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PECTIN LYASE PRODUCTIVITY BY A UV-IRRADIATED *ASPERGILLUS ORYZAE* MUTANT UNDER CARROT-KOJI PROCESS

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ABSTRACT

Pectin lyase has several applications in different industrial areas. The enzyme has been produced by batch fermentation while the production of mutant pectin lyase has been improved by using carrot-koji fermentation. The present work deals with the stimulation in pectin lyase activity by a UV-irradiated *Aspergillus oryzae* mutant-auxotroph under carrot-koji process. The physical mutagenesis was induced using ultraviolet radiations. The effect of different distance from UV source (5-30 cm) and different exposure time (10-60 min) was investigated. The final mutant derivative UV-t30 was able to produce 9.26 U/ml of pectin lyase which was significantly higher than the wild-type. Two stage submerged fermentation was carried out while using carrot peelings as a substrate. The wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* showed the highest production by using 2 and 1.5 g carrot peelings, respectively 48 h after incubation (seeded with 8% inoculum). The enzyme was activated by $CaCl_2$ and $(NH_4)_2SO_4$ whereas it was inhibited in the presence of Tween-80. The enzyme was further activated by mutant strain UV-t30 while inhibited by wild-type ISL-9 using KNO_3. After optimization of parameters for enzyme activity, the potent mutant showed a 1.3-fold increase in the enzyme activity as compared to the wild-type. The study proved that carrot peel has nutrients which enabled *A. oryzae* to produce pectin lyase in koji process.

Keywords: *Aspergillus oryzae*, pectin lyase, mutant strain, UV radiation, auxotroph formation, koji process.

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INTRODUCTION

The enzyme pectin lyases (*EC 4.2.2.10*) are known as pectinase that have ability to degrade esterified pectin without methanol production through β-elimination into small molecules (Usha *et al.,* 2014). The β-elimination precedes the formation of 4,5-unsaturated 6-O-methylated galacturonide molecule in non-reducing end of one of the cleavage products (Irshad *et al.,* 2014; Zeuner *et al.,* 2020). It acts directly on the pectin without requirement for the past activity by diverse enzyme of pectinolytic complex (Atalla *et al.,* 2019). Molecular weight of pectin lyase lies in between 30-40 kDa excluding pectin lyase from *Pichia pinus* and *Aurebasidium pullulans*, their molecular mass is ~90 kDa (Sharma *et al.,* 2013). Pectin lyases are divided into two classes e.g., acidic pectin lyase and alkaline pectin lyase (Jayani *et al.,* 2005). Pectin lyase has large industrial applications such as clarification, cold stabilization, extraction of fruit juices, maceration of the plant tissues, saccharification of the biomass, degumming of the plant fibers, cotton scouring, improve fiber quality, reduction in cationic demand of the pectin solutions in the paper processing (Gummadi and Kumar, 2008), treatment of the industrial wastewater, oil extraction, remove off peels, liquefaction, gelation (Sharma *et al.,* 2013). Pectin lyase is produced by microorganisms at the higher level because of the numerous advantages of microorganisms. Pectin lyase produced by following fungi such as *Aspergillus fumigatus, A. niger, A. flavus, A. oryzae, A. ochraceus, A. sydowii, Penicillium spp., Trichoderma viridae, T. harzianum, Pseudomonas viridiflava, P. fluorescences, Pythium splendens* (Usha *et al.,* 2014). Yeast such as *Candida spp*. and *Saccharomyces cerevisiae* can produce pectin lyase (Gainvors *et al*., 1994). Bacterial pectin lyase have also been characterized from a wide range of bacterial species particularly *Bacillus subtilis* (Swain and Ray, 2010) and *Geobacillus stearothermophilus* (Demir *et al.,* 2011). Strain improvement is an important part in the process development for microbial products. These improvements are introduced in the target through mutagenesis which leads to the increase in the productivity or decrease in the process cost. It can be carried out by using physical agents or by employing chemicals agents (Sreeju *et al.,* 2011), but scientists preferred mostly ultraviolet radiations to improve microbial strain (Huang *et al.,* 2019).

