Original Research

STRAIN IMPROVEMENT OF ACCLIMATIZED STRAIN BY MUTAGENESIS FOR HYPER PRODUCTION OF ENDOGLUCANASE

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ABSTRACT

Cellulose is an un branched glucose polymer composed of an β-1, 4 glucose units linked by a β-1, 4-D-glycosidic bond.Cellulases are high molecular colloidal protieneous bio-catalyst. Industrial cellulases represent numbers of cellulases, cellobiase and related enzyme of completely non uniform nature ranging in a molecular weight of 10,000 to 4 million. The extracellular cellulolytic enzyme system consists of three major enzyme components: Endoglucanase (EC 3.2.1.4, 1.4-β-D glucanohydrolases), exoglucanases or cellobiohydrolases (EC 3.2.1.19, 1, 4-β-D glucancellobiohydrolases) and β-glucosidase or cellobiases (EC 3.2.1.21, β-D-glucosideglucohydrolases). Endoglucanase (EC 3.2.1.4, 1.4-β-D glucanohydrolases) is commercially produced and widely applied in textile industry as a biopolishing agent and have considerable economic importance. The biopolishing of textile products are carried out at about 45 ˚C to 55 ˚C temp and pH 4.2 to 4.5, occasionally certain critical biopolishing is also carried out at neutral pH value.Endoglucanase biocatalyst activity is reported in certain bacteria and numerous fungi among which strains of *Trichoderma*and, *Aspergillus*are used for commercial enzyme production. These microbial strains are natively saprophytic, acidophilic, mesophilic. For sustainable, economical and potential endoglucanase production at secondary screening, the microbial community requiresmutation for hyper production ofthe enzyme after acclimatization. For the desired screening, the microbial community was exposed to U.V. radiation for hyper production of endogluconase applying mutagenesis process. During the experiment, the bulk cell mass was exposed to U.V. light under precise experimental condition.After exposure, culture wasexamined for the utilization zone on Carboxyl Methyl Cellulose agar plate using congo red reaction and the acceleration of desired enzymatic activity was determined. The mutagenesis was conducted for about six month and the developed attenuated microbial strain was characterized for sustainable, hyper enzyme production of endoglucanase.

KEYWORDS : Cellulase, Acclimatized, Mutation, Primary marker, U.V. rays.

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INTRODUCTION

Lignocellulose is major renewable natural resource and abundantly present on the biosphere. It accounts about 60 to 80 % of polysaccharide which accounts about 60 -80% of polysaccharide contain of the earth. It has been approximated that 7.2 X 10^{11} tons of cellulose is reserved in plant and by plant metabolites around 4 X 10^{10} tons are generated[1]. Cellulose is an unbranched glucose polymers composed of a β -1, 4 glucose units linked by a β -1, 4-D- glycosidic bond [2]. The extracellular cellulolytic enzyme system consists of three major enzyme components: Endoglucanase (EC 3.2.1.4, 1.4-β-D glucanohydrolases), exoglucanases or cellobiohydrolases (EC 3.2.1.19, 1, 4-β-D glucancellobiohydrolases) and β-glucosidase or cellobiases (EC 3.2.1.21, β-D-glucosideglucohydrolases[3]. Cellulases are high molecular colloidal proteineous bio-catalyst. Industrial cellulases represent numbers of cellulases, cellobiase and related enzyme of completely non uniform nature ranging in a molecular weight of 10,000 to 4 million[4]. These enzymes are categories on the basis of their pH optimum for cellulolytic activity as Acid stable cellulase – more effective in pH range 4.5 to 5.0, Neutral stable cellulase – bioreacting at neutral pH 6.8-7.2 and alkaline stable cellulase – catalyzed bio-reaction above pH 7.8. The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic action by endoglucanase (E.C. 3.2.1.4), which hydrolyzed intramolecular β -1,4-glucosidic bond of cellulose chain randomly to produce new chain ends, the exoglucanase called cellobiohydrolase (E.C. 3.2.1.91) which processively cleave the formed chain at the both polar ends to release soluble disaccharide cellobiose or monosaccharide glucose and β-glucocidase (E.C. 3.2.1.21) complete the hydrolysis of cellulose, these three cellulotic enzymesprocesses occurs simultaneously [5]. Cellulases are considerable important commercial enzyme and at production and consumption rest next to amylase and protease in global commercial market. Due to their broad spectrum catalytic ability, they are used in large scale bioconversion reactions. For commercial production of cellulases at sustainable, economical state, hyper biosynthetic fast growing microbial strains are under prime requirement. Numerous research reports are available for the screening and development of cellulolytic microbes for industrial production through adaptation, mutagenesis, microbial recombination and genetic engineering .The industrially important microbial strains for a particular product are developed under a specialized screening programme, categorized as primary screening and secondary screening process[6].

