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Screening of phytate hydrolysis *Bacillus* sp. isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase

[Topraktan fitat hidrolize eden *Bacillus* sp. 'lerin taranması ve fitaz üretimi üzerine bazı besinsel ve fiziksel faktörlerin optimizasyonu]

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ABSTRACT

Objective: To isolate phytase producing *Bacillus* sp. from soil samples of Turkey, and optimize the growth conditions for maximum production of phytase.

Material and Methods: The screening of isolates was performed on phytase screening medium. The best producer was selected. Phytase activity was determined by measuring the amount of liberated inorganic phosphate. Optimal culture conditions and fermentation parameters for phytase production were assessed.

Results: 236 *Bacillus* sp. strains isolated. The best phytase producing strain showed higher enzyme yield in the presence of w*heat bran and* lactose as carbon source, meat extract as organic nitrogen source, $CaCl_2$ as metal source. 0.3% as phytate concentration was found to be best. In the physical parameters, the best results was obtained at 35°C, pH 7.5, 200 rpm as agitation rate, 2-4% as inoculum size and 48 h as inoculum age. A new medium was obtained by optimizing the incubation conditions of phytase production from *Bacillus* sp. EBD 9-1. In this medium, enzyme yield was enhanced 62% compared to basal medium.

Conclusion: The present study suggests that the novel *Bacillus* sp. phytase enzyme may have wide industrial application, and can be used as an animal feed additive.

Key Words: *Bacillus*, Isolation, Screening of phytase, Optimization

Conflict of Interest: The authors declare that there is no conflict of interest.

ÖZET

Amaç: Türkiye'nin toprak örneklerinden fitaz üreten *Bacillus* sp. 'lerin izole ve fitazın maksimum üretimi için üreme şartlarının optimize edilmesidir.

Gereç ve Yöntem: İzolatların taranması fitaz tarama ortamında gerçekleştirilmiştir. En iyi üretici seçilmiştir. Fitaz aktivitesi ortaya salınan inorganik fosfatın miktarı ölçülerek belirlenmiştir. En iyi kültür koşulları ve fermantasyon parametreleri fitaz üretimi için değerlendirilmiştir.

Bulgular: 236 *Bacillus* sp. suşu izole edilmiştir. En iyi fitaz üreten suş yüksek enzim verimini karbon kaynağı olarak buğday kepeği ve laktoz, organik azot kaynağı olarak meat ekstrakt, metal kaynağı olarak $CaCl_2$ varlığında göstermiştir.% 0.3 fitat konsantrasyonu en iyi olarak bulunmuştur. Fiziksel parametreler içinde, en iyi sonuçlar 35°C'de, pH 7.5'de, çalkalama oranı olarak 200 rpm'de, inokülüm miktarı olarak % 2-4 'de ve inokülüm yaşı olarak 48 saat'de elde edilmiştir. *Bacillus* sp. EBD 9-1'den fitaz üretimin inkübasyon şartlarının optimize edilmesiyle yeni bir ortam elde edilmiştir. Bu ortamda, enzim verimi temel ortamla karşılaştırıldığında % 62 artmıştır.

Sonuç: Mevcut çalışma yeni *Bacillus* sp. fitaz enziminin geniş endüstriyel uygulamaya sahip olabilirliğini ve hayvan yemi katkı maddesi olarak kullanılabilirliğini önermektedir.

Anahtar Kelimeler: *Bacillus*, İzolasyon, Fitaz Taranması, Optimizasyon

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Phytases have been one of the focal enzymes for nutrition, enviromental protection, and human health during the past two decades. Phytases (E.C.3.1.3.8. inositol hexaphosphate phosphohydrolase) sequentially cleave orthophosphate groups from the inositol core of phytate or phytic acid, the major chemical form (60–90%) of phosphorus in plants [1].

Phosphorus is one of the necessary mineral nutrients for animals during their growth, reproduction and calcification of the bones [2]. Animals do not possess the enzyme phytase; hence they cannot derive the nutrient phosphate from phytate. Phytate is also negatively charge, therefore it can strongly chelate with cation (Ca, Mg, Zn) to form insoluble salt and inhibit some enzymes (tripsin, amylase, acid phosphatase), thus influencing the absorption and digestion of this mineral by animal and reducing the bio-availability [3]. The undigested phytate and the unabsorbed inorganic phosphorus in monogastric derived faeces have the potential to cause phosphours pollution in areas of intensive animal production, leading to eutrophication [2]. Phytases have been commercialized as animal feed supplements and, it could resolve some of the problems caused by phytate in animal feeds [4].

On the other hand, it is also reported that lower inositol phosphate derivatives can have health benefits in the protection against colon cancer, arteriosclerosis, neural tissue, and coronary heart diseases. Technical improvements by adding phytases during food processing have been reported for breadmaking, production of plant protein isolates, corn wet milling and the fractionation of cereal bran [5-7].

Phytase is sold as a GRAS product and complies with current FAO/WHO and FCC recommendations for food grade enzymes [8].

