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Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oilcakes as substrates

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Abstract

Commercially available oil cakes such as coconut oil cake (COC), sesame oil cake (SOC), palm kernel cake (PKC), groundnut oil cake (GOC), cottonseed oil cake (CSC) and olive oil cake (OOC) were used as substrates for phytase production in solid-state fermentation using three strains of *Rhizopus* spp., namely *Rhizopus oligosporus* NRRL 5905, *Rhizopus oryzae* NRRL 1891 and *R. oryzae* NRRL 3562. COC was the most preferred substrate, in general, for all the three strains; GOC and PKC resulted in comparable enzyme titers with *R. oryzae* NRRL 1891 but CSC and OOC poorly supported the cultures in producing phytase. *R. oryzae* NRRL 1891 produced the highest titers of phytase on COC (30.1 U enzyme per gram dry substrate, U/gds), followed by SOC (28.9 U/gds). Mixed substrate fermentation using COC and SOC in the ratio 1:1 (w/w) further enhanced enzyme production by *R. oryzae* NRRL 1891 to 35 U/gds. An incubation time of 72 h, initial moisture of 52% and an inoculum of 1 ml was the optimum cultural conditions for the production of phytase in mixed substrate fermentation. Supplementation of the fermentation medium with 1% glucose increased phytase activity to 52 U/gds. Addition of ammonium nitrate at 0.5% concentration resulted in further enhancement of the enzyme tires to 64 U/gds. Thus, mixed substrate fermentation using COC and SOC resulted in more than two-fold increase in phytase production under optimized conditions (64 U/gds phytase in comparison to 30.1 U/gds by COC individually). Results obtained appear to be of commercial significance showing the potential of oilcakes and mixed substrate fermentation for phytase production.

Keywords: Mixed substrate fermentation; Oilcakes; Solid-state fermentation; Phytase; *Rhizopus* spp.; Process optimization; Feed enzyme

1. Introduction

Oil meals/oil cakes such as coconut oil cake (COC), sesame oil cake (SOC), cotton seed cake (CSC), groundnut cake (GOC), palm kernel cake (PKC), olive oil cake (OOC), etc. are useful source of protein and energy for livestock. They are commonly used in animal feed, especially for ruminants. India is one of the world's leading oilseeds producers. Total production currently stands at over 25 million tonnes per annum while the exports account for over 4.3 million tonnes of oilmeals, valued at US$ 800 million annually [1]. Oil cakes have high nutritional value, as they possess high protein content (ranging from 15 to 50%). They are economically cheap, stable and dependable sources available in large quantities throughout the year.

COC is obtained from the kernel of coconut fruit. It is a valuable source of energy for monogastrics when it contains a high content of residual oil since they can easily digest short chain fatty acids. Most COC encountered has18–23% protein content [2]. Sesame seeds contain high levels of phytic acid (5%). SOC is being used as a valuable ingredient up to 5% in well-formulated poultry feed due to its rich sulphur amino acid and essential fatty acid content [3]. CSC ranks third in the total oilseed meal produced in the world. It has protein content of 41% and a fibre content of 11% [3]. It lacks essential amino

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acid and is poorly balanced. Good quality GOC is an excellent source of protein. However, there are high chances of mycotoxin contamination in it [4]. PKC has comparatively low protein content among the oil meals. It has high fibre content and poor amino acid digestibility, which restricts its use as animal feed for monogastrics but is best-suited for ruminants [5]

Phytase (EC 3.1.3.8) is a phosphomonoesterase capable of hydrolysing phytic acid to inorganic orthophophate (Pi) and a series of lower esters of myo-inositol, thereby, releasing free myo-inositol [6]. Phytate is a main storage form of phosphorus in legumes, cereal grains and oilseeds [7], which is not readily utilized by monogastrics. When excreted, it is broken down and cause eutrophication in farming areas. The potential problem of phosphorus pollution could be addressed by the nutritional standpoint before the feed becomes manure. An environmentally friendly way could be adopted to reduce the nutrients excreted by the animals, instead of seeking a method for recycling it. Supplementation of phytase to the feedstuff could reduce Pi release and focus on nutritional benefits and aid environmental problems.

