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Original Research article

GAMMA RAYS MUTAGENESIS OF A*SPERGILLUS NIGER* FOR HYPERPRODUCTION OF MUTAROTASE

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ABSTRACT

Pure culture of wild type of *Aspergillus niger* was subjected to gamma rays mutagenesis at different dose rates (40 to 120 k.Rads). The mutant strains of *Aspergillus niger* were isolated and selected by random screening method. The selected mutant derived strains were compared with parent type of *Aspergillus niger* for enhanced production potential of mutarotase. A mutant strain of *Aspergillus niger* with maximum production potential of mutarotase was finally selected. The enzyme activity of wild type strain was 4.33 U/mL whereas the mutant derived strain showed significantly higher enzyme activity (22.0 U/mL). It is concluded that gamma irradiation of *Aspergillus niger* can be used for hyperproduction of mutarotase for its ultimate use in clinical diagnostic kits.

Keywords: *Aspergillus niger*, Gamma rays, Mutagenesis, Hyperproduction of mutarotase

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INTRODUCTION

Mutarotase (aldose-1-epimerase) is a key enzyme of carbohydrate metabolism that catalyzes the inter-conversion of α- into β-anomers of hexoses such as glucose and galactose [1]. It allows the rapid conversion of α-D-glucose to β-D-glucose. The enzyme is a monomer with molecular weight of 40,000 kDa and works at a broad pH range of 4-8 [2]. However, the optimum pH for its maximum activity is 7.4 at 40-43 °C [3]. The specific activity of mutarotase was found to be 1.5 times higher in the nuclei (122 U/g dry weight) than that in the cytoplasm (80 U/g dry weight) when isolated with non-aqueous solvents. It is suggested that mutarotase may be involved in the metabolism of D-glucose in the nuclei [4]. The major

sources used for the production of mutarotase are: *Penicillinum notatum*, *Escherichia coli* [2] and *Aspergillus niger* [5].

The capacity of filamentous fungi is one of the key features in considering them as a host for producing higher value recombinant therapeutic proteins [6]. Toyoda *et al*. (1983) reported the activity and specific activity of crude rat kidney mutarotase as 6.05 U/mg and 0.57 U/mg, respectively [3]. Zia (2002) reported that the activity and specific activity of crude bovine cortex was 0.529 U/mL and 0.227 U/mg, respectively [7]. Varalakshmi *et al.* (2009) screened five fungal isolates for the production of α-amylase using both solid-state and submerged fermentation [8]. They observed that the best amylase producer among them was *Aspergillus niger* JGI 24 strain, which yielded the maximum enzyme production by solid-state fermentation (SSF) on wheat bran. Further attempts to enhance the enzyme production by UV induced mutagenesis showed that the survival rate decreased with increase in duration of UV exposure. The partial purification of the enzyme using ammonium sulphate fractionation resulted in 1.49 fold increase in the enzyme activity. Gamma rays have been used for the mutagenesis of *Aspergillus niger* for hyperproduction of amylase as well as for industrial enzyme fermentation [9, 10]. However little is known about their effects on the hyperproduction of mutarotase. The present study was conducted to investigate the impact of gamma rays mutagenesis of *Aspergillus niger* to obtain mutants with hyper-production of mutarotase.

MATERIALS AND METHODS

The study was conducted in the Enzyme Research Laboratory, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan. The test microorganism used in the study was wild type of *Aspergillus niger* [11]. The stock culture was maintained on potato dextrose agar (PDA) media (containing potato starch 2.0g, glucose 2.0g, agar 2.0g, urea 0.3g, $ZnSO_4.7H_2O$ 0.001g, KH_2PO_4 0.008g, KCl 0.015g, $MgSO_4.7H_2O$ 0.05g, in 100 ml medium). The pH of the media was adjusted at 4.0 with 1M HCl /1M NaOH solution.

Strain improvement technique

Mutagenesis was carried out using gamma rays produced from Cobalt-60 (Co^{60}) as a source of gamma radiation. Vogel's media (KH_2PO_4 0.5g, NH_4NO_3 0.2g, $(NH_4)_2SO_4$ 0.4g, $MgSO_4$ 0.02g, peptone 0.1g, trisodium citrate 0.5g, yeast extract 0.2g, glucose 50% (w/v), in 100 ml of media), having pH 5.5, was prepared and spores of *Aspergillus niger* were transferred with the help of a syringe in the flaks containing Vogel's media. These flasks were kept on rotary shaker at 150 rpm at 30°C for 72 hours and the growth was confirmed by slide test.

