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RANDOM MUTAGENESIS FOR ENHANCED PRODUCTION OF ALPHA AMYLASE BY *ASPERGILLUS ORYZAE* IIB-30

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Abstract

The current study deals with the isolation of novel mutant of *A. oryzae* for higher alpha amylase production. The alpha amylase producing *A. oryzae* strain IIB-30 was subjected to physical (UV) and chemical (Nitrous acid and EMS) mutagenic treatments to enhance the amylolytic potential of parental strain. After mutagenesis isolates were screened both qualitatively and quantitatively. Among these, EMS-20 exhibited highest enzyme activity (280±0.9 U/ml). This mutant showed 2.1 fold increased activity over the parental strain in terms of enzyme production. The result showed the EMS was effective mutagenic agent for stain improvement.

Introduction

The starch degrading enzyme alpha amylase is extensively found in nature. This extracellular enzyme randomly hydrolyses α-1,4 glucosidic linkages throughout the starch molecule producing mono saccharides and oligosaccharides such as glucose, maltose, and alpha limit dextrin (Omemum *et al*., 2005; Bhanja *et al*., 2007; Leman *et al*., 2009; Haq *et al.,* 2010). Alpha amylases are gaining more importance because their range of application has expanded in many fields including medicinal, clinical, and analytical chemistry. In addition to their use in starch saccharification they are also employed in food, textile, detergent, baking, brewing and paper industries (Abdullah *et al*., 2011). These are chief enzymes used in starch processing industries for hydrolysis of starch into simple sugar components. Alpha amylase can be derived from several sources such as plants, animals and microorganisms, but production from first 2 groups is limited for several reasons. Filamentous fungi have been well known for secreting starch degrading enzymes. The capability of filamentous fungi to produce large quantities of extracellular protein has made them appropriate for the industrial enzyme production. Different spp. of *Aspergillus* including *Aspergillus niger*, *Aspergilus tamarii*, *Aspergillus awamori* and *Aspergillus oryzae* have attain attention to obtain several types of hydrolytic enzymes like lipase, alpha amylase, amyloglucosidase and protease. However, *A. oryzae* is the organism of choice because of its ubiquitous nature, non fastidious nutritional requirements and high productivity of alpha amylase (Abe *et al.*, 1988; Archer & Wood, 1995; Agger *et al*., 2001; Zangirolami *et al*., 2002; Shafique *et al*., 2009, Malik *et al.*, 2011a). Increasing utility of alpha amylase in several industries pose a greater pressure on increasing enzyme production on local scale and exploration of new rapid processes (Carlsen *et al*., 1996; Ramachandran *et al*., 2004; Kathireasan & Manivanan, 2006; Gupta *et al*., 2008).

The selection of mutant strains of alpha amylase in the presence of 2-deoxy-D-glucose is a technique to get more efficient enzyme producing cells (Annos & Blaschek, 1991). Traditional approaches for strain improvement including ultraviolet (UV) radiation,and employing of alkylating agents such as N-methyl N-nitro N-nitroso guanidine (NG), ethyl methane sulphonate (EMS) and nitrous acid to get superior mutants have been proved fruitful by subjecting the microorganisms to these mutagens, followed by appropriate selection and screening of the survivors (Szafraniec *et al*., 2003). However, strain improvement is trial and error process involving laborious procedure. Rational selection techniques are most effective and commonly have a biochemical basis (Elander 1982). In Qualitative screening prior to laboratory fermentations, rational selection is attained by the use of methods permitting visual identification of higher mutations. The selection of α amylase producer by means of the zone size of starch hydrolysis is an example. However, it is not in any way be associated with the quantity of alpha amylase obtained because of the activity of starch hydrolysis of other amylolytic enzymes such as glucoamylase. Hence the isolation of better producer of alpha amylase by employing starch plate can only be partially selective (Kuek & Kidby, 1984). Mutant strains of *A. oryzae* were found to be best for enzyme production compared to wild strain. It was studied that a mutant strain of *A. oryzae* showed more dextrinizing and saccharogenic activity than the parental strain. In case of mutagenic application to the wild strain, better initial improvement can be expected. A best mutant for alpha amylase production can be obtained by irradiating the fungal strain to the UV irradiation and then successive treatment with mutagenic chemicals like NG, EMS etc. (Spohr *et al*., 1998; Qirang & Zhao, 1994; Azin & Noroozi, 2001; Malik *et al*., 2011b).

