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Enhanced phytase production by *Aspergillus niger* mutants in solid state fermentation

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Abstract

The present research work was conducted to improve the phytase production by genetic alteration of *Aspergillus niger* with induced mutagenesis using solid state fermentation. Strain improvement was carried out in the presence of ultra violet (UV) irradiation and ethyl-methane sulphonate (EMS) [0.5% v/v] treatments for various time intervals. We reported an improved strain of *Aspergillus niger* designated as UV-3 mutant producing a zone of hydrolysis of about 40 mm, in comparison to wild strain (26 mm). The highest enzyme activity was found to be 547.64 IU/g for UV-3 mutant followed by EMS-4 mutant (492.23 IU/g) compared to wild strain which showed 406.45 IU/g of enzyme activity. There was 1.35-fold increase in phytase production after mutation studies of *Aspergillus niger*. Phytase was applied as poultry feed additive and given to broiler chickens for 5 weeks. The results exhibited that there was increase in body weight gain (BWG) of chicks for experimental group (2028 g) in comparison to control group (1903 g). Thus, physical and chemical mutagenesis was proved as an effective technique for the improvement of strain and ultimately for enhanced and economical phytase production for different industrial applications.

Keywords: *Aspergillus niger*; induced mutagenesis; phytase production; solid state fermentation; UV irradiation & EMS treatment.

1. Introduction

Phytic acid (*myo*-inositol hexakis-phosphate) is the major storage form of organic phosphorus. It is found in legume seeds, cereal grains, nuts and oilseeds. These seeds and grains are the major ingredients of commercial animal feeds (Shah *et al*., 2009; Sandhya *et al*., 2015; Kumar *et al*., 2017). Monogastric animals like poultry and fish, do not have adequate quantity of intrinsic phytases in their digestive tract to degrade the phytic acid complexes present in their feed. As a result, the undigested phytate phosphorus is released by these animals as waste material in their fecal matter which creates severe environmental pollution and eutrophication in water bodies (Singh & Satyanaryana, 2010; Yao *et al.,* 2011; Sandhya *et al*., 2015).

Moreover, phytic acid makes insoluble complexes with proteins and many important metal ions i.e. magnesium, calcium, iron and zinc and, thus reduces the bioavailability of these vital nutrients and acts as an anti-nutritional factor (Rao *et al*., 2009; Bakri *et al*., 2018).

Phytases (*myo*-inositol hexakis-phosphate phosphohydrolase) (EC 3.1.3.8 and 3.1.3.26) are the enzymes that belong to a sub-class of histidine acid phosphatases. Phytase produces different substances i.e. lower *myo*-inositol phosphate, free inorganic phosphate (Pi) and potentially chelated minerals, and in some cases, free *myo*-inositol by catalyzing the hydrolytic splitting of phytic acid in stepwise reactions. When phytase is added in poultry feed, more inorganic phosphorus and other vital minerals can become available. The amount of phosphorus, excreted through the manure can also be minimized (Rasul *et al*., 2019; Zaheer *et al*., 2019).

Phytases have very profound role in the animal feed and various food industries because these enzymes enhance the digestion and absorption of phosphorus and certain other poorly available nutrients including copper, manganese, iron and zinc in the monogastric diet supplements (Munir & Maqsood, 2013; Vasudevan *et al*., 2017) and play important role in increasing the body weight gain (BWG) and growth performance of these animals. Phytases also decrease the concentration of phosphorous in the animal's excrement, which can otherwise cause environmental pollution. Phytases as animals feed additive can thus be used as substitute for expensive di-calcium phosphate and reduce the cost of animal feed (Dahiya, 2016; Jatuwong *et al*., 2020).

Phytase has been obtained from different organisms like fungi, bacteria, yeasts, plants and animals. Among various fungi, filamentous fungi like *Aspergillus niger* is being investigated as an excellent source of phytase. It has ability to grow on different agro-wastes under solid state fermentation process and can produce cost effective phytases (Salmon *et al*., 2012; Singh *et al*., 2015; Ahmad *et al*., 2017; Bakri *et al*., 2018).