Selection of potential doses for mutation

Five different doses of gamma radiation were selected, which were 40, 60, 80, 100, and 120 k.Rad (0.4 to 1.2 kGy). As the impact of dose depends on time, so 40, 60, 80, 100 and 120 k.Rads treated samples were collected after, 1, 2, 3 and 4 days. After treating spores with mutagen, 10 fold dilutions of spores were spread on PDA plates and kept in an incubator for 72 hours. After the corresponding time period, the colonies were counted and the survival curve was drawn. Colonies were restricted with Triton X-100 (2%), used in PDA media. 2-deoxy-D-glucose was used as a selective marker in PDA media, which is a structural analogue of glucose, for the selection of hyper-producing mutant strains.

Mutarotase Enzyme Assay

Spore suspension was prepared by mixing the plate culture with 5ml water. Two ml of each spore suspension was seeded into 100 ml of fermentation media having pH 6.0 (sucrose 3g, potato starch 5g, yeast extract 0.2g, $NaNO_3$ 0.2g, $MgSO_4.H_2O$ 0.05g, $CaCl_2$ 1 g in 100 ml of media). These flasks were incubated on rotary shaker at 30°C for 24 hours. Growth of the fermentation media was homogenized, centrifuged and the supernatant was used as enzyme sample.

At time zero, 0.1 ml of enzyme solution was added into 9.9 ml of EDTA buffer and then exactly 100 mg of α-D-glucose was rapidly dissolved in this mixture. The rotation was determined at 1 minute intervals for 10 minutes and then after 5 minutes interval until 30 minutes and lastly at 15 minutes interval until its rotation became constant [7]. The enzyme activity was calculated and compared with the activity of wild type strain of *Aspergillus niger*. The activity was calculated from the standard mutarotase curve prepared by adding fixed amounts of alpha-D-glucose (Fig. 1). The data so collected was subjected to statistical analysis and the mean values for native wild type and mutagenized strains were compared [12].

RESULTS AND DISCUSSION

The γ-rays induced mutagenesis of pure wild type *Aspergillus niger* was carried out to obtain the mutant strains for hyperproduction of mutarotase.

Formation of Kill curve

Initially a kill curve was prepared using gamma radiation using Co^{60} as a source of mutagenesis. An optimum gamma-ray dose was calculated at which 80% killing or alternatively 20% survival was obtained. The results are shown in Table-1.

