Isolation and statistical experimental design for the optimization of phytase production by a newly isolated strain, *Aspergillus Terreus (OP028905)*

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Abstract- The present study was designed to produce phytase by screening the isolated strains of indigenous fungi and their substrates. It was performed on 6 substrates to find the higher activity of phytase which was obtained from 11 fungal isolates. A highly phytase producing strain was screened along with substrate; furthermore, the isolated strain was identified as *Aspergillus terreus* (OP028905) by 18SrRNA gene sequencing. Wheat bran was selected as a substrate using the One Variable at a Time (OVAT) strategy. Physico-chemical parameters were optimized through statistical designs, Response Surface Methodology (RSM) and Taguchi in solid-state fermentation. Central Composite Design (CCD) was selected through RSM and applied to analyze the relationship between the tested variables (temperature, pH, time period, inoculum size, moisture content, substrate concentration, and nitrogen source). RSM phase-I exhibited **192 IU/mL**; phase-II exhibited **245 IU/mL**, while Taguchi method showed **155 IU/mL** as maximum activity. RSM exhibited 36% more phytase production than Taguchi method. Among these designs RSM phase-II exhibited maximum activity. Conclusively, the optimized conditions based on RSM came out as temperature 50˚C, pH 8.0, inoculum size 2mL, time period 1 day, moisture content 60%, substrate concentration 10g, and nitrogen source (0.2mg).

Keywords: fungal isolates, RSM, Taguchi, solid-state fermentation

I. INTRODUCTION

In current era, industry involved exorbitantly in producing frugal and cost-effective enzymes particularly in developed countries [1]. The trend to utilize industrial enzymes is increasing for its low energy input, non-toxicity, less time processing, lucrative, and environment friendly characteristics [2]. Phytase (EC 3.1.3.8) is an important phosphatase because of its mechanism of action and significant importance in food industry (human and animal food processing) [3]. Numerous fungal species have been known for phytase production [4]. The fungal phytase hydrolyzes phytates in upper part of the stomach and intestine in a wide range of pH values (Neira-Vielma, Aguilar, Ilyina, Michelena-Álvarez, & Martínez-Hernández, 2022). *Aspergillus terreus* is a filamentous fungus which has been widely studied for its ability to produce various enzymes, including phytase [5]. Phytase enzyme catalyzes the reaction using phytate as a substrate and releases minerals and phosphorus to make sure their availability in the body for their bioavailability[6]. Aspergillus species is the chief producer of phytase having generally recognized as safe (GRAS) status with potential applicability in feed industry [7]. Among different sources the fungal source is more desired because of its thermo-stability, extra-cellular enzyme secretion and broad pH range [8]. The demand of a cost-effective process for phytase production is increasing day by day; surely, this research is going to be helpful in this regard.

Inositol hexa-phosphate (IP6) or phytates ($C_6H_{18}O_{24}P_6$) are the salt form [9] of phytic acid, foremost storing structure of phosphorus in the plants, including oil seeds [10], cereals, grains which comprise almost 75-80% of the overall Phosphorus present in oil seeds, cereals, and legumes. Phytate chelates a number of metal ions with it especially makes bond with calcium, magnesium, and potassium along with phosphorus which is 50-85% of the total phosphorus of the seed [11]. Phytate has phosphorus locking nature, that's why it leads to the reduction of phosphorus and cations bioavailability [12].

highly recognized in scholar fraternity and form a core part of PhD curriculum. Research scholars publish their research work in leading journals to complete their grades. In addition, the published research work also provides a big weight-age to get admissions in reputed varsity.

Solid state fermentation (SSF) is an ideal approach for the idyllic production of crude industrial enzymes, and generally preferred due to the production of highly concentrated crude enzymes at low costs [13]. SSF permits the usage of renewable agricultural and industrial residues as substrates for abundant production of microbial enzyme [14]. Culture conditions in SSF are analogous to the natural habitat of filamentous fungi. These conditions enable the fungi to grow and excrete a great deal of enzyme mass by controlling the physicochemical factors i.e., organism type, substrate, moisture, inoculum size, and temperature [15] .

Parameter optimization is a tedious task due to the connection of various physical and chemical factors which influencing fermentation. Present investigation applied one variable at a time (OVAT) methodology for fungal and substrate screening.