In the last few decades, there is an increasing demand of phytase in different fields particularly as an animal feed additive, therefore, the ultimate goal is the cost-effective production of phytase by hyper secretory strains (Shah *et al*., 2009). For this purpose, serious attentions are required towards qualitative and quantitative improvement methods. Quantitative enhancement methods include the exploitation of different mutagens for improvement of strain and optimization of fermentation conditions for the higher yield of enzyme, as the amount of enzyme produced by wild culture is low (Bhaskaran *et al*., 2018).

In view of industrial applications, the objective of current investigation was to enhance the phytase production by strain improvement of filamentous fungus *Aspergillus niger* through mutagenesis technique using two different mutagens (UV irradiation and Ethyl methane sulphonate).

2. Materials and methods

2.1. Procurement and maintenance of microbial cultures

Different microbial strains i.e. *Penicillium commune*, *Rhizopus oligosporus*, *Aspergillus niger*, *Trichoderma viride* and *Saccharomyces cerevisiae* were obtained from the Microbiology Laboratory, PCSIR Laboratories complex, Lahore. These microbes were preserved on Potato

Dextrose Agar (PDA) slants at 4°C. For culturing, a loop full of spores from pure microbial culture was transferred into each Potato Dextrose Agar (PDA) slant aseptically and incubated at 35ºC for 5-7 days in an incubator. The slants showing suitable growth of microbial strains were selected for subsequent studies.

2.2. Inoculum preparation

For preparing inoculum, sterilized distilled water (10 ml) was added into five days old culture slant and the fungal mycelia were scrapped with the help of inoculation loop, under aseptic conditions. To homogenize the spore suspension, it was agitated mildly for 2 minutes. A hemocytometer was used to adjust the number of spore (1×10^7 spores/ml).

2.3. Screening of microbial strains and fermentation media

For the screening purpose, five microbial strains i.e. *Penicillium commune*, *Rhizopus oligosporus*, *Aspergillus niger*, *Trichoderma viride* and *Saccharomyces cerevisiae* were cultured on ten different fermentation media (Table 1). The best microbial strain and fermentation medium were selected for maximum phytase production using solid state fermentation process.

Table 1. Composition of the different fermentation media (M) for phytase production

Ingredients (g)	**Fermentation medium (M) (g)**									
	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M 10**
Wheat Bran	100	-	-	-	-	-	-	-	-	-
Rice Bran	-	100	-	-	-	-	-	-	-	-
Cotton Seed Meal	-	-	100	-	-	-	-	-	-	-
Wheat Straw	-	-	-	100	-	-	-	-	-	-
Rice Polish	-	-	-	-	100	-	-	-	-	-
Soybean Meal	-	-	-	-	-	100	-	-	-	-
Corn Cobs	-	-	-	-	-	-	100	-	-	-
Corn Husk	-	-	-	-	-	-	-	100	-	-
Rice Straw	-	-	-	-	-	-	-	-	100	-
Rice Husk	-	-	-	-	-	-	-	-	-	100
Urea	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$MgSO_4.7H_2O$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
KCl	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$FeSO_4.7H_2O$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

2.4. Conditions for UV and EMS Mutagenesis

Spore suspension of *Aspergillus niger* was prepared by serial dilution method from 5 days old fungal slant. From 7th dilution (10^{-7}), 1 ml of the spore suspensions were poured in the uncontaminated petri plates and exposed to UV irradiation (240 nm) at a distance of 15 cm for

variable time durations i.e. 15, 30, 45, 60, 90 and 120 minutes in a UV chamber except for control (without UV irradiation treatment).

For chemical mutagenesis, a stock solution of 0.5% (V/V) Ethyl methane sulfonate (EMS) was prepared. 1 ml of spore suspension from 7th dilution (10^{-7}) and 1 ml of EMS (5 µl/ml) solution were mixed in various test tubes and incubated for various time intervals (3, 6, 9, 12 and 24 hrs) at room temperature. Potato Dextrose Agar (PDA) in molten form (45°C) was then poured into each petri plate containing mutated spore suspensions under sterilized conditions and placed in an incubator at 37°C for five to seven days until sporulation of fungal culture. The petri plates showing least percentage of survival rates of fungal colonies were chosen for further studies.

2.5. Selection of best mutant strain for enhanced phytase production

All the selected colonies of *Aspergillus niger*, after various mutagenic treatments, were streaked on the phytase screening medium (PSM) plates. Composition of PSM is given in Table 2. These plates were incubated for three to five days at 37°C and zone of hydrolysis were observed after every 24 hours. The selection of hyper-secretory mutants was made on the basis of enhanced zone of hydrolysis after same incubation period and by analyzing enzyme activity after solid state fermentation batch.

