STRAIN IMPROVEMENT AND ASSESSMENT OF CULTURAL CONDITIONS FOR IMPROVED BIOSYNTHESIS OF PECTINASE USING *PENICILLIUM NOTATUM*

Ali Nawaz[1], Mahnoor Hussain[1], Marium Munir[2], Hamid Mukhtar[1] and Ikram ul Haq[1]

[1]*Institute of Industrial Biotechnology (IIB), GC University Lahore, Pakistan*
[2]*Department of Biotechnology, Lahore College for Women University Lahore, Pakistan*
Corresponding author email: alinawazgcu@gmail.com

ABSTRACT

Efficient pectinase producing fungi is the need of an hour for number of industries such as feed, textile, paper, pulp and food. The objective of the current study was to increase the production of pectinase from *Penicillium notatum* MH-61 through random mutagenesis using physical and chemical methods followed by growth parameters' optimization. Wild strain of *Penicillium notatum* MH-61 was subjected to UV irradiation (60 min), nitrous acid (0.9 M) and ethidium bromide (0.5 mg/mL) treatments. Mutants obtained were screened for pectinase production using mineral salt agar medium by observing zones of clearance. Later, the mutant strains showing better zones of clearance were analyzed for estimation of pectinase using solid-state fermentation employing sugarcane bagasse as substrate. UV treated mutant strain MH-UV9 exhibited highest pectinase activity i.e. 5.1 ± 0.10 U/mL/min as compared to that of wild type i.e. 2.15 ± 0.02 U/mL/min. The cultural conditions such as incubation time, pH and temperature for pectinase production by wild (MH-61) and mutant (MH-UV9) strains under solid state fermentation were also optimized. The enhanced activity of pectinase using UV mutant MH-UV9 i.e. 5.98 ± 0.05 U/mL/min was obtained using fermentation medium of pH 5.0 at 30°C after an incubation period of 120 h. The whole process resulted in 2.37 fold increase in pectinase production as compared to that of wild strain.

Keywords: Mutagenesis, Pectinase, *Penicillium notatum*, Solid State Fermentation.

INTRODUCTION

Pectinases include a group of enzymes that degrade pectin, a major component present in middle lamella and primary cell walls of higher plants (Mojsov, 2016). The structure of pectin is composed of three pectic elements namely rhamnogalacturonan-I, homogalacturonan and substitutable galacturonans (Benoit *et al.*, 2012). Polygalacturonases (PG) belong to class Hydrolases that break α-1, 4 glycosidic linkages between galacturonic acid units of pectic acid by hydrolysis. They include endo-PG (EC 3.2.1.15) that catalyze hydrolysis of pectic acid randomly and exo-PG (EC 3.2.1.67) that catalyze terminal cleavage of polygalacturonic acid from non-reducing end by hydrolysis (Garg *et al.*, 2016).

Pectinases from more than 30 different genera of microbes have been reported, for example, molds such as *Aspergillus, Fusarium, Penicillium, Rhizopus;* yeast species such as *Saccharomyces, Kluyveromyces* and bacterial species including *Bacillus* and *Erwinia* (Favela-Torres *et al.*, 2006). Yeast and fungi are increasingly being used for enzyme production with the development of biotechnology and contribute 50% of industrial enzymes (Saranraj and Naidu, 2014). Microorganisms are selected for pectinase production on the basis of various features, such as, the type of fermentation, genetic properties of strain, thermal stability and pH of enzymes. Most extensively used genera for pectinase production include *Penicillium, Aspergillus* and *Erwinia* (Favela-Torres *et al.*, 2006).

Pectinase production can be carried out either by solid state or submerged fermentation. Mostly, filamentous fungi are employed for the production of pectinolytic enzyme mixtures at commercial level as they have better ability to colonize and penetrate the substrate, therefore, solid-state fermentation (SSF) is regarded more appropriate for fungal growth. Several studies have reported that higher production of pectinase is obtained by SSF than submerged fermentation (SMF). Cultural conditions such as incubation time, temperature and pH can further be optimized to increase enzyme activity (Favela-Torres *et al.*, 2006).

