International Research Journal of Pharmaceutical and Applied Sciences
Available online at **www.irjpas.com**
Int. Res J Pharm. App Sci., 2012; 2(2):79-91

IRJPAS **ISSN-2277-4149**

Research Article

STRAIN IMPROVEMENT OF *ASPERGILLUS NIGER* FOR GLUCOAMYLASE BY PHYSICAL AND CHEMICALMUTAGENS

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(Received: 14 March, 2012; Accepted: 20 April, 2012; Published: 29 April, 2012)

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ABSTRACT

The purpose of present investigation is to enhance Glucoamylase production by subjecting *Aspergillusniger* to strain improvement by random mutagenesis using Ultra-Violet (UV) irradiation, Ethyl Methyl Sulphonate (EMS) and Ethidium bromide treatment. mutants were screened on the basis of enzyme assay by spectrophotometer using Dinitrosalicylicacid reagent (DNS).UV-18 mutant produced 18.11 U/ml/min, EMS-4 mutant produced 14.93 U/ml/min, Ethidiumbromide-13 mutant produced 18.31U/ml/min and EMS&EB-18 mutant produced 18.84 U/ml/min glucoamylase, compare to wild strains UV-18,EMS-4,EB-13 and EMS&EB-18 mutant strains were found to produce 2-4 fold more enzyme .thus these findings have more impact on enzyme economy for biotechnological applications of microbial amylases .

Key words: *Aspergillusniger* ,Strainimprovement, Glucoamylase, Ethidiumbromide, Ethylmethylsulphonate

INTRODUCTION

Glucoamylase is an enzyme of great importance in the starch industry. It has the ability to hydrolyze starch, thus converting it into glucose. Glucose is a necessary compound in various food industries[1].

Glucoamylase is also widely used in brewing, paper, food, pharmaceutical and textile industries[2]. Food wastes can also be used for Glucoamylase production by *Aspergillusniger* by the process of both submerged and solid state fermentation, which then

can be employed in glucose and brewery industry[3]. Glucoamylase occurs in microorganisms as an extracellular enzyme and it has characteristic property of hydrolyzing ∝-1, 4 and ∝-1, 6 linkages of the saccharides formed by the action of other amylases on starch. Glucoamylase hydrolyze terminal non-reducing ∝-1,4-glucopyranose[4].The production of glucoamylase can be increased to many folds by inducing mutation in the wild strains. It has been reported that the mutant strain of *Aspergillus niger* has a better ability for the Glucoamylase production. The *Aspergillus* strains can be treated with UV irradiations or chemicals such as N-methyl, N-nitro, N-nitrosoguanidine, dimethyl sulphate, EMS,ethidium bromide and nitrous acid to induce mutation and for the improvement of Glucoamylase production[5].Characterization of Glucoamylase produced by a mutated fungus showed that it possessed a high activity towards raw starch Glucoamylase produced by the mutants formed after γ –rays treatment of the parent strain showed that its properties were improved and the production of ethanol was enhanced[6].The mutant strains of *Aspergillus* sp.were produced by combined mutation, produced Glucoamylase which was capable of converting maltodextrins into glucose[7]. Multiple and alternate mutations have a great effect on the yield of glucose. The specific activity of Glucoamylase and its thermostability were increased and resulted generally in increased glucose yield. Enzyme production and their properties were compared in several *Aspergillus* mutants. The mutant produced quite a high level of glucoamylase as compared to the wild strain but regardless of the strain used, the composition and properties of enzyme complex were similar for all glucoamylase components[8]. The growth in the form of small pellets favoured glucoamylase production while larger pellets lowered the amount of glucoamylase produced not match with results (Table 2A) .The effect of *Aspergillus niger* strain, inoculum type and addition of inducers was studied and it was reported that Glucoamylase production was greatly influenced by fungal strain and inoculum type but the inducers had no effect[9].The aim of the present study is to improve the strain potential for Glucoamylase

production through chemical and physical mutagenesis.

MATERIALS AND METHODS

Improvement of strain: *Aspergillus niger* strain was improved by ultraviolet radiation and mutagenic agents. Alternate treatments of ethidium bromide and EMS explain were also given to the parental strain. Two mutagens were used at a time. The strain was treated with ethidium bromide and then with EMS. Mutagenic treatment with UV was given to parental strain[10]. Ethidium bromide was used for the induction of mutation in the parental strain[11]. The induction of mutation was done by alternate treatment of the conidial suspension by two mutagens (ethidium bromide and EMS)[12].

