Screening of Agro residues for the Production, Purification and Characterization of phytase enzyme from *Aspergillus ficuum* MTCC 7591

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Abstract: ***This work focus on the production of phytase from Aspergillus ficuum (MTCC 7591), which is one of the industrial important strain and also thermo stable in nature. Agro residues like rice bran (RB), wheat bran (WB), cotton seed oil meal (CSOM), coconut oil meal (COM), peanut oil meal (POM) and sesame oil meal (SOM) were used as a substrate for production of phytase through solid state fermentation (SFF). Among which coconut oil meal (22.4 U/gds & 38.45 U/ gds) shows maximum enzyme activity followed by wheat (17.86 U/gds & 32.56 U/gds) and sesame seed (19.6 U/gds & 25.36 U/gds). Phytase yield was increased with addition of salt solution to the substrate. The mixed substrate of coconut oil cake and wheat bran shows higher activity compare to individual titer value of 43.56 U/gds. The purification was done using ammonium sulphate precipitation and dialysis. The partial purified enzyme was subjected to SDS-PAGE analysis and it was found to be approximately 65kDa.***

Keywords:(WB), (MTCC 7591), (CSOM), (SOM) (SFF). (POM) PAGE

I. INTRODUCTION

One of the important pig and poultry feeding, phosphorus is present in the form of phytic acid and phytate. Phytic acid is the main storage form of phosphorus in cereals, nuts, oil cakes and legumes [1], [2]. Due to its anti-nutritive effect, it chelates the various metal ions like Ca^{2+}, Mg^{2+}, Zn^{2+}, and forms complex with amino acids, proteins [3]. The lack of phytic acid degrading enzymes reduces the absorbing capacity of the phosphorus, protein and other mineral of the monogastric animals [4]. The undigested phytic acid is excreted as an insoluble complex containing various minerals and nutrients from these animals, which contributes mineral deficiency and phosphorus pollution in soil. Rapid algal growth (eutrophication) due to the surface run off reduces the oxygen in the water leading to the death of fish, it tends to affect the food chain.

Phytase is an enzyme used to hydrolysis the phytic acid into their myo-inositol phosphate derivatives that have a novel metabolic effect [5]. Phytase can be produced by various sources like bacteria, fungi and yeast. In which *Aspergillus* sp plays main role in product formation through SSF process. The produced enzyme was acid and thermo

tolerance with higher yield compare to submerged fermentation [6].

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In this work *A.ficuum* was used for the enzyme production. The main objective of this work is to screen various agro-residues like wheat bran (WB), rice bran (RB), coconut oil meal (COM),sesame oil meal (SOM), cotton oil cake (CSOM), and peanut oil meal (POM) for high production of phytase enzyme, purified and characterized the enzyme for subsequent application in food industry.

II. MATERIALS AND METHODOLOGY

Screening of microorganism for phytic acid degradability

The organism was grown in PSM media consist of 1.5% agar with varying concentration of Sodium phytic acid (0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.6. The inoculated culture plate were kept at 30ºC for 48 hrs, from which the strain was selected, as a result of the maximum zone of clearance [7], [8]**.** The plates were incubated at The quantitative experiments were conducted with 50 ml of PSM broth, with different concentration of Phytic acid sodium salt (0.1%-0.6% w/v) was taken in 250 ml of Erlenmeyer flask [9]**.** Autoclave at 121ºC for 15 min is done for complete sterilization and the flasks were cooled, inoculated with spore suspension of *A.ficuum* (MTCC7591). The supernatant was collected and assayed for extracellular phytase activity [10], [11]**.**

Raw material selection and analysis of protein and reducing sugar

Agro residues like rice bran, wheat bran, cotton seed oil meal, coconut meal, peanut meal and sesame oil were purchased from local market in Tiruppur district. The raw material was ground and sieved using a 40mm mess sieve. The soluble protein content, reducing sugar content was measured[12], [13].

Solid state fermentation and enzyme extraction

Experiments was carried out using Erlenmeyer flasks (250 ml) containing 5 g of agro residue to which 1 ml of a salt solution containing 5 g/L of NH_4NO_3, 0.5 (1:1) g/L of K_2HPO_4/Na_2HPO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, and 0.5 g/L of NaCl [8] is added. The autoclaved flasks were incubated with spore suspension at 30°C. 50 ml of 0.1% Tween 80 which is added to the fermented residue was used for the extraction of enzyme. Flasks were mixed well using magnetic stirring for 30 min and the suspension was filtered and centrifugation. The phytase activity for the obtained supernatant (crude extract) was assayed[14]**.**

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Enzyme activity

The phytase enzyme activity was measured by the ammonium molybdate blue method [15] with some modifications. 8% sodium phytic acid is used as a substrate to determine enzyme activity. The reaction was carried out at an optimum temperature of 50ºC, pH of 5.0 with duration of 30 min. The absorbance was measured in UV-spectrophotometer at 380 nm. Under standard assay conditions, one unit of phytase activity is be defined as the amount of enzyme that liberates 1 µ mol of inorganic phosphate per min [16], [17].

