OPTIMIZATION APPROACHES FOR ENHANCED PRODUCTION OF PHYTASE FROM THE HALOPHILIC BACTERIUM *COBETIA MARINA* STRAIN 439

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ABSTRACT

Phytase catalyzes the breakdown of complex organic forms of phosphorus into simpler forms by sequential hydrolysis of phosphate-ester bonds to release inorganic phosphate. Supplementation of feed with bacterial phytase can therefore improve the bioavailability of phosphorus and trace elements. In the present study, the production of phytase by a strain of Cobetia marina isolated from the lye of the Burgas salt pans and the optimization of culture conditions to enhance phytase production are reported. The effects of physical conditions and nutrients on phytase production were investigated. Physical parameters studied included fermentation time, inoculum size, agitation, pH and temperature. Maximum growth, as well as enzyme production (15.3 U mg^{-1}) were recorded after 72 h incubation period. To increase the level of enzyme production, different culture conditions were applied and the optimum temperature and pH for phytase production were found to be 35^{o}C and pH 7.5, respectively. Nutrient factors studied included carbon source, nitrogen source, substrate type and concentration, NaCl concentration and metal ions (salts). Our studies showed that the optimum phytase production was achieved using a mineral medium supplemented with: 1.5 % maltose, 0.5 % $NaNO_3$, 0.7 % sodium phytate and 7 % NaCl. Among the metal ions tested K^+, Mn^{2+} and Fe^{2+} were found to have no effect on enzyme production. However, the removal of Ca^{2+} and Mg^{2+} from the production medium, lead to a decrease in strain growth and phytase production. Metal ions like Co^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+}, Ag^{2+} and the various detergents tested, caused a significant inhibition on phytase production. As a result of the optimization approaches applied the production level of phytase was enhanced 2.7-fold and (40.5 U mg^{-1}) compared to the initial enzyme yield.

Keywords: *Cobetia marina, optimization, phytase production, sodium phytate.*

INTRODUCTION

Phytases (E.C.3.1.3.8. inositol hexaphosphate phosphohydrolase) are a class of enzymes that catalyse the hydrolytic degradation of phytic acid to free inorganic phosphorus and low molecular weight myo-inositol phosphate esters [1]. A very large amount of organic phosphorus in plants, soils, and in foods and feeds containing sorghum, glutinous rice, bran, and other ingredients is in the form of phytic acid (also known as inositol hexaphosphate) [2]. Phytate (phytic acid) is known as a dietary inhibitor that chelates trace elements like calcium, magnesium, iron and zinc. Phytate can also combine protein and vitamins as insoluble complexes to reduce their utilization efficiency, activity, and digestibility and prevents them from being bioavailable

to monogastric animals, including humans, since they lack the enzyme phytase in their digestive tract [3]. The addition of phytases to animal feed increases the bioavailability of phosphorus and minerals by degrading phytic acid, thereby eliminating its anti-nutritional effect and ensuring balanced nutrition. In addition, phytases included as feed additives lead to a reduction in the amount of undigested phytate in manure in areas with intensive livestock farming, which significantly reduces the negative environmental impact [4]. Phytase enzymes play an important role not only in the animal feed industry, but also in the food industry, and have the potential to improve the general health of people. The use of phytase in food processing has enhanced bread making, plant protein isolate synthesis, maize wet milling, and cereal bran separation [5]. The role of microbial phytases in plant nutrition has also been extensively studied. Plants require phosphorus for their various fundamental processes such as photosynthesis, flowering, fruiting, and maturation. Significantly high phosphorus levels are required for cell division and in the development of meristematic tissues. It also promotes the growth of roots and helps in nitrogen fixation. Phytate mineralization is observed by many microorganisms and can be implemented in plant systems for induced agricultural sustainability [6]. It has been shown that the introduction of bacterial phytases into the rhizosphere improves the utilization of phosphorus from soil phytates, indicating a role for these enzymes in plant nutrition. The action of bacterial phytases in the rhizosphere of plants prevents the formation and eliminates chelated phytates, which bind metals and trace elements, making them unavailable to plants. The phytase from the culture liquid of rhizobacteria *B. amyloliquefaciens* FZB45 was shown to promote seed germination and growth of maize under phosphate starvation when phytate was present in the medium [7]. Using the culture liquid of soil bacteria as biofertilizers may be an economic and environmentally friendly way to substitute the mineral phosphorus fertilizers and limit their application [8]. Inositol produced by the gradual degradation of phytic acid by phytase also has great application value in the feed, food, medical and other industries, as inositol can be added to feed as a biological promoter for aquaculture and livestock, and can also be added to feed as a nutritional supplement [2]. Some myoinositol phosphates were found to be efficient in the protection against complications caused by diabetes, as well as for treatment of chronic inflammations and cardiovascular diseases. They also were found to possess antitumor properties [8].

