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ACKNOWLEDGEMENTS. I thank the Space Applications Center, Indian Space Research Organisation, Ahmedabad, India for providing the financial support (EPSA/GHCAG/GSD/WP/15/2017) for the research. I acknowledge Dr Harsh Bhu for fruitful discussions on the initial draft of this paper and anonymous reviewers for suggesting the improvements in the manuscript. I also thank the Head, Department of Geology, M. L. Sukhadia University for providing the necessary research facilities.

Received 25 June 2021; revised accepted 17 August 2021

doi: 10.18520/cs/v121/i7/962-966

# Increased xylanase activity in *Aspergillus niger* through mutation

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***Aspergillus niger*** **is used for xylanase production on agricultural waste as substrate under broth culture. Rice straw and sugarcane bagasse have been the most potential substrate for xylanase production. Two different mutagens were used: UV radiation for different time durations and 5-bromouracil of different concentrations. Mutants so formed were selected on the basis of morphological and colony characteristics. Selected mutants were checked for their stability, requirement of amino acid and xylanase activity. Tested mutants showed 4-fold increase in xylanase activity from wild type.**

**Keywords:** Agricultural waste, mutation, rice straw, sugarcane bagasse, xylanase.

MICROBIAL enzymes are one of the fastest growing fields in biotechnology as these enzymes have several industrial applications[1]. Most common enzymes include cellulase, mannase, arabinase, xylanase, etc. Hemi-cellulose is the second most abundant renewable biomass in nature after cellulose. Xylan is the major hemi-cellulose component and accounts for 20–35% of plant cell wall dry weight[2]. Xylan provides potential raw materials for obtaining fermentable sugars which can be converted into several valuable products. Xylanases are hydrolytic enzymes which cleave the $\beta$-1,4 backbone of the hemi-cellulose. Xylanases and associated debranching enzymes are produced by a large variety of microorganisms including bacteria, actinomycetes, yeast and fungi[3].

Xylan consists of beta-1,4-linked D-xylopyranose as a backbone and short chains of o-acetyl, $\alpha$-L-arabinofuranosyl, and $\alpha$-D-glucuronyl residues. Enzymes such as endo-1,4-beta-xylanase, beta-D-xylosidase, beta-D-xylosidase, alpha-L-arabinofuranosidases, alpha-glucoronidases and acetyl esterases act synergistically to hydrolyse xylan. Pre-treatment with enzymes (mainly cellulase-free xylanase) has been reported as an environmentally benign and less expensive[4] method that aids in the spread of hardwoods and softwoods[5].

Enzymatic conversion of hemi-cellulose produces high value products like furfural, xylitol, biofuels and in artificial low calorie sweeteners[6]. Breakdown of hemicelluloses in wheat flour makes the dough softer and easier to knead[7]. Micro-organisms such as bacteria and fungi producing xylanases are more favoured as they are easily available and structurally stable as well as can also be easily genetically manipulated. It has been reported that fungi are more efficient in producing xylanase in comparison to bacteria, however some species of *Bacillus* have been reported to secrete important extracellular enzymes and protein in the medium.

Mutation plays an important role in improving fungal strains for specific work. Mutation may take place spontaneously or may be induced. Spontaneous mutation is slow and their rate depends on the growth conditions of organism and the mutation frequency is low[8]. Induced mutation could be carried out either by physical or chemical methods. Physical mutation could be carried out by UV radiation, gamma radiation or X-rays[9] whereas the chemical mutation is carried out using EtBr (ethidium bromide), 5-bromouracil, EMS (ethyl methyl sulphonate), etc. Xylanase activity of *A. niger* has been increased up to 118% by mutation. UV radiation has been reported as very effective in industries and do not require any equipment. As a result, the current study aims to increase xylanase activity in *A. niger* by mutation in order to investigate its industrial potential[10]. As it has been reported that xylanase is produced by different *Aspergillus* species such as *A. nidulans*, *A. niger* and *A. oryzae*. Different strains of *A. niger* have been reported for the production of Xylanase through mutagenesis. Mutagenesis involves two different methods which includes physical and chemical; in the physical method fungus is treated with UV radiations for different time intervals and in the chemical method fungus is grown on the medium containing chemical mutagen such as 5-bromouracil, ethylmethyl sulphonate, etc. In the commercial development of microbial fermentation processes, industrial strain enhancement is critical. According to Rowlands[11], mutagenic methods can be tweaked in

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terms of mutagens and dose. Effects can be taken into account, and mutagenesis can be intensified or guided to produce the highest frequency of suitable mutant types among the isolates to be tested. Most of the research on increasing xylanase activity by mutation and protoplast fusion have been conducted on mesophilic and thermophilic strains acquired from composting materials, agricultural wastes and natural environments.

