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Optimization of Phytase Production by *Bacillus* sp. (HCYL03) under Solid-State Fermentation by Using Box–Behnken Design

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HIGHLIGHTS

- *Bacillus sp* (HCYL03) was capable of producing phytases under solid-state fermentation.
- Wheat bran, residue of the rolled milled wheat grain, showed, indeed, potential to be used in phytases production.
- Using wheat bran to attain bioproducts can reduce some social/environmental issues.

Abstract: Phytase production by *Bacillus* sp. (HCYL03) isolated from soil was investigated by solid-state fermentation (SSF) using wheat bran as substrate. After screening, morphological and molecular identification, *Bacillus* sp. (HCYL03) was selected for experiments due to high phytase activity. The SSF conditions were optimized by using one-variable–at-a-time strategy. A Box–Behnken design was employed to investigate the optimization of the most significant variables affecting the enzyme production. Maximum enzymes production was observed after 72 hrs with inoculums 2mL at 45^{o}C and pH 5. Maximum partial purification was obtained with 60% ammonium sulfate. Protein contents in crude extract and partially purified enzyme were found to be 9.58 and 3.95 mg/mL, respectively. Optimum temperature and pH of the partially purified phytase after characterization were found to be 45^{o}C and 5, respectively. The study of different metal ions sources revealed that Ca^{2+} and Mg^{2+} ions registered the highest enzyme productivity) but Zn^{2+}, Cu^{2+}, Cd^{2+}, $Mn^{2+}$and Fe^{2+} ions exhibited inhibitory effect on phytase activity. Briefly, the *Bacillus* sp. (HCYL03) production of phytase utilizing the low-cost agro-residue wheat bran as substrate is an innovative and cheap way for the production of this important enzyme and opens a new way for researchers to investigate this dome.

Keywords: phytase; wheat bran; response surface methodology; solid-state fermentation.

INTRODUCTION

Phytate, also known as phytic acid, stores about 60-90 percent of the inorganic phosphorus found in fruits, oil seeds, and cereals. When phytate is hydrolyzed, it provides inorganic phosphorus and minerals to germinating seeds [1]. Phytate forms complexes with divalent and trivalent cations, starch, proteins and lipids, making phosphorus unavailable for utilization; therefore, phytic acid is known as an anti-nutritional factor [2].

Phytases (myo-inositol hexakisphosphate phosphohydrolases) are a class of phosphatases that catalyze the hydrolysis of phytates to *myo*-inositol, inositol phosphate, and inorganic phosphates. Phytase enzymes are reported in plants, animals, bacteria and fungi [3]. Animals like poultry, pig and fish cannot hydrolyze phytate as they do not have gastrointestinal phytase. As a result, inorganic phosphate is supplemented in their feed in order to fulfill phosphate requirement. This increases costs and contributes to phosphate pollution problems. The utilization of phytase enzymes in broilers feed resulted phosphate assimilation in the feed ingredients, slump phosphate in the manure and consequently reach the environment [4]. Several studies reported that supplemental phytase enzymes in poultry diets can be used to hydrolyze anti-nutritive phytic acid and this enzyme also increased utilization of phosphorus from phytic acid [5.6].

As literature indicated that yeast, fungi and various bacterial strains produced phytase enzymes which can be applied at industrial level [7]. Phytase enzymes are therefore an imperative industrial enzyme and a topic of broad research. In the most recent years, production of phytase by fermentation has grown up, due to the advantages of method, both in economic and practical perspectives as better product recovery, low-technology cultivation equipment, higher product concentration, and lower plant operation cost [2].

Presently, the majority of commercial SSF phytase is produced by growing the fungus *Aspergillus niger* on wheat bran, which provides both a surface area for microbial attachment and carbon and nitrogen nutrients from xylan and protein [8]. However, bacterial phytases displayed some distinct advantages in terms of their stability and resistance to proteolysis over phytases synthesized by fungi [9]. In fact, research has shown that SSF production of phytase by *E. coli* is economically feasible when process conditions are optimized to enhance enzyme yields utilizing low-cost substrates [10]. Very little information has been published that details *Bacillus* sp. phytase production under SSF conditions. Keeping in view this background, present study was designed to evaluate the effects of SSF process conditions on phytase yield from *Bacillus* sp. cultivated on a wheat bran substrate.

MATERIALS AND METHODS

Sample collection

Crude soil samples (n=50) were collected in sterile bags from different areas of Pakistan and brought at Industrial Biotechnology Laboratory, PMAS Arid Agriculture University Rawalpindi, Pakistan for isolation of bacteria.

Isolation and screening of phytate hydrolyzing bacteria

Two grams (g) soil was suspended in 10 mL distilled water and stirred. The suspension was filtered by Whatman filter paper and dilutions were made. Tenfold serial dilutions of each sample was plated on Phytase Screening Media (PSM) and incubated at 37^{o}C. The PSM was prepared by dissolving 3.0g glucose; 1.0g tryptone; 1.0g sodium phytate ($C_6H_9Na_9O_{24}P_6$); 0.3g $CaCl_2$; 0.5 g $MgSO_4$; 0.04 g $MnCl_2$; 0.0025 g $FeSO_4$; and 15 g agar in 1.0 Liter of distilled water. Following incubation, strains showing zone of phytate hydrolysis (>6 mm diameter) were selected, isolated and stored at -20^{o}C in 15 % glycerol till further analysis. On the basis of qualitative and quantitative enzymatic analysis, seven potent phytate-hydrolyzing strains were selected for further studies [11].

