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Isolation, Identification and Nutritional Optimization of Phytase Producers from North Gujarat using Ovat Approach

Madhvi[a], Hardik Shah[b*]

[a]*Assistant Professor Mehsana Urban Institute of Science, Ganpat University, Mehsana,384012, India*
[*b]*Associate Professor Mehsana Urban Institute of Science, Ganpat University, Mehsana,384012, India*

Abstract

Soil samples collected from twelve distinct locations across the North Gujarat region were systematically screened to isolate efficient phytase-producing bacteria. A total of 42 isolates exhibiting positive phytate hydrolysis were obtained and characterized based on their morphological features. Among these, three isolates DSS1, CDS1 and BGS3 demonstrated superior hydrolytic capabilities and were therefore selected for detailed nutritional optimization using a one-variable-at-a-time (OVAT) approach. Nutritional optimization revealed isolate-specific preferences for substrate components. Wheat bran and yeast extract supported the highest phytase yields in DSS1 and CDS1, whereas rice bran combined with ammonium sulphate was optimal for BGS3. Metal ion supplementation further enhanced enzyme production, with Ca^{2+} significantly improving phytase activity in DSS1 and BGS3, while Mg^{2+} served as the most effective enhancer for CDS1. Overall, this study highlights the diversity of indigenous phytase-producing bacteria in North Gujarat soils and underscores the importance of tailored nutritional optimization to maximize enzyme production for potential biotechnological and feed industry applications.

Keywords: Nutritional parameter, Optimization, Phytase, north Gujarat.

1. Introduction

Phytate (myo-inositol hexakisphosphate) is a major anti-nutritional factor in plant-based feed ingredients such as cereals and legumes, particularly affecting monogastric animals, which lack sufficient endogenous phytase to degrade it efficiently. As a result, a significant fraction of phosphorus in these diets remains bound in phytate and is excreted, contributing to environmental phosphorus loading and increasing feed costs (Urbano et al., 2000). In addition, phytate strongly chelates essential mineral cations (e.g., Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}) and binds proteins, further reducing their bioavailability (Zhou & Erdman, 1995; Webb, 2013). To mitigate these problems, phytases (myo-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.26 / EC 3.1.3.8) catalyze the stepwise dephosphorylation of phytate into lower inositol phosphates and inorganic phosphate. These enzymes belong to different classes, including purple acid phosphatases (PAP), β-propeller phytases (βPP), and histidine acid phosphatases (HAP), and have become important feed additives to improve nutrient utilization (Mullaney & Ullah, 2003). In recent years, microbial phytases, especially those derived from bacteria, have gained increasing prominence than their fungal counterparts in the food and feed industries due to their favourable catalytic properties such as high substrate specificity, proteolytic resistance, and stability under diverse pH and thermal conditions (Jatuwong et al., 2020; de Oliveira Ornela & Souza Guimarães, 2019; Jorquera et al., 2018). A recent review highlights how engineered microbial phytases enhance mineral bioavailability, improve phosphorus utilization, and reduce phosphorus excretion, all of which contribute to more sustainable animal nutrition (Joudaki et al., 2023). The commercial and environmental relevance of phytase use is also reflected in recent market data: the feed phytase market is expanding rapidly, driven by its role in reducing inorganic phosphate supplementation and minimizing phosphorus pollution (Business reserach Insights, 2025-2030). In livestock systems, bacterial phytases are being used to improve nutrient digestibility and reduce emissions. For instance, supplementing a biosynthetic bacterial 6-phytase at 2,000 to 5,000 FTU/kg in the diets of lactating dairy cows significantly improved phosphorus and protein digestibility while lowering fecal phosphorus excretion

*Corresponding Author
E-mail address: hss02@ganpatuniversity.ac.in

(Dersjant-Li et al., 2023). Similarly, in aquaculture, microbial phytases have been shown to enhance phosphorus retention, growth, and nutrient utilization in species such as Atlantic salmon (Terrey et al., 2024). Beyond phosphorus, phytase supplementation also improves the utilization of other nutrients. In poultry, studies have shown that phytase not only releases phosphorus, but also increases amino acid, mineral, and energy digestibility, and reduces gut irritation benefits that extend beyond its traditional role. Moreover, recent advances in probiotic and recombinant phytase production open new frontiers: for example, genetically engineered Lactococcus lactis strains expressing phytase have been proposed as probiotic feed additives to deliver the enzyme in the gut (Bandari et al., 2024; Prajapati & Shah, 2022). Given this growing interest and the increasingly sophisticated uses of phytase, there is a critical need to explore novel phytase-producing strains and optimize their production. In this context, the present study focuses on the isolation of phytase-producing bacteria from diverse environments in North Gujarat and the systematic optimization of nutritional parameters to enhance their phytase yield for potential application in feed and food industries.

