polyphosphate granules in the vacuoles of *H. fabianii* J640 and *H. anomala* J224-1 were observed by fluorescence microscopy (data not shown), indicating that these strains accumulate phosphorus as polyphosphate in the vacuoles. The amount of total phosphorus per mutant cell was 2.2–3.5 times larger than that in the parent strains (Table 4). In addition, the amount of polyphosphate eluted from the mutants was 3–5 times higher than that from the parent strains.

Of the four mutants isolated from *H. fabianii* J640, PFW4 grew the fastest and removed the largest amount of phosphorus, so this mutant was used for further experiments.

Table 4 The content of phosphorus in the yeast strains

Strains	PO ₄ of elution ^a (mg P/l-OD ₆₆₀)	Poly-P of elution ^a (mg P/l-OD ₆₆₀)	T-P of elution ^a (mg P/l-OD ₆₆₀)	T-P of cells ^b (mg P/l-OD ₆₆₀)
<i>H. fabianii</i> J640 (wild strain)	0.35	0.59	1.12	1.89
<i>H. fabianii</i> J640 PFW1	0.64	2.88	3.50	6.59
<i>H. fabianii</i> J640 PFW2	0.54	2.76	3.32	5.29
<i>H. fabianii</i> J640 PFW3	0.49	3.16	3.67	6.46
<i>H. fabianii</i> J640 PFW4	0.71	2.33	2.98	4.71
<i>H. anomala</i> J224-1 (wild strain)	0.55	1.30	2.09	3.86
<i>H. anomala</i> J224-1 PAW1	0.38	4.03	4.60	8.34

Each strain was incubated in 5 ml of high-P YPD medium with shaking for 24 h.

^a Elution of yeast cells following heating at 70°C for 1 h. PO₄-P and T-P were assayed by standard methods; poly-P was hydrolyzed to PO₄-P by heating at 100°C for 7 min with 1 N HCl, then assayed by standard methods.

^b Total phosphorus content of the yeast cells was assayed by standard methods.

Removal of phosphorus from *shochu* wastewater

Cell growth for *H. fabianii* J640 and the mutant PFW4 (Fig. 2a) and *H. anomala* J224-1 and the mutant PAW1 (Fig. 2b) in sweet potato *shochu* wastewater were almost same. Changes in DTN, DOC, and pH were also almost the same (data not shown). On the other hand, the mutants (PFW4 and PAW1) could remove about 95% of DTP from the wastewater (36 h: Fig. 2c,d), while the parent strains removed only about half. Similar results were obtained with barley *shochu* wastewater (data not shown).

Figure 3 shows the concentration of inorganic phosphate ($\text{PO}_4\text{-P}$) and other phosphorus compounds in sweet potato and barley *shochu* wastewater following cultivation of each yeast strain for 36 h. Sweet potato and barley *shochu* wastewater contained 30% and 45% organic phosphorus compounds of DTP, respectively. In contrast, the parent strains removed only 50% to 70% of $\text{PO}_4\text{-P}$ and 30% to 50% of organic phosphorus compounds. The mutants efficiently removed not only inorganic phosphate ($\text{PO}_4\text{-P}$) but also organic phosphorus compounds.

The ratio of C/N removal was almost the same between parents and mutants (around 100:5.4~6.5), but the ratio of