Nutritional and environmental conditions play a critical role in pectin lyase production (Afifi *et al.,* 2002). Pectin lyase production has been reported (Batool *et al.,* 2013; Atalla *et al.,* 2019) from numerous microbes by using synthetic and agroindustrial residues as substrate. The present study was accomplished for the research work on stimulation in pectin lyase activity by UV-irradiated *A. oryzae* mutant auxotrophs under carrot-koji

process. Pectin lyase production through this method has not been reported yet; therefore, future optimization study is a pre-requisite before scaling up investigation. However, more work on development of UV-irradiated *A. oryzae* auxotrophs, screening of fungal auxotrophs for pectin lyase activity and optimization of carrot-koji fermentation for better enzyme production is required to get an insight into the koji-process.

MATERIALS AND METHODS

The experimental study had been carried out from 25th September to 25th February (2019-2020) in Research Lab. 5 & 6 of Institute of Industrial Biotechnology, Government College University, Lahore (Pakistan). The chemicals used in this study were bovine serum albumin (BSA), monobasic potassium phosphate (KH_2PO_4), Tween-80 and dioctyl sodium sulphosuccinate (MOT). These were of maximum possible purity.

Organism: The wild-type *Aspergillus oryzae* (ISL-9) was obtained from culture collection of Institute of Industrial Biotechnology (IIB), GC University Lahore. The strain was grown for 3-5 days and maintained on potato dextrose agar (PDA) slants at 30°C in a cooled incubator. The slant having maximum hyphal growth and sporulation was stored at 4°C and sub-cultured every 2 weeks.

Pre-treatment of substrates: Carrot (*Daucus carota* subsp. *sativus*) peelings were used as substrate in pectin lyase production from *A. oryzae*. Fresh carrot peelings were taken from the local market of Lahore (Pakistan) and oven dried at 60°C for 60 min. Once the peelings were moisture free, they were crushed into a fine granular form (Atalla *et al*., 2019). Wheat bran was used as carbon source and oven dried at 50°C for 60 min.

Inoculum preparation: Conidial suspension of *A. oryzae* was prepared from an agar slant (3-days old culture) by adding 10 ml of sterile MOT aseptically to the slant culture. The inoculum needle was used to break conidial clumps and a tube was swirled to obtain a homogenous suspension. Hemocytometer was used to count the number of spores in the inoculum.

Improvement of selected strain of *A. oryzae*: For improved enzyme production, mutagenesis was induced in the strain after exposure through UV radiation, described by Huang *et al*. (2019). Conidial suspension of *A. oryzae* was prepared in phosphate buffer (pH 7.2). Then exposed the conidial suspension to UV radiation at different distances from UV source (5-30 cm) and different exposure time (10-60 min). After UV mutagenesis, approximately 1 ml of the treated conidial suspensions was taken from stock and inoculated to the PDA plate that was supplemented with 0.01% (w/v) pectin. The plates were then incubated at 30°C for 3-4 days with daily monitoring. After UV treatment, the exposed cultures were kept under dark to avoid photoreactivation.

Fermentation technique: Production of pectin lyase was carried out aseptically by using two stage koji fermentation. Carrot peelings were used as raw substrate and wheat bran as additional carbon source for enzyme production. All experimental treatments were performed in 250 ml Erlenmeyer flasks containing 0.5 g of granular carrot peels moistened with 50 ml distilled water. Flasks were plugged with cotton plug and autoclaved at 121°C (15 psi) for 15 min. After autoclaving, 5 g of wheat bran was transferred to the flasks and autoclaved again. After sterilization, the medium was cooled down at room temperature and seeded with 2% (v/w) of 3-days old spore suspension (wild-type ISL-9 and mutant strain UV-t30) of *A. oryzae* under aseptic conditions. The flasks were placed at shaking incubator at 30°C for 72 h (160) rpm. All the fermentation experiments were run in a set of three parallel replicates.

Analytical techniques: A crude enzyme was extracted by centrifuging the fermentation media using refrigerated centrifuge (SIGMA, 2-16k, Germany) at 3000 rpm (4°C) for 15 min.

