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# EFFECT OF MUTAGENS ON LOCAL FUNGAL ISOLATE OF JABALPUR REGION FOR OVER-EXPRESSION OF GLUCOAMYLASE

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***Abstract:*** The present investigation was conducted to study effect of mutagenesis for increased glucoamylase productivity by local soil fungi of Jabalpur region. The fungus was subjected to mutagenesis using ultraviolet radiation, nitrous acid and ethidium bromide treatment for increased glucoamylase production. After the treatment and 7 repeated subculture, expression of enzyme activity by the mutant SSP#16NA3 was found to be irrevocable. The glucoamylase production in soild state fermentation by mutant isolate (SSP#16NA3 mutant) was done in solid substrate (rice bran) in standard basal medium at temperature 27°C with *p*H 6.0 and incubated for 96 h. The potent mutant isolate showed nearly 3 fold increase in enzyme activity as compared to wild strain. This local fungal isolate showing enhanced glucoamylase activity can be exploited for achieving economy in starch processing industries.

**Keywords:** mutagenesis, ultraviolet radiation, nitrous acid, ethidium bromide, soild state fermentation, mutant isolate

## 1. INTRODUCTION:

Starch is one of the most important naturally occurring polymers and appears to be the cheapest future raw material of alcohol industry (Sandhu *et al.* 2016). The soil fungi act as a source of industrially important and relevant enzymes like glucoamylase, protease, lipase etc. This enzyme plays important role in food industries for saccharification of starch and other related oligosaccharides. Starchy biomass appears to be the cheapest future raw material of alcohol industry in view of rising cost and deteriorating quality of fermentable molasses. The use of fungi for the hydrolysis of starch is preferred in recent years; therefore significant research exists for its use in industrial biotechnology. The conversion of starch and starchy waste materials traditionally acquires acid hydrolysis but nowadays hydrolysis by enzymes has advantages because of its specificity. Glucoamylase (exo-1, 4-α-D-glucanglucanohydrolase, EC 3.2.1.3 with synonym amyloglucosidase) is the major starch-degrading enzyme (Kumar et al 2009) secreted by fungi. They act on the polymers from the non reducing chain ends and release β- D glucose residues (Antranikian 1992) and hydrolyze α -1, 4 glycosidic linkages in raw or soluble starches and related oligosacharides, producing β-glucose.

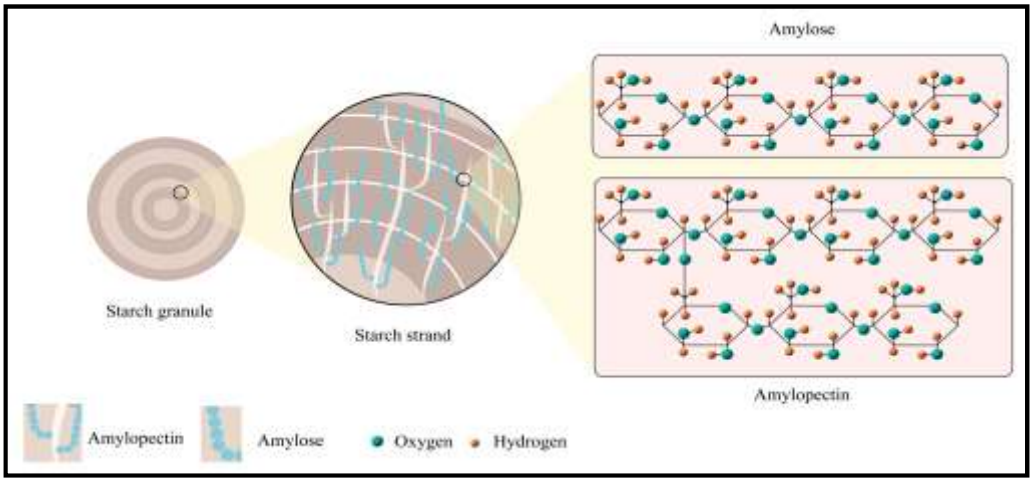


**Fig. 1** Structure of starch amylose and amylopectin (Aydemir *et al*. 2014)

In recent years, glucoamylase for industrial activities have received very little attention because of its high cost production. It is therefore, very necessary, that efforts are made to produce amylases using microorganisms grown on cheaper substrates (Sandhu *et al* 2016). A number of industries that depend on glucose as raw materials will receive a great boost when amylases, particularly, glucoamylase are produced in commercial quantities by fungi using local and cheap substrates. Glucoamylase shows its applications in many industries like food, beer, baking, alcohol and production of glucose syrups with a high amount of fructose. The present work was designed with aim of improvement of potent fungal strain by mutagenic (Ultraviolet radiation, nitrous acid and ethidium bromide) treatment (Chand *et al*.2005). Thereafter study of glucoamylase production of mutant isolate was done by solid state fermentation.

