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Optimizing the biosynthesis of phytase and chitinase produced by Bacillus subtilis NBIMCC 2353 by changing the carbon and phosphorus sources and the composition of the nutrient medium

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Abstract

In the present study, optimization opportunities the biosynthesis of the enzymes phytase and chitinase produced by *Bacillus subtilis* NBIMCC 2353 have been presented. Data from experiments with 10 sources of carbon and phosphorus have been presented. The most suitable of them and their necessary concentration to achieve maximum phytase activity have been established. It has been shown that a decrease in the phosphorus present in the medium leads to an increase in the enzyme activity, with the most active phase at a temperature of 60°C.

Three variants of nutrient media for the cultivation of the strain Bacillus subtilis NBIMCC 2353 have been studied in order to increase the biosynthesis of chitinase: standard culture medium for reproduction and maintenance of the producer; standard culture medium with added 0.5% fresh mycelium of Ganoderma lucidum; standard nutrient medium with added 0.5% chitin from shrimp shells. The chitinase activity of the 48th hour was determined, which increased significantly with the addition of natural components containing chitin to the nutrient medium.

Key words: enzyme activity, phytase, chitinase, *Bacillus subtilis*

1.Introduction

Phytase (Myo-inositol hexakisphosphate phosphohydrolase), EU 3.1.3.8 and EU 3.1.3.26) are a subfamily of the acidic histidine phosphatases hydrolyzing phytic acid (myo-inositol hexakisphosphate) with the release of one or more phosphoacid groups. In mature seeds of monocotyledonous and dicotyledonous plants, phytic acid is the major phosphorus form of repository, representing 60-90% of all organic phosphorus. Because of its strong chelation ability, it is regarded as an anti-nutritional factor as it forms insoluble complexes with important metal ions such as calcium, zinc, magnesium, iron, etc., reducing their bioavailability and digestibility. Organically bound phosphorus in phytic acid is not metabolised by monogastric animals such as pigs, birds, fish, and humans. The absence of enzyme phytase in these organisms leads to the release of organically bound phosphorus in nature. In areas with intensive livestock farming, this is a serious environmental problem that can be solved by reducing the phytinic acid content of cereals by enzymatic hydrolysis and increasing their bioavailability. Fitases are synthesized by microorganisms belonging to: bacteria of the genus *Aerobacter* [1], *Bacillus subtilis* [2,3], *Enterobacter* [4], *Klebsiella* [5]*; Aspergillus spp.* [6,7] *and yeasts of the genus Arxula* [6,8], *Candia crusei* [6], *Candida melibiosica 2491* [9,10,11], *Clavispora, Debaryomyces, Hanseniaspora, Kluyveromyces, Metchnikowia, Schwanniomyces, Pichia* [8,12], *Rhodotorula* [13], *Saccharomyces* [6,8] *Schwanniomyces* [8,12], *Torulaspora* (8).

Chitinases (E.C.3.2.1.14) are hydrolytic enzymes capable of hydrolyze the chitin polymer (N-acetyl-D-glucosamine), which is a major ingredient of the cell walls of fungi, exoskeletons of insects and crustaceans to oligomers. Fungi, bacteria, insects, crustaceans and higher plants produce chitinases with potential application in the biocontrol of plant pathogenic fungi, of insects as well as in the field of biotechnology [14, 15, 16]. Bacteria of the *Bacillus species* have been shown to produce chitinases [17]. Many studies have been conducted in recent decades in connection with the possible potential for the use of this bacterium as a biofungicide. The production of chitinases from a microbial source represents particular interest due to the wide range of their applications [18, 19]. Bacteria can produce chitinases in the absorption of chitin as sources of carbon and nitrogen. Wang et al., (2006) [20], indicate that the strain Bacillus subtilis W-118 separates chitinase when cultured in a medium containing shrimp powder and crab shells as the main source of carbon.

Bacillus subtilis is a gram-positive, non-pathogenic bacterium with pronounced chitinase activity, which has a number of advantages over other bacteria, expressed in the ability to form endospores, enduring extreme pH, temperature and osmotic conditions. In addition, it does not contain endotoxins and is therefore considered safe [21, 22].

