

Research Article

Heterologous expression of phytase in *Schizochytrium* sp. as a fortified feed additive for the Livestock industry

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Abstract

Phytates present in plant-derived feed can chelate nutrients and reduce their bioavailability for monogastric animals such as poultry and swine. The addition of hydrolase, phytase can alleviate this problem but is hindered by its cost. The goal of the current study is to clone, express and purify the phytase gene from *Bacillus* sp. (DS11) into *Schizochytrium* sp. ATCC 20888 is also a good producer of Docosahexaenoic acid (DHA). This is expected to enhance animal nutrition and reduce phosphate pollution. The DNA sequence analysis using multiple sequence alignments showed significant similarity to the phytase gene from *Bacillus* sp. (DS11). Subsequently, specific primers were designed based on the consensus sequence of the *Bacillus* phytase gene obtained from sequencing. The coding DNA sequence was determined to have a length of 1152 base pairs. Phytase gene was successfully cloned into the pRI201-AN DNA vector and transformed into *Schizochytrium* sp. Screening on G418 plates showed 53 resistant colonies and from this 11 prominent colonies were chosen for further testing. Out of this, 8 colonies tested positive, with colony PCR having 1.5 kb with a phytase activity of 1.77 U/ml of crude lysate. Further purification with Ni-NTA affinity chromatography provided a specific activity of 15.59 U/mg. This appears to be the first ever reported recombinant phytase produced in *Schizochytrium* sp. The phytase recommendations are 250U/Kg of feed preparation for broiler & swine diets. It was also determined that 72.64 U/5.2 gm of wet biomass and 1.80% of w/w microalgae would fulfil these requirements per kg of feed preparation.

Keywords: Assay, Cloning, Docosahexaenoic acid, Omega-3-fatty acid, Phytase, Phytic acid, *Schizochytrium* sp

INTRODUCTION

Phytic acid or Phytate, (myo-inositol hexakis dihydrogen phosphate)(cation salt) is an anti-nutrient in the feed industry. It accounts for more than 80% of phosphorus with minerals in seeds, edible legumes, cereals, and nuts in phytic acid form (Maga,1982; Vohra,2003; Lott *et al.*, 2000 These cereals and seeds are the key ingredients in animal feed for growth and nutrition (Afinah, 2010). Phytic acid is mainly found as phytate because of its strong binding affinity to cations. These metal complexes with divalent cations make phytic acid a potent chelator of several critical dietary minerals such as Ca^{2+} , Mg^{2+} , Zn^{2+} , or Fe^{2+} . Ruminant animals

use phytic acid as a source of phosphorus due to the phytase secretion by their gut bacteria (Mullaney, 2000). Nevertheless, monogastric animals do not produce Phytase, hence, supplementation of phosphate (PO_4^{3-}) in their diets is necessary (Cangussu *et al.*, 2018). The livestock industry uses exogenous inorganic phosphate to meet the nutritional requirements of these animals. It is not only cost-intensive but also disturbs the ecology as the undigested phytate or excess inorganic phosphate increases phosphorus levels in the environment, leading to eutrophication in nearby lakes and ponds (Reddy *et al.*, 2017)

Phytase catalyzes the release of phosphate(PO_4^{4-}) from phytate by generating less-phosphorylated myo-inositol

derivatives. Based on the specific motif and 3D structure, Phytases have been classified into four classes: Histidine Acid Phosphatases (HAP) (N-terminal active site motif RHGX₃RP and C-terminal HD motif.), Beta-propeller Phytase (BPP) Protein which consists mainly of β -sheets and resembles a six-blade propeller with six Ca^{2+} , Cysteine Phosphatase (CP), (sequence motif HCXXGXXR (T/S), Purple Acid Phytases (PAP) (Seven conserved residues in five conserved motifs: DXG, GDXXY, GNH(D/E), VXXH and GHXH) (Bozzo, 2002; Antonyuk., 2014). The ENZYME database also classified phytases into three groups based on the position in the carbon ring, where dephosphorylation of phytate initiates 3-phytase: EC 3.1.3.8; 6-phytase: EC 3.1.3.26; 5-phytase: EC 3.1.3.72. HAP, PAP, and CP are acidic phytases, whereas BPP from bacillus is alkaline. These phytases are derived from microorganisms, animals, and plants. *Bacillus* phytases are shown to entirely nullify phytate's ability to chelate metal ions and release phosphates from the inositol ring. In this regard, *Bacillus* sp. are used as feed additives for animals with neutral digestive tracts of some aquatic species, poultry and swine, since these enzymes have a neutral optimal pH. These phytases are also stable at high temperatures and meet in the manufacturing process of pelleting the feed (Singh *et al.*, 2018; Zhao, 2021)