Identification of phytase producing bacterial isolates

Morphological characteristics of bacterial culture were observed by performing specific tests. The selected bacterial isolates were recognized by cell morphology via microscopic examination, colony morphology (size, shape and pigmentation) and biochemical characterization.

Molecular identification of phytase positive strains

The bacterial isolates that showed positive results were further identified molecularly. Their DNA was extracted, followed by PCR amplification carried out in a thermo cycler based on the methodology as described by Gupta [12]. The process of 16S rRNA gene amplification was performed using universal primers. Forward primer 785F (GGATTAGATACCCTGGTA) having 18 bases and reverse primer 907R (CCGTCAATTCMTTTRAGTTT) having 20 bases.

The purified product of PCR was used for sequencing of their genome. Molecular identification of bacterial strains was carried out by 16S rRNA gene sequence analysis performed by Macrogen Company (Macrogen Co, South Korea), using ABI 3730XL DNA Analyzers. For this purpose, bacteria cells were grown under appropriate conditions to late logarithmic phase. After centrifugation, high-molecular-weight genomic DNA was extracted and analyzed using ABI 3730XL DNA. Nucleotide blast was performed and phylogenetic analysis was done for the molecular identification through Clustal W and Clustal X version 2.0. Out of seven potent phytate- hydrolyzing strains, the strain with highest phytase activity was identified as *Bacillus* sp. (HCYL03) and was used in further studies.

Solid-state fermentation

Wheat bran was used as substrate for the production of phytase enzyme. Commercial quality wheat bran was obtained from local market, dried and ground in a grinder. Phytase Production by SSF was carried out in 250 mL Erlenmeyer flasks, 10g of substrate was placed in 250mL Erlenmeyer flasks and moisture was optimized as 60 %. After autoclaving at 121ºC for 15 min, the flasks were inoculated with different volumes of inoculums, pH, temperature and incubation periods as per layout of optimization experiments.

Physico-chemical optimization

Box Behnken Design (BBD) of response surface methodology (RSM) was used for Physico-chemical optimization of fermentation parameters. These include fermentation period (24 to 72 hrs), pH (4 to 10), inoculums size (2 to 8 mL) and incubation temperatures (25 to 45°C).

After optimization of physical parameters, PSM broth was supplemented with lactose & maltose (0.1 to 0.5 %), and peptone, tryptone & urea (0.1 to 0.5 %) as carbon and nitrogen sources, respectively in order to check their effect.

Enzymes extraction

Crude enzyme was extracted by mixing the contents of each flask with 50 mL distilled water. The mixture was placed in shaking incubator WIs-20R at 120 rpm and 37°C for 1 h. Crude enzyme was filtered through filter paper and subjected to centrifugation in Centurion Scientific K241R centrifuge at 1000 rpm for 15 min at 4ºC to remove impurities. The supernatant obtained was used for phytase assay.

Phytase Assay

Enzyme activity was calculated by a calibration curve over the range 5-25 µg/mL orthophosphate .Activity was expressed in unite per mililitre (U/mL). According to Heinonen and Lahti [13], one unit of phytase is defined as the amount of enzyme releasing 1 µmol of inorganic phosphorus per ml per minute under the assay conditions.

Protein contents determination

Concentration of total protein was determined by Bradford method [14]. Absorbance was measured at 595 nm through *UV*-visible *spectrophotometer*. Protein concentration was calculated based on a convenient standard curve using bovine serum albumin (Sigma-Aldrich).

Partial Purification of Phytase Enzyme

Partial purification of phytase was carried out by $(NH_4)_2SO_4$ precipitation. About 20 to 80 % $(NH_4)_2SO_4$ powder was added in 2mL of each crude extract and left overnight at 4ºC. These crude extract were then subjected to centrifugation at 10,000 rpm for 10 minutes. Enzyme assay was done for supernatant.

Characterization of partially purified Phytase

Temperature stability of phytase was tested by subjecting the enzyme to different temperatures i.e, 25, 35, 45, 55 and 65^{o}C for 1 hour. The optimum pH for the activity was determined by mixing equal volumes of Glycine–HCl, sodium acetate, Tris–HCl and Glycine–NaOH buffers at different pH ranging from 2.0 to 10.0. Phytase activity was determined after incubation for 6 h at 37°C.

Metal ion effect on phytase activity

The effect of metal ions on the enzyme activity was separately investigated with the addition of chloride salts of Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+} and Cd^{2+} directly to the standard reaction mixture in a final concentration of 1, 5, and 10 mM. Enzyme activity determined in the absence of metal ion was defined as 100%.

Validation of response surface methodology optimum conditions

Fermentation was carried out in triplicate under optimum conditions as suggested by RSM to validate the results. These results were compared with RSM predicted values. If validation and RSM values are close to each other, then model was accepted.