Table 2. Composition of phytase screening medium (PSM)

Ingredients	Concentrations (g/L)	Ingredients	Concentrations (g/L)
Glucose	5	$FeSO_4.7H_2O$	0.01
Phytic acid	3	KCl	0.5
NH_4NO_3	5	$MnSO_4.4H_2O$	0.01
$MgSO_4.7H_2O$	0.5	Agar	20

2.6. Fermentation studies

The fermentation medium (10 g rice polish, 0.5% NH_4NO_3, 0.1% KCl, 0.1% $FeSO_4.7H_2O$ and 0.1% $MgSO_4.7H_2O$) was taken in 250 ml Erlenmeyer flask, after amended with 10 ml distilled water. The medium was autoclaved for sterilization at 121°C for 15 min. After cooling at room temperature, 10% (v/w) of fungal inoculum was added, and kept in an incubator at 37°C for five days.

After incubation, 0.2 M citrate buffer, 50 ml (pH 5.5) was poured to each fermented flask, placed in a water bath shaker at 37°C and shaken for 1.5 hours at 200 rpm. Double layered muslin cloth was used for filtration and centrifugation of filtrate was done at 10,000 rpm for 15 min in a centrifuge machine. Supernatant was collected as crude enzyme extract and phytase activity was measured.

2.7. Phytase assay

Phytase activity was measured by estimation of inorganic phosphorus released from substrate (sodium phytic acid solution), according to the modified methods of McKie & McCleary (2016). Reaction mixture, containing 1% (w/v) phytic acid solution (pH 5.5) and enzyme extract 0.2 ml each, was taken in a test tube and placed in a water bath for 15 min at 37°C.

Then, 0.4 ml of trichloroacetic acid (TCA) 15%, was added to stop the reaction. Afterwards, 2 ml colour reagent was added in the test tube containing above mixture (0.2 ml) and 1.8 ml double distilled water, and incubated at 50°C for 15 min. Absorbance of the liberated inorganic phosphorus was measured at 655 nm.

One-unit phytase activity is defined as the quantity of enzyme required to release 1 μ mole inorganic phosphorus per ml per min using the standard assay conditions.

2.8. Application of phytase as poultry feed additive

The phyatse was checked as poultry feed additive and its effect on the growth performance of broiler chickens was analyzed.

For this purpose, a 5-week feeding trial containing 30 chicks was undertaken with three dietary treatment groups i.e. T0 (control group) without any phytase in the basal diet**,** T1 with phytase @ 1000 IU/Kg diet, T2 with phytase @ 2000 IU/Kg diet. Composition of basal diets given to birds g/100 g, maize 65.65, soybean meal 24, corn gluten meal 3, soybean oil 3, lime stone 1.2, dicalcium phosphate 1.6, vitamin-mineral premix 1, salt 0.3, lysine 0.15, methionine 0.1. Phytase was mixed in liquid form in the feed. Phytase produced from *Aspergillus niger* using solid state fermentation was used as feed supplement for the better growth of chicks.

2.9. Statistical analysis

All the experiments were set up in a completely randomized design (CRD) with three replicates. Analysis of variance of all parameters were computed using COSTAT computer package (CoHort Software, 2003, Monterey, California).

3. Results

The present study includes the screening of microbial strains and fermentation media. It also includes mutational studies of *Aspergillus niger* for enhanced and cost-effective production of phytase using solid state fermentation. The outcome of this study revealed improved and economical production of enzyme by mutant strains compared to wild type.

3.1. Screening of microbial strains and fermentation media for phytase production

Different microbial strains and fermentation media were screened for maximum phytase production. Results shown in Figure 1 indicate that *Aspergillus niger* and M5 fermentation medium produced maximum phytase.

