Pak. J. Bot., 52(5): 1809-1813, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-5(29)

STRAIN IMPROVEMENT BY RANDOM MUTAGENESIS OF *ASPERGILLUS TAMARII* RMLC-10 FOR IMPROVED BIOSYNTHESIS OF POLYGALACTURONASE

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Abstract

Increasing and inevitable applications of polyagalacturonase in food and poultry feed industries urged the researchers to develop improved fungal strains for its industrial production. Polygalacturonase producing a wild strain of *Aspergillus tamarii* RMLC-10 was exposed to physical i.e. UV rays and chemical, i.e. Ethyl methanesulfonate (EMS), sodium azide and nitrous acid treatment to develop a mutant strain. This resulted in the development of forty-five variant strains. Among these, sodium azide treated a mutant strain of *Aspergillus tamarii* SA-11 showed a maximum increase in enzyme activity i.e. 32.01±0.09 U/ml compared to wild strain RMLC-10 with the enzyme activity of 24.57±0.04 U/ml. Effect of different cultural conditions such as incubation time, temperature and medium pH on polygalacturonase activity of wild and mutant strains was assessed. This showed an increase in enzyme activities of both wild (27.18±0.02 U/ml) and mutant (34.67±0.06) strains after an incubation time of 72 h at 30℃ in a fermentation medium of pH 6.

Key words: Mutagenesis, Pectinase, Optimization, Biosynthesis.

Introduction

Pectinase is a group of enzymes that include polygalacturonase (EC 3.2.1.15), pectin esterase (EC 3.1.1.11) and pectin lyase (EC 4.2.2.2). These enzymes differ in their mode of action and act synergistically to degrade pectin that is an important component of plant tissues (Ire & Vinking, 2016). Polygalacturonase (PG) is one of the widely used and most studied enzyme that catalyzes the hydrolysis of α-1,4-glycosidic bonds present between galacturonic acid units of pectin. It has two forms, endo-polygalacturonase (endo-PG) and exo-polygalacturonase (exo-PG). Endo-PG randomly acts on the polygalacturonic acid backbone and release oligogalacturonic acid residues while exo-PG acts at non-reducing ends of polygalacturonic acid chains and release monogalacturonic acid units (Zaslona & Trusek-Holownia, 2015).

Pectinases are produced by many plants and microorganisms. They are being widely used in industries that's why they have great economical value. Bacterial species such as *Clostridium sp., Bacillus sp*. and *Pseudomonas sp.* produce pectinases (Prathyusha & Suneetha, 2011). It has been reported that almost all pectic enzymes that are used in industries are being produced using fungi such as *Aspergillus sp., Fusarium sp., Penicillium sp., Sclerotium sp.* and *Rhizopus sp.* (Yannam *et al.*, 2014). Among fungi, the genus *Aspergillus* holds great importance in biotechnological applications especially in fermentation industries because of its natural ability to produce different metabolites, enzymes, organic acids and antimicrobial compounds (Cyrus & Juwon, 2016).

Currently, fermentation is a widely used method for the production of valuable products by microorganisms using organic waste (Jahan *et al.*, 2017). The production of polygalacturonase depends on many factors such as the type of fermentation process, microorganism being used as the source of enzyme, strain improvement and environmental parameters like pH, temperature and aeration (Ire & Vinking, 2016).

Strain improvement includes genetic modification of microorganisms to enhance their potential for biotechnological applications. Random mutagenesis using physical i.e. UV rays, X-rays, gamma rays and chemical mutagens such as Ethyl methanesulfonate (EMS), sodium azide and nitrous acid is a cost-effective method for obtaining an improved strain for industrial purposes (Ademakinwa *et al*., 2017). It involves inserting random mutations into the genome of the desired organism and then selection of mutant strains having desired characteristics. It is a hit and trial method as many unintended mutations can also be caused other than desired mutations (Derkx *et al.*, 2014). Many factors influence the process of mutagenesis such as the type of mutagen, dose of mutagen, treatment duration and type of microorganism. Usually, as the mutagen concentration is increased, the organism is more severely affected (Srivastava *et al*., 2011).