*A. niger*, isolated in the Microbiology laboratory of Plant Science department has been used for xylanase production.

Minimal medium was used with the following composition – $MgSO_4 \cdot 4H_2O$ (0.25 g); $K_2HPO_4$ (0.50 g); starch (7.5 g); agar (10.250 g) in 500 ml distilled water. Complete medium was also used in this study, the composition was the same as minimal medium excepting it also includes all amino acids.

5-Bromouracil, a base analogue and UV radiation have been used as mutagen. Different concentrations of 5-bromouracil, i.e. 200, 400, 600 and 800 µg were used to treat the freshly grown mycelium/spores of *A. niger*. 5 ml of spore suspension was treated by UV radiation for different time intervals from 30 to 180 min with a gap of 30 min each. For mutant selection, 1 ml of spore suspension was plated with both complete and minimum medium after treatment. Mutants differ from wild type in their morphology, colony pattern, diameter (as shown in Figure 1), colour, size of somatic and reproductive structures were selected and tested for requirement of specific amino acids for the growth and xylanase activity. Stability of the mutants was checked by successive culturing.

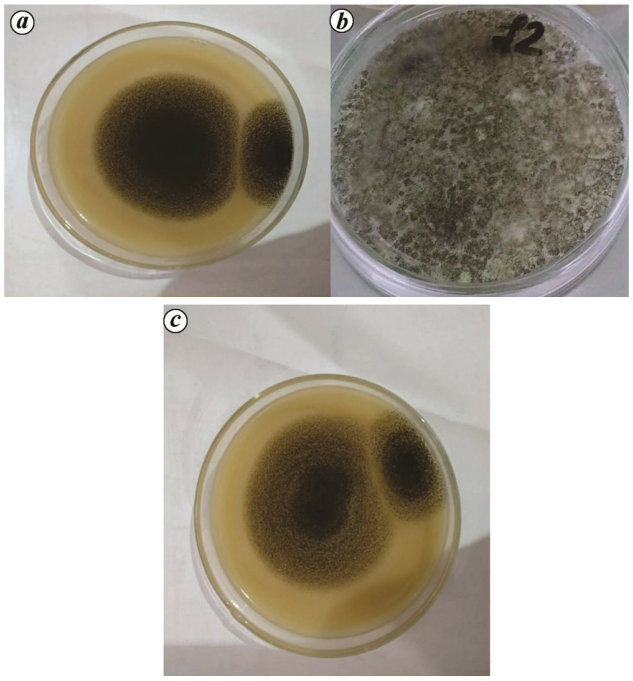


**Figure 1.** ***a***, *Aspergillus niger* wild; ***b***, *A. niger* after treatment with physical mutant, i.e. UV radiation; ***c***, *A. niger* after treatment with chemical mutant, i.e. 5-bromouracil.

25 ml YpSs broth medium supplemented with 1 g of rice straw powder was used for the production of xylanase. The above medium was inoculated with the test fungus and incubated at 30°C and pH 6.0 for 5 days at constant temperature incubators. Triplicates were taken for each strain of *A. niger* wild type and mutants. After incubation, culture was centrifuged at 5000 rpm for 5 min and the supernatant was used as enzyme to evaluate xylanase activity by DNS method[12,13]. The enzyme activity was evaluated using the following formula:

$$\text{Enzyme activity} = \text{OD} \times 1/\text{enzyme volume} \times 1/\text{substrate volume} \times 1/\text{incubation time} \times \text{retention coefficient}$$

1% xylan in acetate phosphate buffer with pH 6.0 was used as a substrate.

Standard curve was prepared using stock solution of 1 mg/ml of xylose. One ml of different concentrations from 0 to 200 µg of xylose prepared from stock solution in different test tubes and boiled after adding 3 ml of DNS reagent. OD was taken at 540 nm against spectro-zero after cooling it.