Phytases are abundantly found in nature. The primary sources include plants, microbes (bacteria and fungi), and some animal tissues [6]. Some of the phytase producing microorganisms include bacteria, such as *Bacillus* [4, 9 - 11], *Pseudomonas* [12], *Escherichia coli* [13]. Fungi or yeasts that produce phytases include the following: *Sporotrichum thermophile*, *Penicillium purpurogenum*, *Aspergillus oryzae*, *Humicola nigrescens*, *Aspergillus flavus*, *Zygosaccharomyces, Pichia kudriavzevii,* and *S. cerevisiae* [14].

Phytases of microbial origin are preferred because of their large-scale production and their potential in biotechnological applications. Phytase is of great industrial importance and there is a constant interest in isolating new microbial strains producing phytase.

The aim of this study was to involve the *Cobetia marina* strain 439 (under nucleotide accession number LN849908) which had shown the highest phytase synthesis compared to the other strains tested and in view of the phytase industrial importance to optimize the production parameters and culture conditions to obtain the highest yield of phytase enzyme.

EXPERIMENTAL

Bacteria and enzyme activity

In our previous study, twelve phytase-producing bacterial strains were isolated from the Burgas and Pomorie salt pans, Bulgaria. The screening for extracellular phytase production was carried out according to Palla et al., using phytase screening medium (PSM) contained (w/v): 1.5 % glucose, 1.0 % sodium phytate, 0.2 % $CaCl_2$, 0.5 % $(NH_4)_2SO_4$, 0.05 % KCl, 0.05 % $MgSO_4$, 0.001 % $FeSO_4$, 0.001 % $MnSO_4$, 10 % NaCl, 3 % agar (pH 7.2) [15]. After incubation for 72 h at 35°C, colonies that were surrounded by clear zones on the PSM were selected and flooded with 2 % (w/v) aqueous cobalt chloride ($CoCl_2$) solution and incubated at room temperature for 30 min. A freshly prepared mixture (1:1) of 6.25 % (w/v) ammonium molybdate $(NH_4)_6Mo_7O_{24}$ and 0.42 % (w/v) ammonium heptavanadate (NH_4VO_7) solutions were added to the plates. The plates were incubated at room temperature for 5 min [16]. The colonies that

maintained the zone of hydrolysis were selected and their phylogenetic affiliation was determined by 16S rRNA gene sequence analysis and the nucleotide sequences were deposited in EMBL under nucleotide accession numbers LN849896 - LN849910 [17].

In this study, the selected strains were cultivated in PSM broth in flasks (20 mL medium in 100 mL flasks) on an orbital shaking incubator (150 rpm) for 120 h at 30°C and the growth was monitored by measuring the optical density at 660 nm (OD_{660nm}). After incubation the liquid culture was centrifuged at 10 000 rpm for 20 min. The supernatants were then used as the source of extracellular phytases and used for the phytase activity assay. Sodium phytate as substrate was used for phytase activity assay.

