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Enhanced Arsenic Accumulation by Engineered Yeast Cells Expressing *Arabidopsis thaliana* Phytochelatin Synthase

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ABSTRACT: Phytochelatins (PCs) are naturally occurring peptides with high-binding capabilities for a wide range of heavy metals including arsenic (As). PCs are enzymatically synthesized by phytochelatin synthases and contain a $(\gamma\text{-Glu-Cys})_n$ moiety terminated by a Gly residue that makes them relatively proteolysis resistant. In this study, PCs were introduced by expressing *Arabidopsis thaliana* Phytochelatin Synthase (AtPCS) in the yeast *Saccharomyces cerevisiae* for enhanced As accumulation and removal. PCs production in yeast resulted in six times higher As accumulation as compared to the control strain under a wide range of As concentrations. For the high-arsenic concentration, PCs production led to a substantial decrease in levels of PC precursors such as glutathione (GSH) and γ-glutamyl cysteine (γ-EC). The levels of As(III) accumulation were found to be similar between AtPCS-expressing wild type strain and AtPCS-expressing *acr3*Δ strain lacking the arsenic efflux system, suggesting that the arsenic uptake may become limiting. This is further supported by the roughly 1:3 stoichiometric ratio between arsenic and PC2 ($n=2$) level (comparing with a theoretical value of 1:2), indicating an excess availability of PCs inside the cells. However, at lower As(III) concentration, PC production became limiting and an additive effect on arsenic accumulation was observed for strain lacking the efflux system. More importantly, even resting cells expressing AtPCS pre-cultured in Zn^{2+} enriched media showed PCs production and two times higher arsenic removal than the control strain. These results open up the possibility of using cells expressing AtPCS as an inexpensive sorbent for the removal of toxic arsenic.

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KEYWORDS: heavy metals; bioremediation; arsenic; phytochelatin; biosorbents

Introduction

Arsenic (As) is an extremely toxic metalloid pollutant affecting the health of millions of people throughout the world (Nriagu and Pacyna, 1988). Arsenic exposure, either due to geochemical enrichment or industrial processes, is associated with many major health disorders such as increased risk of hypertension (Chen et al., 1995), skin, lung, and bladder cancer (Karagas et al., 1998). Arsenic exists as arsenate, As(V), and arsenite, As(III), with the latter being more toxic of the two (Norman, 1998). Their toxicity is attributed to substitution of pentavalent arsenate As(V) for phosphate and the affinity of trivalent As(III) for protein thiol groups, protein–DNA and DNA–DNA cross-linking. Recently it has been suggested that As(III) acts as an endocrine disruptor binding to hormone receptors interfering normal cell signaling (Kaltreider et al., 2001). Arsenite-stimulated generation of reactive oxygen species, known to damage proteins, lipids, and DNA, is probably the direct cause of the carcinogenicity (Liu et al., 2001).

Given its extreme toxicity, the regulatory limit of arsenic in the USA is currently set at 10 ppb. Conventional water

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treatment technologies, such as coagulation, and precipitation, do not offer specificity, and require water chemistry alteration along with the addition of other chemicals (Clifford et al., 1998). Current technologies such as absorption on activated alumina, polymeric anion exchange (Chwirka et al., 2000), and sorption by iron oxide coated sand particles (DeMarco et al., 2003) are more effective for As(V) than As(III) as these methods all require prior oxidation of As(III) to As(V) before treatment (Driehaus et al., 1995; Wilkie and Hering, 1998). As a result, these methods are mainly used for small-scale removal because of the relatively high cost.

Bioremediation using microbes is often considered as a cost effective and efficient way of removing heavy metals (Mejare and Bulow, 2001). Several attempts have been made to enhance the intracellular heavy metal content of microorganisms by expressing metal-chelating peptides such as metallothioneins (MT) (Ma et al., 2001; Sar and D'Souza, 2001; Say et al., 2003; Sousa et al., 1996, 1998), however, they generally lacked specificity and affinity for As. Recently, it was shown that specific arsenic accumulation in *E. coli* can be achieved by expressing an arsenic-binding protein arsR (Kostal et al., 2004). However, the use of *E. coli*, which has been associated with many infections, is generally not considered safe. This report demonstrates the use of engineered baker's yeast as an effective biosorbent for As.