2. MATERIAL AND METHODS

Soil samples were collected from 12 distinct locations across the North Gujarat region, each representing ecologically diverse habitats to increase the likelihood of isolating phytase-producing microorganisms. Details of sampling sites, sample sources, number of isolates obtained, and isolate designations are provided in Table 1. Wheat bran extract agar, previously standardized in our laboratory (Madhvi & Shah, 2024), was used for preliminary screening of phytase-producing microorganisms. Soil samples were serially diluted and spread-plated onto the screening medium. After incubation, isolates exhibiting clear zones of hydrolysis around the colonies were considered positive for extracellular phytase activity (Sreedevi & Reddy, 2012).

Hydrolysis efficiency (HE) was calculated using the formula:

$$\text{Hydrolysis efficiency (in \%)} = \frac{[\text{Zone Diameter(Z)} - \text{Colony Diameter(C)}]}{\text{Colony Diameter (C)}} * 100$$

Isolates displaying distinct hydrolysis zones were subjected to morphological characterization. Based on both qualitative (zone size, hydrolysis efficiency) and quantitative phytase assays, the three most promising isolates DSS1, CDS1, and BGS3 were selected for further optimization studies.

Optimization of nutritional factors on phytase production

Wheat bran extract broth was used as the basal fermentation medium, containing all standard components, with individual medium constituents varied to determine optimal conditions. The study encompassed the optimization of carbon sources, nitrogen sources, and metal ions. Phytase production under each condition was quantified using a previously reported assay method (Madhvi & Shah, 2024). To assess the effect of carbon sources on phytase production, various agricultural residues—wheat bran, rice bran, cottonseed oil cake, corn meal, and groundnut meal—were evaluated. Each fermentation flask was supplemented with 2% (w/v) of an individual substrate or a 1:1 combination of two substrates. Cultures were incubated for 72 hours, after which phytase activity was quantified using the standard enzymatic assay. For nitrogen source optimization, the fermentation medium was supplemented with either organic or inorganic nitrogen sources at 1% (w/v). Inorganic nitrogen sources included ammonium nitrate, sodium nitrate, and ammonium sulphate, while organic nitrogen sources included peptone, urea, and yeast extract. To evaluate the influence of metal ions on phytase production, the medium was supplemented with individual metal salts at a final concentration of 10 mM. The monovalent and divalent cations assessed included $CaCl_2$, $CoCl_2$, $FeSO_4$, KCl, NaCl, $MgSO_4$, and $MnSO_4$. For each parameter examined, enzymatic assays were meticulously conducted following the methodology outlined above, ensuring rigorous scientific precision. Furthermore, to ascertain the robustness of the findings, all experiments were conscientiously repeated in triplicate (n=3), thereby enhancing the statistical reliability of the observations. The average values along with their standard errors are shown in graph. A one-way ANOVA and Post-hoc tukey test in R studio were performed to determine significance of every parameter in SmF for phytase production. The significance was estimated at 95% confidence level and the P-value at 0.05, values less than 0.05 are supposed to show a significant effect on phytase production.

3. RESULTS AND DISCUSSION

3.1 Isolation and morphological identification of phytase producer

In the present study, soil samples were collected from 12 distinct locations and screened for phytase-producing bacteria based on the zone of hydrolysis. The site details, geographical locations, sample sources, number of positive isolates, and the designations assigned to each isolate are summarized in Table 1. A total of 42 isolates exhibiting measurable hydrolysis zones were identified as potential phytase producers. Their colony diameter, zone of clearance, and hydrolysis efficiency are presented in Table 2. All positive isolates were further examined for morphological characteristics, with the results summarized in Table 3. Based on qualitative plate assays (Table 2) and quantitative phytase activity reported previously , isolates DSS1, CDS1, and BGS3 were selected for detailed nutritional optimization studies (Madhvi & Shah, 2024).