Statistical analysis

Box–Behnken design [15] was followed for the production of phytase by *Bacillus* sp. (HCYL03).The RSM under Box Behnken Design was used for designing experiments using JMP® (It is a statistical discovery tool from SAS Institute Inc.). This design is useful for optimizing a small number of variables at a few levels. This design was used at three levels; low, central and high (−1, 0, and +1). The data was then subjected to analysis of variance (ANOVA) followed by regression equation using the statistical software mentioned above to describe the level of phytase Y produced as a function of parameters under study [16]. Enzyme activity data was presented as Mean ± S.D and analyzed through one-way ANOVA.

RESULTS AND DISCUSSION

Selection of substrate

Wheat bran is a residue of the rolled milled wheat grain. The main layers of wheat bran are pericarp, aleurone and testa tissue [17]. Wheat bran was selected as substrate for SSF as it serve as best carbon source for bacteria. Moreover, it may provide enough nutrients like proteins (13-18%), starch (14-25%), non-starch carbohydrates (55-60%), fats (3-4%) and minerals (3-8%) required for high enzymes production [17]. Literature revealed that the phytase enzyme was inducible, and the presence of phytate, wheat bran in the medium is necessary for enzyme formation [18].

Bacterial Strain

After morphological and molecular procedure our strain was identified as *Bacillus* sp. (HCYL03).The 16S rRNA Gene Sequence was 99.29% similar to *Bacillus* sp. (HCYL03). As this strain showed maximum phytase activity among all potent strain, so it was selected for further investigation. Identification was made using 16S rRNA, a powerful tool for deducing evolutionary relationships and phylogenetic among eukaryotic organisms, bacteria and archaebacteria [19].

Optimization of phytase production

For enhanced production of phytase enzyme, both physical and nutritional parameters were optimized through RSM. Positive interaction among these parameters leads to enhanced production of phytase (Figures 1a-b; 2a-d). The temperature, time and pH are found significantly affecting parameters according to the ANOVA (Table 1). Temperature, time and pH have positive effect on phytase production. The quadrate effects of time and pH are found significant, but the quadratic effect of temperature is non-significant.

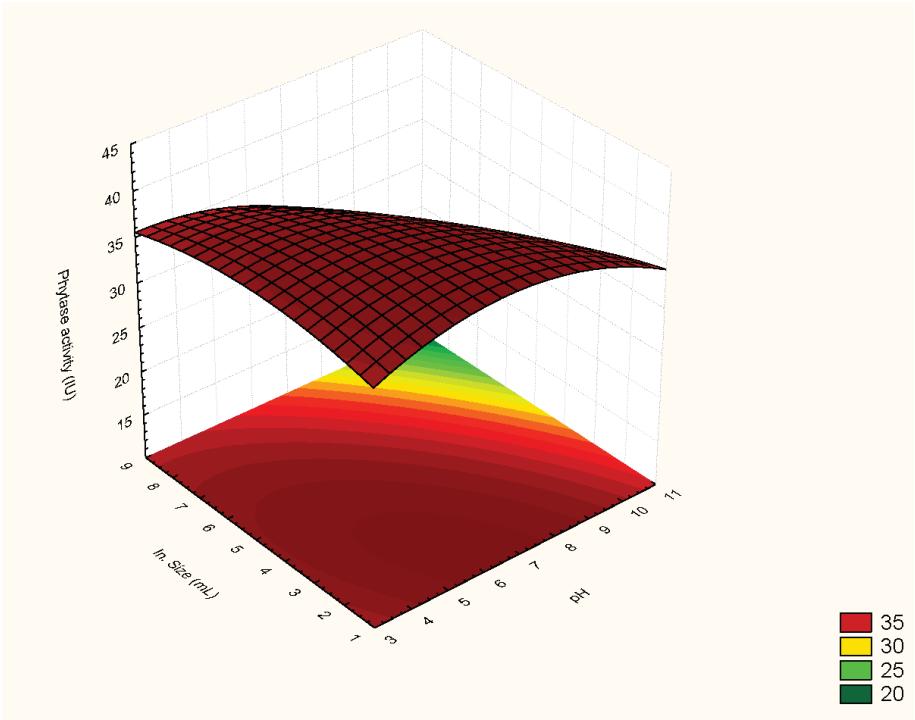

Figure 1-a. Response surface plot and its contour plot of Phytase production by *Bacillus subtilis* HCYL07 showing interaction effect of pH and inoculum size.

