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Screening of Phytase Overproducing Strains in *Aspergillus* spp. by UV Mutagenesis

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Phytases (*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8) are enzymes which catalyze the hydrolisys of phytate into myo-inositol and inorganic phosphates. Phytases are found in plants and a variety of microorganisms. *Aspergillus* species were treated with 254 nm of UV irradiation for the screening of phytase overproducing mutant strains. At 15 minute irradiation, the survivals of population were less than 5%, and UV irradiation time was decided at 20 minute for the isolation of mutant strains. Four UV mutant strains in *A. oryzae* (YUV-47, -169, -341, -511) and six in *A. ficuum* (FUV-17, -36, -69, -193, -317, -419) were isolated on PSM media containing ammonium phosphate. The specific enzyme activities of *A. ficuum* mutants are 110 to 140% higher than that of wild type.

KEYWORDS: ***Aspergillus ficuum, Aspergillus oryzae*, Phytase, UV irradiation**

Phosphate is required as the mineral sources in organisms. It is one of essential elements for plants, and acts on most biochemical reaction of animal in the body. The main form of phosphate is called as phytate, an organic chemical compounds. Phytate is the major phosphate storage form of plant seeds, such as corn, oilseed, meal, cereal grains, legumes, which are the principle components of animal feeds (Wang *et al.*, 1980). Monogastric animals, such as pig and poultry are unable to metabolize phytate and largely excrete it in their manure. The presence of phytate in animal feeds for chickens and pigs is also undesirable since the phosphate moiety of phytate act as an anti-nutritional agents by chelating essential minerals, possibly protein and vitamin, rendering the nutrients unavailable (Maga, 1982). This consequently contributes to phosphorus pollution problems in areas of intensive livestock production. Many researches have been processed for the removal of phytate in many directions; heating, autoclaving (Chang *et al.*, 1977), soaking (De Boland *et al.*, 1976), fermentation (Mandal *et al.*, 1972), and chemical treatment (Brooks and Morr, 1984), but none of them are successful. The enzymatic hydrolysis of phytate into less-phosphorylated *myo*-inositol derivatives is desirable. Attempts to enzymatically hydrolyze phytate have been made to improve the nutritional value of feed and to decrease the amount of phosphorus excreted by animals (Bitar *et al.*, 1972). Phytase (*myo*-inositol hexakis phosphate phosphohydrolase, EC 3.1.3.8) is enzyme which catalyzes the hydrolysis of phytate into *myo*-inositol and inorganic phosphates (Nelson *et al*,. 1968; Ullah, 1988b; Fig. 1). Phytases are found in plants and in a variety of micro-organism including *Bacilli*, yeasts and filamentous fungi (Shieh and Ware, 1968; Howson and Davis, 1983). Addition of phytase in livestock enhances phosphate utilization from phytate and reduces orthophosphate excretion of animals. Phytase production of *Aspergillus* species, *A. oryzae and A. ficuum*, are influenced by nitrogen source, ammonium ions, especially ammonium phosphate, are markedly superior to nitrate for the phytase production. In this studies, phytase high-producing strains were isolated by UV irradiation with filamentous fungi *Aspergillus* species, *A. oryzae* and *A. ficuum*, and analyzed their characteristics.

Materials and Methods

Strain and medium

Strains were *Aspergillus oryzae* (YTH-1, *arg*B mutants; Hahm and Batt, 1988) and wild type of *Aspergillus ficuum* (NRRL 3135). For the growth of strains and spore harvest, YPD media (yeast extract 10 g/L, peptone 20 g/L, D-glucose 20 g/L) and YPS media (yeast extract 10 g/L, peptone 20 g/L, sucrose 20 g/L) and minimal media, Czapek-Dox media (bacto sacchrose 30 g/L, sodium nitrate 3 g/L, di-potassium phosphate 1 g/L, magnesium sulfate 0.5 g/L, potassium chloride 0.5 g/L, ferrous sulfate 0.01 g/L) were used. For the phytase activity assay, PSM media (Phytase Screening Media, D-glucose 15 g/L, calcium phytate 5.0 g/L, $MgSO_4$-$7H_2O$ 0.5 g/L, KCl 0.5 g/L, $FeSO_4$-$7H_2O$ 0.5 g/L, $MnSO_4$-$4H_2O$ 0.01 g/L, agar 15 g/L, nitrogen source; $NH_4H_2PO_4$ 5.0 g/L) were

