Proc Zool Soc (Jan-June 2013) 66(1):27–35
DOI 10.1007/s12595-012-0057-9

RESEARCH ARTICLE

Optimization of Fermentation Conditions for Phytase Production by Two Strains of *Bacillus licheniformis* (LF1 and LH1) Isolated from the Intestine of Rohu, *Labeo rohita* (Hamilton)

Tanami Roy · Goutam Banerjee · Suhas Kumar Dan · Arun Kumar Ray

Received: 10 August 2012 / Revised: 8 December 2012 / Accepted: 17 December 2012 / Published online: 8 March 2013

Abstract The culture conditions for extracellular production of phytase by two strains of *Bacillus licheniformis* (LF1 and LH1) isolated from the proximal and distal intestine of rohu (*Labeo rohita*) were optimized to obtain maximum level of phytase. Both the strains were cultured TSA broth for 24 h at 37 ± 2 °C, when average viable count of 9.75 × 10^7 cells ml^{-1} culture broth was obtained. This was used as the inoculum for the production medium. Sesame (*Sesamum indicum*) oilseed meal was used as the source of phytic acid (substrate). The effects of moisture, pH, temperature, fermentation period, inoculum size, different nitrogen sources, vitamins and surfactants on phytase production by these two strains were evaluated. Phytase yield was highest (1.87 U in LF1 and 1.57 U in LH1) in solid-state fermentation. Enzyme production in both the isolates increased in an optimum pH range of 5.5–6.5. Minimum phytase production was observed at 50 °C, while maximum production was obtained at 40 °C. To standardize the fermentation period for phytase production, production rate was measured at 12-h intervals up to 120 h. Enzyme production increased for 72 h of fermentation in both strains, and decreased thereafter. The enzyme production increased with increased inoculum size up to 3.0 percentage points for the strain LF1 and up to 2.0 % for the strains LH1. Ammonium sulphate as the nitrogen source was most effective in LF1, while beef extract proved useful to maximize enzyme production by LH1.

T. Roy · G. Banerjee · S. K. Dan · A. K. Ray (✉)
Fisheries Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan 731 235, West Bengal, India
e-mail: aray51@yahoo.com

Keywords Phytase · Fish gut bacteria · *Bacillus licheniformis* · Fermentation conditions · Optimization · *Labeo rohita*

Introduction

Phytase (E.C.3.1.3.8. myo-inositol hexaphosphate phosphohydrolase) is a hydrolytic enzyme that initiates the release of phosphate from phytic acid (myo-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate), which is the predominant form of phosphorus in cereal grains, oilseeds and legumes (Reddy et al. 1982). The presence of phytic acid in feed is undesirable as it chelates nutritionally important divalent cations like potassium, magnesium, zinc, iron, calcium and copper, and proteins and amino acids, thereby rendering them biologically unavailable to the animal (Harland and Morris 1983). Thus, the inclusion of plant proteins in fish diets may cause increased phosphorus discharge into the environment and a reduction in growth resulting from the decreased bioavailability of minerals (Baruah et al. 2004). The phytate phosphorus that is excreted into the environment is acted upon by microorganisms that release the phosphorus, causing pollution in terms of algal growth (Baruah et al. 2004). The ruminants digest phytic acid through the action of phytases produced by the anaerobic gut fungi and bacteria present in their ruminal microbiota. However, phosphorus contained in phytate (inositol hexaphosphate) is not digestible to fish because they lack the endogenous enzyme (phytase) necessary to release phosphorus (Cho and Bureau 2001). However, supplemental inorganic phosphate does not diminish the antinutritive effect of phytic acid. One of the effective methods to diminish the antinutritive effect of phytic acid is to hydrolyse it using the enzyme phytase

(Francis et al. 2001; Cao et al. 2007; Gatlin III et al. 2007). The inclusion of microbial phytase in the feed is an approach to increase phytate phosphorus bioavailability and thereby reduce the use of inorganic phosphorus supplements (Liener 1994; Sardar et al. 2007; Cao et al. 2008).

Although phytases have been detected in several species of bacteria, yeasts and fungi (Greiner and Konietzy 2006; Li et al. 2008; Roy et al. 2009; Khan and Ghosh 2012), industrial production of phytase currently utilizes the soil fungus, *Aspergillus*, on which considerable research has been conducted (Ullah 1988a, b; Volfovà et al. 1994). The microbiota that have not been examined so far are the aerobic/facultative anaerobic symbiotic bacteria from fish gut, which are highly active in enzyme production involved in digestion (Bairagi et al. 2002; Ghosh et al. 2002; Saha et al. 2006). Moreover, it is important to realize that any single phytase may never be able to meet the diverse needs for all commercial and environmental applications. Therefore, there is ongoing interest in screening microorganisms, including bacteria from different sources, for novel and efficient phytase.

The investigation of the autochthonous phytase-producing bacteria in the gastrointestinal tracts of 10 freshwater culturable teleosts were determined and different bacterial strains from selected fish species were isolated in a pure culture and a comparative assay of extracellular microbial phytase activity by these isolates were conducted. The selected strains were further assayed quantitatively for phytase activities. The most promising phytase-producing bacteria were identified on the basis of both phenotypic characteristics and 16S rDNA sequence analysis (Roy et al. 2009).