Determination of pectin lyase activity: Assay of pectin lyase was performed by the method described by Soares and Silva (1999). Enzyme (0.5 ml) was incubated for 1 h with 0.5 ml of pectin (0.5%), 1 ml of 50 mM Tris HCl buffer (pH 8) and 1 ml of 0.2 mM $CaCl_2$, respectively. After 1 h, absorbance was measured at 548 nm against blank solution

Enzyme activity unit: One unit of pectin lyase activity was defined as the amount of enzyme present in 1 ml of original enzyme solution which released 1 µM of galacturonic acid in 1 min.

$$= \frac{A \quad o \ e \quad s \quad \times s \quad f}{Ti \quad o \ in \quad S \quad f}$$

$$= \frac{C \quad (\mu M/m) \ o \ s}{A}$$

Determination of protein content: Bovine serum albumin (BSA) was used for protein determination after Bradford (1976). Absorbance was measured at 595 nm.

Statistical analysis: The comparison of treatment effects was performed by one-way ANOVA (Spss-9, version-4) and the protected least significant difference method after Snedecor and Cochran (1980). Significance difference had been shown as Duncan's multiple ranges, among the replicates in the form of probability (<p>) values.

RESULTS AND DISCUSSION

Strain improvement by induced mutagenesis using ultraviolet radiations: The effect of induced mutagenesis of *A. oryzae* ISL-9 by ultraviolet radiations at different distances from UV source (5-30 cm) and for defined exposure time (10-60 min) for better pectin lyase production in batch culture is shown in Table 1. When spore suspension was exposed at 5 cm distance from UV source, the mutant produced 5.62 U/ml of pectin lyase. An increase in production was noted when the spore suspension exposed at 15 cm distance from UV source i.e., 7.22 U/ml. Huang *et al.* (2019) also reported 9.99% increase in pectinase production by mutated strain R-7-2-4 of *A. tubingensis*. In the present study, optimal time for UV exposure was also evaluated. The enzyme activity increased significantly after 30 min of UV treatment (9.26 U/ml). The selected mutants UV-t30 were stored for further experiments.

Parametric optimizations for pectin lyase production: The effect of different substrate level (0.5-3 g) on pectin lyase production by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* in batch culture is shown in Fig. 1. As the substrate level was increased, a raise in the enzyme activity was observed. At the substrate level 2 g and 1.5 g, highest enzyme activity was achieved by wild-type ISL-9 (10.02 U/ml) and mutant strain UV-t30 (12.61 U/ml), respectively. As increase in the substrate level above optimal, resulted in the decline of the enzyme activity of both strains. After optimum substrate level, all active sites of enzyme are filled, thus increase in substrate concentration had no effect on the enzyme activity (Kent, 2000; Silva *et al.,* 2002). In the similar study, Atalla *et al*. (2019) used carrot peel as a substrate and yielded pectin lyase production. However, Koser *et al*. (2014) reported an enzyme activity of 875 U/ml, by using lemon peels as pectin source.

Table 1: UV induced mutagenesis in *A. oryzae* for enhanced pectin lyase production.

UV irradiation	Strain coding	PL activity (U/ml)
Distance (cm)		
5	UV-d5	5.62
10	UV-d10	6.18
15	UV-d15	7.22
20	UV-d20	6.21
25	UV-d25	4.85
30	UV-d30	4.11
Exposure time (min)		
10	UV-t10	6.83
20	UV-t20	8.19
30	UV-t30	9.26
40	UV-t40	8.61
50	UV-t50	7.92
60	UV-t60	6.79

Carrot peeling (0.5 g), wheat bran (5 g), inoculum size (2%), time of incubation (72 h).

