



Cellulase and Xylanase Activities of Mutants of *Neurospora crassa* Induced with Leaf Extract of *Abroma augusta* L.

Apurba Lal Ray* and Tahsina Rahim

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

Abstract

Neurospora crassa, a non-pathogenic filamentous fungus produces hydrolytic enzymes (cellulase, xylanase, etc.) and is capable to convert lignocellulosic materials (cellulose, xylan) into simple sugars. Carboxymethylcellulase (CMCase) and xylanase activities were evaluated using 4 morphological mutants of *N. crassa* induced with leaf extract of *Abroma augusta* L in comparison to the wild type (Ema). Enzymatic activity was expressed as International Unit (IU). It was found that *N. crassa* mycelia secrete cellulases (CMCase) and xylanases to the culture supernatant. Wild *N. crassa* (Ema) showed highest cellulase activity (0.066 IU) and mutant ro 197 showed lowest (0.013 IU) activity. In case of xylanase, wild showed highest (0.313 IU) and mutant ro 197 showed lowest (0.088 IU) activity. These enzymatic assays revealed that wild and mutants of *N. crassa* possess detectable cellulase and xylanase activity.

Key words: Mutants, *Neurospora crassa*, Enzyme activities

Introduction

Some microorganisms (especially filamentous fungi) possess an efficient hydrolytic system capable of performing several roles, such as conversion of lignocellulosic material to essential metabolites for growth. Usually, these fungi secrete a pool of enzymes, including amylases, cellulases (cellobiohydrolases, endoglucanases), hemicellulases (xylanases), β -glycosidases, and lignin-peroxidases. Some fungal species of the genus *Aspergillus*, *Candida* and *Neurospora* are of special interest due to their ability to produce hydrolytic enzymes, such as cellulases, amylases and chitinases, which are of special interest due to their importance in biotechnological processes (Benoliel *et al.*, 2005).

Fungi play a major role in recycling cellulose, which is a β -1, 4-linked glucose polymer. An important feature of this molecule is its crystalline structure; elementary fibrils are stiffened by both inter- and intra-chain hydrogen bonds, which result in a sufficiently packed structure that prevents penetration, not only by enzymes, but also by small molecules, such as water. However, some regions, called amorphous, are sufficiently spacious to permit penetration by larger molecules, including cellulases (Lynd *et al.*, 2002).

Xylan is a component in plant cell walls, being the second most abundant polysaccharide found in nature. It consists of a heteropolysaccharide containing substitute groups of

acetyl, 4-O-methyl-D-glucuronosyl and α -arabinofuranosyl residues linked to the backbone of β -1, 4-linked xylopyranose units (Subramaniyan and Prema, 2002). The xylan layer is covalently linked to lignin and interacts non-covalently with cellulose and thus protects the fibers against degradation by cellulases (Beg *et al.*, 2001).

Neurospora crassa is a non pathogenic filamentous fungus of the class ascomycetes. *N. crassa*, the pink bread mold, is of particular interest to biologists because of its use in the study of genetic and metabolic pathways. Now-a-days, plant extracts are used for the study of antifungal activity (Haque and Shamsi, 1996, 1997) and induction of mutation (Mozmader *et al.*, 2000, 2002). In this study, an attempt was taken to produce mutants of *N. crassa* by using leaf extract of *Abroma augusta* L. (as natural chemical mutagen). The mutants were then used for production of cellulase and xylanase activities.

Materials and Methods

Mutation in *N. crassa* (Ema, 5297) was induced by using leaf extract of *A. augusta* L. The wild type strain of *N. crassa* was obtained from Microbial Genetics Stock, Department of Botany, University of Dhaka. Vogel's minimal medium (Vogel, 1956) was used for culturing *N. crassa* in the test