Microalgae are nutritive and are rich sources of many vitamins, omega acids, and anti-oxidants, easy for scale-up in cGMP conditions, and belong to generally regarded as a safe (GRAS). Hence, microalgae were selected as an ideal recombinant expression system for phytase production. Moreover, microalgae are already used as feed in the poultry and fish industries. Microalgae, *Schizochytrium* sp. are potential alternatives to conventional omega 3 PUFA (polyunsaturated fatty acid) sources. It has a high content of docosahexaenoic acid, DHA, which has been used commercially (Alok *et al.*, 2021). Omega-3 fatty acids help in development and metabolism, growth and productive performance, immune response and anti-

oxidative properties, improving meat quality, bone growth, development, fertility rates, and semen quality in poultry and swine industries. These are linked to health benefits against human disorders and diseases (Alagawany, 2019). *Schizochytrium* sp. has numerous advantages like high growth rate, proper post-translational modifications, low-cost medium, and the ability to survive extreme environmental conditions such as temperature, light, and salt which thereby reduces the contamination rate (Hu, Xuechao *et al.*, 2021). The present study aimed to clone, express, and purify the phytase gene in *Schizochytrium* sp. which can serve both as a feed and phytase source, thereby reducing the feed cost and increasing livestock farming.

MATERIALS AND METHODS

Designing of primer from the consensus sequence of *Bacillus* phytase gene

Five Phytase gene sequences from *Bacillus* species were obtained from Genbank of NCBI (WP_088037351, WP_02405493.1, WP_201488970.1, ABL86758.1, and WP_014305231.1). (<https://www.ncbi.nlm.nih.gov/genbank/>) Multiple sequence alignment and primers were designed for phytase gene using SnapgeneV.6.2.1. to amplify the gene and identify the phytase gene by PCR from the isolated gDNA of BAB -3372 (Hui *et al.*, 2017) using the forward primer 5'CGCCATATGAATCATTCAAAAACACTTTTGTAAAC-3' and reverse primer 5'CGAGCTCTCAG-TGGTGGTGGTGGTGGTGTGTTCGCTTCTGTCGGT CATT 3'. Amplification was analyzed using Thermal Cycler (Eppendroffflexilid, Nexus gradient) with the final reaction volume of 50 μ l. The PCR mixture consisted of 100 ng of Genomic DNA samples, 1 μ M of the primers, 200 μ M dNTPs, 200 μ M $MgCl_2$, 1U of Hi-fidelity DNA polymerase (F530XL Phusion, ThermoScientific), and 5 μ l of 10 \times PCR buffer. Parameters included the Denaturation step at 95 $^{\circ}$ C, for 5 minutes, for 30 cycles at 95 $^{\circ}$ C, 1 minute, 61 $^{\circ}$ C, 1 minute, and 72 $^{\circ}$ C, 1 minute. The final extension of the program was set at 72 $^{\circ}$ C for 5

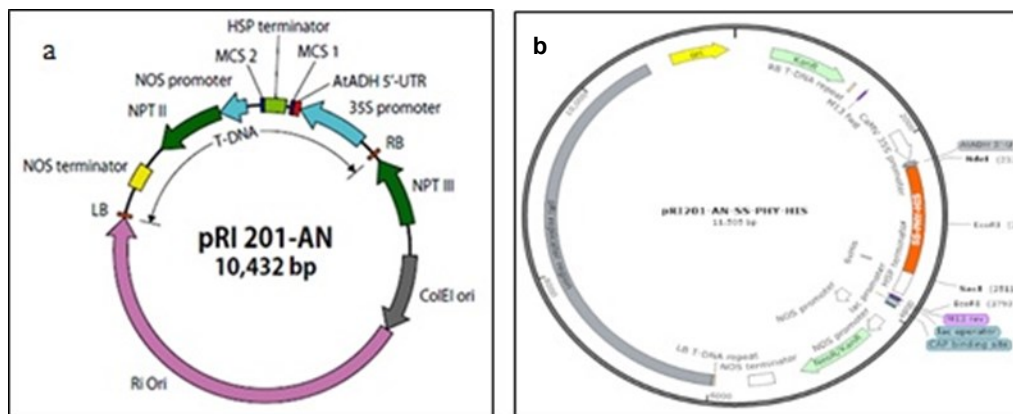


Fig. 1. a. Vector map description of pRI201-AN ; b. pRI201-AN-SS-phy-His

minutes and was confirmed by Agarose gel Electrophoresis. The quality of Genomic DNA was also assessed.