Table 1: Details of site, location and source for sample collection

S. no	Sample Code	Site	Location	Source	No. of phytase positive isolates identified	Designation
1	ADC	Himmatnagar	23.4547° N, 72.5258° E	Soil from agricultural area near Aabad Dairy	4	ADC1 to ADC4
2	CDSAS	Patan	23.8500° N, 72.1210° E	Dairy Farm area soil	4	CDSAS1 to CDSAS4
3	ASC	Cheradu	23.4537° N, 72.5308° E	Maize Field Soil Cheradu	4	ASC1 to ASC4
4	DSS	Sanganpur	26.8192° N, 75.7660° E	Dump site Sanganpur	3	DSS1 to DSS 3
5	CDSS	Sanganpur	23.5114829 °N, 72.4517037 °E	Cattle Dung Shed Sanganpur	3	CDSS1 to CDSS3
6	MFS	Ganpat University	23.4471° N, 72.4983° E	Microforest region	4	MF1 to MF4
7	CDBFS	Meu	23.5006° N, 72.5131° E	Cattle Dung Bajri field Soil Meu	4	CDBFS1 to CDBFS 4
8	CDS	Kherva	23.5442° N, 72.4421° E	Cattle Dung Shed, Kherva	2	CDS1 to CDS2
9	LGS	Mansa	29.9995° N, 75.3937° E	Lemon Garden Soil	2	LGS 1 to LGS 2
10	OGS	Gozariya	23.4792° N, 72.5648° E	Soil of Biofertilizer Company, Gozaria	3	OGS 1 to OGS3
11	BGS	Gozariya	23.4997° N, 72.5710° E	Garden Soil, Gozariya	5	BGS1 to BGS5
12	MP	Mehsana	23.5984° N, 72.4087° E	Dump site at seasonal river	4	MP1 to MP4

Table 2: Hydrolysis efficiency of phytase producing isolates on WBEAM plates

S.no.	Isolates	Zone Diameter (in mm)	Colony Diameter (in mm)	Hydrolysis Efficiency (%)
1	ADC1	20	11	81.82
2	ADC2	12	7	71.42
3	ADC3	19	11	72.73
4	ADC4	6	4	50.00
5	ASC1	20	8	150.00
6	ASC2	8	5	60.00
7	ASC3	12	8	50.00
8	ASC4	11	7	57.14
9	BGS1	10	5	100.00
10	BGS2	22	15	46.67
11	**BGS3**	**40**	**13**	**207.69**
12	BGS4	10	4	150.00
13	BGS5	12	9	33.33
14	CDBFS1	20	14	42.86
15	CDBFS2	9	5	80.00
16	CDBFS3	8	5	60.00
17	CDBFS4	13	8	62.50

S.no.	Isolates	Zone Diameter (in mm)	Colony Diameter (in mm)	Hydrolysis Efficiency (%)
18	**CDS1**	**30**	**11**	**172.73**
19	CDS2	18	10	80.00
20	CDSAS1	15	7	114.29
21	CDSAS2	13	9	44.44
22	CDSAS3	16	9	77.78
23	CDSAS4	14	8	75.00
24	CDSS1	28	11	154.55
25	CDSS2	15	7	114.29
26	CDSS3	10	6	66.67
27	**DSS1**	**42**	**14**	**200.00**
28	DSS2	14	8	75.00
29	DSS3	9	6	50.00
30	LGS1	13	6	116.67
31	LGS2	11	8	37.50
32	MF1	13	8	62.50
33	MF2	9	5	80.00
34	MF3	11	6	83.33
35	MF4	10	4	150.00
36	MP1	10	5	100.00
37	MP2	17	10	70.00
38	MP3	16	9	77.78
39	MP4	10	5	100.00
40	OGS1	10	5	100.00
41	OGS2	9	4	125.00
42	OGS3	14	9	55.56

