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Research article

MULTISTEP MUTAGENIC STRAIN IMPROVEMENT IN *ASPERGILLUS CARBONARIUS* TO ENHANCE PECTINASE PRODUCTION POTENTIAL

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ABSTRACT: Pectinase is one of the most important commercially synthesised enzyme having its application in several industrial sectors like food and beverages, fruit clarifications etc. *A.carbonarius* has the capacity to produce Exo-pectinase 50 U/ml by submerged fermentation process as per the previous study. The present study describes the improvement of previously identified fungal strain *Aspergillus carbonarius* for enhancement of pectinase production by inducing mutations using physical and chemical mutagens. Aiming to increase the potentiality in pectinase production, the parental strain was treated for three times with four mutagens - UV irradiation, Colchicine, Hydrogen peroxide and Ethidium bromide to obtain mutants. Mutants were selected based on higher enzyme activity, improved growth rates and varied morphology with increased pectinase production. All the surviving mutants were assessed quantitatively after first mutagenic treatment. The stability of the best mutants was tested by repeating the exposures for two times to obtain 3rd generation mutants. These mutants were tested quantitatively to assess the pectinase production. Of all the best mutants E8 showed maximum activity producing 65U/ml pectinase enzyme compared to wild and sister mutants. The wild strain of *A. carbonarius* is a low pectinase producing organism as per literature. This strain was successfully mutated to increase the productivity rate to 1.8 fold in comparison to wild strain. This overproduction and strain stability may be due to repeated mutagenic treatments.

Keywords: *Aspergillus carbonarius*; mutations; pectinase; UV mutations; Colchicine; Hydrogen peroxide; Ethidium bromide.

INTRODUCTION

Fungi have a great ability to degrade polysaccharide constituents of plant biomass like pectin located in middle lamella of plant cell wall [Luciana, et. al., 1999].They are the potential source of pectinolytic enzymes with scientific, commercial and economical interest.

Pectinolytic enzymes are of great commercial value among various depolymerising enzymes. Various industrial applications like food processing, textile processing, degumming of plant fibres, extraction of pigments from plant materials, preparation of cellulose fibres for linen, jute and hemp manufacture [Whitaker, 1984], agro waste treatment and fruit juice treatment, coffee & tea fermentation, oil extraction, paper pulp treatment, waste water treatment, bleaching of paper, adding poultry feed and in the alcoholic beverages and food industries [Bharadwaj, et. al., 2010] are done using these enzymes. Fungi can produce both intracellular as well as extracellular enzymes. Fungi are heterotrophic organisms, hence they depend on carbon compounds synthesized by other living organisms. Small molecules like mono, disaccharides, fatty acids and amino acids can easily pass through but for breaking down of larger complex compounds like pectin, fungi secrete extra cellular enzymes. It is well known that compared to intracellular enzymes, the extra cellular enzymes can be easily extracted. Procedures of intracellular enzyme extraction are a time taking and cost effective [Hankin, et.al., 1975]. *Aspergillus carbonarius* is a large sized black coloured spore forming filamentous ascomycete having a capacity to produce exopectinase enzyme [Kavitha, et.al., 2000]. This organism is a common inhabitant of decaying fruit and vegetable wastes.

Extensive research for high potential enzyme preparations from a large variety of fungi has been conducted from years together, but it couldn't reach to the requirements of the industrialists. Hence, strain improvement by mutations is a successful method employed for increasing the efficiency of wild fungal strains, but it is largely a trial and error process involving several laborious steps of procedures in performance [Iftikhar, et.al., 2010] .The rarity in obtaining positive results and hence the failure of subsequent workers in reproducing them is the main obstacle in acceptance of mutations [Robert, et.al., 1940].

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The main aim of this work is to induce mutations and develop a mutant strain of higher exopectinase secreting potential than its parent strain. In a previous paper, we described the screening, isolation and characterisation of an exopectinase producing fungi, *Aspergillus carbonarius* in SbF. Media formulation was done using various fruit wastes of which *A.carbonarius* showed maximum enzyme activity of 48 U/ml in groundnut hull – groundnut oil cake (GHG) media [Sabika, et.al., 2012]. In this paper a multistep mutagenesis was employed and the increase in production levels of Exopectinase by the mutants of same fungus in SbF with similar media composition by comparing the mutants developed with the wild as well as neighbouring sister mutant strains was reported.