Cloning and plasmid pRI201-AN DNA construction

The Plasmid DNAPRI201-AN (Fig.1a) is a binary vector for high-level expression of a foreign gene in dicotyledonous plants, with 10432 bp ds-DNA circular. The amplified phytase gene (DS11) was digested and cloned into pRI201-AN vector between NdeI and SacI restriction sites (Cheng *et al.*, 2012). The phytase gene is named *Bacillus* signal sequence (ss) Phytase(phy) with Histidine tag (HIS) as (SS-Phy-HIS). Furthermore, the resulting construct was named pRI201-AN-SS-Phy-HIS (Fig.1b)

The phytase gene was digested and purified using a gel purification elution kit. (Nucleospin Gel and PCR clean-up kit, Macherey Nagel). Purified pRI201-AN and Inserts (SS-Phy-His) concentration was estimated and ligated (2011A T4 DNA ligase, Takara bioscience) within the ratio of 1:3. The ligation mixture was transformed into *E. coli* Top10 chemically competent cells (C4010) and selected on an antibiotic plate containing 50mg/ml of Kanamycin and incubated at 37°C overnight (Cheng, *et al.*, 2011). The plasmid was isolated from the selected colonies and confirmed with restriction digestion using *Nde1* and *Sac1*. (Nucleospin plasmid, 250 prep, Macherey Nagel).

Culture conditions of *Schizochytrium* sp.:

Schizochytrium sp. ATCC 20888 was procured from American Type Culture Collection (ATCC, VA, USA). The strain was preserved as 20% (v/v) glycerol stocks kept at -80°C. The frozen ampoules were thawed by transferring 50µl onto a plate containing ATCC 662 seawater medium and incubated at 28°C. The viability was observed after 2- 4 days of incubation. The *Schizochytrium* sp. was cultured in 50ml medium with composition (g/L): yeast extract 10, glucose 30, peptone 2, and sea salt 15 in a 250ml flask; with pH 6.0 before autoclaving at 121°C for 20 min and incubated at 25°C, 200 rpm for 48 hrs.

Antibiotic sensitivity of *Schizochytrium* sp.

To select the appropriate binary vectors for transformation, the antibiotic sensitivity of *Schizochytrium* sp. was determined. Microalgae were grown on Yeast extract peptone dextrose (YPD) agar with 1.5% sea salt at different concentrations of G418 disulfate (TCI) of 100µg/ml and 300 µg/ml, and the streaked plates were incubated at 28°C for five days. The antibiotic-resistant clones were subsequently streaked on higher concentration plates such as 600µg/ml and 1000µg/ml plates.

Transformation of *Schizochytrium* sp. by Electroporation

The developed clone pRI201-AN-SS-Phy-HIS was

used for transformation by electroporation of *Schizochytrium* sp. ATCC 20888. It was carried out according to the modified methods of Adachi *et al.* (2017) using Gene Pulser II Electroporation System of Bio-Rad (Hercules, CA, USA). *Schizochytrium* sp. was cultured in 100 ml YPD media with 1.5% sea salt and incubated at 30°C, 200rpm, for 48 hr. The culture was harvested at the exponential stage and centrifuged at 5000 rpm, 4°C for 5 min. Cells were re-suspended in 0.4 mL of 0.2% of sea salt solution in 1.5 ml Eppendorf tube with 250 mg of 1mm sterilized glass beads with 5% PEG8000 (Polyethylene glycol) and vortexed by Vortex Genie II mixer for 10s, to break the cell walls. These treated cells were collected at different time points and then washed with 0.2% sea salt solution once and then with 50 mM sucrose three times. 10 µL of plasmid DNA with 5 to 10µg was added to transform 100µL-200 µL of treated cells by electroporation at 2000V field strength, one pulse, 200 Ω resistance, 50 µF capacitance, and 2 mm cup.

Screening of recombinant *Schizochytrium* sp. clones on G418

Rich media with composition (g/L): glucose 40, peptone 5, yeast extract 10, and sea salt 20 was added to the transformation mix and incubated for 12 hr at 30°C with 200 rpm. 200 µl of the sample was plated on YPD, 5% Sea salt, 1.5% Agar, and 300µg/ml G418 disulfate and incubated at 30°C for 4 days. Colonies were selected based on size and reselected on YPD + 5% sea salt media, 600µg/ml, and 1000 µg/ml of G418 disulfate.