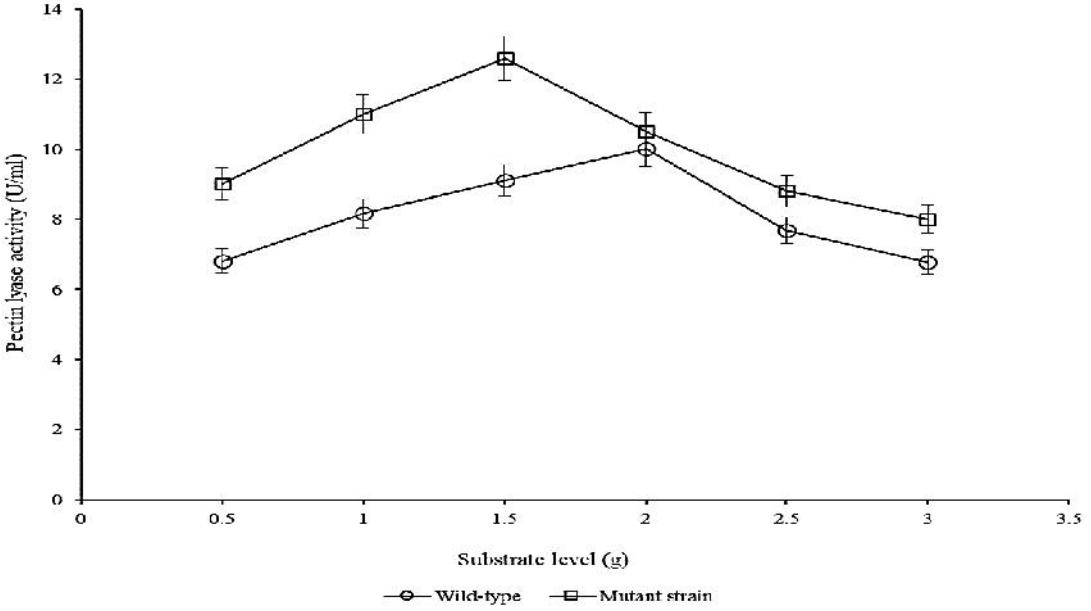

Fig. 1: Effect of different substrate level on pectin lyase production by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

* Time of incubation (72h), inoculum size (2%)

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at p≤0.05 from each other.

Fig. 2 shows the effect of incubation period (24-96 h) on the production of pectin lyase by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* in batch culture. An enhanced enzyme activity was recorded after 48 h incubation, shown by mutant strain UV-t30, which was 1.3-fold higher than the wild-type. Incubation period (48 h) was considered optimal for further studies. The low activity at large incubation period could be due to imbalance of microbial growth with the nutrient availability (Batool *et al*., 2013). In the similar study, Usha *et al*. (2014) optimized 48 h of incubation time for pectin lyase activity. However, an incubation time of 72 h was considered optimal in a study by Esmail *et al*. (2013). Similar kind of studies has also been reported by Sandri and Silveira (2018).

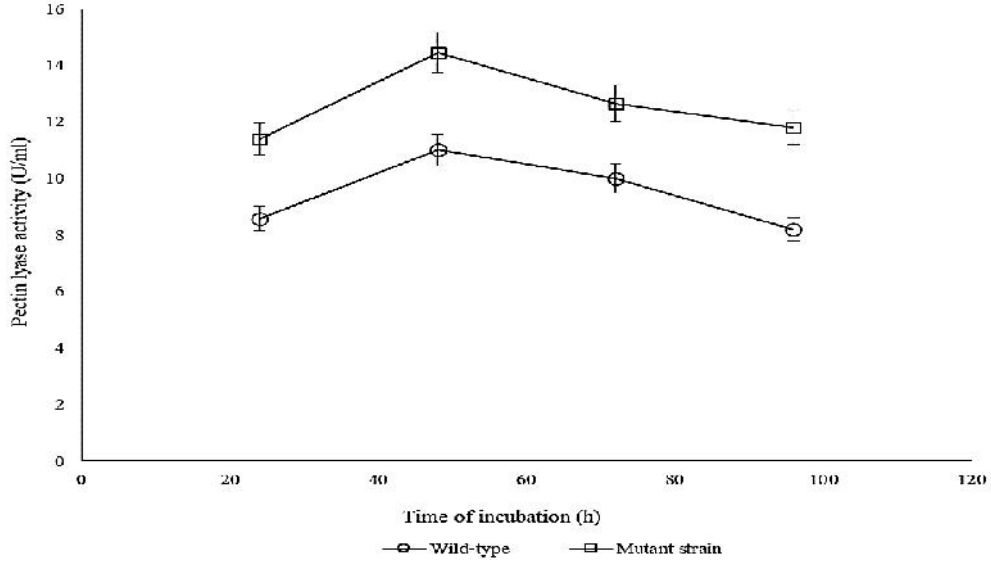

Fig. 2: Effect of incubation period on pectin lyase activity production by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

* Carrot peel (2 g) for wild-type however carrot peel (1.5 g) for mutant strain, inoculum size (2%).