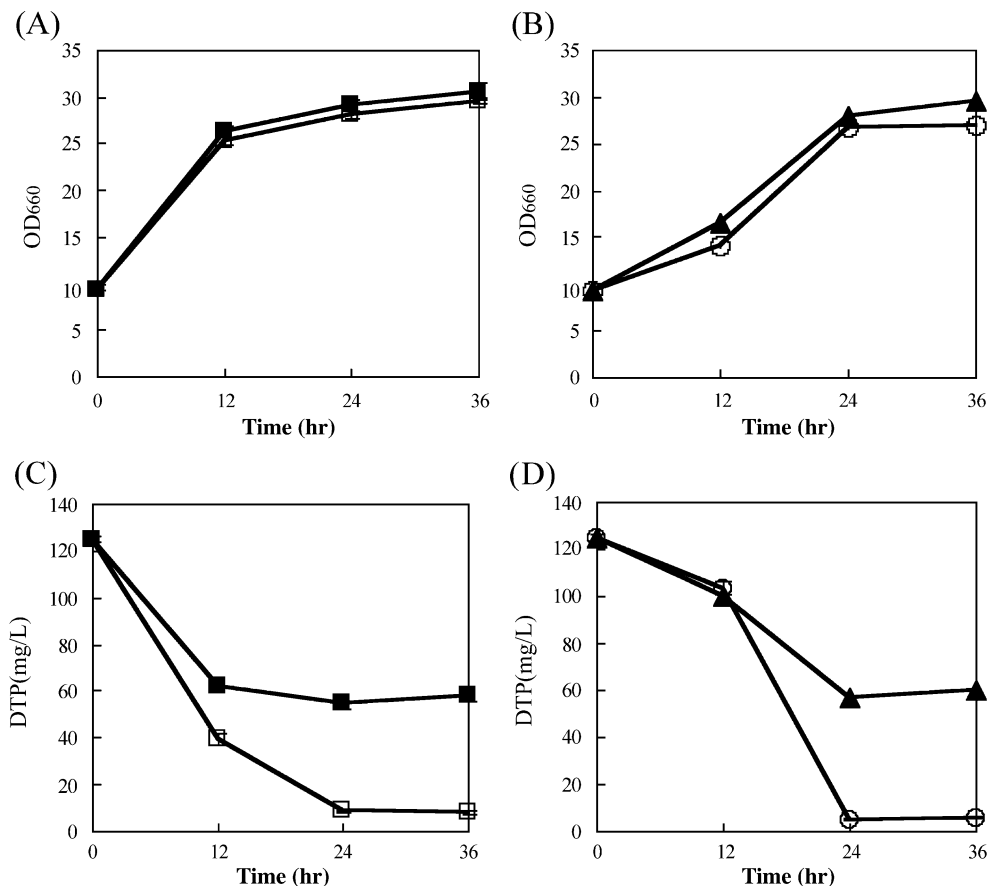
C/P removal by the mutants (around 100: 2.8~3.3) was twice that of the parent strain (around 100: 1.5~1.8) in each *shochu* wastewater (Tables 5).

Discussion

In this study, we bred two wastewater treatment yeasts, *H. fabianii* J640 PFW4 and *H. anomala* J224-1 PAW1, that accumulated 2.2 to 3.5 times more phosphorus than the parent strains.

In *S. cerevisiae*, the *PHO* regulatory pathway senses phosphorus concentration signals and regulates the uptake of phosphorus (Oshima 1997; Auesukaree et al. 2004). The major phosphate transporters, such as Pho84p, and acid phosphatases, such as Pho5p, are regulated in parallel by the *PHO* regulatory pathway. In this study, we showed that *PHO* mutants that constitutively express *PHO84* and *PHO5* could take up phosphorus at twice the rate of the wild-type strain. We also showed that in two wastewater treatment yeast strains, *H. fabianii* J640 and *H. anomala* J224-1, acid phosphatase activities were regulated by the extracellular phosphorus concentration. We believe that some mutation

Fig. 2 Growth (a, b) and DTP uptake (c, d) of wild-type strains and mutants. Wild-type strain *H. fabianii* J640 (filled square) its mutant PFW4 (empty square) and wild-type strain *H. anomala* J224-1 (filled triangle) and its mutant PAW1 (empty circle) were incubated in 5 ml of YM medium with shaking for 48 h. The cells were harvested and inoculated in 50 ml of wastewater ($\text{OD}_{660}=0.2$) in a 200-ml flask and grown at 30°C with shaking at 120 rpm



in the regulatory pathways of *H. fabianii* J640 PFW4 and *H. anomala* J224-1 PAW1 causes phosphate transporters and acid phosphatase to be constitutively expressed, leading to enhanced uptake and accumulate of phosphorus.

In bacteria, the *pho* regulon regulates the uptake of phosphorus (Amemura et al. 1985). Kato et al. (1993) created recombinant *Escherichia coli* that overexpressed genes encoding polyphosphate kinase (*ppk*) and the phosphate-specific transport system (*pstSCAB*). This strain accumulated high levels of polyphosphate with phosphorus accounting for 16% of its dry weight. However, the recombinant strain grew too slowly to be practical for treating wastewater.

