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ORIGINAL PAPER

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Cloning and expression of fungal phytases in genetically modified strains of *Aspergillus awamori*

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Abstract In an effort to produce phytases cost-effectively, and to determine the efficiency of a novel expression system, the genes for *Aspergillus awamori* (*phyA*) phytase and *Aspergillus fumigatus* (*phyA*) phytase (a putative thermostable enzyme) were cloned and overexpressed in *A. awamori*. Regulation of phytase expression was achieved by separately placing the genes under the transcriptional control of the glucoamylase A (*glaA*) promoter of *A. awamori*. A gene fusion strategy was employed that involved the insertion of a hexapeptide Kex-2 protease cleavage site between the native glucoamylase and heterologous proteins and allowed for the efficient secretion and processing of the resultant chimeric proteins produced in this system by an endogenous Kex-2 protease. The genes for both of the above-mentioned phytases have already been cloned; however, this is the first report of either of the two phytases being fused with the glucoamylase gene, placed under the transcriptional control of the *glaA* promoter and overexpressed in *A. awamori*. Following transformation of *A. awamori* with separate expression vectors (one for each phytase), induction of phytase expression in submerged culture was effected by utilisation of a starch-containing medium. Optimisation of heterologous protein production in small shake-flask cultures involved changes in medium constituents. Maximum phytase expression levels of 200 phytase units (PU) ml^{-1} and 62 PU ml^{-1} for recombinantly expressed phytases from *A. awamori* and *A. fumigatus*, respectively, were obtained in crude fermentation extracts. Subsequent process scale-up to 4 l batch fermentation yielded phytase production levels comparable to those obtained on small scale. The enzyme yields herein reported demonstrate that the expression system developed and the host strain utilised were capable of expressing phytase at levels comparable to, or exceeding, previously reported data.

Keywords *Aspergillus awamori* · *Aspergillus fumigatus* · Gene expression · Phytase · Submerged culture

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Introduction

Phosphorous is an essential element for the growth and development of all organisms, playing key roles in skeletal structure and in vital metabolic pathways too numerous to mention. As such, all animal diets must contain adequate amounts of this element. The negative effects of phosphorous-deficient diets on livestock performance are multifold and well documented [5], and include reduced appetite, bone malformation and lowered fertility. The principal storage form of phosphorous in feedstuffs of plant origin is the hexaphosphate ester of *myo*-inositol, more commonly known as phytic acid, which is the mixed salt of *myo*-inositol hexaphosphoric acid [7]. This molecule accounts for up to 80% of the phosphorous in grains and seeds [18]. Phytic acid, and the salts and esters of phytic acid, are considered to be anti-nutritional factors as they chelate essential minerals and interact with proteins, thereby decreasing their bioavailability [4].

Phytase is an enzyme that hydrolyses phytate to liberate inositol and inorganic phosphorous. Phytase activity is widespread in nature and has been reported in plant and animal tissues, and in a variety of microorganisms [6,14]. Monogastric animals such as pigs and poultry have very low levels of phytase in the intestine, with the result that phytic acid phosphorous is essentially unavailable to them and is thus excreted. To counteract this, an external source of phosphorous must be supplied in sufficient quantity to meet the daily requirements of the animal. Together, these factors result in the environment becoming overloaded with

phosphorous in areas of intensive livestock production [33]. The phytic acid excreted in the manure of feed animals is enzymatically hydrolysed by soil and water microorganisms. The phosphorous released is transported into rivers and lakes and, if introduced in high quantities, causes eutrophication [3]. In contrast to monogastrics, phytate phosphorous is readily available to ruminants, as certain microorganisms present in the rumen produce phytase, catalysing the conversion of phytate to inositol and available inorganic phosphate [4].

Dietary supplementation with microbial phytase is well established as an effective and practical method of improving phytate digestibility and increasing phytate-phosphorous utilisation in production animals [15,23]. The use of phytase as an enzyme in animal feed supplementation sets certain demands upon the properties of the enzyme. In particular, the enzyme should withstand high temperatures such as those involved in the pelleting of animal feeds [11]. During the pelleting process, temperatures may transiently reach 90°C [34]. A heat-stable phytase able to withstand temperatures up to 100°C over a period of 20 min with a loss of initial enzymatic activity of only 10% was described by Pasamontes et al. [19]. However, *Aspergillus fumigatus* phytase has not been accepted as thermostable by all researchers. Some maintain that the enzyme is not thermostable but has a remarkable ability to refold completely into a native-like, fully active conformation after heat denaturation [34].