Phytase activity was determined by measuring the amount of liberated inorganic phosphate. The reaction mixture consisted from 100 µL of enzyme solution with 900 µL of 0.5 % Na phytate in 0.1 M Tris-HCl buffer, containing 7 % NaCl, (pH 7.2). The enzyme reaction was carried out at 30°C for 30 min and then the reaction was stopped by adding 750 µL of 5 % trichloroacetic acid. The liberated phosphate was measured at 700 nm after adding 1500 µL of colour reagent, which is prepared freshly before using by mixing (4:1) of 2.5 % ammonium molybdate solution in 5.5 % sulfuric acid and of 2.5 % ferrous sulfate solution. One unit of phytase activity was defined as to liberate 1 µmol of phosphate per minute under the assay condition. A control reaction was carried out with the absence of the enzyme. A standard curve (using 0.5 - 10 µg mL^{-1} KH_2PO_4) was made by treating standard phosphate solutions without added phytase under the same conditions [16].

The total protein content was measured by the Lowry assay [18]. A standard curve was prepared using bovine serum albumine (BSA) as a standard protein with concentrations ranging from 0 - 1000 µg mL^{-1}. After establishing the concentration of protein in different enzyme samples, their specific activity was evaluated.

Cobetia marina strain 439 showed the highest phytase activity compared to the other strains tested.

Optimization of physical factors affecting phytase production from *Cobetia marina strain* 439

To study the effect of incubation period, the bacterial culture *Cobetia marina strain* 439 was inoculated in PSM broth and incubated at 30°C for 120 h at 150 rpm. The samples were harvested at regular time intervals of 24 h and cell growth as well as enzyme activity was measured.

The bacterium was grown in PSM broth until OD_{660} of 0.5 and the cultivation was carried out at different inoculum sizes ranging (0.5 %, 1 %, 1.5 % and 2 %). The flasks were incubated at 30ºC for 72 h. Samples were taken for phytase activity and cell growth.

Effect of agitation condition was carried out at the following shaking rates - 0 rpm, 80 rpm, 100 rpm, 150 rpm, 180 rpm and 200 rpm. The flasks were inoculated at 30ºC for 72 h. Growth and phytase activity were recorded.

The effect of temperature was evaluated by incubating the bacterial culture at various temperatures (30, 35, 40, 45, 50, 55, 60 and 65°C) at pH 7.0. Phytase activity and cell growth were performed after 72 h of incubation.

The optimum pH of the culture medium was determined in the range of 5.0 - 9.0. The pH of the PSM broth was adjusted using 1 N HCl or 1 M NaOH. The flasks were incubated at 35°C for 72 h. Samples were taken for phytase activity and cell growth.

Optimization of nutritional factors on phytase production from *Cobetia marina* strain 439

Various carbon sources (1.5 % w/v), such as fructose, sucrose, maltose, lactose, mannose, starch, sorbitol and glycerol, have been evaluated for their effect on phytase production by replacing of the original carbon source (glucose) in the production medium.

The influence of nitrogen sources (0.5 % w/v) on phytase production was investigated by using organic (peptone, yeast extract, tryptone) and inorganic (ammonium chloride, ammonium nitrate, potassium nitrate, sodium nitrate) nitrogen sources. These nitrogen sources were used to replace the ammonium sulphate, available in the medium.

Different sodium phytate concentrations (0.3, 0.5, 0.7, 1.0, 1.5 and 2.0 % w/v) were used to find the most appropriate concentration.

The test bacterium *Cobetia marina* strain 439 was isolated from lye, collected from Burgas salt pans (33 % salinity), and hence the effect of various concentrations (0, 3, 5, 7, 10 and 15 % w/v) of sodium chloride was tested in phytase production.

The effect of metal salts had been studied as one of

these salts ($MgSO_4$, KCl, $CaCl_2$, $FeSO_4$ or $MnSO_4$) was removed while the others were used in the concentrations described above in the PSM broth. The study was conducted and as a control with all four salts that were contained in the production PSM broth [19].

Different trace elements (0.1 % w/v) Co^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+}, Ag^{2+}, including EDTA were assessed for their influence on growth and phytase production. The effect of different surfactants (Tween 20, Tween 80, Triton X 100 and sodium dodecyl sulfate (SDS)) on phytase production was studied by incorporating them into the PSM broth at a concentration of 0.1 % (w/v).

Data analysis

All the experiments were carried out independently in triplicate. The statistical analysis was performed using Microsoft Office 365 Excel 2020 software.