Mutation

A mutation is a permanent change of the nucleotide sequence of the genome of an organism, virus, or extrachromosomal DNA or other genetic elements. Mutations result from damage to DNA which is not repaired or to RNA genomes (typically caused by radiation or chemical mutagens), errors in the process of replication, or from the insertion or deletion of segments of DNA by mobile genetic elements. Mutations may or may not produce discernible changes in the observable characteristics (phenotype) of an organism[7].

Four classes of mutations are (1) spontaneous mutations (molecular decay), (2) mutations due to error prone replication bypass of naturally occurring DNA damage (also called error prone translesion synthesis), (3) errors introduced during DNA repair, and (4) induced mutations caused by mutagens.

METHODOLOGY

Physical mutagenesis: UV Irradiation:

To developed hyper endogluconase production, selected fungal strain coded 5 was irradiated under UV radiation through standardize mutagenesis protocol.

Among fungi, induction of hyper production expression due to mutagenesis is under extreme low probability and hence as primary marker, antifungal resistance was selected to eliminate the non-mutational wild type fungal population which was subsequently followed for the enrichment of desired marker strain for the aimed purpose.

Development of primary marker

Suitable antifungal agents were selected and their minimal inhibition concentration was determined for selected fungal variant.

(A) Selection of Antifungal agent

Four broad spectrum antifungal agents, Griseofulvin, Nystin, Flucanozole and Pymaricin were tested for their inhibitory action to selected potent fungal strains.

Standardized cup-bored agar plate technique was used to qualify selected antifungal agent at concentration of 100 µg per ml to 800 µg per ml as primary marker for the aimed purposed. The comparative study of inhibitory zone size of each antibiotic at lowest concentration was critically carried out for the selection of suitable antifungal agents as a marker in mutagenesis experiment.

(B) Determination of MIC of Flucanozole for coded 5 strain

The Flucanozole was verified for its MIC dose for strain coded 5 by standards antibiotic broth assay technique with the gradient of 50 µg per ml to 600 µg per ml. For mutagenesis MIC + dose was selected.

Mutagenesis:

Physical mutagenesis process was conducted with U.V. irradiation.

- U.V. chamber was sterilize by spraying formaldehyde-ethanol mixture and was kept close for about 18 to 24 hours.
- The culture exposure container was kept upon a magnetic stirrer keeping a distant of about 15 to 18 inch from U.V. illuminating source having a wavelength of 254 nm.
- Under aseptic condition approx 50 ml of fungal conidia suspension (10^{11} spore count/ml) was added into 200 mm diameter petri dish and magnetic stirrer was on for smooth, gentle shaking of the suspension.
- The U.V. bulb was on, simultaneously more than 1.0 ml of spore suspension was withdrawn into the 10 × 100 mm tubes and marked as '0'.
- With the help of sterile pipette at the interval of every 1 minute, U.V. irradiated suspensions were collected into individually tube and marked as 1,2,3,4,…..40.
- The sample collected tubes were kept in a dark box to avoid photoregeneration.
- After the completion of an experiment aseptically 1.0 ml of U.V. irradiated suspension was diluted to 10.0 ml with sterile distilled water.
- For desire mutant, 1.0 ml from diluted suspensions were cultured by pour plate techniqueusing CarboxyMethylCellulose (CMC) mineral media with slightly higher dose then MIC of Flucanozole. (Composition Glucose 2.0 % ; CMC 0.2 % ; $(NH_4)_2SO_4$ 0.3 % ; KH_2PO_4 0.05 % ; $MgSO_4$ 0.005 % ; $CaCl_2$ 0.005 %.)
- The prepared plates were incubated at 30 $\pm$ 2 ºC for 4–5 days.
- For the determination of survival curve against the ultraviolet irradiation or time duration of exposure, the above step was repeated only difference is media prepared without Flucanozole (Table 1)[8].