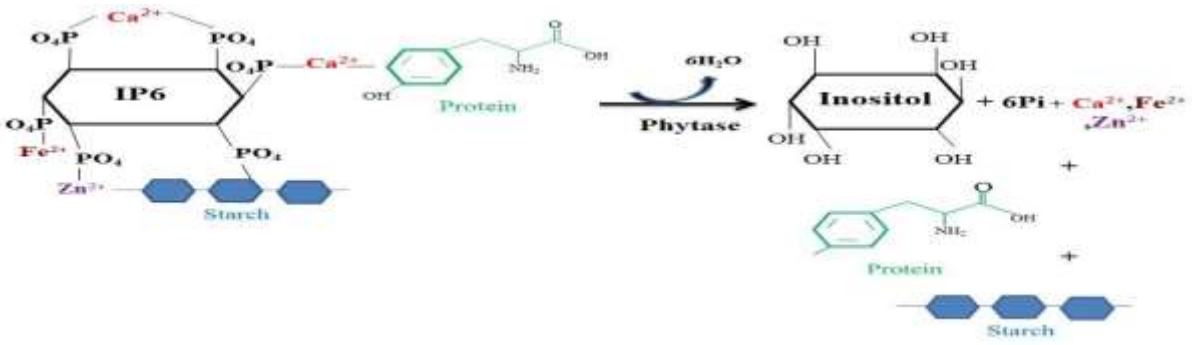


Figure-1: Phytase catalyzing the biochemical reaction: Phytate (IP6) forms a complex with mineral ions, protein, and starch. Phytase is hydrolyzing the phytate and releasing Ca, Fe, Zn, P, protein and starch molecules in order to make them biologically available.

OVAT methodology is significant for determining a particular condition (only 2 variables) with less experimental trials [16]. In an initial stage of an experiment OVAT is a preferred choice because of lower production cost with maximum enzyme production and easy handling. Substrate and fungal screening are two critical parameters that are kept as variables and remaining physico-chemical parameters persist constant.

After determining hyper phytase producing fungus and substrate, remaining physico-chemical parameters were optimized with statistical design. When number of parameters increases, their evaluation of interactions and effects gain further complexity. For cost-effective phytase production of with optimal conditions in less time, low expenses, efficient data interpretation using a small number of experiments, the statistical method (Response Surface Methodology and Taguchi method) is a worthy strategy [17]. With the help of Orthogonal Arrays (OAs) and Taguchi method, the number of required experiments decrease as compared to that of required by application of other statistical factorial design. RSM is an influential and information based mathematical model having a set of statistical tools. It identifies interactions between numerous variables with fewer experimental trials. It is easy to use and practically viable. RSM has substantial popularity for the section of industrial application as well [18].

In the present scenario, this study was designed to examine the lucrative production of fungal phytase utilizing cheap source. Phytase is non-toxic in nature, cost effective and eco-friendly [19]. The strains were screened to determine the phytase activity. Later, these strains were identified on the base of gene-sequencing. For molecular identification, 18S RNA sequencing was performed by Macrogen Company, Korea. A suitable strain having hyper-producing phytase ability on a suitable substrate was identified. The selected strain was allowed to grow on waste material in solid state fermented conditions optimized statistically.

II. MATERIAL AND METHODS

Strain Maintenance

Almost, 11 fungal strains were collected from rotten vegetables, fruits, and soil in the area of head Sagar (Punjab, Pakistan). The samples were given names BT-1 to BT-11. Serial dilution was performed for each sample to ensure the culture purity. In Laminar Flow Hood, a drop (full of fungal spores) was dropped on a sterile glass petri plate, containing PDA solid media, in which 1% of phytic acid solution was added for the maintenance of fungal growth. Plates were incubated at 35°C±1 for 3 days (72 hrs) with pH 4.5 [20]. The obtained pure culture of organism was preserved in agar slants of solid potato dextrose (PDA) in sterile test tubes tightly covered with sterilized cotton swab and aluminum foil at 30°C (Reilly et al., 2018). The slants were preserved at 4°C±1 in refrigerator for further usage. These fungal cultures were re-cultivated periodically at optimal growth conditions as needed.

Inoculum Preparation

The fungal spore suspension culture was prepared in sterile Erlenmeyer flask with liquid PDA medium containing all the carbon and phosphate sources vital for fungal growth [21]. The pH of the suspension was maintained at 4.5, autoclaved at 121°C, PDA broth media was inoculated with loopful fungal spores in laminar flow-hood from grown culture to ensure culture purity and placed in shaking incubator at 120rpm, 30°C for 72 hours to get a sufficient homogeneous spore suspension. The Hemocytometer method was performed to ensure the spore's count10^7-10^8 spores/mL. To maintain the accuracy of the experiments fresh inoculum was prepared for every experiment [22].

Biomass and Organism Screening through OVAT Methodology

Before the parameters optimization through statistical analysis there is a need to perform a classical method for the selection of best substrate and organism for the higher phytase production. This experiment was designed according to OVAT strategy [23]. To sort out the best substrate for the fungal growth, different agricultural wastes were procured from local fields, flour milling industry, food processing industry, and market (wheat husk, wheat bran, green chick pea leaves and pods guava leaves, rice polish, rice husk). Each experiment was performed in 250 mL Erlenmeyer flask with 5 g of wheat bran and 60-65% distilled water and autoclaved along with substrate. After cooling, it was inoculated with 3 mL vegetative form of fungus (inoculum) and incubated at 35°C for 5 days [24]. Standard curve was designed to find out unknown concentration of phytic acid by the action of phytase enzyme. Total 132 (66×2) trials were performed to

screen out higher phytase producing fungus and most suitable substrate. Later OVAT, Taguchi method and Response Surface Methodology were performed to optimize physico-chemical parameters.