Microbial enzymes have the enormous advantage of being able to be produced in large quantities through the application of established fermentation techniques. Enzymes production is closely controlled in microorganisms and therefore, to improve its productivity, these controls can be exploited and modified. (Ray et al. 2007). It is well known that production of phytase from fungi can be influenced by a number of factors, such as the amount of phosphate in the medium, carbon source, oxygen supply and inoculum size (Shieh and Ware 1968; Howson and Davis 1983; Ebune et al. 1995). Ebune et al. (1995) demonstrated that addition of 0.5 % Tween-80 or sodium oleate to the culture medium enhanced phytase production by *A. ficuum* in a solid-state fermentation. They also observed that glucose concentration in the medium had an effect on enzyme production. In continuous culture, Lambrechts et al. (1993) found that the level of phytase production by *Schwanniomyces castellii* increased with the increase of pH (when the pH of medium was over 6.0) and dilution rate. Therefore, attention has been focused on studying the phytase activity and phytase enzyme production by several microorganisms in various products as well as in various environments. To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favourable for the microorganism for over-production of the desired metabolite. An elaborate investigation is therefore, required to establish the optimum conditions to scale up enzyme production in an individual fermentation process (Ray et al. 2007). In the present investigation, the environmental and nutritional parameters for fermentation were optimized to enhance phytase production by two different strains LF1 and LH1 of *Bacillus licheniformis* isolated from the gut of rohu, *Labeo rohita*.

Materials and Methods

Microorganisms and Growth Medium

Two strains of *B. licheniformis*, LF1 and LH1 isolated from the gastrointestinal tract of rohu, *L. rohita* (Roy et al. 2009) were identified as potent phytase producers. Both strains were cultured in 4 % tryptone soya broth for 24 h at 37 ± 2 °C when an average viable count 9.75×10^7 cells ml^{-1} culture broth (determined by spread plate technique) was obtained. This was used as the inoculum for the production medium, as required.

The composition of modified phytase screening medium (MPSM) as described by Howson and Davis (1983) is: glucose, 10.0 g l^{-1}; $(NH_4)_2SO_4$, 1.0 g l^{-1}; urea, 10 g l^{-1}; citric acid, 3.0 g l^{-1}; sodium citrate, 2.0 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 1.0 g l^{-1}; sodium phytate, 3.0 g l^{-1}; 1 M Tris buffer (pH 8.0), 100 ml l^{-1}; $FeSO_4 \cdot 7H_2O$, 0.1 g l^{-1}; biotin, 50 mg l^{-1}; thiamine–HCl, 20 mg l^{-1}; and agar, 20.0 g l^{-1}. For the preparation of MPSM, 0.3 g of sodium phytate was dissolved in 10 ml of deionized H_2O sterilized separately and then combined with 90 ml of sterilized sodium phytate-free MPSM. The composition of the basal medium (pH 7.0) is (g l^{-1}): NaCl, 5.0; K_2HPO_4, 1.0; peptone, 1.0; glucose, 1.0; phenol red, 0.012.

Quantitative Enzyme Assay

The quantitative assay of phytase was performed following Engelen et al. (1994). Liquid media (broths containing MPSM without agar) were used for the quantitative assay of the phytase. Seed culture of the selected strains was performed in TSA broth for 24 h. The liquid production media of 25 ml were inoculated with 0.5 ml of the inoculum obtained from the seed culture. The culture flasks were incubated in a shaker incubator for 72 h at 37 °C. The contents were centrifuged (10,000×*g*, 10 min, 4 °C) and the cell-free supernatant was used for an enzyme assay. The colour that developed due to phytase activity was

determined spectrophotometrically at 415 nm. One phytase unit (U) was defined as the amount of enzyme per millilitre of culture filtrate that released 1 μg of inorganic phosphorus per minute.

Fermentation Conditions

Fermentation was carried out at pH 7.0, 37 ± 2 °C, for 72 h, if not stated otherwise.

Optimization of Moisture Content in Fermentation Process

To determine the optimum moisture content in the fermentation process, the microorganisms were cultured in MPSM, which was prepared by moistening sodium phytate with a basal medium. The moisture content of the fermentation medium varied from 5 to 100 %.

Optimization of pH and Temperature

The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium at different levels in the range of pH 5.0–10.5. In order to determine the effective temperature for phytase production by the selected strains, fermentation was carried out at 25, 30, 37, 40, 45, and 50 °C.

Optimization of Fermentation Period for Phytase Production

Fermentation period is an important parameter for enzyme production by microorganisms. Some microorganisms produce maximally in their exponential growth phase, whereas some in their stationary growth phase. In this experiment, fermentation was carried out from 24 to 120 h, and production rate measured at 12-h intervals.

Optimization of Inoculum Size for Fermentation Process

The inoculum volume was optimized for maximal enzyme production by the microorganisms. The fermentation medium was inoculated with 1.0, 2.0, 3.0, 4.0, and 5.0 % seed culture (tryptone soya broth) and incubated in still culture at 37 °C.

Effect of Nitrogen Sources on Phytase Production

To detect the appropriate nitrogen source for phytase production by the isolates, the fermentation medium was supplemented with five inorganic (ammonium nitrate, ammonium chloride, ammonium sulphate, potassium nitrate and sodium nitrate) and five organic (arginine, L-asparagine, tryptophan, tyrosine and beef extract) nitrogen compounds at 0.2 % level, replacing the prescribed nitrogen source of the fermentation medium.