Colony PCR confirmation of recombinant *Schizochytrium* sp.

The colony PCR method was assayed for rapidly screening colonies of transformed *Schizochytrium* sp. that were grown on selective media with 300µg/mL of G418 disulfate (TCI). The desired clone construct (pRI201-AN-SS-phy-His) was verified and amplified by a standard method (Bergkessel *et al.*, 2013). The Genomic DNA was prepared using AmpReady@Instant Genomic DNA preparation Kit from 30M Genomics India. PCR master mix was prepared by including the following components (per reaction 25µl): Taq master mix 12.5 µl (Takara RR310A); Forward vector-insert overlapping primer (10 µM), 0.5 µl; Reverse Insert - Vector overlapping primer (10 µM), 0.5 µl; dH₂O, 10.5 µl. 1µl of template gDNA (≥100ng). PCR was performed using an Eppendorf PCR Thermal Cycler. The cycling conditions were 98°C, 1 min, followed by 30 cycles of 95°C, 30 s, 58°C, 30s and 72°C, 1m. From the resulting PCR products, the phytase gene amplified band was observed in Agarose gel electrophoresis with a 1Kb DNA ladder. PCR Positive Clones were subcultured and observed that the host growth declined due

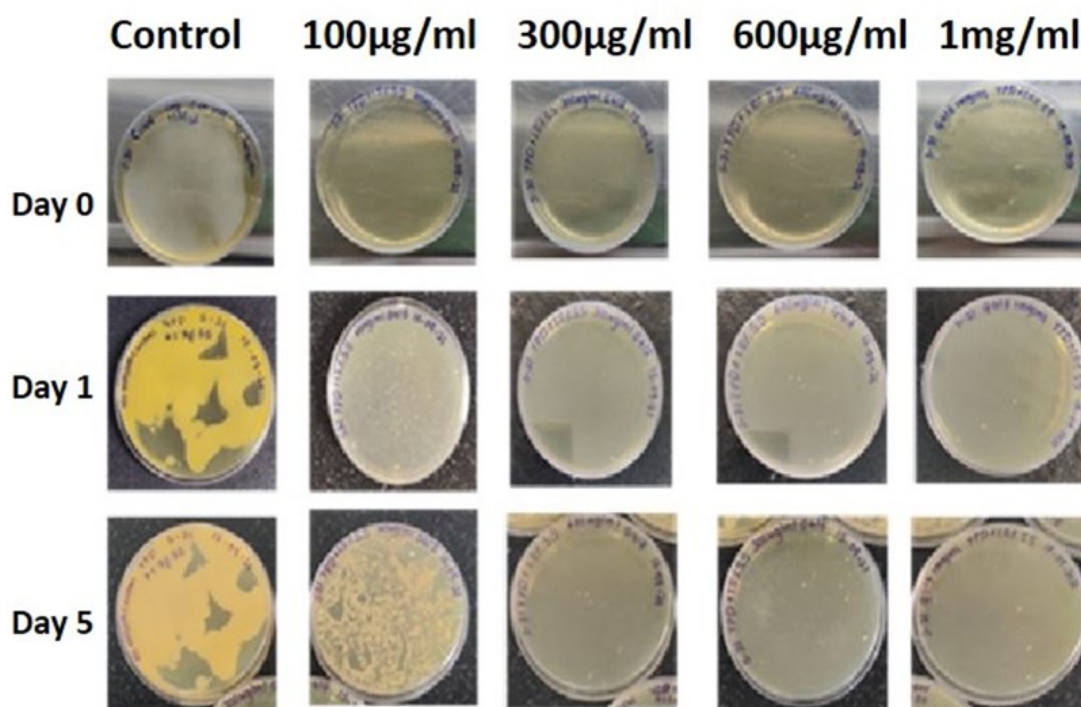


Fig. 2. Antibiotic sensitivity of G418 disulfate on *Schizochytrium sp.*

to the selection pressure.