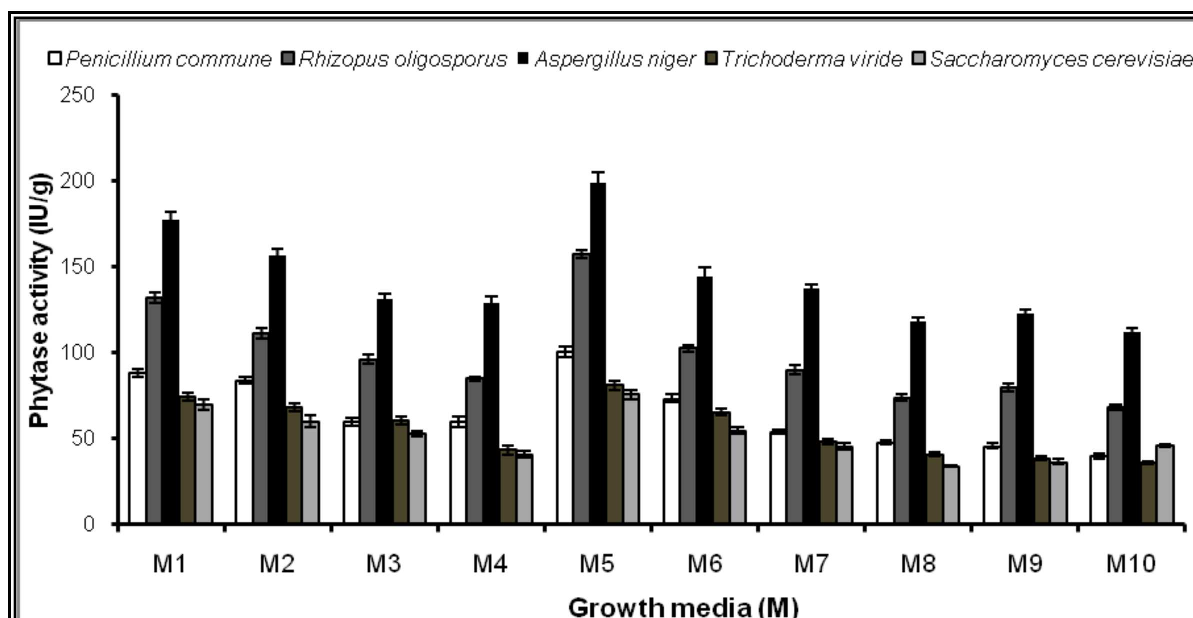

Fig. 1. Screening of microbial strains and fermentation media for phytase production

3.2. Mutagenesis by UV irradiations and EMS treatments

For physical and chemical mutagenesis, the wild strain of *Aspergillus niger* was characterized by exposing to different doses of resistance to UV irradiation i.e. 15-120 min and to EMS treatment for different duration of time i.e. 3-24 hrs, in separate experiments. The results indicated that the survival rate of fungus decreased gradually, it was 77.55% after 15 min and reduced to 8.16% and 2.04% after 90 min and 120 min of exposure time, respectively for UV irradiation (Figure 2 a), whereas for EMS treatment, a survival rate of 81.57% was achieved after 3 hrs, and it became 10.52% after 12 hrs and decreased to 0% after 24 hrs of exposure time (Figure 2 b).

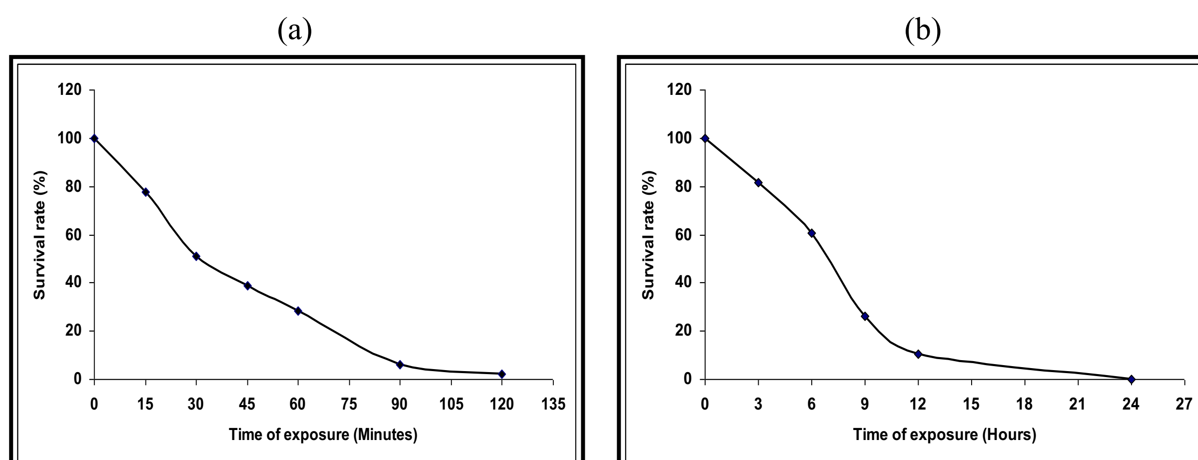

Fig. 2. Survival curves for colonies of *Aspergillus niger* at different exposure time of (a) UV radiations & (b) EMS treatment