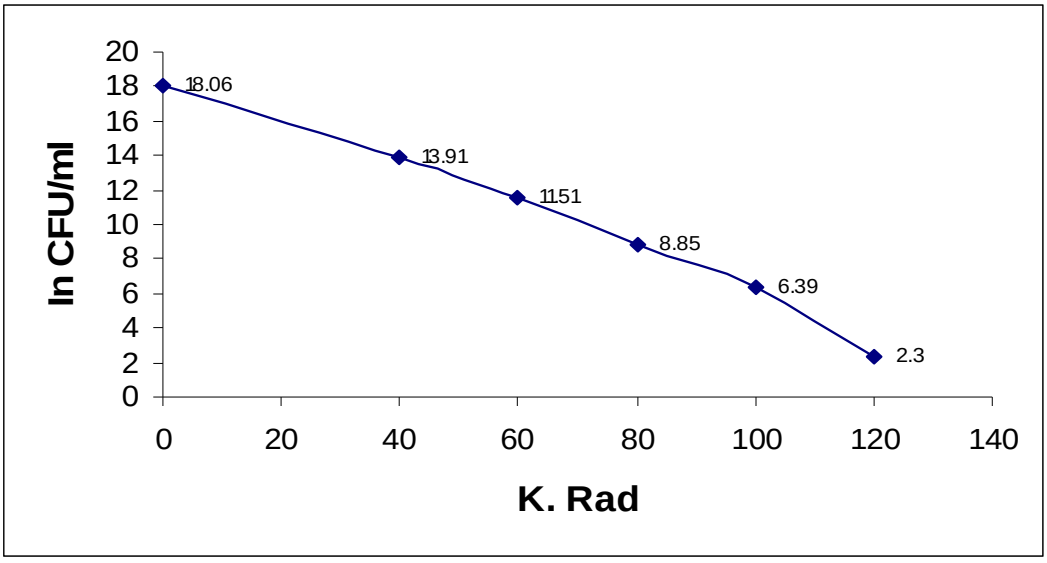

Fig.1 Kill curve for *Aspergillus niger* spores exposed to γ-rays

Mutants of *A. awamori* were isolated with enhanced production of extra-cellular xylanase and β-xylosidase by using NMT chemical mutagen as reported by Smith and Wood (1991)[13] and a 90%

kill rate from the spore suspension was obtained [13]. The present study however, reports the effect of gamma rays as physical mutagen on pure wild *Aspergillus niger* for the hyperproduction of mutarotase. The curve used is shown in Fig.1. Usami *et al*., (2001)[14] carried out an investigation to obtain high citric acid yield from *A. niger* by Co^{60} gamma irradiation [14]. The spore suspensions were exposed to various doses ranging from 1.75 x 10^4 to 21.0 x 10^4 k.Rad and the survival rate and mutation rate were measured. When each sample was subjected to an irradiation dose of 10 x 10^4 k.Rad or more, the survival rate was less than 4% and the mutation rate was about 17%. The proportion of repressed sporulation types to all mutation rates increased after nitrogen mustard treatment along with UV irradiations.

Table1: Evaluation of Dose related mutagenic effects of Gamma Rays

Sr. No.	Dose (k.Rad)	Survival Rate (%age)	Mutagenic Rate (%age)
1.	**1.75 x 10^4**	**80**	**0.5**
2.	**3.50 x 10^4**	**50**	**3.0**
3.	**7.00 x 10^4**	**20**	**10**
4.	**10.50 x 10^4**	**4**	**17**
5.	**13.50 x 10^4**	**3.8**	**17.2**
6.	**17.00 x 10^4**	**3.5**	**17.5**
7.	**20.50 x 10^4**	**3.3**	**18.5**
8.	**24.00 x 10^4**	**3.0**	**20**

Table 2 Comparison of relative increase Mutarotase enzyme activity & specific activity in different studies

Enzyme Activity	Zia, (2002)		Toyoda *et al.*, (1983)		Rodrigues *et al.*, (2009)		Xiong *et al.*, (2004)		*Present Study	
	Native	Mutant	Native	Mutant	Native	Mutant	Native	Mutant	Native	Mutant
Activity (IU)	.529 (38% rise)	-	6.05	-	5.24	-	4.78	-	4.33	22.02 *
Specific Activity (U/mg)	0.227	-	0.570	-	.260	-	0.42	-	0.391	2.00*
%age increase	40%	-	32%	-	37%	-	43.3	-	19.7*	19.55

*This work has been carried out first time under local conditions

Selection of *Aspergillus niger* mutants

After mutagenesis, the dilutions were made and 0.1mL of that was placed on PDA media and the numbers of colonies were restricted to 30 or less. The use of triton X-100 (2%) was found to be the best for colony restriction and clearance. Triton X-100 at 0.01% has already been used to limit the colony size, to facilitate the screening and isolation of mutants of *Trichderma reesei* enhanced cellulase production [15]. In the present study 2% triton X-100 proved to be optimum

for colony restriction whereas 0.1% sodium taruroglycocholate was used as colony growth restrictor, for hyper xylanolytic mutant of *Fusarium oxysporum* [16].

Triton X-100 (2%) was used to restrict the colonies to merge with each other and to obtain colonies of *A. niger* in reasonable sizes which could be isolated easily. Valuable results were obtained by using triton X-100 as colony restrictor although the difference of microorganism was significant yet, it was successful. In the present study, it was found that maximum utilizable dose of mutagenic rays was 7 x 10^4 k.Rad having 20% survival and 10% mutagenic rate. The results are shown in Table-I. In order to produce the depressed mutants for enzyme production, 2-deoxy D-glucose was used at 1mg/mL concentration. The γ-rays treated spores were spread on PDA plate with 2-deoxy D-glucose. All the selected mutants along with wild type of *Aspergillus niger* were used for the hyperproduction of enzyme mutarotase. Shaker flasks carrying 3% sucrose as substrate in 100mL of fermentation medium were inoculated with 2mL of spore suspensions from the selected potential mutant strains. These were kept in rotary shaker at a speed of 120 rpm at 30^{o}C for 24 hours.