Culturing of recombinants and purification by Ni NTA Affinity chromatography

A single colony of recombinant *Schizochytrium sp.* was inoculated in 10 ml fresh liquid media containing 150 ml of YPD +1.5% Sea salt media with 300 µg/ml of G418 and kept in a shaker incubator for 8 h at 37°C. Once the seed culture reached the exponential stage observing the absorbance of 0.6 -1.0, 3% of the culture was inoculated in a 500ml baffled flask containing YPD media. The flask was incubated at 200 rpm at 28°C for 4 days in a shaker incubator and the culture was harvested by centrifugation at 5000 rpm for 10 min at 4°C. The obtained pellet of 5.2g was resuspended in 100ml of Tris buffer with 1mM of PMSF. The cells were lysed by passing through Panda plus Homogenizer at 900 bar for 3 cycles. After every lysis cycle, the cells were observed under a microscope for lysis confirmation. The lysate was centrifuged and filtered using a 0.45 µm filter. Purification of the recombinant Phytase with a His tag was carried out on 20 ml of Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography matrix (Cytiva, USA) connected to the Fast Protein Liquid Chromatography (Cytiva Akta, USA). Before loading the supernatant, 20 ml of the Ni-NTA agarose column was equilibrated with buffer A (50mM Tris-HCl, 1mM PMSF, pH 7.2). The serial washes were performed with buffer B (25mM of Imidazole in 50mM Tris buffer with 1 mM of PMSF, pH 7.2). The protein was eluted with buffer A and buffer B with 25-500mM Imidazole in a gradient

(Dechow, 1989). The collected elution fractions were analyzed for phytase activity.

Phytase activity of recombinant *Schizochytrium sp.*

Phytase activity was quantified as described by Heinenon and Lahti, 1981 with modification. 100 µL of each fraction was added to 500 µL of 44.1mM sodium phytate in acetate buffer to start the catalytic reaction. The reaction mixture was incubated at 37 °C for 10min. Positive control was carried out under the same conditions by adding 100 µL of a Commercial phytase, instead of the recombinant phytase. The reaction mixture was incubated at 37 °C for 10min. Then, 4 mL of freshly prepared acetone-acid-molybdate, colorimetric solution (Acetone, 5 N Sulphuric acid (H₂SO₄), and 5% Ammonium molybdate; 2:1:1 v/v/v) was added, and absorbance was measured at 400nm. This method was chosen since it directly detects the yellow phosphomolybdic acid without reduction to molybdenum blue. The calibration curve was prepared with inorganic phosphate (KH₂PO₄) in the range (0.15–2.5 µmol). The phytase activity (U/mg of Protein) is defined as the µmol of phosphate released per min under the standard assay conditions.

RESULTS

Genomic DNA isolated from *Bacillus sp* DS11 and phytase gene was PCR amplified and cloned into a plant expression vector (pRI 201) and transformed into

Table 1. Transformation mix with Electroporation parameters with colony count

Label	Transformation Mix	Pulse length (Time) ms	Voltage	Number of colonies
TF-1	200µl cells+7.5ug pDNA	3.1	2kv	13
TF-2	150µl cells+10ug pDNA	3.3	2kv	2
TF-3	100µl cells+5ug pDNA	4.3	2kv	40
TF-4	200µl cells	4.2	2kv	No growth

Schizochytrium sp.

The eluted volume of Genomic DNA was 50µl with a concentration of 0.4µg/µl. The quality of the DNA was measured with Optical Density (OD in nm) at A260/280 and obtained 1.8 and A260/230 absorbance ratio was found to be 1.7. In different species, the DNA extraction efficiency may vary by the A260/A280 as reported: 1.84-2.02 for *Anabaena* sp., 1.88-2.12 for *Nodularia spumigena*, and 1.75-1.90 for *Nostoc* sp. The consensus obtained from the aligned five sequences was found to be ~1152 bp. The first four sequences showed 100% similarity with *Bacillus* sp., including N-terminal and C-terminal. The purified PCR fragment was ligated with a pRI 201-AN vector and transformed into *E.coli* Top10 chemically competent cells (C4010). The transformed *E.coli* culture was grown in kanamycin plates of 50µg/ml. The plates with positive clones were screened and digested with restriction enzymes for further confirmation. The electroporation transformation mix of *Schizochytrium* sp. with developed plasmid growth are given in Table 1. The results showed that TF-3 had a maximum of 40 colonies and TF-2 a minimum of 2 colonies. It was also observed that the cell without plasmid inhibited growth. The colonies' growth patterns were as TF-3>TF-1 >TF-2 >TF-4.

