Valorization of *Lemna minor* Leaves via Solid-State Fermentation by a Fish-Gut *Bacillus subtilis* for Aquafeed Application

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Abstract— *This study aimed to enhance the nutritional value of the freshwater macrophyte Lemna minor (duckweed) for use as an aquafeed ingredient through Solid-State Fermentation (SSF). A phytase-producing bacterium, Bacillus subtilis (HM352551), isolated from the gut of the teleost fish Labeo bata, was used as the fermenting agent. Key SSF parameters were optimized for maximum phytase yield. The highest phytase activity of 15.26 ± 0.09 U/g was achieved after a 10-day incubation at 35°C, with an initial substrate moisture content of 50% and a moistening media pH of 7.0. An inoculum size of 4% (v/w) also yielded high activity (14.28 ± 0.11 U/g). Proximate composition analysis of the fermented leaf meal revealed a significant increase ($p < 0.05$) in crude protein, lipid, ash, and mineral content (Na, K, Ca, Mg, P, Zn, Fe, Cu, Mn). The levels of all essential amino acids increased. Concurrently, there was a significant reduction ($p < 0.05$) in antinutritional factors, including crude fibre, phytic acid, trypsin inhibitor, and tannin. Concentrations of heavy metals (Pb, Cd, Cr, Ni) were also reduced. The results demonstrate that SSF using a host-derived gut bacterium is an effective strategy for the bioconversion of low-cost aquatic weeds into a nutritionally enhanced, safer, and sustainable component for aquafeed formulations.*

Keywords— Bacillus subtilis, phytase, Lemna minor, solid-state fermentation, aquafeed, antinutritional factors.

I. INTRODUCTION

In recent years, diverse terrestrial and aquatic macrophytes have been incorporated into carp diets as partial replacements for costly fishmeal [1]. As protein is a critical component for fish growth, alternative plant protein sources have been explored from the early days of freshwater aquaculture [2]. Duckweed, *Lemna minor*, is considered a promising natural feed for carps [3,4] due to its relatively high protein content, favourable amino acid profile, and small size [5]. Its leaves contain low fibre, and the cell walls have low lignin content [6]. Duckweed is also a source of trace minerals like potassium and phosphorus, as well as pigments such as carotenes and xanthophylls [7].

However, plant-based ingredients contain antinutritional factors (ANFs) like tannins, phytic acid, trypsin inhibitors, and saponins, which reduce nutrient digestibility and bioavailability [8]. Solid-State Fermentation (SSF) using exo-enzyme-producing microorganisms is an effective method to reduce ANFs [8]. SSF is often preferred over submerged fermentation due to its higher product concentration, lower energy and wastewater output, simpler operation, and reduced space requirements [9].

Indigenous phytase-producing bacteria from fish guts are advantageous for fermenting plant materials. Their enzymes can offer precise activity, protease resistance, and high catalytic efficiency compared to fungal alternatives [9]. Using invasive aquatic weeds as an SSF substrate provides economic benefits via low-cost biomass and enables sustainable nutrient recovery.

The primary objective of this research was to ferment *Lemna minor* leaves using an autochthonous fish-gut bacterium, *Bacillus subtilis* (HM352551), to degrade ANFs and improve nutrient bioavailability, thereby evaluating its potential as a beneficial ingredient in aquaculture feeds.

II. MATERIALS AND METHODS

2.1 Microorganism and Inoculum Preparation:

The phytase-producing bacterium used was isolated from the gut of *Labeo bata* and identified as *Bacillus subtilis* (GenBank Accession No. HM352551) via 16S rRNA gene sequencing [10]. The culture was maintained on modified phytase screening medium (MPSM) agar [11]. Inoculum was prepared by growing the culture in MPSM broth at 35°C for 48 hours, yielding a suspension containing approximately 5.6×10^7 cells mL^{-1}.