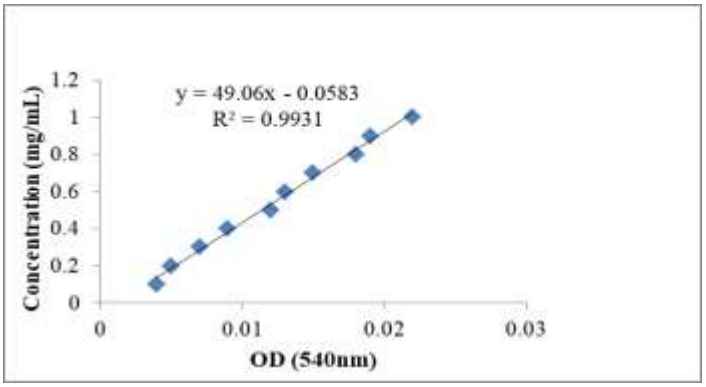


Figure-2: Standard Curve of KH_2PO_4 (mg/mL)

Taguchi Method

Taguchi method was applied for the parameter's comparison that how much parameter difference affects the phytase production. This could be characterized as a regulatory figure denoting how procedure has been impacted by defects in the product. In our study, after biomass and fungal screening, Taguchi L18 a factorial design was applied to test the effect of 7 factors with three levels each (3^7) to optimize phytase production. This method reduces the trial numbers from 324 to just 18 trials with variability of parameters i.e., temperature (30, 40, 50 °C), pH (3, 5, 8), inoculum size (3, 4, 5 mL), time period (3, 5, 7 days), moisture content (30, 60, 90 %), substrate concentration (3, 5, 8 g), and Nitrogen source i.e., ammonium sulfate (0.1, 0.2, 0.3 mg).

Response Surface Methodology (RSM)

This approach was applied on physico-chemical parameters (incubation time, pH, temperature; moisture level, additional carbon sources and nitrogen sources, and inoculum size) to study their interactive effect and to get maximum level phytase production in solid-state fermentation. Experiment was designed in MINITAB software through Central Composite Design (CCD). RSM was applied in two experimental phases for experimentation ease. Four out of seven parameters (temperature, pH, inoculum size, time period) were put in one statistical design; remaining three (Moisture Level, Nitrogen Source, Substrate Concentration) were put in the other [25].

Enzyme Extraction and Assay

The crude extract was extracted by mixing fermented material, inside the Erlenmeyer flask, with distilled water (three times more than the weight of fermented material). Then flasks were incubated in a shaking incubator (200 rpm) for 1 h at 35˚C to ensure the proper mixing of enzyme in the water [26]. Then the material was filtered with the help of muslin cloth; filtrate was centrifuged in 1.5 mL eppendorf tube (10,000 × g, for 10 min, at 4˚C) ; the clear cell free supernatant was collected and analyzed for enzymatic assay [27]. To check the maximum activity of phytase in the classical and statistical experimentation, enzyme assay was performed. 0.5 mL (centrifuged) enzyme was taken in an autoclaved sterile glass test tube; 0.5 ml 1% phytic acid solution was added to it. This solution was placed in an incubator at 20˚C for 5 minutes. Afterward, 500µl of 15% TCA was added to stop the reaction. Along with this, 300µl of distilled water and 0.9µl of chromogenic reagent (H_2SO_4 3mL, Ammonium Molybedate 0.5mL, and Ascorbic Acid 1mL) were added. The whole experimental mixture was incubated at 50˚C for 30 minutes. Spectrophotometer analysis was performed to check enzyme activity at 540 nm optical density [28]. The unit of phytase activity is defined under assay conditions as the amount of phytase that releases 1 µmol of inorganic phosphorus per mL of culture filtrate under assay condition per minute [29]. The OD was put in the regression equation to get the enzyme activity. The regression equation is as follows

$$y = 49.06x - 0.0583 \qquad (1)$$

Here y comes out as enzymatic activity in mg/mL and x is the optical density which was obtained from spectrophotometer analysis [30].

Enzyme activity converted from mg/mL to µg/mL/min by using the following formula:

$$\frac{\frac{\mu g}{mL}}{minute} = \frac{Activity\ (\frac{mg}{mL}) \times 1000}{volume\ used\ in\ activity\ assay \times assay\ time} \qquad (2)$$

To make values in U/mL following equation was applied to enzyme activity in µg/ml/min:

$$\frac{\frac{\mu mol}{mL}}{min}(U) = \frac{activity\ in\ \frac{\frac{\mu g}{mL}}{minute}}{Molar\ Mass\ of\ \mathrm{KH2PO4}} \qquad (3)$$

Statistical Analysis

Three replicates were run under each treatment. The means and standard errors of means were calculated for each treatment. Furthermore, Analysis of Variance (ANOVA) and regression techniques were used to see the significant difference among varying levels of each parameter; it also predicted that how the enzymatic activity depended upon varying levels of different parameters [31].