3.3. Screening of best mutant strain of *Aspergillus niger* for best phytase production

All the selected colonies of *Aspergillus niger* with minimum rate of survival were streaked on the phytase screening medium (PSM) plates and put in the incubator for 3-5 days at 37°C. The selection of best mutants was carried out on the basis of large zones of hydrolysis. Among UV mutants, largest zone of hydrolysis of 40 mm was formed by UV-3 followed by UV-1 (34 mm) and UV-5 (30 mm) than control (26 mm) as shown in Table 3 & Figure 3. Among EMS mutants, EMS-4, EMS-2 and EMS-1 producing 37 mm, 32 nm and 27 mm zones of clearance, respectively (Table 3 & Figure 4).

Table 3. Comparison of zones of clearance produced by wild and UV & EMS mutant strains of *Aspergillus niger*

Sr. No.	Strains	Zones of clearance (mm)
	Wild	26
1	UV-1	34
2	UV-2	29
3	UV-3	40
4	UV-4	21
5	UV-5	30

Sr. No.	Strains	Zones of clearance (mm)
	Wild	26
1	EMS-1	27
2	EMS-2	32
3	EMS-3	ND
4	EMS-4	37

(UV: Ultra violet irradiations), (EMS = Ethyl methane sulphonate; ND: Not detectable)

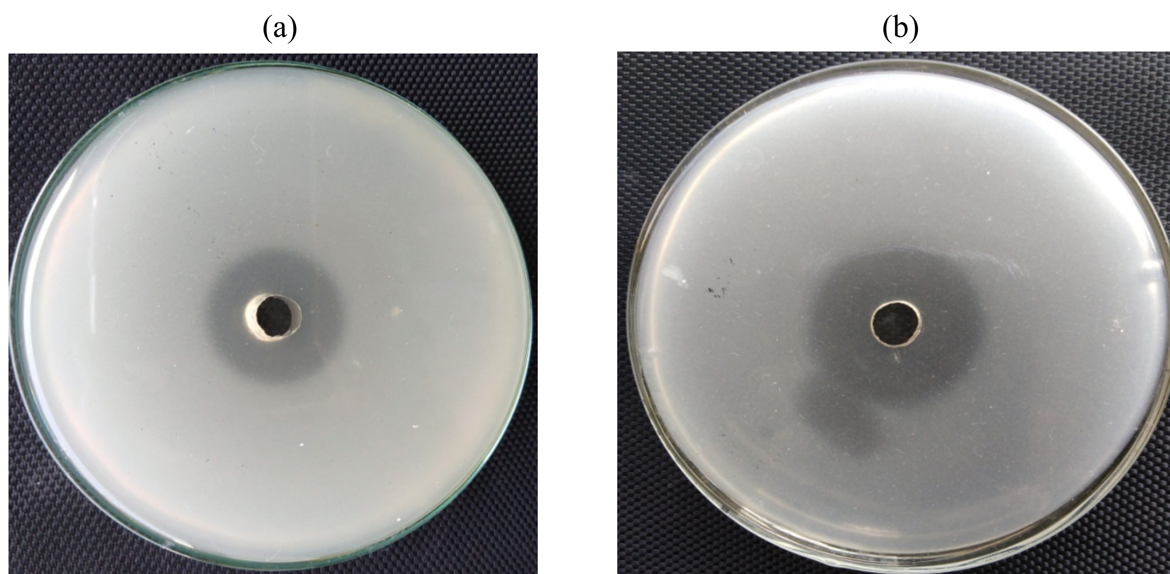

Fig. 3. Zones of clearances shown by (a) control (wild strain) and (b) UV-3 mutant on phytase screening medium after incubation at 37°C for 3-5 days