Phytochelatins (PC) found in many plants and yeasts (Rauser, 1995; Zenk, 1996), are naturally occurring peptides which contain multiple repeats (from 2 to 11) of the γ-Glu-Cys moiety terminated by a Gly residue. PCs are enzymatically synthesized by PC synthase from glutathione (GSH) (Cobbett, 2000), which requires heavy metal presence for its activation (Ha et al., 1999; Vatamaniuk et al., 2000). PCs have been shown to bind heavy metals such as cadmium, mercury, and lead (Maitani et al., 1996), and unlike MTs, PCs are known to bind to arsenite with high affinity by forming complexes such as PC_2-As-PC_2 via their thiol groups (Schmoger et al., 2000).

Saccharomyces cerevisiae is a promising microorganism that can be engineered for As removal as it is generally considered as safe, and the mechanisms of arsenic resistance (Ghosh et al., 1999) have been extensively studied during the last decade. *S. cerevisiae* has a natural arsenic detoxification system which is mediated not only by export of As(III) by the membrane transporter Acr3p (Wysocki et al., 1997) but also by transport of arsenite–glutathione complex into the vacuole by the ATP-binding cassette transporter Ycf1p (Ghosh et al., 1999). Recently a tobacco PC synthase was expressed in *S. cerevisiae* with reported increased tolerance for Cd and As. The same report also demonstrated restored As tolerance in a strain lacking Ycf1p (Kim et al., 2005). Although little comparison on arsenic accumulation has been made, all reports have demonstrated increased arsenic resistance with the production of PCs (Ha et al., 1999; Hartley-Whitaker et al., 2001; Li et al., 2004). We hypothesize that the production of PCs in yeast would result in an increase in arsenic accumulation.

In this paper, we report enhanced arsenic accumulation and removal by engineered yeast cells expressing *Arabidopsis thaliana* phytochelatin synthase (AtPCS). We show that AtPCS expression in *S. cerevisiae* results in PC synthesis and sixfold increase in arsenic accumulation. Even resting cells previously grown on zinc enriched media can be used as biosorbents to remove arsenic. These results lay the foundation for the use of engineered PC biosynthesis to enhance the intracellular arsenic content of yeast and their use as a low-cost biosorbent for arsenic removal.

Materials and Methods

Strains and Cell Culture

The *S. cerevisiae* strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and the corresponding *acr3Δ* strain 15616 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 acr3::URA3*) (Open Biosystems, Huntsville, AL) were used for AtPCS expression. *E. coli* strain JM109 (e14−(McrA−) *recA1 endA1 gyrA96 thi-1 hsdR17* (*r*K− *m*K+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacIqZΔM15*]) (Stratagene, La Jolla, CA) was used for DNA manipulations. Yeast transformations were performed as described previously (Ito et al., 1983).

Plasmid pYES3-AtPCS1::FLAG was kindly donated by Dr. Philip Rea at University of Pennsylvania, Philadelphia (Vatamaniuk et al., 1999). The gene for AtPCS was under the control of strong yeast promoter PGK and flanked by a FLAG tag for easy detection on western blot using a monoclonal anti-FLAG tag antibody (Sigma, St. Louis, MO).

Yeast strains were grown in minimal SG media (2% galactose, 0.5% bacto-casamino acid, and 0.67% yeast nitrogen base) supplemented with auxotrophic requirements when required. *E. coli* strain was grown in Luria–Bertani (LB) medium (29) containing 100 μg/mL of ampicillin (Sigma).

Protein Expression Analysis

Expression of phytochelatin synthase (PCS) was verified by Western blotting (Sambrook et al., 2001). Cells were harvested, washed, concentrated to an optical density (600 nm) of 10 in SDS gel-loading buffer and lysed by boiling at 95°C for 10 min. Proteins were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with a monoclonal anti-FLAG tag antibody overnight (Sigma), washed with TBS buffer (20 mM Tris, 0.5 M NaCl) with 0.05% (v/v) Tween 20 and incubated with alkaline phophatase conjugated goat anti-mouse immunoglobulinG (Invitrogen, Carlsbad, CA) for 2 h. After washing three times with TBS buffer with 0.05% (v/v) Tween 20, blots were developed using a western plus kit form Amersham Biosciences Piscataway, NJ. A prestained protein standard marker (Kaleidoscope

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Standards (Bio-Rad, Hercules, CA)) was used to determine protein molecular weights.