Molecular Identification

The pure form of grown fungal strain (maximum phytase producing) were identified through molecular screening method i.e., DNA sequencing. The fungal strain was sent to the experts (Macrogen) for sequencing. The genes of isolates were sequenced using primer (5′-3′) FP-(GTA GTCATATGCTTGTCTC, RP-TCCGCAGGTTCACCTACGGA. Sequence assemblage was performed through software Bioedit. The sequence result was identified on the basis of percentage (%) similarity; it was compared with known sequences on the online server, National Center for Biotechnology Information in the Genbank, by using the Basic Local Alignment Search Tool (BLAST) with a search set (18S ribosomal RNA sequences from fungi type and reference material) and was optimized for highly similar sequences/ megaBLAST. A phylogenetic tree was made in robust tree software by using the neighbor-joining method with Bootstrap analysis to obtain evolutionary relationships of taxa.

III. RESULTS AND DISCUSSION

Biomass and Organism Screening through OVAT Methodology

The enzyme assay was performed, at optimized physical conditions, with this extracted filtrate to find out the maximum production. The biomass and the fungal strain were screened out with classical method or OVAT (one variable at one time) on the basis of high phytase production under solid state fermentation [32]. The highly phytase producing fungus was come out to be BT6 and the substrate was wheat bran; these were picked up for further experimentation. The maximum production came out to be 448 IU/mL by using wheat bran and BT6 strain. Other substrates as wheat husk, rice husk, rice polish, chickpea leaves and pods and grass show phytase production in descending order. The physico-chemical parameters affect the phytase productivity significantly in fermentation process [33] that's why the present study was designed to optimize physico-chemical parameters to achieve higher level of phytase production by using OVAT, Taguchi and RSM methodologies. Wheat bran has been reported previously as the most effective substrate for phytase production [34]. He reported an investigation with wheat bran as the highest phytase producing biomass and strain as *Aspergillus niger* with an activity 208.30 ± 0.22 U/gds in solid state fermentation conditions. Another study reported the production of xylanase (89.39 IU/mL) by indigenous fungal strain of *Aspergillus niger* , with the use of corncob as a substrate [35].

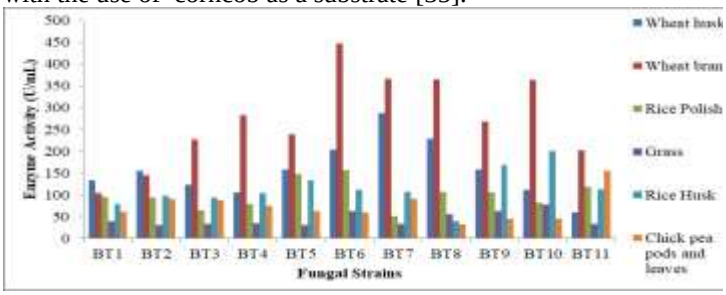


Figure-3: Screening of biomass and fungal strain on the basis of high phytase production

Optimization of Physico-chemical Parameters through Taguchi Method

The Taguchi method of DOE evaluates the main and interface effects of the factors individually and in groups. Out of 18 trials, the trial number 5 shows the maximum phytase production 155 IU/mL with temperature 30 ºC, pH 5, inoculum 4mL, time period 7 days, substrate concentration 3 g, and nitrogen source (ammonium sulfate) 0.1 mg. All of these parameters were variant in three figures or numbers which are demonstrated in the following table. Taguchi method is a powerful and efficient design that operates over a variety of conditions optimally and consistently in which the key concern is parameter design. For the quality characteristics analysis Taguchi used S/N i.e., signal to noise ratio instead of standard deviation. This relates the mean with standard deviation in the way that if standard deviation decreases, mean also decreases and vice versa [36]. B. Singh (2017) reported an investigation on phytase production by *Aspergillus oryzae*, with the use of Taguchi design SBS50. designed Taguchi method for screening of physico-chemical parameters (temperature, pH, incubation time, feed ingredients) and their interaction for in-vitro intrinsic phytase activity of feed (rye, wheat, and barley) in poultry science [37]. They made a comparative study; Taguchi method was reported as sufficient and resource saving method which is a substitute of full factorial design. They reported an investigation using Taguchi approach for the improved production of enzyme (tannase) by *Klebsiella pneumoniae* using Indian gooseberry leaves [38].