Fungal phytase is being widely used as an animal feed due to its high yield and acid tolerance [8] in comparison to the bacterial enzyme. Filamentous fungi are potent tools in solid-state fermentation (SSF) [9–14]. *Aspergillus* species, namely *Aspergillus ficcum* [9], *Aspergillus oryzae* [10], *Aspergillus niger* [11], *Aspergillus fumigatus* [12], *Aspergillus carbonarius* [13], etc. have commonly been used for the production of phytase. Some species of *Rhizopus* spp. such as *Rhizopus oryzae*, *Rhizopus oligosporus* and *Rhizopus stolonifer* [14] also produce phytase. *Rhizopus* spp. has historically been employed in the fermentation of tempeh, an ancient Indonesian food made of soybean. It is also used in the production of cheese, organic acids, etc.

Crude phytase produced by generally regarded as safe (GRAS) strains cultured on feed supplements (oilcakes) in SSF could be fed directly to monogastric animals along with their feed ration. It could serve as a value-added supplement as it not only provides phytase for the animals, but also contain fungal proteins, sugars and some accessory enzymes [15,16].

The objective of this work was to evaluate the potential of different oilcakes such as COC, SOC, CSC, GOC, PKC and OOC for the production of phytase in SSF using strains of *Rhizopus* spp.

2. Materials and methods

2.1. Microorganisms

R. oligosporus NRRL 5905, *R. oryzae* NRRL 1891 and *R. oryzae* NRRL 3562 were used in the study. These were grown on potato–dextrose-agar at 30 °C, stored at 4 °C and sub-cultured fortnightly.

2.2. Substrates

Wheat bran (WB), COC and SOC were obtained from a local market in Trivandrum; GOC and CSC were purchased from markets in Rajkot (Gujarat) and Delhi, respectively. OOC and PKC were procured as gifts from Greece and Malaysia, respectively.

2.3. Preparation of inoculum

Ten millilitres of distilled water containing 0.1% Tween-80 was transferred to a sporulated (7 days old) PDA slant culture. The spores were dislodged using the inoculation needle under aseptic conditions and the suspension, with appropriate dilution was used as inoculum.

2.4. Solid-state fermentation

Five grams of dry substrates were taken into a 250 ml Erlenmeyer flask and to this a salt solution (2 ml) containing (g/l), NH_4NO_3, 5 g; NaCl, 1 g; $MgSO_4{\cdot}7H_2O$, 1 g; and distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121 °C at 15 psi for 15 min. Fermentation was carried out at 30 °C for 72 h with initial moisture of 50% and inoculum size of 1 ml (6×10^7 spores). All the experiments were done in two sets and averages values are reported.

2.5. Dry weight determination

The dry weight of the samples was determined by drying them in a hot air oven at 80 °C for 24 h.

2.6. Enzyme extraction

Crude enzyme was extracted by mixing a known quantity of fermented matter with distilled water containing 0.1% Tween-80 on a rotary shaker (180 rpm) for 1 h. The suspension was then centrifuged at 7000 rpm at 4 °C for 15 min and the supernatant was used for enzyme assay.

2.7. Phytase assay

Phytase activity was determined by the method described by Harland and Harland [17]. The reaction mixture consisted of 1 ml of 0.1 M $MgSO_4{\cdot}7H_2O$, 2.4 ml of 6.82 mM phytic acid (prepared with 0.2 M sodium acetate buffer, pH 5.15) and 0.6 ml properly diluted crude enzyme solution to make the total reaction volume of 4 ml. The reaction was carried out at 55 °C for 60 min in a temperature-controlled water bath. After incubation, 1 ml of the reaction mixture was transferred to a test tube containing 0.5 ml 10% trichloroacetic acid to stop the reaction. To this, Taussky–Schoor reagent (2.5 ml) was added and blue colour developed was measured spectrophotometrically at 660 nm. One unit of phytase is defined as the amount of enzyme releasing

1 μmol of inorganic phosphorus per ml per minute under the assay conditions.

2.8. Estimation of soluble protein

Soluble protein concentrations were determined in the aqueous extract of fermented matter by the method of Lowry et al. [18] using bovine serum albumin as standard.