The antibiotic screening of transformant *Schizochytrium* sp. cells growth in YPD 300µg of G418 disulfate resulted in obtaining 57 colonies, only 40% were selected based on the size and subcultured 600µg/ml and 1000 µg/ml of G418 disulfate (Fig.2). In the *Schizochytrium*

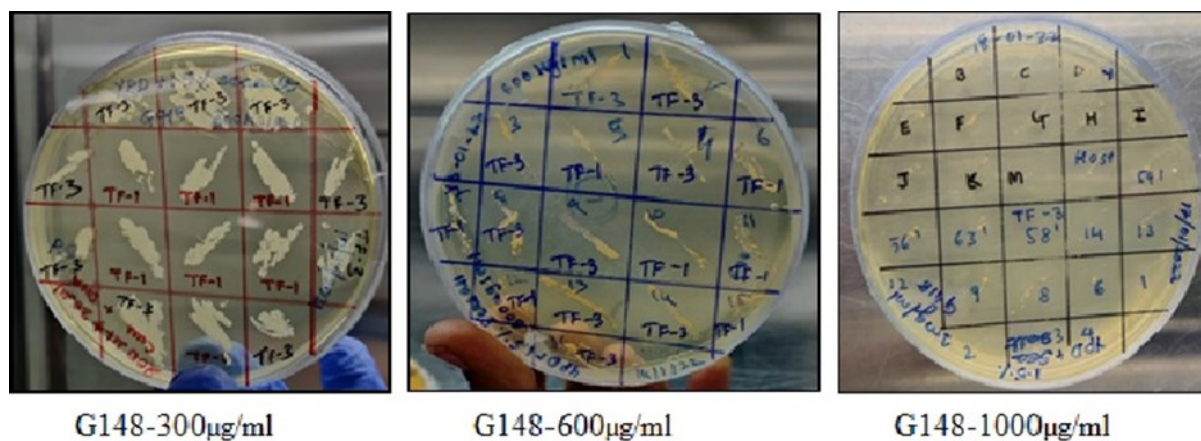
sp. with 600µg/ml and less (stressed) growth was observed (Fig. 3)

Colony PCR of a 1.29 kbp Phytase gene was performed using a PCR-ready mix and analyzed on 1% agarose gel. Recombinant clones were detected from all transformed cells containing vector + insert (pRI201-AN-SS-phy-His) clone 6-9, lane 10 (1kb ladder) clone 11-14 at the same time, and no product was amplified from cells transformed with only vector, i.e., without insert (pRI201-AN) (sample 1-5). No amplification was also observed from the host genomic DNA and non-transformed cells. Positive clones were used for further experiments. Reproducibility was confirmed by repeated culture of transformants in both liquid and solid medium with antibiotics and obtained stable transformants, as shown in Fig. 4.

The decline or inhibition of growth was observed with 1000ug/ml and the Host cell's growth declined due to selection pressure after 96 hr at 28°C at all three concentrations, as shown in Fig. 5. The recombinant phytase was purified by Ni²⁺ affinity chromatography. Most phytase activity was observed in fraction eluted with 350mM to 400mM imidazole fraction. Eluted Phytase activity (1.77U/ml) correlated with the protein concentration (0.23mg/ml) is shown in Fig. 6.

DISCUSSION

Phytase is a phosphatase enzyme used as a feed additive to release phosphorus from phytate and helps ab-

**Fig. 3.** Effect of selection Pressure on Recombinant *Schizochytrium* sp. Phytase clones

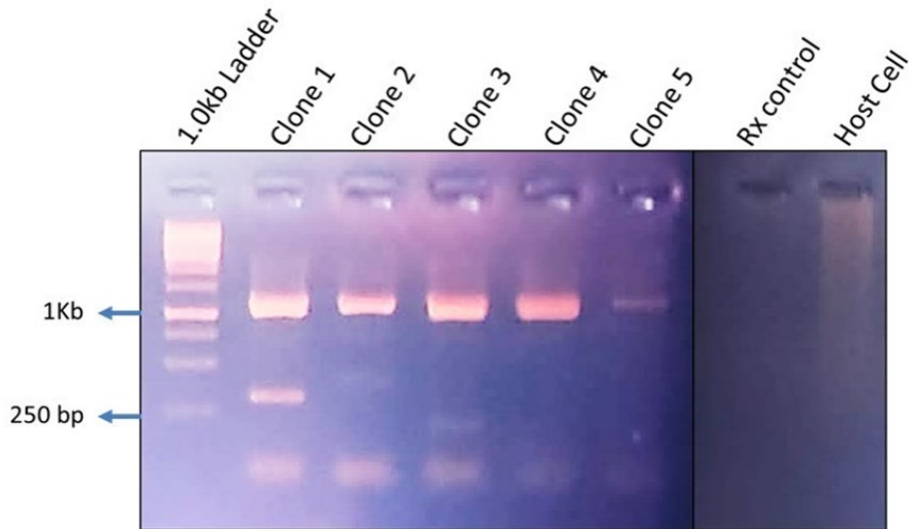


Fig. 4. Colony PCR: 5 colonies were Positive with the expected size of 1.29 kb fragment.