Table 2: Hydrolysis efficiency of phytase producing isolates on WBEAM plates

Isolates	Size	Shape	Margin	Surface	Consistency/ Texture	Elevation	Opacity	Pigmentation	Gram Reaction
ADC1	Big	Irregular	Undulated	Rough	Dry	Flat	Opaque	Nil	Positive
ADC2	Big	Curled	Irregular	Rough	Dry	Flat	Opaque	Nil	Negative
ADC3	Big	Filamentous	Irregular	Rough	Dry	Flat	Opaque	Nil	Positive
ADC4	Small	Round	Entire	Smooth	Moist	Raised	Translucent	Nil	Positive
ASC1	Big	Irregular	Undulated	Dull	Dry	Flat	Translucent	Redish	Negative
ASC2	Small	Irregular	Undulated	Dull	Dry	Flat	Opaque	Nil	Negative
ASC3	Big	Round	Entire	Rough	Mucoid	Raised	Translucent	Nil	Positive
ASC4	Big	Irregular	Undulated	Rough	Mucoid	Flat	Translucent	Pinkish	Negative
BGS1	Small	Round	Entire	Smooth	Dry	Flat	Opaque	Creamy	Positive
BGS2	Big	Round	Entire	Glistening	Sticky	Raised	Transparent	Nil	Positive
BGS3	Small	Round	Entire	Smooth	Wet and soaked	Elevated	Translucent	Nil	Positive
BGS4	Small	Round	Entire	Smooth	Butyrous	Raised	Transluscent	Nil	Positive
BGS5	Big	Irregular	Undulated	Smooth	Sticky	Flat	Transparent	Nil	Negative

Isolates	Size	Shape	Margin	Surface	Consistency/ Texture	Elevation	Opacity	Pigmentation	Gram Reaction
CDBFS1	Big	Round	Round	Rough	Dry	Flat	Opaque	Nil	Positive
CDBFS2	Small	Irregular	Undulated	Rough	Dry	Flat	Opaque	Nil	Negative
CDBFS3	Small	Irregular	Undulated	Dull	Dry	Flat	Translucent	Nil	Positive
CDBFS4	Big	Round	Entire	Smooth	Sticky	Raised	Translucent	Nil	Negative
CDS1	Big	Irregular	Undulated	Rough	Wet and soaked	Flat	Translucent	Pink	Positive
CDS2	Big	Irregular	Irregular	Dull	Dry	Convex	Opaque	Nil	Positive
CDSAS1	Big	Round	Entire	Dull	Dry	Flat	Opaque	Nil	Positive
CDSAS2	Big	Irregular	Irregular	Smooth	Dry	Convex	Transparent	Nil	Negative
CDSAS3	Big	Irregular	Irregular	Rough	Dry	Flat	Opaque	Nil	Positive
CDSAS4	Big	Filamentous	Irregular	Glistening	Mucoid	Raised	Transparent	Nil	Positive
CDSS1	Big	Curled	Entire	Smooth	Sticky	Raised	Transparent	Yellowish	Negative
CDSS2	Big	Curled	Entire	Smooth	Sticky	Raised	Transparent	Nil	Negative
CDSS3	Small	Round	Entire	Smooth	Sticky	Flat	Translucent	Nil	Positive
DSS1	Small	Round	Entire	Smooth	Moist	Flat	Translucent and white	No	Positive
DSS2	Big	Round	Entire	Smooth	Moist	Raised	Transparent	Nil	Positive
DSS3	Small	Irregular	Irregular	Rough	Dry	Flat	Transparent	Nil	Positive
LGS1	Small	Round	Entire	Rough	Dry	Flat	Transluscent	Creamy	Positive
LGS2	Big	Irregular	Undulated	Rough	Dry	Flat	Opaque	Nil	Positive
MF1	Big	Filamentous	Irregular	Rough	Dry	Flat	Opaque	Nil	Negative
MF2	Small	Filamentous	Irregular	Rough	Dry	Raised	Opaque	Nil	Positive
MF3	Small	Round	Entire	Smooth	Wet and soaked	Convex	Transparent	Nil	Positive
MF4	Small	Irregular	Irregular	Smooth	Dry	Flat	Transluscent	Nil	Negative
MP1	Small	Irregular	Irregular	Rough	Dry	Raised	Transluscent	Nil	Positive
MP2	Big	Irregular	Irregular	Rough	Dry	Flat	Opaque	Nil	Negative
MP3	Big	Round	Entire	Rough	Sticky	Raised	Transluscent	Nil	Positive
MP4	Small	Round	Undulated	Smooth	Moist	Raised	Transparent	Nil	Positive
OGS1	Small	Round	Entire	Rough	Dry	Convex	Transparent	Yellowish	Negative
OGS2	Small	Round	Entire	Smooth	Wet and soaked	Convex	Translucent	Nil	Positive
OGS3	Big	Round	Entire	Smooth	Wet and soaked	Raised	Opaque	Nil	Positive