Table-1: Response Table for Means

Level	Temperature	pH	Inoculum	Time period	Moisture content	Substrate concentration	N source
1	76.97	37.84	65.62	27.21	47.94	67.67	68.06
2	61.14	79.26	68.06	65.01	71.68	56.81	63.34
3	41.75	62.77	46.18	87.64	60.24	55.38	48.47
Delta	35.22	41.42	21.88	60.44	23.74	12.30	19.59
Rank	3	2	5	1	4	7	6

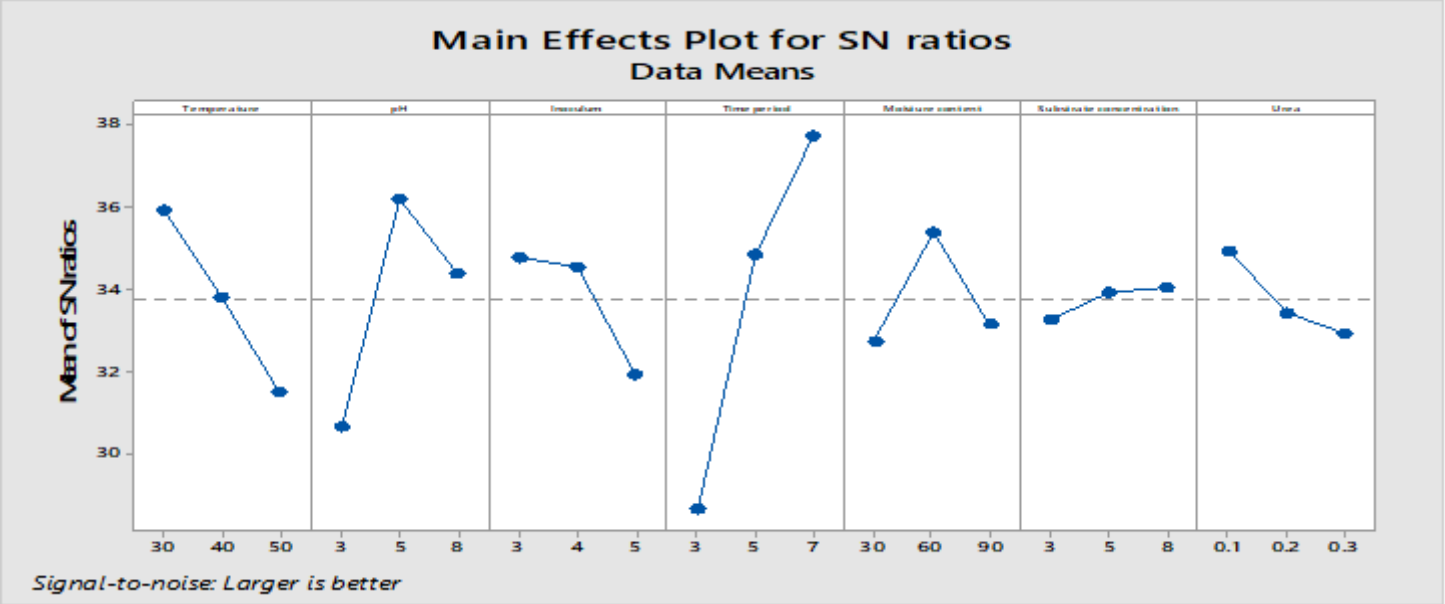


Figure-4: Plot between means of signal to noise ratios (y-axis) for overall phytase production (blue lines and points) and physico-chemical parameters (x-axis).

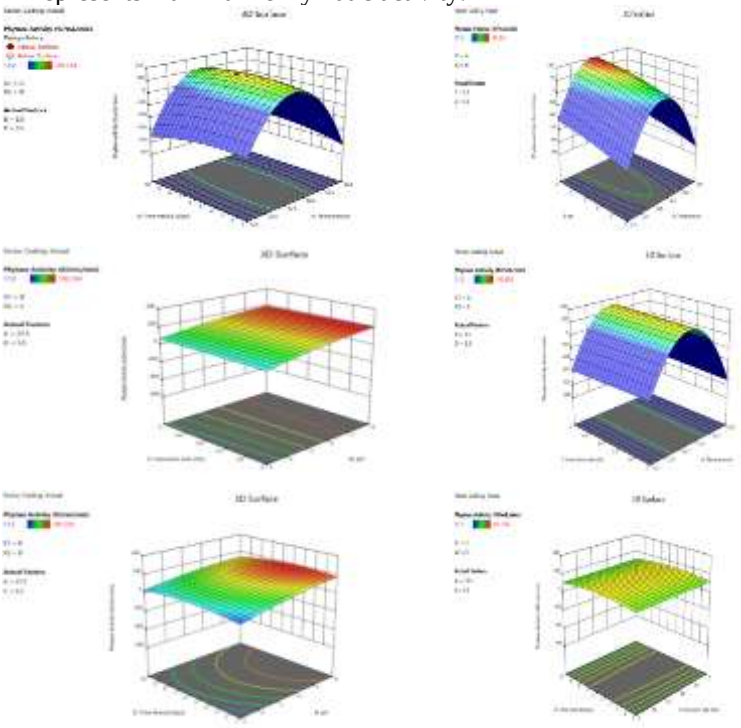
Time period has highest effect on signal to noise ratio and substrate concentration has least effect, while temperature, pH, incubation time, moisture content and urea has moderate effect.