The high-P accumulating yeasts *H. fabianii* J640 PFW4 and *H. anomala* J224-1 PAW1 efficiently removed not only

Table 5 Ratio of the removal of carbon, nitrogen, and phosphorus (C/N/P) in cultivated and cultivated barely sweet potato *shochu* wastewater

Strains	C	N	P
Cultivated sweet potato <i>shochu</i> wastewater			
<i>H. fabianii</i> J640 (wild strain)	100	5.7	1.6
<i>H. fabianii</i> J640 PFW4	100	5.4	2.8
<i>H. anomala</i> J224-1 (wild strain)	100	5.8	1.5
<i>H. anomala</i> J224-1 PAW1	100	5.8	2.9
Cultivated barely <i>shochu</i> wastewater			
<i>H. fabianii</i> J640 (wild strain)	100	6.0	1.8
<i>H. fabianii</i> J640 PFW4	100	6.3	3.3
<i>H. anomala</i> J224-1 (wild strain)	100	6.5	1.8
<i>H. anomala</i> J224-1 PAW1	100	5.8	3.3

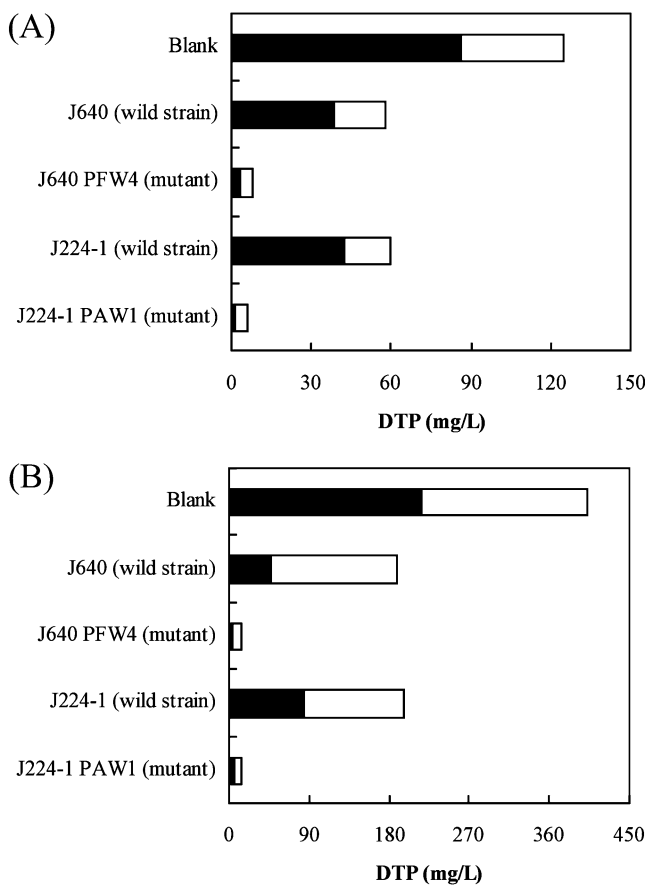


Fig. 3 The concentration of phosphorus and its composition of cultivated wastewater (**a** sweet potato *shochu* wastewater, **b** barley *shochu* wastewater). Each strain was inoculated in 50 ml of wastewater ($OD_{660}=0.2$) in a 200-ml flask and grown at 30°C with shaking at 120 rpm for 36 h. PO₄-P (black bars) and organic phosphorus compounds (white bars) were assayed by standard methods

inorganic phosphate, but also phosphorus compounds (Fig. 3). Phosphorus is imported into yeast cells through the high/low affinity phosphate transporter as inorganic phosphate. In the process of breeding high-P accumulating yeasts, we selected mutants that, unlike their parent strains, secreted acid phosphatases in high phosphorus medium. The rationale was that the secreted acid phosphatase would hydrolyze phosphorus compounds to inorganic phosphate, which would be easier for the yeasts to take up.

Because the sludge of *H. fabianii* J640 PFW4 and *H. anomala* J224-1 PAW1 accumulate high level phosphorus (Table 4), it would be utilized for producing phosphorous and nitrogenous animal feed and/or plant fertilizers. In addition, the sludge of these strains accumulated phosphorus as polyphosphate granules. By heating the sludge to 70°C, the phosphorus was easily eluted to the liquid phase from the cells (Kuroda et al. 2002). Phosphate and polyphosphate can be precipitated from this solution adding CaCl₂ (Kuroda et al. 2002). The recovered phosphorus has a high degree of purity and would be useful in various industrial applications.

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