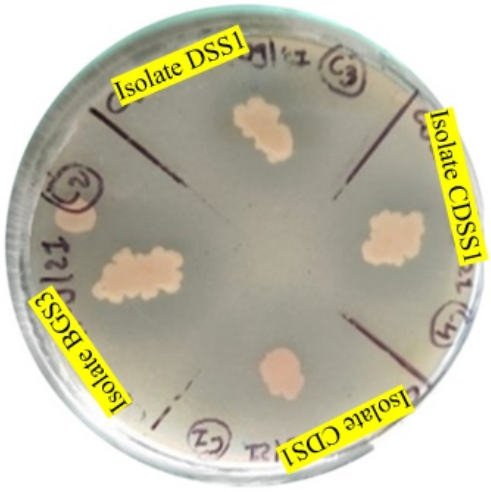

Fig.1: Zone of hydrolysis produced by phytase

3.2 Effect of nutritional parameter on phytase production

Variability in phytase production is well documented both across microbial genera and among species within the same genus under identical cultural conditions. This emphasizes the need for isolate-specific optimization strategies to achieve maximal enzyme yields. In our previous work, physical parameters for phytase production were optimized for isolates DSS1, CDS1, and BGS3. Building upon that foundation, the present study investigates the influence of carbon sources, nitrogen sources, and metal ions on phytase production in these isolates. Agro-industrial by-products are widely recognized as economical and nutrient-rich substrates for enzyme production (Pires et al., 2019). Accordingly, several commonly used substrates including wheat bran, rice bran, groundnut meal, cottonseed oil cake, and maize meal were compared. Wheat and rice brans, known for their high phytate content and suitability for microbial growth, supported robust phytase production (Ravindran

et al., 2018). The current findings align with earlier reports showing that wheat bran promotes maximum phytase yield in many Bacillus isolates, including DSS1 and CDS1. Interestingly, BGS3 displayed its highest enzyme productivity when supplemented with rice bran (Fig. 2) (Shah et al., 2009), consistent with prior observations in Bacillus sp. C43, where wheat bran and rice bran were favorable substrates, individually or in combination (Sreedevi & Reddy, 2012). In Pseudomonas fluorescens wheat bran has been reported to produce maximum phytase production among the various carbon sources and this is due to the presence of high phytate content in aleurone layer of these grain (Tungala et al., 2013). Similar trends have been noted in fungi and bacteria, where wheat bran—alone or blended with substrates such as linseed oil cake or groundnut cake—enhances phytase synthesis (Mussa et al., 2023; Qasim et al., 2017; Rani & Ghosh, 2011). Conversely, studies on Enterobacter aerogens reported rice bran as a superior substrate (Muslim et al., 2018), underscoring species-specific preferences.

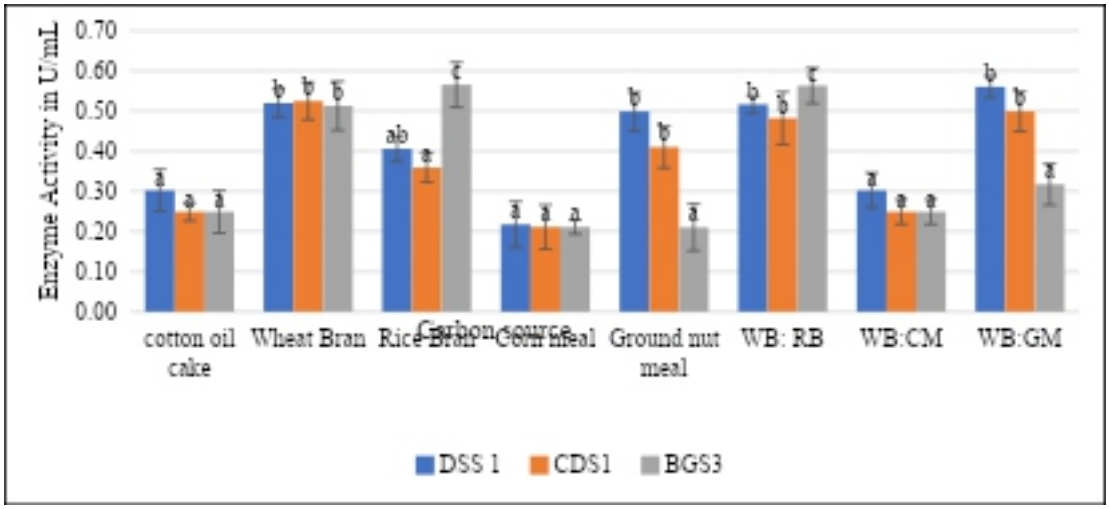

Fig.2: Effect of carbon source on phytase production.
One way ANOVA revealed significant effects of carbon source and strain, as well as a significant interaction between them. (Post-hoc) Tukey's HSD comparisons showed that Rice Bran and WB: RB under the BGS3 strain yielded significantly higher activity.

