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RANDOM MUTAGENESIS OF *ASPERGILLUS SP.* FOR ENHANCED PRODUCTION OF PECTINASE

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Abstract
Pectinases are extensively used in the food sector to produce and clarify fruit juices enhance the cloud stability of fruit and vegetable nectars and eliminate haze from wines. The aim of the current study was the strain improvement of Aspergillus sp. AB-15 and optimization of culture conditions such as incubation period, temperature and pH for enhanced enzyme production. Random mutagenesis was carried out by subjecting Aspergillussp. AB-15 to nitrous acid (0.9M) and UV exposure (70 min) treatments. This resulted in the development of twelve mutant strains out of which nitrous acid mutated strain IIB-NA6 showed the highest pectinase activity i.e. 8.505±0.05 U/ml/min as compared to the wild strain having activity of 1.97±0.08U/mL/min. The optimization of cultural conditions for wild and mutant strains was also carried out. Both mutant IIB-NA6 and wild AB-15 strains showed maximum pectinase activity at 30ºC using a fermentation medium of pH 5 after five days of incubation (120 h). This study revealed that strain improvement and cultural conditions optimization resulted in a 4.3-fold increase in pectinase production using a mutant strain of Aspergillus sp. (IIB-NA6).

Keywords: *Biosynthesis, Enzyme, Fermentation, Genetic manipulation, Mutagenesis, Pectin*

INTRODUCTION

Pectic substances are hydrolyzed by pectinases. The largely studied pectinolytic enzymes are pectin esterases, lyases, polygalacturonases and protopectinases (1). They consist of α (1→4) linked D-galacturonic acid residues (2). Plants, fungi and bacteria contains pectinases (3). These enzymes work on pectin. Pectin is the important component present in middle lamella of plant's cell wall (4). Pectin is homopolymeric which is product of poly-α-(1,4)- galacturonic acid (partially methylated). It may also consists of D-apiose, L-fucose, D-xylose and D-glucuronic acid residues (5).

They are divided into three groups on basis of their mode of action. Pectic acid is produced when de-esterification of methoxyl group of pectin is catalyzed by pectin esterase. Hydrolases catalyze hydrolytic cleavage of α-1, 4- glycosidic linkage in pectin and pectic acid. Lyasescatalyze α-1, 4- glycosidic linkage respectively and as a result by trans-elimination reaction, methyl galacturonates and unsaturated galacturonates are produced.Animals, microorganisms and plants can produce pectinases. The species of *Bacillus*, *Pediococcus*, *Lactobacillus* and *Leuconostoc* are predominant in producing pectinases. Among fungi, *Rhizopus sp.*, *Trichoderma sp*. and *Aspergillus sp*. are predominant (6). *Aspergillus sp*. is responsible for hydrolysing pectic elements. They produce extracellular enzymes (7).

Pectinases are produced by submerged and solid state fermentation. Solid state fermentation is carried out in absence of water while submerged fermentation takes place in presence of water. Solid state fermentation is more common in producing pectinases because of simplicity and high productivity (8). For solid state fermentation, *Aspergillus sp*. has some advantages of higher enzyme titre and increased productivity (9).

For enhanced production of pectinases, random mutagenesis is considered an efficient method. In microbes by using different chemical or physical methods, mutagenesis induces genetic variability. Among

chemical methods, chemicals such as ethidium bromide and nitrous acid are used while physical method involves the use of gamma or ultraviolet radiations. The production of enzymes enhanced due to UV treatment method. UV irradiation causes thymine dimmer formation that destroys DNA structure (10). Nitrous acid is also a strong mutagenic agent that converts cytosine to uracil, guanine to xanthine and adenine to hypoxanthine (11). Ethidium bromide effects DNA replication and transcription (12).

The cultural conditions for maximum production of enzyme should be optimized. The important parameters to be optimized are temperature, pH and incubation time. Pectinases are used in wine and fruit juice preparation (13). Their first commercial application was observed in 1930. These enzymes enhance juice clarification and yield.

MATERIALS AND METHODS

MICRO ORGANISM

The culture bank of Institute of Industrial Biotechnology (IIB), Govt. College University, Lahore, provided the strain of *Aspergillus sp.* (AB-15). The strain was streaked and incubated for two days at 30°C. It was sub-cultured after fourteen days to keep the microbes viable (14).

