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Comparative Evaluation of Submerged and Solid-State Fermentation for Enhanced Phytase Production by *Aspergillus niger* strain RA401

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ABSTRACT
An essential industrial enzyme called phytase (EC 3.1.3.8) is utilized extensively in animal nutrition to increase phosphorus bioavailability and lessen phosphorus pollution in the environment. The synthesis of phytase by *Aspergillus niger* strain RA401 under submerged fermentation (SMF) and solid-state fermentation (SSF) was compared in this work. By altering the incubation period, beginning pH, temperature, carbon and nitrogen supplies, and inoculum size, SMF optimization was done one factor at a time (OFAT). Agro-industrial wastes were used as solid substrates by SSF. Under SSF, wheat bran promoted the highest amount of phytase synthesis. Under ideal circumstances, SSF produced substantially more phytase activity than SMF overall. The results show that SSF is an excellent, economical, and ecologically friendly method for the *Aspergillus niger* strain RA401 to produce phytase.

1. INTRODUCTION:

Myo-inositol hexakisphosphate, or phytic acid, is the main form of phosphorus stored in oilseeds, legumes, and grains. The lack of endogenous phytase causes monogastric animals to poorly digest phytic acid, despite the fact that it is plentiful in plant-based feed ingredients. This results in decreased mineral bioavailability and increased phosphorus excretion into the environment (Singh & Satyanarayana, 2011). Thus, in the livestock and poultry industries, microbial phytases have become essential feed additives.

Due to their capacity for external secretion, acidophilic enzyme characteristics, and adaptability to a variety of fermentation systems, filamentous fungus, especially Aspergillus niger, are regarded as effective phytase producers among microbial sources (Vats & Banerjee, 2004). Fermentation-based techniques are the mainstay of commercial phytase production; the most commonly used methods are solid-state fermentation (SSF) and submerged fermentation (SMF).

The benefits of SMF, which involves microbial growth in liquid media, are easy scale-up and exact control over environmental factors. But it frequently necessitates refined substrates and produces a lot of wastewater (Pandey et al., 2001). SSF, on the other hand, reduces catabolite suppression, increases enzyme titers, and effectively uses agro-industrial leftovers by using moist solid substrates devoid of free-flowing water, which closely resembles the natural habitat of filamentous fungi (Singhania et al., 2010).

Phytase production by Aspergillus niger RA401 was optimized under SMF, agro-industrial residues were screened for phytase production under SSF, and a thorough comparison of SMF and SSF was conducted to determine the best fermentation method.

2. MATERIALS AND METHODS:

2.1 Source of the fungal strain:

Previously isolated from rotten fruits, the fungal strain employed in this study was kept in the culture collection of the Chaitanya (Deemed to be University) Biochemistry Laboratory in Himayath nagar (V), Moinabad (M), Ranga Reddy District, Hyderabad. Based on molecular identification and microscopic features, the isolate was previously known to be Aspergillus niger strain RA401. The internal transcribed spacer (ITS) region of rDNA was

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amplified and sequenced to obtain molecular confirmation. The nucleotide sequence was then uploaded to the NCBI GenBank with accession number MN153032.

2.2 Effect of incubation time:
The fungal culture was cultivated in the chosen basal medium and incubated at 30 ± 2 °C to find the ideal incubation time. Samples were taken out at intervals of 24, 48, 72, 96, and 120 hours. Enzyme activity was measured after the culture filtrate was gathered. Enzyme production typically rose with incubation duration and peaked in the late exponential or early stationary phase. Following this, a decrease was noted as a result of either proteolytic enzyme breakdown or nutrient depletion.

2.3 Effect of pH:
Before sterilization, the pH of the fermentation medium was adjusted using 1 N HCl or 1 N NaOH throughout a range of 3.0–8.0 in order to examine the impact of initial medium pH on enzyme synthesis. Enzyme activity was measured after the inoculation flasks were incubated under normal conditions. Fungal metabolism, enzyme secretion, and nutrient solubility are all significantly influenced by pH; Aspergillus species typically produce more phytase when the pH is acidic.

2.4 Effect of Incubation Temperature:
Fermentation was done at various temperatures (25, 30, 35, 40, and 45 °C) in order to maximize temperature. The ideal incubation time was followed by a measurement of enzyme activity. Enzyme production, protein stability, and microbial growth rate are all impacted by temperature.

2.5 Effect of Carbon Sources:
Each of the different carbon sources—glucose, sucrose, lactose, starch, and maltose—was added to the basal medium at the same proportions (1% w/v) PDA supplemented with calcium phytase. The impact of every carbon source on the synthesis of enzymes was assessed. By means of catabolite suppression or induction processes, carbon sources can greatly control enzyme biosynthesis and act as energy substrates.