MATERIALS AND METHODS

Microorganism:

The culture of *Aspergillus carbonarius* used in this work was isolated previously qualitatively by standard plate screening method [Sabika, et.al., 2012]. In the present data, this strain was improved through UV and various chemical mutagens in terms of pectinase production using submerged fermentation in 250 ml Erlenmeyer flasks.

Subculture and maintenance of microorganism

The strains were sub cultured on SDA slants and incubated for 72 h at 30°C. The sub cultured strains were maintained in a refrigerator at 4°C in the laboratory by conventional methods and sub cultured at regular intervals.

Strain Improvement

For the sake of culture improvement, the identified pectinolytic strain was treated in two steps with four mutagens for obtaining a potential and stable mutant:-

Ultraviolet Irradiation - physical mutagen [Vijaya lakshmi, et.al., 2010]
Colchicine - chemical mutagen [Bhargavi, et.al., 2010]
Hydrogen peroxide - chemical mutagen [Aldo, et.al., 2006]
Ethidium bromide - chemical mutagen [Chand, et.al., 2005]

Multistep mutagenesis

A eukaryotic system generally has a natural tendency of reverting back to its wild form. Hence the selected mutant strains were again subjected to mutagenesis following same methodology and the selected mutant strains of second generation pectinolytic mutants
were cultivated in liquid culture medium [Sudarshana, et.al., 2012].

In the first step, the wild strain was exposed to various mutagens and estimated for pectinolytic activity. The first generation mutants having better pectinolytic activity were selected. In the second step of mutagenesis, the identified best mutants were treated with the same mutagen at same concentration for two times [Sudarshana, et.al., 2012]. The obtained third generation mutants were screened out for improved pectinolytic activity [Muhammad, et.al., 2011].

Physical mutagen treatmen

YEP selective media [Rupinder, et.al., 2005] of following composition (g/L) was prepared and plated in sterile petri plates for growth of the fungal isolate.

Pectin – 10; yeast extract – 5; Congo red - 0.125% [Hyuk Woo, et.al., 2007]; Agar – 2%; pH – 6.2

A loop full of parental strain (3 days old culture) was inoculated into the plate and was exposed to ultraviolet radiation for 10, 20, 30, 40, 50, 60, 70, 80, 90,100,110 and 120 min time interval under beam of UV lamp(235 nm) (GERMICIDAL LAMP (VL-G),UV tube T- 15C 15W 254 nm, VILBER-LOURMAT). The distance between lamp and the petri plate was adjusted to 10 cm for each trail to obtain 95% death rate [Vijaya lakshmi, et.al., 2010]. On completion of predetermined time the plates were retained in dark for overnight to prevent photo reactivation of mutants.

Chemical mutagen treatment

Similarly, selective media was prepared and 5 ml of molten agar was mixed with different concentrations of Colchicine, Hydrogen peroxide and Ethidium bromide mutagens ranging from 1 - 10µg and inoculated with parental strain [Chand, et.al., 2005].

Selection of mutants

After exposure/treatment with mutagens, the plates were incubated at 28^0C ±2 and were examined regularly at an interval of 24 hrs. The surviving colonies were selected and examined for characters including growth rate that was measured as linear growth (mm), number of colonies and sporulation in each plate that counted as colony forming unit (CfuX10^{-5}) [Abdel- Latif, et.al., 2010] and their pectinolytic effect on the selective media.

The cultural and morphological variations in between mutant and parental strain were also observed. The Cfu count was recorded to plot a survival/kill curve. Further all the strains were tested quantitatively for enzyme production by shake flask culture fermentation.

Quantitative analysis of mutants

To find out the positively mutated strain of the fungus, submerged fermentation was performed using previously determined groundnut hull – groundnut oil cake (GHG) media. The obtained mutants and the parental strain were inoculated into the GHG media (pH-6.2) and all the flasks were incubated in orbital shaking incubator at 120 rpm for 5 days at 30^0C±2 temperature. The extracted cell free filtrate was further assayed to determine the potentiality in enzyme secretion of the mutated strains. The obtained results were compared with the parental/wild strain to estimate the increase in pectinase production.