In the current study, isolate DSS1, CDS1 and BGS3 gives the higher production of phytase in the presence of yeast extract, however the isolate BGS3 gives the higher phytase productivity if ammonium sulphate was used as nitrogen source (Fig.3). Although, for B. altudinis F15 it was reported that organic source of nitrogen in production media results in higher phytase production as compared to inorganic source, and hence found yeast extract as the ideal supplement for phytase production in media (Suliasih et al., 2022), results were further supported by Shanmugam et al, for phytase production by Bacillus subtilis (MD and Kalaichelvan, 2012), Bacillus subtilis 168 (Chen et al., 2015), Bacillus subtilis DR6 (Singh et al., 2013) and other phytase-producing bacteria, which achieved optimal phytase production with the addition of yeast extract (Boukhris et al., 2015).

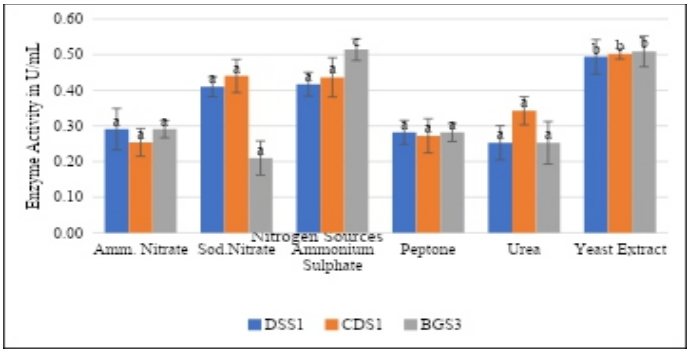

Fig. 3: Effect of nitrogen source on phytase production
Based on one way Anova nitrogen source significantly affects phytase production, strain has no overall significant effect. The interaction effect between nitrogen source and strain is significant, hence the effectiveness of nitrogen sources varies depending on the strain. Tukey's HSD (Post-hoc) analysis revealed BGS3 ammonium sulphate delivers significantly higher phytase activity.

Zailan et al. (2021) isolated four different bacterial strains from a Malaysian hot spring and demonstrated that yeast extract as a nitrogen source yielded higher phytase production than peptone. Similarly, other researchers (e.g., Muslim et al., 2018; Wang et al., 2004) have reported that organic nitrogen sources like yeast extract can enhance phytase production. In contrast, in the current study on Bacillus subtilis BGS3, ammonium sulphate (an inorganic nitrogen source) gave the highest phytase yield. This finding aligns with those of Mussa et al. (2023), who found that Enterobacter asburiae (RS1) and Klebsiella pneumoniae (RS8) showed maximal phytase production in the presence of 1% ammonium sulfate. Similarly, Roy & Ghosh (2014) reported high phytase activity in Klebsiella spp. and Shigella spp. when ammonium sulphate was used. Moreover, Suliasih & Widawati (2022) found that in Enterobacter cloacae, inorganic nitrogen resulted in better phytase production than organic nitrogen sources. Parallel observations have been made in fungi, Aspergillus niger (Suleimenova et al., 2016) and other fungal species (A. fumigatus, A. flavus, Mucor rouxii, Penicillium purourogenum) also show significant enhancement of phytase production under inorganic nitrogen (Sadaf et al., 2022). Additionally, Azeem et al. (2015) found that in A. ficuum, ammonium sulfate was superior to ammonium nitrate for enzyme production. Taken together, these findings suggest that nitrogen form is a key determinant in modulating phytase synthesis, likely because different nitrogen sources influence bacterial (and fungal) metabolism and enzyme-production pathways differently, as also suggested by Muslim et al., (2018). Minerals or metal ions are commonly added to production media not just to maintain ionic balance, but also because they play critical roles in enzyme structure and function. The activation or inhibition of enzyme production by different metal ions underscores their importance, especially for metal-dependent enzymes (Vohra & Satyanarayana, 2003). In our study, B. subtilis BGS3 and DSS1 isolates showed maximal phytase production in the presence of Ca^{2+}, whereas in CDS1, phytase production was enhanced by Mg^{2+} (Fig. 4), suggesting a stimulating effect of these metal ions on enzyme synthesis. Conversely, Fe^{2+} and Co^{2+} ions caused a marked reduction in phytase production, indicating inhibitory roles. These observations are consistent with prior work: for example, Sreedevi & Reddy (2012) found that Ca^{2+} strongly enhances phytase production in Bacillus sp. C43, and Demirkan et al. (2014) similarly reported that $CaCl_2$ was more favorable than $MgSO_4$ or NaCl, while $FeSO_4$ was inhibitory. Underlying these effects, the biochemical dependence of phytase on calcium has been well documented: B. subtilis phytase loses activity upon metal ion removal (e.g., via EDTA), but is reactivated most effectively by Ca^{2+}, with only weak recovery by other divalent ions (Kerovuo et al., 2000). Structurally, crystal-structure studies of Bacillus alkaline (β-propeller) phytases reveal that Ca^{2+} ions are coordinated both in the active site and in binding with the phytate substrate, contributing to substrate recognition, proper folding, and catalytic efficiency (Kerovuo et al., 2000). In particular, reported Ca^{2+} increases the thermostability of such phytases: in one Bacillus phytase (YCJS), the melting temperature (T_m) rose significantly when Ca^{2+} was present, compared to the apo-enzyme (Zeng et al., 2011). On the other hand, inhibitory effects of Fe^{2+} and other metal ions may partly arise from non-specific mechanisms: as reviewed, certain metal ions can precipitate with phytate, reducing the available substrate for the enzyme rather than directly binding to or denaturing the enzyme (Konietzny & Greiner, 2002). Thus, the observed inhibition in our isolates may reflect both direct effects on the enzyme's conformation and indirect effects via substrate–metal interactions.