Mixed substrate fermentation:

The WB and COM served as the best substrate for phytase production. This two substrate and a mixture of this two (1:1 w/w) were taken to estimate the enzyme production.

Purification and characterization of the enzyme

Phytase enzyme extracted from fermented media of *Aspergillus ficuum* was filtered through double layer muslin cloth. In order to remove the debris, the resulted filtrate was centrifuged at 10,000 rpm for 15 minutes at 4ºC. Ammonium sulfate were added to precipitating the protein followed by dialysis against distilled water. The dialyzed sample was passed through the DEAE cellulose [19]**.** The purified enzyme was subjected to SDS-PAGE; the gel gets stained with Coomassie brilliant blue R-250. The molecular weight standards were co-electrophoresed [20], **.** and de-staining solution (25% methanol and 10% glacial acetic acid) until a clear background was obtained that was changed at 3-4 h interval [5]**.**

III. RESULT AND DISCUSSION:

Aspergillus ficuum (MTCC 7591) was subcultured using potato dextrose agar and maintained in agar slants and plate. Initially, it showed white color mycelia and later it changed to dark green colored dense mycelia mat in the slant and agar plate. The colonies produce asexual conidio spores by which the organism reproduces itself and the white apron of hyphal growth was observed at the initial stage of log phase and the packets of conidio spores of dark green appearing above the whitish base on the agar plate.

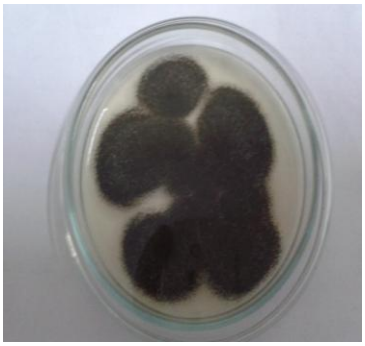

Fig. 1 Subcultured *Aspergillus ficuum* MTCC 7591 on potato dextrose agar slant and plate.

Screening of organism for phytase production

Screening of organism was done using PSM media consist of varying concentration of phytic acid (0.1%-0.6%) with 1.5% agar were incubated at 30ºC for 48 hrs.

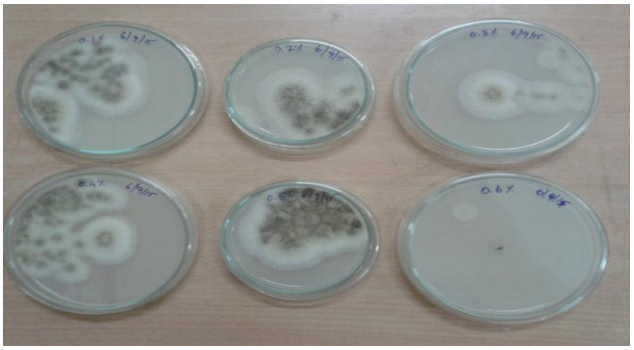

Fig. 2 *Aspergillus ficuum* MTCC 7510 cultured grown in different concentration of phytic acid.

In the qualitative analysis, the culture was grown in PSM media with different concentration of phytic acid sodium salt (0.1%-0.6% w/v). There was a gradual increase in the growth of microbes but in 0.5% of phytic acid concentration was observed maximum zone of clearance while in 0.6% there was no growth of organism due to inhibition of phosphate**.**

Optimization studies using phytic acid:

For quantitative analysis, the culture was grown in PSM media without agar. The inoculated submerged sample was taken after 72 hrs and tested for phytase enzyme activity. The phytase enzyme activity was increased with a further increase in phytic acid concentration. In submerged fermentation, the phytase enzyme activity was higher in 0.5% of phytic acid added to the medium but in case of 0.6% phytic acid concentration, there is a sudden decrease in phytase activity due to feedback inhibition. Dephosphorylated products inhibit the phytase enzyme activity by binding to the active site**.**