## 2. MATERIALS AND METHODS

The fungal isolates used in the current study were obtained from soil samples of Jabalpur region (Sandhu *et al* 2018). They were subjected to primary and secondary screening for glucoamylase activity. The primary screening was done by starch hydrolysis test (Abe *et al* 1988). This was followed by secondary screening (Pandey *et al.* 2000) which was done by solid state fermentation using rice bran as substrate in mineral media. Among the isolates, potential isolate SSP#16 showing maximum zone and glucoamylase activity was selected for further study of mutagenic treatment.

**Improvement of selected potent isolate by mutagenesis:**
The potent amylolytic fungal isolate SSP#16 was exploited further for strain improvement using UV light and chemical mutagenic treatment in order to attain a fungal strain giving high titers of glucaomylase activity. The exposed cells were incubated in potato dextrose agar for 5 days at 27°C. The number of survivors was counted with reference to control. The colonies that survived in both mutagenic treatments were randomly selected and re-examined for primary and secondary screening).

**a) Treatment with UV Light**
Mutagenic treatment with UV light was used as given by Haq *et al.* (2002). The spore suspension (1 mL) was serially diluted upto $10^{-6}$ times. These diluted suspensions (10 mL) was transferred to the sterilized Petri-plates and then placed under UV lamp (emitting the energy of 1.6x10 J/min$^2$ /s) for 5, 10, 15, 20, 25 and 30 min at a distance 30 cm. The plate without UV exposure was taken as control. After different time interval, 0.5 mL of the spore suspension was spread plated on different Petri-plates containing potato starch agar medium (wrapped with carbon paper to prevent from photo reactivation). These plates were then placed in the incubator at 27±1ºC for 4-5 days. The survived colonies from UV light treated plates were picked up randomly for screening of mutants.

**b) Nitrous Acid Treatment**
Nitrous acid treatment was given to the selected fungal isolate according to method given by Carlton & Brown (1981). The spore suspension was prepared by adding 10 mL of 0.8% Tween 80 solution on slants containing fungal spores. This spore suspension (1 mL)was suspended in a mixture of 0.1 mL of sodium nitrite (0.3 M) and 0.9 mL of acetate buffer (*p*H 4.5), which generates mutagenic agent (nitrous acid). The spore suspension was incubated in this mixture for 5, 10, 15, 20, 25 and 30 min. At each time interval, 5 mL of 0.2 M phosphate buffer (*p*H 7) was added to stop the reaction. The suspension without nitrous acid was taken as control. After specific time interval, tubes were centrifuged at 6000 rpm for 15 min. The supernatant was discarded to eliminate nitrous acid and pellet was re-suspended in 1 mL phosphate buffer. This step was repeated thrice and suspended in similar buffer. The treated cells were cultured on PDA plates and incubated at 27±1ºC for 5 days. The survived colonies from treated plates were picked up randomly for screening of mutants.

**c) Ethidium Bromide Treatment**
Ethidium bromide (EtBr) was used as mutagenic agent as given by Michaelis *et al.* (1971) at concentration 0.01 mg/mL, 0.03 mg/mL and 0.05 mg/mL. 10 mL of each concentration of ethidium bromide was transferred to respective sterilized centrifuged tube containing 5 mLof spore suspension. The tubes containing suspension were kept at room temperature for different timeintervals such as 15, 30, 45 and 60 min. After the fixed time interval, the tubes were centrifuged at 6000 rpm for 15 min. The

fungal cells formed a pellet along the wall of the tube. The supernatant containing ethidium bromide was discarded. To each tube, 5 mL of sterilized distilled water was added and was vortexed for 2 min. The tubes were re-centrifuged to remove the traces of ethidium bromide from fungal cells. The process of washing was repeated thrice. 10 mL of sterilized distilled water was added to each tube and were shaken to form spore suspension. The tube without ethidium bromide treatment was taken as control in the experiment. This treated suspension (0.5 mL) was transferred to the PDA plates, which were then rotated clock-wise and anti-clock-wise to spread it evenly. The Petri-plates were incubated at27±1ºC for 5 days. The survived colonies from treated plates were picked up randomly for screening of mutants.