0.9 ml of substrate and 0.1 ml of buffer was incubated at 50°C for 30 min in a test tube. 3 ml of DNS reagent was added and boiled for 5 min and was taken to set zero at 540 nm.

0.2 ml enzyme and 1.8 ml substrates were taken in a test tube and kept at 50°C for 30 min in test tube. After reaction, 1 ml of the reaction mixture was taken in a test tube, 3 ml of DNS reagent was added and boiled for 5 min. After cooling, OD was taken at 540 nm against spectro-zero.

Mutants formed by physical and chemical mutation were selected and named according to the source of mutagens. Mutants differed significantly from wild type in terms of morphology, colony layout, diameter and colour (Table 1).

Mutants obtained differ in the requirement of amino acid (Table 2) and has been characterized as auxotrophic mutants, which will be used for further improvement in xylanase activity through protoplast fusion.

Xylanase activity of the wild type and mutants has been undertaken and results have been presented in Table 3. All the tested mutants showed an increase in xylanase activity over wild type; however mutant AnB-3 showed

**Table 1.** Morphological difference *Aspergillus niger* wild and mutants

| Isolates | Characteristics |
|---|---|
| Wild | Dark black, dense colony, outer colony black |
| Mutants | |
| AnB-1 | Light black, sparse colony, outer whitish black |
| AnB-2 | Light black, sparse colony, outer whitish black |
| AnB-3 | Light black, sparse colony, outer whitish black |
| AnU-1 | Black, scattered colony, outer creamish white colony |
| AnU-2 | Black, scattered colony, outer creamish white colony |

**Table 2.** Growth of mutants on minimal medium in presence/absence of different amino acids

| Amino acid | Wild | AnB-1 | AnB-2 | AnB-3 | AnU-1 | AnU-2 |
|---|---|---|---|---|---|---|
| Cysteine | + | + | + | + | + | + |
| Arginine | + | + | – | – | – | + |
| Lysine | + | – | + | + | + | – |
| Valine | + | – | + | + | + | + |
| Glycine | + | + | – | – | – | – |
| Proline | + | + | – | + | + | + |
| Serine | + | + | + | – | + | + |
| Histidine | + | – | + | + | – | + |
| Methionine | + | + | – | + | – | – |
| Tryptophan | + | + | + | + | + | + |

(+) Growth with presence of amino acid; (–) No growth with absence of amino acid.

**Table 3.** Xylanase activity of *A. niger* wild and mutant strain

| Isolates | Day | Source | Enzyme activity (IU/ml) | % Increase |
|---|---|---|---|---|
| Wild | 5 | – | 691.48 ± 0.76 | – |
| AnB-1 | 5 | Chemical | 1163.04 ± 0.52 | 68.19 |
| AnB-2 | 5 | Chemical | 1091.87 ± 0.67 | 57.88 |
| AnB-3 | 5 | Chemical | 1289.18 ± 0.88 | 86.43 |
| AnU-1 | 5 | Physical | 1182.79 ± 0.31 | 71.05 |
| AnU-2 | 5 | Physical | 998.37 ± 0.84 | 44.38 |

the highest activity of 1289.18 IU/ml followed by AnU-1 (1182.79 IU/ml) on day 5, showed an increase of 86.43% and 75.05% over wild type.

Mutants of *A. niger* differ in their culture characteristics and in requirement of amino-acid, they were regarded as auxotrophic mutants. Many workers have also produced auxotrophic mutants in the same procedure[14–16]. Mutants selected showed an increase in xylanase activity (Table 3). Amongst all the mutants, mutants AnB-3 and AnU-1 exhibited 86.43% and 71.05% increase in enzyme activity. It may be due to improved change in gene sequence which resulted in the formation of more active protein. However, more work is needed to look for industrial applications of this xylanase. Further work to improve xylanase activity by fusing these auxotrophic strains using protoplast fusion is under progress[17,18].

*A. niger* wild and mutants formed during the present study have been proved to be more effective in utilization of rice straw waste, thus helpful in minimizing pollution caused by burning of rice straw. Mutants formed were more effective in utilization of rice straw than the wild *A. niger*.


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ACKNOWLEDGEMENT. We are grateful to the Council of Science and Technology, UP, for providing financial assistance for above work.

Received 18 May 2021; revised accepted 23 August 2021

doi: 10.18520/cs/v121/i7/966-968