With this in mind, the aim of this study was to isolate *phyA*-encoding genes from *Aspergillus awamori* and *A. fumigatus* and induce expression of these enzymes in the host strain *A. awamori*. Two expression vectors containing the distinct phytase genes amplified by PCR from genomic DNA of *A. awamori* and *A. fumigatus* were constructed. These constructs were used to transform *A. awamori*, resulting in the generation of genetically modified strains in which the fungal phytases were placed under the control of the glucoamylase A (*glaA*) promoter from *A. awamori*. Screening of positive transformants for production of active phytases resulted in selection of two clones, one generated from each vector, that secreted significant levels of extracellular phytase into the medium.

Materials and methods

Strains and plasmids

A. awamori ATCC 76060 was obtained from the American Type Culture Collection (Manassas, Va.) and *A. fumigatus* CBS 148.49 was obtained from the Centraal Bureau voor Chimmelcultures (CBS) Fungal and Yeast Collection, Utrecht, The Netherlands. The expression vector pGEXA was designed by R. Murphy, Alltech Ireland. *Escherichia coli* strain Inv′αF, and the plasmid PCR 2.1 were from Invitrogen (Carlsbad, Calif.). All manipulations for the growth and storage of *E. coli* were carried out according to standard procedures [25].

Chemicals and reagents

All materials used were of molecular biology grade or the highest grade available. All bacterial and fungal culture media reagents were supplied by either Difco (Detroit, Mich.) or Sigma-Aldrich, (St. Louis, Mo.). Lodex 5 maltodextrin was obtained from Cerestar, Neuilly-sur-Seine, France. Multi-purpose agarose, ampicillin, RNase A, restriction endonucleases, DNA-modifying enzymes, digoxigenin (DIG) system components, *Taq* DNA polymerase and polymerase chain reaction (PCR) reagents were obtained from Roche (Nutley, N.J.). All other chemicals, reagents and molecular weight markers for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Sigma Aldrich.

Nucleic acid manipulations

DNA was isolated from 200 mg lyophilised mycelia grown as described by Rasmussen et al. [22]. All nucleic acid manipulations were based on the methods of Sambrook et al. [25]. Total RNA was isolated from ground mycelia using the Ultraspec-II RNA Isolation System (Biotecx, Houston, Tex.) according to the manufacturer's instructions. For northern analysis, mRNA was isolated from total RNA using a mRNA isolation kit (Sigma-Aldrich). Capillary blotting was used to transfer electrophoretically resolved mRNA from agarose gels to nitrocellulose membranes for northern hybridisation analysis. Detection of mRNA species of interest was carried out using the DIG detection system according to standard protocols as outlined by the manufacturer (Roche). Hybridisation of DIG-labelled RNA probes was carried out as previously described [25] and the resultant signal on the nucleic acid blot was detected using immunological techniques as follows. An anti-DIG-antibody-alkaline phosphatase conjugate was allowed to bind to the hybridised probe. This signal was then detected with a chemiluminescent alkaline phosphatase substrate and appeared as a dark band on standard X-ray film.

Isolation of phytase genes from *A. awamori* and *A. fumigatus*

Two genes encoding phytase were amplified by PCR, one from genomic DNA of *A. awamori* ATCC 76060 and the other from genomic DNA of *A. fumigatus* CBS 148.49. The 5′ oligonucleotides were so designed as to place the gene in frame when subcloned into the expression vector pGEXA immediately downstream from the glucoamylase gene sequence, thus placing the gene under the control of this highly efficient starch-inducible promoter. The nucleotide sequences of the primers utilised (incorporating the restriction sites indicated) are as follows. The *A. awamori phyA* gene was isolated using primers based on the previously published sequence of *Aspergillus niger phyA* phytase [29]: 5′ end oligonucleotide (Phytp2) 5′ GATATCGCAGTCCCCGCCTCGAGAAATCAA 3′ (*Eco*RV), 3′ end oligonucleotide (Phybm2) 5′ ATCGATTAAGC-AAAACACTGCGCCCAATCACC 3′(*Cla*I). Through utilisation of a published nucleotide sequence for the coding region of the putative thermostable *A. fumigatus phyA* gene [19], specific 5′ and 3′ oligonucleotide primers were constructed: 5′ end oligonucleotide (Fum5B) 5′ GCTGATATCAAGTCCTGCGATACGGTAGAC 3′ (*Eco*RV), 3' end oligonucleotide (Fum3B) 5′ GATATCTCAAC-TAAAGCACTCTCCCCA 3′ (*Eco*RV).