2.9. Biomass estimation

Fungal biomass estimation was carried out by determining the *N*-acetyl glucosamine released by the acid hydrolysis of the chitin, present in the cell wall of the fungi [19]. For this, 0.5 g (dry wt. basis) of fermented matter was mixed with concentrated sulphuric acid (2 ml) and the reaction mixture was kept for 24 h at room temperature (30 °C). This mixture was diluted with distilled water to make 1 N solution, autoclaved (15 psi for 1 h), neutralized with 1 N NaOH and made to 100 ml with distilled water. The solution (1 ml) was mixed with 1 ml acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 ml) was added followed by the addition of 1 ml Ehrlich reagent and incubated at 65 °C for 10 min. After cooling, the optical density of the reaction mixture was read at 530 nm against the reagent blank [20]. Glucosamine (Sigma) was used as the standard. The results are expressed as mg glucosamine per gram dry substrate (gds).

2.10. Evaluation of substrates and strains of Rhizopus spp. for the production of phytase

Different oilcakes such as COC, SOC, CSC, GOC, PKC, OOC and WB were evaluated for the production of phytase. Fermentation was carried out with conditions as mentioned above. Likewise, phytase production by three different *Rhizopus* spp. was compared.

2.11. Mixed substrate fermentation

The best two substrates that yielded high phytase activity were selected for further experiments. Similarly the best strain was used for mixed substrate fermentation. Thus, COC and SOC were used in combinations (1:1, 1:2, 1:3, 1:4; COC:SOC) for phytase production using *R. oryzae* NRRL 1891.

2.11.1. Optimization of process parameters for the production of phytase

SSF was carried out to study the effect of various physico-chemical parameters required for the optimum production of phytase by *R. oryzae* NRRL 1891 using the mixed substrate comprising COC and SOC (1:1 (w/w)). Studies were carried out to optimize incubation time (24, 48, 72, 96 and 120 h), initial moisture content of the substrate (48, 52, 56, 60, 64, 68 and 72%), inoculum size (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml), effect of supplementation of carbon sources at 1% (w/w) concentration (starch, sucrose, lactose, maltose, glucose and mannitol) and nitrogen sources (organic and inorganic) at 1% (w/w) concentration (peptone, corn steep solids, urea, yeast extract, ammonium sulphate, ammonium nitrate and sodium nitrate).

Since glucose and ammonium nitrate showed a positive influence on enzyme production by the fungal culture, it was thought desirable to explore their suitable concentrations for enhanced phytase yields. Thus, both of these were supplemented in the SSF media at different levels (0.5. 1.0, 1.5, 2.0, 2.5 and 3.0%). Experiments were also conducted to study the influence of supplementation of phosphorus (in the form of KH_2PO_4) at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% concentration.

3. Results and discussion

3.1. Evaluation of individual substrates and strains

Selection of a suitable substrate for the production of enzyme is a primary-key factor and an extremely significant step. Substrates provide the required energy and substratum for the fungus to grow and produce the desired metabolite [23]. The results of this study showed that enzyme production by all the three strains on COC were higher than the other substrates studied (Fig. 1). Maximum phytase activity was obtained when SSF was carried out by *R. oryzae* NRRL 1891 (30.1 U/gds; Fig. 1). SOC resulted 29.8 U/gds phytase, followed by PKC and GOC, which resulted almost comparable yields. However, the strain produced very low titres of enzyme on OOC and CSC, the lowest being with OOC. The capability of a fungus to produce a product in large amount is correlated with the nature and nutrient-availability of the substrate. This was the apparent reason in the above finding. It was interesting to note that the other strain of *R. oryzae*, i.e. NRRL 3562 also yielded high enzyme titres on COC (27.6 U/gds of phytase). *R. oligosporus* NRRL 5905 produced 20.9 U/gds on SOC (Fig. 1).

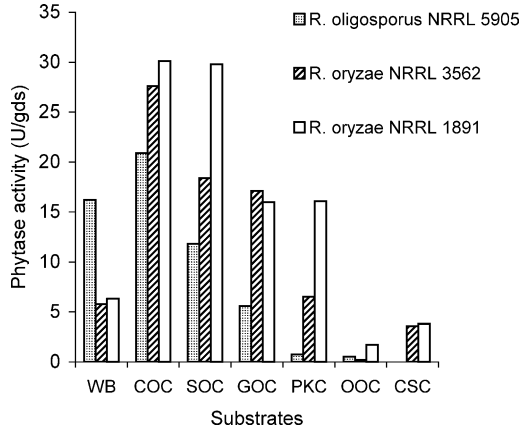

Fig. 1. Evaluation of substrate and strains for the production of phytase.