**Primary and Secondary Screening of mutants**

The selected mutants were evaluated separately for primary screening (starch hydrolysis test by Abe *et al* 1988) and secondary screening (glucoamylase production in solid state fermentation by Pandey *et al.* 2000). From the various mutated colonies, the colonies were primarily screened on the basis of zone of clearance on starch agar plates to check the zone of clearance. Colonies showing larger zone of clearance than the parental strain were selected and subjected for the secondary screening by solid state fermentation

**Stability Studies**

Among the different mutants, the promising mutant isolate was used for assessment of stability in zone of clearance and enzyme production after repeated subcultures. It was performed under uniform conditions via starch hydrolysis assay method and solid state fermentation to determine consistent enzyme production. The stability of selected mutant was determined by successive 7 repeated sub-culturing on potato dextrose agar medium plates incubated at 27±1ºC for 5 days (Mishra *et al.* 2014). After each subculture, mutant was tested for its ability to show consistent zone of clearance (starch hydrolysis test) and consistent production of glucoamylase by solid state fermentation.

## 3. RESULTS AND DISCUSSION

The present study was aimed at the physical and chemical treatment of mutagenesis and selection of potent mutant isolate. The criterion for the selection of mutagens was on the basis of research data available for enhancement of enzyme activity. The mutagens showing low toxicity, easy accessibility and with no ethical issues were selected for the mutation treatment in the present study (Abdullah *et al.* 2013; Mishra *et al.* 2014; Ghani *et al.* 2013; Radha *et al.* 2012; Kumar *et al.* 2009; Imran *et al.* 2012).The potent fungal isolate SSP#16 showing maximum glucoamylase activity during screening was exposed to physical (UV treatment) and chemical mutagens (Nitrous acid and EtBr) to further enhance the glucoamylase production. After physical and chemical treatment, out of large number of colonies, the survivor mutant colonies were randomly selected for the selection of desired mutant with high enzyme activity. The selected colonies were investigated by primary screening (starch hydrolysis) and secondary screening (glucoamylase production by SSF). The mutants giving utmost zone of clearance range were further assessed for stability studies.

**1. Ultraviolet (UV) Treatment**

The potent fungal isolate was subjected to Ultraviolet (UV) ray treatment for varying time intervals for the selection of mutant fungi showing higher glucoamylase production. After exposure for 5, 10, 15, 20, 25 and 30 min, plating was done on potato dextrose agar plates. These plates were incubated at 27ºC for 5 days. The colonies that survived in mutagenic treatment were randomly selected and examined for starch hydrolysis test. Primary screening of the selected colonies was achieved on selective isolation medium (in starch agar plates). About 42 colonies were selected and screened for larger zone of starch hydrolysis. Of all the isolates investigated, 14 mutant isolates designated UV10, UV17, UV18, UV19, UV20, UV21, UV22, UV23, UV24, UV25, UV26, UV27, UV28, UV29 showed larger zone of clearance as compared to the control (wild) as depicted in Fig. 2. Among these isolates, the highest zone of clearance 34.63±0.52 mm was shown by mutant UV18 which is followed by mutant UV22 and UV24 and rest of the mutants showed minor change in zone of clearance. The results show that increase in UV exposure caused decline in enzyme activity. Similar studies showed significant increase in amylase production by treatment of *Aspergillus flavus NSH9* with mutagen UV irradiation (Ruaida *et al.* 2021) and reported 1.3 times more glucoamylase activity as compared to contol.

**2. Nitrous acid treatment:**

The potent fungal spore suspension was exposed to nitrous acid treatment by incubating in nitrous acid (acetate buffer *p*H 4.5 containing sodium nitrite**)** for 5, 10, 15, 20, 25 and 30 min. After exposure, the cells were plated on PDA plates and incubated at 27ºC for 5 days. About 30 mutant strains were screened for larger zone of starch hydrolysis in the Petri-plates. Fig.3 reveals that the mutants showed significant variation in zone diameter and selected five isolates designated NA3, NA6, NA12, NA13 and NA16 showed increase in zone of clearance as compared to the control. Among these isolates, the highest zone of clearance 35.83±0.68mm was shown by mutant NA3 which is followed by mutant NA12 and NA16. These selected mutants NA3, NA6, NA12, NA13 and NA16 were further subjected to secondary screening and stability studies. Abdullah *et al*. (2013) studied random mutagenesis for enhanced production of α amylase by *Aspergillus oryzae* HB-30. The UV, Nitrous acid and ethyl methane sulphonate was used and it was found that the use of nitrous acid and ethyl methane sulphonate enhance the amylolytic potential of the parental strain. Similar reports by Rubinder *et al*. (2002); Azin and Noroozi, (2001) showed the use of nitrous acid as potent mutagen for enhanced enzyme production.