Optimization of Physico-chemical Parameters through RSM

Response Surface Methodology Phase I

The 1st phase of Response Surface Methodology (RSM) was designed under Central Composite Design (CCD) for four physical parameters (temperature, pH, inoculum size and time period) those were put to be optimized for maximum phytase production. The trial no. 22 gives the maximum phytase production i.e., 192 IU/mL at temperature 50 ºC, pH 8, inoculum size 8 and time period 1 day. The results revealed the wide-ranging variations in phytase production among trials, which could be a result of combination of different cultural conditions and their interactive effects. Previous studies have already confirmed an effective economic enhanced phytase production using the package of Minitab® software for medium optimization [39]. Kumari & Bansal, 2021 achieved 2.5-fold enhanced phytase production from *Aspergillus niger* NT7 following a statistical methodology (RSM) in solid state fermentation [40]. Primarily, they identified critical parameters using OVAT strategy; further, modeling and optimization were performed through RSM with 5g what bran, 2 % mannitol, 0.5 % ammonium sulfate, pH 4.3 at 35°C after 5 days of fermentation. Ibarruri, Cebrián, and Hernández (2019) optimized SSF conditions through RSM to maximize the value of fermented brewer's spent grain from two *Rhizopus sp* [41]. SSF has been used in industry over half century ago and still applied for the low cost production of enzymes, antibiotics, secondary metabolites, biofuels, and vitamins with less labor [33]. With optimized growth, system conditions and resolving issues related to scaling up production SSF can be used efficiently to yield a variety of human needs without producing any environmental pollution [42].

Figure-5: RSM Phase-I. 3-D Response Surface Graphs, exhibiting effect of interaction between physico-chemical parameters on Phytase Production (IU/mL) in Solid State Fermentation. Blue color exhibits lowest activity and red color represents maximum enzymatic activity.



The 3-D graphs indicate interaction between two different parameters and phytase activity. Phytase activity was plotted on y-axis, one parameter is on x-axis and the other one is on z-axis. Optimum phytase production is indicated by peak and shape of the 3D graph for the two interactive parameters [43].

Table-2: RSM Phase-I, Analysis of Variance (ANOVA) for phytase production using Central Composite Design

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	14	107435	7673.9	247.54	0.000
Linear	4	45257	11314.3	364.97	0.000
Temperature	1	812	811.6	26.18	0.000
pH	1	42915	42915.0	1384.32	0.382
Inoculum size	1	25	25.2	0.81	0.000
Time Period		273	273.5	8.82	0.010
Square	4	18060	4515.0	145.64	0.000
Temperature * Temperature	1	9321	9320.6	300.66	0.000
pH*pH	1	8289	8289.4	267.39	0.002
Inoculum size*Inoculum size	1	2	1.5	0.05	0.828
Time period * Time Period		1137	1137	36.68	0.000
2-Way Interaction	6	2138	365.4	11.50	0.000
Temperature*pH	1	5	5.5	0.18	0.680
Temperature*Inoculum size	1	76	76.3	2.68	0.138
Temperature* Time period	1	238	238.3	7.69	0.014
pH * Inoculum size		1033	1032.9	33.32	0.000
pH * Time Period		758	758.4	24.46	0.000
Inoculum * Time Period		2	2.3	0.07	0.791
Error	15	465	31.0		
Lack-of-Fit	10	465	46.5	*	*
Pure Error	5	0	0.0		
Total	29	107900			

SS= sum of squares, DF= Degree of Freedom, MS= means sum of squares

ANOVA of RSM Phase-I

P value for the model was 0.000, represents model's significance which means that the phytase production is significantly influenced by the model parameters. The coefficient of determination (R-sq=97.09) and correlation coefficient (adjusted R-sq=99.17) for RSM phase-I was more than 97% which means that the models fitted better with observations and it is able to predict the optimization of parameters in the model [44]. The linear interactions appeared ($p<0.05$) except for pH in linear and for inoculum size in square interaction.