2.2 Substrate Collection and Processing:

Lemna minor leaves were collected from local water bodies in Burdwan, West Bengal, India (23°12' N, 87°45' E). The fresh leaves were oven-dried at 70°C for 48 hours, ground into a fine powder using a laboratory blender, and stored as *Lemna* Leaf Meal (LLM) for use as the solid fermentation substrate.

2.3 Solid-State Fermentation (SSF):

Five grams of dry LLM was placed in a 250 mL Erlenmeyer flask. The substrate was moistened with 3 mL of MPSM broth (lacking sodium phytate and agar) and additional distilled water to achieve the desired final moisture level. The flask was plugged with cotton and sterilized at 121°C and 15 psi for 20 minutes. After cooling, the substrate was aseptically inoculated with 1 mL of bacterial suspension. Fermentation was carried out at 35°C for 72 hours. All experiments were performed in triplicate.

2.4 Optimization of SSF Parameters:

SSF was conducted to maximize phytase production and phytate hydrolysis. The following parameters were optimized: incubation temperature (25–50°C), initial pH of the moistening medium (pH 5–9), fermentation period (24–144 hours), inoculum size (1–5 mL), and initial substrate moisture content (10–100%).

2.5 Enzyme Extraction and Phytase Assay:

After fermentation, phytase was extracted from the solid substrate [8]. Phytase activity was assayed using sodium phytate as the substrate [12]. One unit (U) of phytase activity was defined as the amount of enzyme required to release 1 µg of inorganic phosphorus per minute under assay conditions. Activity was expressed as units per gram of dry substrate (U/g). The soluble protein in the crude extract was estimated by the Lowry method [13] using bovine serum albumin as the standard.

2.6 Proximate, Mineral, and Antinutrient Analysis:

Proximate composition (crude protein, lipid, fibre, ash) of raw and fermented LLM was analyzed using standard AOAC methods [14]. Mineral elements (Na, K, Ca, Mg, P, Zn, Fe, Cu, Mn) were analyzed by atomic absorption spectrophotometry (PerkinElmer Analyst 700) and flame photometry. Tannin [8], phytate [15], and trypsin inhibitor activity [16] were determined. Amino acid profiles were analyzed using an automated amino acid analyzer (Shimadzu-10AS). Fatty acids were quantified by GC-MS (Shimadzu GC-MS-QP2010).

2.7 Statistical Analysis:

All data are presented as mean ± standard error (SE) of three replicates. A t-test was used to compare means between raw and fermented LLM. One-way ANOVA followed by the Student-Newman-Keuls test was applied to analyze the effect of different SSF parameters on phytase yield. Analyses were performed using SPSS Version 16.0, with significance accepted at $p < 0.05$.

III. RESULTS

3.1 Optimization of SSF Conditions for Phytase Production:

Phytase production was significantly influenced by all tested parameters ($p < 0.001$, ANOVA). Activity increased with incubation time, reaching a maximum of 12.39 ± 0.09 U/g on day 10, followed by a decline (Fig. 1d). The optimum initial pH of the moistening medium was 7.0, yielding 12.41 ± 0.10 U/g (Fig. 1b). A sharp decline occurred at higher or lower pH. The optimum incubation temperature was 35°C, resulting in the highest activity of 15.21 ± 0.09 U/g (Fig. 1c). The initial substrate moisture content of 50% was optimal, producing 15.26 ± 0.09 U/g (Fig. 1a). An inoculum size of 4 mL (equivalent to 4% v/w) gave maximum activity (14.28 ± 0.11 U/g), with a decline at higher inoculum levels (Fig. 1e).