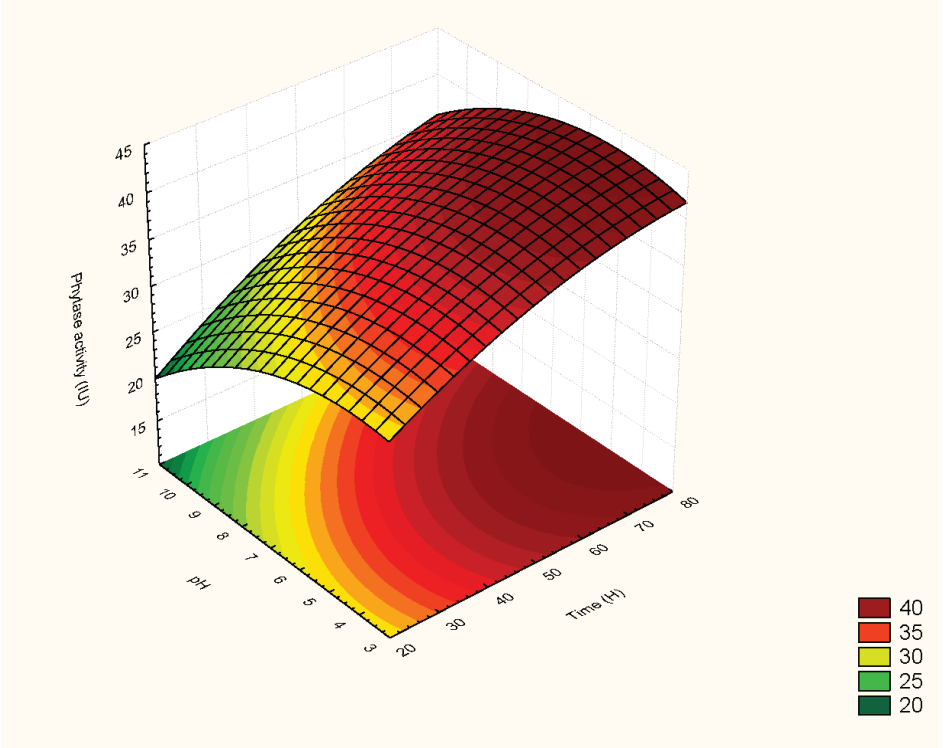

Figure 1-b. Response surface plot and its contour plot of Phytase production by *Bacillus subtilis* HCYL07 showing interaction effect of time and pH.

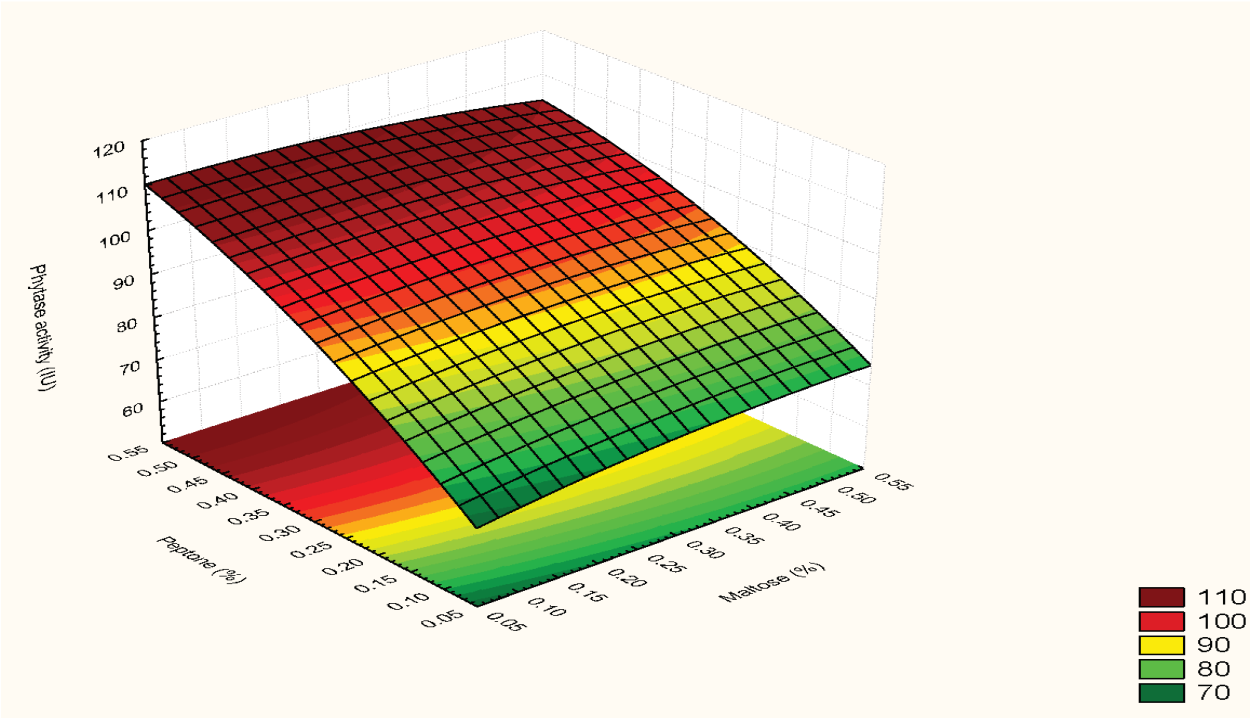

Figure 2-a. Response surface plot and its contour plot of Phytase production by *Bacillus subtilis* HCYL07 showing interaction effect of maltose and peptone.