Strain improvement can be achieved successfully by random mutagenesis that involves exposing microorganisms to chemical (nitrous acid, ethidium bromide) or physical (UV) mutagenic agents. Mutagens mainly target nitrogenous base sequence of DNA and the type and concentration of mutagen can be optimized to increase the number of desirable mutant strains (Ribiero *et al.*, 2013). UV irradiation causes mutation in DNA sequence through excitation of electrons in molecules that leads to pyrimidine dimer formation as extra bonds are formed between adjacent pyrimidines. Nitrous acid alters base pairing by causing oxidative deamination of cytosine or adenine. Frame shift mutations are caused by ethidium bromide as it intercalates into the double-stranded DNA

(Suribabu *et al.,* 2014). These mutations can be beneficial for microorganisms and the mutant strain may become better adapted to otherwise unfavorable environment and may exhibit better biocatalysis for industrial purposes (Ho and Ho, 2015).

Pectinases are used in many industrial operations and constitute one fifth of the enzyme market worldwide. They are used for maceration of fruit pulps and clarification of fruit juices in food industries. The rate of tea fermentation can be enhanced by using pectinases and they are also used to separate the coating of coffee beans for coffee fermentation. Pectinases reduce the cationic demand from peroxide bleaching in paper and pulp industry by depolymerizing polygalacturonic acid. Pectinases can be used for extraction of oils from citrus fruits as emulsifying characteristics of pectin that interfere with oil collection from extracts of citrus peel are eliminated by pectinases. The waste water released from fruits processing industries contain pectic elements that can be removed by using pectinases making its disposal more suitable for activated sludge treatment. Pectinases can also be used for purification of plant viruses (Bhardwaj *et al.,* 2017).

MATERIALS AND METHODS

Microorganism

Penicillium notatum strain MH-61 was taken from the culture bank of Institute of Industrial Biotechnology, Government College University Lahore. It was stored at 4°C and sub-cultured on malt agar after 2 weeks to maintain the viability (Oumer and Abate, 2018).

Strain Improvement

Spore suspension of concentration 1.2 x 10^7 spores per mL was prepared for strain improvement using a 48 h old culture of fungal strain MH-61 (Haq *et al.,* 2014).

UV treatment

Fungal strain was exposed to UV light for different time intervals i.e. 10, 20, 30, 40, 50, 60, 70 and 80 min after inoculating 0.1 mL spore suspension at the center of malt agar plates that were placed 8 cm away from UV light (30W). After completing specific time intervals of UV exposure, incubation of plates was carried out at 30°C for 48 h. Survival curve was plotted and mutagenesis was carried out by repeatedly exposing the fungal strain to UV irradiation for particular time interval at which complete inhibition of fungal growth was observed (Ghazi *et al.,* 2014).

Nitrous Acid treatment

Pellets obtained after centrifugation of 1 mL spore suspension at 6000 rpm for 10 min were washed thrice using phosphate buffer (0.1 M, pH 7). Washed pellets were then treated with sodium acetate buffer (0.1 M, pH 4.2) and different concentrations of sodium nitrite (0.7 M - 1.2 M). After thorough mixing of reaction mixture for 10 min, 0.3 mL phosphate buffer (0.1 M, pH 7) was added to stop the reaction. Spore suspension (0.1 mL) was inoculated on malt agar plates and incubation was carried out at 30°C for 2 days (Haq *et al.,* 2014).

Ethidium Bromide treatment

Spore suspension (250 µL) was centrifuged at 6000 rpm for 10 min and different concentrations of ethidium bromide (0.1 mg/mL to 0.7 mg/mL) were added for mutagenesis after washing pellets obtained with phosphate buffer (0.1M, pH7). Malt agar plates were inoculated with 20 µL of ethidium bromide treated spore suspension and incubated at 30°C for 2 days (Naeem *et al.,* 2018).

Screening of mutant strains

For primary screening, mutant colonies obtained were inoculated on petri plates having Mineral Salt Agar medium and 0.1% 2-deoxy-D-glucose. After incubation of plates at 30°C for 48 h, they were flooded gently with potassium iodide-iodine solution to analyze pectinase activity of mutant strains (Usha *et al.,* 2014). Solid state fermentation of selected mutant strains was then carried out for secondary screening to estimate pectinase activity (Phutela *et al.,* 2005).