In recent years, interest in phytase and chitinase produced from bacterial sources has been increasing. *Bacillus subtilis* is a probiotic microorganism, which is used in agriculture and the production of functional foods in animals and humans. Proteolytic, cellulase and chitinase enzymes produced by this strain have been mainly studied. There are insufficient data on the phytase and chitinase produced by *Bacillus subtilis* and their activity, which is the subject of the present study [3]. The main goal of the experiments is to optimize the biosynthesis of the enzymes phytase and chitinase from the producer strain *Bacillus subtilis* by changing the carbon and phosphorus sources and the composition of the nutrient medium.

2. Materials and methods

Experiments were performed to optimize the biosynthesis of the enzymes phytase and chitinase produced by the producer strain *Bacillus subtilis* NBIMCC 2353. The starter culture was purchased from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC).

Optimization of enzyme phytase biosynthesis

Nutrient medium. A medium with the following composition (g / l) was used: meat extract - 10, peptone - 10, NaCl - 2.

Sources of carbon and phosphorus. Ten carbon sources were studied - fructose, glucose, sucrose, galactose, starch, lactose, cellulose, mannose, raffinose, arabinose in a concentration of 2%. The influence of the inorganic phosphorus source KN_2PO_4 and the organic sodium phytate was studied. The concentration of KN_2PO_4 was varied from 0.2 to 5 mmol, and of sodium phytate - from 0.02 to 0.5 mmol.

Cultivation conditions. Cultivation was carried out in 400 ml Erlenmeyer flasks at 25% fill and at 30 ° C for 48 hours on a BS / 4 shaker apparatus with an amplitude of 100 rpm.

Method for determination of phytase activity: The enzymatic activity of phytase was determined by measuring the amount released inorganic phosphate released by hydrolysis of sodium phytate at pH 5.5, according to the modified method of Georgiev and Gargova (2007) [10]. The optical density of the solution was measured at 415 nm. One unit of phytase activity (PhA) represents the amount of enzyme

that catalyzes the hydrolysis of sodium phytate with formation of 1μmol of inorganic orthophosphate in 1 min.

Optimizing the biosynthesis of the enzyme chitinase

Nutrient medium. For the experimental experiments were used:

• **Standard nutrient medium for propagation and maintenance of strain *Bacillus subtilis* NBIMCC 2353,** (g / l): 8 - FOS (fructooligosaccharides); Meat extract - 10.0; Peptone - 10.0; NaCl - 5.0; Agar - 20.0; 1.5 g yeast extract; 1.3 g K_2HPO_4, 0.3 g $MgSO_4$. Distilled water - 1l. The components were dissolved in distilled water and sterilized in an autoclave for 20 minutes at 121°C and a pressure of 1 atm. In order to obtain vegetative reproduction, cultivation was performed in 500 ml Erlenmeyer flasks, containing 100 ml of growing medium for 24 hours at 30°C on a BS/4 circular shaker apparatus at 100 rpm.

• **Standard nutrient medium with added 0.5% fresh mycelium from *Ganoderma lucidum***:
Fresh mycelium from the medical mushroom *Ganoderma lucidum* was grown laboratory at the Institute of Cryobiology and Food Technology - Sofia. Erlenmeyer flasks of 250 ml were used, the amount of the culture medium together with the inoculum of *Bacillus subtilis* NBIMCC 2353 cells is 100 ml. Initial cell concentration is $0,4x10^7$ CFU/ml.

• Standard culture medium with added 0.5% chitin from shrim shells (Sigit - Aldrich):
Erlenmeyer flasks of 250 ml were used, the amount of the culture medium together with the inoculum of *Bacillus subtilis* NBIMCC 2353 cells is 100 ml. Initial cell concentration is $0,4x10^7$ CFU/ml.

Method for determination of chitinase activity: The cultural medium and biomass were centrifuged at 10,000 rpm; 10 minutes at 4°C to obtain a cell supernatant containing extracellular enzymes. The enzymatic activity of chitinase was assayed using 750 μL of 1% colloidal solution of chitin and 750 μL of supernatant from the culture medium and incubated at 30 °C for 2 hours. To the suspension was added 500 μL of 3,5-dinitrosalicylic acid reagent and heated at 100°C for 5 minutes. The absorption of the solution was measured at a wavelength of 540 nm [23]. One unit of chitinase enzyme activity is defined as the number of enzymes that hydrolyze chitin to release 1 μg of N-acetyl-D-glucosamine per hour.

