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ORIGINAL PAPER

A thermostable phytase from *Bacillus* sp. MD2: cloning, expression and high-level production in *Escherichia coli*

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Abstract Phytase is used as a feed additive for degradation of antinutritional phytate, and the enzyme is desired to be highly thermostable for it to withstand feed formulation conditions. A *Bacillus* sp. MD2 showing phytase activity was isolated, and the phytase encoding gene was cloned and expressed in *Escherichia coli.* The recombinant phytase exhibited high stability at temperatures up to 100°C. A higher enzyme activity was obtained when the gene expression was done in the presence of calcium chloride. Production of the enzyme by batch- and fed-batch cultivation in a bioreactor was studied. In batch cultivation, maintaining dissolved oxygen at 20–30% saturation and depleting inorganic phosphate below 1 mM prior to induction by IPTG resulted in over 10 U/ml phytase activity. For fed–batch cultivation, glucose concentration was maintained at 2–3 g/l, and the phytase expression was increased to 327 U/ml. Induction using lactose during fed-batch cultivation showed a lag phase of 4 h prior to an increase in the phytase activity to 71 U/ml during the same period as IPTG-induced production. Up to 90% of the total amount of expressed phytase leaked out from the *E. coli* cells in both IPTG- and lactose-induced fed-batch cultivations.

Keywords Alkaline phytase · *Bacillus* sp. · Fed-batch cultivation · Protein secretion

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Introduction

Phytase is an important enzyme used as an additive in animal feed. The enzyme catalyses the sequential hydrolysis of phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate; IP6) present in plant material to less phosphorylated myo-inositol derivatives with concomitant release of inorganic phosphate [28]. The phosphate is made available to monogastric animals, such as pigs, chickens and fish, that do not produce phytase and also their microflora cannot degrade phytate. This eliminates the need for external addition of phosphorous to the feed, which incurs costs and also contributes to environmental pollution [3, 23, 43]. Removal of phytate from the feed also has other advantages in terms of overcoming its negative nutritional effects such as decrease in the bioavailability of vital minerals [20, 21, 27, 30], impairing the function of digestive enzymes or decrease in the digestibility of feed protein [2, 3, 17]. For the phytase to be effective, it needs to be thermostable enough to withstand the high temperatures used during feed preparation.

A wide variety of phytases are known from different organisms; however, the focus has been on the enzymes of fungal origin, such as *Aspergillus* spp. [36, 38], which are significantly active at low pH as in stomach where phytate exists in a metal-free form but has low thermostability [19, 22, 28, 45]. A few phytases, known as alkaline phytases, which degrade phytate that is present as a metal-phytate complex in plants, have been reported from *Bacillus* spp. [3, 7, 12–14, 37] and pollen of some plants such as *Typha latifolia* [8] and *Lilium longiflorum* [35]. Such phytases can potentially be used for treatment of the animal feed prior to feeding (i.e., during feed mixing, pelleting and storage).

Several phytase genes have been successfully cloned and expressed in different microbial hosts, corn kernels of

transgenic maize and saliva of transgenic pigs [5, 12, 13, 17, 19, 39]. *Escherichia coli* is often the primary choice as host microorganism for production of recombinant proteins. Some of the earlier attempts with expression of *Bacillus* phytases in *E. coli* have resulted in production of inactive enzyme in the form of inclusion bodies [12, 33]. This entails additional steps for recovery of the active protein [33]. Kim et al. [15] have reported production of *Bacillus* sp. DS11 phytase to 20% content of the total soluble protein in *E. coli* BL21(DE3) using the pET-22b(+) vector with the inducible T7 promoter.

Many strategies have been devoted to the establishment of high cell density processes to increase the level of production of recombinant target proteins by *E. coli*. Most of them are related to controlling the substrate concentration at certain levels in order to avoid overflow metabolism and the resultant acetate accumulation under glucose excess conditions [1, 10, 18, 31]. Åkesson and coworkers [1] have previously proposed a feeding strategy controlled automatically by the dissolved oxygen level such that aerobic conditions are maintained in spite of the limitations in oxygen transfer, i.e., the feeding rate is decreased when maximum oxygen transfer capacity is reached in the bioreactor.

In this study, we report cloning and expression of a thermostable alkaline phytase from a newly isolated *Bacillus* sp. MD2 in recombinant *Escherichia coli*, and its production by high cell density cultivation using a strategy based on dissolved oxygen level to control stirrer speed and to regulate feeding to avoid anaerobic conditions and acetate accumulation.