Response Surface Methodology Phase-II

In RSM Phase-II, physico-chemical parameters (moisture content, substrate concentration and nitrogen source) were optimized with the previously optimized significant parameters for maximum phytase production [45]. In the Phase-II, the 60% moisture content, 10g substrate concentration and 0.2 mg ammonium sulfate supported maximum phytase production which was more than RSM Phase-I i.e., 245 IU/mL. In a study [46] , RSM potentially enhanced phytase production from *Aspergillus ficuum* by parameter's optimization. Another study [47] testified that RSM increased about 3 fold phytase production in SSF by *Aspergillus niger* NCIM 563, by utilizing wheat bran. *A. ficuum* was grown on wheat straw for enhanced phytase production with two-step experimental design (OVAT and RSM). Three factors were picked up as most effective factors trough OVAT strategy and were put into RSM which resulted in an upsurge phytase production 16.46±0.56U/gds [48]. Recently, *Aspergillus terreus* fsp-4 producing thermo-stable phytase was reported which exhibited highest activity at 40 ºC [49].

Figure-6: RSM Phase-II. 3-D Response Surface Graphs are exhibiting effect of interactions among physico-chemical parameters on phytase production (IU/mL) in Solid State Fermentation. Blue color exhibit lowest activity and red color represents maximum enzymatic activity.

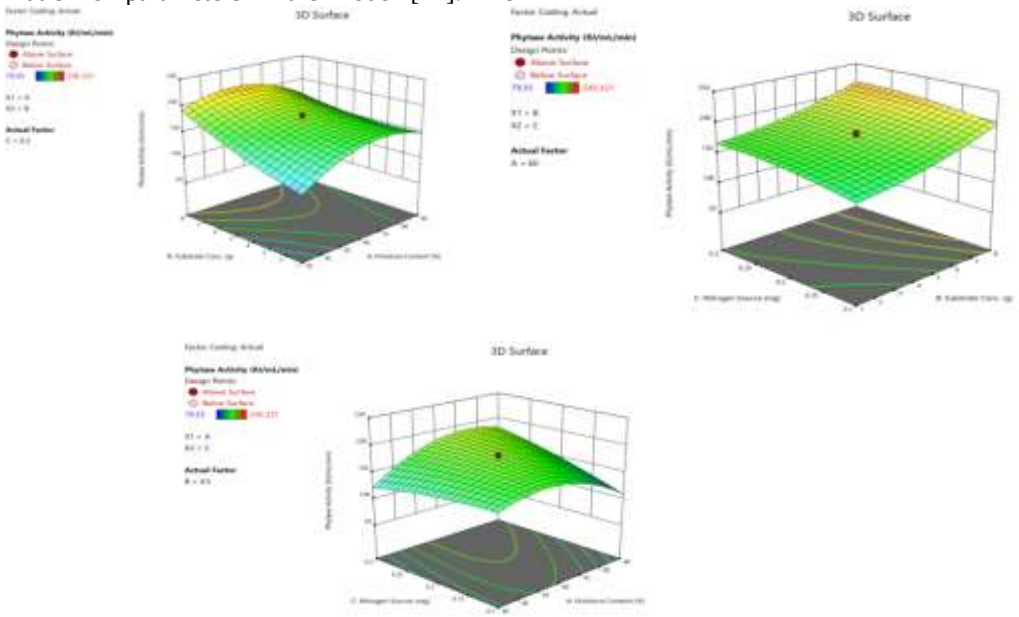


Table-3: Analysis of Variance (ANOVA) for RSM-II

Source	DF	Adj SS	Adj MS	F-V
Model	9	34501.8	3833.5	140
Linear	3	9122.4	3040.8	111
Moisture content	1	364.5	364.5	13.
Substrate concentration	1	7005.9	7005.9	256
Nitrogen source	1	1752.1	1752.1	64.
Square	3	14406.2	4802.1	175
M.C*M.C	1	14095.7	14095.7	515
S.C*S.C	1	498.1	498.1	18.
Nitrogen source*Nitrogen source	1	458.6	458.6	16.
2-Way Interaction	3	6951.6	2317.2	84.
M.C*S.C	1	2109.5	2109.5	77.
M.C*N.S	1	4833.4	4833.4	176
S.C*N.S	1	8.7	8.7	0.:
Error	10	273.7	27.4	
Lack-of-Fit	5	186.1	37.2	2.:
Pure Error	5	87.6	17.5	

SS= sum of squares, DF= Degree of Freedom, MS= means sum of squares

ANOVA of RSM Phase-II

The experimental data was fitted to a quadratic equation with phytase activity 245 IU/mL, as a function of the concentration of substrate, pH, moisture content, time period, inoculum size, nitrogen source, and temperature. Both models represented the analysis of variance [50]. P value for the RSM Phase-II was also 0.000; it represents model's significance which means that the phytase production is significantly influenced by the model parameters. The test of lack of fit was insignificant (value = 0.214) representing good fitness of the model with the experimental data. The coefficient of determination (R-sq=95.27) and correlation coefficient (adjusted R-sq=98.50) for RSM phase-II was more than 97% which means that the models fitted better with observations. The 1-Way and the 2-Way interactions appeared to be effective as well (P<0.05).