Mutarotase Activity:

The mutarotase activity of pure wild type of *Aspergillus niger* was 4.33 U/ml whereas the mutant derived strain obtained in this study showed 22.02 U/ml. The specific rotation of the enzyme was 57° with 225 μM of α-D-glucose concentration. The time related mutarotase activity of *Aspergillus niger* is shown in Fig. 1. This concentration of α-D-glucose was determined by the standard curve of α-D-glucose that was plotted between specific rotation and α-D-glucose concentration. The activity of the mutant derived strain was greater than that of wild type strain because of mutation.

Toyoda *et al.*, (1983)[3] reported the activity and specific activity of crude rat kidney mutarotase as 6.05U/mL and 0.57U/mg, respectively [3]. While another study by Zia (2002)[7] reported the activity and specific activity of crude bovine cortex as 0.529U/mL and 0.227U/mg, respectively [7]. Both of these studies reported lesser activities than observed in the present study. The major reason of such a difference may be due to the microbial source and its induced mutagenesis for enhanced production. Xiong *et al.* (2004)[17] developed a very efficient method combined with U.V. irradiation and the nitroso-guanidine method, selection of biochemical mutants resistant to metabolic inhibitors (2-deoxy-D-glucose, antimycin A, sodium ortho-vanadate and sodium azide) for improvement of ribonuclease production by *A. niger* [17]. The most active strain (*A. niger* SA- 1320 strain) resistant to sodium azide was obtained, which had 43.3% increase in RNase production in comparison with the parent strain. Rodrigues *et al*. (2009)[18] conducted the production of citric acid (CA) through a careful strain selection process by means of physical and chemical optimization and mutation [18]. The best results (445.4 g of CA/kg of Crude Product (CP)) were obtained with sugarcane molasses and 4% methanol (v/w). The mutagenesis induction of *A. niger* LPB-BC strain was performed with UV irradiation. Eleven mutant strains were tested in Solid State Ferment (SSF) where two mutants showed a higher CA production when compared to the parental strain. *A. niger* LPB B3 strain produced 537.6 g of CA/kg of CP on the 6th day of fermentation, while *A. niger* LPB B6 strain produced 616.5 g of CA/kg of CP on the 4th day of fermentation, representing a 19.5% and 37% gain, respectively.

There was 84% increase in the production of mutarotase from gamma rays induced mutagenesis; the most active mutant strain (*A. niger* M-4) was isolated on 2-deoxy- D-glucose selective medium). The divergence is due to the difference in the product and mutagen. Moreover, the

selective marker is also different. The activity of mutarotase of crude *Aspergillus niger* mutant resulted 22.0 U/mL. The specific rotation of enzyme was 57° with 225 μM of α-D-glucose concentration. This concentration of α-D-glucose was determined by the standard curve of α-D-glucose that was plotted between specific rotation and α-D-glucose concentration. The activity of mutant strain was greater than the wild type due to mutation. In the present research work, triton X-100 (2%) was used in PDA plates as colony restrictor, which proved to be the best colony restrictor for fungal growth. The comparison between different studies regarding the hyperproduction of mutarotase is shown in Table 2. The hyper-produced mutants of *Aspergillus niger* for mutarotase were isolated on the selective medium having 2-deoxy D-glucose as a marker and showed a 5.11 fold increase in production as compared to wild type. The difference is only due to change in the product and mutagen used. Gamma rays are strongly mutagenic and cause living cell mutation. So an attempt was made to get the advantage of mutagenesis obtaining mutant isolates with high yield of mutarotase. Overall the results of the present study showed 5.11 fold (84%) increase in mutarotase activity in mutant derived strain of *A. niger* as compared to pure wild type. This divergence may be attributed to differences in product and change in the radiation source and therefore such results are in line with the previously reported results [3, 8].

CONCLUSION:

In conclusion, the mutant derived strain of *Aspergillus niger* obtained in the present study represents a rich source of mutarotase, so its application in the development of diagnostic kits or biosensors would be of great value in the field of medical sciences.

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