Solid state fermentation

Fermentation medium was prepared after Phutela *et al.,* (2005) using pectin (1%), sucrose (0.314 %), $FeSO_4$ (0.029%), KH_2PO_4 (0.65%), $(NH_4)_2SO_4$ (1.2%) and urea (0.3%) as diluent (25 ml) and sugarcane bagasse (5 g) as

solid substrate. Spore suspension (1 mL) was inoculated in fermentation medium and incubated at 30°C for 4 days (Phutela *et al.*, 2005).

Enzyme extraction

Enzyme was extracted by adding 25 mL phosphate buffer in flasks and incubating them at 160 rpm for 60 min in shaking incubator. The filtrate obtained after filtering fermentation medium with muslin cloth was centrifuged for 10 min at 6000 rpm. The supernatant obtained was used for carrying out enzyme assay (Phutela *et al.*, 2005).

Pectinase Assay

Pectinase activity was determined after modification of method demonstrated by Oumer and Abate, (2018). Control and experimental tubes were prepared by taking 500 µL pectin and 300 µL phosphate buffer in both tubes and 200 µL of enzyme was only added in experimental tube. Incubation of tubes was performed at 30°C for 10 min in shaking water bath and after incubation, enzyme (200 µL) was added in control tube. Blank was prepared with 1 mL distilled water. Pectinase activity was determined by adding DNS (1 mL) in all tubes and absorbance was recorded at 540 nm using spectrophotometer. One unit of pectinase activity was defined as the concentration of enzyme that releases 1 µmoL of galacturonic acid per minute of the reaction under standard assay conditions.

$$Pectinase\ activity\left(\frac{\frac{U}{mL}}{min}\right)=\frac{\left[Concentration\ \left(\frac{mg}{mL}\right)x\ Dilution\ factor\ x\ 1000\right]}{[Molecular\ weight\ of\ galacturonic\ acid\ x\ Incubation\ time]}$$

Optimization of cultural conditions

Optimum conditions for pectinase production were determined by carrying out solid state fermentation of wild and mutant strains at different incubation periods i.e. 3, 4, 5, 6 and 7 days; different temperatures such as 20°C, 25°C, 30°C, 37°C and 40°C and different pH values ranging from 4.0, 5.0, 6.0, 7.0 and 8.0 (Patil and Chaudhari, 2010).

Statistical analysis

The computer statistical software (SPSS16) was used for statistical analysis of results. Significant difference among replicates has been presented as Duncan's multiple range tests in the form of probability (p) values (Duncan, 1955). 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

Strain improvement by UV irradiation

Penicillium notatum MH-61 was subjected to ultraviolet (UV) treatment for different time intervals i.e. 10, 20, 30, 40, 50, 60, 70 and 80 min. Fungal growth was completely inhibited at 60 min UV exposure. This time of exposure was selected and used repeatedly for mutagenesis of *Penicillium notatum* MH-61. After successive rounds of UV exposure, five mutant strains were obtained. Among all, the highest pectinase activity was exhibited by MH-UV9 i.e. 5.1 ± 0.10 U/mL/min as compared to that of wild strain i.e. 2.15 ± 0.02 U/mL/min (Fig. **1**). Increase in pectinase activity after UV treatment of wild strain indicates that irradiation causes mutation in genetic makeup of wild strain by changing the structure of pyrimidines i.e. cytosine and thymine bases present in DNA. This leads to pyrimidine dimers formation that affects DNA replication (Suribabu *et al.*, 2014). The current findings indicate 2.37 times increase in pectinase activity which is better than the findings of Kamalambigeswari *et al.*, (2018) who reported 1.1 fold increase in pectinase activity by mutant strain of *A. niger* after UV exposure of 60 min. The reason for this variation might be the difference in fungal species used. However, Heerd *et al.*, (2014) reported similar results with 2.4 fold increase in pectinase production using improved strain of *A. sojae*.