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at p≤0.05 from each other.

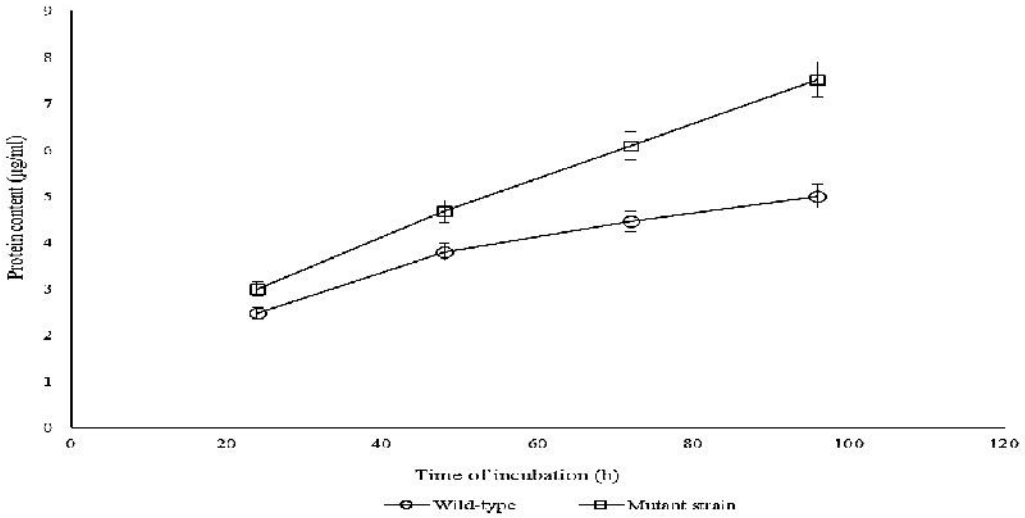

Fig. 3: Determination of protein contents over different incubation period of *A. oryzae* under carrot koji process.

*Enzyme extract (0.1 ml), 5 ml Bradford's reagent, at 30°C for 20 min.

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at p≤0.05 from each other.

Fig. 3 shows the effect of incubation period on determination of protein content in batch culture by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae*. At 24 h incubation, both strains ISL-9 and UV-t30 showed minimum protein content. After 72 h incubation, highest protein contents were shown by mutant strain UV-t30, which was 1.5-fold higher than the wild-type ISL-9 and considered as optimal. Protein content has been increases with time due to the secretion of microbial proteins like enzymes, hydrolyzed peptides, and other nitrogenous microbial components like chitin (Oseni and Akindahunsi 2011). Batool *et al*. (2013) investigated protein estimation by Biuret method using BSA as standard.

The effect of inoculum size (2-12%) on enhanced pectin lyase production by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* in batch culture was evaluated in Fig. 4. The data suggested that the increased inoculum size encouraged pectin lyase activity. At the 8% inoculum size, the highest enzyme activity was recorded by the mutant strain UV-t30 (20.34 U/ml) which was significantly higher than the wild-type (13.42 U/ml). A decline in the activity was observed due to the nutritional imbalance, when the inoculum size was further increased, produced highest growth that led to the autolysis of the cell (Mendez-Vilas, 2016; Pili *et al.,* 2017). In the similar study, Atalla *et al*. (2019) reported 8% inoculum size to be optimal for pectin lyase production by *Penicillium expansum* RSW-SEP1. However, Safia *et al*. (2014) investigated that 1% inoculum size to be optimal for *A. oryzae* pectin lyase.