Materials and methods

Bacterial strains, plasmids and media

Isolate MD2 was obtained from an excrement sample in Hanoi (Vietnam) on meat peptone agar (MPA) medium containing 1% sodium phytate. *Escherichia coli* strains DH5α and BL21(DE3) were purchased from Invitrogen (Carlsbad, CA) and Novagen (Madison, WI), respectively. The cloning vector pJET1.2 and expression vector pET-22b(+) were purchased from Fermentas (MBI Fermentas, Germany) and Novagen, respectively. pE10C2 plasmid was constructed in this study from pET-22b(+) and the phytase gene from *Bacillus* sp. MD2. The recombinant *E. coli* strain was cultivated either in LB medium supplemented with 100 μg/ml of ampicillin or synthetic glucose-minimal medium composed of (per liter): 10 g glucose, 2 g $(NH_4)_2SO_4$, 4.807 g K_2HPO_4 and 1.067 g $NaH_2PO_4.H_2O$, 0.5 g $(NH_4)_2HC_6H_5O_7$, 2 mM $MgSO_4$, 2 ml trace element solution (composed of 0.5 g $CaCl_2$, 16.7 g $FeCl_3$, 0.18 g $ZnSO_4.7H_2O$, 0.16 g $CuSO_4.5H_2O$, 0.15 g $MnSO_4.H_2O$, 0.18 g $CoCl_2.6H_2O$, and 20.1 g sodium EDTA per liter) [9], and 100 mg ampicillin.

Identification of *Bacillus* sp. MD2

Genomic DNA was extracted from the isolate MD2 according to Sambrook et al. [34]. The 16S rRNA gene of isolate was amplified and sequenced using a forward primer 8–27f (AGAGTTTGATCCTGGCTCAG) and a reverse primer 1492R (GGTTACCTTGTTACGACTT). The 16S rDNA sequence was compared with sequences available in public databases. Sequencing was done using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocols. Sequence reactions were electrophoresed using the ABI PRISM® 3100 DNA sequencer.

Cloning and expression of a phytase gene from the isolate MD2

Genomic DNA was extracted from the isolate MD2 following the method of Sambrook et al. [34]. The phytase encoding gene was amplified from the genomic DNA using a forward primer Phyf (TATAAAGCTTCTGTCTGATC CTTATCATTTTACCG) and a reverse primer Phyr (TCTCTCGAGTTTTCCGCTTCTGTCGGTCAG), which were designed based on the phytase gene sequence of closest organisms identified by 16S rDNA sequence similarity search. The gene amplification was performed by polymerase chain reaction (PCR) using 5 μl of *Pfu* DNA polymerase buffer (Fermentas), 1.25 units of *Pfu* DNA polymerase (Fermentas), 1.25 units of *Vent* DNA polymerase (New England BioLabs), 1 μg genomic DNA and 0.25 μM primers in a total volume of 50 μl. The reaction was run for 15 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. Finally, an additional post-cycle extension was carried out for 7 min at 72°C. The amplified fragments were purified and cloned into blunt cloning vector pJET1.2 and transformed to *E. coli* DH5α competent cells. After colony PCR screening, plasmids were purified from the insert positive clones by alkaline lysis method and then digested with *Hind*III/*Xho*I. The released inserts were re-cloned into expression vector pET-22b(+) to construct pE10C2 that was transformed into *E. coli* BL21(DE3) cells. All the cloning steps were done according to Sambrook et al. [34].

Shake flask cultivation of the recombinant *E. coli*

The *E. coli* BL21 (DE3) cells harboring the recombinant vector pE10C2 were grown on a LB-agar plate containing 100 μg/ml of ampicillin at 30°C. A single colony was picked from the plate and cultured for 5 h in 5 ml LB broth containing 100 μg/ml of ampicillin at 30°C and 200 rpm.

This culture was used to inoculate 100 ml of the same medium, which was incubated under similar conditions in a gyratory shaker incubator. When the optical density (OD) of the culture at 620 nm was about 0.7, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. Culture samples (3 ml) were withdrawn at different time intervals for analysis of enzyme activity in extracellular, cytoplasmic and periplasmic fractions according to Novagen pET System manual (http://www.novagen.com). The extracellular activity was determined in the cell-free supernatant obtained after centrifugation of the sample at 8,000 g for 10 min at 4°C. The cell pellet was washed with physiological saline and resuspended in 30 mM Tris–HCl buffer pH 8 containing 20% (w/v) sucrose and 5 mM $CaCl_2$, and then EDTA was added to a final concentration of 1 mM. After gentle shaking at room temperature, the cells were harvested by centrifugation and subjected to osmotic shock by re-suspending in ice-cold 5 mM $MgSO_4$, and shaken for 10 min on ice. After centrifugation, periplasmic activity was determined in the supernatant, while the remaining cell pellet was re-suspended in 0.1 M Tris–HCl buffer, pH 7, containing 5 mM $CaCl_2$, and the cytoplasmic content was released by four rounds of sonication. All the assays were performed in triplicates.