* Corresponding author: E-mail: : apurba.anirban@gmail.com

tube and also to study the mutagenic effect on culture plate. Sorbose minimal medium (SM) was used for single colony isolation. To test mutagenic effect of *A. augusta* leaf extract on the growth of *N. crassa*, different concentration of the extract was prepared with 40% sterilized distilled water. Mutagenic effect was determined by measuring the radial growth of mycelia of Ema upto 30 hours. For mutation in *N. crassa*, 4 ml of *A. augusta* leaf extract was taken into a centrifuge tube. 1 loop of conidia of Ema was taken into the tube and was shaken for homogenous solution. The tube was kept for 2 hours for mutation. After centrifugation the solution above conidia was poured out from the centrifuge tube. The same procedure was repeated 3 times. Then 1 ml of distilled water was added with the treated conidia remaining at the bottom of the centrifuge tube and the tube was shaken well. The sterilized Petri dishes were marked and 1 drop of the suspension was taken accordingly. 10 ml of molten SM media was added to a Petri dish and were shaken gently to mix the suspension and media. The plates were kept inside the incubator at 25° C for 24 hours for growth of conidia. A number of well separated colonies were isolated from each Petri dish by cutting agar blocks from the conidial colony and were inoculated into small tubes containing VM media. After 5 days, all the cultures were observed and classified by comparing their characters with wild type Ema.

Study of enzyme activities

For study of enzyme activities of wild and mutants of *N. crassa* (Ema), the organisms were grown on VM liquid medium separately in which glucose was replaced by other carbon sources such as carboxymethylcellulose (CMC) and xylan at 0.5% (w/v) concentration. Incubation was carried out at 30° C for 3 days for mycelial growth. The mycelial biomass and other non-soluble material in the culture filtrates were separated by centrifugation at 15,000 rpm for 5 min. The clear supernatants were used for enzyme assays. For this purpose, standard curves of reducing sugar for CMCase and xylanase were prepared. For this reason, 1.8 ml substrate solution [from 1% (w/v) CMC and 1% (w/v) xylan, in 50 mM citrate buffer, pH 6.5] was taken separately in appropriately labeled test tubes (1 test tube for control and 5 for standards). In case of control, 0.2 ml distilled water and in case of standards (5 tubes) 0.2 ml standard sugar (glucose and xylose) was added separately and the solutions were mixed with the help of a vortex mixer. Standard sugar was prepared at 0.1, 0.2, 0.3, 0.4, 0.5 ml glucose and xylose stock separately with 0.9, 0.8, 0.7, 0.6, 0.5 ml citrate buffer in 5 separate test tubes. In each case, total volume was 1 ml.

From each tube (1 ml) 0.2 ml standard sugar (glucose and xylose separately) was added in 5 standard test tubes. In case of assays (control and samples) 0.2 ml supernatant of cultures was added with 1.8 ml substrate (CMC and Xylan separately). 3 ml DNS reagent was added into each test tube and mixed thoroughly by a vortex mixer. In case of sample assays, DNS reagent was added after incubation (30 min for CMC and 5 min for xylan) but DNS reagent was added immediately with each test tube of control assay made for each sample. The test tubes were kept in a boiling water bath for 15 min. The test tubes were cooled under running tap water for 3 min. 6 ml distilled water was added to each of the test tubes. The content of test tubes was mixed thoroughly. Reducing sugars were determined by measuring the absorbance at 540 nm using the blank as reference. Hydrolytic activity was calculated by measuring the amount of reducing sugars released from CMC and xylan. The amount of reducing sugar produced was measured by the DNS method (Miller, 1959) using glucose and xylose as standards respectively for CMCase and xylanase activities. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol reducing sugar equivalent per minute under the assay conditions.

Enzyme activities were calculated from the following formula:

$$\text{i) CMCase activity in IU} = \frac{\text{Reducing sugar (mg/ml)} \times 1000}{\text{Incubation time (30 min)} \times 180}$$

$$\text{ii) Xylanase activity in IU} = \frac{\text{Reducing sugar (mg/ml)} \times 1000}{\text{Incubation time (5 min)} \times 150}$$

Results and Discussion

A. augusta is a medicinal plant and its leaf contains taraxerol and B-sitosterol (Ghani, 2003). Leaf extract of *A. augusta* showed considerable mutagenic effect on *N. crassa*.