Standardization of Suitable Vitamin Source in the Fermentation Medium

To ascertain whether vitamins have some role in enzyme production by the isolates, six common vitamins namely, folic acid, riboflavin, thiamine, pyridoxine, pantothenic acid and cyanocobalamin were tested. In this experiment, MPSM was prepared with 10 % moistening agent and modifications of fermentation conditions were made on the basis of the results of the previous experiments. The stock solutions of vitamins were sterilized separately by filtration (Jena G5) and then added aseptically to the sterile medium at concentration of 0.01, 0.10, 1.00 μg ml^{-1} for each vitamin.

Influence of Surfactants in the Fermentation Medium on Phytase Production

To investigate the influence of surfactants on phytase production, the fermentation was carried out using surfactants namely, Tween-80, Teepol, sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA) at 0.05, 0.10 and 0.20 % concentrations. The stock solution of these additives was sterilized separately by filtration. The solutions were aseptically added to the sterile medium.

Statistical Analysis

The data were subjected to analysis of variance using Origin 6.1 software. Duncan's multiple range test (Duncan 1955) was employed to test differences among means. The significance of differences was tested at the significance level $P = 0.05$.

Results and Discussion

Regardless of the fermentation process that is used to grow cells, it is necessary to monitor and control parameters starting from the selection of optimum carbon and nitrogen sources and including inoculum volume, moisture content, pH, temperature, incubation period etc. (Ray et al. 2007). Changes in one of these parameters can have a dramatic effect on the yield of cells and the stability of protein product. The high rate of metabolism supports the critical period of metabolite production. Consequently, adequate and timely supply of carbon and nitrogen can be key factors affecting peak productivity levels and their duration.

The meaning of optimization in this context needs careful consideration of the environmental and nutritional parameters for growth and production (Winkler 1991).

Medium formulation is the foremost step for designing successful laboratory experiments for yield enhancement. The medium constituents must satisfy the elemental requirement for cell biomass and metabolite production; hence there must be adequate energy supply for biosynthesis and cell maintenance. The first step to consider is an equation based on the stoichiometry for growth and product formation. Thus, for an aerobic fermentation the reaction is as follows:

$$\begin{aligned}\text{Carbon and energy source} + \text{nitrogen source} + \text{oxygen} \\ + \text{other requirements} = \text{biomass} \\ + \text{products} + CO_2 + H_2O + \text{heat}.\end{aligned}$$

This equation should be expressed in quantitative terms for economical designing of the medium to control the unspent nutrients. Thus, it is possible to calculate the minimal nutrient quantities that are needed to produce a sufficient amount of biomass. Substrate selection for enzyme production in a solid state fermentation (SSF) process depends upon several factors, mainly related with substrate cost and availability and thus may involve screening several agro-industrial residues. In the course of this study, sesame (*S. indicum*) oil seed meal was considered as substrate for fermentation. In a SSF process, the solid substrate not only supplies nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate that provides all the required nutrients to the microorganisms growing in it should be considered as the ideal substrate. In the present experiment, glucose was used as the standard carbon source for optimizing phytase production (Ray et al. 2007).

Optimization of Moisture Content in Fermentation Process

Phytase yield was highest (2.25 U in LF1 and 2.01 U in LH1) when the moisture content in the fermentation medium was 10 %. However, the production of phytase by the two strains LF1 and LH1 of *B. licheniformis* at 10 % moisture content was not significantly different from that at 5 % (Fig. 1a). The requirement of water for growth and metabolic activities of microorganisms and the consequent potential of the water activity of the medium in controlling fermentation processes are well established (Hahn-Hägerdal 1986). SSF is distinct from submerged fermentation since microbial growth and product formation occur at or near the surface of the solid substrate particle having low moisture contents. Hence, it is crucial to provide optimized water content to the fermenting substrate. In the present study, it was observed that 10 ml of distilled water was sufficient to moisten 100 g sodium phytate to give high enzyme titers. It appears therefore, that 10 % moisture content of the medium volume was optimum for phytase production by the two strains LF1 and LH1 of *B. licheniformis.*

Optimization of pH and Temperature

The strain LF1 exhibited highest phytase activity at pH 5.5 whereas the activity was highest at pH 6.5 in case of LH1. However, in case of LH1, phytase activity at pH 6.5 was not significantly different ($P < 0.05$) from the activity at the pH 7.0. Production was much less up to pH 8.0, and declined again beyond pH 8.5 in case of LF1 and in case of LH1, production was much less up to pH 9.0, and declined again beyond pH 9.0. It was found that phytase activity increased under acidic pH rather than under alkaline pH (Fig. 1b). Most organisms grow optimally within a wide pH range. The pH has a profound effect on the production of the enzyme. In the present study, maximum phytase activity was recorded at pH between 5.5 and 6.5. For phytase production, the optimum pH of most bacteria and fungi ranged from 5.0 to 7.0. Most microbial phytases, especially those of fungi origin, show the pH optimum at 4.5 to 5.5; some bacteria show a pH optimum at 6.5 to 7.5. For *Aerobacter aerogenes* (Greaves et al. 1967), *Pseudomonas* sp. (Irving and Cosgrove 1971), *E. coli* (Greiner et al. 1993), *S. ruminantium* (Yanke et al. 1999), *L. amylovorus* (Sreeramulu et al. 1996), the optimum pH for phytase production was recorded in the range of 4.0 and 5.5. The pH optimum of *Enterobacter* sp. 4 (Yoon et al. 1996) and *Bacillus* sp. DS11 (Kim et al. 1998) was in the neutral range (7.0–7.5). In the present study, maximum phytase activity was recorded at pH 5.5 and 6.5 for LF1 and LH, respectively.