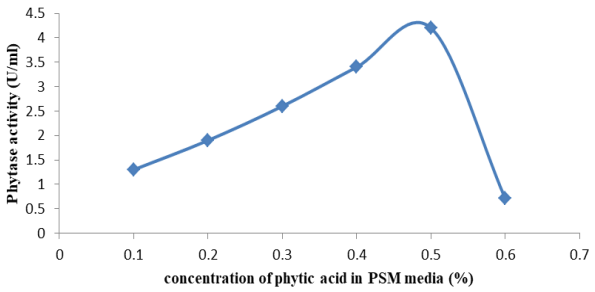

Fig. 3 Phytase activity in PSM media with various concentration of phytic acid

Agriculture residues soluble protein estimation by Lowry's methods

Lowry's is one of the most widely used methodologies to find out protein concentration of unknown samples. Protein content present in agriculture residue was found out by Lowry's test using BSA as a standard.

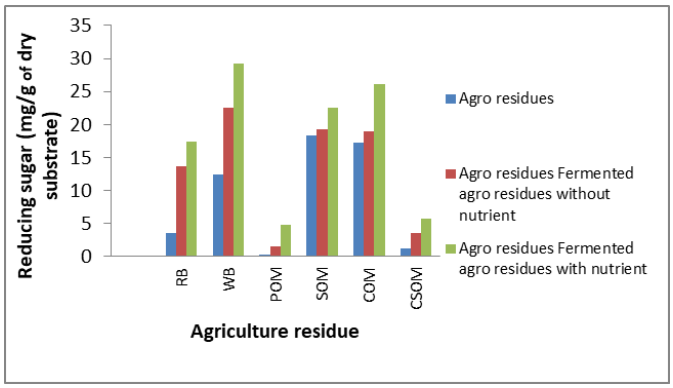

Fig. 4 Protein content present in agriculture residues

The order of protein content present in agriculture resides fermentation with nutrients COM > WB > POM >SOM >CSOM> RB. The protein content was high in coconut oil cake and wheat bran compare to other agriculture residues. It was found that in raw agriculture residues soluble protein content was less compared to fermented agriculture residues without and with nutrients. After fermentation, the protein content was high due degradation of phytic acid which tends to release chelated proteins and extracellular enzyme. Added nutrients tend to improve the microorganism growth thereby increased protein content was observed [21], [22].

Reducing sugar estimation by DNS method

Aspergillus ficuum has grown on carbon-rich substrates like monosaccharide and polysaccharides. The reducing sugar present in agro-residues enhances the growth of the organism and the result showed as the order of WB> COM > SOM >RB >CSOM> POM.

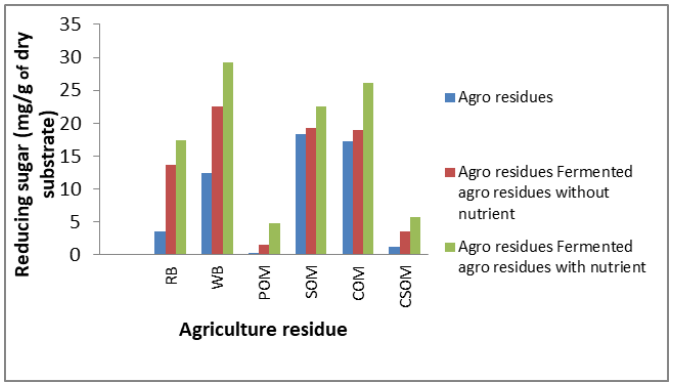

Fig. 5 Reducing sugar content of agriculture residues

Phytase activity after solid state fermentation:

The agriculture residues were taken as a solid substrate to grow *Aspergillus ficuum*, after 72 hrs the enzyme was extracted and tested for phytase enzyme activity.

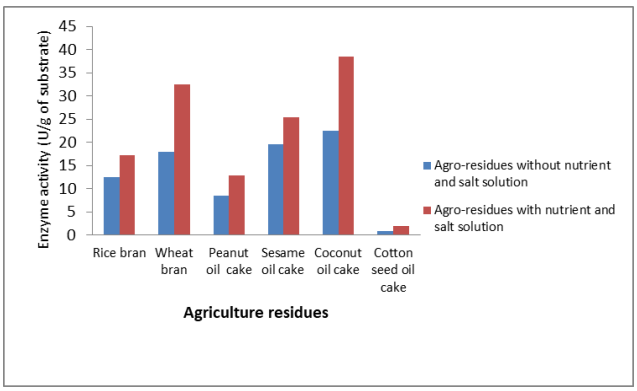

Fig. 6 Phytase activity in fermented agro-residues

Six agro-residues were taken for substrate screening among which coconut oil cake shows maximum enzyme activity followed by wheat and sesame seed.