RESULTS AND DISCUSSION

Bacteria and enzyme activity

The phytase activity of tested strains was determined using agar petri dishes with phytase screening medium (PSM) and was detected as a halo zone around the colonies producing the enzyme. A total of 12 bacterial strains were detected as extracellular phytase producers [17]. In the present study, quantitative screening of phytase-producing halophilic bacterial strains was conducted. *Cobetia marina* strain 439 had the highest phytase activity (14.5 U mg^{-1}), while *Halomonas smyrnensis* strain 18 had the lowest phytase activity (1.3 U mg^{-1}) as shown in Table 1.

Table 1. Phytase activity (U mg^{-1}) of the tested halophilic bacterial strains.

Species	Isolate number	Phytase activity, U mg^{-1}
Halomonas smyrnensis	18	1.3 ± 0.05
Halomonas eurihalina	20	4.7 ± 0.03
	27	2.5 ± 0.07
	U1	4.2 ± 0.05
Halomonas halophila	U2	9.8 ± 0.03
Cobetia marina	437	10.5 ± 0.05
	439	14.5 ± 0.05
Chromohalobacter canadensis	21	3.5 ± 0.07
	28	2.1 ± 0.03
Salinivibrio costicola	23	5.8 ± 0.03
Nesiotobacter exalbescens	24	3.3 ± 0.03
Virgibacillus salarius	434	8.3 ± 0.03

Further studies aimed to optimize the enzyme production process and to obtain a phytase with increased activity. In this way, the phytase produced by *Cobetia marina* strain 439 can serve as a feed additive in animal diets, as well as to reduce the problem of phosphorus pollution in livestock production areas.

Optimization of physical factors on phytase production from *Cobetia marina* strain 439

Cultivation time plays an important role in maximizing enzyme production. The present results showed that maximum enzyme activity was obtained in the stationary phase of growth, during the 72nd h of incubation at 30^{o}C with pH 7.0 and 150 rpm (Fig. 1a). Prolongation of fermentation resulted in a slight decrease in phytase activity**.** The enzyme activity by *Cobetia marina* strain 439 was not in parallel with cell growth. Similar results have also been reported by other researchers [9, 20].

The effect of inoculum size of the *Cobetia marina* strain 439 was clarified and maximum phytase production was recorded with the inoculum size of 1.0 % at 72 h, 150 rpm and 30^{o}C and pH 7.0. Using 0.3 % inoculum, the phytase activity was 7.0 U mg^{-1} at an optical density of 0.7. As the percentage of inoculum used increased, both cell growth and phytase activity increased reaching maximum levels with 1.0 % inoculum. Further inoculum size increase led to a decrease in the monitored parameters. This result is expected because at low inoculum level, bacterial growth may be reduced, resulting in insufficient amount of biomass and prolonging the time for bacterial culture to enter the stationary phase of growth. This in turn increases the time required for substrate consumption and synthesis of the phytase enzyme. On the other hand, at high inoculum concentration, the bacteria grow rapidly, and nutrients present in the medium become insufficient. aeration problem arises, as well as a rapid change in pH of the medium [21]. This is a probable reason for the lower phytase activity when using 2.0 % inoculum (Fig. 2b).

When the effect of agitation speed was examined, it was found that *Cobetia marina* strain 439 grew optimally

and produced phytase with the highest activity at 180 rpm (Fig. 1c). Low agitation rates lead to uneven oxygen distribution, which can inhibit the growth of *Cobetia marina* strain 439, and bacterial cells can settle to the bottom, reducing the access to nutrients and oxygen. This is probably due to the observed growth and phytase activity values recorded at 0, 50 and even 100 rpm. As the stirring rate increases, the growth of *Cobetia marina* strain 439 improved. The cells remained suspended which improved their metabolic activity and at 180 rpm maximum phytase activity (24 U mg^{-1}) was recorded. At higher stirring speeds the enzyme activity appeared to be lower (19 U mg^{-1}) and the bacterial cells grew less.