Inoculum

Conidial: Inoculation was done by conidia from the slant culture (3-5 days old). The conidial suspension was prepared by adding 10 ml of 0.005% Monoxal 0.T (Di-octylester of sodium sulpho succinic acid) to the slant having profused growth of conidia. The inoculating needle was used to break the conidial clumps. Then the tube was shaken vigorously to form a homogeneous conidial suspension. The density of the conidial suspension was measured on a haemocytometer (Neubauer Precicdor HBG, Germany).The counting chamber was a ruled glass slide with a cover which holds a definite volume of a fluid. The conidia in a square (0.1 mm depth) were counted under microscope and then the number of conidia in one ml of the conidial suspension was counted. It was found to be $1.2x10^6$ conidia/ml.

Vegetative: Twenty five ml of fermentation medium g/L (Starch, 10.0; Lactose, 10.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4.7H_2O$, 2.0; $CaCl_2.2H_2O$, 2.0; KH_2PO_4, 1.50; K_2HPO_4,0.1; Distilled water, 1000 ml; pH 5.5) was added in 250 ml conical flask, cotton plugged and was then sterilized. One ml of conidial suspension prepared as mentioned above was poured to the flask with the help of a pipette under aseptic conditions. The flask was incubated at 30^oC for 24 hours in an incubator shaker shaking at 200rpm.

Fermentation technique

Shake flask: Twenty five ml of fermentation medium was poured in each 250 ml conical flask and were cotton plugged. The flasks were sterilized in an autoclave at 121^{o}C, 15 lb/inch^{2} for 15 minutes. After cooling the medium at room temperature, one ml of the conidial inoculum was added in each flask under aseptic condition. These flasks were then placed in the rotary incubator shaker (Model: 10*400-xx2c, SANYO, Gallen Kamp PLC, UK) rotating at 200 rpm for 72 hours at 30^{o}C. After 72 hours, the fermented broth was filtered and filtrate was used for the estimation of Glucoamylase. All the experiments were run parallel in triplicate.

Dry cell mass: Fermented broth was filtered from a pre-weighed filter paper. The residue left on the filter paper (cell mass) was oven dried at 100^{o}C overnight and then placed in a desiccator. It was then reweighed.

Enzyme assay: The assay of Glucoamylase was carried out according to the method of Cadwell[13]. One unit of activity is the amount of enzyme, which liberates one mg of glucose per hour from 5% soluble starch. The enzyme activity was then converted into U/ml/min by applying the following formula.

$$\frac{\text{Sugar released}}{\text{Molecular wt. of glucose x time of incubation}} \times 100$$

One ml of the diluted enzyme extract was added to 1.0 ml of 5% soluble starch solution prepared in acetate buffer (pH 4.8). The enzyme substrate mixture was incubated at 60^{o}C for one hour. Then 2 ml of Dinitrosalicylic acid reagent (DNS) was added to each test tube. The test tubes were placed in boiling water for 5 minutes and cooled at room temperature. The contents of the test tubes were diluted up to 20 ml with distilled water.The transmittance of mixture was observed at 546 or 540 nm on spectophotometer. The transmittance was converted to mg of glucose from standard curve.

Table 1A. Mutants selected after the treatment of ultraviolet radiations for Glucoamylase production.