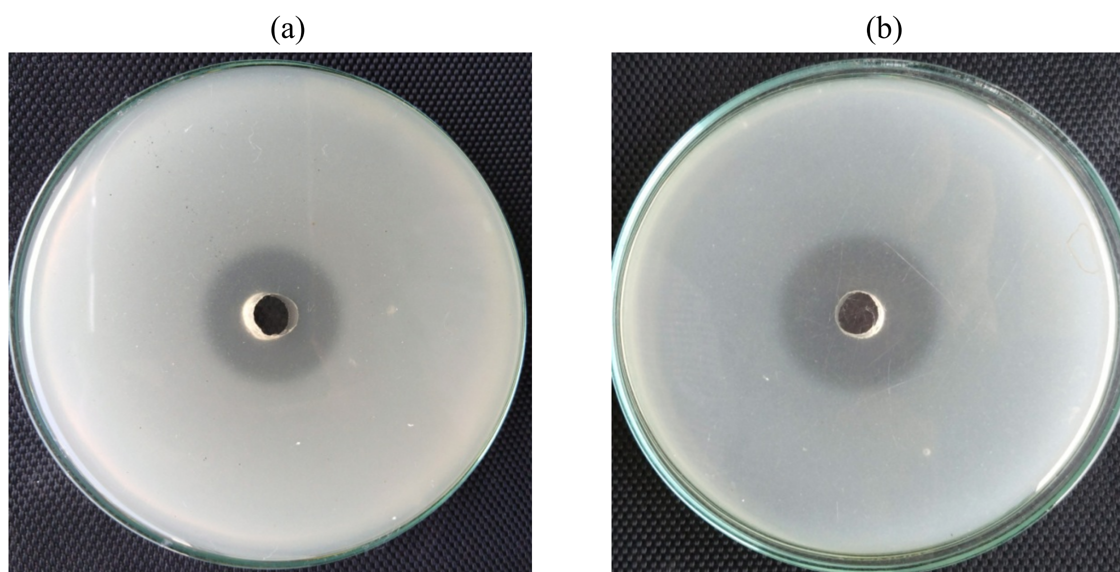

Fig. 4. Zones of clearances shown by (a) control (wild strain) and (b) EMS-4 mutant on phytase screening medium after incubation at 37°C for 3-5 days

3.4. Compararison of phytase production by wild and selected mutant strains of *Aspergillus niger*

All positive mutants (selected on the basis of large zones of hydrolysis on phytase screening medium) were further studied for their potential to produce maximum phytase enzyme by solid state fermentation process at pre-optimized culture conditions and best phytase producer was find out. Among the various positive mutants, the maximum production of phytase was (547.64±7.27 IU/g) given by UV-3 mutant followed by EMS-4 (492.23±5.59 IU/g) and UV-1 (481.54±7.14 IU/g) compared to wild strain (406.45±6.18 IU/g) (Figure 5).

Thus, UV-3 mutant designated as *Aspergillus niger* UV-3 was found to be most hyper secretory (547.64±7.27 IU/g) mutant strain amongst all mutated and wild strains (406.45±6.18 IU/g) as shown in Figure 5. It gave about 1.35-fold increased phytase production compared to the wild strain using similar fermentation conditions. A comparison of other mutagenesis reports with current study is given in Table 4.