These enzymes have been isolated from fungi, yeast, bacteria and protozoa [1]. Bacterial phytases and phytase-producing bacteria is as well as their potential biotechnological applications. Especially, *Bacillus* sp., *E.coli*, *Pseudomonas* sp., *Citrobacter* sp., are the best alternative to produce the enzyme [9].

Bacillus phytases have been studied extensively because of the immense potential of these enzymes having unique characteristics, feasibility of mass production for market and applicability in animal feed [10]. Hence phytase has a great industrial significance, and there is an ongoing interest in isolation of new microbial strain producing phytase and optimization of this enzyme [11].

The principal goal of this study is to isolate of *Bacillus* sp. strain with higher phytase-producing capacity from different soil samples. Various parameters of nutritional and physical factors were tested on the enzyme production. The present investigation was aimed at optimization of growth conditions which have been predicted to play a significant role in enhancing the production of phytase.

Materials and Methods

Screening and culture conditions

Soil samples of 30 different cities of Turkey were collected. For the screening of the *Bacillus* sp. strains with higher phytase-producing capacity from soil samples, 0.25 g soil was suspended in 10 ml of 0.85% sterile NaCl solution. It was incubated at 60 °C for 30 min and the *soil* suspension was subjected to serial dilution and inoculated onto nutrient agar plates. The *inoculated plates* were incubated for 24 h at 37 °C.

For determination of the genus of the selected strains (about 300) morphology and biochemical tests were studied, and genus of the selected strain was identified as *Bacillus* (about 236) [12]. The *Bacillus* sp. strains to be screened for phytase production were cultivated individually on modified phytase screening medium (PSM) [13] containing (g/L) Glucose 20, Na-phytate 4, $CaCl_2$ $2H_2O$ 2, NH_4NO_3 5, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.7H_2O$ 0,01, agar 15 at 37 °C for 96 hours. After incubating, depending on the zone of clearance the strain that yielded a high level of phytase was selected. The selected culture was maintained on nutrient agar medium and stored at 4 °C.

To select the best liquid production medium for phytase activity, five culture media [13, 14, 15, 16] was used and, compared. The best medium contained (% w/v): Dextrose 0.5; peptone 1; yeast extract 0.5; $MgSO_4$ 0.1; CaCl2 0.1, sodium phytate 0.1 (pH 7.0) [15, TS medium]. The precultures were cultivated in LB medium for 18 h. Then, overnight cultures with OD_{600}=0.3 were inoculated at 1% in enzyme production media (150 mL in 500 mL Erlenmeyer flasks) and incubated at 37 °C for 16, 24, 32, 48, 56, 72, 80 and 96 h in a shaking incubator (150 rpm). At the end of each period, the cultures were centrifuged (3461 *xg*, 10 min) and the supernatants were used for determination of phytase activity. Bacterial biomass was determined by measuring optical density at 600 nm.

The strain that yielded a high level of phytase and the medium were selected for further experiments.

Phytase activity assay

Phytase activity was determined according to the method described by Choi *et al.* (2001) [14]. 0.1 ml of enzyme solution with 0.9 ml of 2 mM sodium phytate in 0.1 M Tris–HCl buffer (pH 7.0) was carried out at 37 °C for 10 min and then the reaction was stopped by adding 0.75 ml of 5% trichloroacetic acid. The liberated phosphate was measured at 700 nm after adding 1.5 ml of color reagent, which is prepared freshly before using by mixing four volumes of 2.5% ammounium molybdate solution in 5.5% sulfuric acid and one volume 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 phosphate calibration curve was made by treating standard phosphate solutions of 0–100 µM KH_2PO_4 without added phytase under the same conditions as described above.

Effect of nutritional factors on phytase production

Various carbon sources such as glucose, fructose, sucrose, maltose, lactose, *glycerol, potato starch, corn starch, wheat bran and wheat starch* evaluated for their effect on phytase production by replacing dextrose in the production medium.

Organic and inorganic nitrogen sources chosen for the study were *tryptone,* peptone, meat extract, yeast extract, skim milk, and corn step liquor; NH_4NO_3, KNO_3, $(NH_4)_2SO_4$, $NaNO_3$ and NH_4Cl. These nitrogen sources were used to replace the organic and inorganic source available in the medium.

The culture medium was supplemented with the following metal salts such as $MgSO_4$, $LiSO_4$, $FeSO_4$, $CaCl_2$, KCl, NaCl, $MnSO_4$ and $ZnSO_4$.

Different sodium phytate concentrations (0.05, 0.1 (control), 0.2, 0.3, 0.4 and 0.5 % w/v) were used to optimize the best concentration.

The each flask was inoculated with 1% inoculum and incubated at 37 ºC for 48 h. Phytase activity and biomass were tested. The optimum carbon, nitrogen sources and metal ions were found by analyzing the results of phytase production

Effect of physical factors on phytase production

Some parameters (temperature, pH, agitation, inoculum size and inoculum age (days)) were studied for its influence in phytase production in basal medium.

The effect of temperature was evaluated by incubating the reaction mixtures at different temperatures such as 30, 35, 40 and 45 ºC in production medium with optimal pH of 7.0. Phytase activity and biomass were performed after 48 h of incubation.

pH in the range of 4.0–8.0 were examined for their effect on phytase production by the selected isolate grown in production medium. The pH of the medium was adjusted using 1 N HCl or 1 N NaOH. The flasks were incubated at 37 ºC for 48 h. Samples were taken for phytase activity and biomass.