Mutant No.	Exposure time (minutes)	Glucoamylase Production(U/ml/ min)	Dry cell mass (g/l)	Mycelial Morphology
1	30	12.11 ± 0.46	10.73 ± 0.93	Medium Pellet
2	35	12.82 ± 0.76	10.76 ± 1.16	Medium Pellet
3	35	13.41 ± 1.27	10.95 ± 0.78	Medium Pellet
4	40	13.53 ± 1.40	10.82 ± 1.33	Medium Pellet
5	50	17.56 ± 1.27	11.58 ± 1.40	Medium Pellet
6	50	10.82 ± 0.46	10.41 ± 0.74	Medium Pellet
7	50	12.57 ± 0.29	10.67 ± 0.99	Medium Pellet
8	50	14.38 ± 0.45	10.22 ± 0.81	Medium Pellet
9	55	11.44 ± 0.83	10.62 ± 1.51	Medium Pellet
10	55	15.12 ± 0.23	10.72 ± 1.51	Medium Pellet
11	60	17.12 ± 1.42	11.43 ± 0.81	Medium Pellet
12	60	13.41 ± 0.55	10.38 ± 0.99	Medium Pellet
13	60	12.73 ± 0.81	10.48 ± 1.33	Medium Pellet
14	65	11.80 ± 1.61	10.44 ± 0.99	Medium Pellet
15	65	10.94 ± 76	10.25 ± 0.72	Medium Pellet
16	70	10.54 ± 0.42	10.36 ± 0.70	Medium Pellet
17	70	14.42 ± 1.15	11.42 ± 1.16	Medium Pellet
18	**70**	**18.11 ± 1.41**	**11.84 ± 0.95**	**Medium Pellet**
19	70	15.14 ± 0.36	11.22 ± 0.74	Medium Pellet
20	75	10.82 ± 0.29	10.35 ± 0.94	Medium Pellet
21	80	13.45 ± 0.64	10.81 ± 0.94	Medium Pellet
22	80	12.39 ± 0.25	10.58 ± 0.72	Medium Pellet
23	80	12.84 ± 0.52	9.87 ± 0.80	Medium Pellet
24	85	13.42 ± 1.14	10.45 ± 0.34	Medium Pellet
25	85	11.39 ± 1.27	10.38 ± 0.94	Medium Pellet
26	85	11.45 ± 1.40	10.42 ± 0.81	Medium Pellet
27	90	12.23 ± 1.14	10.38 ± 1.42	Medium Pellet
28	90	13.23 ± 1.14	10.29 ± 1.32	Medium Pellet
29	90	12.42 ± 0.45	10.34 ± 1.51	Medium Pellet
30	90	10.58 ± 0.29	9.88 ±1.33	Medium Pellet

Table 1B. Range of mutants selected after U.V treatment for Glucoamylase production.

Subgrouping of mutants	Number of mutants	Range of Glucoamylase (U/ml/min)
GI	18	10.0 -13.0
GII	9	13.1 -16.0
GIII	3 More	than 16.0

Each value is an average of three replicates ± denotes standard deviation among replicates.
Initial pH 5.5 .
Incubation period 72 h,
Incubation temperature 30 ± 2°C

Table 2A. Mutants selected after treatment with Ethidium bromide for Glucoamylase production .

Mutant No.	Exposure time (minutes)	Glucoamylase production(U/ml/min)	Dry cell mass (g/l)	Mycelial morphology
1	35	14.34 ± 1.46	10.82 ± 1.50	Small Pellet
2	40	17.43 ±. 0.30	11.51 ± 0.52	Medium Pellet
3	40	14.24 ± 0.25	11.25 ± 0.32	Small Pellet
4	50	17.54 ± 1.27	12.01 ± 1.41	Small Pellet
5	50	14.39 ± 1.08	10.74 ± 1.59	Small Pellet
6	50	15.12 ± 1.14	12.34 ± 1.12	Small Pellet
7	50	17.39 ± 1.46	12.34 ± 0.42	Medium Pellet
8	55	15.32 ± 0.29	11.31 ± 1.45	Medium Pellet
9	55	14.39 ± 0.64	10.94 ± 0.72	Medium Pellet
10	55	16.25 ± 0.36	11.24 ± 1.59	Medium Pellet
11	60	14.82 ± 1.61	10.82 ± 0.72	Medium Pellet
12	60	15.11 ± 1.27	11.42 ± 1.36	Medium Pellet
13	**60**	**18.31 ± 1.54**	**12.54 ± 0.74**	**Small Pellet**
14	60	16.32 ± 0.20	11.84 ± 0.72	Medium Pellet
15	65	12.46 ± 0.21	10.62 ± 1.32	Medium Pellet
16	65	14.94 ± 1.91	10.92 ± 1.59	Medium Pellet
17	65	15.16 ± 0.92	11.42 ± 1.30	Medium Pellet
18	65	14.83 ± 0.55	10.78 ± 1.10	Medium Pellet
19	70	11.92 ± 0.36	10.05 ± 0.55	Medium Pellet
20	70	15.32 ± 0.79	10.92 ± 1.44	Medium Pellet
21	70	14.83 ± 0.40	11.51 ± 1.41	Medium Pellet

Table 2B. Range of mutants selected after ethidium bromide treatment for Glucoamylase production.

Subgrouping of mutants	Number of strains	Range of Glucoamylase (U/ml/min)
GI	2	10-13.0
GII	13	13.1-16.0
GIII	6	More than 16.0

Each value is an average of three replicates, ± denotes standard deviation among replicates.
Initial pH 5.5
Incubation period 72 h
Incubation temperature 30 ± 2°C

Table 3A. Mutant strains selected after EMS treatment for Glucoamylase production.