The order of substrate suitability was COM > WB >SOM >RB >POM >CSOM.

COM is a valuable source for the production of enzyme compare to all other residues due to presence of short chain fatty acids even the few reports suggested the use of wheat bran [21]. On the other hand, our work found the maximum yield using coconut meal compare to wheat bran and it was also reported by Bogar *et al* in 2003 [23]. COM produce good anchorage for the organism growth with the higher yield of biomass and phytase enzyme activity is high in case of coconut oil cake compare to all other substrates [24], [25]

In Sesame oil cake is rich in sulfur amino acid, essential fatty acid and phytic acid content. So the biomass production and the phytase activity are high. Peanut cake is rich in protein. However, mycotoxin contamination is of high chances in it. Maybe due to mycotoxin presence, it restricts the microorganism growth on the substrate thereby it limits the phytase enzyme production [26].

Cotton oil seed cake contains 40% of fiber and 11% of protein content and it is poorly balanced due to the lack of essential amino acid. As a result, the organism growth and phytase production are less compared to all other substrates.

Estimation of biomass in solid state fermentation:

Table. 1 Biomass concentration in fermented agro-residues

Agro residues	Agro-residues without nutrient and salt solution (mg/gds)	Agro-residues with nutrient and salt solution (mg/gds)
Rice bran	144	168
Wheat bran	182	216
Peanut oil meal	133	153
Sesame oil meal	231	246
Coconut oil meal	247	254
Cotton seed oil cake meal	152	170

Mixed substrate fermentation for phytase production:

From the earlier study, it was confirmed that wheat bran and coconut oil meal served as the best substrate for phytase production. This two substrate and a mixture of this two (1:1 w/w) were taken to estimate the enzyme production. Among which mixed substrate of wheat bran and coconut oil cake showed a higher titer value of enzyme compare to the individual titer value.

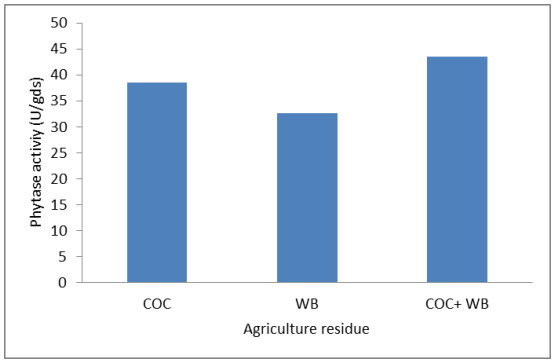

Fig.7 Phytase activity on various substrates

Characterization of phytase enzyme

The molecular weight of phytase enzyme:

The subunit molecular weight and homogeneity of the purified phytase was estimated by SDS-PAGE.

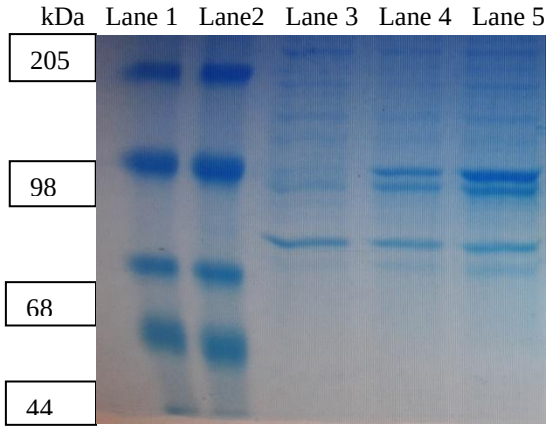

Fig. 8 Electrophoresis of purified phytase by SDS-PAGE analysis – molecular weight marker standards (Lane1, Lane 2), partially purified enzyme (Lane 3), Crude enzyme(lane 4&5).

From this result, it could be regarded as homogeneous. It reveals that the molecular weight of the purified phytase was approximately to be 65 kDa. Due to glycosylation property, phytase enzyme from the filamentous fungi would have various ranges of molecular weight between 65-120 kDa [21]. From the obtained results, it can be concluded that the purified phytase from *A.ficuum* MTCC has undergone glycosylation. *A.ficuum* NTG-23 organism reported the molecular weight of 65.5 kDa.

IV. CONCLUSION:

The present report supported the superiority of coconut oil cake in comparison with the wheat bran for phytase synthesis. The phytase enzyme was purified by various analytical techniques and SDS-PAGE was performed. The molecular weight of the produced phytase enzyme was 65 kDa. The growing economic stress on animal feed can be overcome by producing it from agriculture residues as a substrate and it would be environmental friendly approach. Microbial source offer techno economical feasibility for the production of phytase and its application.

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