Batch- and fed-batch cultivations of recombinant *E. coli* in a bioreactor

A 3 liter bioreactor (Chemoferm FCL-B-3, Hägersten, Sweden) containing 2.5 l of minimal medium was autoclaved for 40 min at 121°C. After cooling, the sterilized medium was supplemented with sterilized solutions of glucose, trace elements, $MgSO_4$ and ampicillin, respectively, to the desired concentration. The medium was inoculated with 100 ml culture of *E. coli* BL21(DE3) harboring pE10C2 grown in the same medium for 16 h at 30°C with shaking at 200 rpm. For batch cultivations, phosphate buffer in the medium was replaced by 50 mM Tris-maleate buffer pH 7.0.

Cultivations in the bioreactor were performed at 30°C with stirring at an initial speed of 200 rpm. The dissolved oxygen (DO) level was monitored using a DO probe, and when reduced to 30% saturation, the stirrer speed was increased automatically, and the DO was thereafter controlled at 20–30% saturation. The pH of the culture was maintained by addition of 3.57 M ammonia solution, which was controlled by a signal from the pH probe in the bioreactor. The volume of the ammonia solution consumed during the cultivation was recorded. The concentration of glucose was monitored off line in duplicates every 30 min by Accucheck glucose test strips. Inorganic phosphate (P_i) in the fermentation broth was monitored in triplicates at regular time intervals according to the method of Shimizu [37] (see ahead under phytase activity assay). For induction of the phytase production, the IPTG or lactose was added to the bioreactor to a desired concentration when the concentration of inorganic phosphate had decreased to less than 1 mM.

Fed-batch cultivation was started using a continuous feed of a solution containing 50% (w/v) of glucose, 50 ml/l of 1 M $MgSO_4$ and 10 ml/l of trace element solution in order to maintain the glucose concentration at 2–3 g/l in the bioreactor.

Phytase activity assay

The phytase activity was assayed according to the method described by Shimizu [37]. Unless otherwise mentioned, a mixture of appropriately diluted enzyme sample and sodium phytate (1.5 mM) dissolved in 100 mM Tris–HCl buffer, pH 7, containing 5 mM $CaCl_2$ was incubated at 70°C for 20 min. The reaction was stopped by addition of an equal volume of 15% trichloroacetic acid (TCA) solution. The precipitate obtained was removed by centrifugation, and 500 µl of the clear supernatant was transferred to an Eppendorf tube to which was then added 500 µl ammonium molybdate reagent (containing 1:4 mixture of 2.7% $FeSO_4$ and 1.5% ammonium molybdate in 5.5% H_2SO_4). After 5 min incubation at room temperature, absorbance at 700 nm was read, which was used to calculate the concentration of P_i from a standard curve made using sodium dihydrogen phosphate solution in the concentration range of 0–600 µmol/l P_i. All the samples were assayed in triplicates. One unit of phytase activity was defined as the amount of enzyme that releases 1 µmole of P_i per minute under the assay conditions.

Protein analysis

The purity of the phytase samples prepared from the cultivation broth was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12.5% (w/v) polyacrylamide.

Protein concentration was measured by bicinchoninic acid method using bovine serum albumin as standard. The recorded values were an average of measurements of triplicate samples.

Results and discussion

Isolation and identification of phytase positive strain

The isolate MD2 showing positive phytase activity (0.2 units/ml) was obtained from a sample of infant excreta after 3 days of incubation at 37°C in MPA medium containing

1% sodium phytate. The 1,407-nucleotide-long 16S rRNA gene sequence (GenBank accession number GU143091) of the isolate showed the highest similarity (99%) to *Bacillus subtilis*, *B. amyloliquefaciens* and *B. velezensis* sequences.

Sequence analysis of *Bacillus* sp. MD2 phytase

The phytase encoding gene (GenBank accession number GU143090) of *Bacillus* sp. MD2 was amplified and sequenced so that the amino acid sequence could be deduced. The sequence comprised a mature peptide of 354 amino acids, and like other phytases of *Bacillus* origin, it lacked RHGXRXP, a conserved motif at catalytic site of acidic phytases [28]. The sequence is highly similar to that of *ts-phy* from *Bacillus amyloliquefaciens* DS11 [15], the only difference being at positions 81 and 148 where Ala and Asn in *ts-phy* are replaced by Val and Asp, respectively. Close similarity is also seen with *phyC* from *Bacillus subtilis* VTTE68013 [12], the sequences differing by eight residues.