The effect of different temperature values on the phytase production from *Cobetia marina* strain 439 was evaluated at 72 h, 180 rpm and pH of 7.0. The optimum temperature for phytase production was recorded at 35°C (26 U mg^{-1}). Further rise in temperature, decreased the production of phytase and the minimum phytase activity was observed at 55°C (3.2 U mg^{-1}). At a temperature above 55°C, the growth of bacterial culture significantly decreased (Fig. 1d).

pH is one of the most important physical factors controlling fermentation processes. In the present study, the effect of pH on phytase production revealed that pH 7.5 was optimal for phytase production (27.3 U mg^{-1}), respectively at 72 h, at 35°C and 180 rpm. At pH 9.0 and 6.0, phytase production was slower than other pH values and was about 35 % and 32 % of the maximum production, respectively (Fig. 1e).

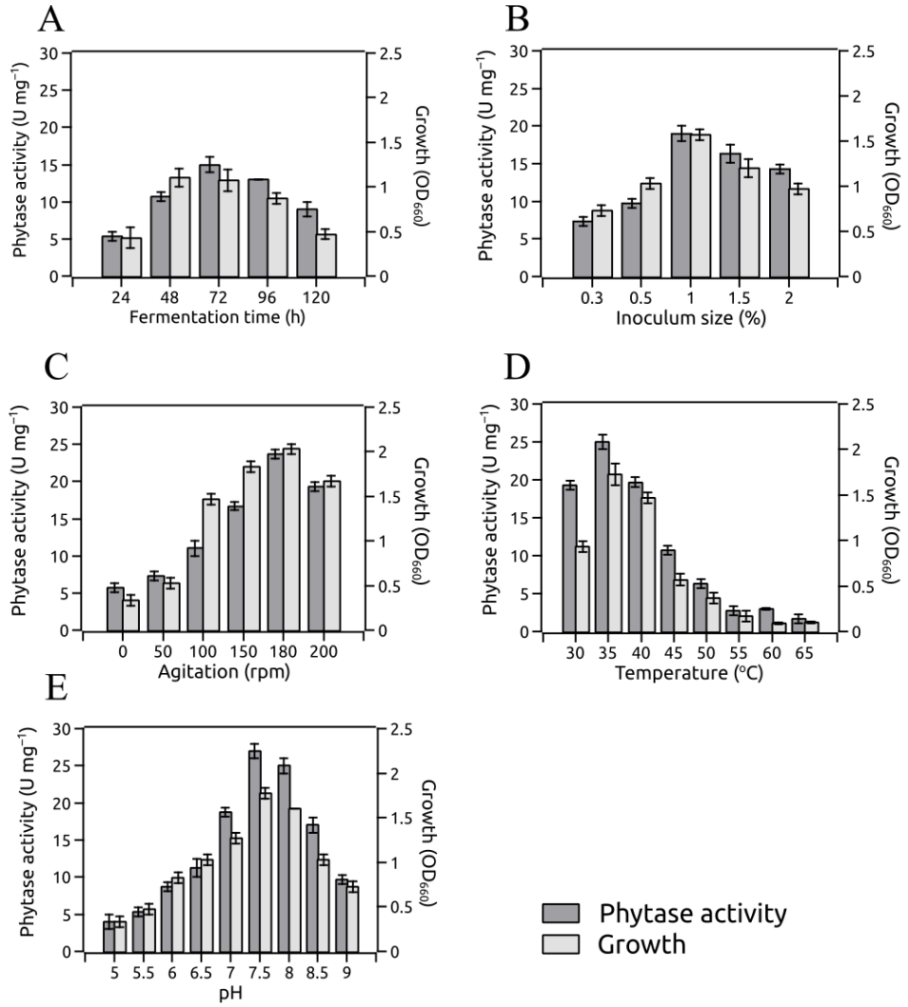

Fig. 1. Optimization of physical factors on growth and phytase production from *Cobetia marina* strain 439. (a) - the effect of cultivation time; (b) - the effect of inoculum size; (c) - the effect of agitation; (d) - the effect of temperature; (e) - the effect of pH.

Optimization of nutritional factors on phytase production from *Cobetia marina* strain 439

In this study, eight carbon sources were examined for their effectiveness. Maltose (40.5 U mg^{-1}) had the greatest influence on phytase production. Most carbon sources improved phytase production relative to the control - glucose (27.8 U mg^{-1}), e.g. sucrose (29.2 U mg^{-1}) and fructose (35.3 U mg^{-1}) also had a significant effect on phytase production (Fig. 2a).