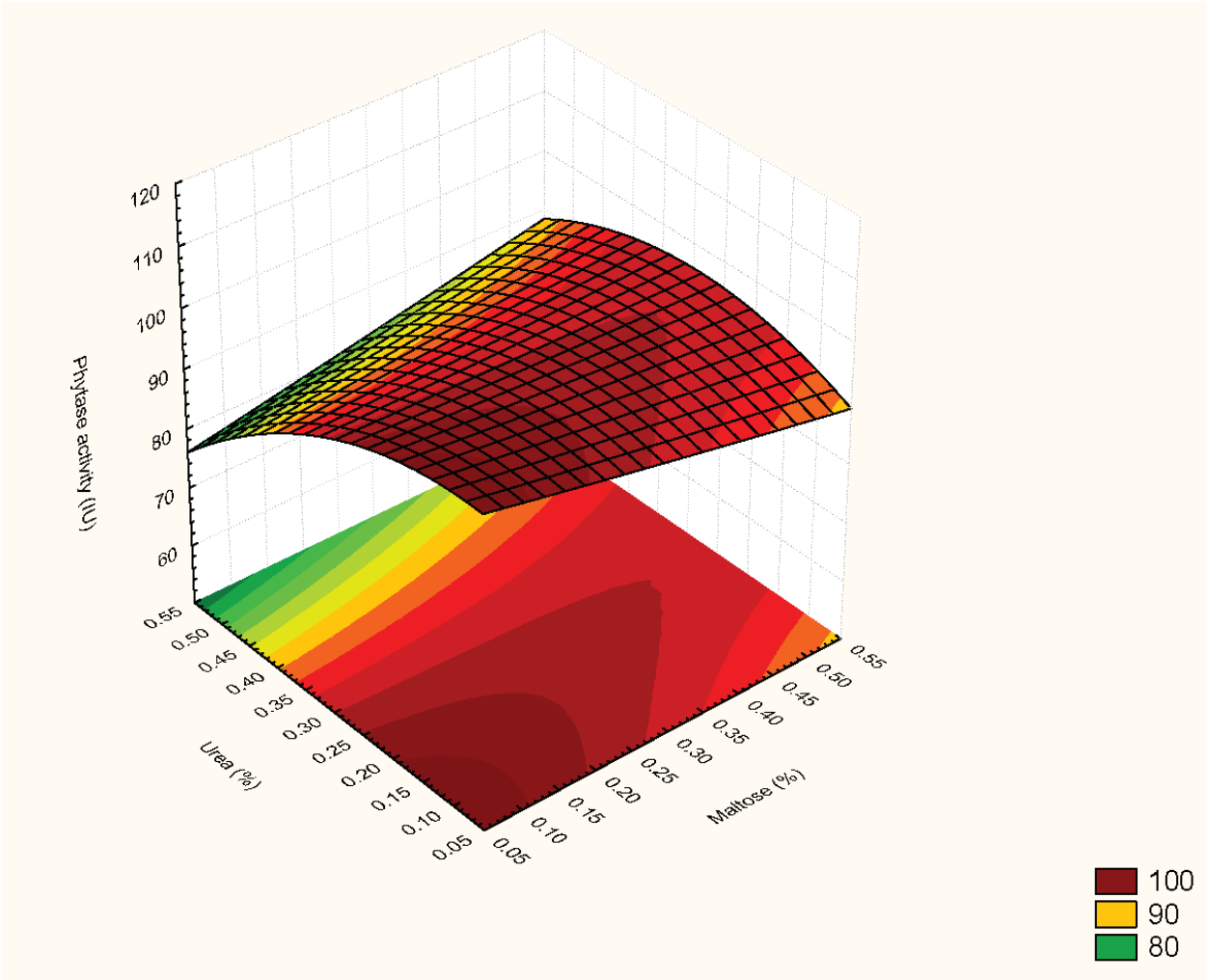

Figure 2-b. Response surface plot and its contour plot of Phytase production by *Bacillus subtilis* HCYL07 showing interaction effect of maltose and urea.