2.6 Effect of Nitrogen Sources:
By adding organic (yeast extract, peptone) and inorganic (ammonium sulfate, ammonium nitrate, sodium nitrate) nitrogen sources to the medium, the impact of various nitrogen sources was investigated. After adding nitrogen sources in equal amounts, the activity of the enzymes was measured. Fungal growth, protein synthesis, and enzyme production are all impacted by the availability of nitrogen.

2.7 Screening of Agro-Industrial Wastes for Enzyme Production under Solid-State Fermentation (SSF)
A variety of agro-industrial residues were screened using solid-state fermentation (SSF) as inexpensive substrates for increased enzyme production by Aspergillus niger strain RA401. SSF is thought to be beneficial for the generation of fungal enzymes because it closely resembles the natural habitat of filamentous fungi.

2.8 Choosing the Best Substrate:
The best substrate for more optimization research under SSF was determined to be the agro-industrial waste that produced the maximum enzyme activity. Better substrate utilization, increased product concentration, and decreased catabolite repression are all factors linked to increased enzyme synthesis in SSF.

Because these circumstances are ideal for the synthesis of phytase, optimization ranges were chosen.

Using conventional techniques based on the release of inorganic phosphate from sodium phytate, phytase activity was measured. The efficiency of SMF and SSF processes is compared using phytase activity as a key metric. The process's effectiveness led to its selection for large-scale production, which facilitated additional research.

Protein Estimation: Lowry's method was used to determine the total protein content

3. RESULTS AND DISCUSSION:

3.1 Selection of fungal strain:
The fungal strain used in this study was previously grown in the culture collection of the Chaitanya (Deemed to be University) Biochemistry Laboratory at Himayathnagar (V), Moinabad (M), Ranga Reddy District, Telangana, after being isolated from rotten fruits. The isolate was previously recognized as *Aspergillus niger* strain RA401 by molecular identification and microscopic characteristics. The nucleotide sequence was added to the NCBI GenBank with accession number MN153032, and molecular confirmation was obtained through amplification and sequencing of the internal transcribed spacer (ITS) region of rDNA. The verified and conserved *Aspergillus niger* strain RA401 was subcultured on potato dextrose agar (PDA) slants and stored at 4°C for future studies (Klich, 2014; Nilsson et *al*., 2019).

3.2 Optimization of Phytase Production under SMF:
To optimize different physicochemical parameters impacting the phytase-producing Aspergillus niger strain's ability to produce enzymes, submerged

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fermentation (SMF) was used. One-factor-at-a-time (OFAT) optimization was used, in which one parameter was changed while all other factors remained unchanged.

3.3 Screening of Agro-Industrial Substrates under SSF:

The initial pH had a significant impact on the synthesis of phytase; at pH 5.0, the highest activity was seen. The acidic optimum supports the A. niger phytase and is consistent with previous findings that phytase gene expression and secretion are enhanced by acidic pH (Singh & Satyanarayana, 2011). Maximum phytase synthesis was found at 50 °C through temperature optimization, indicating that the enzyme is thermally tolerant at SMF circumstances. Among nitrogen sources, yeast extract promoted the highest phytase production, whereas carbon source screening revealed glucose to be the most efficient substrate. The best biomass development and enzyme yield were obtained with an inoculum size of 1×10^6 spores mL^{-1}.

3.4 Comparative Evaluation of SMF and SSF:

Under ideal circumstances, SSF produced phytase activity that was roughly 1.6 times greater than SMF. Reduced catabolite repression, greater oxygen transport, and decreased water activity are the reasons for the increased enzyme production in SSF. By using inexpensive substrates and producing little wastewater, SSF also provides financial benefits.

4. CONCLUSION:

The study clearly demonstrates that SSF is superior to SMF for phytase production by *Aspergillus niger* RA401. The use of wheat bran as a substrate under SSF significantly enhanced enzyme yield, highlighting the potential of SSF for sustainable industrial phytase production.

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Table 1: Summary of Processing Parameters for Submerged Fermentation (SMF) of Phytase

Parameter	Optimized / Typical Range	Selected / Optimal Condition	Justification
Basal medium	PDB, Czapek–Dox, modified mineral medium	Modified Czapek–Dox	Provides defined carbon and nitrogen sources suitable for controlled optimization
Carbon source	Glucose, sucrose, starch, maltose (1–3%, w/v)	Glucose (1.5–2.0%)	Supports rapid biomass formation and enzyme secretion
Nitrogen source	$NaNO_3$, $(NH_4)_2SO_4$, yeast extract, peptone (0.1–0.5%)	$NaNO_3$ (0.3%)	Nitrate nitrogen favors extracellular phytase synthesis
Phytate inducer	Sodium phytate (0.05–0.5%)	0.1–0.2%	Induces phytase gene expression
Initial pH	4.0–6.5	5.0–5.5	Acidic pH enhances phytase production and enzyme stability
Inoculum size	10^5–10^7 spores mL^{-1}	1×10^6 spores mL^{-1}	Ensures uniform growth without excessive biomass competition
Inoculum age	3–7 days	5 days	Actively germinating spores yield higher enzyme levels
Temperature (°C)	28–37	30–32	Optimal for fungal metabolism and protein secretion
Agitation speed (rpm)	100–200	150	Enhances oxygen transfer while preventing shear damage
Incubation time (h)	48–144	72–96	Peak phytase accumulation before proteolytic degradation
Working volume	20–30% of flask volume	25%	Maintains adequate oxygen availability
Dissolved oxygen	Aerobic	Non-limiting	Essential for filamentous fungal growth
Trace elements	Mg^{2+}, Fe^{2+}, Mn^{2+} (ppm)	Standard mineral salts	Stabilize enzyme activity and metabolic pathways
Enzyme harvest	Culture filtration + centrifugation	10,000 × g, 10 min	Efficient recovery of extracellular phytase