Table I. Radial growth of *Neurospora crassa* Ema on VM agar plates containing leaf extract of *Abroma augusta*

Concentration of extract (ml)	Radial mycelial growth (in cm) after different time intervals			
	21 h	24 h	27 h	30 h
0 (Control)	2.30	2.80	3.20	3.70
0.5	0.80	1.20	1.50	1.70
1.0	0.60	0.90	1.20	1.40
2.0	0.40	0.55	0.70	0.90
4.0	0.20	0.30	0.40	0.50

It was found that the mutagenic effect increased with the increase of the leaf extract concentration in VM medium (Table I). The mutagenic effect was evaluated by measuring the radial growth of mycelia of the fungal strain Ema on VM agar plates. Lower radial growth indicated higher mutagenic effect.

After mutation in *N. crassa* (Ema) conidia with leaf extract of *A. augusta*, the mutants were classified into 4 morphological mutant groups (Table II). These were albino (al 123), vigorous (vg 115), fluffy (fl 128) and ropy (ro 197). Among the 4 morphological mutant groups, the ropy mutant was the most prevalent (34%) while fluffy represented the lowest (17%) type of mutants. These 4 mutants were used for extra-cellular cellulase (CMCase) and xylanase production in submerged cultures.

Table II. Classification and Nomenclature of *Abroma augusta* leaf extract induced mutants of *Neurospora crassa* according to Mozmader *et al.* (2000)

Group	Name of the mutant	Characteristics	No. of mutant	Frequency (%)
A	Albino	Both conidia and mycelia are colourless	8	19.51
B	Vigorous	Profuse conidial growth touches the plug	12	29.27
C	Fluffy	Profuse mycelial growth	7	17.07
D	Ropy	Mycelia form rope like structure and conidia pink	14	34.15

Enzyme activities were calculated by the amount of reducing sugar (Table III and IV) obtained from standard curve prepared for CMCase and xylanase (Fig. not shown).

Table III. Reducing sugar of CMCase obtained from standard curve

Name and number of mutants	Assay sample (OD)	Assay control (OD)	OD of CMCase	Reducing sugar of CMCase (mg/ml)
Ema	0.280	0.098	0.182	0.36
al 123	0.124	0.081	0.043	0.085
vg 115	0.076	0.029	0.047	0.09
fl 128	0.094	0.006	0.088	0.17
ro 197	0.154	0.118	0.036	0.07

From this study, it was found that *N. crassa* secretes cellulases (CMCase) to the culture supernatant, since activity was detected in mycelium cultures of the fungus. The cultures grown on CMC yielded detectable activities. Wild *N. crassa* (Ema) showed highest cellulase activity (0.066 IU), fl 128

showed nearest (0.031 IU) and mutant ro 197 showed lowest (0.013 IU) activity (Table V). But this cellulase activity of wild and mutants is lower than the activity of wild as well as UV ray induced anthranilic acid mutants of *N. crassa* as reported earlier by Rahim (2001). The reason is perhaps the double incubation time (7 days) for mycelial growth of the earlier work than the present study. Nevertheless, the data suggest that *N. crassa* probably prefers soluble (CMC) sources as like insoluble substrates (similar to those found in nature).

Xylanase activity was present in mycelium form. In case of xylanase, Ema showed highest (0.313 IU), vg 115 showed nearest (0.249 IU) and ro 197 showed lowest (0.088 IU) activities (Table V). The xylanase activity was found to be the most important hydrolytic activity, being related to the

Table IV. Reducing sugar of xylanase obtained from standard curve

Name and number of mutants	Assay sample (OD)	Assay control (OD)	OD of xylanase	Reducing sugar of xylanase (mg/ml)
Ema	0.148	0.041	0.107	0.235
al 123	0.158	0.125	0.033	0.07
vg 115	0.106	0.021	0.085	0.187
fl 128	0.189	0.127	0.062	0.136
ro 197	0.035	0.005	0.030	0.066

hydrolysis of hemicellulose. Xylan is the main component of hemicellulose (Thomson, 1993).

The enzymatic assays of this study indicate the presence of a hydrolytic system in *N. crassa*, which was able to grow in media containing cellulose or xylan as the sole carbon source. Besides, these findings revealed that mutation is not deleterious in all cases but advantageous for pure research and applied field.