STRAIN IMPROVEMENT

INOCULUM PREPARATION

Two days old *Aspergillus sp.* slant was obtained and sterilized distilled H_2O (10 mL) was taken into test tube. Spores were then suspended by using sterilized loop (inoculating loop). Spore suspension having 1.2×10^7 concentrations was selected for further studies (15).

NITROUS ACID MUTATION

Two days old *Aspergillus sp.* slants were obtained and its spore suspension was prepared. The spore suspension (1 mL) was added to 6 eppendorf tubes by using micropipette and centrifuged for ten minutes (at 6,000 rpm). The pellet obtained was washed with buffer (phosphate buffer of pH 7.0). It was treated with sodium acetate buffer (0.1 M), along with $NaNO_2$ of different concentrations of 0.7 M to 1.2 M and mixed vigorously (for ten minutes). By adding 0.3 mL of phosphate buffer, the reaction was stopped. The 100 µL of the spore suspension was transferred to malt agar plates. It was incubated for two days at 30°C (16).

UV TREATMENT

Two days old *Aspergillus sp.* was obtained. The spore suspension was prepared in sterilized distilled H_2O and dilution (100 µL) was inoculated on malt agar plates. Through UV lamp, these plates were then exposed to UV radiations (at a distance of 8 cm) with different time intervals (such as 10, 20, 30, 40, 50, and 60 min). The plates were then removed and covered to prevent reversion of mutation (on exposure to light). Then survival curve was plotted, and the time at which fungal growth was inhibited completely was selected and it was then repeatedly used to obtain mutant strain (17).

Pectinase activity of mutant strains was analysed by flooding plates with potassium iodide-iodine solution after inoculation of mutant colonies on plates containing mineral salt agar and 2-deoxy-D-glucose (0.1%) after 48 hrs. incubation at 30°C (18). Secondary screening was performed by solid state fermentation (19).

INOCULUM PREPARATION

Sterilized distilled water 10 ml was added in fungal slants, aseptically. With the help of sterilized inoculating loop, the spores were scratched to make homogenous spore suspension (20).

SOLID STATE FERMENTATION

Spore suspension was inoculated in sterilized fermentation medium. The flask was incubated for four days at 30°C in static incubator (21).

ENZYME EXTRACTION

After four days of incubation, phosphate buffer (25 ml) was added in fermented flask and incubated for one hour at 30°C in shaking incubator. After one hour of incubation, the crude enzyme was filtered using muslin cloth and centrifuged (SIGMA laboratory centrifuge 3K30) at 6000 rpm (for 10 min). Pellet was discarded and for determination of enzyme activity, supernatant was used. (22).

PECTINASE ASSAY

For enzyme assay, phosphate buffer 300 µL and 500 µL of pectin (substrate) were added in control tube and experimental tube have 200 µL enzyme along with 300 µL phosphate buffer and 500 µL of pectin (substrate). In blank tube, distilled water (1mL) was added. The experimental and control tubes were then incubated (at 30ºC) in shaking water bath (Model: WSB-30) for 10 min. After incubation, 200 µL enzyme was added in control tube and one mL DNS was added in all test tube to stop the reaction of enzyme with substrate. These were then placed in water bath (for 10 minutes). Test tubes were then cooled (with cold water) and H_2O (5mL) was added, if colours were too dark. From spectrophotometer, absorbance was taken (at 540 nm) (23).

ENZYME UNIT

One unit of pectinase activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute under standard assay conditions and expressed as units per mL (U/mL/min) (24).

CALCULATIONS OF ENZYME UNITS

$$\text{Pectinase activity (U/mL/min)} = \frac{\left[\text{concentrationfromgraph}\left(\frac{\text{mg}}{\text{mL}}\right)\text{xdilutionfactorx 1000}\right]}{\left[\text{Molecularweightofgalacturonicacidxincubationtime}\right]}$$

OPTIMIZATION OF CULTURAL CONDITIONS

In order to obtain maximum production of pectinase, wild and mutant strains were subjected to optimization of cultural parameters.

pH AND TEMPERATURE OPTIMIZATION

The fermentation media pH was varied from 5 to 8 (25). Temperature was varied to check its effect on production of enzyme. The fermentation (solid state) was carried out at five different temperatures such as 25°C, 30°C, 37°C, 40°C and at room temperature (26).