Molecular Identification

The 18S rRNA sequencing was carried out using primer (5′-3′) FP - GCCTGTCTCAAAGATTAAGCC, RP-CACCTACGGAGACTTTGTTAC. Sequence assemblage and alignment was performed through software Bioedit. After coting's assembling, the sequencing results were identified on the base of percentage similarity. These sequencing results were also likened with already known sequences in the Genbank, by using the BLAST of the NCBI server, to get homologues for the phylogenetic analysis. The identified sequence was submitted to bankit-NCBI (https://www.ncbi.nlm.nih.gov/BankIt) submission tool database under Accession No. SUB11831568 H220323-R03_E07_06_NS1.ab1 OP028905. A Phylogenetic tree was obtained with an online server named phylogeny.fr with one click mode which constructs the phylogenetic tree there to find out most close evolutionary relationship of taxa which comes out with *Aspergillus terreus* (Colmán, Alves, da Silva, & Barreto, 2018; Dereeper et al., 2008; Matwa & Sundaramoorthy). Many investigations were reported; however, *Aspergillus* was the best producer of phytase. Various species of *Aspergillus* were screened out with 18S rRNA gene analysis with phylogenetic affiliations [51]. The gene sequence for *Aspergillus terreus* is given below:

GATCCTTCATGTCTAGTATAAGCACTTTATACTGTGAAA
CTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGAT
AGTACCTTACTACATGGATACCTGTGGTAATTCTAGAG
CTAATACATGCTAAAAACCTCGACTTCGGAAGGGGTGT
ATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCT
TGGTGATTCATAATAACTTAACGAATCGCATGGCCTTG
CGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACT
TTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGG
GTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGC
CTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGA
CAATAAATACTGATACGGGGCTCTTTTGGGTCTCGTAA
TTGGAATGAGTACAATCTAAATCCCTTAACGAGGAACA
ATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATT
CCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAA
AAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCCGGTC
CGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTCCTT
CTGGGGAACCTCATGGCCTTCACTGGCTGTGGGGGGAA
CCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAG
CAGGCCTTTGCTCGAATACATTAGCATGGAATAATAGA
ATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCG
CCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTAT
TCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGA
CTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCAT
TAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATC
AGATACCGTCGTAGTCTTAACCATAAACTATGCCGACT
AGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCA
CCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGGA
GTATGGTCC

18S ribosomal RNA gene, partial sequence

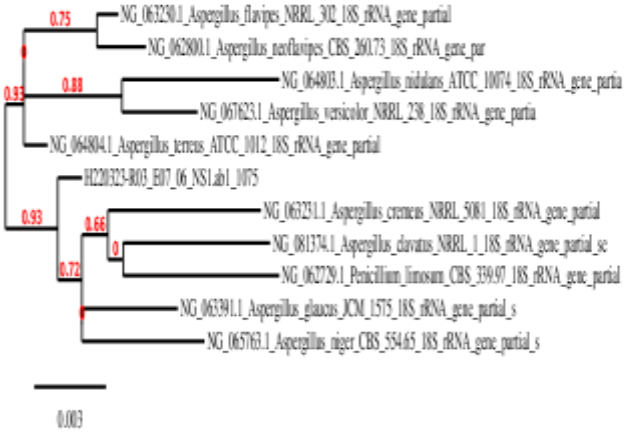


Figure-7: Phylogenetic and evolutionary relationships taxa of *Aspergillus terreus*

Based on molecular characterization, the isolate BT6 was identified as *Aspergillus terreus* (Accession No.OP028905), with 99.44% similar to *A. terreus* (Accession No.NG_064804) ITS sequence. Phylogenetic tree is showing the relationship of phytase producing fungus *Aspergillus terreus*, ITS sequences with reference sequences obtained through BLAST analysis. The sequence alignment was performed using the Bioedit and tree was constructed using Neighbor joining with algorithm using online server [52]. The tree was rooted using H220323-R03_E07_NS1.ab1_1075 *Aspergillus terreus*, a phytase producing fungus, as the out group. Matwa and Sundaramoorthy (2020) reported phytase production from *Aspergillus terreus*.

IV. CONCLUSIONS

Fungal sample has been isolated successfully and identified on the molecular basis as *Aspergillus terreus* (OP028905). Wheat bran, as an agricultural waste was utilized as a substrate for

phytase production in SSF. The organism appeared to be a potential candidate for enhanced phytase production in SSF and physico-chemical parameters were successfully optimized by using Response Surface Methodology (Central Composite Design) for enhanced phytase production. RSM phase-I exhibited as maximum activity **192 IU/mL**; phase-II exhibited **245 IU/mL**, while Taguchi method showed **155 IU/mL**. RSM exhibited 36% more phytase production than Taguchi method. Conclusively, the optimized conditions for enhanced phytase production were temperature 50˚C, pH 8.0, inoculum size 2mL, time period 1 day, moisture content 60%, substrate concentration 10g, and nitrogen source (0.2mg). This investigation suggested very simple methodology in economical production of phytase.

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