The effect of temperature on phytase production by the bacterial isolates is depicted in Fig. 1c. In both the cases, minimum phytase yield was observed when fermented at 50 °C, while maximum yield was at 40 °C. Like pH, temperature is one of the most important parameters essential for the success of a fermentation reaction. Microorganisms grow slowly at a temperature below or above the normal growth temperature because of a reduced rate of cellular production (Ray et al. 2007). If the growth temperature is too high but not lethal, there may be a premature induction of target protein expression. For phytase production by the strains LF1 and LH1, 40 °C was found to be most effective. Production started to decline after further increase in temperature. At lower temperature, substrate transport across the cells is suppressed and lower product yields are attained. At higher temperature, the maintenance energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic

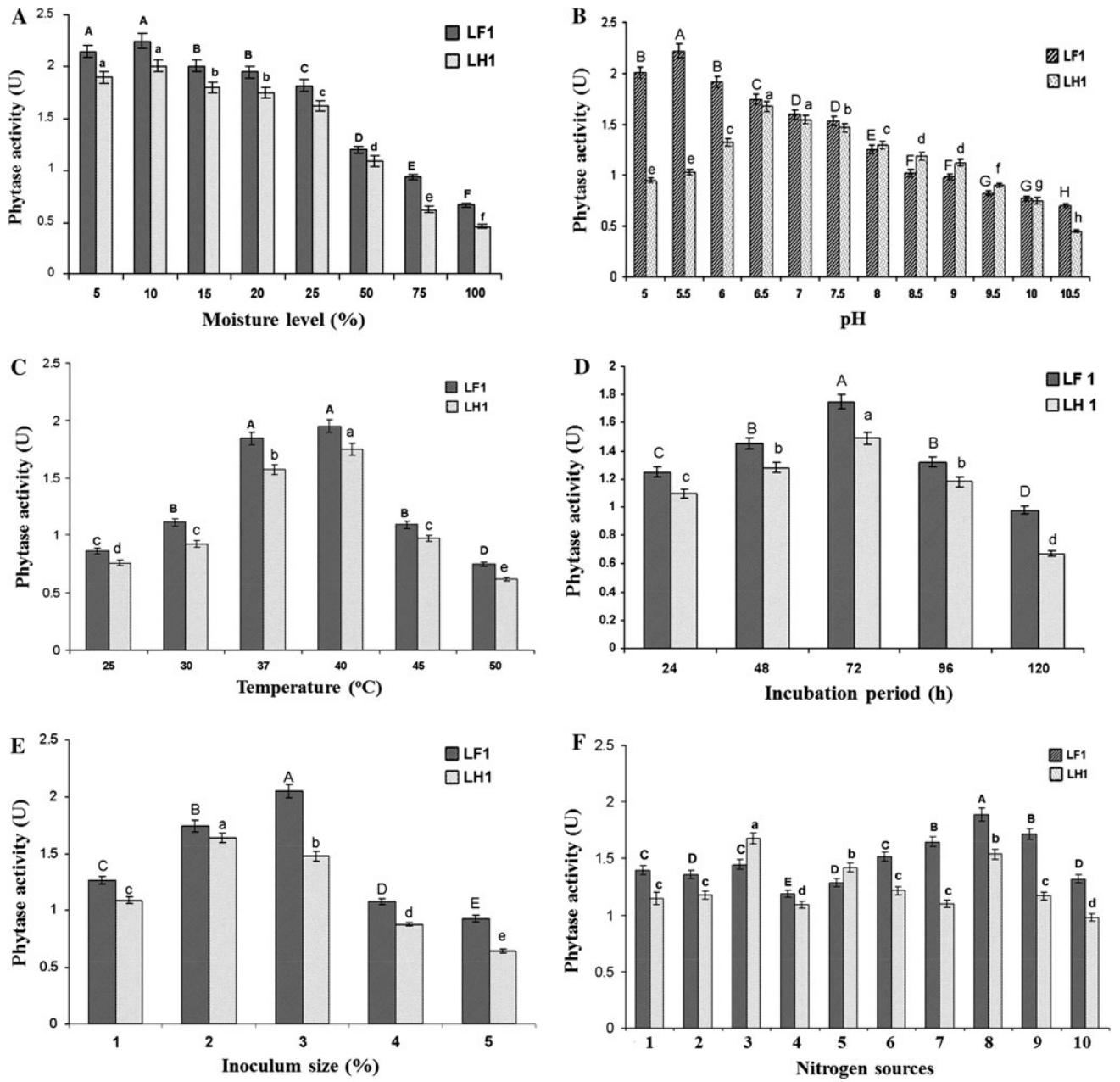

Fig. 1 Effect of different parameters on phytase production by two strains of *Bacillus licheniformis,* LF1 and LH1. **a** Moisture content, **b** pH, **c** temperature, **d** incubation period, **e** inoculum size, and **f** different nitrogen sources. [*1* Arginine, *2* asparagine, *3* beef extract, *4* tryptophan, *5* tyrosine, *6* NH_4NO_3, *7* NH_4Cl, *8* $(NH_4)_2SO_4$, *9* KNO_3, *10* $NaNO_3$]. *Error bars* show deviation among three replicates. Means with *different letters* are significantly different ($P < 0.05$). (U µg of inorganic phosphorus liberated ml^{-1} of enzyme extract min^{-1})