OPTIMIZATION OF INCUBATION TIME

At different incubation periods (72, 96, 120, 144 and 168 h), the fermentation was carried out to check the effect of incubation time on enzyme production (27).

STATISTICAL ANALYSIS

For the statistical analysis of results, computer statistical software was used. Significant difference among the replicates has been presented as Duncan's multiple range tests in form of probability (p) values (28).Y-error bars in figures indicate the standard deviation (±S.D) among the three parallel replicates which differ significantly at ≤ 0.05.

RESULTS AND DISCUSSION

RANDOM MUTAGENESIS

UV IRRADIATION

The wild strain of *Aspergillus sp.* AB-15 was exposed to ultraviolet treatment for ten different time intervals such as 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min (Fig. 1a). It was observed that fungal growth was completely inhibited at 70 min UV exposure. For mutagenesis of *Aspergillus sp.* AB-15, this exposure time (70 min) was used. Hence, seven mutant strains were found after repeated rounds of UV exposure for

70 min. The maximum pectinase activity as compared to wild strain i.e. 1.97±0.13 U/ml/min was shown by mutant strain IIB-UV3 i.e. 3.944±0.09 U/ml/min as shown in figure 1. The dimer formation in pyrimidines is caused by UV irradiation such as thymine dimers. The photoproduct is induced by UV irradiation, in which carbon molecules (4 and 6 'C' molecules of adjacent pyrimidines) get covalently linked (29). Kamalambigeswari *et al.,* (30) observed 1.1% increase in pectinase activity after exposing *Aspergillus niger* strain for 60 min to UV which is contradictory to the finding of current study that showed 2% increase in the pectinase activity after 70 minutes of exposure time. Yin *et al.,* (31) reported 73% increase in pectinase activity from *Fusarium oxysporum* after exposing to UV irradiation for 60 min. The difference in pectinase production improvement might be due to the increased exposure time in current study. Another reason can be the difference in the fungal strains applied. **Cultural Conditions:** pH of medium: 5; Temperature of incubation: 30ºC; Incubation time: 120 h.

NITROUS ACID

The different concentrations of nitrous acid such as 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2 M were used to check their effect on *Aspergillus sp.* (Fig. 1b). AB-15 for enhanced pectinase production. The fungal growth was not observed at 0.9 M conc. Five mutant strains were found after repeatedly treating the wild strain with nitrous acid of 0.9 M concentration. The maximum pectinase activity was shown by IIB-NA6 i.e. 8.505±0.05 U/ml/min, while lowest activity was shown by IIB-NA7 i.e. 1.27±0.17 U/ml/min as compared to wild strain having activity of 1.97±0.08 U/ml/min as shown in figure 2. Mala *et al.,* (32) and Karanam and Medicherla (33) reported 2.53 times and 1.26 times increase in lipase activity using *Aspergillus niger* and *A. japonicas,* respectively. **Cultural Conditions:** pH of medium: 5; Temperature of incubation: 30ºC; Incubation time: 120.