Materials and Methods

Organism: The *Aspergillus oryzae* (IIB-30) used in the current study was obtained from GCU culture bank.

Induction of mutation

Minimal inhibitory concentration of 2-deoxy-D-glucose: In order to find out the minimal inhibitory concentration (MIC), parental strain was grown on starch agar medium along with 2-deoxy-D-glucose (0.0-0.5%) at 30ºC (Azin & Noroozi, 2001).

Ultraviolet (UV) irradiation: From the parental fungal isolate (5 day old culture), 1ml of the conidial suspension was added to a cotton wool plugged Erlenmyer flask containing 25ml of sterilized medium. The conidia were allowed to grow at 30°C on a shaking incubator with 200 *g* for about 6 h to get fresh growing fungal mycelia. Five milliliter of the medium containing mycelial suspension was poured to a sterilized Petri plate and these mycelia were exposed to ultraviolet (UV) irradiation for 15-75 minutes under the beam (λ=253 nm and 220 V at 50 c/s) of UV lamp. The mycelial suspension was treated with radiation intensity of 1.2×10^2 J/m^2/s. The distance b/w the sample and lamp was kept 8 cm for all trials to get more than 95% death rate (Azin & Noroozi, 2001).

Nitrous acid treatment: A solution of $NaNO_2$ (0.07-0.3M) prepared in acetate buffer (pH 4.5) was transferred to conidia of *A. oryzae* which were already washed and centrifuged (Carlton & Brown, 1981). After fixed intervals the solution was thoroughly shaken. Fivefold dilution of 1.0 ml of solution is made in 0.2 Mphosphate buffer (pH 7.1) to stop the reaction. A control without nitrous acid was run parallel. After specific time interval, for 15 min the tubes were centrifuged at 6000 *g*. To eliminate nitrous acid from the conidia the supernatant was discarded and ten mililiter of sterilized phosphate buffer was poured in all test tubes. For further elimination of traces of nitrous acid the tubes were re-centrifuged and this practice was repeated thrice. After washing the conidia were suspended in similar buffer.

EMS-treatment: Various conc. (25-150µl) of ethyl methane sulphonate (EMS) were transferred to each centrifuge tubes having 4ml of conidial suspension and shaken to form a homogeneous mixture. After fixed intervals the conidia were centrifuged and washed in phosphate buffer for three times. The EMS treated conidia were re-suspended in same buffer.

Selection of mutants: After mutagenic agents treatment, 100 µl of all suspension containing treated conidia was aseptically poured to the seprate Petri plates having starch agar medium supplemented with g/l; Triton X-100 (5.0), 2- deoxy-D-glucose (above the MIC of parent strain). The plates were incubated at 30ºC and were examined regularly after 3-4 day to study the growth pattern. The colonies were selected qualitatively; showing the bigger starch hydrolytic zone compared to parental strain and was allowed to grow on PDA slants for culture maintenance. These colonies were then tested quantitatively for the production enzyme using submerged fermentation.

Inoculum preparation

Conidial inoculums: Conidia from 3-4 day old slant cultures were used for inoculation. The conidial suspension was prepared in sterilized 0.005% dioctyl ester of Sodium sulpho succinic acid (Monoxal O.T). Ten milliliter of sterilized Monoxal O.T was transferred to each slant having profuse conidial growth on its surface. An inoculating needle was used to break the clumps of conidia. The test tube was shaken vigorously to make a homogeneous suspension. The numbers of conidia were counted with the help of a Haemacytometer. Each milliliter of the suspension contained 2.6×10^6 CFU.