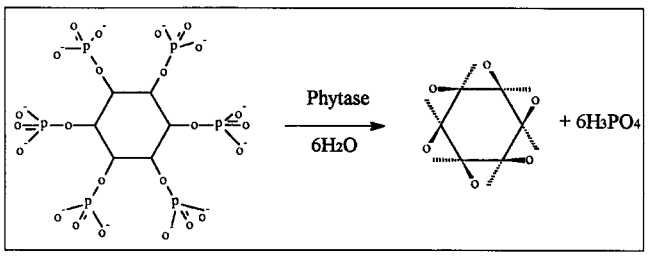

Fig. 1. Hydrolysis of phytate into *myo*-inositol and inorganic phosphates with phytase.

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used.

UV irradiaton

For the isolation of phytase overproducing strains in *A. oryzae* and *A. ficumm,* around 10^6/m*l* conidia were irradiated with UV light of 254 nm at a distance of 20 cm. Proper irradiation time was selected after survived populations were analyzed at every 5 min irradiation. UV-treated spores of *A. oryzae* and *A. ficumm* were spreaded on PSM media (Marisa and Rudy, 1994).

Plate assay for phytase

Phytase plate assays were performed on PSM media adjusted pH 5.5. UV-treated spores were cultured at 30°C for 2~3 days and phytase high producing mutants, which produced larger clear zone on PSM media than control, were selected. They transferred to YPD media for 1 day and each one was analyzed on PSM media at 30°C after one day incubation.

Culture media assay for phytase

Selected mutant strains of *Aspergillus* were cultured in YPD broth at 30°C for 3 days and then mycelia were recovered by filtration. Mycelia were incubated in PSM broth at 30°C for 2 days. The culture media were obtained by filtration. Phytase activities in culture media were performed as follow. Add 10 μl of crude culture media and 420 μl of substrate solution with 2 mM sodium phytate (0.1848 g of sodium phytate in 100 m*l* of 0.1 M Tris-Cl, pH 7.0) in reaction tube and mixed well. After reacted on 37°C for 30 min, reactions were stopped with 500 μl of of 5% TCA solution and incubated in ice for 10 min. 4 m*l* of reagent A (10% ascorbic acid, 2.5% ammonium molybdate, 6 N H_2SO_4) was added and reacted on 37°C for 30 min. After that, sample were analyzed by spectrophotometry (DU 650, Beckman, U.S.A) at 820 nm (Shimizu, 1992; Engelen *et al.*, 1994). Standard curve were composed with inorganic phosphate (K_2HPO_4) by Fiske-Subbarow's methods (Fiske and Subbarow, 1925).

Protein assay

Protein assay was perfomed by Bio-Rad protein microassay methods (Bradford, 1976, Ca #500-0006, BIO-RAD). 800 μl of sample and 200 μl of 5X dye regent were mixed and reacted for 5 min at room temperature. Absorbance at 595 nm was measured with Microplate Reader (Ca #550, Bio-Rad). Standard curve were composed with bovine serum albumin (BSA).

Results and Discussion

UV irradiation

When *A. oryzae* and *A. ficuum* conidia were treated with UV irradiation at 254 nm at a distance of 20 cm, their populations reduced about 70% for 5 min irradiation. At 15 min irradiation, the populations decreased about 93~96%, and after that, no more decrease was observed (Fig. 2). Therefore, UV irradiation time was decided at 20 min for the isolation of phytase high-producing mutant strains.

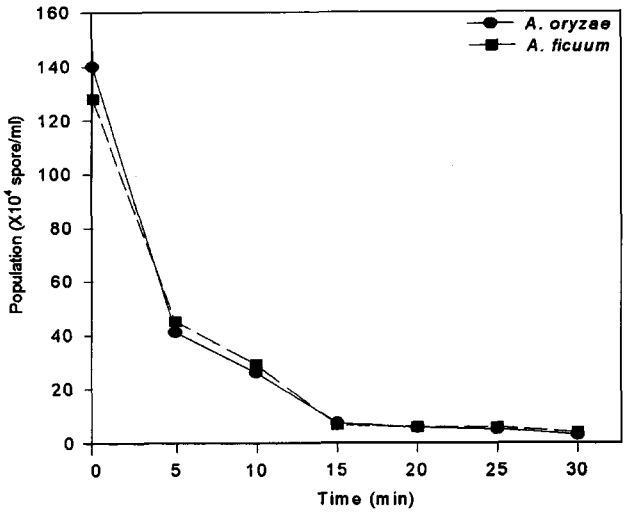

Fig. 2. Survival curve of *Aspergillus* spores by UV-irradiation.