Arsenic Accumulation by Growing Cells

Yeast cells were inoculated into 20 mL of SG medium containing the appropriate auxotrophic supplements and arsenite (20 μM or 500 ppb) in a 125-mL flask at an initial OD_{600} of 0.1 from overnight grown seed cultures. At different time points, cell densities were measured and one milliliter samples were taken for arsenic analysis. For whole-cell arsenic contents, cells were washed with 5 mM HEPES buffer containing 0.8% NaCl for 3 times before drying at 65°C for 24 h. The dried cell pellets were digested with 100 μL of concentrated nitric acid for 2 days (modified from Sriprang et al., 2003). The total arsenic content was measured using atomic adsorption spectroscopy (Perkin Elmer, Inc., Waltham, MA). Arsenic was determined at 193.7 nm in a graphite furnace.

Arsenic Accumulation and Removal by Resting Cells

Cells were grown as described above except in the presence of 100 μM $ZnCl_2$. Cells were washed and resuspended in prewarmed TB74S buffer (50 mM Tris, 150 mM NaCl; pH 7.4). Arsenite was added at the concentrations stated in experiments. The whole-cell arsenic content was determined as described above.

GSH, γ-EC, and PC Analysis

Cells were cultured as described above. The derivatization procedure with monobromobimane (mBBr) using fluorescence detection was adapted from Sneller et al. (2000). Cells were harvested, washed with 5 mM HEPES buffer (pH 7.1) containing 0.8% NaCl, and freeze-dried. Twenty milligrams of the lyophilized samples was resuspended in 1 mL of 6.3 mM diethylenetriamine pentaacetic acid (DTPA) with 0.1% trifluoroacetic acid (TFA). Thio-containing peptides, γ-glutamylcysteine (γ-EC), GSH, and PCs, were extracted by vortexing eight to ten times with 0.3 mL of glass beads (400–600 μm) for 60 s and putting on the ice for 60 s. The homogenates were centrifuged and supernatants were filtered. Two hundred fifty microliters of the samples was mixed with 450 μL of 200 mM 4-(2-hydroxy-ethyl)-piperazine-1-propane-sulfonic acid buffer (pH 8.2) with 6.3 mM DTPA and 10 μL of 25 mM of mBBr. After 30 min of derivatization at 45°C in the dark, 1 M of methane sulfonic acid was added to stop the reaction. The peptides were separated on a reverse phase Gemini C18 column (pore size, 110 Å; particle size, 5 μm; dimension, 4.6 × 150 mm, Phenomenex, Torrance, CA) by a binary linear gradient elution program that employed methanol with 0.1% (v/v) TFA and water with 0.1% (v/v) TFA as the mobile phase. The column was equilibrated with 12% methanol with 0.1% TFA and eluted by a gradient from 12% to 100% methanol over 60 min at a flow rate of 0.5 mL/min. Fluorescence was monitored by an Agilent 1200 Series HPLC fluorescence detector using an excitation wavelength of 380 nm and an emission wavelength of 470 nm. GSH and γ-EC standards were acquired from Sigma and PC2 was purchased from GenScript Corporation (Piscataway, NJ). Peaks obtained for GSH, γ-EC, PC2, PC3, and PC4 standards were used to identify the corresponding peaks of the samples. Standard curves for GSH, γ-EC, and PC2 were used to calculate GSH, γ-EC, and PC2 concentrations of the samples.

Results

Expression of AtPCS and PC Synthesis

Plasmid pYES3-AtPCS1::FLAG, which contains the *AtPCS* gene under control of a constitutive PGK promoter, was used for AtPCS expression. A single band of the expected size (56 kDa) was recognized on the Western blot from cells carrying this plasmid using a monoclonal antibody against the FLAG epitope (Fig. 1A) (Li et al., 2004). When grown in the presence of As(III), synthesis of different PCs (PC2, PC3, and to some extent PC4) was detected with PC2 being the most abundant form (Fig. 1B). In contrast, cells not carrying *AtPCS* showed no PC production (Fig. 1C).