Shake flask studies: Twenty five milliliter of the fermentation media containing (g/l) Starch 10 $MgSO_4.7H_2O$ 0.005, $CaCl_2.2H_2O$ 0.2, $FeSO_4$ 0.1, $(NH_4)_2SO_4$ 2, was transferred to separate 250 ml cotton plugged conical flasks. The flasks were sterilized in an autoclave for 15 min and cooled at room temperature. One milliliter of inoculum was transferred to each flask. The flasks were placed in the orbital shaking incubator for incubation at 30°C with shaking speed of 200 *g*. After 72 h of incubation, content of flasks were filtered and filtrate was used for the estimation of enzyme activity while the residue was used for the estimation of cell mass. All the experiments were run parallel in triplicates.

Estimation of alpha amylase: The estimation of alpha amylase was carried out according to the method of Rick & Stegbauer (1974). "One unit of activity was that amount of enzyme, which in 10 min liberates reducing group from 1% Lintner's soluble starch corresponding to 1mg of maltose hydrate." The enzyme activity was determined by taking 1 ml of diluted filtrate in a test tube. One milliliter of starch solution (1%) was also added into it. A blank was run parallel by replacing the filtrate with 1 ml of distilled water. After incubation of 10 min at 40°C, the reducing sugar liberated was measured at 546 nm by the DNS method (Miller, 1959) using maltose as a standard.

Determination of mycelial morphology: Mycelial morphology was determined on an aliquot extended on the Petri plates followed by pellet diameter (Moreira *et al*., 1996). For rounded pellets, if the diameter was less than 0.5mm, they were categorized as fine pellets, between 0.5-2 mm as small pellets, between 2-3mm as intermediate pellets while those above 3 mm were referred to as large pellets.

Estimation of dry cell mass (DCM): Dry cell mass was estimated by filtering the culture broth by means of pre weighed Whatman filter paper. Mycelia were carefully washed with water and dried in oven at 105°C for 2 hours. The dry cell mass was weighed and calculated as g/l by subtracting the initial weight from the final weight.

Statistical analysis: Treatment effects were compared by the method of Snedecor & Cochran (1980). Post-Hoc Multiple comparison tests were applied under one-way ANOVA. Significance has been presented in the form of probability ($p<0.05$) values.

Results and Discussion

In order to increase the alpha amylase production, different isolates were obtained by irradiating the conidia of *A. oryzae* IIB-30 with different doses of UV light (15-75 min). The data of screening of isolates after UV treatment is given in Table 1. A total of 32 strains were isolated by observing bigger zones of starch hydrolysis in Petri plate compared to parental strain. Of all the isolates tested, UV-20 gave maximum enzyme production (150±1 U/ml). It was due to the fact that UV irradiation possibly changed the structure of DNA by photolysis i.e, formation of pyrimidine dimers. The structural alteration in DNA was associated with the activity of the enzyme. Thymidine-thymidine dimer probably promoted mycelial growth in the form of round pellets and subsequently enzyme activity, which resulted in greater secretion of enzyme from the mycelial cells (Ali *et al*., 2002). UV mutant of fungi showed more enzyme production compared to parental strain as reported by Azin & Noroozi, (2001), Rubinder *et al*., (2002), Ellaiaha *et al*., (2002) and Karanam & Medicherla, (2008).

The UV-20 mutant was subjected to alternate treatment with nitrous acid and EMS for further improvement in the enzyme production. Table 2 shows screening of nitrous acid treated isolates.NA-15 gave maximum enzyme productivity (280±0.9 U/ml).The reason may be that it brings alterations in the structure of DNA by replacing the amino group with hydroxyl group at carbon no.6. EMS-20 gave 2.1 fold alpha amylase production than the parental culture (Table 3). It might be due to EMS affect the DNA by the addition of ethyl group to the bases which result mispairing of bases and causing the mutation. UV and nitrous acid were commonly used for strain improvement as reported by Azin & Noroozi (2001) and Rubinder *et al*., (2002). The data of Table 1.1, 2.1 and 3.1 showed number of survivors in each case (UV, NA, EMS) respectively.