Screening of phytase overproducing mutants

To isolate phytase overproducing strains in *A. oryzae* and *A. ficuum*, UV treated mutants were analyzed by plate assay. Four phytase overproducing mutants, YUV-47, -169, -341, and -511 in *A. oryzae* and six mutants, FUV-17, -36, -69, -193, -317, and -419 in *A. ficuum* were isolated. According to the phytase plate assay, mutants of *A. oryzae* had about 120~135% of higher enzyme activities than YTH-1 strain and mutants of *A. ficuum* had about 140~170% of higher enzyme activities than wild type (Table 1). In the results of analysis on enzyme activity with culture media of selected

Table 1. Ratio of phytase plate assay

Strain	Mutants	Colony (cm^2)	Clear zone (cm^2)	Ratio	%
A. oryzae	YW[a]	8.04	12.57	1:1.56	100
	YUV-47	9.12	14.82	1:1.63	125.8
	YUV-169	9.00	14.60	1:1.62	123.6
	YUV-341	9.62	15.72	1:1.63	134.7
	YUV-511	9.10	15.00	1:1.65	130.2
A. ficuum	FW	2.23	8.13	1:3.65	100
	FUV-17	2.97	11.87	1:3.90	150.8
	FUV-36	3.14	12.57	1:4.00	158.8
	FUV-69	3.57	13.87	1:3.89	174.6
	FUV-193	3.15	11.65	1:3.70	144.1
	FUV-317	3.18	12.98	1:4.08	166.1
	FUV-419	2.87	12.17	1:4.24	157.6

[a]YW; *A. oryzae* YTH-1, YUV-#; *A. oryzae* UV mutant number, FW; *A. ficuum* NRRL3135, FUV-#; *A. ficuum* UV mutant number.

Table 2. Phytase specific enzyme activity

Strains	Conc. of secreted protein (mg of protein)	Specific enzyme activities (μM of monophosphate/min/X)		
		Secreted protein (mg of protein)	Wet mycelium (g of wet mycelia)	Culture media (ml of culture media)
YW[a]	0.0576	10.19	0.05	0.013
YUV47	0.0883	6.94	0.046	0.0143
YUV169	0.089	6.13	0.044	0.0127
YUV341	0.0778	8.47	0.045	0.014
YUV511	0.0675	8.75	0.046	0.0141
FW	0.0458	14.34	0.066	0.0134
FUV17	0.0387	16	0.068	0.0135
FUV36	0.0528	11.46	0.055	0.0138
FUV69	0.0552	16.36	0.114	0.0188
FUV193	0.0568	10.06	0.112	0.0146
FUV317	0.0473	15.02	0.099	0.0171
FUV419	0.0363	16.76	0.082	0.0148

[a]YW; *A. oryzae* wild type, YUV-#; *A. oryzae* UV mutants number, FW; *A. ficuum* wild type, FUV-#; *A. ficuum* UV mutants number.

mutants, FUV-69 mutant of *A. ficuum* showed highest enzyme activity. In *A. oryzae,* mutants showed a little higher actiities than YTH-1 strain. Between *A. oryzae* and *A. ficuum,* none of *A. oryzae* mutants showed the higher activities than *A. ficuum* mutants.

Secreted total proteins were analyzed with culture media of selected mutants. All of *A. oryzae* mutants, YUV-47, -169, -341, and -511, secreted more proteins than YTH-1 strain and *A. ficuum* and its mutants. *A. oryzae* mutants secreted about 120 to 150% of more proteins than YTH-1 strain and *A. ficuum*'s mutants, about 110~125% more proteins than wild type. *A. oryzae* mutants showed higher growth rate than YTH-1.

Based on the enzyme activities per mg of secreted protein, *A. ficuum* mutants, FUV-17, -69, and -419 showed higher enzyme activities than wild type. In contrast with *A. ficuum* mutants, *A. oryzae* mutants didn't show higher enzyme activities than YTH-1. It was due to higher growth rate and more secreted proteins in *A. oryzae* mutants. Similar results were obtained with the wet mycelia weight basis. When the enzyme activities of mutants were analyzed based on the ml of culture media, *A. ficuum* mutants, FUV-69 and -317, had higher enzyme activities than wild type and *A. oryzae* mutants YUV-47 had slightly higher enzyme activity than YTH-1 strain (Table 2).

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