Strain improvement by nitrous acid treatment

Effect of nitrous acid on *Penicillium notatum* MH-61 was observed for enhanced production of pectinase using different concentrations (0.7, 0.8, 0.9, 1.0, 1.1, 1.2 M) of nitrous acid. At 0.9 M nitrous acid concentration, no fungal colonies were observed. Wild strain was repeatedly treated with this concentration of nitrous acid and eight mutant strains were obtained. All of the mutant strains exhibited increase in pectinase production. However, mutant strains MH-NA4, MH-NA7 and MH-NA11 showed better pectinase activity i.e. 3.66 ± 0.05, 3.57 ± 0.12, 3.46 ± 0.08

U/mL/min, respectively as compared to other mutant strains (Fig. **2**). Since microorganisms are susceptible to DNA modification, random mutagenesis is an effective method for strain improvement. Nitrous acid causes oxidative deamination of nitrogenous bases by removing amino groups from adenine, cytosine and guanine (Suribabu *et al.*, 2014). This leads to mispairing between altered bases. According to the current findings, pectinase activity was increased 1.70 times after using nitrous acid as chemical mutagen. Hadj-Taieb *et al.* (2002) observed the secretion of exo and endopectinases from *Penicillium occitanis* fifty times more than the wild type when subjected to nitrous acid mutagenesis. Sharma *et al.* (2017) also reported 11% increase in lipase yield from *Cunninghamella* sp. using nitrous acid as a mutagen. This shows effectivity of nitrous acid as a mutagen for achieving enhanced product formation.

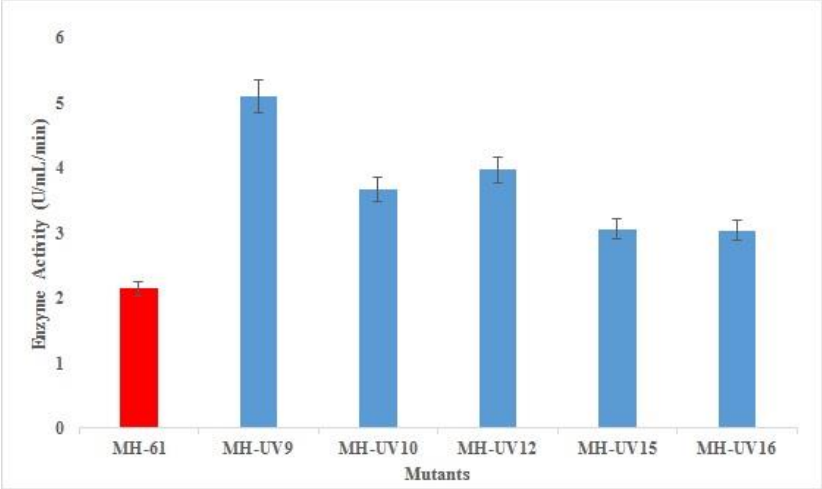

Fig. 1. Pectinase activity of wild along with mutant strains of *Pencillium notatum* obtained after UV irradiation. (Cultural Conditions: pH of medium: 6; Temperature of incubation: 30°C; Incubation time: 96 h)

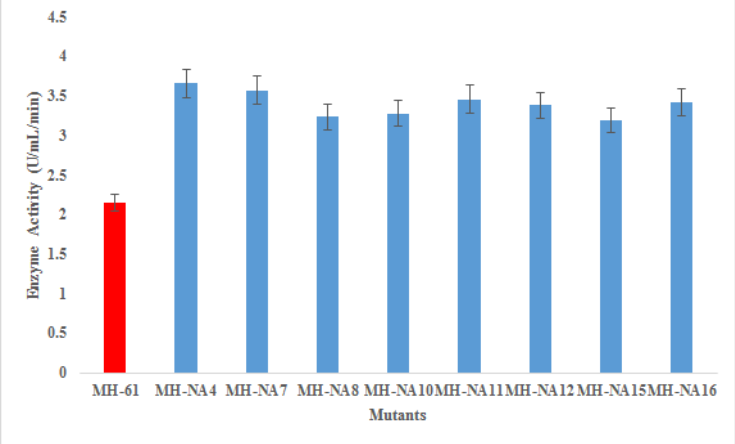

Fig. 2. Enzyme activity of parent strain and mutant derivatives of *Penicillium notatum* obtained after nitrous acid treatment. (Cultural Conditions: pH of medium: 6; Incubation temperature: 30°C; Incubation time: 96 h)