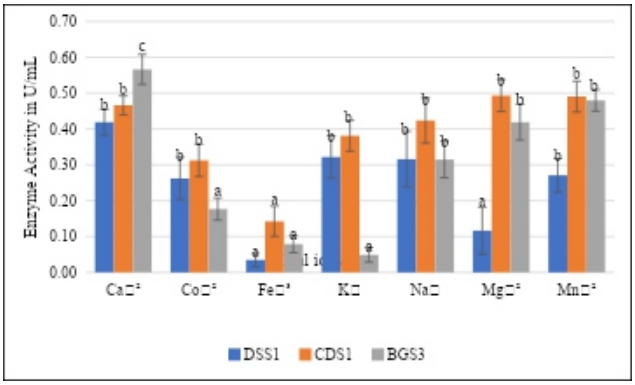

Fig.4: Effect of metal ions on phytase production A one- way ANOVA showed that both metal ion and strain significantly affect activity, with a significant interaction, indicating that the relative performance of metal ions depends on the strain. Tukey's HSD (Post-hoc) analysis revealed Calcium (Ca^{2+}) supports higher activity in the BGS3 strain.

Many prior studies similarly validate the strong positive influence of Ca^{2+}, particularly calcium chloride, on phytase production. For example, Bacillus amyloliquefaciens DS11 requires Ca^{2+} for its phytase activity: its enzyme is uniquely calcium-dependent, hydrolyzing only the calcium-phytate complex, and both unbound Ca^{2+} and phytate can act as competitive inhibitors (Oh et al., 2001). In B. amyloliquefaciens DS11, calcium also plays a structural role: removing it destabilizes the enzyme, while adding Ca^{2+} helps it refold even after heat denaturation (Shim & Oh, 2012). These findings support observations in other Bacillus species (Oh et al., 2001; Shimizu, 1992) that Ca^{2+} significantly enhances phytase production. On the other hand, some studies report that metal ions do not always stimulate phytase activity particularly in fungal systems. For instance, Qasim et al. (2017) found that in Aspergillus tubingensis, the addition of common divalent metal ions did not significantly change phytase activity (Qasim et al., 2017). One plausible explanation for this lack of stimulation is the formation of insoluble metal–phytate