Pectinases are mainly used for fruit juice extraction and clarification as they can reduce the viscosity and turbidity of fruit juices. They are also used for pectin-free starch and animal feed production, tea and coffee fermentation and extraction of vegetable oils. They also have their application in textile industries for bio-scouring of cotton and ramie fibers and in paper industries for bio pulping of papers. They are also used for wastewater treatment discharged from food industries. They are also used to degrade plant cell walls for tissue culture and for purification of plant viruses (Pasha *et al*., 2013). To meet the increasing demand of pectinases, in view of their widespread applications, there is a need for improved polygalacturonase producing strains. Therefore, the current study focused was on improved strain development using efficient mutagens. Furthermore, optimal fermentation conditions were determined for enhanced production of the enzyme.

Materials and Methods

Microorganism: *Aspergillus tamarii* strain RMLC-10 isolated from spoiled chiku was used for strain improvement (Munir *et al*., 2018).

Strain Improvement: *Aspergillus tamarii* strain (RMLC-10) 48 hours old slant was poured with 10 ml of saline water. Conidia were scratched gently using sterilized inoculating loop aseptically. Serial dilution of conidial suspension was carried out to obtain 1.4 x 10^7 conidia per mL which were used for mutagenesis (El-Aziz, 2013).

UV mutagenesis: One mL of conidial suspension was exposed to UV light (245 nm) at a distance of 10 cm for different time exposures of 10, 20, 30, 40 and 50 minutes after the method of Shakibaie *et al*., (2018).

Chemical Mutagenesis: One ml conidial suspensions were subjected to 1% (w/v) sodium azide, 1 M Nitrous acid and 0.1% EMS treatment for variable time periods i.e. 10, 20 and 30 min. After that, conidial suspensions were centrifuged at 10000 rpm for 15 minutes and the pellet was subjected to washing with phosphate buffer. After washing, pellets were re-suspended in one mL of the same buffer (Kamalambigeswari *et al*., 2018).

Mutant screening: Subsequently, the conidial suspension was poured on to a potato dextrose agar plates having 0.1% 2-deoxy-D-glucose and allowed to incubate at 30°C for 72 hours. The fungal colonies with less than 1% survival rate were selected as mutant strains (El-Aziz, 2013).

Submerged fermentation: Fermentation medium used by Fratebianchi *et al*., (2017) with a modification in carbon source (Chiku peel powder was used instead of glucose) was used for polygalacturonase production using both wild and mutant strains. Conidial suspension (1 mL) was inoculated in fermentation medium and incubated at 30°C for 72 h with shaking speed of 200 rpm. After incubation, the fermentation broth was filtered using muslin cloth and filtrate obtained was used for polygalacturonase enzyme activity.

Polygalacturonase assay: Pectinase activity was determined after modification of the method demonstrated by Oumer & Abate, (2018). 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: Different fermentation conditions i.e. incubation time (24, 48, 72, 96 and 120 h), temperature (30, 35, 40, 45 and 50℃) and pH (5, 6, 7 and 8) were also analyzed for their effect on polygalacturonase production employing wild and mutant strains.

Statistical analysis

The SPSS 16.00 statistical software was used for statistical analysis of results. 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