**3. Ethidium bromide (EtBr) treatment:**

The conventional mutagen Ethidium bromide (EtBr) was adopted to develop mutant for enhanced production of glucoamylase.The treatment was done by the exposing the fungal spore suspension with (0.01, 0.03 and 0.05 mg/mL) EtBr solution. The cells were incubated in this mixture for 15, 30, 45 and 60 minute at room temperature. After exposure, the cells were plated on PDA plates and incubated at 27ºC for 5 days. The colonies were selected on the basis of larger zone of starch hydrolysis in the Petri-plates. Fig. 4 depicts that the colonies showed variation in zone diameter and the colonies designated as EtBr3, EtBr5, EtBr6, EtBr7, EtBr10 and EtBr11 exhibited higher zone of clearance diameter as compared to wild isolate The highest zone of clearance 31.16

± 0.58mm was shown by mutant EtBr6 which is followed by mutant EtBr5 and EtBr7. The rest of the selected colonies showed minor or no variation in zone diameter. Similarly, Ruaida *et al* 2021 reported 1.7 times higher glucoamylase activity with 5 µg/mL EtBr in sublethal concentration.

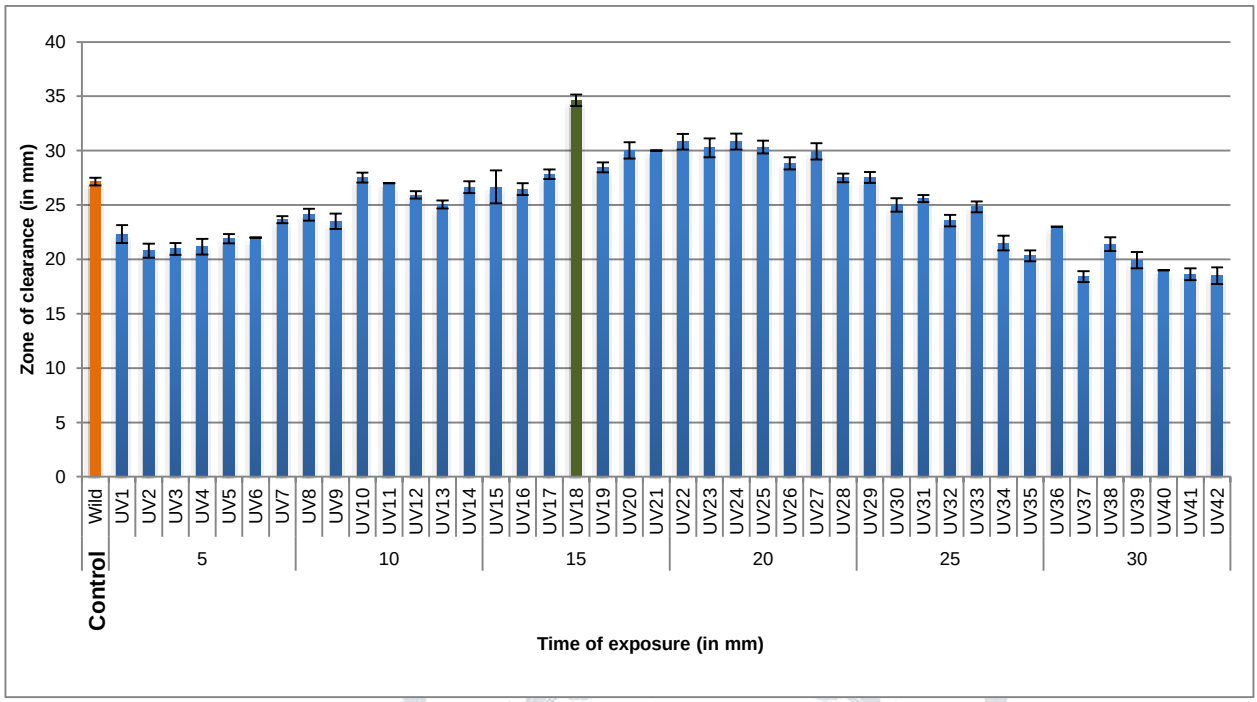


**Fig 2: Effect of UV radiation on glucoamylase activity of the mutant isolates**

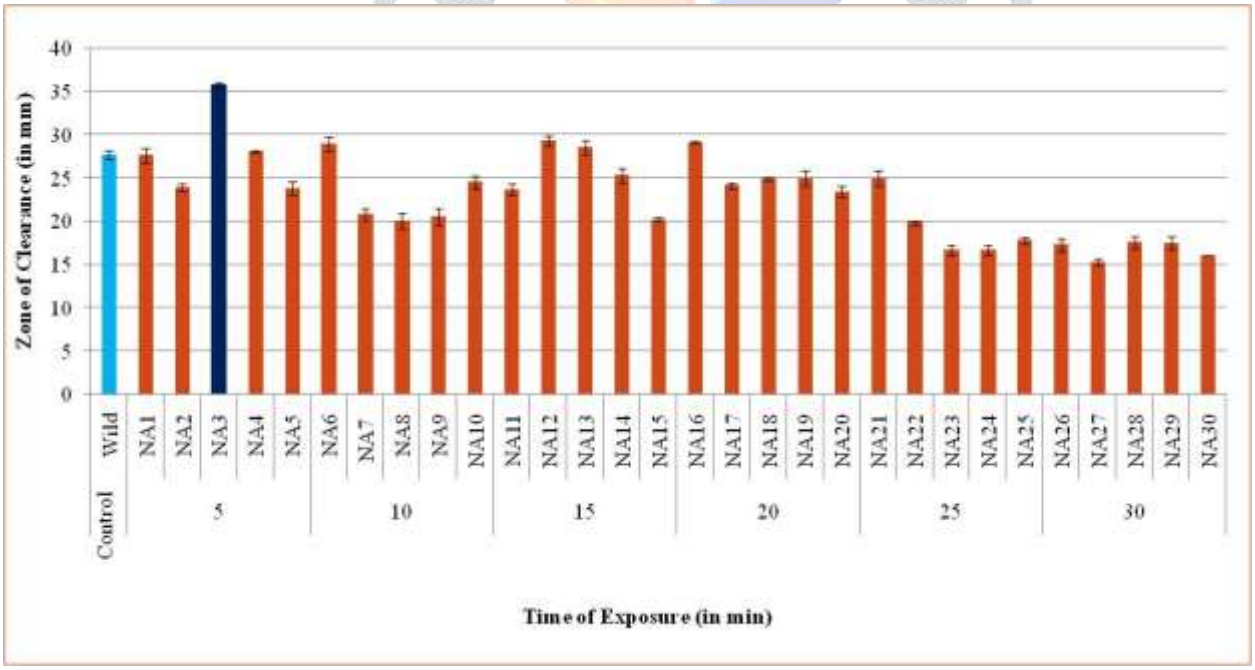


**Fig 3: Effect of Nitrous acid on glucoamylase activity of mutant isolates**

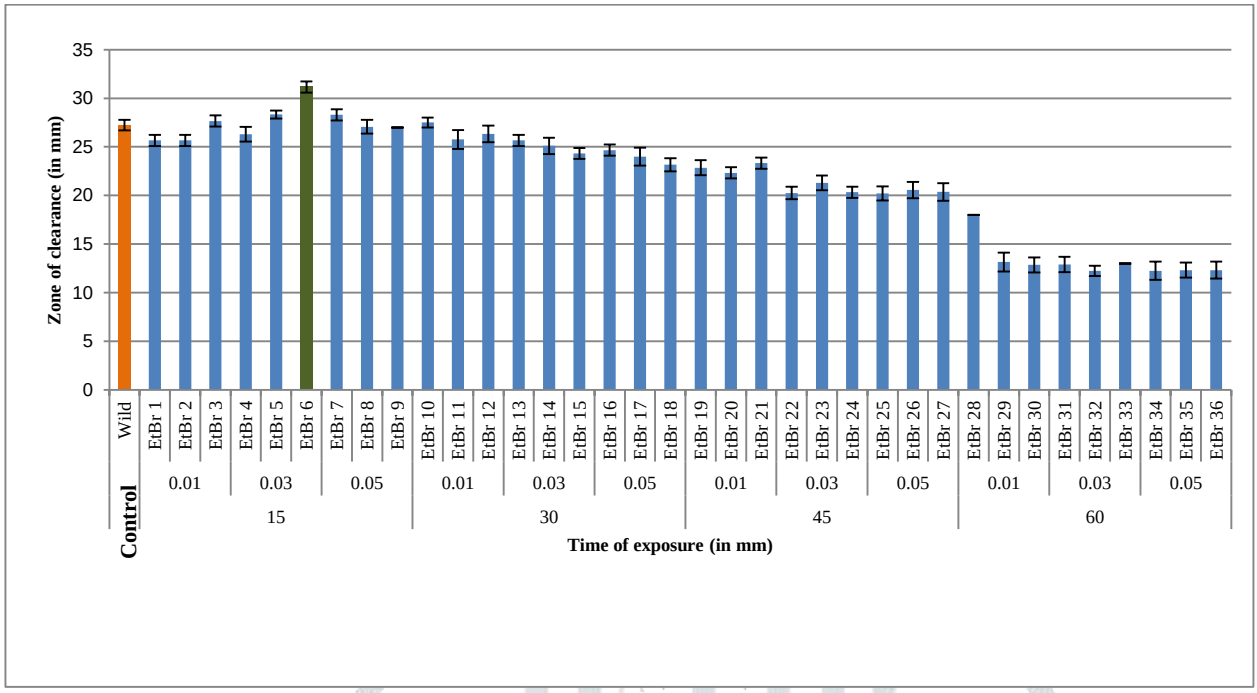


**Fig 4: Effect of Ethidium bromide on glucoamylase activity of mutant isolates**

**Screening of selected mutant colonies**

The selected mutants during primary screening were evaluated separately for enzyme production in solid state fermentation to examine the increase in glucoamylase activity. The mutants were selected on the basis of larger zone size from the various mutagenic treatment processes. The evaluation for secondary screening was done on basal media (rice bran moistened with mineral media) and enzyme activity was determined after 96 h of incubation at 27ºC. Based on level of glucoamylase produced by solid state fermentation, it was observed that all the selected mutants UV17, UV18, UV22, UV24, UV27, NA3, NA6, NA12, NA13, NA16, EtBr3, EtBr5, EtBr6, EtBr13 and EtBr15 exhibited increased glucoamylase activity with respect to control (wild) isolate. Among these mutants, the maximum glucoamylase activity (12.15 U/mL/min) was observed with NA3 mutants, UV18 exhibited 11.04 U/mL/min glucoamylase activities whereas EtBr6 showed 8.94 U/mL/min glucoamylase activity. Fig. 5 depicts that the glucoamylase activity varied notably for the mutants with higher zone of clearance. Similar results were reported by Raju *et al* 2012 for the screening of fungal colonies treated with physical and chemical mutagens.