A typical PCR reaction contained the following: 1 μg total genomic DNA or 50 ng plasmid DNA; primers (500 ng each); $MgCl_2$ (2.5 mM); $(NH_4)_2SO_4$ reaction buffer at 1× concentration; dNTPs (500 μM each) and sterile distilled water to 98 μl. The enzyme (2 U) was added after an initial denaturation step. An automated DNA thermal cycler (Perkin Elmer Cetus, Foster City, Calif.) was used for amplification of PCR products using standardised techniques as outlined by the manufacturer. Typically, amplification by PCR consisted of the following: a 2 min

denaturation step for total genomic DNA or 4 min for plasmid DNA at 94°C, followed by 1 min at 85°C for addition of the polymerase. Subsequent to the addition of the polymerase, a total of 30 cycles of 1 min at 94°C, 1 min at 60°C and 1–2 min at 72°C was carried out. A final 10 min step at 72°C was employed to ensure homogeneity of the amplified fragments. The DNA fragments obtained were subcloned separately into the shuttle vector PCR 2.1 and nucleotide sequencing of these plasmids was carried out using M13 forward and M13 reverse primers. Analysis of the resultant sequence data was performed using the BLAST sequence analysis package available online at the NCBI homepage (http://www.ncbi.nlm.nih.gov) and the two DNA fragments were confirmed to be two distinct phytase genes. Alignment of the PCR-generated phytase sequence obtained from *A. awamori* 76060 with DNA sequences encoding phytases in the database revealed that this phytase was 97% homologous to the *phyA* phytase of *A. niger* (accession number Z16414). Similarly, the PCR-generated fragment amplified from *A. fumigatus* CBS 148.49 displayed 98% homology to the *A. fumigatus* phytase *phyA* gene (accession number U59804).

The confirmed phytase genes were subsequently excised from the shuttle vectors using the appropriate restriction enzymes. Following electrophoresis, DNA fragments of interest were gel purified using an agarose gel DNA extraction kit obtained from Sigma-Aldrich. Ligation of these DNA fragments separately into *Stu*I-digested alkaline phosphatase-treated vector pGEXA yielded two distinct expression constructs, namely pGEXAPhyA (for *A. awamori* phytase) and pGEXAFum (for *A. fumigatus* phytase). A gene fusion strategy was employed that involved the insertion of a hexapeptide Kex-2 protease cleavage site between the native glucoamylase and heterologous proteins and allowed for the efficient secretion and processing of the resultant chimeric proteins produced in this system by an endogenous Kex-2 protease. The expression vectors also contained a selectable marker (*sh-ble*) encoding phleomycin resistance and allowed for the efficient selection of transformants that had successfully incorporated the expression construct into the genome. Nucleotide sequencing results verified the integrity of the ligation junction on each expression vector and confirmed the presence of the gene fragment in the correct reading frame for expression.

Growth and transformation of *A. awamori*

The host strain *A. awamori* ATCC 76060 strain used in this study was grown and maintained as previously described by Ward et al. [32]. The expression constructs were subsequently used to transform *A. awamori* according to methods described previously [26,32]. Positive transformants were identified by their ability to grow in the presence of 100 μg ml^{-1} phleomycin.

PCR analysis of positive transformants

PCR analysis was carried out on genomic DNA from putative transformants to confirm integration of plasmid DNA into the *A. awamori* genome. A top strand primer (pGEXseq5′: 5′ GTGGCCGAGTATCGTGGC 3′), which anneals to an upstream region 5′ from the ligation junction, was utilised. The bottom strand primer used (Phybm2 or Fum3B outlined above) was homologous to the 3′ end of the appropriate phytase gene depending on the expression vector from which the transformants were generated. Control PCR reactions were also performed on genomic DNA from untransformed *A. awamori* (negative control), and on plasmid DNA from the appropriate expression vector containing the phytase gene of interest (positive control).

Expression of recombinant phytases

Induction of phytase expression was carried out by growth of transformants in medium containing (g l^{-1}) soluble starch: 100; yeast autolysate: 64; tri-sodium citrate: 70; $MgSO_4 \cdot 7H_2O$: 2; $(NH_4)SO_4$: 15; $NaH_2PO_4 \cdot 2H_2O$: 7. Small scale cultures were grown at 30°C in 250 ml shake flasks containing 50 ml inducing medium on a rotary shaker set at 200 rpm. Larger scale cultures were grown in a 5 l batch fermenter with a 4 l working volume, the operating parameters of which are outlined in the text.