pathway (Aiba et al. 1973) resulting in maximum production. Phytase from *B. subtilis* (Powar and Jagannathan 1982), *E. coli* (Greiner et al. 1993), *Klebsiella aerogenes* (Tambe et al. 1994), *Enterobacter* sp. 4 (Yoon et al. 1996), *K. oxytoca* MO-3 (Jareonkitmongkol et al. 1997) and *Selenomonas ruminantium* (Yanke et al. 1999) were optimally active in the temperature range between 50 and 60 °C. The optimum temperature for phytase production of *Mitsuokella jalaludinii*, a bacterial species from the rumen of cattle was 39 °C (Lan et al. 2002).

Optimization of Fermentation Period for Phytase Production

Since fermentation duration is crucial, it is also important to find out the optimum period for enzyme production. Some organisms are reported to produce maximally in the log phase of growth, whereas some at their stationary phase. In the present investigation, however, maximum phytase production by LF1 and LH1 was obtained at 72 h fermentation and decreased thereafter (Fig. 1d).

Optimization of Inoculum Size for Fermentation Process

Enzyme production in both the strains increased gradually up to 3 % inoculum size, but decreased thereafter. In the present experiment, maximum phytase activity was observed at 3 % inoculum level for LF1, and in case of LH1, it was at 2 % inoculum level. However, phytase activity decreased at lower and higher inoculum (v/v) concentrations (Fig. 1e). The culture used to inoculate the fermentation medium must be in a healthy, active state and of optimum size, possibly minimizing the length of log phase, thus in its high rate for substrate conversion. The inoculum quantity normally used is between 3 and 10 % of the medium volume (Lincoln 1960; Meyrath and Suchanek 1972; Hunt and Stieber 1986). A relatively large inoculum volume may be used to generate the maximum production in as short a time as possible, thus increasing the vessel productivity. The physiological condition of the inoculum, when it is transferred to the next culture stage, can have a major effect on fermentation performance. The optimum transfer time must be determined so that the inoculation with an ideal culture can be achieved. Lincoln (1960) stressed that bacterial inoculum should be transferred in the logarithmic growth phase when the cells are still metabolically active. Inoculum age is particularly important in the sporulating bacteria, because sporulation is induced at the end of the logarithmic phase and the use of an inoculum containing high percentage of spores would result a long log phase in subsequent fermentation. To determine the optimum inoculum dose and the time of inoculum transfer in the present experiment, the inoculum was transferred after 24 h of growth, i.e., in its log phase. In this experiment, for LF1, maximum phytase activity was observed at 3 % inoculum level, and in case of LH1 it was 2 % inoculum level. Phytase activity decreased at lower and higher inoculum concentrations (Gulati et al. 2006).

Effect of Nitrogen Sources on Phytase Production

The results of the effect of various nitrogen sources on phytase production revealed that $(NH_4)_2SO_4$was most effective for the strain LF1, whereas beef extract was most effective for the strain LH1 (Fig. 1f). Most industrially used microorganisms can utilize inorganic or organic nitrogen sources. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates and as amino acids, protein or urea. It was found that the growth was faster with the supply of organic nitrogen, and a few microorganisms also were found to have absolute requirement for amino acids (Ray et al. 2007). However, amino acids are more commonly added as complex organic nitrogen sources which are non-homogenous, cheaper and readily available. Inorganic nitrogen source such as ammonium sulphate (0.1 %) was used for phytase production by *Pseudomonas* sp. (Irving and Cosgrove 1971), *Enterobacter* sp. 4 (Yoon et al. 1996) and *S. castellii* (Lambrechts et al. 1992). Casein hydrolysate (1 %) and $(NH_4)_2SO_4$ (0.1 %) were used as nitrogen sources for phytase production by *B. subtilis* (Powar and Jagannathan 1967). In the present study, ammonium sulphate proved to be the best for LF1 and beef extract was most effective for the strain LH1.

Standardization of Suitable Vitamin Source in the Fermentation Medium

In case of standardization of suitable vitamin source in the fermentation medium, pyridoxine and pantothenic acid and cyanocobalamin did not have any positive effect on phytase production by the isolates. But at 1.00 μg ml^{-1} concentration of thiamine, both the strains (LF1 and LH1) yielded increased amount of phytase (Table 1). Besides carbon and nitrogen sources, some microorganisms require additional trace elements and vitamins for growth and enzyme production. For phytase production by yeasts, the screening medium contained vitamins and trace elements (Segueilha et al. 1992).The addition of trace elements was not needed for phytase production by *Bacillus subtilis* (Powar and Jagannathan 1967) and *Bacillus* sp. DS11 (Kim et al. 1998).Vitamins, particularly B-vitamins are known to have distinct roles for extracellular enzyme production by microorganisms. Therefore, it was very much necessary to determine their role on phytase production by the isolates. In the present study, thiamine at a concentration of 1.00 μg ml^{-1} increased the phytase production whereas pyridoxine, pantothenic acid and cyanocobalamin did not have any positive effect on enzyme production by the isolates.