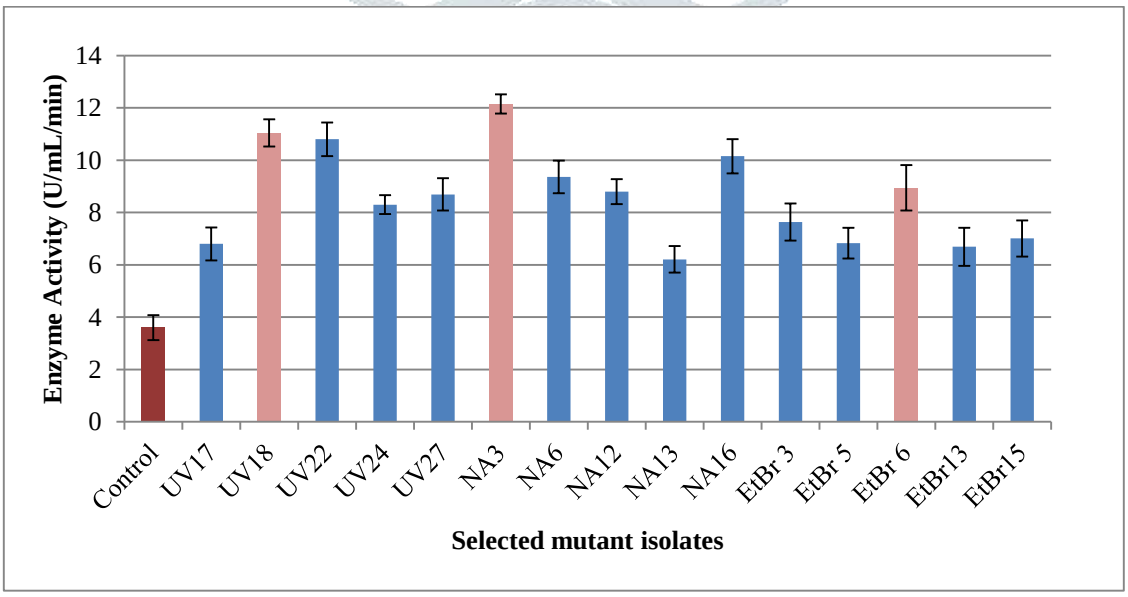


**Fig. 5:** Screening of selected mutants for glucoamylase activity

**Stability studies of mutant**

Among the different selected mutants, the promising mutant isolates SSP#16UV18, SSP#16NA3 and SSP#16EtBr6 were used for assessment of stability in zone of clearance and enzyme production after 7 repeated subcultures. It was performed under uniform conditions via starch hydrolysis assay method and solid state fermentation to determine consistent enzyme production. The stability of selected mutant was determined by successive 7 repeated sub-culturing on potato dextrose agar medium plates incubated at 27±1ºC for 5 days. After each subculture, mutant was tested for its ability to stably show zone of clearance (starch hydrolysis test) and production of glucoamylase by solid state fermentation (Mishra *et al.* 2014). However, the mutant NA3 showed consistency in the production of the enzyme after repeated subcultures. The zone of clearance and enzyme activity remained almost same for all the subcultures. Figure 6 and Fig.ure 7 depicts consistent glucoamylase activity in case NA3 mutant. The stable enzyme activity was sustained even after 7 repeated subculture showing expression of enzyme activity by the mutant **SSP#16 NA3** to be irrevocable.