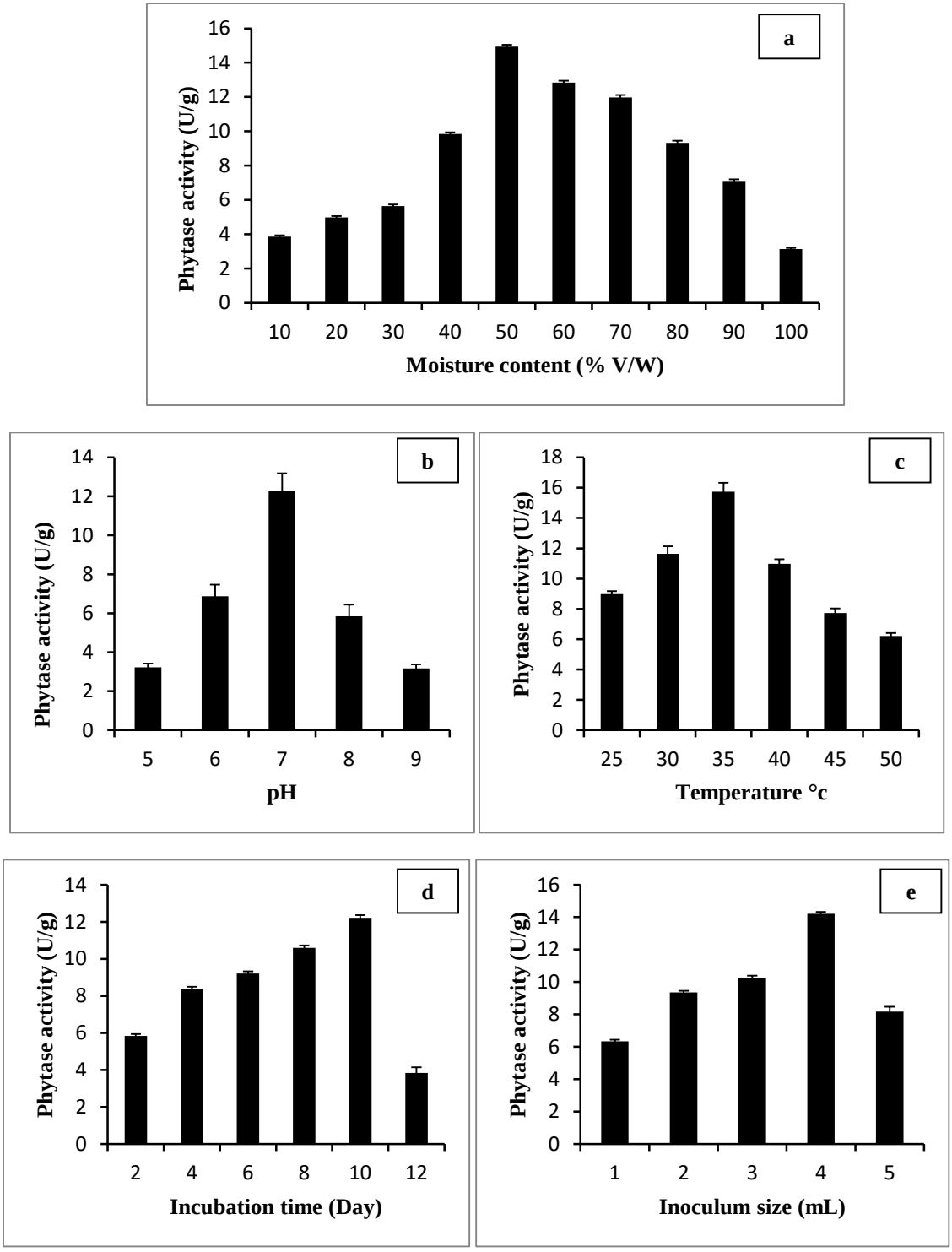

FIGURE 1: Effect of different initial moisture content (a), initial pH of moistening media (b), temperature (c), incubation period (d), and inoculum size (e) on phytase production in solid-state fermentation (SSF). Bars with different alpha plates are statistically significant ($p < 0.001$; Student-Newman-Keuls test)