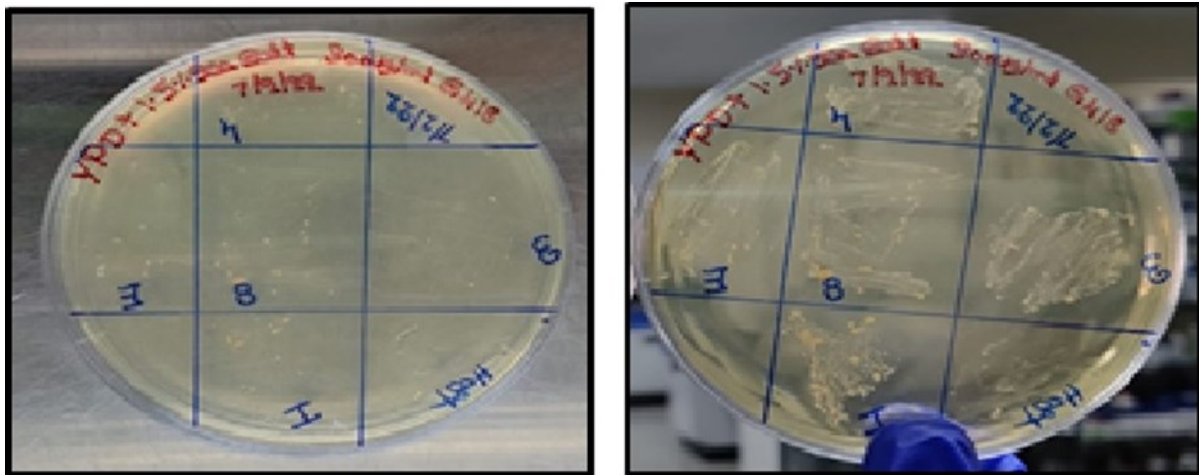


Fig. 5. PCR Positive Clones were subcultured along with Host on YPD agar, 5% sea salt media with 300µg/ml concentration and observed that the Host growth declined due to the selection pressure

sorption in monogastric animals. Phytase hold a revenue share of 83.6% in the global market in the animal feed industry in 2015 and accounted for annual sales of US\$ 350 million (Reddy, 2015). Ranjan and Satyanarayana (2016) reported that phytase was added to about 70% of monogastric animal feed. Saadi, (2021) and Moza (2021) also isolated phytase genes using PCR; the sequences were similar to the present outcomes. Most of the alkaline phytase-coding genes from *Bacillus* species are also identical when correlated with each other (Kim,1998), while phytase-coding genes of Fungi are in the range of 1176 bp to 1638 bp(Corrêa, 2020).

To select an appropriate binary vector for transformation, we first determined the antibiotic sensitivity of *Schizochytrium* sp. The results revealed that 300 µg/mL G418 could fully inhibit the *Schizochytrium* sp. growth on YPD Agar with a 1.5% sea salt plate. Therefore, G418 was expected to be a suitable selec-

tive agent, and the binary vector pRI201-AN encodes the neomycin phosphotransferase II (NPT II) gene which confers resistance to G148 for transformation. Huang (2021) also studied the antibiotic resistance of *Schizochytrium* sp. with G148 and the finding was in line with the present study.

Hence, it was confirmed that the transformants were accumulated within the fatty acids profile of *Schizochytrium* sp. Earlier literature reported that particle bombardment is appropriate for the transformation of *Thraustochytrium aureum* ATCC 34304, *Parietichytrium* sp. TA04Bb, and *Schizochytrium* sp. 204-06 m (Sakaguchi, et al., 2012). Another study by Cheng (2012) reported that the *Agrobacterium tumefaciens* mediated transformation efficiently transferred foreign genes and processes in *Schizochytrium* sp. It was also reported that electroporation had been shown to enable the transformation of algae with the cell wall and cyanobacteria such as *Chlamydomonas reinhardtii*, *Nanno-*