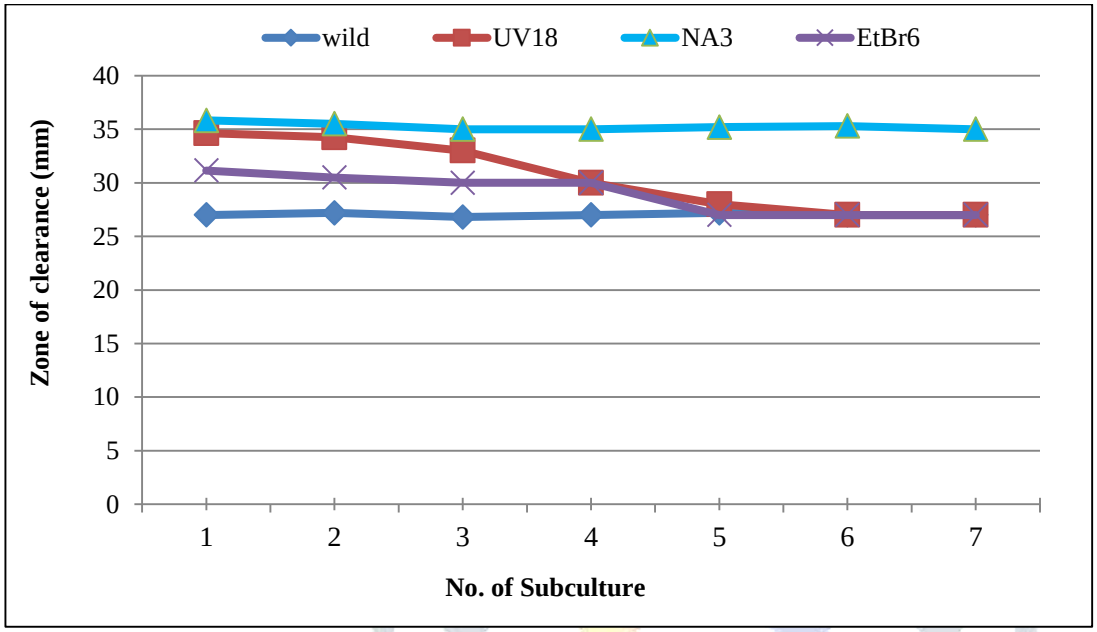


**Fig. 6:** Stability studies of selected mutants for starch hydrolysis test

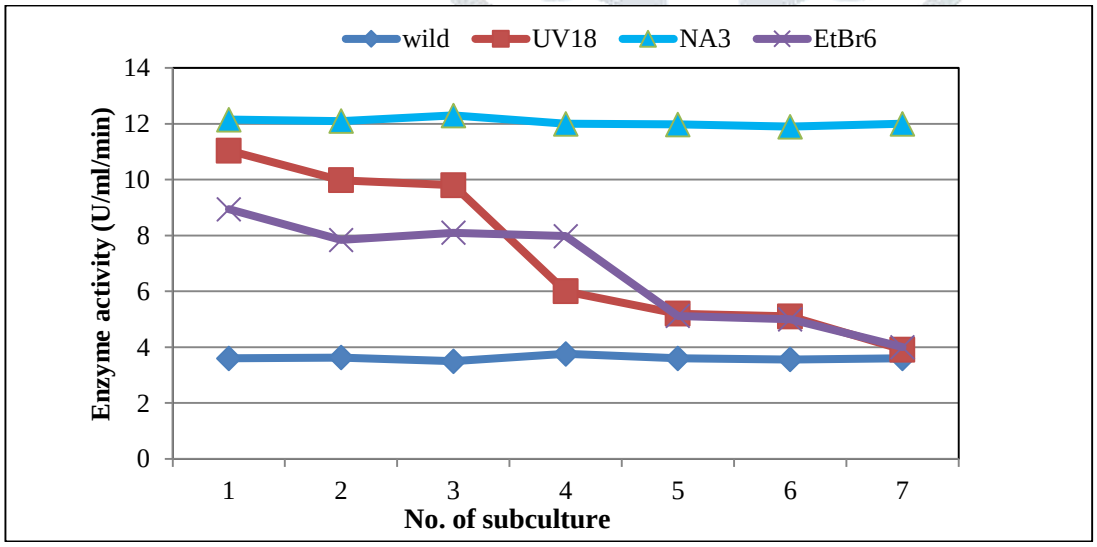


**Fig. 7:** Stability studies of selected mutants for solid state fermentation

**STUDY OF GLUCOAMYLASE PRODUCTION IN SOLID STATE FERMENTATION**

The glucoamylase production in soild state fermentation by mutant isolate (**SSP#16 NA3 mutant**) was done as shown in Table 2. The solid substrate (rice bran) was used in standard basal medium at temperature 27°C with *p*H 6.0 and incubated for 96 h. The samples were withdrawn every 24 h interval and after fermentation, the fermented medium was homogenized with 0.1M citrate phosphate buffer (*p*H 5.5) and filtered using Whatman paper no. 1 to separate the cell free supernatant from used substrate (rice bran) with cell biomass. The supernatant obtained was centrifuged at 10000 rpm for 20 min and again filtered. The glucoamylase activity was determined according to Cori (1955) and the reducing sugars liberated were determined by Dinitrosalicylic (DNSA) method (Miller *et al.* 1959).

| S. No. | Parameters | |
|---|---|---|
| 1. | Basal Media | Rice Bran with Mineral Media |
| 2. | Moisture level | 1: 2 |
| 3. | Incubation temperature | 27ºC |
| 4. | *p*H | 6.0 |
| 5. | Incubation temperature | 96 hrs |