3.2 Effect of Fermentation on Substrate Composition:

Fermentation under optimal conditions significantly altered the composition of LLM (Table 1). Crude protein increased by 9.74%, lipid by 15.30%, and ash by 23.05%. Crude fibre content decreased substantially by 39.87%. All analyzed macro- and micro-elements showed significant increases ($p < 0.05$). Concentrations of the antinutritional factors phytic acid, tannin, and trypsin inhibitor activity were reduced by 70.91%, 67.68%, and 57.50%, respectively. The levels of all essential and non-essential amino acids increased significantly. The content of individual saturated (SFA), monounsaturated (MUFA), and

polyunsaturated (PUFA) fatty acids also increased. Furthermore, the concentrations of heavy metals (Pb, Cd, Cr, Ni) were reduced.

TABLE 1

PROXIMATE COMPOSITION AND CONCENTRATION OF DIFFERENT MINERAL IONS , ANTINUTRITIONAL FACTORS AMINO ACIDS AND FATTY ACIDS IN RAW AND FERMENTED *LEMNA MINOR* LEAF MEAL (LLM)

Parameters	Raw	SSF processed	% Increase / Reduction (↓)
Nutrients			
Crude Protein	17.87 ± 0.08	19.61 ± 0.11	9.74
Crude Lipid	1.83 ± 0.03	2.11± 0.05	15.30
Crude Fibre	11.16 ± 0.05	6.71 ± 0.04	39.87 ↓
Crude Ash	3.21 ± 0.05	3.95 ± 0.05	23.05
Macro elements (g/kg)			
Ca	14.74 ± 0.11	15.82 ± 0.12	7.32
K	8.7 ± 0.18	9.4 ± 0.21	8.33
P	6.5 ± 0.09	7.41± 0.11	13.85
Na	2.24 ± 0.06	2.50 ± 0.05	11.75
Mg	5.1 ± 0.03	5.34 ± 0.03	4.65
Microelements (mg/kg)			
Fe	46 ± 0.09	53 ± 0.09	15.22
Zn	18 ± 0.17	19.05 ± 0.11	5.82
Mn	17 ± 1.05	18.40 ± 1.03	8.23
Cu	2.2 ± 0.04	2.46 ± 0.04	12.03
B	12.24±0.08	13.12±0.09	7.23
Mo	0.3±0.02	0.4±0.02	33.33
Heavy Metals (mg/kg)			
Pb	2.29 ±0 .05	1.96 ± 0.07	8.30↓
Cd	0.79 ± 0.04	0.67 ± 0.03	15.19↓
Cr	5.73±0.06	4.81±0.02	16.06↓
Ni	3.11±0.03	2.48±0.03	20.26↓
Antinutritional factors (g %)			
Phytate	1.26 ± 0.03	0.64 ± 0.03	70.91 ↓
Tannin	0.99± 0.03	0.32 ± 0.02	67.68 ↓
Trypsin inhibitor	1.20 ± 0.04	0.51 ± 0.03	57.50 ↓
Amino acid Composition (g/100g protein)			
Alanine	7.4 ± 0.03	7.8 ± 0.04	5.41
Arginine	3.9 ± 0.04	4.2 ± 0.03	7.70
Cysteine	1.2 ± 0.05	1.4 ± 0.05	16.67
Glutamic acid	12.2 ± 0.05	12.5 ± 0.05	2.46
Methionine	1.7 ± 0.04	1.9 ± 0.03	11.76
Leucine	8.6 ± 0.05	8.9 ± 0.05	3.49
Valine	6.4 ± 0.03	6.7 ± 0.04	4.69
Lysine	5.8 ± 0.04	6.1 ± 0.05	5.17
Phenyl alanine	6.3 ± 0.05	6.6 ± 0.04	4.76
Glycine	4.4 ± 0.05	4.7 ± 0.03	6.81
Aspertic acid	11.1± 0.04	11.4 ± 0.06	2.70