Sodium azide mutagenesis: *Aspergillus tamarii* strain RMLC-10 conidia were subjected to EMS, Sodium azide, Nitrous acid and UV irradiations exposure for strain improvement to enhance polygalacturonase production (Fig. 1). EMS treatment resulted in nine, sodium azide treatment in seventeen, nitrous acid treatment in twelve and UV irradiation exposure in seven variant strains. Among these, maximum activity was depicted by sodium azide treated *Aspergillus tamarii* mutant strain SA-11 which is 32.01±0.09 U/ml. Sodium azide can directly affect the genetic makeup of the fungi and might result in the incomplete synthesis of some genes. This may also cause the breakdown of the already existing gene which may result in a loss in expression of the product as seen in the case of variants with negative results compared to the parent strain. The more polygalacturonase activity in sodium azide treated mutant strain compared to other mutagens treated strains, can be attributed to the effect of sodium azide on the precursors in the metabolic pathway which resulted in the enhanced expression of polygalacturonase (Cyrus & Juwon, 2015). Similar finding were reported by Rajeshkumar & Ilyas (2011) and Liang *et al*., (2015) who reported a mutation in the *Aspergillus fumigatus*, *Penicillium* sp. and *Aspergillus niger* for the enhanced formation of variable bio-products.

Effect of Incubation time: Different incubation times such as 24, 48, 72, 96 and 120 h were analyzed for their effect on polygalacturonase production using both wild (RMLC-10) and mutant (SA-11) strains of *Aspergillus tamarii*. It is noted that both wild and mutant strains gave best polygalacturonase activities i.e. 24.57±0.10 and 32.01±0.08 U/ml, respectively, after 72 hours of incubation time. Further increase in the incubation time resulted in decreased polygalacturonase activities for RMLC-10 and SA-11 (Fig. 2). Initially, time is required by the microorganism to reach to its exponential phase where maximum production of enzymes can be achieved. The decrease in enzyme activity after 72 h might be due to initiation of stationary and decline phases of microbial growth which resulted in constant or decreased product formation (Meletiadis *et al*., 2001). Phutela *et al*., (2005) reported the production of polygalcturonase after 72 h of incubation time using *Aspergillus fumigatus.* Maldonado & Saad (1998) also validate our results who reported polygalacturonase production by *Aspergillus niger* after 72 h of incubation.