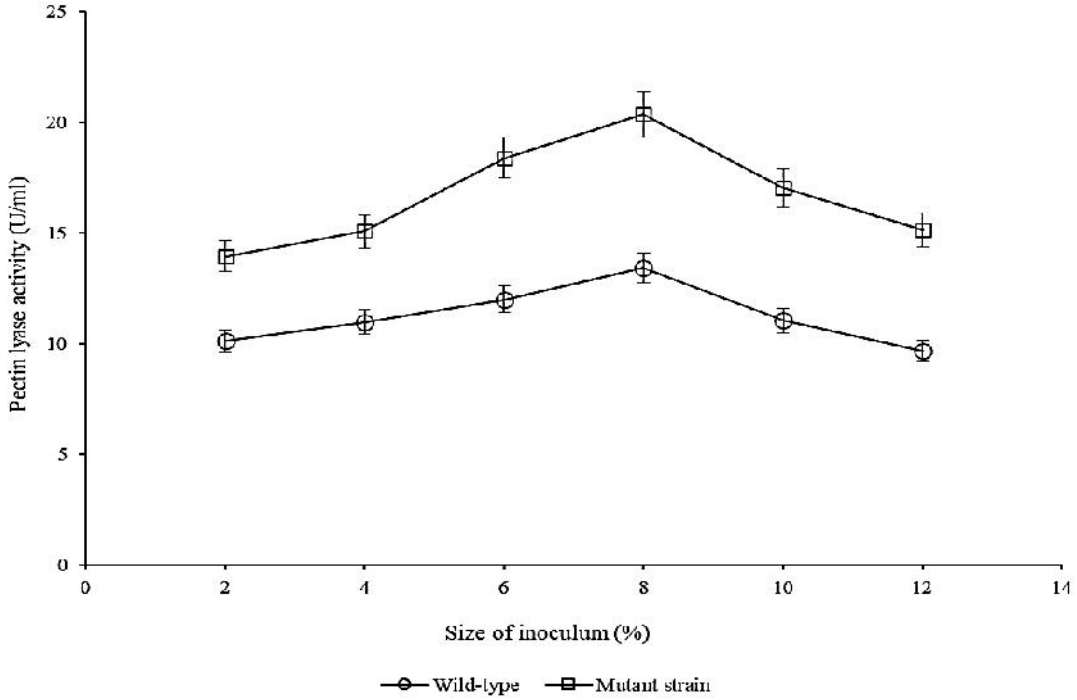

Fig. 4: Effect of different inoculum size on pectin lyase production by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

* Carrot peel (2g) for wild-type however carrot peel (1.5 g) for mutant strain, incubation period (48 h)

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at p≤0.05 from each other.

Role of various additives on pectin lyase activity: The evaluation of the effect of different concentrations (4-24 mM) of $CaCl_2$ on pectin lyase activity is shown in Fig. 5. In the present study, it was found that $CaCl_2$ act as stimulator for the enzyme activity. A steady increase in the activity was recorded with increase of $CaCl_2$. At 8 mM concentration of $CaCl_2$, mutant strain UV-t30 showed highest pectin lyase activity of 23.45 U/ml which was 1.29-fold higher than wild type ISL-9. In the similar study, Poturcu *et al*. (2016) investigated the role of $CaCl_2$ on pectin lyase activity and found to have a stimulatory effect on the enzyme. However, Pedrolli and Carmona (2009) examined the role of $CaCl_2$ on enzyme activity and have an inhibitory effect on the enzyme.

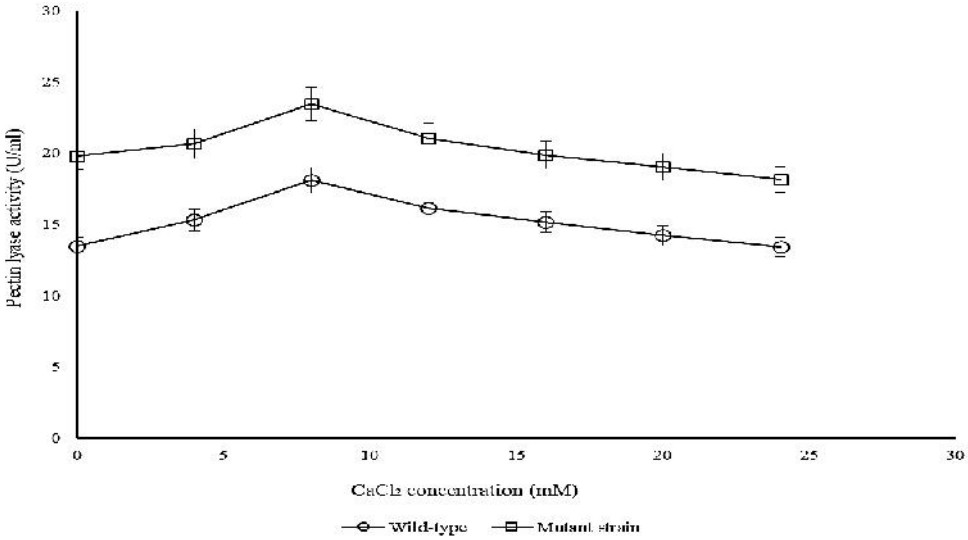

Fig. 5: Effect of different concentrations of $CaCl_2$ on pectin lyase activity by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