Random mutagenesis by ethidium bromide treatment

Penicillium notatum MH-61 was treated with ethidium bromide (0.1 – 0.7 mg/mL) for increase in pectinase production. Nine mutants were obtained when parent strain was treated with 0.5 mg/mL concentration of ethidium bromide. Pectinolytic activity of all mutant strains was enhanced as compared to that of parent strain. Maximum pectinolytic activity was obtained by mutant strain MH-EB8 i.e. 3.97 ± 0.09 U/mL/min followed by MH-EB9 i.e. 3.73 ± 0.11 U/mL/min and MH-EB12 i.e. 3.71 ± 0.11 U/mL/min (Fig. **3**). Pectinase activity of mutant MH-EB8 was enhanced by 1.84 folds as compared to wild strain might be due to the action of ethidium bromide as an intercalating agent as it has a planar structure that gets inserted between the nitrogenous bases of DNA (Suribabu *et al.*, 2014). This mutation may result in strain improvement for enhanced production of specific enzyme. Kamalambigeswari *et al.*, (2018) subjected *A. niger* to ethidium bromide (6 mg/mL) treatment for 60 min and recorded 1.44 times increase

in pectinase yield as compared to wild strain that contradicts with our results which showed 1.84 times increase in the activity of pectinase by *Penicillium notatum* The reason might be that *Penicillium notatum* used in current study was more vulnerable to mutagenesis by ethidium bromide even at low concentration as compared to that of *A. niger* used by Kamalambigeswari *et al.,* (2018).

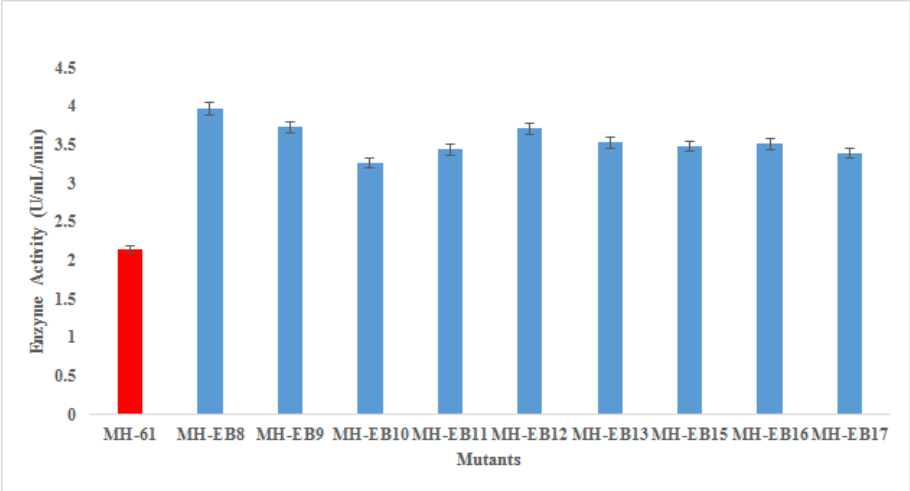

Fig. 3. Pectinase production by wild and mutant strains of *Penicillium notatum* after ethidium bromide treatment. (Cultural Conditions: pH of medium: 6; Temperature of incubation: 30°C; Incubation time: 96 h)

Optimization of incubation time

Wild strain of *Penicillium notatum* MH-61 and its mutant strain MH-UV9 were analyzed for pectinase production employing different incubation periods of 72, 96, 120, 144 and 168 h using solid state fermentation. Highest pectinase activity i.e. 5.12 ± 0.03 U/mL/min by mutant strain MH-UV9 was achieved after 120 h incubation and further increase in incubation period resulted in decrease in pectinase activity (Fig. **4**). Similar pattern was observed in case of wild strain. As the substrate concentration is depleted over time, the product formation becomes constant and does not change with increasing incubation period (Kent, 2000). However, Abbasi *et al.* (2011) and Martin *et al.* (2004) reported 192 h as the optimum incubation period for pectinase production by *Penicillium notatum* which contradicts to present study. This might be due to variation in solid state fermentation conditions as 70% and 67% moisture content for fermentation medium was maintained in the previous studies of Abbasi *et al.* (2011) and Martin *et al.* (2004) whereas in the current research, 80% moisture content was maintained. Amin *et al.* (2013) observed maximum activity of pectinase after 72 h incubation which contradicts current study. The contradiction might be due to difference in strains of *Penicillium notatum* used.