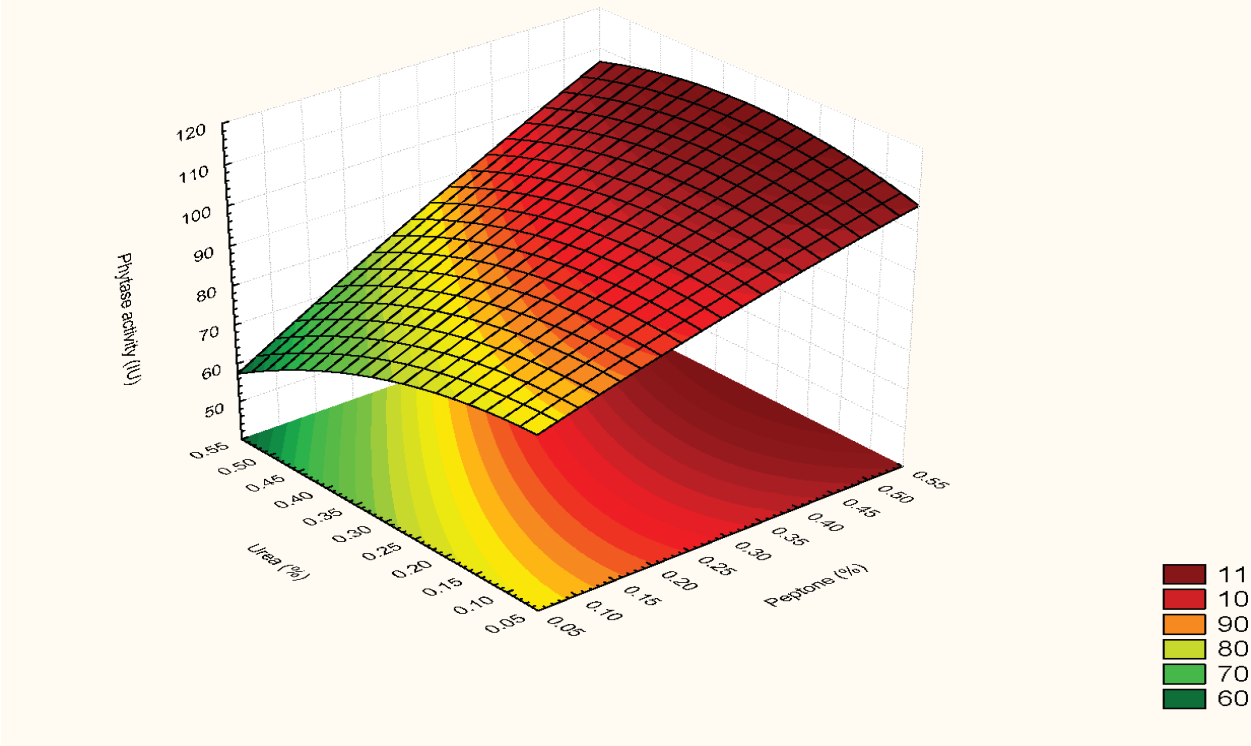

Figure 2-c. Response surface plot and its contour plot of Phytase production by *Bacillus subtilis* HCYL07 showing interaction effect of urea and peptone.