Table 2: Summary of Processing Parameters for Solid-State Fermentation (SSF) of Phytase

Parameter	Optimized / Typical Range	Selected Condition	Justification
Substrate type	Wheat bran, rice bran, corn cob, groundnut oil cake	Wheat bran (example)	Rich in phytate, carbohydrates, and minerals; supports fungal growth and enzyme induction
Particle size	250–500 µm	~350 µm	Provides high surface area while maintaining porosity and oxygen transfer
Moisture content (%)	50–75	60–70	Ensures adequate water activity and nutrient diffusion without limiting aeration
Initial pH	4.5–6.0	5.0–5.5	Acidic pH favors *Aspergillus* growth and phytase induction
Inoculum size	10^5–10^7 spores g^{-1} substrate	10^6 spores g^{-1}	Ensures rapid colonization and minimizes contamination

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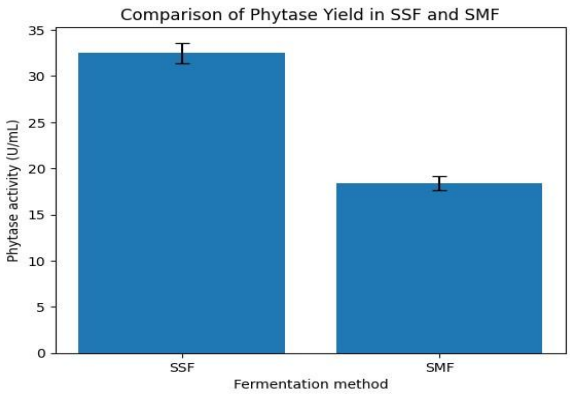
Incubation temperature (°C)	28–37	30–35	Optimal for fungal metabolism and enzyme biosynthesis
Incubation time (h)	48–144	72–120	Maximum phytase accumulation before nutrient depletion
Aeration	Passive	Natural diffusion	Maintains oxygen supply without mycelial damage
Nitrogen supplementation	0.1–0.5% (w/w)	$(NH_4)_2SO_4$ (0.2%)	Enhances protein synthesis and enzyme secretion
Inducer (phytate)	0.1–0.5% (w/w)	Sodium phytate (0.2%)	Induces phytase gene expression
Mineral salts	Mg^{2+}, Fe^{2+}, Mn^{2+} (trace levels)	$MgSO_4$ + $FeSO_4$	Stabilizes enzyme structure and activity
Enzyme extraction buffer	Citrate buffer (0.05 M)	pH 5.0	Maintains phytase stability during extraction
Extraction time (min)	20–60	30	Ensures maximum enzyme recovery

Table 3: Summary of Optimized Solid-State Fermentation (SSF) Conditions for Phytase Production by *Aspergillus niger* RA401

Parameter	Optimized Condition	Justification / Observation
Substrate	Wheat bran	Supported the highest phytase activity due to high phytate content and nutrient availability
Particle size	250–500 µm	Ensured adequate surface area, porosity, and oxygen transfer
Moisture content	60–70%	Facilitated optimal nutrient diffusion and fungal growth
Initial pH	5.0	Favored acidophilic nature and maximal phytase synthesis
Inoculum size	1×10^6 spores g^{-1} substrate	Enabled rapid substrate colonization and minimized contamination
Incubation temperature	30–32 °C	Optimal for fungal metabolism and enzyme biosynthesis
Incubation time	72–96 h	Corresponded to peak phytase accumulation before nutrient depletion
Fermentation system	Solid-state fermentation (SSF)	Mimics natural fungal habitat and enhances enzyme yield
Enzyme extraction buffer	Citrate buffer (0.05 M, pH 5.0)	Maintained enzyme stability and ensured maximum recovery

Table 4: Comparison of Submerged Fermentation (SMF) and Solid-State Fermentation (SSF) for Phytase Production

Fermentation method	Phytase activity (U/mL) (Mean ± SD, n = 3)	Relative activity (%)
SSF	**32.5 ± 1.1**	**100.0**
SMF	18.4 ± 0.8	56.6



Comparison of Submerged Fermentation (SMF) and Solid-State Fermentation (SSF) for Phytase Production

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