Effect of agitation condition was carried out at following shaking rate, 0 rpm, 50 rpm, 100 rpm,150 rpm (control), 200 rpm and 250 rpm. The flasks were inoculated at 37 ºC for 48 h. Biomass yield and phytase were recorded.

The bacterium was grown in basal medium until OD_{600} of 0.3 and the cultivation was carried out at different inoculum sizes ranging (1% (control), 2%, 3%, 4% and 5%). The flasks were incubated at 37 ºC for 48 h. Samples were taken for phytase activity and biomass.

Phytase activity and biomass was measured by incubating production medium seeded with different inoculum age (18 h control, 1, 2 and 3 days) at 37 ºC. Phytase and biomass were determined for all the samples.

A new medium including the best contitions with nutritional and physical factors for phytase production were improved, and the novel *Bacillus* sp. isolate was grown in this modified medium for 48 h. Biomass yield and phytase was recorded, and compared with basal medium.

For statistical analysis, standard deviation for each experimental results and student's t-test was calculated using Excel Spread-sheets available in Microsoft Excel. Results presented in this study are means of three independent determinations. Barscor respond to standard deviation.

Results and Discussion

236 *Bacillus* sp. strains isolated from soil samples were determined on the basis of morphological and biochemical characteristics (Table 1). It is generally, accepted that the primary habitat of the aerobic endospore-forming bacilli is the soil. Since most *Bacillus* species can effectively degrade a series of biopolymers (proteins, starch, pectin, etc.), they are assumed to play a significant role in the biological cycles of carbon and nitrogen [17].

The phytase activities of all strains were assayed using phytase screening medium (PSM), and exhibited as diameter of clear zone in mm. Total 36 *Bacillus* sp. strains were found as extracellular phytase producer. Among *Bacillus* sp. strains, nineteen isolates showed weak clear peripheral hydrolytic zones (2-4 mm), thirteen isolates showed moderate clear peripheral hydrolytic zones (5-8 mm), four isolates showed high clear peripheral hydrolytic zones (9-11 mm). One isolate as named *Bacillus* sp. EBD 9-1 displaying the largest clearing zone (11 mm) was selected (Fig. 1), and then checked for quantitative test of phytase in five different liquid media. Enzyme activities in culture media [13, 14,15 (PSM medium), 16] were 70 IU/ml at 24 h, 160 IU/ml at 72 h, 185 IU/

Table 1. Morphological and biochemical tests for identification of *Bacillus* genus.

Test	Result
Shape	Rods
Gram staining	+
Spore formation	+
Motility	+
Starch hydrolysis	+
Catalase	+
Indol Production	-
Nitrate Reduction	+
Gas Production from Glucose	-
Acid Production from Glucose	+

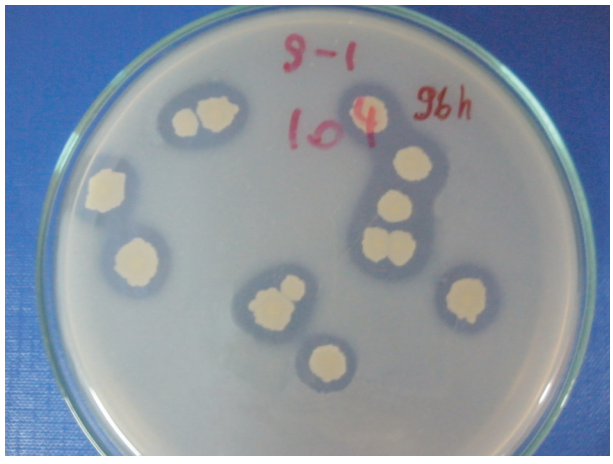

Figure 1. Clear zone by phytase-producing *Bacillus* sp. EBD 9-1 on PSM plate after 96 hour.

ml at 72 h and 130 IU/ml at 72 h, respectively. Among tested media, TS medium [15] was the best than other. In this medium, the maximum phytase activity (210 U/ml) was attained after 48. Maximum enzyme activity was obtained at the stationary phase of growth phase, and continued during the stationary phase. Biomass was 0.7, and 0.67 at 32 h and 48 h, respectively. The enzyme activity by *Bacillus* sp. EBD 9-1 was not in parallel with cell growth. Similar results have also been reported by other researchers. The maximum production of phytase *Bacillus* sp. KHU-10 [18], *Bacillus subtilis* (natto) [19] were reported to be reached during the stationary phase. It may be that the phytase is not required during balanced growth and may be synthesized in the response to a limitation in some nutrient. Because the synthesis of the enzyme was started as soon as the growth rate began to fall [18].

It was reported that twelve phytase producing thermophilic bacteria were isolated from the rhizosphere of leguminous plant methi (*M. falcata*). Of the twelve isolates, *Bacillus laevolacticus* strains designated as Tj1, Tj3, Tj4, and Tj6 produced 0.158, 0.216, 0.202, and 0.283 U/ml phytase, respectively [20].