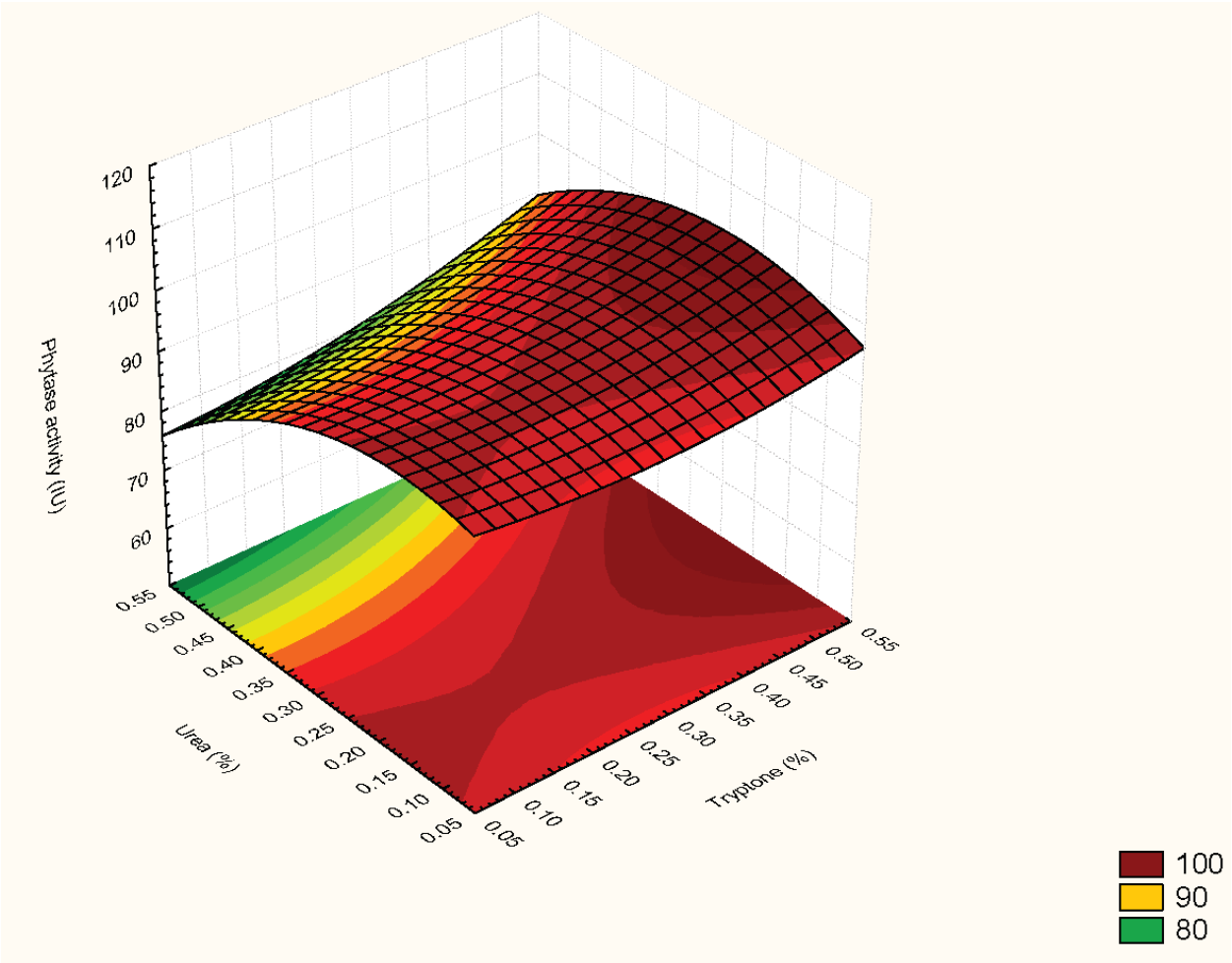

Figure 2-d. Response surface plot and its contour plot of Phytase production by *Bacillus subtilis* HCYL07 showing interaction effect of urea and tryptone.

Table 1. Analysis of Variance, culture conditions.

Effect	SS	Degree of freedom	MS	F	P
intercept	8.46407	1	8.46407	23.5062	0.000512
Time	8.47482	1	8.47482	23.5361	0.000510
$Time^2$	3.15165	1	3.15165	8.7527	0.013010
pH	5.68392	1	5.68392	15.7852	0.002185
pH^2	9.67540	1	9.67540	26.8703	0.000302
Temperature	2.94861	1	2.94861	8.1888	0.015472
$Tempreture^2$	1.35194	1	1.35194	3.7546	0.078753
inoculum	0.05762	1	0.05762	0.1600	0.696787
$Inoculum^2$	0.06009	1	0.06009	0.1669	0.690736
Time*pH	10.08050	1	10.08050	27.9953	0.000256
Time*temp	0.00000	1	0.00000	0.0000	1.000000
pH*temp	0.00000	1	0.00000	0.0000	1.000000
Time*inoculum	0.01453	1	0.01453	0.0403	0.844470
pH*inoculum	62.60407	1	62.60407	173.8624	0.000000
Temp*inoculum	0.00000	1	0.00000	0.0000	1.000000
Error	3.96086	11	0.36008		

Interaction among physical parameters

The results of RSM indicated that higher pH and incubation period have positive interaction. With increasing pH and incubation period resulting enhanced phytase activity as shown in Figure 1-a. The pH and inoculum volume both are important for microbial growth and enzyme production. A positive interaction was observed between these two parameters. Maximum enzyme activity was recorded at pH 5 and 2 mL inoculums size (Figure 1-b). Intact enzymes possess both cationic and anionic groups often at their active sites at given pH. Any change in pH may alter ionic form, three dimensional structure and activity of enzyme. It is worth noting that phytase activity at optimum physical parameters was 45 U/mL and many folds higher than that obtained without optimization (1.7 U/mL).

Interaction among nutritional parameters

Addition of carbon and nitrogen sources are displayed substantially affecting parameters according to the ANOVA (Table 2). Enhanced phytase production was noted after addition of carbon (maltose) and nitrogen (peptone, urea and tryptone) sources (Figures 2a-d). Positive interaction exhibited between peptone and maltose. Peptone and urea interaction also indicated a positive impact on phytase production. Interaction between urea and maltose also enhanced phytase activity. Similarly enhanced phytase production was seen by interaction between urea and tryptone. Optimized levels for the growth of *Bacillus* sp. HCYL03 were found to be 0.1%. 0.3%. 0.5%. 0.5 % and 0.3 % for lactose, maltose, peptone, tryptone and urea, respectively using RSM.

Table 2. Analysis of Variance, C and N Sources Levels

Effects	SS	Degree of freedom	MS	F	P
intercept	1804.126	1	1804.126	348.8238	0.000000
Lactose	0.555	1	0.555	0.1074	0.752722
Lactose ^{2}	0.026	1	0.026	0.0050	0.945354
Maltose	5.091	1	5.091	0.9843	0.354176
$Maltose^2$	0.088	1	0.088	0.0170	0.900051
Peptone	64.240	1	64.240	12.4206	0.009672
$Peptone^2$	7.813	1	7.813	1.5106	0.258753
Tryptone	14.003	1	14.003	2.7074	0.143879
$Tryptone^2$	14.184	1	14.184	2.7424	0.141691
Urea	8.350	1	8.350	1.6144	0.244476
$Urea^2$	57.256	1	57.256	11.0703	0.012639
Lactose*Maltose	4.695	1	4.695	0.9077	0.372458
Lactose*Peptone	8.873	1	8.873	1.7155	0.231619

Cont. Table 2

Maltose*Peptone	67.150	1	67.150	12.9833	0.008702
Lactose*Tryptone	0.513	1	0.513	0.0991	0.762084
Maltose*Tryptone	17.026	1	17.026	3.2920	0.112489
Peptone*Tryptone	15.611	1	15.611	3.0184	0.125894
Lactose *Urea	0.462	1	0.462	0.0893	0.773783
Maltose*Urea	251.450	1	251.450	48.6173	0.000217
Peptone*Urea	298.049	1	298.049	57.6271	0.000127
Tryptone *Urea	67.058	1	67.058	12.9655	0.008731
Error	36.204	7	5.172		

The experiments in triplicate were conducted to validate the phytase production on predicted optimal level by RSM. The observed enzyme activity on these levels was 118 U/mL is very close to the predicted activity (120.72 U/mL). An optimum carbon and nitrogen ratios are required for microbial growth and subsequently enzymes production. Enzymes production was increased because of ready availability of carbon and nitrogen. The nitrogen and carbon were freely available for microbe as compared to bound form in substrate. At low carbon and nitrogen levels, the microbe is nutrient starved and high concentration makes the substrate unfavorable for microbes through altering its texture.