Arsenic Content and Analysis of Different Metabolites in PC Production

The ability of engineered cells to accumulate As(III) was investigated by the addition of 20 μM sodium arsenite to the growth medium. Cells expressing AtPCS showed a sixfold increase in the intracellular arsenic content (Fig. 2B) over the control strain BY4742 and correlated well with the increase in PC production (Fig. 2C). The presence of PCs also protected the cells from As(III) toxicity as the final density for cultures expressing PC was improved by 20% (Fig. 2A). Both As accumulation and PC production showed no saturation, suggesting continuous PC production and subsequent intracellular As(III) accumulation. The low level of PC2 detected for the control strains is likely the result of native PC production by *S. cerevisiae* as reported previously (Kneer et al., 1992). As expected, strain 15616 lacking the efflux protein Acr3p showed enhanced arsenic accumulation as compared to the control strain BY4742. However, the intracellular As contents were very similar in both strains with AtPCS expression as opposed to the expected higher As accumulation in 15616 lacking the efflux system. This result suggests that other factors such as the uptake of As(III) may be limiting the additional accumulation.

To investigate the effect of PC production on the different precursors in PC synthesis, the intracellular γ-EC and GSH contents were analyzed by HPLC. The levels of γ-EC and GSH decreased in cells expressing AtPCS. In contrast, cells