Influence of Surfactants in the Fermentation Medium on Phytase Production

It is known that surfactants can regulate enzyme synthesis and secretion. Han and Gallagher (1987) reported that the addition of surfactants such as polyoxyethylene ethers and sodium oleate to liquid culture medium markedly increase the level of phosphatase production in *Aspergillus ficuum*. Tween-80 and sodium oleate have been shown to increase the rate of phytase production in *A. ficuum* NRRL 3135, whereas Triton X-100 has a negative effect on the process (Ebune et al. 1995). The stimulating effects of surfactants on fungal enzyme production are due to the action of surfactants on cell permeability (Ebune et al. 1995). However, 0.5 % and 1.0 % Tween-80 and 1.0 % Triton X-100 did not have any effect on phytase production of

Table 1 Effect of vitamins on phytase production by the strains LF1 and LH1 of *Bacillus licheniformis*

Vitamins	Concentration (μg ml^{-1})	Phytase (μg inorganic phosphorus liberated ml^{-1} of enzyme extract min^{-1})	
		Bacillus licheniformis LF1	*Bacillus licheniformis* LH1
Folic acid	0.01	0.87^{f} (±0.025)	0.77^{g} (±0.022)
	0.10	1.19^{e} (±0.034)	1.92^{c} (±0.055)
	1.00	1.84^{c} (±0.053)	2.47^{b} (±0.054)
Riboflavin (B_2)	0.01	1.22^{e} (±0.035)	1.27^{f} (±0.071)
	0.10	1.33^{e} (±0.038)	1.42^{e} (±0.036)
	1.00	1.36^{d} (±0.039)	0.64^{h} (±0.041)
Thiamin (B_1)	0.01	1.49^{d} (±0.043)	1.18^{f} (±0.018)
	0.10	2.85^{b} (±0.082)	1.45^{e} (±0.034)
	1.00	3.07^{a} (±0.088)	2.80^{a} (±0.042)
Pyridoxine (B_2)	0.01	0.78^{f} (±0.023)	0.66^{h} (±0.081)
	0.10	0.76^{g} (±0.022)	0.64^{h} (±0.019)
	1.00	0.91^{f} (±0.026)	0.62^{h} (±0.018)
Pantothenic acid (B_5)	0.01	0.83^{f} (±0.024)	0.78^{h} (±0.017)
	0.10	0.71^{g} (±0.025)	0.61^{i} (±0.022)
	1.00	0.59^{h} (±0.017)	0.52^{i} (±0.015)
Cyanocobalamin (B_{12})	0.01	1.09^{e} (±0.032)	0.65^{h} (±0.025)
	0.10	0.85^{f} (±0.025)	0.82^{g} (±0.023)
	1.00	1.15^{e} (±0.033)	1.52^{d} (±0.043)
Without vitamin		1.92^{c} (±0.054)	1.61^{d} (±0.046)

Data are mean value ± SE ($n = 3$). Values with same superscripts in the same vertical column are not significantly different ($P < 0.05$)

Table 2 Effect of surfactants on phytase production by the strains LF1 and LH1 of *Bacillus licheniformis*

Surfactant	Concentration (%)	Phytase (μg of inorganic phosphorus liberated ml^{-1} of enzyme extract min^{-1})	
		Bacillus licheniformis LF1	*Bacillus licheniformis* LH1
Tween-80	0.05	1.37^{b} (±0.040)	1.31^{b} (±0.037)
	0.10	1.16^{c} (±0.034)	1.22^{c} (±0.034)
	0.20	1.05^{d} (±0.034)	1.15^{c} (±0.034)
Teepol	0.05	1.29^{b} (±0.037)	1.04^{d} (±0.029)
	0.10	1.10^{c} (±0.031)	1.00^{d} (±0.028)
	0.20	1.01^{d} (±0.029)	0.99^{d} (±0.040)
SDS	0.05	1.08^{c} (±0.031)	1.02^{d} (±0.029)
	0.10	1.00^{d} (±0.028)	0.97^{d} (±0.028)
	0.20	0.97^{d} (±0.028)	0.85^{e} (±0.024)
EDTA	0.05	0.97^{d} (±0.028)	0.87^{e} (±0.025)
	0.10	0.85^{e} (±0.024)	0.79^{e} (±0.023)
	0.20	0.74^{f} (±0.021)	0.74^{f} (±0.021)
Without surfactants (control)		1.88^{a} (±0.054)	1.62^{a} (±0.045)

Data are mean value ± SE ($n = 3$). Values with same superscripts in the vertical column are not significantly different ($P < 0.05$)

M. jalaludinii. The difference in results may be related to the difference in cell wall structure of fungi and bacteria (Lan et al. 2002). Surface acting agents are also known to increase the membrane permeability of the microorganisms and at the same time leaching of metabolites out of the cell (Abbott and Gledhill 1971). All the four surfactants tested in the present study, Tween-80, Teepol, SDS and EDTA are strong detergents. It was observed that the use of surfactants in the present study did not enhance further enzyme production (Table 2).

Conclusion

This investigation led us to conclude that moisture; pH, temperature, and nitrogen sources play crucial role in phytase production by the two strains LF1 and LH1 of *B. licheniformis*. SSF was suitable for increased phytase production by these organisms. It has been found that phytase activity increased under acidic pH rather than under alkaline pH. Thiamine at 1.00 μg ml^{-1} concentration increased the phytase production, whereas, pyridoxine, pantothenic acid and cyanocobalamin did not have any positive effect on enzyme production by the isolates. Further investigations are required to make use of the full potential of these organisms for phytase production by employing genetic, biochemical and microbial engineering techniques.