Expression and localization of recombinant *Bacillus* sp. MD2 phytase

A plasmid construct (pE10C2) was prepared in which the phytase gene was inserted between the restriction sites for *Hind*III and *Xho*I at the multiple cloning site of pET-22b(+). This construction allows expression of the phytase with *pel*B leader peptide at the N-terminal end, which is expected to facilitate the export of the target protein to the periplasmic space. The recombinant constructs without the leader peptide resulted in most of the phytase being insoluble, and there was very little activity detected in the culture. *E. coli* BL21(DE3) cells, transformed with the recombinant plasmid pE10C2 with the leader peptide, were grown in LB medium supplemented with ampicillin in a shake flask at 30°C and induced for expression of the enzyme activity by addition of 1 mM IPTG. Maximal phytase activity of 1.34 U/ml was obtained at about 4 h after induction. The activity in the cytoplasm and periplasm fractions was seen to decrease after 4 h and was eventually lost while the extracellular activity increased steadily (Figure 1a). The loss of cell-associated activity seems to be due to its complete release into the medium, although some degradation by intracellular proteases cannot be ruled out.

Supplementation of the medium with $CaCl_2$ during induction resulted in expression of higher phytase activity, with maximum activity of 3.6 U/ml being obtained at 10 mM $CaCl_2$. The activity was detected in all three fractions, although the extracellular activity was the most predominant. The periplasmic phytase activity started to decrease after 10 h of induction while the activity in the extracellular fraction increased to about 50% of the total

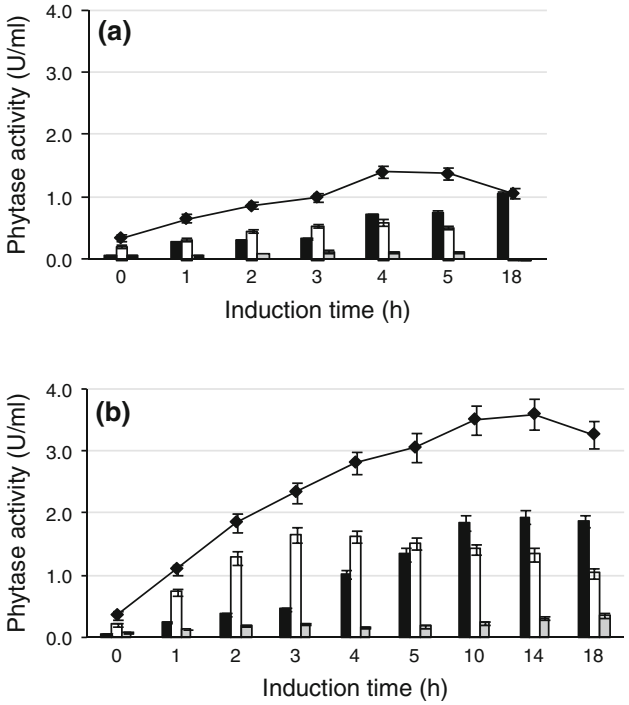

Fig. 1 Distribution of the expressed recombinant phytase in shake flask cultivation of *E. coli* BL21(DE3) at 30°C. Cells grown **a** without $CaCl_2$ and **b** with 10 mM $CaCl_2$ during enzyme induction. Symbols: Total phytase activity (*filled diamond*), and activity in cell free fraction (*filled bar*), periplasmic fraction (*open bar*) and cytoplasmic fraction (*gray bar*)

activity (Fig. 1b). The recombinant phytase was estimated to constitute about 19% of the total soluble protein present in the cells. The higher activity obtained on addition of $CaCl_2$ seems to be due to stimulation of the activity and increased stability of the phytase in the presence of the salt. Increasing the cultivation temperature to 37°C decreased the level of expression, but increased the extent of secretion of the recombinant enzyme into the culture medium (data not shown).

The possibility to secrete the target protein into the extracellular medium provides several advantages for enzyme production including a better folding environment free of cell-associated proteolytic degradation and easier recovery without the need for cell disruption and separation of cell debris [26]. There are several reports on the recombinant proteins directed to the periplasm being exported out of the cells [26, 29, 41]. This has been attributed to the stress on the outer membrane due to periplasmic accumulation of proteins resulting in loss of membrane integrity or triggering of autolytic response caused by change in cultivation conditions. Leakage of the periplasmic enzymes by increasing the growth temperature is likely to be caused by induction of heat-shock response and activation of the phospholipase activity in the outer membrane [41]. There have also been many efforts to engineer *E. coli* to excrete

proteins extracellularly [26]. Recently, secretion of *Bacillus* hydrolytic enzymes using their native signal peptides or that of *E. coli* outer membrane protein, OmpA has been achieved [46]. Miksch et al. [24] have used the coexpression of Kil protein to secrete a recombinant *E. coli* phytase.