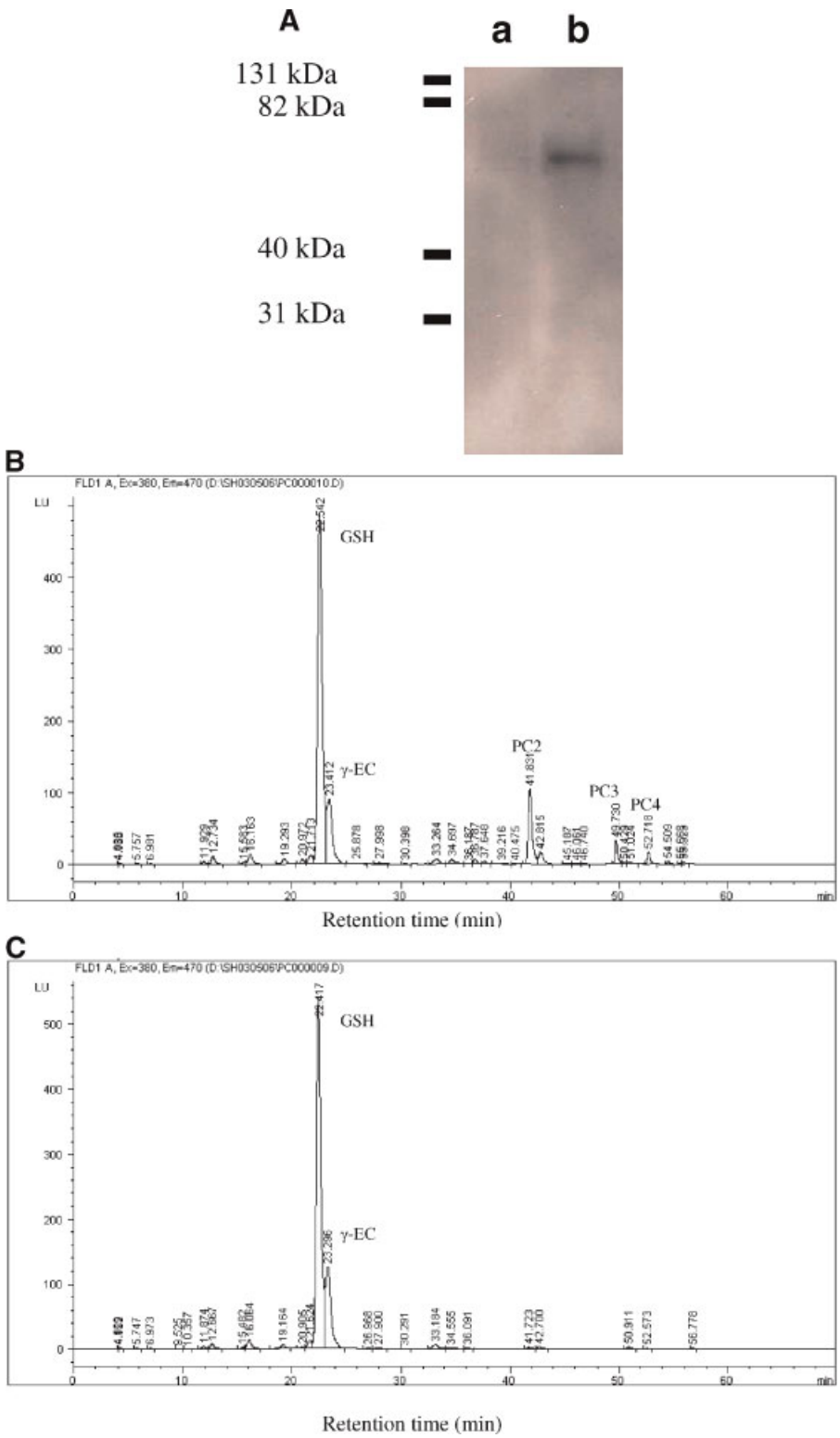

Figure 1. **A**: Western blot analysis of AtPCS expression from strains BY4742 harboring either pYES3-AtPCS1::FLAG (**lane b**) or no plasmid (**lane a**). Production of PCS-FLAG was detected by an anti FLAG-tag antibody. **B**: HPLC analysis of PCs (PC2, PC3, and PC4), GSH and γ-EC from cell extracts of strain BY4742 harboring pYES3-AtPCS1::FLAG grown in the presence of arsenite (20 μM). **C**: HPLC analysis of non-protein thiols from the cell extracts of strain BY4742 grown in 20 μM As(III).

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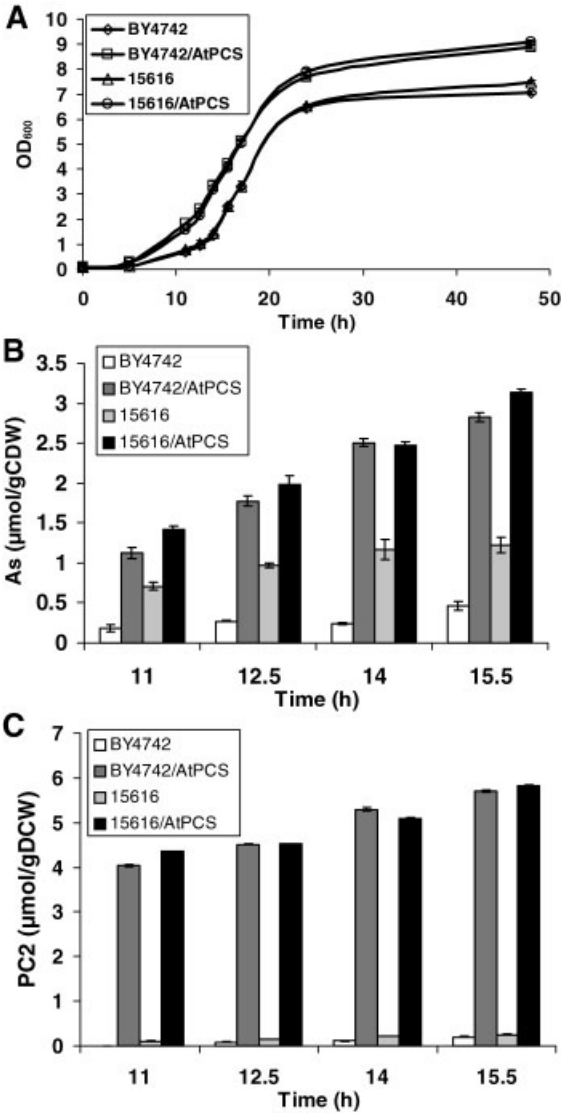

Figure 2. Growth curves (**A**), intracellular specific As contents (**B**) and PC2 contents (**C**) from strains BY4742 (wild-type strain), BY4742/AtPCS (BY4742 harboring pYES3-AtPCS1::FLAG), 15616 (acr3 deletion strain), and 15616/AtPCS (15616 harboring pYES3-AtPCS1::FLAG) grown in 20 μM As(III) enriched media. Data shown are the mean values (+standard deviation) obtained from three independent experiments.