**Table 2: Study of glucoamylase under optimized conditions in solid state fermentation**

**4. CONCLUSION**

In the present scenario, there is an urge to investigate the use of novel methods for enhanced production of enzymes. The traditional approaches for strain improvement (physical and chemical mutagens) have been used and proved to be fruitful to get superior mutants with enhanced enzyme production (Pathak *et al.* 2015). The present study was aimed at the exposure of potent fungal isolate to physical (UV treatment) and chemical mutagens (Nitrous acid and Ehidium bromide). The selected mutant treated with nitrous acid showed almost 3 fold increase in glucoamylase activity as compared to control. The expression of enzyme activity by the mutant SSP#16 NA3 was found to be irrevocable after 7 repeated subculture. The glucoamylase production in soild state fermentation by potent mutant isolate (SSP#16 NA3 mutant) was done in solid substrate (rice bran) in standard basal medium at temperature 27°C with *p*H 6.0 and incubated for 96 h. Thus, the present research reports the strain improvement of glucoamylase producing soil fungi isolated from Jabalpur (M.P) India. The research work was undertaken with the aim of exploiting local fungal isolate of Jabalpur region for industrial applications. The use of agro-industrial wastes residues in the present work emerges as an economical method for glucoamylase production and the local fungal isolate can be exploited for achieving economy in various industrial applications of biotechnological importance.

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