Properties of the recombinant *Bacillus* sp. MD2 phytase

In order to characterize the enzyme, the recombinant *Bacillus* sp. MD2 phytase was purified to homogeneity from the crude cell homogenate by heat treatment, immobilized metal affinity chromatography and gel filtration to yield an enzyme with a specific activity of 39 U/mg. The pure enzyme had a molecular weight of 47.5 kDa and was optimally active at pH 6–7 and 67–73°C. The presence of 2–5 mM Ca^{2+} was essential for the enzyme to be active at 70°C. It was highly stable in the pH range of 3.6–9.6 at room temperature, and exhibited remarkable thermostability in the presence of 5 mM $CaCl_2$, retaining about 40% of the activity at pH 7 after 10 min at 100°C (data not shown). This is a desirable feature for the enzymes for animal feed applications in order to tolerate pelleting temperature of 80–85°C. Indeed, evaluation of the *ts*-phytase from *B. amyloliquefaciens*, to which the MD2 phytase is related, has shown promise for application in feed treatment [16].

A distinguishing feature of the MD2 phytase with respect to the other alkaline phytases that possess strict specificity for phytate substrate [4, 28] was that the enzyme, although being most active with phytate, displayed even some degree of activity with other phosphate substrates, such as ADP, ATP, α-fructose-1,6-diphosphate, *p*-nitrophenyl phosphate and sodium pyrophosphate.

Production of the recombinant phytase by batch cultivation in a bioreactor

Since LB medium does not have a good buffering capacity and is also expensive, a minimal medium, with glucose as carbon source and ammonium sulfate and citrate as the inorganic source of nitrogen, was tested for cultivations. Preliminary investigations with phosphate buffer in the medium led to precipitation of calcium phosphate when the cells were induced in the presence of $CaCl_2$. It was thus replaced by Tris-maleate (50 mM), pH 7, for buffering the medium. Cultivations in shake flasks in this medium resulted in very slow cell growth taking about 5–7 h to reach an OD_{620} of 0.7 prior to induction of the cells. During cultivation in a bioreactor at an initial stirrer speed of 200 rpm, the cells started to grow exponentially after 4 h (Fig. 2) at a maximum specific growth rate μ_{max} of 0.49 h^{-1}. The dissolved oxygen started to decrease after 3 h of cultivation, and when it reached 30% saturation the stirrer speed was increased (around 6 h), and the DO level was

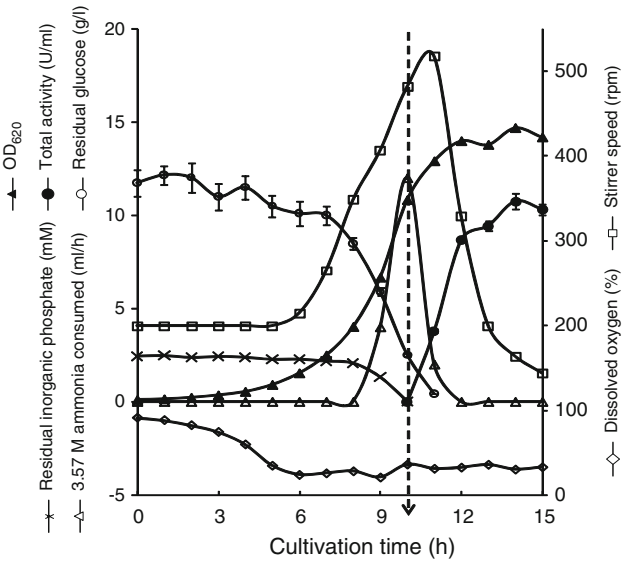

Fig. 2 Batch cultivation of recombinant *E. coli* in 2.5 l of minimal medium with 50 mM Tris-maleate buffer pH 7. Dissolved oxygen, stirrer speed and ammonia consumed are log data from the bioreactor, inorganic phosphate, OD_{620} and total activity were off line measurements in triplicates, while residual glucose was measured off line in duplicates. The arrow shows the induction time

thereafter maintained at 20–30% saturation. Under such conditions, the cells reached an OD_{620} of over 10.8 (cell dry weight of about 3.73 g/l) at 10 h (Fig. 2). At this time, 1 mM IPTG was added as an inducer of phytase production since the level of inorganic phosphate (detected in low concentration in the medium) had dropped to below 1 mM, hence avoiding the risk of calcium phosphate precipitation. Phosphate starvation has previously been used as a strategy for induction of recombinant phytase in *B. subtilis* using a phosphate-starvation inducible *pst*-promoter [13, 44].