Table V. Enzymatic activity of wild and mutants of *Neurospora crassa*

Name and number of mutants	CMCase (IU)	Xylanase (IU)
Ema	0.066	0.313
al 123	0.016	0.093
vg 115	0.017	0.249
fl 128	0.031	0.181
ro 197	0.013	0.088

Sunna and Antranikian (1997) reported that cellulase and xylanase enzymes may be used in fruit juice clarification and vegetable oil extraction. As *N. crassa* is a non-pathogenic filamentous fungus, so cellulase and xylanase enzymes produced by wild as well as different mutants of *N. crassa* can be used for the same purpose.

Conclusion

4 morphological mutants of *N. crassa* were obtained after mutation of *N. crassa* with leaf extract of *A. augusta* L. Carboxymethylcellulase (CMCase) and xylanase activities were evaluated using these mutants of *N. crassa* in comparison to the wild type (Ema). Wild *N. crassa* (Ema) showed highest cellulase activity (0.066 IU), fl 128 showed nearest (0.031 IU) and mutant ro 197 showed lowest (0.013 IU) activity. In case of xylanase, Ema showed highest (0.313 IU), vg 115 showed nearest (0.249 IU) and ro 197 showed lowest (0.088 IU) activity.

References

- Beg Q. K., Kapoor M., Mahajan L. and Hoondal G. S. (2001) Microbial xylanases and their industrial applications: a review. *Appl. Microbiol. Biotechnol.* **56**: 326-338.
- Benoliel B., Arraes F. B. M., Reis V. C., Siqueira S. J. L., Parachin N. S. and Torres F. A. G. (2005) Hydrolytic enzymes in *Paracoccidioides brasiliensis* - ecological aspects. *Genet. Mol. Res.* **4**(2): 450-461
- Ghani A. (2003) Medicinal plants of Bangladesh (2nd edition).
- Haque T. and Shamsi S. (1996) Activity of certain plant extract against jute stem rot fungus *Macrophomina phaseolina*. *Dhaka Univ. J. Biol. Sci.* **5**(1): 103 -104.
- Haque T. and Shamsi S. (1997) Activity of some plant extracts on growth of sheath rot pathogen *Sarocladium oryzae* (Sawada). W. Rames and D. Hawksa. *J. Asiat. Soc. Bangladesh, Sci.* **23**(1): 153- 155.
- Lynd L. R. Weimer P. J., Van Zyl W. H. and Pretorius I. S. (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **66**: 506-577.
- Miller G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31**: 426-428.
- Mozmader T. I. M. A., Yesmin N. and Jabbar A. (2000) Induction of mutation in *Neurospora crassa* with aqueous extract of garlic (*Allium sativum* L.) and genetical and chromatographic studies of some selected mutants. *Dhaka Univ. J. Biol. Sci.* **9**(2): 143 -150.
- Mozmader T. I. M. A., Keya U. S. and Haque T. (2002) Induction of mutation in *Neurospora crassa* with aqueous, petroleum ether and chloroform extracts of Neem (*Azadirachta Indica* A. Juss) leaves. *Dhaka Univ. J. Biol. Sci.* **11**(2): 167 -173.
- Rahim T. (2001) Genetical and biochemical investigations and protoplast fusion of anthranilic acid mutants of *Neurospora crassa*. PhD thesis. Department of Botany, University of Dhaka.
- Subramaniyan S. and Prema P. (2002) Biotechnology of microbial xylanases: enzymology, molecular biology and application. *Crit. Rev. Biotechnol.* **22**: 33-64.
- Sunna A. and Antranikian G. (1997) Xylanolytic enzymes from fungi and bacteria. *Critical Review in Biotechnology.* **17**(1): 39-67
- Thomson J. A. (1993) Molecular biology of xylan degradation. *FEMS Microbiol. Lett.* **104**: 65-82.
- Vogel H. J. (1956) A convenient growth medium for *Neurospora crassa*. *Microbial Genetics Bull.* **13**: 42- 43.

Received : May 25, 2009;

Accepted : September 16, 2009