Selection of hyper Endoglucanase producing strains

The cultivated mutant colonies were inoculated in the center with almost equal amount on CMC mineral media plates. The plates were incubated at 30 $\pm$ 2 ºC for 4–5 days. After incubation period the petri plates were flooded with Congo red solution (0.1%), and after 5 min the Congo red solution was discarded, and the plates were washed with 1M NaCl solution allowed to stand for 15 to 20 minutes. The clear zone was observed around the colony when the enzyme had utilized the cellulose (Figure 3).

RESULT

Among Griseofulvin, Nystin, Flucanozole and Pymaricin, Fluconozole proved ideal antifungal for the development of primary marker to eliminate non mutated fungal strain. (**Figure 1**). Flucanozole at 600.0µg per ml proved MIC dose for fungal strain.

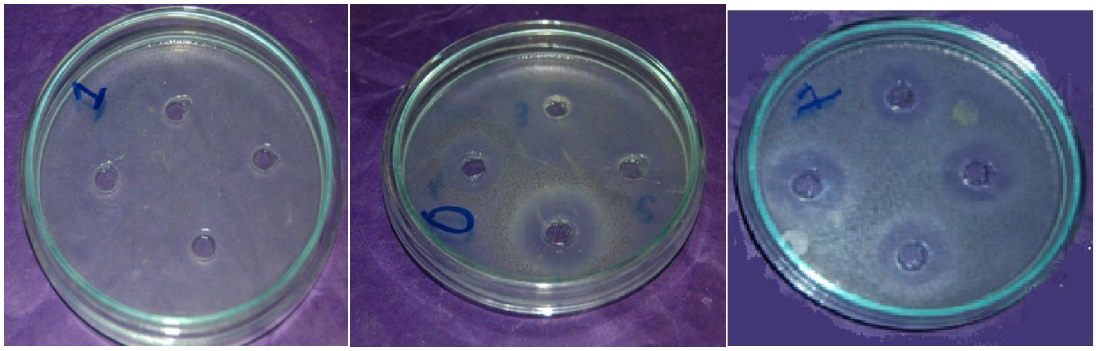


Figure1 : Inhibitory zone by selected antifungal agent

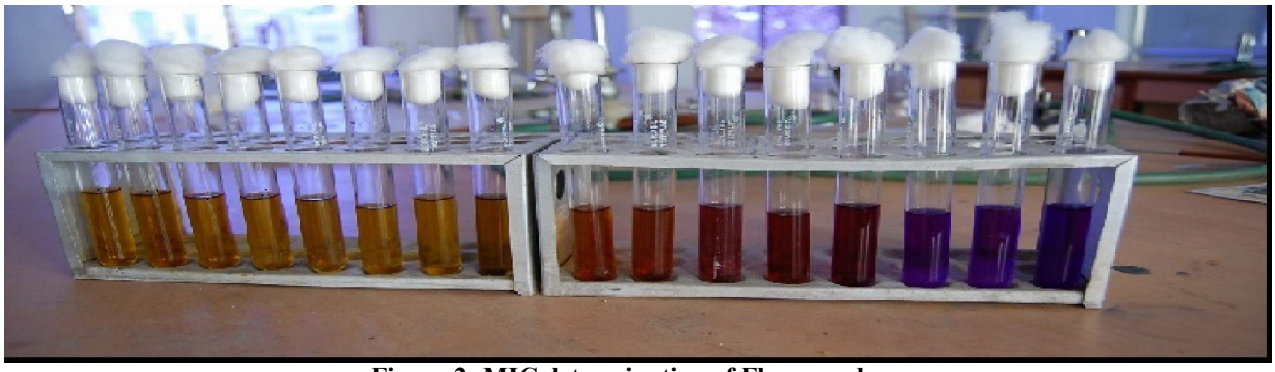
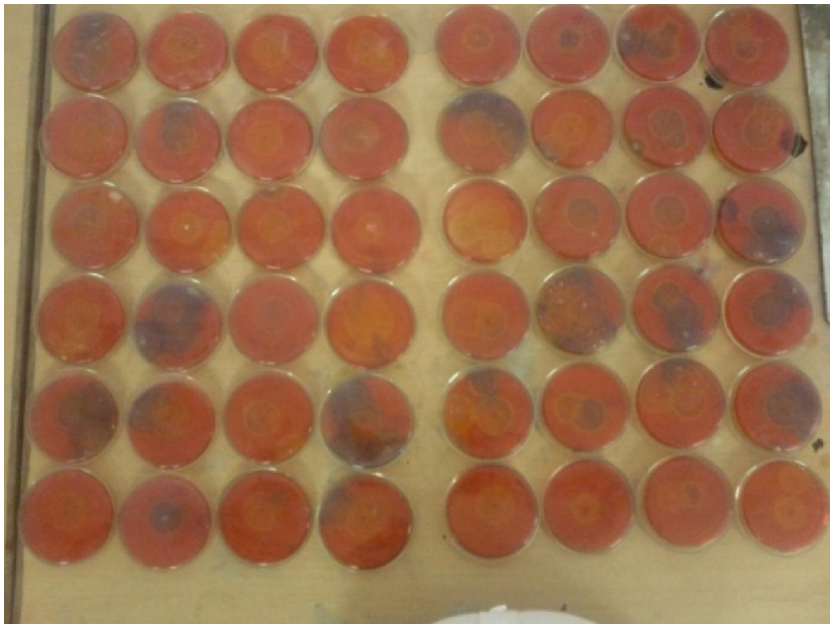