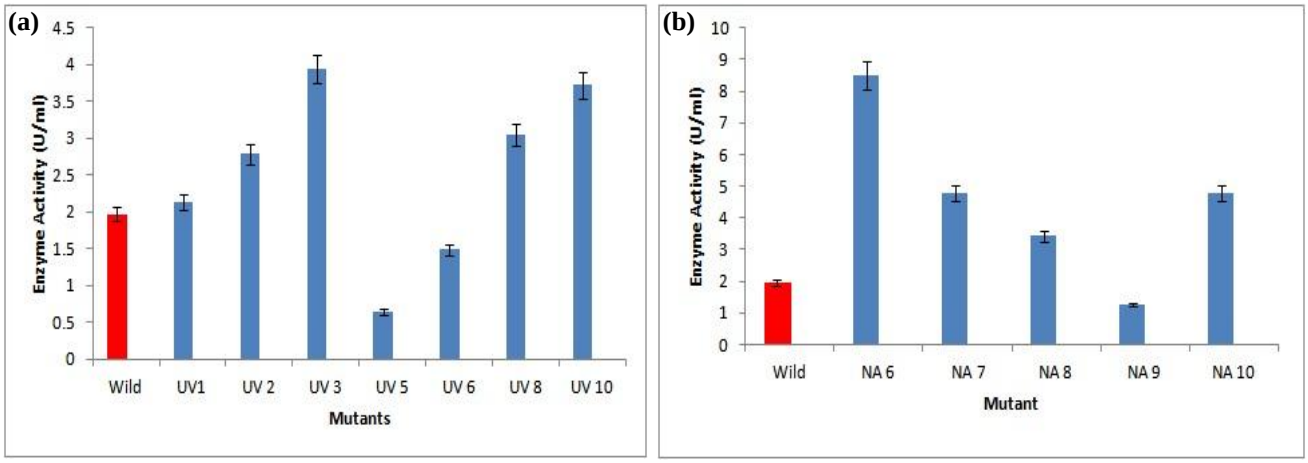

Fig. 1a. Comparative pectinase production analysis of wild and mutant strains of *Aspergillus sp.* obtained after UV irradiation. **1b.** *Pectinase activity (U/mL) of wild and mutant strains of Aspergillus sp. obtained after nitrous acid treatment*

EFFECT OF INCUBATION PERIOD

The mutant (IIB-NA6) and wild (AB-15) strain was used to analyze the effect of different incubation times i.e. 72, 96, 120, 144 and 168 h for enhanced production of pectinase. The maximum pectinolytic activity of mutant i.e. 10.01±0.06 U/ml/min and wild i.e. 3.65±0.15 U/ml/min strains was found after 120 hours (Fig. 2a). Further increase in the incubation time resulted in decreased pectinolytic activity. With the increase in time, the production decreases because the cells reached the decline phase, resulted in low enzyme production (34). The production of enzyme is also affected by nutrient's availability. Darah*et al.,* (35) and Durairajan and Sankari (36) optimized the production of pectinase by *A. niger* and observed highest enzyme activity after 144 h incubation which is not in accordance with the result of current findings, that might be due to difference in fungal strains. **Cultural Conditions:** pH of medium: 5; Incubation time: 120.

OPTIMIZATION OF TEMPERATURE

The mutant strain (IIB-NA6) and wild (AB-15) strain was used to check the effect of different incubation temperatures (20ºC, 25ºC, 30ºC, 37ºC, 40ºC) on pectinase production. The maximum enzyme

activity of mutant and wild strains was observed at 30 ºC i.e. 10.01±0.06 U/mL 4.28±0.02 U/ml, respectively as shown in Fig. 2b. The activity of mutant and wild strain was drastically decreased at 40ºC as shown in Fig. 2b. Temperature plays an effective role in production of enzyme. The lower temperature would result in slower metabolism of cell and high temperature would damage the growth of cells (37). The enzyme denaturation may result in low enzyme activity at higher temperature (38). Abbasi *et al.,* (39) also recorded maximum pectinase production by *A. niger* at 30ºC which is coherent with the current results. **Cultural Conditions:** pH of medium: 5; Incubation time: 120 h