It was obtained phytase-producing strains from soil samples and 71 % of the isolates had phytase activities above 0.01 U/ml [4]. The culture conditions for production of phytase by *B. licheniformis* under shake flask culture were optimized to obtain high levels of phytase (0.267 U/ml) [21]. The production of phytase have been detected after 36 h of cultivation and increased during growth and reached maximum level (109 U/mg) at 48 h [22].

Enzyme formation is largely dependent on the condition of growth of the culture and composition of nutrient medium. The present investigation was aimed at optimization of medium components which have been predicted to play a significant role in enhancing the production of phytase.

The research was focused on the improvement of phytase level. Some carbon, nitrogen sources and metal ions were used for the production of phytase by *Bacillus* sp. EBD 9-1. When dextrose in basal medium was replaced by various carbon sources (glucose, fructose, sucrose, maltose, lactose, *glycerol,* potato starch, corn starch, wheat bran and wheat starch), wheat bran = lactose were the best sources for phytase production (Fig. 2). Wheat bran and lactose (both of, 408 IU/ml), sucrose (388 IU/ml), wheat starch (372 IU/ml), maltose (341 IU/ml), corn starch (322 IU/ml), fructose (284) and potato starch (226 IU/ml) increased the production of phytase by 94%, 84%, 77%, 62%, 53%, 35% and 7% when compared to control (210 IU/ml), respectively. The phytase production was affected by carbon sources in the order: wheat bran = lactose> sucrose> wheat starch> maltose> corn starch>fructose > potato starch> control (dextrose)> glucose> glycerol. The increase in enzyme production was parallel to the growth rate (48 h) (Fig. 2). Glycerol (100 IU/ml) caused reduced phytase synthesis.

Reserachers have been reported different results for the best carbon sources. But, in several similar studies, it showed that wheat bran and sucrose was the best carbon source for phytase production by some *Bacillus* sp.[23-25], however, it poorly supported phytase production in *B. laevolacticus* [20].

In this study, fructose, maltose and sucrose also was very effect on production of phytase. But, Kim (1997) [26] has been reported that phytase of *B.amyloliquefaciens* not induced by glucose, fructose, maltose and sucrose. On the other hand, glucose was found to be the optimum carbon source for phytase activity by new isolate *Bacillus subtilus* strain BPTK4 [22], *Bacillus subtilis* [24], *Lactobacillus amylovorus* B4552 [27]. Sighn *et al*. (2013) [28] recorded that *B. subtilis* DR6 strain had optimum phytase activity on 0.75% glucose + 0.75% sucrose. Various sugars (monosaccharides and disaccharides) have been analyzed, sucrose was able to support maximal phytase expression (0.106 U/ml). Upon combining sucrose and pea flour, the phytase production increased to 0.35 U/ml as compared to 0.299 U/ml when pea flour was the sole carbon source [20].

Nitrogen sources also affected enzyme production. Effects of various organic and inorganic nitrogen sources on production of phytase were investigated (Fig. 3). Results obtained showed that the best nitrogen source for phytase production was meat extract (365 IU/ml) at 48 h and enzyme yield was 73% compare to control (210 IU/ml). The enzyme yields for yeast extract, corn step liquor, and peptone were 66%, 59% and 38%, respectively. Tryptone (200 IU/ml) is also good, but skim milk (46 IU/ml) drastically inhibited protease production. Maximum phytase production were meat extract> yeast extract> corn step liquor> peptone> control> tryptone> skim milk. The increase in enzyme production was parallel to the growth rate for all organic nitrogen sources. On the other hand, inorganic sources did not promote phy-