On GOC, a moderate amount of enzyme was produced, which ranged between 11.8 and 17.1 U/gds by three different strains of *Rhizopus* spp. Comparable results were obtained for PKC (5.6–16.1 U/gds). Interestingly, WB, which has been generally regarded as an ideal substrate for SSF, resulted less enzyme titres in comparison to most of the oilcakes (Fig. 1).

3.2. Mixed substrate fermentation

Mixed substrate fermentation using COC and SOC resulted in an increase in phytase production in comparison to individual substrates (35.1, 28.8, 24.6 and 23.0 U/gds enzyme by mixed substrates at 1:1, 1:2, 1:3 and 1:4 ratios, respectively, in comparison to 30.0 and 29.8 U/gds for COC and SOC individually, respectively, in 72 h; data not shown). Hence, in all subsequent experiments, a mixed substrate consisting of COC and SOC (1:1 (w/w)) was used.

3.3. Biomass growth and phytase production at different periods of fermentation

Fig. 2 shows the evolution of fungal cellular growth as estimated by glucosamine level and phytase production over a period of 120 h. Evidently, there was good correlation between cellular growth and enzyme production and both were maximum after 72 h fermentation (35 U/gds phytase and 57 mg/gds biomass). Incubation beyond this period did not result any further increase in biomass concentration but resulted in decrease in enzyme level. At 120 h, the enzyme activity decreased to 26.09 U/gds. This could be because of the exhaustion of nutrients in the medium.

3.4. Optimization of moisture content and inoculum size for phytase production

Moisture content and inoculum size are the key factors that strongly influence microbial growth and activity in SSF [21,22]. Filamentous fungi, when cultivated on agro-industrial residues during SSF, grow best when the substrate moisture content is generally between 50 and 75% [23]. In the present study, 26.4 U/gds phytase was produced (in 72 h) with substrate initial moisture content of 48%. It increased to 39.5 U/gds with 52% substrate moisture, which was optimum moisture level for phytase production (Fig. 3). Higher moisture levels were not suitable for enzyme production. When initial moisture was 72%, it resulted in drastic reduction in phytase activity (6 U/gds). However, these findings differed widely from the results described by Bogar et al. [15] who found maximum phytase production by *Mucor racemosus* with 75% substrate moisture. Increase in the moisture results in the decrease of enzyme activity, which may be attributed to the phenomenon of flooding of inter-particle space of the substrate. This reduces the growth and proliferation of fungal mycelium [24].

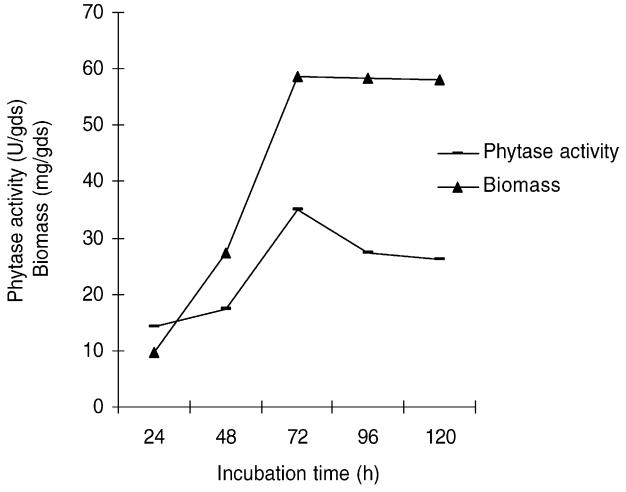

Fig. 2. Phytase activity and growth of *R. oryzae* in mixed substrate fermentation at different time intervals.

An inoculum size of 1 ml (containing 6×10^7 spores) was found to be optimal for phytase production. A decrease in enzyme production was noted when the inoculum size increased (Fig. 3). Enzyme production attains its peak when the nutrients available to the biomass are balanced. Under conditions when there is a misbalance between nutrients and proliferating biomass, it results in decreased enzyme synthesis, which is true with all the microorganisms.