complexes: when metal ions precipitate with phytate, they reduce the availability of free substrate, thereby limiting enzyme action. This mechanism is supported by more recent work showing that metal–phytate complexation, especially with ions like Fe^{2+}, can strongly inhibit phytase hydrolysis by making the substrate less accessible (Sun & Jaisi, 2021). Overall, the differential effects of metal ions in our study highlight that metal ion homeostasis is a critical parameter for optimizing phytase production. Ca^{2+} and Mg^{2+} likely act as cofactors or structural stabilizers in the enzyme, whereas Fe^{2+} and Co^{2+} may disturb either enzyme structure or substrate availability. While many bacterial (especially Bacillus) phytases are strongly activated by Ca^{2+} due to both structural and catalytic roles, fungal phytases may respond differently in some cases showing no enhancement, possibly because substrate availability is compromised by precipitation of metal-phytate complexes. Consequently, based on the various parameters studied under the OVAT approach, isolates giving the best results are compared in Table 4.

Table 4: Maximum phytase produced under different optimized parameters

Phytase act. under optimized cond.	Isolate DSS1	Isolate CDS1	Isolate BGS3
Incubation time	72 hrs (0.52 ± 0.02 U/mL)	72 hrs (0.59 ± 0.02U/mL)	72 hrs (0.62 ± 0.03 U/mL)
Optimum pH	6 (0.59 ± 0.02 U/mL)	6 (0.56 ± 0.02 U/mL)	7 (0.59 ± 0.02 U/mL)
Optimum temp. in °C	40 (0.56 ± 0.02 U/mL)	55 (0.54 ± 0.02 U/mL)	37 (0.58 ± 0.01 U/mL)
Inoculum percent	5% (0.60 ± 0.03 U/mL)	10% (0.62 ± 0.03 U/mL)	5% (0.65 ± 0.03 U/mL)
Carbon source	Wheat bran (0.52 ± 0.02 U/mL)	Wheat bran (0.52 ± 0.02 U/mL)	Rice bran (0.57 ± 0.03 U/mL)
Carbon source in combination	WB:RM (0.52 ± 0.01 U/mL)	WB:GM (0.49 ± 0.02 U/mL	WB: RB (0.56 ± 0.02 U/mL)
Nitrogen source	Yeast Extract (0.49 ± 0.01 U/mL)	Yeast Extract (0.50 ± 0.01 U/mL)	Ammonium sulphate (0.52 ± 0.01 U/mL)
Metal ions	Ca^{2+} (0.42 ± 0.02 U/mL)	Mg^{2+} (0.49 ± 0.02 U/mL)	Ca^{2+} (0.57 ± 0.02 U/mL)

3. CONCLUSION

Phytase is a crucial enzyme with broad applications across human health, animal nutrition, plant growth promotion, and environmental management. Its primary role in hydrolysing phytic acid not only enhances the bioavailability of essential micronutrients such as phosphorus, calcium, zinc, and iron but also mitigates the antinutritional effects associated with phytate-rich diets. In human and animal health, phytase supplementation has been widely recognized for improving mineral absorption, digestive efficiency, and overall nutritional status. In agriculture, phytase-producing microorganisms contribute to plant growth through enhanced phosphorus mobilization in soil, reducing reliance on chemical fertilizers. Additionally, phytase has emerging applications in bioremediation, particularly in mitigating phosphorus pollution caused by agricultural run-off, thus supporting environmental sustainability. Despite its wide-ranging benefits, the production of phytase using purified substrates remains costly. Therefore, employing low-cost, sustainable alternatives such as agricultural residues as carbon and nitrogen sources is essential to make large-scale enzyme production economically viable. Achieving a high enzyme yield requires optimizing both nutritional and environmental conditions for microbial growth. Even slight variations in physical parameters (e.g., temperature, pH, inoculum size) or nutritional inputs (e.g., carbon and nitrogen sources, metal ions, inducers) can significantly influence microbial metabolism and, consequently, phytase biosynthesis. In the present study, optimization experiments revealed that yeast extract and wheat bran served as the most effective nitrogen and carbon sources for isolates DSS1 and CDS1, whereas ammonium sulphate and rice bran supported maximal phytase production in BGS3. Among the tested metal ions, Ca^{2+} acted as a strong stimulator for isolates DSS1 and BGS3, while Mg^{2+} enhanced phytase production in CDS1. Based on comparative analysis among 42 isolates, three potent phytase producers were identified, and their production profiles were thoroughly optimized. Notably, isolate BGS3 exhibited the highest and most consistent phytase yield under optimized conditions (0.57+0.02 U/mL), making it the most promising candidate for subsequent statistical optimization and potential scale-up.

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