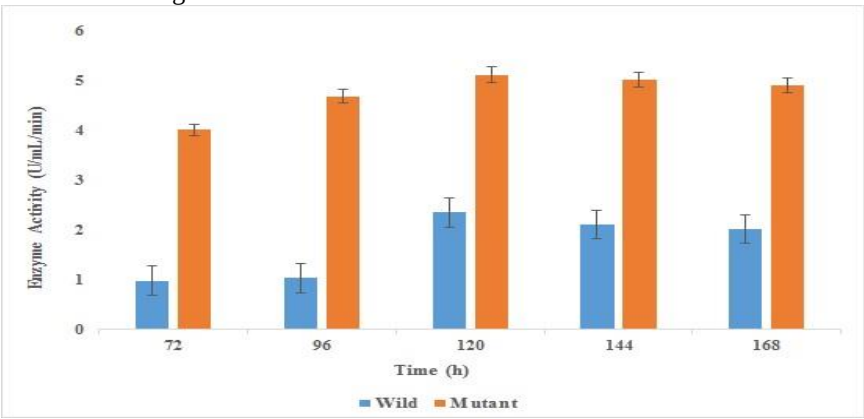

Fig. 4. Effect of different incubation times on pectinase activity of wild and mutant strain. (Cultural Conditions: pH of medium: 6; Incubation temperature: 30°C)

Effect of temperature

Effect of different incubation temperatures (20°, 25°, 30°, 37° and 40°C) on pectinase production of both strains i.e. wild *Penicillium notatum* MH-61 and mutant strain MH-UV9 was determined. Pectinase production was

enhanced gradually from 20°C to 25°C and reached to maximum at 30°C i.e. 5.11 ± 0.19 U/mL/min and 2.35 ± 0.11 U/mL/min and then decreased to 1.18 ± 0.05 U/mL/min and 0.54±0.02 U/mL/min at 40°C by mutant strain MH-UV9 and wild strain MH-61, respectively (Fig. **5**). When temperature exceeds the critical temperature value, the rate of enzyme denaturation exceeds rapidly and enzyme activity is greatly reduced because the kinetic energy of molecules becomes greater than the activation energy and bonds maintaining the three dimensional structure of enzyme are broken down (Germann and Stanfield, 2002). The results of Amin *et al.*, (2013) are in accordance with current study who reported high pectinase activity from *Penicillium notatum* at 30°C by solid state fermentation. Similar results might indicate that microbes used in all mentioned studies are mesophilic. Patil and Chaudhari, (2010) reported maximum production of pectinase by *Penicillium notatum* at 35°C by submerged fermentation that slightly contradicts with current research. This might be due to difference in strains of *Penicillium* used as well as the source from where strains were isolated. Banu *et al.* (2010) and Mathew *et al.* (2008) observed maximum enzyme activity by *Penicillium notatum* at 50°C and 60°C, respectively due to thermophilic nature of fungal strains used.