As seen in Fig. 2, growth became limited with time due to depletion of glucose that led to an increase in dissolved oxygen and decrease in the stirrer speed. The cell growth decreased (μ_{max} of 0.13 h^{-1}) and stopped after 12 h of cultivation (2 h after induction by IPTG) (Fig. 2), and the maximal phytase activity of 10.3 U/ml was obtained after 4 h of induction. The amount of phytase activity released into the medium remained constant over time, and much of the expressed phytase was accumulated in the cytoplasm. As a result, after 4 h of induction, the relative fraction of the extracellular activity was reduced to about 25%, and the cytoplasmic activity increased to about 42% of the total recombinant phytase (Fig. 3). This change in the distribution behavior of the recombinant phytase could be due to cultivation of cells under low phosphate conditions, which leads to induction of the synthesis of periplasmic phosphate-binding proteins that occupy most of the export sites available in *E. coli* [29]. As a result, the export of the

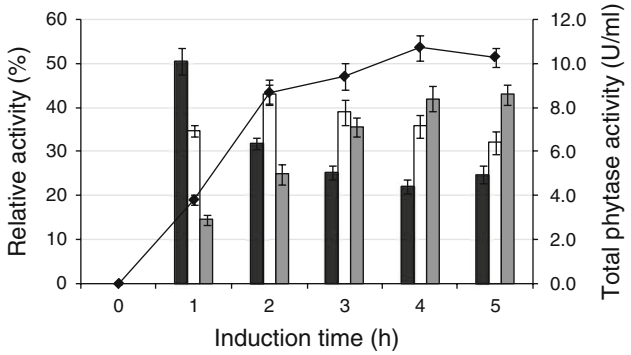

Fig. 3 Distribution of recombinant phytase in 2.5-l batch cultivation: relative activity in the extracellular (*filled bar*), periplasmic (*open bar*) and cytoplasmic (*gray bar*) fraction, and total phytase activity (*filled diamond*)

recombinant protein out of the cells gets limited, and it starts to accumulate inside the cells.

Production of the recombinant phytase by fed-batch cultivation and induction by IPTG

As batch cultivation has its own drawback of nutrient limitation leading to very low cell density, fed-batch cultivation was designed using the minimal medium to prolong the exponential phase of *E. coli* and to get high cell density. It was possible to use phosphate buffer (50 mM, pH 7) for buffering the medium since the larger number of cells obtained during fed-batch should be able to consume the larger amount of the phosphate as nutrient. Cultivation conditions were initially similar to those used for batch cultivation described above. The stirrer speed started to increase at about 7 h of cultivation to compensate for the decrease in dissolved oxygen and reached a maximum of 1,000 rpm after 14 h cultivation time. Glucose concentration was reduced to <3 g/l after 8.5 h, and thereafter a continuous feed of glucose was applied to the reactor according to a programmed feeding profile to maintain the glucose concentration at 2–3 g/l [1, 18, 31]. This resulted in an extension of the exponential phase of the cultivation, μ_{max} of 0.47 h^{-1} between 4–16 h of the cultivation, and increase in cell density to an OD_{620} of 117.6 (cell dry weight of 33.7 g/l) at 16 h (Fig. 4). This corresponded with a decrease in P_i concentration to below 1 mM, and the culture was induced by the addition of 1 mM IPTG and 10 mM $CaCl_2$. The cells continued to grow at a significantly lower growth rate (μ_{max} of 0.1 h^{-1}) after induction to reach an OD_{620} of 188 (49.6 g cell dry weight/l).

A total phytase activity of 327 U/ml (6,600 U/g cell dry weight) was obtained after 5 h of induction, which is about 32 times higher than the production during batch cultivation (Fig. 5a), and is the highest reported so far for recombinant phytase production in a bacterial system (Table 1).