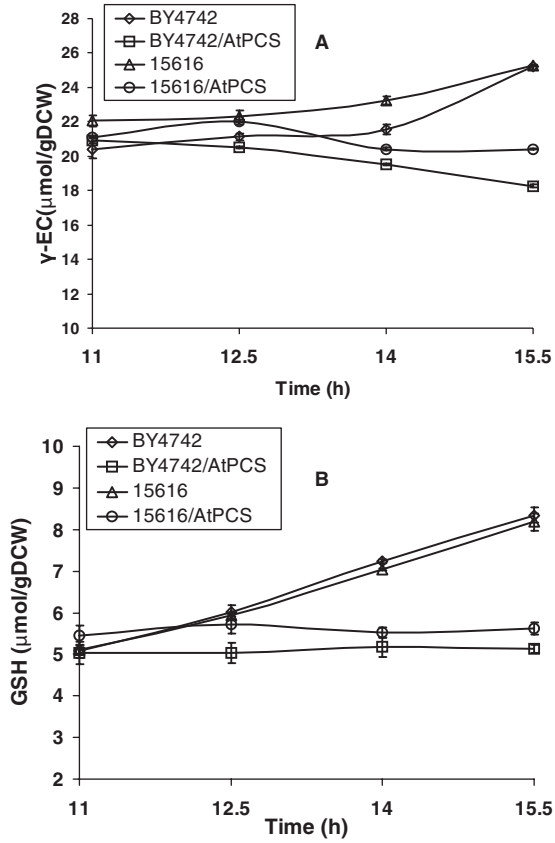

Figure 3. The intracellular (**A**) γ-EC and (**B**) GSH contents from strains BY4742 (wild-type strain), BY4742/AtPCS (BY4742 harboring pYES3-AtPCS1::FLAG), 15616 (acr3 deletion strain), and 15616/AtPCS (15616 harboring pYES3-AtPCS1::FLAG) grown in 20 μM As(III) enriched media. Data shown are the mean values (+standard deviation) obtained from three independent experiments.

without AtPCS expression showed a continuous increase in γ-EC and GSH levels (Fig. 3A and B). These results strongly indicate that γ-EC and GSH levels are decreased due to PC synthesis and PC production is primarily responsible for the increase in intracellular As(III) accumulation. These results also confirm that the availability of GSH in yeast is not limiting PC synthesis as there is a sufficiently high level of intracellular GSH.

To further investigate the utility of AtPCS-expressing growing yeast cells at lower As(III) concentrations commonly found in the environment, similar accumulation experiments were performed, in the presence 500 ppb As(III). Consistent with the metal-dependent activation of AtPCS, the PC2 levels were fivefold lower (Fig. 4B) and continued to decrease because of declining As(III) concentration with time. Even at this lower PC2 concentration, cells expressing AtPCS accumulated sevenfold higher arsenic (Fig. 4) than the control BY4247. However, a similar increase in As(III) accumulation was observed for the acr3Δ strain 15616, indicating that As efflux may play a more important role in the overall accumulation at the lower As concentration. As a result, an additive effect on As(III) accumulation was observed for the acr3Δ strain 15616 expressing AtPCS, resulting in significant removal of the added As(III). This result is particularly encouraging, indicating the usefulness of the approach for practical applications.

Arsenite Accumulation and Removal Using Resting Cells as Biosorbents

One potential utility of AtPCS-expressing cells is the use of resting cells as biosorbents for arsenic accumulation and removal. Unfortunately, the heavy metal requirement for PC