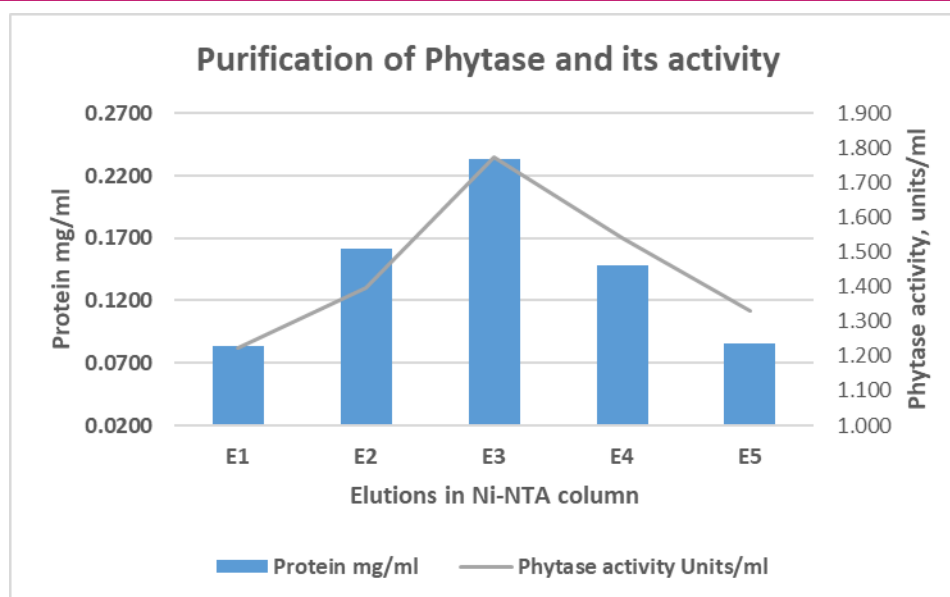


Fig. 6. Purification of Phytase and its activity with protein concentrations

chloropsis sp., *Phaeodactylumtricornutum*, *Nostoc punctiforme*, and *Monoraphidium neglectum* (Shimogawa et al., 1998; Chen, 2019; Zhang, 2014; Holmqvist et al., 2009; Jaeger, et al., 2017). Agitation with glass bead, DNA delivery by PEG is simple, inexpensive and fast. The transformation efficiency (10^3 cells/ μ gDNA) has been reported when the initial cell concentration was (10^8 cell/mL), with (2μ g DNA) (Kindle, 1990).

Schizochytrium sp. grew with 600μ g/ml and reduced stressed growth was observed. These results were corroborated by Zhang et al. (2018) and Fernanda et al. (2016), who used the same strategy to select resistant recombinants. Wang (2019) also picked the transformants that could thrive in highly resistant concentrations. According to the current findings of phytase activity with 1.77U/ml, the Ni-NTA tags utilized in purification had no effect on the phytase enzyme's effectiveness (Dionisio, 2011, Dokuzparmak & Sirin, 2017, Trivedi, et al., 2022). Recombinant phytase exhibited specific activity of 15.59 U/mg, pH of 5.5, and temperature of 35°C . Specific activity was high compared to the previous reports, such as *A. niger* as a famous commercial phytase (specific activity 100 U/mg) (Huang, 2006 Trivedi, et al. 2022). Previously phytase was expressed in *Chlamydomonas reinhardtii*, but they did not purify the protein for activity analysis. This study measured the activity of the partially purified phytase to eliminate any potential effects of crude biomass on the assay. Similar reports were also observed by Hakim (2013) and Padmavathi et al. (2018).

Conclusion

Phytase gene was isolated from *Bacillus* sp. DS11 was cloned and transformed into *Schizochytrium* sp. for

application in the animal nutrition industry as a feed supplement. *Schizochytrium* sp., with high concentration of DHA has been marketed as microalgae for aquaculture feed with brand names AlgaMac and Docosa Gold. Martek produces DHA algal oil from *Schizochytrium* sp, which is known to contain approximately 37% of DHA and 16% of EPA (w/w) and secured GRAS certification (GRN000137) from the US- FDA in 2003 as a food ingredient at 1.5gm DHA/day. Earlier reports revealed that adding 500 to 1,000 units/kg of phytase to feed a diet is equivalent to 1% dicalcium phosphate or 0.18% phosphorus supplementation. Commercial phytase recommendations are about 250 Units per Kg of feed to broiler chicken diets to improve their overall growth and development. In the present study, 72.64 units/5.2 gm of wet biomass was obtained and 1.80% of microalgae will fulfil the phytase requirement and DHA nutrition. Thus, the present study, which emphasized developing a phytase expressing *Schizochytrium* sp, would effectively hydrolyze phytate to inorganic phosphate and fulfil the animal requirements of inorganic phosphate, minerals and omega-3 fatty acids, alleviating phosphorus environmental pollution.

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