3. Results and discussion

The conducted experiments show that from the studied carbon sources, the highest phytase activity is reported in arabinosis - 300 PhA / l, respectively. For fructose, the phytase activity is 200 PhA / l (Table 1), and for the others carbon sources no enzymatic production is detected. The data obtained show that arabinose is most suitable for phytase biosynthesis.

Table 1: Influence of carbon source on phytase activity

№	Carbon source	PhA/l
1	Fructose	200
2	Glucose	0
3	Sucrose	0
4	Galactose	0
5	Potato starch	0
6	Lactose	0
7	Cellulose	0
8	Mannose	0
9	Raffinose	0
10	Arabinosis	300

Fermentation experiments were conducted for phytase biosynthesis of *Bacillus subtilis* strain producer with different concentrations of 1, 2, 3, 4 and 5% of the carbon source of arabinose. Enzymatic activity of 400 PhA/l was found to be observed at a concentration of 1%. In the variants with concentrations of 2, 3, and 4%, the same phytase activity values were obtained - 300 PhA / l. A decrease in phytase activity was observed in the culture fluid with 5% arabinose concentration (Table 2).

Table 2: Influence of arabinose concentration on phytase production

Arabinose content%	PhA/l
1	400
2	300
3	300
4	300
5	200

According to the literature, the phytase biosynthesis is limited by the phosphorus content of the nutrient medium. In this connection, after phosphate ions have been deposited with the components of the nutrient medium, the phosphorus content of KH_2PO_4 and sodium phytate has varied as phosphorus sources. By increasing the phosphorus content to a concentration of 0.2 mmol, corresponding to 0.62 mg%, Pi is aimed at increasing the phytase biosynthesis (Figure 1).

The total phosphorus concentration is determined by the amount of deposited and residual after precipitation (0.076 mmol), i. E. the actual phosphorus content is 0.276 mmol (0.86 mg% Pi). With increasing phosphate content in the culture medium, the enzyme activity decreases. It should be noted that in an environment where no further phosphorus is added, phytase activity is lower by almost 50% of the maximum reading.

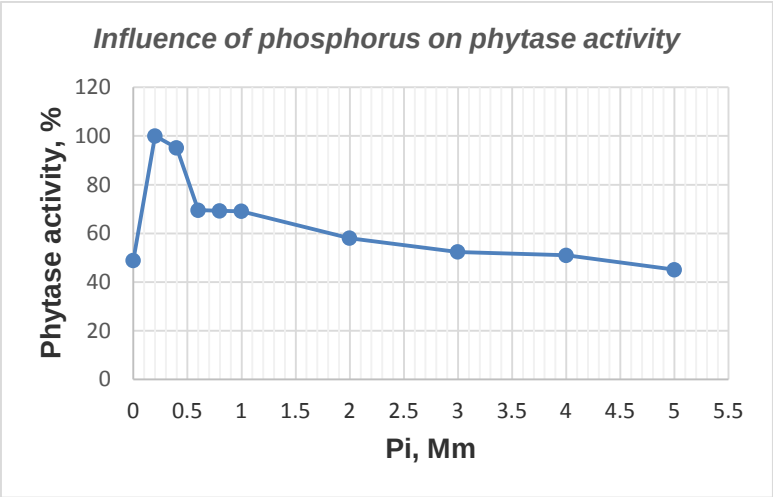

Figure 1: Influence of phosphorus on phytase activity

Experimental experiments were conducted in the temperature range from 37°C to 85°C. It has been found that the optimal temperature for decomposition of the enzyme produced by *Bacillus subtilis* in sodium phytate substrate is 60°C, after which its activity is sharply declining (Figure 2).