Phytase assay and SDS-PAGE analysis of culture supernatants

Enzyme-containing culture extracts were examined for phytase activity using a modification of the assay described by Heinonen and Lahti [10]. Essentially, 0.5 ml aliquots of enzyme sample, suitably diluted in 5 mM sodium acetate buffer pH 5.5, were added to 0.5 ml substrate solution (2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer pH 5.5) and incubated for 10 min at 37°C. The reaction was stopped by addition of 2 ml ice-cold colour stop solution (10 mM ammonium molybdate:5 *N* sulphuric acid:acetone, 1:1:2) followed by the addition of 100 μl 1 M citric acid. Assays were carried out in triplicate with triplicate blanks. Following incubation and subsequent substrate hydrolysis, enzyme activity was quantified from the amount of orthophosphate released by the hydrolysed substrate upon determining the increase in absorbance at 380 nm. The results of the unknowns were compared to a standard curve prepared with inorganic phosphate (K_2HPO_4) and phytase activities of test samples were determined. Phytase assay results from fermentation samples were expressed as phytase units per millilitre supernatant (PU ml^{-1}). One PU represents the quantity of enzyme liberating 1 μmol inorganic phosphate per minute from 2.5 mM sodium phytate at pH 5.5 and 37°C. Cell-free extracts were obtained by centrifugation of culture supernatants at 16,000 *g* for 10 min. These extracts were then desalted using CENTRI-SPIN columns (Princeton Separations, Adelphi, N.J.) according to the manufacturer's instructions. Proteins were quantified by the method described by Bradford [2] using a protein assay reagent kit (Sigma-Aldrich) with bovine serum albumin (BSA) as standard. SDS-PAGE analysis was carried out according to the method of Laemmli [12] on a 1.5 mm thick, 7.5% (w/v) or 12% (w/v) separating gel with a 4% (w/v) stacking gel using a Bio-Rad Protean II vertical slab gel electrophoresis system.

Results

Expression in *A. awamori*

In this study, the gene products of interest were expressed as glucoamylase fusion proteins to aid folding and passage of the heterologous protein through the secretory pathway. Post-translational cleavage of the chimeric proteins yielded active phytase.

Identification of phytase-producing clones

A total of 30 transformants generated from each expression vector were screened for production of active phytases over a 9 day period in inducing medium containing 10% (w/v) starch. The transformants secreting the highest level of extracellular phytase identified as pGP209 (*A. awamori phyA* phytase) and pGF11 (*A. fumigatus phyA* phytase), produced using expression vectors pGEXAPhyA and pGEXAFum, respectively, were selected for further studies.

Confirmatory PCR and northern hybridisation analysis

PCR analysis indicated that the respective expression constructs had integrated into the *A. awamori* genome, as indicated by the presence of a unique DNA fragment of the appropriate size (~1.5 kb) in positive transformants, which was absent from the untransformed host strain (data not shown). Northern hybridisation analysis was performed to determine if correct transcriptional control was being effected by the regulatory elements of the expression vectors employed. Spores (1×10^6) from transformants pGP209 and pGF11, and the untransformed host strain *A. awamori* were inoculated into inducing medium and grown for 7 days following which total RNA was extracted. To serve as negative controls, total RNA was also prepared from uninduced cultures of the above strains. mRNA from uninduced and induced cultures of transformants pGP209 and pGF11 and the host strain *A. awamori* was electrophoresed on duplicate 1.2% (w/v) denaturing agarose gels. The mRNA was subsequently transferred to nitrocellulose membranes by capillary action. One blot was probed with a DIG-labelled RNA probe specific for the *A. awamori phyA* gene, while the other was probed with a DIG-labelled RNA probe specific for the *phyA* gene from *A. fumigatus*, i.e. cross-probing was essentially performed. In the case of transformants generated using expression vector pGEXAPhyA, the predicted size of an intact mRNA transcript encoding the glucoamylase-phytase fusion protein is approximately 3.5 kb. Correctly processed mRNA transcripts from induced pGEXAFum transformants have an expected size of approximately 3.4 kb. Figure 1 shows that a single transcript of the predicted size was detected on each blot, indicating the stability of the mRNA in this expression system. Efficient regulation of the glucoamylase promoter led to the detection of a signal in mRNA from the induced transformants only, with no signals being evident in mRNA from uninduced transformants. The absence of a signal in mRNA from induced and uninduced controls demonstrates the efficacy of the probes and, as is evident from Fig. 1, there was no cross-reactivity between probes for the individual phytases.