Acknowledgments We are grateful to Indian Council of Agricultural Research for financial support in the form of a Research Project.

References

Abbott, B.J., and W.E. Gledhill. 1971. The extracellular accumulation of metabolic products by hydrocarbon degrading microorganisms. *Advances in Applied Microbiology* 14: 249–388.

Aiba, S., A.E. Humphrey, and N.F. Millis. 1973. *Biochemical engineering*, 2nd ed. New York: Academic Press.

Bairagi, A., K.S. Ghosh, S.K. Sen, and A.K. Ray. 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquaculture International* 10: 109–121.

Baruah, K., N.P. Sahu, A.K. Pal, and D. Debnath. 2004. Dietary phytase: an ideal approach for a cost-effective and low polluting aquafeed. *NAGA, World Fish Center Quarterly* 27: 15–19.

Cao, L., W. Wang, C. Yang, W. Yang, J. Diana, A. Yakupitiyage, Z. Luo, and D. Li. 2007. Application of microbial phytase in fish feed. *Enzyme and Microbial Technology* 40: 497–507.

Cao, L., Y. Yang, W.M. Wang, A. Yakupitiyage, D.R. Yuan, and J.S. Diana. 2008. Effect of pretreatment with microbial phytase on

phosphorus utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture Nutrition* 14: 99–109.

Cho, C.Y., and D.P. Bureau. 2001. A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. *Aquaculture Research* 32(Suppl 1): 349–360.

Duncan, D.B. 1955. Multiple range and multiple *F*-tests. *Biometrics* 11: 1–42.

Ebune, A., S. Al-Asheh, and Z. Duvnjak. 1995. Effect of phosphate, surfactant and glucose on phytase production and hydrolysis of phytic acid in canola meal by *Aspergillus ficuum* during solid state fermentation. *Bioresource Technology* 54: 241–247.

Engelen, A.J., F.C. van der Heeft, P.H.G. Randsdorf, and E.L.C. Smit. 1994. Simple and rapid determination of phytase activity. *Journal of AOAC International* 7: 760–764.

Francis, G., H.P.S. Makker, and K. Becker. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199: 197–227.

Gatlin III, D.M., F.T. Barrows, P. Brown, K. Dabrowski, T.G. Gaylord, R.W. Hardy, E. Herman, G. Hu, Å. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E.J. Souza, D. Stone, R. Wilson, and E. Wurtele. 2007. Expanding the utilization of sustainable plant products in aquafeeds: A review. *Aquaculture Research* 38: 551–579.

Ghosh, K., S.K. Sen, and A.K. Ray. 2002. Characterization of bacilli isolated from gut of rohu, *Labeo rohita*, fingerlings and its significance in digestion. *Journal of Applied Aquaculture* 12: 33–42.

Greaves, M.P., G. Anderson, and D.M. Webley. 1967. The hydrolysis of inositol phosphates by *Aerobacter aerogenes*. *Biochimica et Biophysica Acta (BBA) Enzymology* 132: 412–418.

Greiner, R., U. Konietzny, and K.D. Jany. 1993. Purification and characterization of two phytases from *Escherichia coli*. *Archives of Biochemistry and Biophysics* 303: 107–113.

Greiner, R., and U. Konietzy. 2006. Phytase for food application. *Food Technology and Biotechnology* 44: 25–140.

Gulati, H.K., B.S. Chadha, and H.S. Saini. 2006. Production and characterization of thermostable alkaline phytase from *Bacillus laevolacticus* isolated from rhizosphere soil. *Journal of Industrial Microbiology and Biotechnology* 34: 91–98.

Han, Y.W., and D.J. Gallagher. 1987. Phytase production by *Aspergillus ficuum* on semi-solid substrate. *Journal of Industrial Microbiology and Biotechnology* 2: 195–200.

Hahn-Hägerdal, B. 1986. Water activity: A possible external regulator in biotechnical processes. *Enzyme Microbial Technology* 8: 322–327.

Harland, B.F., and E.R. Morris. 1983. Phytate: a good or a bad food component. *Nutrition Research* 15: 733–754.

Howson, S.J., and R.P. Davis. 1983. Production of phytate-hydrolyzing enzyme by some fungi. *Enzyme and Microbial Technology* 5: 377–382.

Hunt, G.R., and R.W. Stieber. 1986. Inoculum development. In *American Society of Microbiology*: *Manual of industrial microbiology and biotechnology,* ed. A.L. Demain and N.A. Solomon, 32–40. Washington DC: American Society for Microbiology.

Irving, G.C.J., and D.J. Cosgrove. 1971. Inositol phosphate phosphatases of microbiological origin. Some properties of a partially purified bacterial (*Pseudomonas* sp.) phytase. *Australian Journal of Biological Sciences* 24: 547–557.

Jareonkitmongkol, S., M. Ohya, R. Watanbe, H. Takagi, and S. Nakamori. 1997. Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3. *Journal of Fermentation and Bioengineering* 83(4): 393–394.

Khan, A., and K. Ghosh. 2012. Characterization and identification of gut-associated phytase-producing bacteria in some freshwater fish cultured in ponds. *Acta Ichthyologica et Piscatorial* 42(1): 37–45.