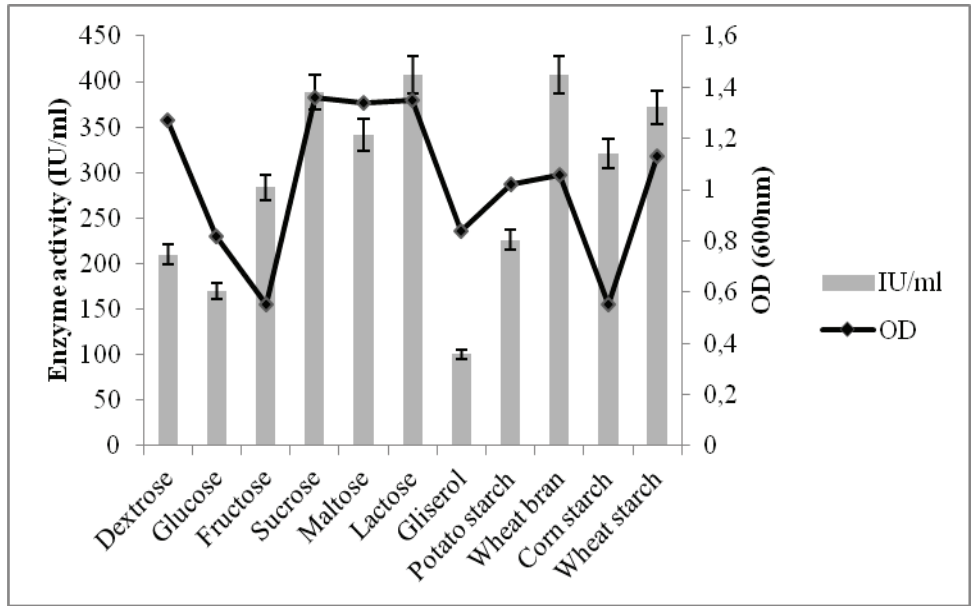

Figure 2. Effect of carbon sources on phytase production by *Bacillus* sp. EBD 9-1. Carbon sources were used as 0.5% in TS medium. The each flask was inoculated with 1% overnight culture (OD_{600}=0.3), and incubated at 37 ºC for 48 h in a shaking incubator (150 rpm). Results are means of three independent determinations. Bars correspond to standard deviation.

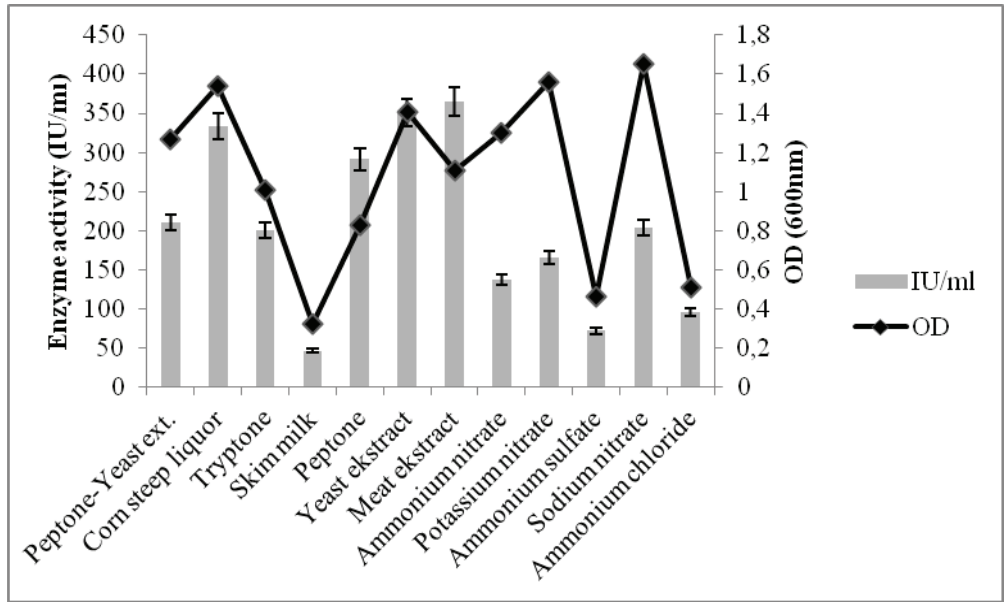

Figure 3. Effect of organic and inorganic nitrogen sources on phytase production by *Bacillus* sp. EBD 9-1. Organic and inorganic nitrogen sources were used as 0.8% in TS medium. The each flask was inoculated with 1% overnight culture (OD_{600}=0.3), and incubated at 37 ºC for 48 h in a shaking incubator (150 rpm). Results are means of three independent determinations. Bars correspond to standard deviation.

tase production compare to control. Bacterial growth was also very low (Fig. 3). Maximum enzyme production were control> $NaNO_3$> KNO_3> NH_4NO_3> NH_4Cl> $(NH_4)_2SO_4$. Among imorganic nitrogen sources $NaNO_3$ (203 IU/ml) was best than others. The effects of organic and inorganic nitrogen sources on phytase production by *Bacillus* sp. have been reported in the literature. Some researchers have been reported that organic or inorganic nitrogen sources influenced enzyme production. It reported peptone and beef extract as a nitrogen sources was best for *Bacillus subtilis* KHU-10 [18]. Yeast extract for *Bacillus* sp [21,22] was the best nitrogen source that induced the production of phytase. Gulati *et al*. (2007) reported beef extract, peptone and tryptone was no effect on phytase production by *Bacillus laevolacticus* [20]. Malt extract used as a source of nitrogen gave the highest phytase production for *Bacillus subtilis* MJA [29]. However, some inorganic nitrogen sources gave

better enzyme production. NH_4NO_3 [21] and $NH_4H_2PO_4$ [20] showed dramatic increase in phytase production.

Metal ions in media are an important factor that affects enzyme production due to act as inducers. The effects of some metal ions on phytase activities were investigated. $CaCl_2$ (282 IU/ml) was found to be the optimal metal source for phytase production (Fig.4). Enzyme yield was 34% compare to control (210 IU/ml). NaCl (223 IU/ml) and $MgSO_4$ (209 IU/ml) also had a positive effect for production. But, there was no significant effect of others salts. There are many such reports on the effect of metal ions on phytase production by bacteria and fungi. Similar result was reported that $CaCl_2$ was an important salt for phytase production by *B.amyloliquefaciens* [30], *Bacillus* sp. DS11 [23], *Bacillus* subtilis (natto) [19], *Bacillus subtilis* [24]. Some cations such as Fe^{2+}, Zn^{2+} and Cu^{2+} caused around 50% inhibition of enzyme activity [31]. In this study, $FeSO_4$ also caused inhibition of enzyme production.