Sample extraction followed by fermentation

Upon completion of SbF, the cell free extract was assayed for enzyme activity after removal of residual fungal growth by filtration using pre determined Whatmann (no-1) [Praveen Kumar, et.al., 2008] filter papers followed by centrifugation at 6000rpm for 10 min. The resulting supernatant was assayed for pectinolytic activity.

Analytical determination

Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method; this method was given by Miller [Miller, 1959]. To1ml of 1% pectin substrate buffer (pH 4.5), 0.5 ml of enzyme extract was added. The reaction mixture was incubated at 50°C for 30 min. After 30min, 1.5 ml of DNS reagent was added to the reaction mixture and were shaken to mix the contents. The test-tubes were heated to 90^0C in the boiling water bath for 10 -15 min, cooled and 5 ml of distilled water was added to the contents of each tube. The absorbance was measured at 575 nm using digital colorimeter (Systronics). The controls were maintained parallel to tests. The enzyme activity was measured as 1 unit of enzyme activity is equal to 0.01 moles of mono galacturonic acid released.

RESULTS AND DISCUSSION

A mutation is a permanent change in the DNA sequence of a particular gene and mutagenesis is the source of all genetic variations. Many works have been carried out in order to find better pectinase producers by mutation and gene modification methods [Abdul, 2008; Durrands, 1988; Leuchtenberger, 1991]. *A.carbonarius* was subjected to mutations for strain development as it is a low pectinase producing organism [Kavitha, et.al., 2000]. The present work deals with exposure of *A. carbonarius* to UV irradiation and chemicals Colchicine, Ethidium bromide and H_2O_2 with an expectation of higher pectinase production.

Morphological examination of the mutants:

The parental/wild strain of *Aspergillus carbonarius* is a very fast growing organism showing profuse growth with heavy sporulation on 3rd day of incubation with well defined colonies. Each colony showed clear, aerial, very long, spiking hyphae with large, black coloured spores within 3 days of incubation.

Morphological changes in the mutants

Growth was observed till 120 min of UV exposure and 1 – 10 µg concentrations of all chemical mutagens but the growth was very scanty as the time interval and concentrations increased. The spiking, very long hyphae and large circular sized conidia lost their appearance along with reduction in extension and branching of hyphae. Instead, very short hyphae with dark greenish – black, small sized conidia were observed which were tightly bound to the agar media. Difficulty in picking up the colonies was observed during sub culturing. Clear zone of lysis was observed around the colony on the 2nd day of incubation.

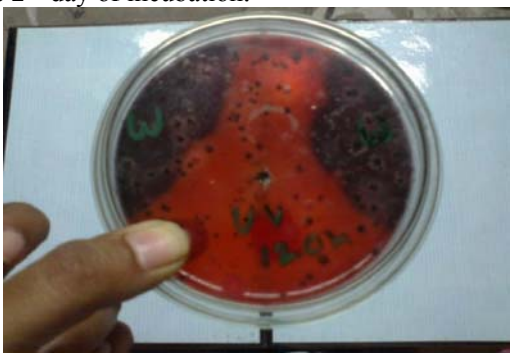

Fig: 1 Picture showing difference in mutant and wild strain growth in production activity

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Quantitative estimation of mutants

All the mutant strains survived after intense UV and chemical treatment but the number of colonies gradually decreased with slight variations. A survival graph was plotted based on the number of colonies generated.