Mutant No.	Exposure time (minutes)	Glucoamylase production(U/ml/min)	Dry cell mass (g/l)	Mycelial morphology
1	40	13.14 ± 1.99	10.92 ± 1.14	Medium Pellet
2	45	14.32 ± 1.40	11.52 ± 1.12	Medium Pellet
3	50	10.12 ± 0.94	12.25 ± 0.89	Medium Pellet
4	**60**	**14.93 ± 0.67**	**10.38 ± 1.27**	**Medium Pellet**
5	60	12.41 ± 0.88	11.05 ± 0.89	Medium Pellet
6	65	11.44 ± 1.32	11.94 ± 0.85	Small Pellet

Table 3B. Range of mutants selected after EMS treatment for Glucoamylase production.

Subgrouping of mutants	Number of strains	Range of Glucoamylase (U/ml/min)
GI	3	10.0-13.0
GII	3	More than 13.0

Each value is an average of three replicates,± denotes standard deviation among replicates.
Initial pH 5.5
Incubation period 72 h
Incubation temperature 30 ± 2°C

Table 4A. Strains selected after alternate treatment of ethidium bromide andEMS for Glucoamylase treatment.

Sr. No.	Glucoamylase production (U/ml/min)	Dry cell mass (g/l)	Mycelial morphology
1.	12.52 ± 0.84	10.48 ± 1.15	Medium Pellet
2.	15.01 ± 1.20	11.75 ± 1.20	Medium Pellet
3.	14.55 ± 1.34	10.97 ± 1.42	Medium Pellet
4.	18.42 ± 0.88	12.84 ± 1.45	Medium Pellet
5.	17.28 ± 1.62	12.59 ± 0.95	Medium Pellet
6.	17.34 ± 0.55	12.28 ± 0.94	Medium Pellet
7.	12.38 ± 0.83	10.48 ± 0.72	Medium Pellet
8.	16.04 ± 0.46	11.72 ± 1.12	Medium Pellet
9.	16.32 ± 0.30	11.53 ± 0.88	Medium Pellet
10.	15.84 ± 0.72	11.24 ± 0.89	Medium Pellet
11.	11.85 ± 1.27	10.36 ± 0.36	Large Pellet
12.	14.34 ± 1.08	10.85 ± 1.14	Medium Pallet
13.	18.25 ± 0.55	12.64 ± 1.34	Small Pellet
14.	18.31 ± 1.42	12.95 ± 0.55	Small Pellet
15.	13.75 ± 0.83	10.76 ± 1.15	Medium Pellet
16.	12.93 ± 0.93	10.58 ± 0.79	Medium Pellet
17.	16.52 ± 0.52	13.81 ± 1.67	Medium Pellet
18.	**18.84 ± 1.50**	**12.92 ± 1.45**	**Very small pellet**
19.	15.78 ± 0.88	12.48 ± 1.18	Medium Pellet
20.	18.01 ± 0.89	12.76 ± 1.55	Small Pellet

Table 4B. Range of mutants selected after alternate treatment of ethidium bromide and EMS for Glucoamylase production.

Subgrouping of mutants	Number of strains	Range of Glucoamylase (U/ml/min)
GI	4	10.0-13.0
GII	6	13.1-16.0
GIII	10	More than 16.0``

Each value is an average of three replicates, ± denotes standard deviation among replicates.
Initial pH 5.5
Incubation period 72 h
Incubation temperature 30 ± 2°C.

RESULTS

Data of table 1A shows the production of Glucoamylase by UV treated strains of *A. niger*. The parental strains of *A. niger* was subjected to UV treatment for different time intervals i.e., from 5-90 min. Thirty mutant strains of *A. niger* were isolated on the basis of bigger zone of starch hydrolysis in the petriplates. These strains were screened for Glucoamylase production in shake flask. The isolates were sub grouped into G-I (10.0-13.0 U/ml/min), G-II (13.1-16.0 U/ml/min) and G-III (more than 16.0 U/ml/min) according to their enzyme productivity (Table 1B). Of all the isolates investigated, maximum enzyme production (18.11 U/ml/min) was obtained by *A. niger* mutant No.18 which was selected