A 0.3% phytate concentration was optimum for maximum phytase synthesis (data not shown).

Environmental conditions could affect the production of extracellular enzymes. Some physical parameters (temperature, pH, agitation, inoculum size and inoculum age) were studied for its influence in phytase production.

As seen be in Fig. 5, incubation temperature 35°C was found to be the best for phytase production. This is expected, since the tested isolate is mesophilic. This organism did good grew at 35°C. Enzyme production at 35 °C was 46% compared to control (37°C). A reduction in enzyme production was observed at 40°C and 45°C (37% and 58%). The production of phytase by *Bacillus* sp. EBD 9-1 was determined to be growth-related, The optimum temperature for the production of phytases for most of the microorganisms lies in the range of 25 to 37°C [32]. It reported *Bacillus* species such as *Bacillus* sp. KHU-10 [14], *Bacillus* sp. DS11 [23], *B.subtilis* [24] produced maximal activity at 37 °C. It has been reported that the new isolated *Bacillus subtilis* produces significant amount of phytase at 32 °C [22]. But, high temperatures have been reported for phytase production. The optimal incubation temperature for phytase production by *B. laevolacticus*, *B.licheniformis* and *Bacillus* sp. DS11 (KCTC 0231BP) was found to be 50 °C [20], 55 °C [21] and 65 °C [33], respectively.

Most isolated phytases have their pH optima in the range of 4.5-6. But, phytase from *Bacillus* sp. have neutral or alkaline pH optima [34]. Various pH, ranging from pH 4.0 to pH 8.0 were tested to determine the optimum pH for phytase production. In this study, the maximum production was observed at pH7.5 (Fig. 6). The phytase production by *Bacillus* sp. EBD 9-1 is growth dependent. Enzyme production and growth were not shown at acidic (pH 4.0) condition. The enzyme production had increased at alkaline pH values. At pH 8.0, enzyme yield was 43%. Similar result has been recorded for *B.licheniformis* (pH 7.5) [21]. The pH 7.0 has been recorded for optimal phytase production by the strain *Bacillus* sp. DS11 (KCTC 0231BP) [33] and *Bacillus subtilis* strain [22]**.** On the contrary, production optimization studies showed that maximum enzyme production by *Bacillus* sp.C43 was obtained at pH 4.0 [35].

Inoculum size plays an important role in fermentation process; in a suitable inoculum size, sufficient amount of nutrient and oxygen will be accessible for growth. In the present investigation, the newly isolated strain grown in basal culture medium with different inoculum size. The results show that the productionof phytase

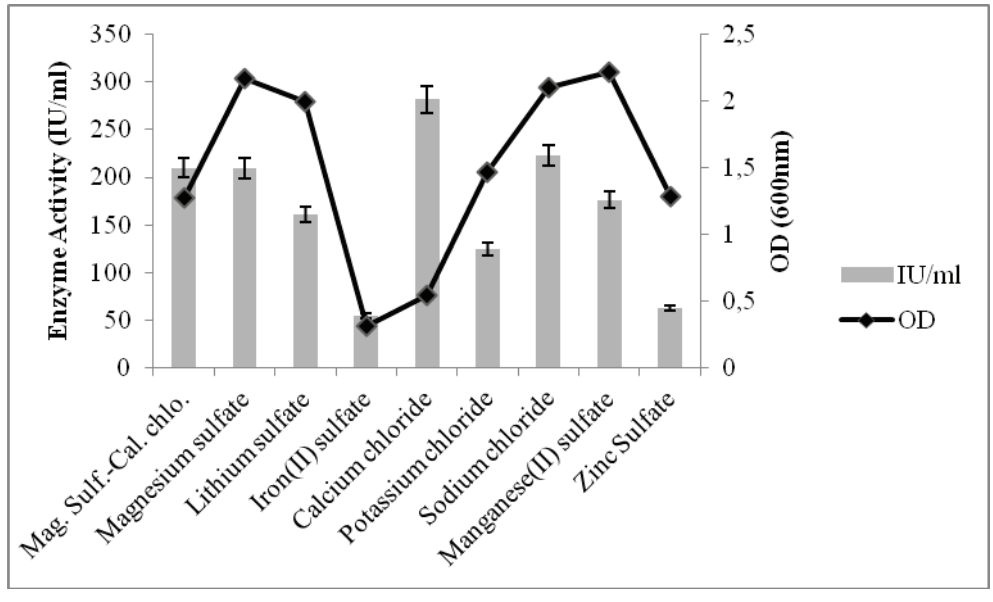

Figure 4. Effect of metal ions on phytase production by *Bacillus* sp. EBD 9-1. Metal sources were used as 0.1% in TS medium. The each flask was inoculated with 1% overnight culture (OD_{600}=0.3), and incubated at 37 ºC for 48 h in a shaking incubator (150 rpm). Results are means of three independent determinations. Bars correspond to standard deviation.