Table 1. Screening of UV isolates of *A. oryzae* IIB-30 for alpha amylase production.

UV irradiated isolates	Exposure time (min)	Enzyme activity (U/ml)	Dry cell mass (g/l)	Mycelial morphology
Parental IIB-30		130 ± 0.1	13 ± 0.1	Large pellets
UV-1	15	120 ± 1.0	12.0 ± 0.1	Large pellets
UV-2		90 ± 0.3	10.5 ± 0.1	Large pellets
UV-3		70 ± 0.2	9.60 ± 0.1	Small pellets
UV-4		101 ± 0.5	11.7 ± 0.1	Large pellets
UV-5		93 ± 0.4	10.7 ± 0.1	Small pellets
UV-6		113 ± 0.7	11.8 ± 0.1	Large pellets
UV-7		126 ± 1.5	12.9 ± 0.2	Large pellets
UV-8		140 ± 1.2	13.2 ± 0.3	Large pellets
UV-9		99 ± 1.0	11.3 ± 0.2	Small pellets
UV-10		106 ± 0.9	11.8 ± 0.2	Large pellets
UV-11		87 ± 0.4	10.2 ± 0.1	Small pellets
UV-12	30	100 ± 1.0	11.0 ± 0.2	Large pellets
UV-13		93 ± 0.8	10.3 ± 0.1	Small pellets
UV-14		117 ± 1.1	11.3 ± 0.1	Large pellets
UV-15		73 ± 0.9	9.60 ± 0.1	Small pellets
UV-16		129 ± 1.4	13.1 ± 0.2	Large pellets
UV-17		82 ± 0.6	10.0 ± 0.1	Small pellets
UV-18		63 ± 0.1	8.50 ± 0.1	Small pellets
UV-19		109 ± 0.8	12.1 ± 0.2	Large pellets
UV-20	45	150 ± 0.3	13.0 ± 0.1	Large pellets
UV-21		136 ± 1.3	13.2 ± 0.2	Large pellets
UV-22		123 ± 1.2	12.7 ± 0.3	Large pellets
UV-23		111 ± 2.0	10.8 ± 0.4	Small pellets
UV-24		28 ± 0.1	5.20 ± 0.1	Small pellets
UV-25		39 ± 0.1	6.60 ± 0.1	Small pellets
UV-26	60	90 ± 0.3	10.8 ± 0.2	Small pellets
UV-27		78 ± 0.5	13.1 ± 0.2	Small pellets
UV-28		80 ± 0.6	10.7 ± 0.1	Small pellets
UV-29		115 ± 1.0	12.0 ± 0.2	Large pellets
UV-30	75	132 ± 1.2	13.1 ± 0.3	Large pellets
UV-31		53 ± 0.2	7.5 ± 0.1	Small pellets
UV -32		43 ± 0.1	7.2 ± 0.1	Small pellets

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates
Incubation time 72 h, pH 6.0, incubation temperature 30°C, agitation rate 160 *g*

Table 1.1. UV treated survivors at different exposure time.

Exposure time	Total number of survivors
15	38
30	25
45	17
60	13
75	2.0

Table 2.1. Nitrous acid treated survivors of *A. oryzae*.

Nitrous acid concentrations (M)	Total number of survivors
0.1	50
0.2	35
0.3	26
0.4	23

Table 2. Screening of nitrous acid treated strains of *A. oryzae* UV-20 for the alpha amylase production.