Protein estimation

Protein content of crude and partially purified phytase was determined by Bradford method. A standard curve was made by drawing graph of different bovine serum albumin concentration against absorbance. Protein quantity was determined by using regression equation. Protein contents in crude extract and partially purified phytase enzyme were found to be 9.58 and 3.95 mL, respectively. Similar results were reported by Ajith and coworkers [20], who found that phytase produced by *Aspergillus foetidus* MTCC 11682 was partially purified by $(NH_4)_2SO_4$ precipitation and Sephacryl S-200HR gel filtration to 23.4-fold (compared to crude extract) with recovery of 13% protein. The presence of high amount of protein in crude extract shows the presence enzymes other than phytase. Purification of phytase was confirmed by low quantity of protein in purified phytase. These results confirm that enzymes other than phytase have been excluded.

Partial purification of phytase

Partial purification was carried out by $(NH_4)_2SO_4$ which was used due to water soluble and high ionic strength. Maximum partial purification was obtained with 60 % $(NH_4)_2SO_4$ precipitation. Maximum precipitation was obtained with 60-80 % $(NH_4)_2SO_4$ as followed by method of Winartia and coworkers [21]. The lesser protein content in partially purified phytase confirmed the removal of unwanted enzymes and purification of phytase. The results of this study are well in international range. Similar results were reported by Borda-Molina and coauthors [22], who attained phytase from *Lactobacillus plantarum* by $(NH_4)_2SO_4$ precipitation. All the precipitates obtained were dissolved in small amounts of 0.1 M Tris-HCl buffer of pH 5.5 and the phytase activities were checked. The active fractions were given for dialysis against the same buffer. The active fractions were pooled and allowed to stand at 4°C. The pure form of the enzyme was thus obtained.

Characterization of Phytase Enzyme

Characterization was carried out for optimum temperature, pH and effect of metal ions. Phytase characterization increases its efficiency in industrial applications.

Optimization of temperature

Maximum phytase activity was seen at 45^{o}C optimum temperature. A gradual increase in activity was observed from 25^{o}C to 45^{o}C, then it start decline and minimum activity was indicated at 65^{o}C. At lower temperature, reduced phytase activity was owing to decreased molecular motion and lesser number of collisions between phytase and its substrate, which ultimately make the reaction slow. Decline in phytase above optimum temperature is due to enzyme denatured and loss of globular structure. These results are concurred with findings of two studies which exhibited that maximum phytase production by bacteria *Anoxybacillus sp. MHW14* and *Streptomyces sp.* recorded at optimum temperature of 45^{o}C [18,23].

Optimization of pH

Optimum pH for phytase activity was observed to be 5 in the present study. Extreme pH may also denatured enzyme. The pH above and below than optimum value change the shape and active site of enzyme. Change in pH also affects charge distribution on amino acid. This make phytase active site non-complementary to its substrate and reaction rate was low above and below optimum pH. These results are agreed with findings of two studies [18,24] which showed that optimum pH for phytase production from *Streptomyces sp.* and *Aspergillus niger S2* was recorded exactly 5.

Effect of metal ion on phytase activity

It is a well known fact that metal ion effect enzyme activity.Ca^{2+} and Mg^{2+} ions in the present study remarkably enhance phytase activity which displayed stimulating effect of these ions. On the other hand, a decline in enzyme activity was observed in the presence of Zn^{2+}, Cu^{2+}, Cd^{2+}, $Mn^{2+}$and Fe^{2+} ions, indicating inhibitory effect of these ions. Phytase inhibition may be due to insoluble metal ion-phytate complexes formation. This might be phytate substrate unavailable for enzyme and hinder enzyme activity. Another study [25] displayed similar results that phytase activity from *Bacillus sp. DS11* was significantly hampered by metal ions such as Cd^{2+} and Mn^{2+}.

CONCLUSION

Waste materials cause a problem all over the world; they always need a new strategy to get rid of it with a benefit target. Millions tons of wheat bran are collected every year as byproducts of industrial work without considerable benefits. This study is opening a new arena of research in the production of *Bacillus* sp. HCYL03 phytase by using wheat bran as substrate. The enzyme was produced under solid-state fermentation and the conditions for enzyme production were optimized by using Box–Behnken design. It is suggested that this work will have great benefit in solving wheat bran waste problem. Also, our research introduced a low cost medium and very simple technique in phytase production which is considered as one of the most important enzymes.

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Conflicts of Interest: The authors declare no conflict of interest.

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