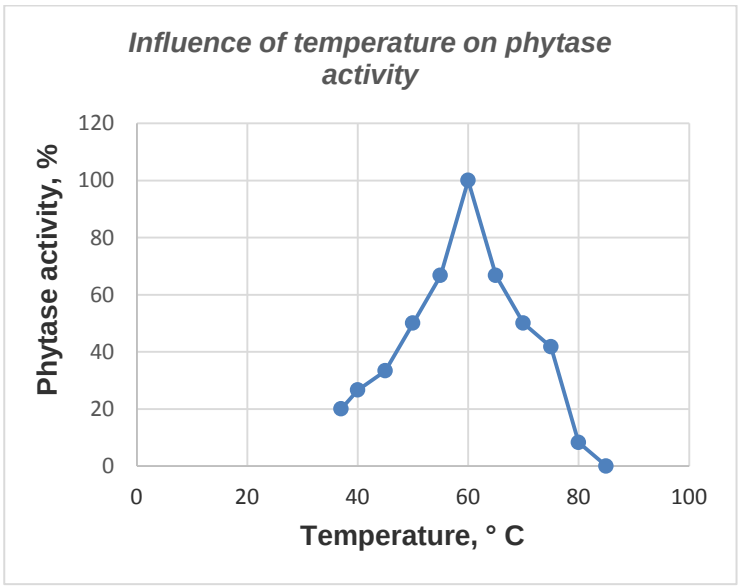

Figure 2: Influence of temperature on phytase activity

Figure 3 shows the values of the enzymatic activity of the chitinase produced by the producer strain *Bacillus subtilis* in three variants of nutrient media: Variant 1 - Standard culture medium for propagation and maintenance of the strain *Bacillus subtilis* NBIMCC 2353; Variant 2 - Standard nutrient medium with added 0.5% fresh mycelium of *Ganoderma lucidum*; Variant 3 - Standard nutrient medium with added 0.5% chitin from shrimp shells.

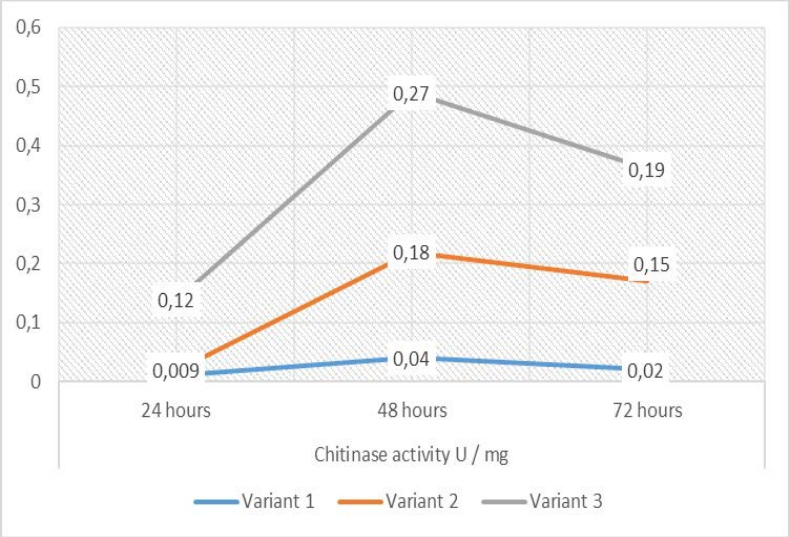

Figure 3: Chitinase activity (U/mg) determined at 24, 48 and 72 hours in three nutrient media variants

The inclusion in the nutrient medium of a natural source containing bound chitin (*Ganoderma lucidum*) leads to an increase in chitinase activity. The highest activity was observed when using purified chitin at the 48th hour from the start of cultivation.

4. Conclusions

The following conclusions can be drawn from the performed experiments:

- From the studied carbon sources, phytase activity is only reported for fructose and arabinose.
- The addition of 1% arabinose to the culture medium stimulates the production of an enzyme phytase. When increasing the percentage of carbon source arabinose, a decrease in enzyme activity is observed.
- It has been shown that precipitation and reduction of the available phosphorus in the medium results in an increase in enzyme phytase activity, the most active phase being at 60°C.
- It has been found that the inclusion in the nutrient medium of a natural source containing bound chitin (Ganoderma lucidum) leads to an increase in chitinase activity. The highest activity is observed with the use of purified chitin.

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