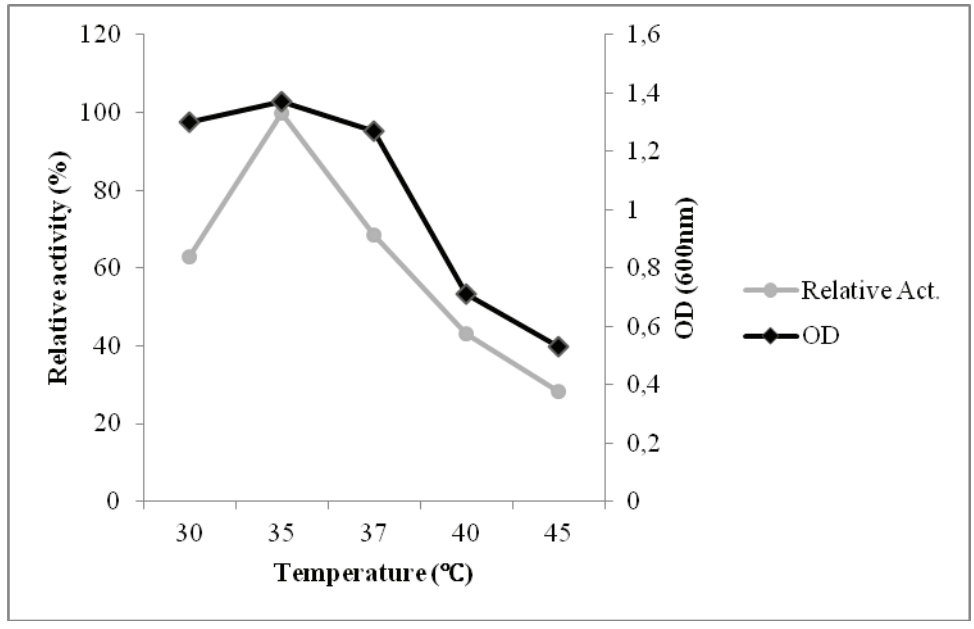

Figure 5. Effect of temperature (ºC) on phytase production by *Bacillus* sp. EBD 9-1. Assay temperatures were 30, 35, 37, 40 and 45 ºC in TS medium. The each flask was inoculated with 1% overnight culture (OD_{600}=0.3), and incubated at 37 ºC for 48 h in a shaking incubator (150 rpm). Results are means of three independent determinations.

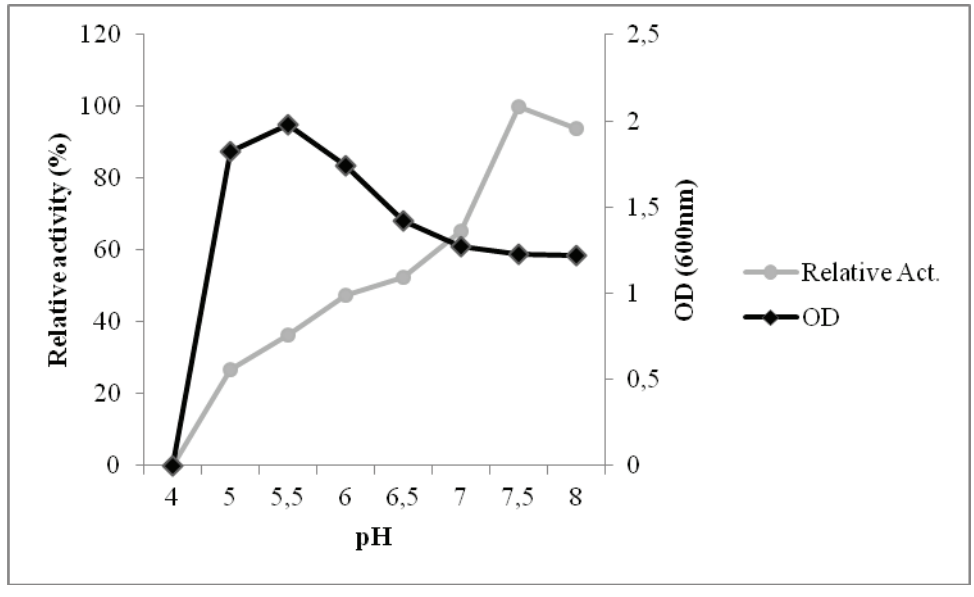

Figure 6. Effect of pH on phytase production by *Bacillus* sp. EBD 9-1. Assay pH ranges were 4.0–8.0 in TS medium. The each flask was inoculated with 1% overnight culture (OD_{600}=0.3), and incubated at 37 ºC for 48 h in a shaking incubator (150 rpm). Results are means of three independent determinations.

increased gradually with the increase of size of inoculum reaching its maximum at range between 2-4%, the enzyme activity was approximately stable (326 IU/ml) (Fig. 7). Phytase yield and biomass formation had parallel. Further increase in inoculum volume (5%) resulted in the decrease of phytase production. Because much increase in inoculum volume caused overcrowding of spore that decreased the enzyme activity. Similar results were also observed. Lata *et al.* [36] showed that the optimum inoculum size was found to be 2%, as compared to 1% inoculum used initially. Maximal phytase yield by *Bacillus laevolacticus* was obtained with 2% (v/v) inoculum size, however, phytase activity decreased at lower and higher inoculum concentrations [20]. Significant enzyme yields were observed with lower inoculum size. But, it was obtained the maximum phytase production at higher inoculum level (10%) [35]. Size of inoculum is an important biological factor. Hence, a balance between the biomass and available materials should be for maximum enzyme production.