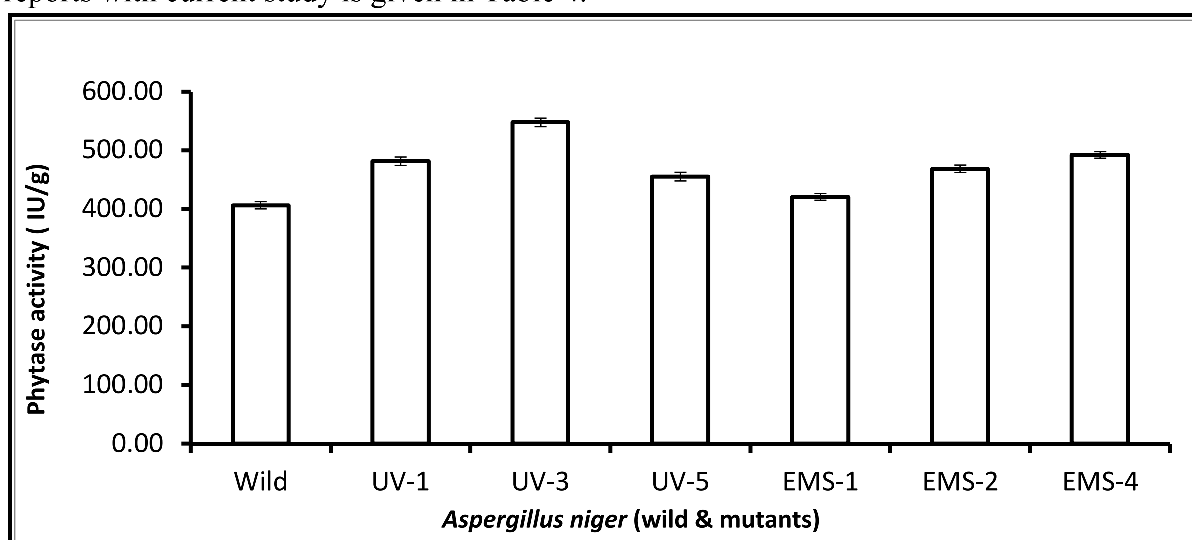

Fig. 5. Comparison of enzyme activities of wild and mutant strain of *Aspergillus niger*. Bars represent standard errors.

Table 4. Comparison of other mutagenesis reports with current study

Mutagens	Microorganisms	Phytase activity		References
		Wild	Mutant	
UV irradiation, Ethyl methyl sulfonate (EMS)	*Aspergillus niger*	406.45 IU/g	547.64 IU/g	Our current study
UV irradiation, Ethyl methyl sulfonate (EMS)	*Aspergillus niger*	6,181 IU/L/D	9,523 IU/L/D	Shah *et al*., 2009
UV irradiation, Ethyl methyl sulfonate (EMS)	*Rhizopus oligosporus*	6.2 U gds^{-1}	31.3 U gds^{-1}	Suresh & Radha (2016)
UV irradiation, Ethidium bromide, Hydroxyl amine	*Aspergillus niger* NCIM1359	254,500 U/l	407,200 U/l	Bhavsar *et al*., 2012
Gamma radiation and Ethyl methyl sulfonate (EMS)	*Sporotrichum thermophile*	6185 U/l/d	19875 U/l/d	Mehmood *et al*., 2019

3.5. Determination of the impact of phytase on the growth performance of broiler chickens

Body weight gain (BWG) of broiler chickens was recorded at weekly interval. Table 5 describes the average body weight gain of chicks after 5 weeks of their age. The results indicated that birds in group T3 had highest average body weight gain (2028 g) compared to other groups.

Table 5. Body weight gain (BWG) g/bird of broiler chickens after 5 weeks

T0	T1	T2
1868	1933	1984
1876	1941	1997
1885	1948	2010
1893	1960	2021
1905	1968	2025
1917	1977	2039
1911	1984	2047
1934	1993	2055
1922	2001	2067
1919	1995	2035
Average 1903	**1970**	**2028**

T0 = Control group (Basal feed without phytase), T1 = with phytase @ 1000 IU/Kg diet, T2 = with phytase @ 2000 IU/Kg diet

4. Discussion

Aspergillus niger was selected as the best strain amongst all the above-mentioned microbial strains for the maximum phytase production using solid state fermentation (SSF) process. Production of phyatse was also obtained by *Aspergillus ficuum* SGA and *Aspergillus niger* CFR 335 (Shivanna & Venkateswaran, 2014) and many other species of genus *Aspergillus* reported by Singh *et al*. (2015); Buddhiwant *et al*. (2016); Thakur *et al*. (2017); Neira-Vielma *et al*. (2018). In the present study, *Aspergillus niger* produced a highest amount of phytase when it was grown in M5 fermentation medium (Rice polish, 0.5% NH_4NO_3, 0.1% $MgSO_4.7H_2O$, 0.1% KCl, 0.1% $FeSO_4.7H_2O$).

In industrial biotechnology, microorganisms have become a major source for the primary and secondary metabolites production, as well as for the synthesis of enzymes and recombinant proteins. Extensive strain improvement studies were carried out, in the beginning by classical mutagenesis and at present by advanced genetic manipulation techniques.