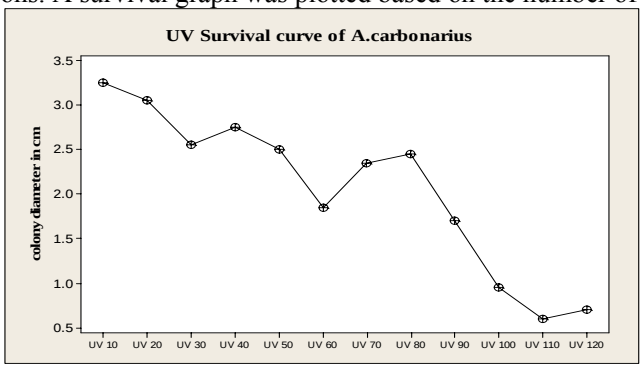

Fig: 2 Graph showing UV survival plot

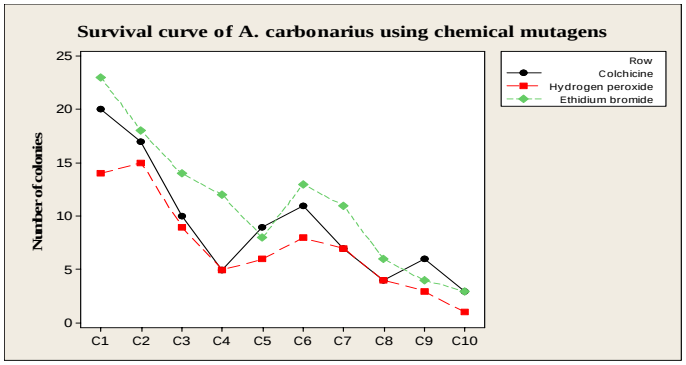

Fig: 3 Graph showing survival plot of three chemical mutagens

All the UV mutants, irrespective of their cultural and morphological characteristics, were tested for improved pectinase production along with parental/wild strain as control. The quantitative tests of the mutants revealed that mutant UV 90 had high titre pectinase production of 45 U/ml compared to wild strain and sister mutants.

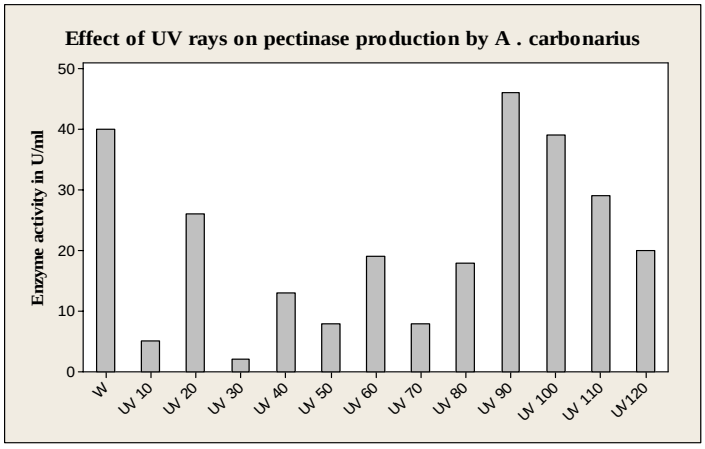

Fig: 4 Graph showing quantitative analysis of UV treated mutants

In order to achieve the best Colchicine mutant, all the mutants were subjected to submerged fermentation along with the wild strain. C2 proved to be the best mutant showing highest pectinolytic activity of 60U/ml compared to parent and sister strains.

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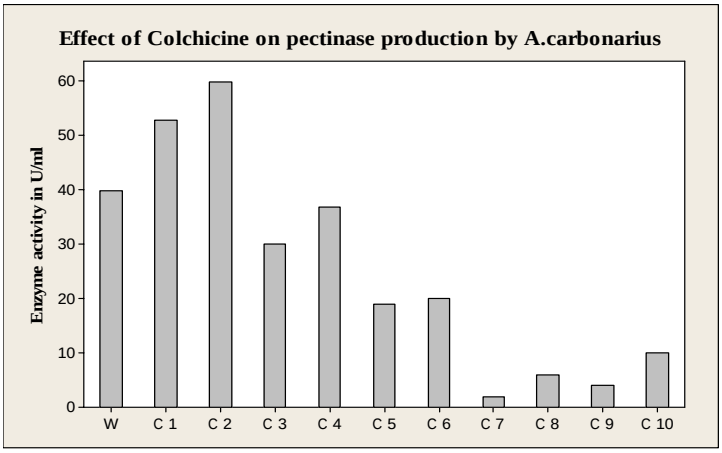

Fig: 5 Graph showing quantitative analysis of Colchicine treated mutants

After treatment with H_2O_2, the potential pectinolytic mutant was screened quantitatively. Significant increase in pectinase production of 46 U/ml was observed in H3 mutant strain.