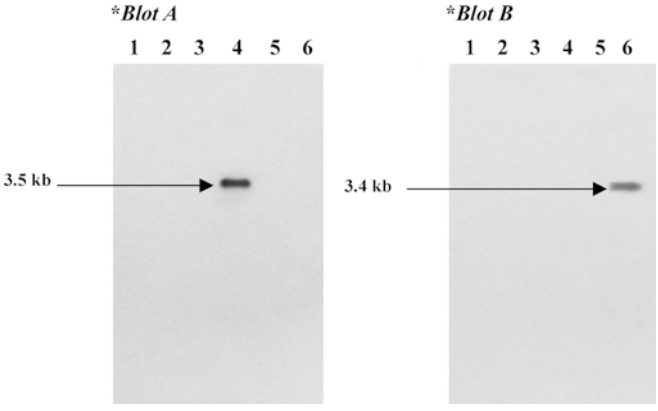


Fig. 1 Northern hybridisation analysis of mRNAs from pGEXAPhyA and pGEXAFum transformants. Lanes: *1* Uninduced host strain *A. awamori*, *2* induced host strain *A. awamori*, *3* uninduced transformant pGP209 (harbouring *A. awamori phyA* gene), *4* induced transformant pGP209 (harbouring *A. awamori phyA* gene), *5* uninduced transformant pGF11 (harbouring *A. fumigatus phyA* gene), *6* induced transformant pGF11 (harbouring *A. fumigatus phyA* gene). Blots *A* and *B* were probed with DIG-labelled RNA probes specific for the *A. awamori phyA* gene and the *A. fumigatus phyA* gene, respectively

Optimisation of heterologous phytase production on small scale

In an effort to optimise the production of biologically active recombinant phytase from the system, a number of strategies were employed. Small scale (50 ml) optimisation studies were performed in submerged culture. To this end, a series of experiments were conducted in which the optimal induction period, optimal starch concentration and type of carbon and nitrogen sources to be utilised were assessed. In an effort to reduce proteolytic degradation of the heterologous protein, the protective effects of supplementation of an already complex inducing medium with increasing concentrations of yeast autolysate and additional protein sources were assessed. Finally, using the growth conditions considered optimal for small scale recombinant phytase production, attempts were made to scale up the production process to 4 l batch fermentation.

Time-course of heterologous phytase production

The time-course of phytase production by transformants pGP209 and pGF11 is presented in Fig. 2. The

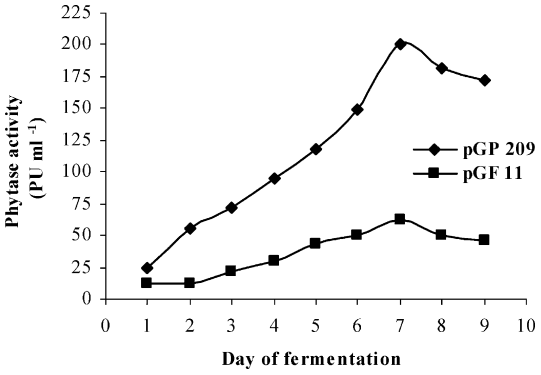


Fig. 2 Time-course of recombinant phytase expression in submerged culture. Results represent means of triplicate assays of samples taken from triplicate flasks on each day of the 9 day induction period

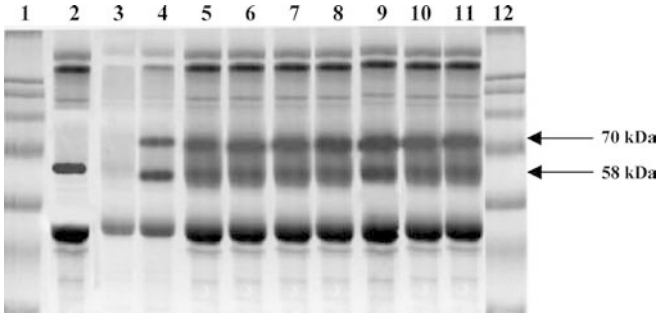


Fig. 3 SDS-PAGE analysis of cell-free extracts from shake flask cultures of pGF11 on 12% (w/v) acrylamide. Lanes: *1* Molecular weight markers (205 kDa, 116 kDa, 97 kDa, 84 kDa, 66 kDa, 55 kDa, 45 kDa and 36 kDa); *2* cell-free extract from untransformed host strain 7 days post induction; *3–11* cell-free extract from transformant pGF11 on days 1–9 post induction, respectively; *12* molecular weight markers

expression levels of heterologous phytase increased on a daily basis until day 7, after which time there was a notable decrease in the level of recombinant phytase produced by both strains. No phytase activity was detected at any time point in the negative control (induced host strain). The highest level of phytase produced was 200 PU ml^{-1} from pGP209 and 62 PU ml^{-1} from pGF11, respectively, on day 7, with enzyme production being linked to growth of the organism. After this period, enzyme production declined rapidly due in part to limiting inducer concentration and also to cellular lysis. Having identified the optimum induction period, all subsequent small scale liquid fermentations were harvested after 7 days. The differences in the phytase activities of the two recombinantly produced enzymes in this study are due to differences in the expression levels of the enzymes, but also to kinetic differences between the two enzymes in question, as observed by Wyss et al. [35]. These authors classified histidine acid phosphatase phytases into two groups: (1) those displaying broad substrate specificity, but low specificity for phytic acid (e.g. *A. fumigatus* phytase) and (2) those displaying narrow substrate specificity and high specificity for phytic acid (e.g. *A. niger* phytase).