Figure 2: MIC determination of Flucanozole

Table 1. Screening of fungal strain mutants for cellulase activity isolated after different time intervals of UV irradiation.

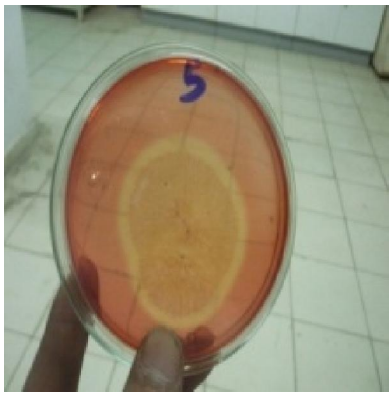
Sr. No.	Exoposure Time (min)	Number of survivor	Number of improved mutant	Range of Zone size in mm
1	0	All	0	-
2	4	87	8	55-57
3	8	79	14	57-59
4	12	65	18	56-59
5	16	51	17	58-61
6	20	34	13	63-68
7	24	21	9	67-69
8	28	12	5	64-66
9	32	6	2	58-60
10	36	4	Nil	-
11	40	Nil	Nil	-

Figure 3 : Screening of mutant strain for hyper endogluconase production using Congo red reaction



The cultivated fungal colonies with highest CMC hydrolyzing zone was regarded as endoglucanase hyper producing potent strain (Figure 4; Table 2).

Figure 4 : CMC hydrolyzing zone with congo red reaction



Acclimatized strain before mutation

Strain after mutation

Table 2 : Difference between acclimatized strain before mutation and after mutation[6]

Strain	Zone in mm with congo red reaction
Strain after acclimatization before mutation	56
Strain after mutation	69

DISCUSSION

The main effect of mutagenic agents (Xrays, UVrays, nitrous acid, dimethylsulfonate, ethyl methane sulfonate (EMS)andacridine mustards) is to induce a lesionin or modification of the base sequence ofDNA molecule; a mutation appears if thislesion remains unrepaired[9]. The mutagenesis wasachieved through UV rays in for highercellulase production. The selection of thesecellulase producing strains was based on thelarger diameter of clear zone surroundingthe colonies on plate screening medium ascompared to wild strain.

In the present study, culturewasmutagenized and genetically modified todevelop a mutant strain capable ofexhibiting hyper production of endoglucanase.This is because of fungal strains have aunique character to pass over theenvironmental stress including chemical andirradiative mutagenesis and are highlysusceptible to various physical as well aschemical mutagenic agents.

CONCLUSION

Secondary screening of hyper endoglucanase producing fungalstrain through mutagenesis proved significant to mutate the fungal strain for hyper biosynthesis of desire enzymes.Thesecondary screened strain could be used for further strain improvement through cell recombination technology for their commercial exploitation for the development of sustainable, ecofriendly, economical large scale production of the enzyme as biopolishing catalyst in textile industries.

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