Kim, Y.O., H.K. Kim, K.S. Bae, J.H. Yu, and T.K. Oh. 1998. Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzyme and Microbial Technology* 22: 2–7.

Lambrechts, C., H. Boze, G. Moulin, and P. Galzy. 1992. Utilization of phytate by some yeasts. *Biotechnology Letters* 14(1): 63–66.

Lambrechts, C., H. Boze, L. Segueilha, G. Moulin, and P. Galzy. 1993. Influence of culture conditions on the biosynthesis of *Schwanniomyces castellii* phytase. *Biotechnology Letters* 15: 399–404.

Lan, Q.G., N. Abdullah, S. Jalaludin, and W.Y. Ho. 2002. Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. *Journal of Applied Microbiology* 93: 668–674.

Li, X., Z. Chi, Z. Liu, K. Yan, and H. Li. 2008. Phytase production by a marine yeast *Kodamea ohmeri* BG3. *Applied Biochemistry and Biotechnology* 149: 183–193.

Liener, I.E. 1994. Implications of antinutritional components in soybean foods. *Critical Reviews in Food Science and Nutrition* 34: 31–67.

Lincoln, R.E. 1960. Control of stock culture preservation and inoculum build-up in bacterial fermentations. *Journal of Biochemistry, Microbial Technology and Engineering* 2: 481–500.

Meyrath, J., and G. Suchanek. 1972. Inoculation techniques—effects due to quality and quantity of inoculum. In *Methods in microbiology*, vol. 7B, ed. J.R. Norris, and D.W. Ribbons, 159–209. London: Academic Press.

Powar, V.K., and V. Jagannathan. 1967. Phytase from *Bacillus subtilis*. *Indian Journal of Biochemistry & Biophysics* 4: 184–185.

Powar, V.K., and V. Jagannathan. 1982. Purification and properties of phytate specific phosphatase from *Bacillus subtilis*. *Journal of Bacteriology* 151: 1102–1108.

Ray, A.K., A. Bairagi, K.S. Ghosh, and S.K. Sen. 2007. Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. *Acta Ichthyologica et Piscatoria* 37(1): 47–53.

Reddy, N.R., S.K. Sathe, and D.K. Salunkhe. 1982. Phytates in legumes and cereals. In *Advances in food chemistry*, ed. C.O. Chichester, E.M. Mrak, and G.F. Stewart, 1–92. New York: Academic Press.

Roy, T., S. Mondal, and A.K. Ray. 2009. Phytase-producing bacteria in the digestive tracts of some freshwater fish. *Aquaculture Research* 40: 344–353.

Saha, S., R.N. Roy, S.K. Sen, and A.K. Ray. 2006. Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes). *Aquaculture Research* 37: 380–388.

Sardar, P., H.S. Randhawa, M. Abid, and S.K. Prabhakar. 2007. Effect of dietary microbial phytase supplementation on growth performance, nutrient utilization, body compositions and haemato-biochemical profiles of *Cyprinus carpio* (L.) fingerlings fed soyprotein-based diet. *Aquaculture Nutrition* 13: 444–456.

Segueilha, L., C. Lambrechts, H. Boze, G. Moulin, and P. Galzy. 1992. Purification and properties of the phytase from *Schwanniomyces castellii*. *Journal of Fermentation and Bioengineering* 74(1): 7–11.

Shieh, T.R., and J.H. Ware. 1968. Survey of microorganisms for the production of extracellular phytase. *Applied Microbiology* 16: 1348–1351.

Sreeramulu, G., D.S. Srinivasa, K. Nand, and R. Joseph. 1996. *Lactobacillus amylovorus* as a phytase producer in submerged culture. *Letters in Applied Microbiology* 23: 385–388.

Tambe, S.M., G.S. Kaklij, S.M. Keklar, and L.J. Parekh. 1994. Two distinct molecular forms of phytase from *Klebsiella aerogenes*: Evidence for unusual small active enzyme peptide. *Journal of Fermentation and Bioengineering* 77: 23–27.

Ullah, A.H.J. 1988a. Production, rapid purification and catalytic characterization of extracellular phytase from *Aspergillus ficuum*. *Preparative Biochemistry & Biotechnology* 18: 443–458.

Ullah, A.H.J. 1988b. *Aspergillus ficuum* phytase: partial primary structure, substrate selectivity, and kinetic characterization. *Preparative Biochemistry & Biotechnology* 18: 459–471.

Volfovà, O., J. Dvořàkovà, A. Hanzlikovà, and A. Jandera. 1994. Phytase from *Aspergillus niger*. *Folia Microbiologica* 39: 481–484.

Winkler, M.A. 1991. Environmental design and time profiling in computer controlled fermentation. In *Genetically engineered protein and enzymes from yeast: production and control. Chap. 4*, ed. A. Wiseman, 96–146. Chichester: Ellis Horwood.

Yanke, L.J., L.B. Selinger, and K.J. Cheng. 1999. Phytase activity of *Selenomonas ruminantium*: A preliminary characterization. *Letters in Applied Microbiology* 29: 20–25.

Yoon, S.J., Y.J. Choi, H.K. Min, K.K. Cho, J.W. Kim, S.C. Zee, and Y.H. Jung. 1996. Isolation and identification of phytase producing bacterium, *Enterobacter* sp. 4 and enzymatic properties of phytase enzyme. *Enzyme and Microbial Technology* 18: 449–454.