SDS-PAGE analysis

SDS-PAGE analysis was performed on cell-free extracts from transformants pGP209, pGF11 and the control host strain taken on each day of the induction period. A unique band of approximately 70 kDa was evident in supernatants obtained from pGF11, which was absent from the control lane (Fig. 3). This correlated with the predicted molecular weight of the phyA gene product from *A. fumigatus* [19,24]. In the case of supernatants obtained from cultures of pGP209, a similar gel to that presented in Fig. 3 was obtained. However, in this case the phytase protein from *A. awamori* had a molecular weight of 84 kDa, which was absent from the control lane (data not shown). This result correlated closely with the molecular weight of *A. niger* phytase from previously published data [33]. As expected, a protein of approximately 58 kDa representing the native glucoamylase A protein was visible in all lanes, including the control lanes (Fig. 3).

Effect of starch concentration/source on expression of recombinant phytase

As the concentration of inducer increased from 10 to 100 g l^{-1}, there was a noticeable increase in the expression of heterologous phytase. Induction of the starch-inducible *glaA* promoter and the consequent expression of phytase in this system was optimal at a starch concentration of 100 g l^{-1}. At higher inducer concentrations, a reduction in the expression level of heterologous phytase was noted. A series of compounds was analysed for their inductive effects using the optimal concentration (100 g l^{-1}) determined above. The most effective starch source to utilise in the inducing medium at a concentration of 100 g l^{-1} was Lodex 5 maltodextrin. The least effective sources of starch at the same concentration were cornsteep solids and cornsteep liquor, both of which resulted in production of very reduced levels of phytase.

Effect of glucose addition

Glucose is a product of the enzymatic hydrolysis of starch by hydrolytic enzymes including glucoamylase. The effect on phytase expression of the addition of glucose to the culture medium at varying concentrations from 0 to 250 g l^{-1} was assessed. The addition of glucose to the medium had a pronounced effect on phytase production by this system. It is clear from Fig. 4 that the expression of phytase was lower at all concentrations of glucose tested compared to unsupplemented cultures. In effect, the higher the concentration of glucose in the medium, the greater the reduction in phytase activity observed.

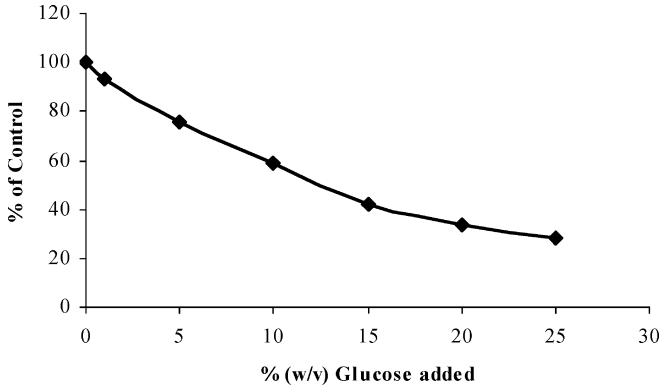


Fig. 4 Effect of glucose addition on heterologous phytase production in shake flask culture. Results represent means of triplicate assays of samples taken from triplicate flasks. Activities expressed as a percentage of the control (no glucose added)

Effect of nitrogen and supplemental protein sources

Efforts to determine the optimal source of nitrogen to utilise in the inducing medium demonstrated the relative importance of this element for the organism. The source of nitrogen utilised in the medium significantly affected growth of the fungus and consequently phytase production. Nitrogen is essential for growth; in its absence, fungal growth was severely affected, culminating in a detrimental effect on phytase production. The most effective source of nitrogen to use in this inducing medium was yeast autolysate, an organic nitrogen source, at a concentration of 6.4% (w/v). In an effort to reduce proteolytic degradation of the heterologous phytases, the protective effect of supplementation of the medium with varying protein sources at a concentration of 20 g l^{-1} was determined. The addition of protein sources such as malt extract and casamino acids to the culture medium had a beneficial effect, culminating in a marginal increase in the levels of phytase produced. In all of the above experiments, the untransformed host strain *A. awamori* was induced alongside the recombinant strains and in no case was phytase activity detected in these culture filtrates.