Regarding the inoculum age, this factor is of importance when using mesophilic organisms due to the relatively low grow rate. In this study, the maximum enzyme production (305 IU/ml) was observed with 2 days old culture (Fig. 7). Use of 72 h old culture as inoculum resulted in decreased phytase activity (162 IU/ml). But, growth was high at 72 h old culture. It suggested that 24 h old actively growing culture resulted in increased phytase activity by *Bacillus laevolacticus*. [20]. Phytase yield and biomass formation were strongly correlated with the inoculum age, indicating strong growth associated phytase production by selected fungal strain [37].

Agitation leads to better dispersion of substrate, nutrients and oxygen in medium. The optimum agitation rate for phytase production (276 IU/ml) was 200 rpm. Significant increase in phytase production had not (Fig. 7). There was a gradual rise in enzyme production and growth with increasing agitation rates. But, enzyme yield at 250 rpm was low, while growth is high. However, it has been observed that higher agitation rates were much better for enzyme production. High agitation observations were also reported for *Enterobacter sakazakii* ASUIA279 (320 rpm) [38] and *Bacillus* sp. DS11 (230 rpm) [39].

Both our results and those of other reserachers have been shown that the phytase production patways of *Bacillus* strains and other bacteria were very different.

In this study, nutritional and physical parameters were optimized for enhanced production of phytase by new isolate *Bacillus* sp. EBD 9-1. By optimizing the incubation conditions of phytase production from this strain was enhanced 62% enzyme yield as compared to basal medium (control).

Biotechnology are using for developing new effective enzymes with improved properties. Here, we reported that phytase enzyme may have wide industrial application, and can be as an animal feed additive.

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Conflict of Interest: The authors declare that there is no conflict of interest.

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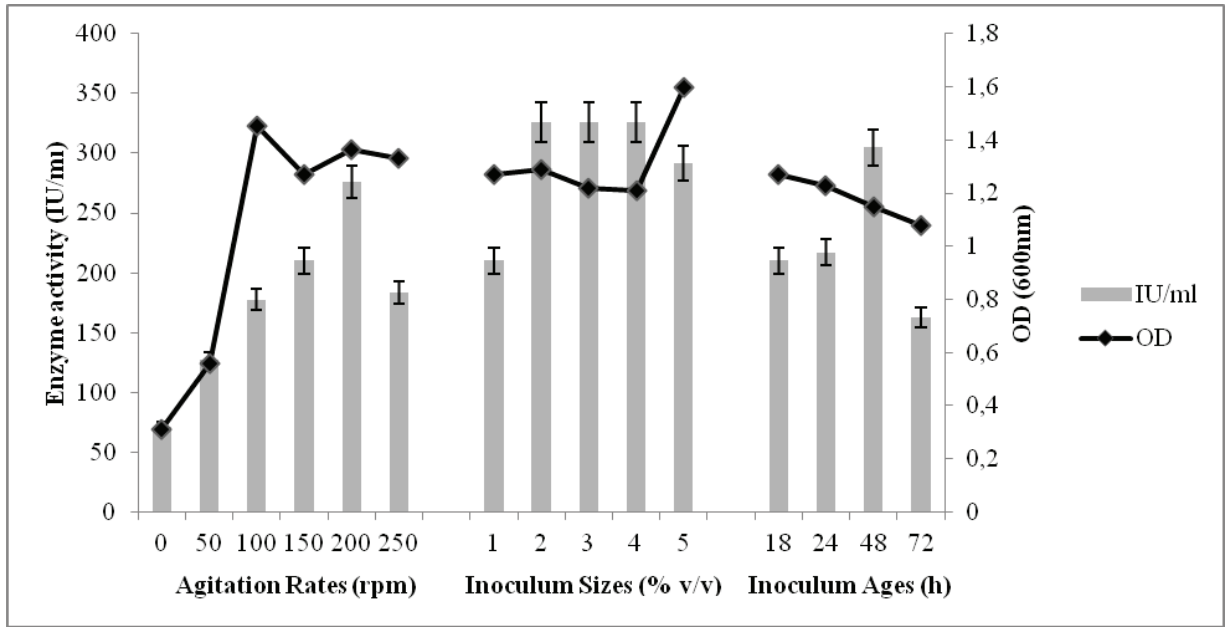

Figure 7. Effects of agitation rate, inoculum size and inoculum age on phytase production by *Bacillus* sp. EBD 9-1. Agitation condition was carried out at following shaking rate 0 rpm, 50 rpm, 100 rpm,150 rpm, 200 rpm and 250 rpm. Inoculum sizes were 1%, 2%, 3%, 4% and 5%. Inoculum ages were 18 h, 1, 2 and 3 days. Each assay was done in TS medium, and incubated at 37 ºC for 48 h. Results are means of three independent determinations. Bars correspond to standard deviation.

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