In the current study, mutation of the wild strain of *Aspergillus niger* was carried out in an attempt to find out such an improved hyper-secretory strain which can produce more enzyme. For physical and chemical mutagenesis, different doses of UV irradiations and EMS treatments were given to the wild strain of *Aspergillus niger*. It was noted that the lethality was increased with the increase in dose of these mutagens, and there was decrease in the number of surviving fungal colonies. Several research workers reported that the number of fungal colonies decreases as UV irradiations exposure time increases (Irfan *et al*., 2011; Suresh & Radha, 2016; Castillo *et al*., 2017).

EL-Bondkly & Keera (2007) performed experiments for mutation of *P. roquefortii* and used different doses of EMS mutagen (100 µl/ml). The results indicated that as the mutagen dose increased, the lethality was also improved. They obtained 20.8% and 7% survival rate after 30 and 60 min of EMS exposure time, respectively.

The selected fungal colonies with lower survival rates were streaked on phyatse screening medium (PSM) plates and fungal strains with large zones of hydrolysis were selected and confirmed for enhanced phytase production in solid state fermentation conditions. In many mutation-based research works, the selection of mutants was carried out due to large zones of hydrolysis on sodium phytic acid medium compared to wild strain (Shah *et al*., 2009; Suresh & Radha, 2016).

Among all hyper secretory mutants, *Aspergillus niger* UV-3 mutant exhibited maximum phytase activity (547.64±7.27 IU/g) compared to wild strain (406.45±6.18 IU/g) and improved phytase yield of about 1.35-fold. A mutagenesis study was conducted using UV irradiation and EMS treatment by Suresh & Radha (2016) for obtaining hyper secretory fungal strain. The results showed that among all the mutant strains, *R. oligosporus* MTCC 1116 (0.3% EMS) depicted 31.3 gds^{-1} phytase activity at 116 hrs, and increased the yield of about 4 fold compared to the wild strain with ADT27 Rice bran variety. Shivana & Govindarajulu (2009) treated *Aspergillus niger* CRF 335 in physical and chemical mutagenesis and found improvement in phytase productivity of mutant strain from 0.85 to 1.26 U mg^{-1}.

Mehmood *et al*. (2019) carried out mutagenesis studies on a fungal strain *Sporotrichum thermophile* using gamma rays and EMS treatment for enhanced phytase production. They reported an improved fungal strain ST20, which showed maximum enzyme activity of 19875 U/l/d compared to wild (6185 U/l/d), with an increase in enzyme productivity of 3.2 time that of wild strain.

Biotechnological application of phytase was performed. Phyatse was used as poultry feed additive and its affect on body weight gain (BWG) of chicks was determined. For this purpose, one control group (T0) without phytase in the feed and two experimental groups (T1, T2) with varying concentrations of phytase in the feed were made (as mentioned in text 2.8). The experimental work was carried out for 5 weeks. The results shown in Table 5 revealed that average body weight gain (BWG) of chicks in experimental group T2 (2028 g) was highest compared to the control group (1903 g).

For biotechnological application of phytase, the effect of phytase was studied on the growth performance of broilers by pre-treating corn-soya diets with microbial phytase by Jatuwong *et al*. (2020). They reported that there was an increase of availability of 60% inorganic phosphorus when microbial phytase was given to broilers fed on low phosphorus diet and phosphorus concentration in the chicken manure was decreased by 50%. The results also indicated the 5.8-13.2% increase in body weight of chickens, after 21 days of phytase supplementation in their diet.

It is obvious from the above-mentioned results that phytase has positive effect on the growth performance of broiler chicken when it was supplemented as poultry feed additive. Where it improves the nutrient contents of the diet by degrading phytic acid and release inorganic phosphorous and important minerals in the poultry feed. Therefore, phytase produced by *Aspergillus niger* using solid state fermentation can be employed successfully as poultry feed supplement.

5. Conclusion

In the present research work, UV irradiation and EMS mutagenesis of *Aspergillus niger* was performed for enhanced and low cost phytase production. UV-3 mutant of *Aspergillus niger* was found as hyper secretory strain that could produce maximum enzymatic activity (547.64 IU/g) compared to wild strain (406.45 IU/g). Due to induced mutagenesis, there was about 1.35-fold of increase in phytase production. Growth performance of broiler chickens was improved (1903 g/bird to 2028 g/bird), when phytase was used as poultry feed additive. Thus, strain improvement strategies, using mutagens like UV irradiation and EMS, were proved to be competent techniques for cost effective and higher-level production of phytase by *Aspergillus niger* using solid state fermentation.

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