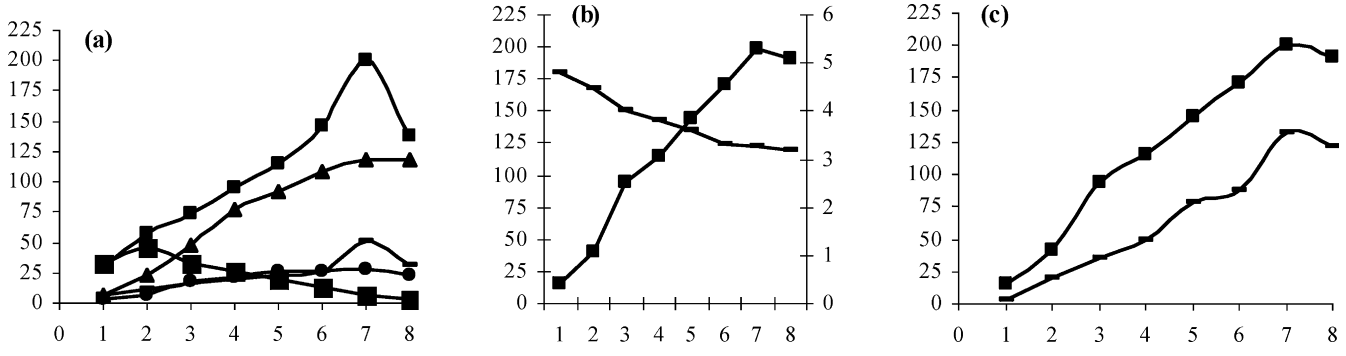
Scale-up

From the foregoing results it was concluded that an inducing medium, containing 100 g starch l^{-1} and 64 g yeast autolysate l^{-1} was optimal for production of phytase by this system. The effects of scale-up on recombinant phytase expression by this system were subsequently determined. For all parameters tested, a starter culture (250 ml culture of transformant pGP209 induced for 4 days in normal inducing medium) was used to inoculate a 5 l batch fermentation vessel with a 4 l working volume of inducing medium.

Effect of agitation intensity

Proper aeration and agitation of the medium is important to keep the medium constituents, microbial cells and oxygen uniformly suspended [31]. The effect of agitation intensity on recombinant phytase production by this system in 4 l batch fermentation was investigated. The aim of the experiment was to determine whether the rate of phytase production was influenced by the degree of agitation employed in the fermentation. Starter cultures (4-day-old) of pGP209 were used to inoculate 5 l batch fermentors with a working volume of 4 l inducing medium containing 10% (w/v) starch and 6.4% (w/v) yeast autolysate. A controlled aeration rate was used in all scale-up attempts. Varying agitation rates were tested in an effort to optimise phytase production. Agitation was performed by two, four-bladed, Rushton turbines. Cell-free extracts were analysed on a daily basis for phytase activity. The agitation rates tested ranged from 50 rpm to 600 rpm and results obtained from fermentations conducted under these conditions are presented in Fig. 5a. One of the main limiting factors to expression of recombinant phytase in this system was the agitation rate. Variations in the intensity of agitation resulted in notable changes in enzyme production, with higher enzyme production generally observed at higher

Fig. 5a–c Optimisation of phytase expression by pGP209 in 4 l batch fermentation. *x-axes* Day of fermentation, *y-axes*: phytase activity [phytase units (PU) ml^{-1}]. **a** Effect of agitation intensity: ● 50 rpm, – 200 rpm, ▲ 400 rpm, small ■ 450 rpm, large ■ 600 rpm. **b** pH analysis of 8-day fermentation: – pH, ■ phytase activity. **c** Effect of pH control: – pH controlled at pH 5.0, ■ no pH control. Results represent means of triplicate assays of triplicate samples from duplicate fermentations



agitation rates. The optimal rate of agitation for phytase production was 450 rpm. Below this rate, phytase production levels declined, while at agitation rates greater than 450 rpm, expression of phytase was dramatically reduced. Microscopic analysis of samples obtained from these fermentations revealed cellular lysis.

Time-course of heterologous phytase production in 4 l batch fermentation

Having optimised the agitation rate best suited to phytase expression in this system, a time-course study to determine the optimal induction period for heterologous phytase expression by recombinant strains pGP209 and pGF11 in a 4 l working volume at 450 rpm was performed. As was the case in small-scale cultures (Fig. 2), the optimal day to harvest large-scale fermentations was also found to be day 7. Maximum phytase activities of 200 PU ml^{-1} and 62 PU ml^{-1} were obtained by fermentation of recombinant strains pGP209 and pGF11, respectively. These expression levels were thus consistent with those levels achieved in small-scale fermentations.