3.5. Effect of supplementation of carbon and nitrogen sources

The impact of supplementation of external carbon and nitrogen sources at 1% concentration on phytase production were studied and the results are shown in Figs. 4 and 5. All the supplemented carbon sources were found to enhance the phytase production. Apparently, the fungal strain had a preferential choice towards monosaccharide, i.e. glucose and produced high titres (maximum) of enzyme (52.7 U/gds). There are other reports, which have also described

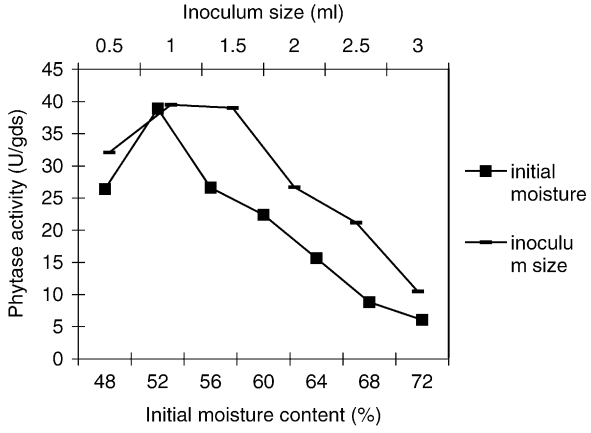

Fig. 3. Optimization of initial substrate moisture content and inoculum size for phytase production.

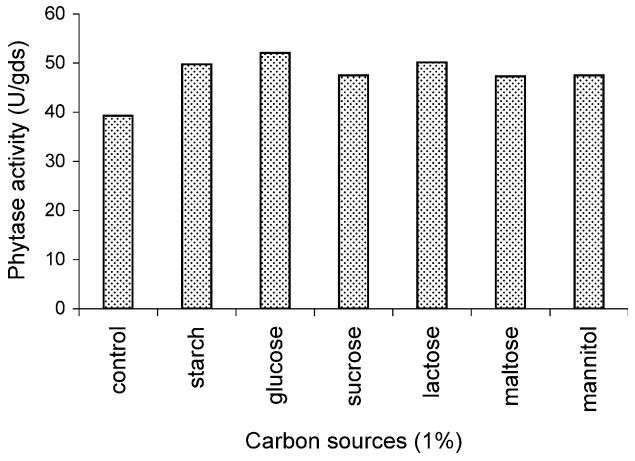

Fig. 4. Effect of supplementation of carbon sources to the mixed substrate on the production of phytase.

similar results [25,13]. Addition of lactose resulted in 50.2 U/gds, while addition of starch resulted in 49.8 U/gds. The remainder of the other carbon sources such as maltose, sucrose and mannitol resulted in the production of 47 U/gds of phytase compared to control 39.3 U/gds (Fig. 4).

Addition of nitrogen sources to the medium showed mixed results on phytase production. Among all the compounds tested, ammonium nitrate was the most suitable as it resulted in the highest enzyme titres (56.5 U/gds). Yeast extract also exerted a similar impact on enzyme production (Fig. 5). Other organic nitrogen sources, viz. peptone marginally enhanced enzyme production but corn steep solid did not show any impact on enzyme production. Inorganic nitrogen sources such as sodium nitrate and ammonium sulphate inhibited enzyme formation by the fungal culture (Fig. 5).

3.6. Effect of concentration of glucose, ammonium nitrate and phosphate on phytase production

Since glucose and ammonium nitrate were found to enhance the enzyme yields, attempts were made to study the impact of their different concentration on phytase production by the fungal culture. Results are shown in Fig. 6. Any decrease or increase in glucose concentration from 1% was not found suitable for phytase production. Higher concentrations of glucose, such as 3% resulted in reduced phytase titres (45 U/gds). Similar findings were reported by other authors [25,26]. However, in the case of ammonium nitrate, reduced concentration, i.e. 0.5% significantly enhanced the enzyme production resulting in 64 U/gds of phytase. With the increase in the concentration, there was gradual decrease in the production of phytase (Fig. 6). Since 0.5% of ammonium nitrate (lowest experimental concentration) was found most suitable, it was thought desirable to further reduce its concentration and study enzyme formation. However, at 0.25% concentration, there was reduction in phytase titres in comparison to 0.5% concentration (data not shown).