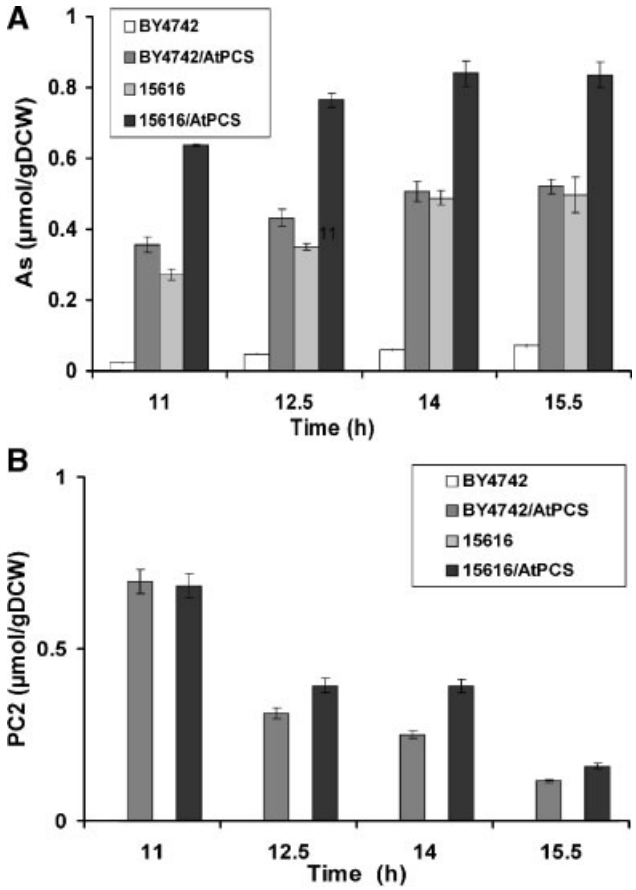

Figure 4. The specific As contents (**A**) and PC2 contents (**B**) from strains BY4742 (wild-type strain), BY4742/AtPCS (BY4742 harboring pYES3-AtPCS1::FLAG), 15616 (acr3 deletion strain), and 15616/AtPCS (15616 harboring pYES3-AtPCS1::FLAG) grown in 500 ppb As(III) enriched media. Data shown are the mean values (+standard deviation) obtained from three independent experiments.

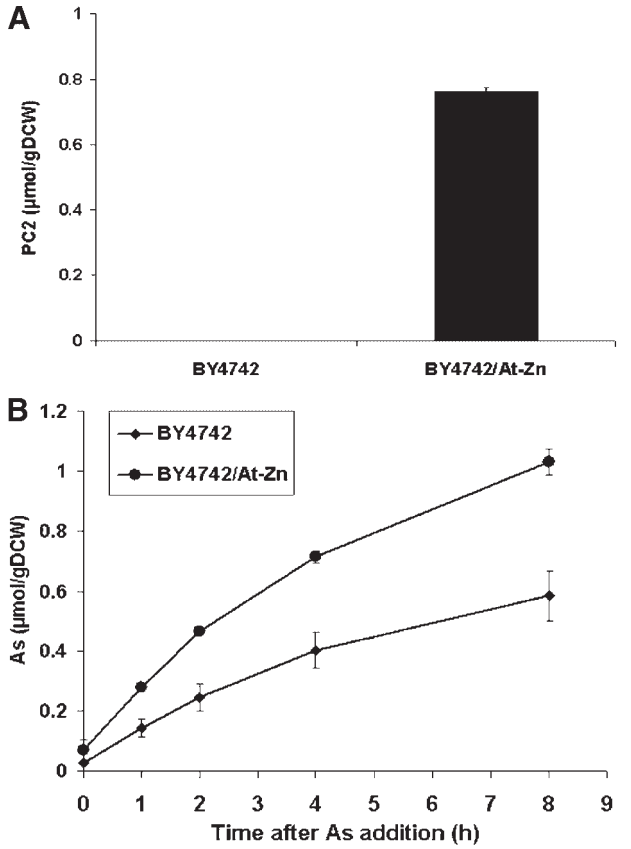

Figure 5. The intracellular (**A**) PC2 and (**B**) As contents from strains BY4742 and BY4742/At-Zn (BY4742 harboring pYES3-AtPCS1::FLAG) pre-cultured in Zn enriched medium. Data shown are the mean values (+standard deviation) obtained from three independent experiments.

synthase activation poses a potential impediment for this strategy. However, it has been reported that Zn can be used to activate PC synthase without any significant intracellular Zn accumulation in AtPCS-expressing *E. coli* (Sauge-Merle et al., 2003). A similar strategy was employed to investigate whether this will enable high-level As accumulation with resting yeast cells. Cells grown in Zn (100 µM) enriched medium were resuspended in As(III) (20 µM) rich buffer and As(III) accumulation was measured at different time points. Consistent with earlier results, cells grown in Zn-enriched media exhibited PC production (Fig. 5A) without any intracellular Zn accumulation (data not shown). However the PC content was fivefold lower than cells grown in As(III), which is in agreement with earlier reports indicating that As is a better activator of PC synthase than Zn (Vatamaniuk et al., 2000). Resting cells expressing AtPCS showed a twofold increase in As accumulation (Fig. 5B). It is interesting to note that even though the intracellular PC content is lower, the maximum accumulation at 8 h was similar to the value obtained with growing cultures, suggesting possible arsenic uptake may be limiting additional As accumulation in growing cultures.