Carbon is an important nutrient and energy source and, in most cases, it determines the rate of product formation. There is evidence that simple and easily digestible sugars can improve phytase production [12]. The production of phytase by *A. niger* NCIM 563 was significantly increased by using fructose and glucose as carbon source [22]. Li et al. reported a phytase-producing marine yeast, *Kodamea ohmeri* BG3, in which production was best in media supplemented with fructose and glucose [23]. Demirkan et al. also indicated that fructose, maltose and sucrose are good carbon sources to increase phytase production by *Bacillus* sp. EBD 9-1 [20]. The mannitol significantly induced phytase production of *Bacillus* sp SP-46 [24].

For the optimal nitrogen source, 0.5 % of different nitrogen sources were used in the fermentation medium (Fig. 2b). The highest yield of phytase was achieved using $NaNO_3$ and yeast extract of 40.3 U mg^{-1} and 35 U mg^{-1}, respectively, compared to the control - $(NH_4)_2SO_4$ - 34 U mg^{-1}. The use of NH_4NO_3 (26 U mg^{-1}) and peptone (21 U mg^{-1}) resulted in significantly higher phytase activity than the use of NH_4Cl (15.5 U mg^{-1}). Similarly, maximum phytase production by *Pseudomonas* sp. AP-MSU 2 [12] and by *Aspergillus niger* [25] was recorded in the medium supplemented with sodium nitrate. The