pH analysis and effect of pH control

To determine the effect of pH on phytase production in this system, a pH versus activity profile for phytase production in 4 l batch fermentation over an 8 day period was generated (Fig. 5b). The cultivation of recombinant strain pGP209 in a 4 l batch reactor without pH control showed many interesting characteristics. The initial pH of the inducing medium was 5.0 and, as the fermentation proceeded, a concomitant drop in pH was noted. Enzyme production began almost immediately and continued to increase on a daily basis until it peaked on day 7. The gradual increase in phytase expression was coupled with a gradual decline in the pH of the medium, which reached its lowest level of pH 3.25 on day 7. This phenomenon is typical of *Aspergillus*, *Rhizopus* and yeast fermentations whereby starch utilisation and the production of acidic by-products results in a decline in the pH of the medium as the fermentation progresses. In an effort to determine the effect of pH control on phytase production by this system, replicate fermentations were set up where the pH of each fermentation was controlled at pH 5.0. A comparison of phytase expression levels from both pH-controlled and non-pH-controlled fermentations is presented in Fig. 5c. These data clearly show that controlling the pH of the fermentation at pH 5.0 had a negative effect on phytase production. Following attempts to control the pH of the fermentation at values below pH 5.0 and at alkaline pH values, poor growth and low phytase production levels were observed (data not shown). In conclusion, higher phytase activities were observed in fermentations where pH control was not implemented.

Discussion

Due to the enormous potential for application of phytase in the animal feed industry, several other researchers have attempted to produce this enzyme cost-effectively. To date *A. niger phyA* phytase has been cloned and overexpressed in several microbial hosts, including *Saccharomyces cerevisiae* [9], *Pichia pastoris* [8] and *A. niger* [27,28,29]. The *phyA* gene from *A. niger* var *awamori* has been cloned and reintroduced into the same strain resulting in several-fold overproduction of phytase [21]. A high level of functional expression of *A. fumigatus* phytase in *P. pastoris* [24] and *A. niger* [19] has also been reported. Following overexpression of *A. niger* pH 2.5 acid phosphatase in the heterologous host *Trichoderma reesei*, transformants producing 240 times more acid phosphatase than the strain from which the gene was originally isolated were obtained [16]. A 2- to 6-fold increase in phytase production was observed by Moore et al. following expression of *S. cerevisiae* and *A. niger* acid phosphatase in *A. oryzae* [17]. In addition, the *phyA* gene from the thermophilic fungus *Thermomyces lanuginosus* has been cloned and overexpressed in *Fusarium venenatum* [1].

This is the first report of either the *A. awamori* or *A. fumigatus* phytase genes being fused with the glucoamylase gene, placed under the transcriptional control of the *glaA* promoter and overexpressed in *A. awamori*. Maximum phytase production levels of 200 PU ml^{-1} and 62 PU ml^{-1} for *A. awamori* and *A. fumigatus* phytases, respectively, observed in crude culture extracts compare very favourably with, and in some cases exceed, previously published data. For example *A. niger* phytase and *A. fumigatus* phytase have been expressed in *P. pastoris* at levels of 25–65 U ml^{-1} and 21–55 U ml^{-1}, respectively [8,24]. The expression levels reported in this study are very significant given the highest phytase expression levels obtained to date in Aspergillus transformants were obtained by van Gorcom et al. [28]. These authors reported maximum phytase production levels of 280 U ml^{-1} when *A. ficuum phyA* was overexpressed in *A. niger*. In addition, the yields presented in this paper are also greater than those obtained in *phyA*-transformed tobacco seeds [20], tobacco leaves [30], soybean seeds [13] and *A. niger* [29].

The recombinantly expressed phytases have been purified, characterised physico-chemically and compared to commercially available phytases (manuscript in preparation). The fungal strains developed show much promise, further advocating *A. awamori* as an efficient host for heterologous protein production. It is anticipated that further increases in the expression levels of these enzymes may be possible through isolation of protease-deficient strains. Ongoing efforts to isolate carbon-derepressed mutants, through a combination of UV irradiation and sodium nitrite/nitrosoguanidine treatment, followed by growth in the presence of the toxic glucose analogue, 2-deoxy-D-glucose, are very

promising. This strategy could potentially generate glucose-insensitive mutants of *A. awamori* capable of phytase expression in submerged culture in the presence of high levels of glucose.

Scale-up of phytase expression in this system from 50 ml shake flask cultures to 4 l batch fermentation proved successful as production levels similar to those observed on a small scale were obtained. Phytase production was dependent on agitation rate, with maximal production observed for both recombinant strains at an agitation rate of 450 rpm when pH control was not implemented. Scope exists for industrial scale optimisation work under submerged fermentation conditions to further increase the expression levels observed. It is reasonable to speculate that by employing some of the strategies outlined above, significant improvements in the production levels of recombinant phytases using this expression system could be achieved. In addition, there remains much scope for production of several other industrially important functional proteins using this system.

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