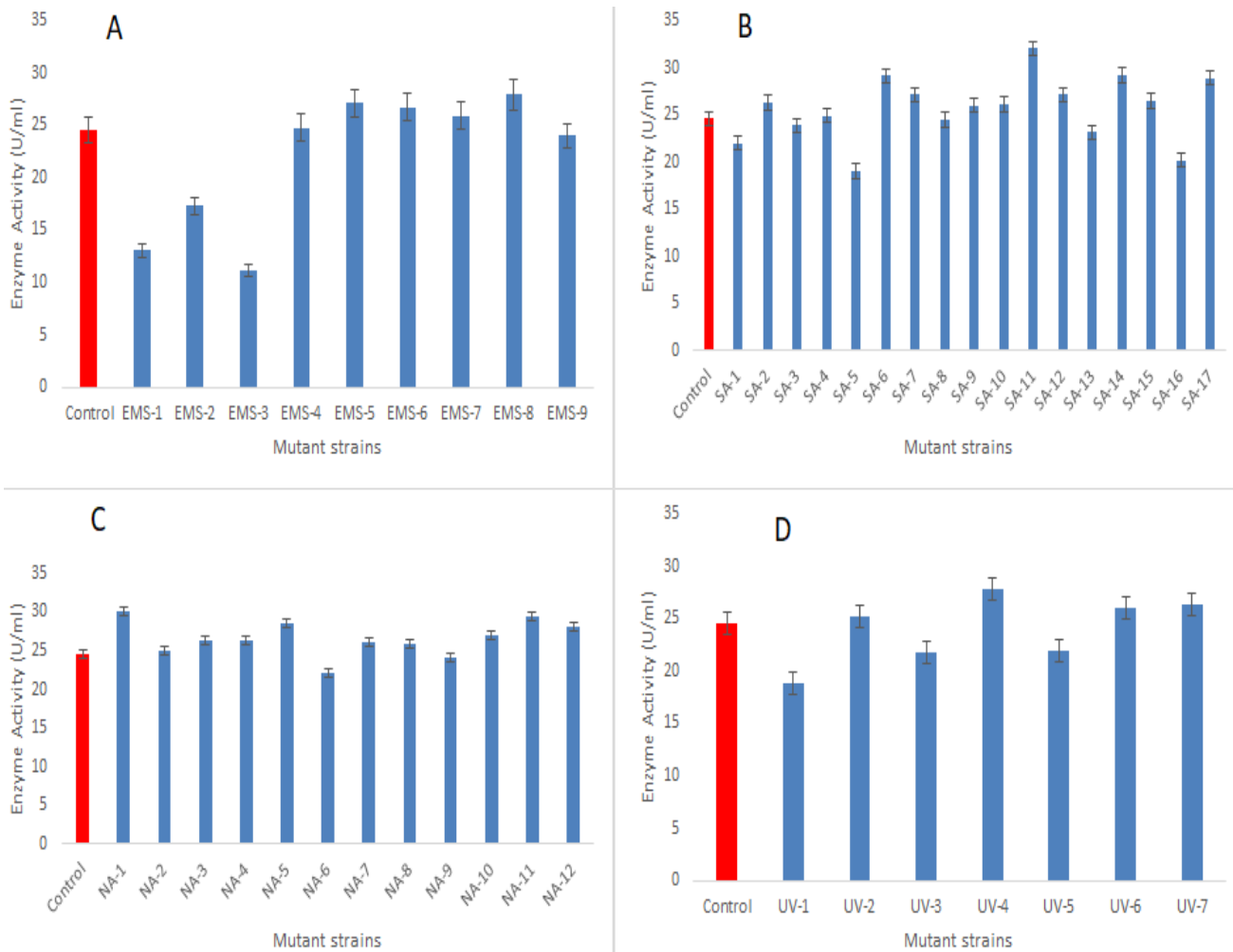

Fig. 1. Mutant strains developed after treatment with EMS (A), Sodium azide (B), Nitrous acid (C) and UV irradiations (D) with their polygalacturonase activities.

Effect of Temperature: Polygalacturonase production by wild and mutant strains of *Aspergillus tamarii* at different temperatures i.e. 25, 30, 35, 40, 45 and 50℃ was analyzed. An initial increase in polygalacturonase activity for both wild i.e., 24.57±0.04 and mutant i.e. 32.04±0.11 strains was observed from 25 to 30℃ as evident from (Fig. 3). However a further increase in the temperature resulted in a continuous decrease in polygalacturonase activity employing wild and mutant strains. Maximum production of the enzyme at mesophilic temperature might be attributed to the isolation of the fungal strain from mesophilic habitat. However, the decreased production of enzyme event at 45℃ shows that the enzyme produced by the strain might remain active up to this temperature for some time. Tari *et al*., (2007) reported the production of polygalacturonase at 30℃ using the *Aspergillus sojae.* However, Martin *et al*., (2004) reported the production of polygalacturonase at 40℃ from *Penicillium* sp. which is contrary to our finding might be due to the isolation of fungi from different habitats.

Effect of medium pH: Fermentation medium initial pH was varied between pH values of 5, 6, 7 and 8 to analyze its effect on the polygalacturonase production using wild and mutant strains of *Aspergillus tamarii*. Among these, maximum polygalacturonase production for wild and mutant strains were obtained as 27.18±0.02 and 34.67±0.06 U/ml, respectively. The polygalacturonase production at other pH values is represented in Fig. 4. Hence, pH 6 was optimized which showed both wild and mutant strains produce polygalacturonase at slightly acidic pH. The activity decreased above and below the optimum pH might be due to ionization of amino acids which affects the polarity of the membrane that disturbs the transport of ions across the cell membrane. Nighojkar *et al*., (2006) and Fontana *et al*., (2005) also reported the polygalacturonase production by fungal strain at acidic pH.

Conclusion

This study concluded that chemical treatment as a better option for strain improvement compared to physical mutagen. Among the chemicals, sodium azide was proved more potent for developing an improved strain for enhanced production of polygalacturonase. Optimisation of cultural conditions was found to have a significant effect on the polygalacturonase production in combination with strain improvement.