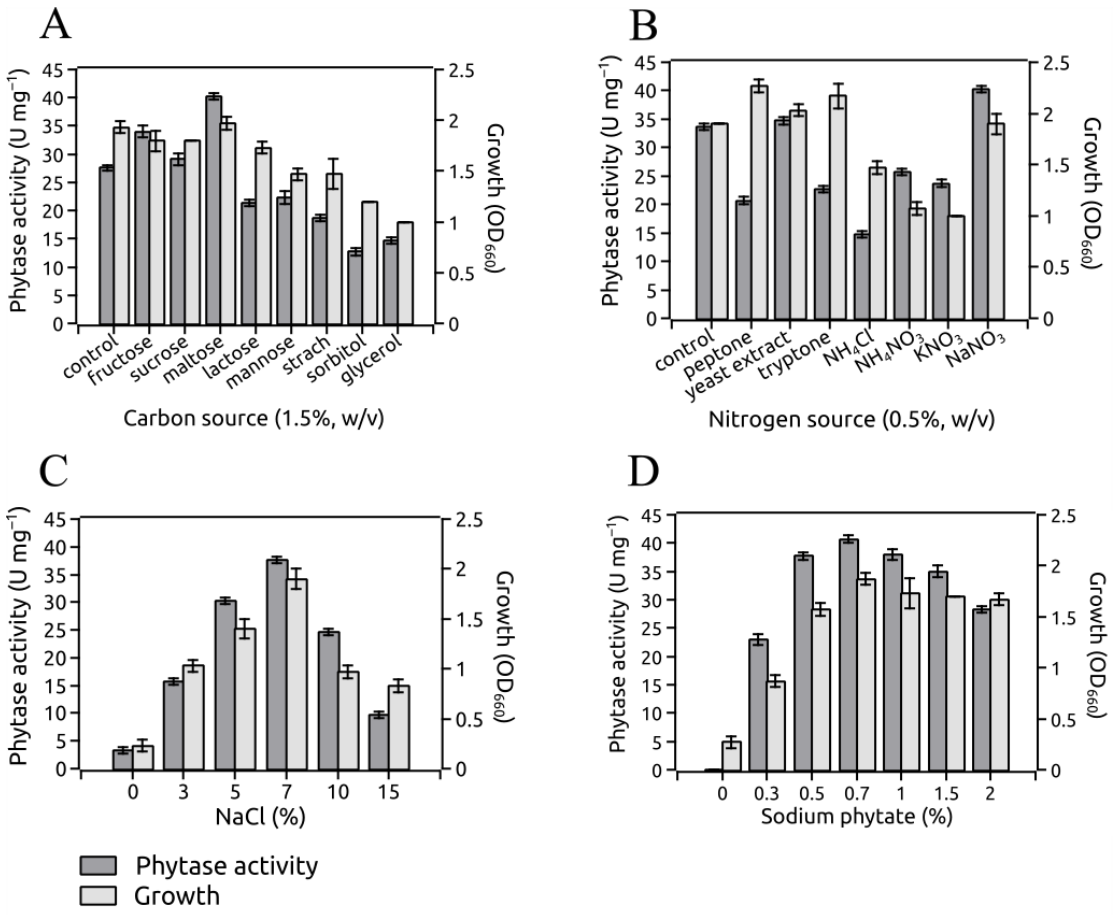

Fig. 2. Optimization of nutritional factors on phytase production from *Cobetia marina* strain 439. (a) - the effect of carbon sources; (b) - the effect of nitrogen sources; (c) - the effect of NaCl concentration; (d) - the effect of sodium phytate concentration.

present study also coincides with the higher phytase production in medium with sodium nitrate.

Peptone and beef extract were reported as best nitrogen sources for *Bacillus subtilis* KHU-10 [9], beef extract, peptone and tryptone had no effect on phytase production by *Bacillus laevolacticus,* but $NH_4H_2PO_4$ caused dramatic increase in phytase production [26]. The simple inorganic nitrogen source may be more convenient to produce extracellular enzymes as compared to the organic sources which have a role in growth promotion. Organic nitrogen sources in the fermentation medium can stimulate the production of proteases, which have a negative effect on total enzyme yield [19].

Very few reports on phytase from halohilic bacteria are available. The present study of the effect of NaCl on phytase production by the halophilic bacterium *Cobetia marina* strain 439 showed that 7 % NaCl gave a maximum phytase production of 38.8 U mg^{-1} (Fig. 2c). At concentrations above 7 % NaCl, the phytase production decreased, but not significantly, with 26 % (10 U mg^{-1}) of phytase production remaining at the highest concentration tested (15 % NaCl). Maximum phytase production at 3 % was reported for *Pseudomonas* AP-MSU 2 [12] and marine yeast *K. ohmeri* BG3 that requires 2 % NaCl for optimum phytase production [23].

A 0.7 % sodium phytate concentration was optimum for maximum phytase synthesis (41.6 U mg^{-1}). Results showed that concentrations of 0.3 to 1.0 % of sodium phytate resulted in significantly higher phytase activity compared with higher concentrations, which showed adverse effect on phytase activity (Fig. 2d).

Metal ions are also important trace elements that have considerable effect on the fermentation of products, especially enzymes. Metal ions proved to be an important factor enhancing phytase production by *Pichia anomala* [27]. In this study, for estimating the effect of metal salts, five metal salts were added to the fermentation medium, which are given as the control in Fig. 3A. Only four of the salts were included in the treatment, and the fifth was removed from the medium. Results showed that there were no significant differences in phytase activity among the treatments and control. However, when Ca^{2+} and Mg^{2+} were removed from the production medium, a decrease in strain growth and phytase production was found. In a similar experiment, however, Qasim et al. found that there were no significant differences in phytase activity between the treatments and the control [19]. There are many such reports on the effect of metal ions on phytase production by bacteria and fungi. Similar results were reported, stating that $CaCl_2$ was an important salt for phytase production by *B. amyloliquefaciens*, *Bacillus* sp. DS11, *Bacillus subtilis* (natto), *Bacillus subtilis* [20]. Sreedevi and Reddy found that the presence of Mg^{2+}, Mn^{2+} and K^+ in the medium for bacterial phytase production significantly increased the enzyme activity [28].