Results recorded in Fig. 6 showed that supplementation of SSF medium with external phosphorus as phosphate was not desirable as it suppressed the enzyme production even at lowest experimental concentration. However, Ebune et al. [25] and El-Batal and Karem [27] found that low levels of phosphate enhanced phytase production. These authors had used different raw materials (canola meal and rapeseed meal) as substrate. In the present experiments, apparently the substrates, i.e. COC and SOC had enough P and external supplementation was not required and rather was harmful.

3.7. Time course of phytase activity and biomass at optimized conditions

A time course study was carried out incorporating all the optimized parameters studied as above. The fermentation media contained mixed substrate (COC + SOC, 1:1 (w/w)), supplemented with 1% glucose and 0.5% ammonium nitrate. The enzyme activity was maximum (64 U/gds) at 72 h compared to 35 U/gds phytase activity before optimization (Fig. 7). Similarly, there was increased production of cellular biomass. Soluble protein content in the fermented samples kept increasing during the course of 120 h of SSF and was maximum 132 mg/gds at the end of fermentation (Fig. 7).

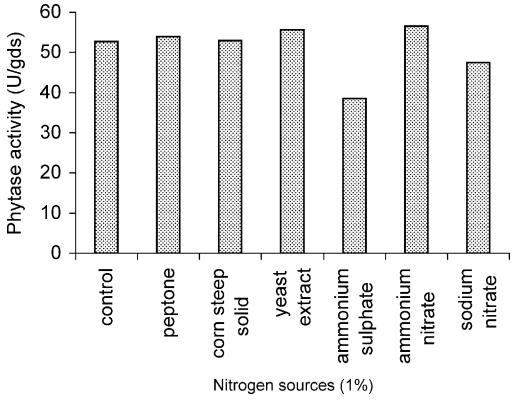

Fig. 5. Effect of supplementation of nitrogen sources to the mixed substrate on the production of phytase.

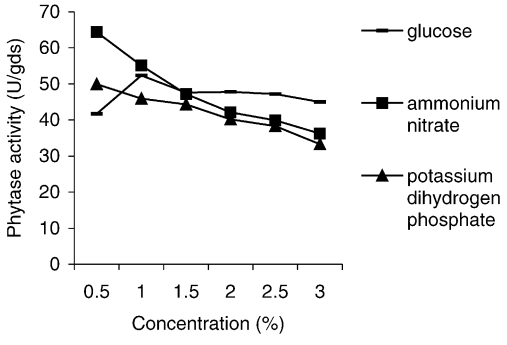

Fig. 6. Effect of addition of different concentrations of glucose, ammonium nitrate and potassium dihydrogen phosphate on the production of phytase.

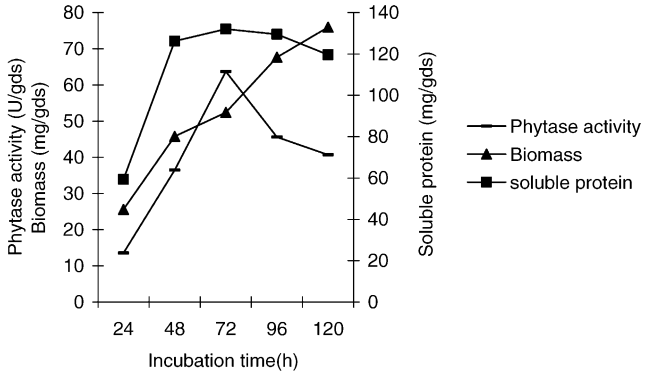

Fig. 7. Growth profile, soluble protein content and phytase activity of *R. oryzae* NRRL 1891 at different periods of incubation.

4. Conclusions

Mixed substrate fermentation employing commercially available oilcakes, viz. COC and SOC showed promising results for phytase production in SSF. Under optimized conditions in SSF, they resulted in more than a two-fold increase in the production of phytase in comparison to the yield by individual substrates. It is envisaged that the fermented matter could be directly fed to the animals (or added as feed ingredient after drying) as it could address the feed digestibility and utilization of phosphorus. We, thus, conclude that oil cakes could be potent substrates for phytase production in SSF.

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