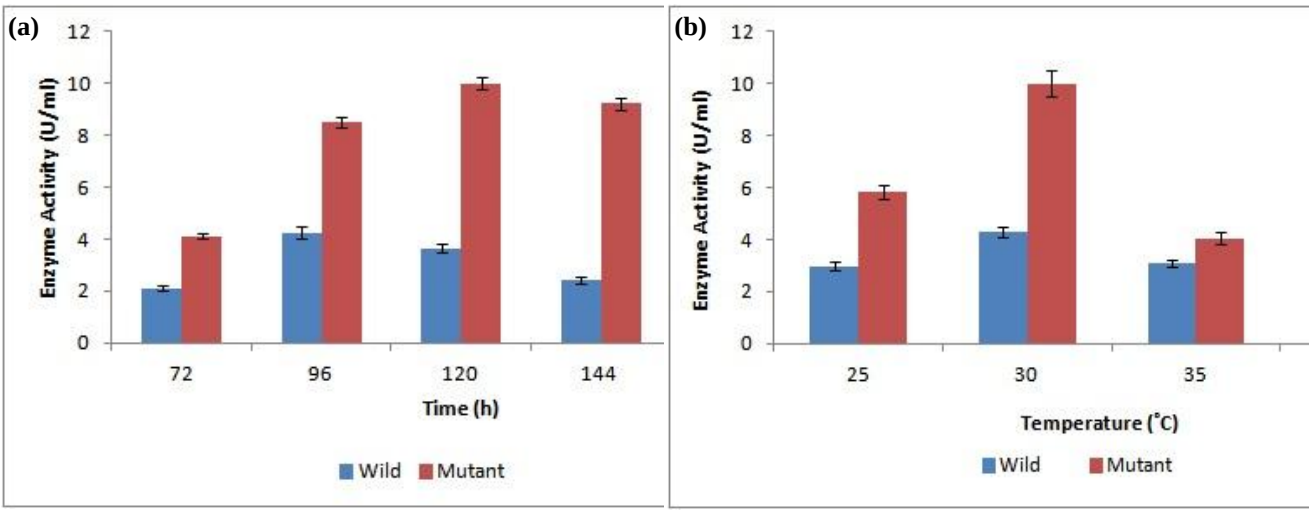

Fig. 2a. Effect of different incubation times on pectinase production using wild and mutant strains of *Aspergillus sp.* (**b).** Temperature effect on pectinase activity (U/mL) of wild and mutant strain of *Aspergillus sp.*

EFFECT OF MEDIUM PH

The effect of pH on the production of pectinase was observed by wild (AB-15) and mutant strain (IIB-NA6) at different pH values such as 4, 5, 6, 7 and 8. The maximum activity of mutant and wild strain was observed at pH 5 i.e., 11.78± 0.05 U/mL, 6.23±0.02 U/mL, respectively (Fig. 3).pH plays a crucial role in production of pectinase. The enzyme may be affected by high or low pH. Many moulds showed maximum pectic enzyme production at acidic pH range (40). Hachemi *et al.,* (41) also analysed highest activity of pectinase *by A. niger* at pH 5 which is in accordance to current study. On the other hand, Debing *et al.,* (42) showed the maximum production of pectinase at pH 6.5 using solid state fermentation from *Aspergillus niger* by using wheat bran as substrate whereas in current study saw dust was used as substrate. **Cultural Conditions:** Temperature of medium: 30ºC; Incubation time: 120 h.

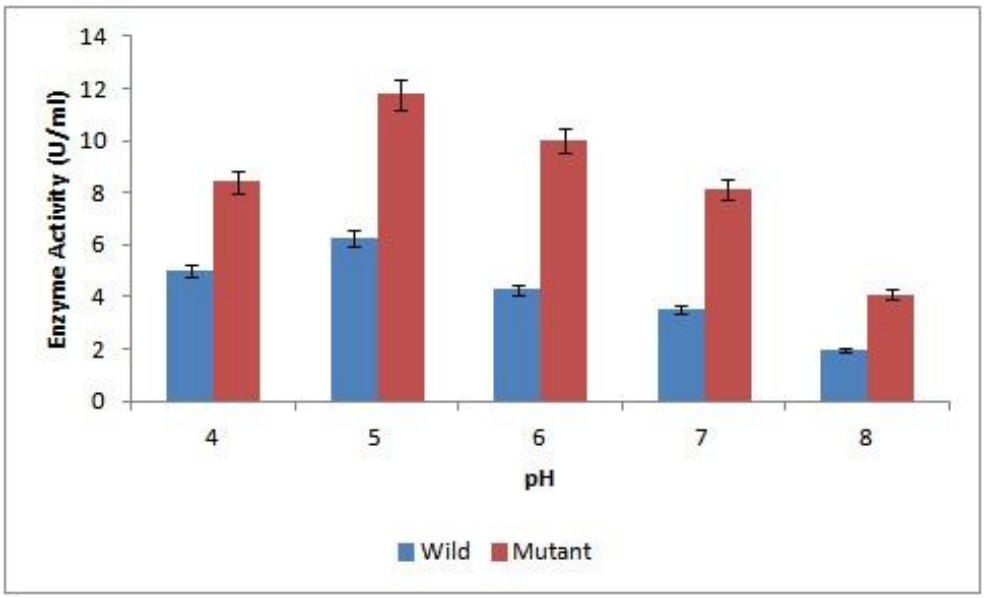

Fig. 3. Effect of different pH on pectinase activity (U/mL) of wild and mutant strain of *Aspergillus sp.*

CONCLUSION

This study concluded that random mutagenesis can be successful strategy for strain improvement to get higher yield of pectinase. UV and nitrous acid treatments were found effective for strain improvement. However, cultural parameters (incubation time, pH and temperature) optimization showed significant effect on enhancing the pectinase activity. The combination of above mentioned strategies can result in development of a better strain for industrial applications.

Conflict of Interest:

Authors have no conflict of interest.

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