Discussion

Attempts to develop useful biosorbents by increasing the heavy metal content of plants, algae or microorganism via genetic engineering have been made in recent years (Ma et al., 2001; Maitani et al., 1996; Sar and D'Souza, 2001). Although significant improvements in heavy metal accumulation have been observed (Li et al., 2000; Pazirandeh et al., 1995) by the overexpression of metal-chelating peptides such as metallothioneins (Sousa et al., 1998) or synthetic phytochelatins (Bae et al., 2000, 2001), the ability of these peptides in arsenite binding has never been confirmed. Naturally occurring phytochelatins are particularly attractive alternatives as they are not subject to extensive proteolysis because of the γ-carboxyamide bond between Glu and Cys, and they are known to bind to arsenic with high affinity (Sauge-Merle et al., 2003). More

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importantly, PC synthesis can be further fine-tuned by the flexibility to engineer the overall biosynthetic pathway.

In this study we demonstrate the utility of engineered *S. cerevisiae* for As bioremediation. Yeast has the upper hand over many bacteria of being considered generally as safe. Expression of AtPCS in yeast led to PCs production and sixfold increase in specific As content of the growing cells in high arsenic enriched media. Strains expressing AtPCS not only accumulated higher As but also showed enhanced growth, leading to only sixfold increase in specific As content as compared to 10-fold increment in volumetric As content. It should be noted that the background arsenic accumulation by the wild-type strains is fairly high as a result of vacuolar sequestration of $As(GS)_3$ complex by the ABC transport protein Ycf1p (Ghosh et al., 1999). As a result, the maximum As level of 3 μmol/g CDW is also twofold higher than that reported for *E. coli* expressing AtPCS (Sauge-Merle et al., 2003), suggesting an improved system for As accumulation using the engineered yeast. Since the supply of precursors such as GSH and γ-EC appears to be sufficient even in the presence of PC synthesis as oppose to the case of engineered *E. coli*, this suggests that *S. cerevisiae* is a better host for such genetic manipulations and the activity of PC synthase can be further improved to increase the PC level and the subsequent As(III) accumulation. The choice of PC synthase is particularly important as expression of a tobacco PC synthase (NtPCS) in yeast did not result in increased arsenic accumulation and production of PCs was not verified (Kim et al., 2005).

It is interesting to note that at high external arsenic concentrations very similar levels of arsenic accumulation was observed between AtPCS-expressing wild type strain and AtPCS-expressing *acr3*Δ strain, indicating that the uptake of arsenic might be a limiting factor. This is further supported by the roughly 1:3 stoichiometric ratio between arsenic and PC2 ($n = 2$) level (comparing with a theoretical value of 1:2), indicating an excess availability of PCs inside the cells and As(III) may become limiting. This bottleneck could be potentially overcome by overexpression of the As(III) transporter Fps1p to increase the rate of As(III) uptake. Overexpression of a mammalian Fps1p analog has already been shown to significantly increase As(III) uptake (Liu et al., 2002), a similar strategy may be combined with PC production to further improve the overall intracellular As accumulation.

AtPCS expressing resting yeast cells pre-cultured in Zn showed PC production and enhanced As accumulation (Fig. 5). Activation of AtPCS without any significant Zn accumulation can possibly be explained by the fact that micromolar heavy metal glutathione thiolates ($Zn.GS_2$) are responsible for phytochelatin synthase activation instead of direct metal binding to the enzyme (Vatamaniuk et al., 2000). This is a major advantage as the displacement of Zn by As will not be a concern for the resting cell system.

In conclusion, the results presented here pave the path for arsenic bioremediation using engineered yeast cells. The ability to independently engineer yeasts cells as biosorbents and their ability to accumulate arsenic rapidly makes this technology particularly promising. Potential applications could include the use of these cells as biofilters or in bioreactors for arsenic removal.

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