Minerals are commonly included in microbial growth media as they can incorporate into the enzyme composition and help maintaining ionic balance. The activation or inhibition of phytase activity by metal ions

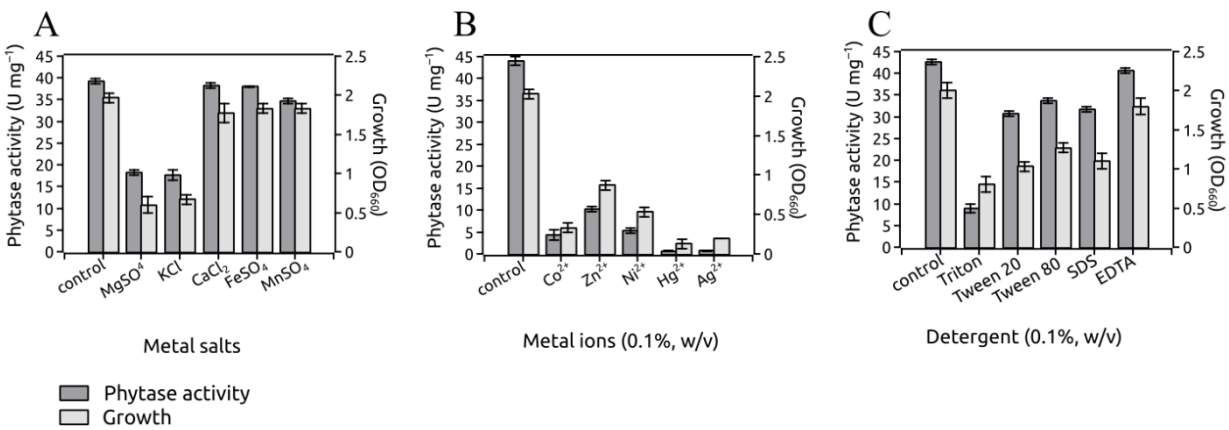

Fig. 3. Effect of metal salts in production medium (A) and effect of additives on phytase production and cell growth (B); (a) - control (all metal salts), minus $MgSO_4$, minus $CaCl_2$, minus KCl, minus $FeSO_4$, minus $MnSO_4$; (b) - metal salts and detergents as additives in 0.1 % w/v.

is explained by their effect on the growth of the bacteria themselves [28].

The influence of metals on the enzyme production was observed by the addition of metallic salts in concentration of 0.1 % w/v to the production medium. Results showed that Co^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+}, Ag^{2+}, strongly inhibited growth as well as phytase production (Fig. 3B). The decrease in the production of phytase in the presence of other metal ions may be attributed to their inhibitory effect on growth or possibly inhibition or inactivation of enzyme itself by these metals' ions.

Surfactants can increase the ratio between the cell wall and the cell membrane which may be the cause of an increase in the rate of release of metabolites from the cells into the fermentation medium [12]. Soni and Khire observed that phytase production by *A. niger* NCIM 1207 was better in a medium supplied with Tween 80 and Triton X-100 [22]. Mandviwala and Khire also claimed that surfactants have an inducing effect on phytase production and observed that phytase production by *A. niger* was higher in a medium supplied with Tween 80 and Triton X-100 [29]. However, similar effects were not observed in our study. Among the various detergents (0.1 % w/v) tested, Triton X-100 strongly inhibited the enzyme production, while SDS, Tween 20 and Tween 80 also showed about 20 % inhibition. EDTA did not affect the enzyme production (Fig.3b).

CONCLUSIONS

The halophilic strain 439 of *Cobetia marina* is a new source of phytase. In this study, nutritional and physical parameters were optimized to enhance phytase production by *Cobetia marina* strain 439. The higher phytase production was achieved with optimized process parameters such as maltose as carbon source, $NaNO_3$ as nitrogen source, incubation temperature 35°C, initial pH 7.5, 0.7 % sodium phytate and 7 % NaCl, for an incubation period of 72 h. By optimizing the incubation conditions, the phytase production of this strain increased 2.7-fold compared to the basal medium. The phytase produced from *Cobetia marina* strain 439 can be used as a feed additive in animal feeds, also to reduce the problem of phosphorus pollution in livestock production areas.

Authors' contributions*: I.B., L.K.: Conceptualization; K.B., Y.G., N.K.: Methodology; N.A.: Validation; K.B., Y.G., N.A., N.K.: Investigation; I.B.: Data curation; I.B.: Writing - original draft preparation; L.K.: Writing - review and editing. All authors have read and agreed to the published version of the manuscript.*

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