after 70 minutes of UV treatment. The production of enzyme following the growth of the organism was found to be highly significant by *A. niger* mutant No.18 and varied significantly ($p<0.05$) than other mutant derivatives. But this mutant was not stable. The mutant strains of *A. niger* was selected after the treatment of parental strain with ethidium bromide (Table 2A). The wild strain of *A. niger* was subjected to ethidium bromide treatment for different time intervals i.e., 30-120 min. Twenty one mutant strains of *A. niger* were selected on the basis of larger zone of starch hydrolysis in the petriplates. Further screening of the strains for Glucoamylase production was done in shake flask. The selected strains were subgrouped into G-I (10.0-13.0 U/ml/min), G-II (13.1-16.0 U/ml/min) and G-III (more than 16.0 U/ml/min) according to their enzyme productivity (Table 2B). Of all the isolates investigated, maximum enzyme production (18.31U/ml/min) was obtained by *A. niger* mutant No.13. Glucoamylase production was found to be highest by *A. niger* No 13.But after subculturing this mutant reverted to its parental enzyme production.

The strains of *A. niger* were screened after the chemical treatment with EMS (Table3A). The parental strains of *A. niger* was subjected to EMS treatment for 30-120 min.Only six mutant strains of *A. niger* were isolated on the basis of bigger zone of starch hydrolysis in the petriplates. These strains were screened for Glucoamylase production in shake flask. The isolates were sub grouped into G-I (10.0-13.0 U/ml/min) and G-II (more than 13.0 U/ml/min) according to their enzyme productivity (Table 3B). Of all the isolates investigated, maximum enzyme production (14.93 U/ml/min) was obtained by *A. niger* No.4. This mutant showed improvement in the production of the enzyme but after subculturing it reverted to its parental enzymeproduction.

The parental strain of *A. niger* was subjected to the alternate treatment of ethidium bromide and EMS and screened for Glucoamylase production (Table 4A). The parental strains of *A.*

niger was subjected to ethidium bromide treatment for 60 min. After that it was subjected to EMS treatment for 60 min. Twenty mutant strains of *A. niger* were isolated on the basis of bigger zone of starch hydrolysis in the petriplates. These strains were screened for Glucoamylase production in shake flasks. The isolates were sub grouped into G-I (10.0-13.0 U/ml/min), G-II (13.1-16.0 U/ml/min) and G-III (More than 16.0 U/ml/min) according to their enzyme productivity (Table 4B). Of all the isolates investigated, maximum enzyme production (18.84 U/ml/min) was obtained by *A.niger* No. 18 (M4 120).

DISCUSSION

The production of enzyme following the growth of the organism was found to be highly significant by *A. niger* M4 120 and varied significantly ($p<0.05$) than other mutant derivatives. Thus,this strain was selected for further studies.The mutant strains of *A.niger* are reported to have better ability for the production of Glucoamylase[14]. Agrawal (1999) reported UV as a potent mutagen. In the present work, the parental strains of *A. niger* were subjected to UV treatment for different time intervals i.e., from 5-90 min. Thirty mutant strains of *A. niger* were isolated on the basis of bigger zone of starch hydrolysis in the petriplates. Of all the isolates investigated, maximum enzyme production (18.11U/ml/min) was obtained by *A. niger* mutant No.18 after 70 min of UV treatment. But this mutant was not stable. It was due the reason that the mutant produced by UV irradiations had undergone back mutations when they were exposed to light[15].

Chemical mutagens like ethidium bromide and EMS gave a stable and viable mutant for production of Glucoamylase. The parental strain of *A. niger* was exposed to the treatments of different chemical mutagens. In the present study the parental strain of *A.niger* was treated with different doses of ethidium bromide for 30-120 min and to EMS treatment for 30-120 min but the mutants

obtained were not stable. It was due to the reason that the DNA of the mutant strains repaired their damaged part during its replication[16]The effect of alternate treatments of different chemical mutagens are more strong than single mutagen. In double mutagen studies the parental strain of *A. niger* was subjected to ethidium bromide treatment for 60 min followed by EMS treatment for 60 min. In this study twenty mutant strains of *A.niger* were isolated on the basis of bigger zone of starch hydrolysis in the petriplates. Of all the isolates investigated, maximum enzyme production (18.84 U/ml/min) was obtained by *A. niger* No. 18 (M4 120). It remained a stable mutant after multiple culture cycles. It was due to the relationship between mutation rate and the amount of dose to the fungi[17]. The productivity increase was more than two fold than the parental strain from 7.46 to 18.84 U/ml./min. This enhancement occurred due to increase in gene copy number and amplification of the DNA region that include gla (glucoamylase) gene[18.]

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