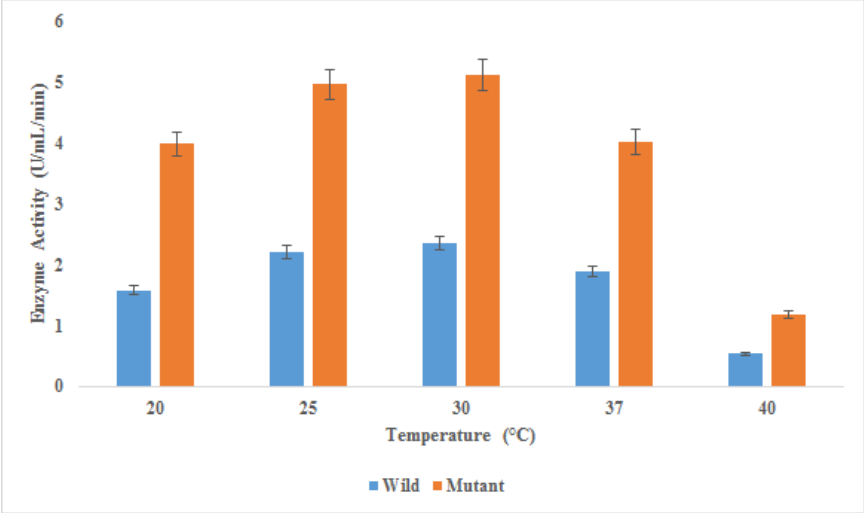

Fig. 5. Optimization of different temperatures on pectinase production using parent (MH-61) and mutant strain (MH-UV9). (Cultural Conditions: pH of medium: 6; Incubation time: 120 h)

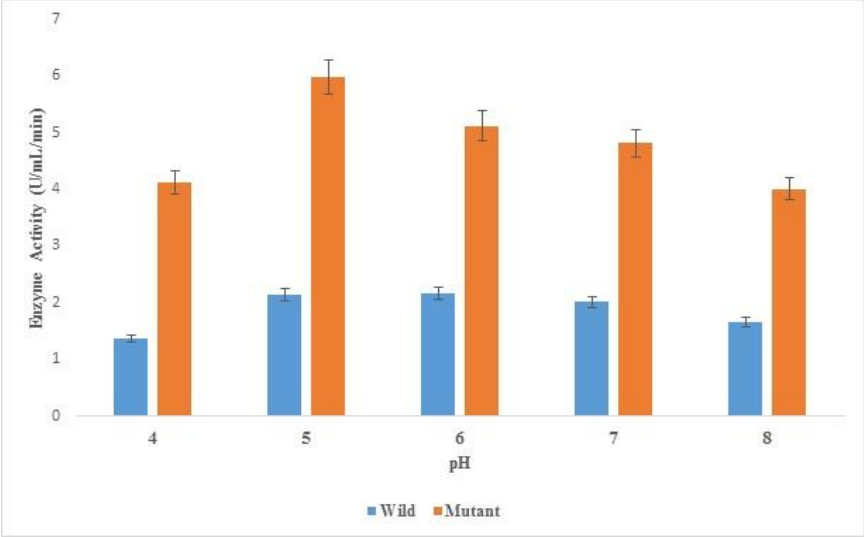

Fig. 6. Effect of different pH on activity of enzyme produced by wild strain (MH-61) and mutant strain (MH-UV9). (Cultural Conditions: Temperature of incubation: 30°C; Incubation time: 120 h)

Optimization of pH of medium

Wild strain *Penicillium notatum* MH-61 and mutant strain MH-UV9 were studied for increase in pectinase production by using fermentation media of different pH i.e. 4.0, 5.0, 6.0, 7.0 and 8.0. The optimum pH for

maximum pectinase activity by mutant i.e. 5.98 ± 0.05 U/mL/min and wild strain i.e. 2.89 ± 0.04 U/mL/min was recorded as 5.0 (Fig. **6**). Further rise or drop in pH led to decreased activity of pectinase. As pH increases or decreases, the electrostatic interactions within the enzyme are altered leading to enzyme inactivation and affect substrate binding to the active site of the enzyme (Nagodawithana and Reed, 2013). Ma *et al.* (2016) reported pH 5.0 as the optimum pH for pectinase production by *Penicillium janthinellum.* This result is consistent with the findings of current study. Similarity in results might be attributed to fungal enzymes slightly acidic optimum pH ((Nagodawithana and Reed, 2013). Banu *et al.* (2010) observed maximum production of pectinase from *P. chrysogenum* at pH 6.5 in submerged fermentation while in current study, solid state fermentation was carried out for pectinase production.

Conclusion

It was concluded from current study that strain improvement by random mutagenesis using physical and chemical methods and optimization of cultural parameters is a successful and cost-effective method for improving the production of Pectinase. This can result in cheaper industrial process as this enzyme is used for various industrial applications such as juice clarification, pulp degradation, paper polishing and increased feed digestibility.

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