Parameters	Raw	SSF processed	% Increase ()/ Reduction (↓)
Histidine	2.4±0.03	2.6±0.04	8.33
Serine	5.2±0.05	5.6±0.05	7.69
Proline	4.5±0.04	4.7±0.05	4.44
Tryotophan	1.35±0.02	1.38±0.03	2.22
Fatty cids (g FAME/100 g crude fat)			
SFA – saturated fatty acids			
C8:0	0.134±0.05	0.142±0.03	5.97
C10:0	0.263±0.02	0.284±0.02	7.98
C12:0	0.210±0.02	0.223±0.02	6.19
C14:0	1.312±0.05	1.411±0.04	7.55
C15:0	0.081±0.03	0.092±0.03	13.58
C16:0	11.96±0.09	12.35±0.06	3.26
C17:0	0.031±0.01	0.036±0.01	16.13
C18:0	4.625±0.04	4.930±0.02	6.59
C20:0	0.046±0.01	0.053±0.01	15.22
C24:0	0.061±0.02	0.068±0.03	11.48
MUFA – monounsaturated fatty acids			
C14:1 c7	0.082±0.01	0.087±0.01	6.58
C15:1 c10	0.081±0.02	0.087±0.01	7.24
C16:1 c7	0.173±0.02	0.184±0.02	6.81
C16:1 c9	0.168±0.01	0.178±0.01	5.99
C16:1 c10	0.554±0.01	0.589±0.02	6.32
C18:1 c11	0.176±0.02	0.191±0.03	8.26
C18:1 c9	5.763±0.05	6.115±0.05	6.11
PUFA – polyunsaturated fatty acids			
C16:2 c9,c12	1.213±0.06	1.313±0.06	8.24
C18:2 c9,c12	11.386±0.08	12.226±0.06	7.37
C18:3 c9,c12,c15	15.269±0.08	16.217±0.05	6.21
C20:2 c11,c14	0.021±0.01	0.022±0.02	5.48

IV. DISCUSSION

The results demonstrate that SSF using *Bacillus subtilis* from fish gut effectively enhanced the nutritional profile of *L. minor*. The optimal pH (7.0) and temperature (35°C) for phytase production align with the neutral/alkaline gut environment of the host fish (*L. bata*) and the mesophilic nature of the bacterium [11]. The decline in activity beyond optimal moisture (50%) and inoculum size (4%) is consistent with reports suggesting impeded aeration and nutrient competition at higher levels [22, 24].

The significant reduction in phytic acid, tannin, and trypsin inhibitor is attributable to the extracellular phytase and other enzymes (e.g., protease, cellulase) produced by *B. subtilis* [11]. The degradation of the fibrous matrix likely contributed to the decrease in crude fibre and the concomitant increase in the relative concentration of protein, lipids, and minerals. The increase in amino acid levels may result from microbial synthesis or the liberation of bound protein fractions. The reduction in heavy metals, though from low baselines, suggests a potential biosorption or biotransformation capability of the bacterial biomass, warranting further investigation.

The use of a host-derived probiotic bacterium for feed processing ensures metabolic compatibility and safety for the target aquatic species. The substantial reduction in ANFs, coupled with improved nutrient density, makes fermented LLM a promising

alternative protein source for carp diets, potentially reducing reliance on fishmeal and the environmental impact of feed production [25, 29].

V. CONCLUSION

This study confirms that solid-state fermentation of *Lemna minor* leaf meal using a phytase-producing *Bacillus subtilis* strain isolated from fish gut is a viable bioprocessing strategy. The process significantly degraded antinutritional factors, improved the bioavailability of nutrients and minerals, and reduced heavy metal content. This approach valorizes a low-cost aquatic weed into a sustainable, nutrient-enhanced ingredient suitable for incorporation into aquafeeds, offering a pathway to reduce feed costs and environmental footprint in aquaculture.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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