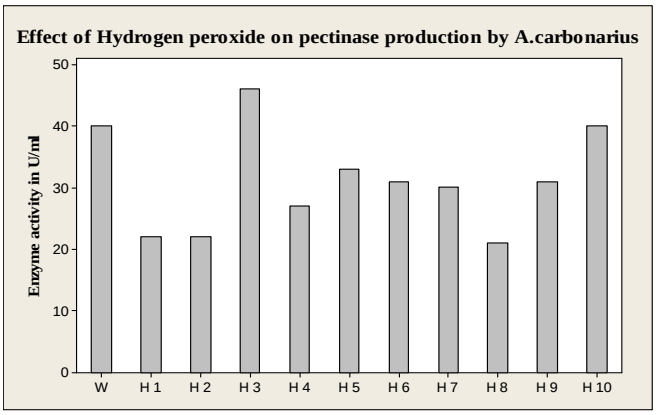

Fig: 6 Graph showing quantitative analysis of H_2O_2 treated mutants

After treatment with lethal mutagen, ethidium bromide, the mutants were tested for hyper pectinase production. The quantitative analysis showed an increase in pectinase production of 65 U/ml by E8 mutant among its variants and parental strain.

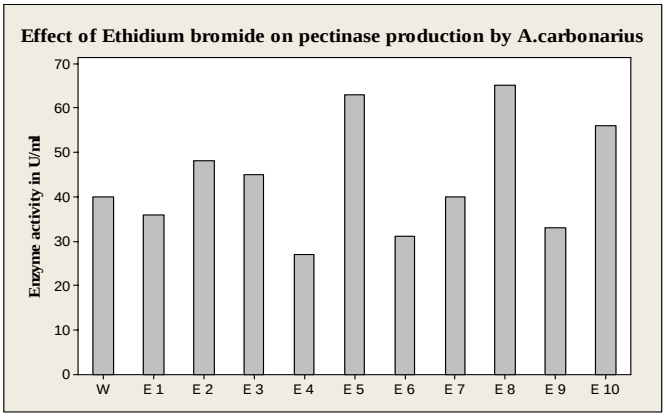

Fig: 7 Graph showing quantitative analysis of Ethidium bromide treated mutants

With a view to enhance hyper pectinolytic efficacy, multi step mutagenesis was employed. All the best mutants identified from various mutagenic treatments in step – 1were again subjected to second level mutagenesis. Four best mutants from different categories were selected and were exposed to same mutagen at same concentration for second and third time. These second generation mutants were quantitatively screened to reveal the hyper pectinolytic activity.

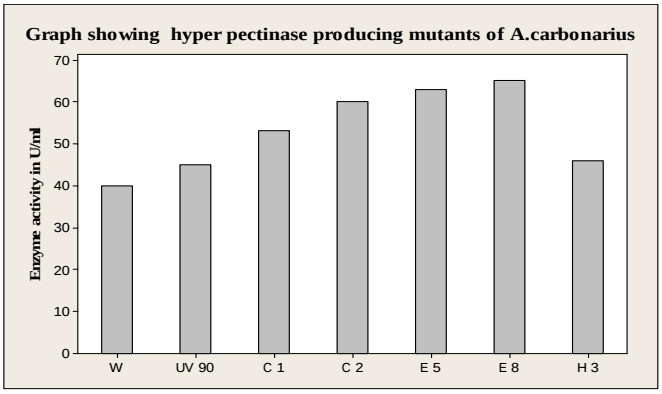

Fig: 8 Graph showing comparative study of best mutants of all mutagens in 3rd generation

Assessment of third generation mutants revealed that mutant E8 showed maximum pectinolytic activity of 65 U/ml enzyme production compared to wild strain and other mutant strains . From this data it can be confirmed that strain has been improved with 1.8 fold increase in enzyme production.

CONCLUSION

The main aim of this project was to mutate and develop a strain capable of secreting high quantities of pectinase enzyme using previously determined production media (GHG). Maximum pectinase production was obtained in the mutant treated with Ethidium bromide of 8µg concentration (**E8**). Various morphological changes were also observed in the mutant compared to wild strain. The stability of the mutant was determined by performing multistep mutagenesis.

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