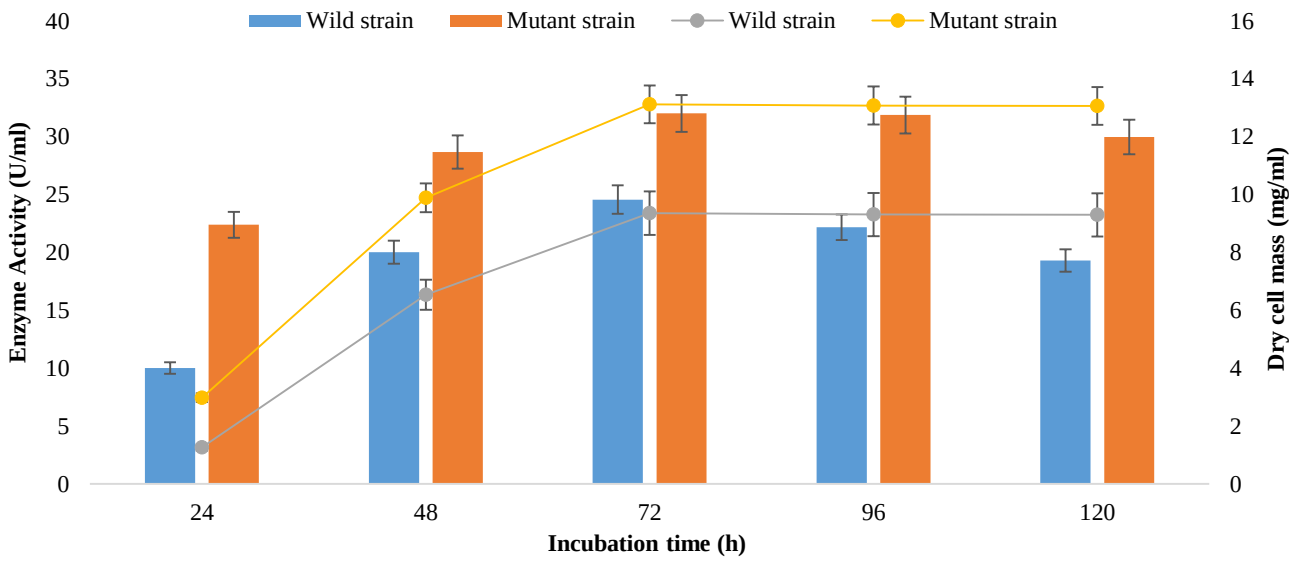

Fig. 2. Effect of incubation time on the production of polygalacturonase by wild and mutant strains of *Aspergillus tamarii*.