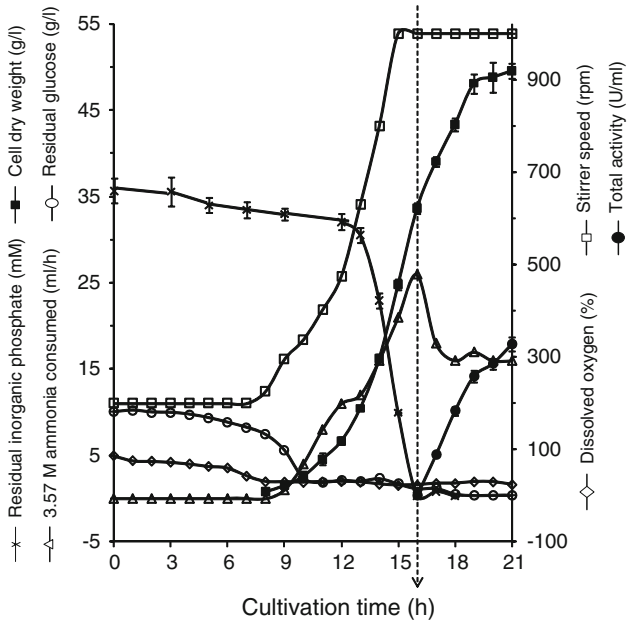

Fig. 4 Fed-batch cultivation of recombinant *E. coli* in 2.5 l synthetic glucose-mineral medium with 50 mM phosphate buffer, pH 7. Dissolved oxygen, stirrer speed and ammonia consumed are log data from the bioreactor; inorganic phosphate, OD_{620}, cell dry weight and total activity were measured off line in triplicates; residual glucose was measured off line in duplicates. The *arrow* shows the induction time

About 85–90% of the expressed recombinant phytase activity was found in the extracellular medium, and the relative enzyme activity in the different cell fractions remained almost constant with time (Fig. 5a). A high level of leakage into the extracellular medium is an advantage for downstream processing of the phytase as there is no need for cell disruption and separation of cell debris, and the amount of host cell proteins is much lower in the extracellular medium. Indeed, the specific activity of recombinant phytase in the cell free fraction of this fed-batch cultivation was 35.3 U/mg total protein, constituting more than 90% of the total protein.

Lactose-induced production of the recombinant phytase during fed-batch cultivation

Induction using lactose has previously been reported to give comparable yields of enzyme activity as with IPTG [6, 42]. Lactose was tested as the inducer of phytase activity in the present system. Preliminary experiments in shake flasks showed that in contrast to induction by 1 mM IPTG, no phytase activity was observed after 1 h of induction with 5–25 mM lactose; however, at 3 h the activity levels were quite comparable with that obtained with IPTG (Table 2). The observed delay in production with lactose as inducer is in accordance with an earlier report [15]. The highest level of production was obtained after

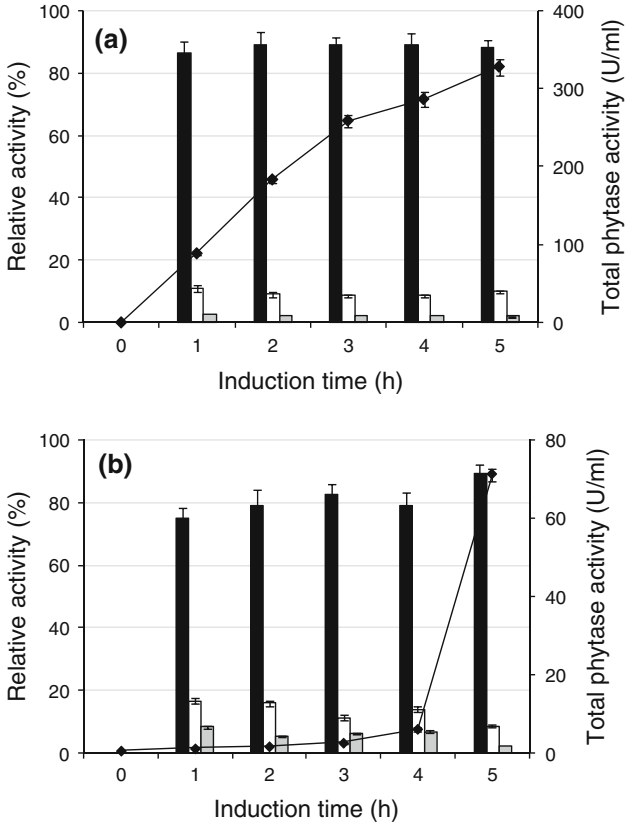

Fig. 5 Distribution of recombinant phytase with time during fed-batch cultivation in 2.5 l culture medium induced by **a** 1 mM IPTG and **b** 20 mM lactose: relative activity in extracellular (*filled bar*), periplasmic and (*open bar*) cytoplasmic (*gray bar*) fractions, and total phytase activity (*filled diamond*)

5 h of induction and was nearly the same at lactose concentrations of 15 mM and above. After 18 h of induction, more than 50% of the phytase activity was found in the extracellular medium.