Nitrous acid treated isolates	conc. of nitrous acid (M)	Enzyme activity (U/ml)	Dry cell mass (g/l)	Mycelial morphology
NA-1	0.1	130 ± 0.2	15.0 ± 0.1	Large pellets
NA-2		140 ± 0.7	15.2 ± 0.2	Large pellets
NA-3		120 ± 0.1	14.0 ± 0.1	Large pellets
NA-4		105 ± 0.6	14.3 ± 0.3	Large pellets
NA-5		138 ± 0.1	14.9 ± 0.1	Large pellets
NA-6		122 ± 0.5	13.8 ± 0.8	Large pellets
NA-7		130 ± 0.7	13.7 ± 0.9	Large pellets
NA-8		128 ± 0.3	13.9 ± 0.3	Large pellets
NA-9		125 ± 0.3	12.7 ± 0.1	Large pellets
NA-10	0.2	124 ± 0.0.2	11.9 ± 0.3	Small pellets
NA-11		129 ± 0.6	13.0 ± 0.3	Large pellets
NA-12		131 ± 0.6	13.8 ± 0.1	Large pellets
NA-13		120 ± 0.1	14.9 ± 0.1	Large pellets
NA-14		115 ± 0.2	9.9 ± 0.4	Small pellets
NA-15	0.3	205 ± 0.5	15.6 ± 0.05	Large pellets
NA-16		132 ± 0.4	11.3 ± 0.1	Small pellets
NA-17		111 ± 0.1	16.1 ± 0.1	Large pellets
NA-18		89 ± 0.4	2.60 ± 0.2	Small pellets
NA-19	0.4	76 ± 0.7	3.80 ± 0.2	Small pellets
NA-20		109 ± 0.5	6.80 ± 0.2	Small pellets
NA-21		102 ±0.1	10.6 ± 0.3	Small pellets

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates

*Incubation time 72 h, temperature 30°C, pH 6.0, agitation rate 160 *g*

Table 3. Screening of EMS treated *A. oryzae* NA15 for the alpha amylase production.

EMS treated isolates	EMS conc. (µl/ml)	Enzyme activity (U/ml)	Dry cell mass (g/l)	Mycelial morphology
EMS_1	25	136 ± 0.1	11.7 ± 0.1	Small pellets
EMS-2		101 ± 0.2	13.9 ± 0.1	Small pellets
EMS-3		125 ± 0.3	11.2 ± 0.2	Large pellets
EMS-4		162 ± 0.2	12.1 ± 0.1	Large pellets
EMS-5		135 ± 0.2	12.6 ± 0.1	Large pellets
EMS-6		101 ± 1.0	12.3 ± 1	Small pellets
EMS-7		168 ± 0.1	15.2 ± 0.1	Large pellets
EMS-8	50	125 ± 0.5	13.2 ± 0.2	Small pellets
EMS-9		168 ± 0.05	14.1 ± 0.1	Small pellets
EMS-10		147 ± 0.3	12.3 ± 0.3	Large pellets
EMS-11		127 ± 0.2	15.3 ± 0.1	Small pellets
EMS-12		100 ± 0.7	12.4 ± 0.4	Small pellets
EMS-13	75	108 ± 0.4	11.7 ± 0.2	Small pellets
EMS-14		187 ± 0.8	12.1 ± 0.1	Large pellets
EMS-15		141 ± 1	11.9 ± 1	Small pellets
EMS-16		118 ± 0.1	7.20 ± 0.2	Small pellets
EMS-17		147 ± 0.5	10.6 ± 0.5	Small pellets
EMS-18	100	163 ± 0.1	16.9 ± 0.1	Large pellets
EMS-19		106 ± 0.1	11.6 ± 0.2	Small pellets
EMS-20		47.0 ± 0.2	5.8 ± 0.2	Small pellets
EMS-21		93 ± 0.4	2.6 ± 0.1	Small pellets
EMS-20	**125**	**280 ± 1**	**0.9 ± 1**	**large pellets**
EMS-23		108 ± 0.1	10.3 ± 0.4	Small pellets
EMS-24		165 ± 0.1	14.1 ± 0.1	Large pellets
EMS-25	150	190 ± 0.3	12.6 ± 0.3	Large pellets
EMS-26		126 ± 0.2	10.2 ± 0.2	Small pellets

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates.
*Incubation time 72h, temperature 30°C, pH 6.0, agitation rate 160 *g*

Table 3.1. EMS treated survivors of *A. oryzae*

EMS concentrations (µl)	Total number of survivors
25	55
50	36
75	25
100	5
125	3
150	2

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