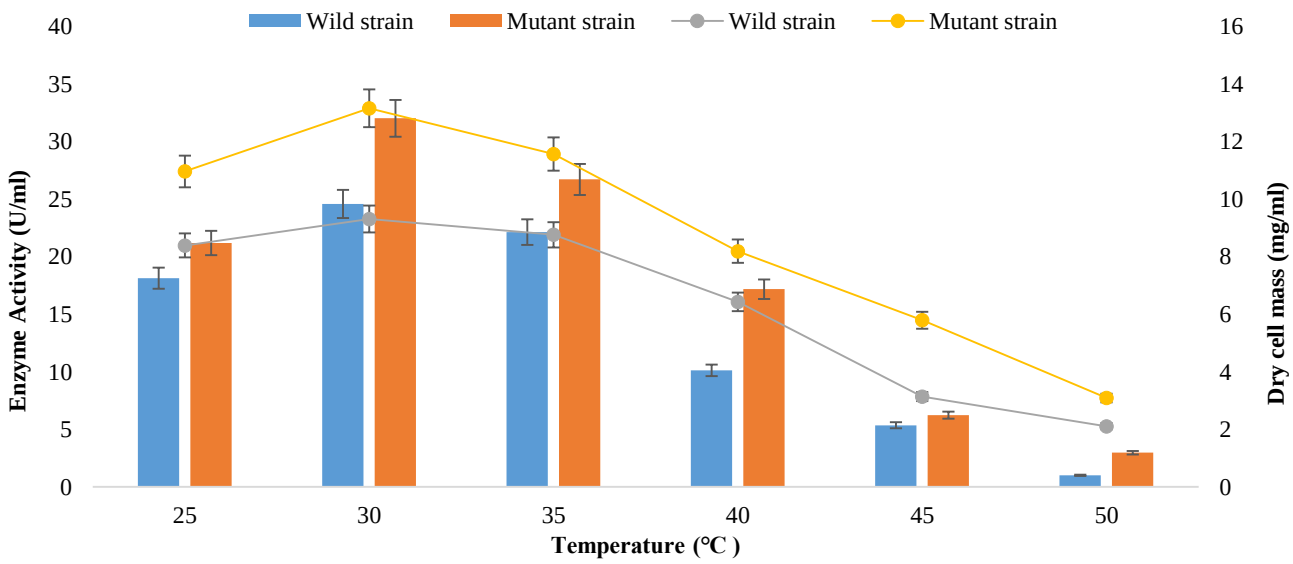

Fig. 3. Effect of temperature on wild and mutant strains of *Aspergillus tamarii* for polygalacturonase production.

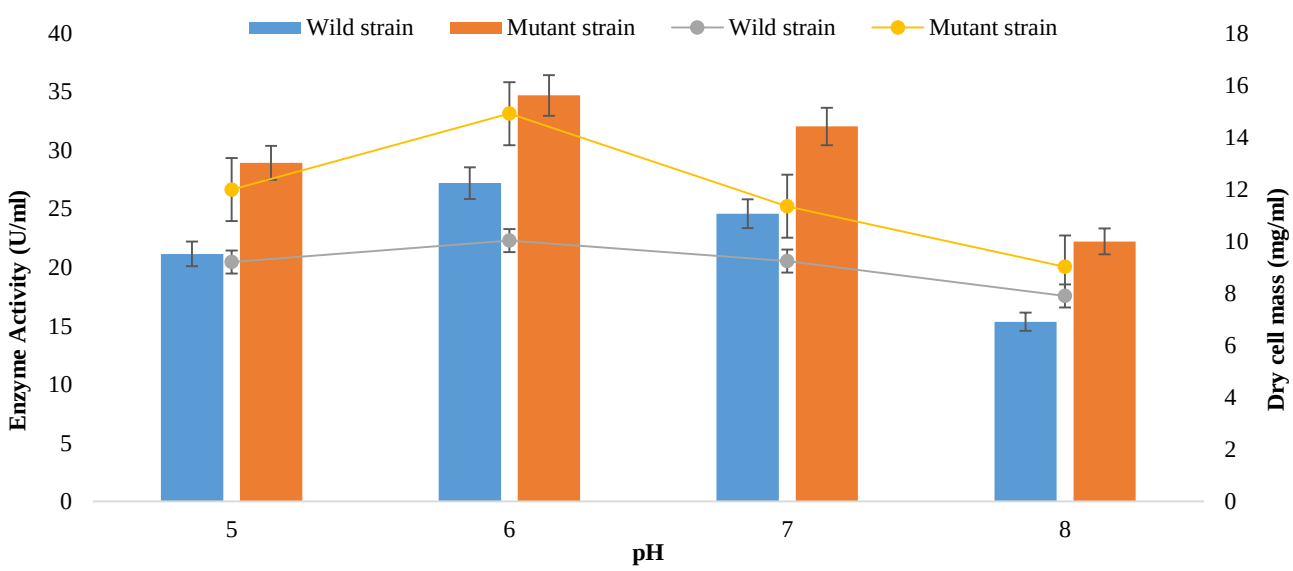

Fig. 4. Effect of fermentation medium initial pH on polygalacturonase production using wild and mutant strains of *Aspergillus tamarii*.

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(Received for publication 2 April 2019)