Production of the recombinant phytase was then studied by fed-batch cultivation with 20 mM lactose as the inducer. The higher concentration of lactose was used assuming that some of it could be consumed by the *E. coli* cells at a low glucose concentration (2–3 g/l) [6]. All the parameters were controlled in the same way as described above for IPTG-induced cultivations. After induction, cells continued growing for 4 h at a maximal specific growth rate of 0.49; however, phytase production was very low in contrast to similar cultivation conditions using 1 mM IPTG as the inducer. Phytase production increased dramatically after 4 h of induction. Delay in enzyme production on lactose induction during fed-batch cultivation of *E. coli* BL21(DE3) cells has been reported earlier [32, 42] and could be explained by the time needed for lactose to be processed to allolactose, an inducer for lac UV5 in *E. coli* chromosome and lac o in pET-22b(+) [11, 25].

Table 1 Comparison of phytase production in batch and fed-batch cultivations in this study with those reported in literature

Cultivation type, inducer	Host strain	Total phytase production (U/ml)	Reference
Batch, IPTG	Recombinant *E. coli*	10.3	This study
Batch, lactose	Recombinant *E. coli*	2.25	[40]
Fed-batch, IPTG	Recombinant *E. coli*	327	This study
Fed-batch, lactose	Recombinant *E. coli*	71	This study
Batch, IPTG	Recombinant *B. subtilis*	2	[17]
Fed-batch, IPTG	Recombinant *B. subtilis*	28.7	[13]
Fed-batch, IPTG	Recombinant *B. subtilis*	48	[44]
Fed-batch, IPTG	Recombinant *E. coli*	120	[18, 24]

Table 2 Expression of phytase activity by recombinant *E. coli* cells induced by different concentrations of lactose

Inducer concentration (mM)	Total activity (U/ml) at different hours after induction				
	0 h	1 h	3 h	5 h	18 h
IPTG, 1 mM	0	1.5	2.50	2.78	2.46
Lactose, 5 mM	0	0	2.05	2.60	2.40
Lactose, 10 mM	0	0	2.12	2.65	2.36
Lactose, 15 mM	0	0	2.33	2.79	2.41
Lactose, 20 mM	0	0	2.28	2.85	2.40
Lactose, 25 mM	0	0	2.31	2.77	2.39

Total activity (U/ml) was the sum of extracellular, periplasmic and cytoplasmic fractions. The enzyme activity was measured in triplicate, and the data show the average value of total phytase activity

Higher cell density (57 g cell dry weight/l) was reached at the end of cultivation due to longer exponential growth phase. However, the total phytase activity after 5 h induction was only 71 U/ml (1.25 U/g cell dry weight), which is approximately 22% of that obtained on induction by IPTG (Fig. 5). This indicated that the conditions used for induction by lactose during high cell density cultivation were not optimal. It has previously been suggested that the residual lactose concentration plays an important role in the induction process; too high concentrations may lead to inhibition, while limitation occurs at concentrations below a critical value [6]. It is thus likely that optimization of the amount of lactose and mode of addition could further increase the phytase activity levels. It is possible that

prolonged incubation (beyond 5 h) could lead to higher enzyme activity in case of both IPTG and lactose-induced fed-batch cultivations but bioreactor capacity posed a limitation.

Distribution of the recombinant phytase during the fed-batch cultivation induced by lactose is shown in Fig. 5b. As in the IPTG-induced cultivation, up to 90% of total phytase activity had leaked out of the cells into the medium. The possibility to use lactose as an inducer for recombinant enzyme production would substantially lower the production cost, especially when added in the form of whey or whey permeate [42].

Concluding remarks

Bacillus sp. MD2 phytase is a highly thermostable enzyme and has good potential as a supplement to feed for monogastric animals. In this study, selection of a suitable expression host and fermentation conditions have resulted in a drastic increase in the production levels of the enzyme. Although large-scale production is normally done using fungal or other microbial hosts that are able to secrete high enzyme amounts, *E. coli* serves as a convenient host for laboratory-scale studies allowing structure-function studies and also evaluation of the product in the desired application. Moreover, extracellular expression is beneficial for avoiding the formation of inclusion bodies or degradation by host proteases, and also lowers the risk of bacterial endotoxins entering the enzyme product.

Currently, the enzyme is being evaluated with regard to feed utilization and animal growth rates. Studies are also on-going at the molecular level to investigate the possibility of increasing the activity of the enzyme in the acidic range.

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