*Carrot peel (2g) for wild-type while carrot peel (1.5 g) for mutant strain, incubation time (48 h) inoculum size (8%).

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other.

The effect of different concentration (0.5-3 mM) of KNO_3 on pectin lyase activity was evaluated in Fig. 6. At the concentration of 2 mM KNO_3, mutant strain UV-t30 exhibited highest enzyme activity of 27.05 U/ml. In the present study, KNO_3 had a positive effect on enzyme activity of mutant strain UV-t30 and was found to have an inhibitory effect on enzyme activity of wild-type ISL-9. K^+ binding enhances the enzyme activity through conformational transitions triggered upon binding to a distant site and act as activator (Vasak and Schnabl, 2016). Afifi *et al*. (2002) investigated the positive effect of K^+ on the enzyme activity. Hamdy (2006) reported that KNO_3 had negative effect on enzyme activity.

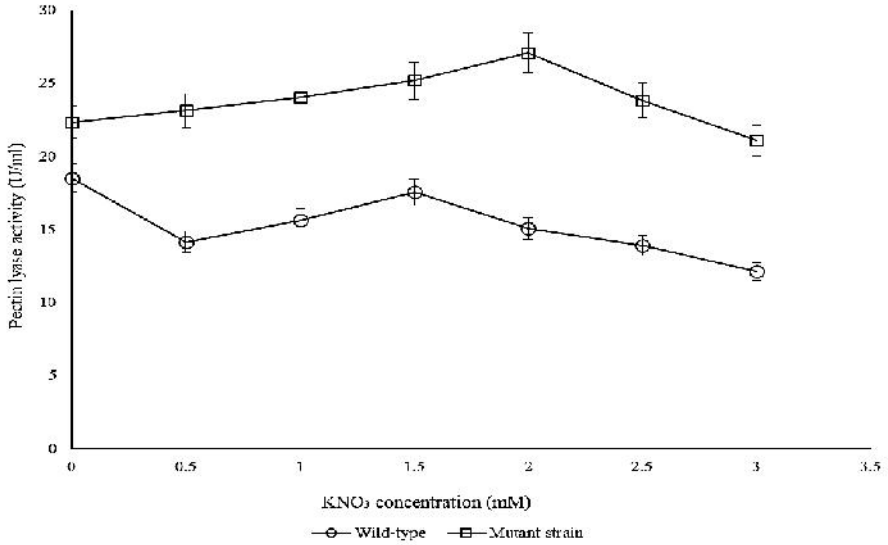

Fig. 6: Effect of different concentrations of KNO_3 on pectin lyase activity by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

*Carrot peel (2g) for wild-type while carrot peel (1.5 g) for mutant strain, incubation time (48 h), inoculum size (8%), $CaCl_2$ (8mM).

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other.

The effect of different concentrations (100-600 ppm) of $(NH_4)_2SO_4$ on pectin lyase activity is shown in Fig. 7. In the present study, it was found that $(NH_4)_2SO_4$ act as a stimulator for the enzyme activity. At the $(NH_4)_2SO_4$ concentration of 200 ppm, the highest enzyme activity of 24.12 U/ml and 28.91 U/ml was exhibited by wild-type ISL-9 and mutant strain UV-t30, respectively. In the similar study, Hamdy (2006) investigated that increased pectin lyase activity with increasing concentration of $(NH_4)_2SO_4$. In another study, Poturcu *et al*. (2016) reported that $(NH_4)_2SO_4$ to have a no effect on enzyme activity. However, Buston *et al*. (2006) investigated the inhibitory role of $(NH_4)_2SO_4$ on pectin lyase activity.

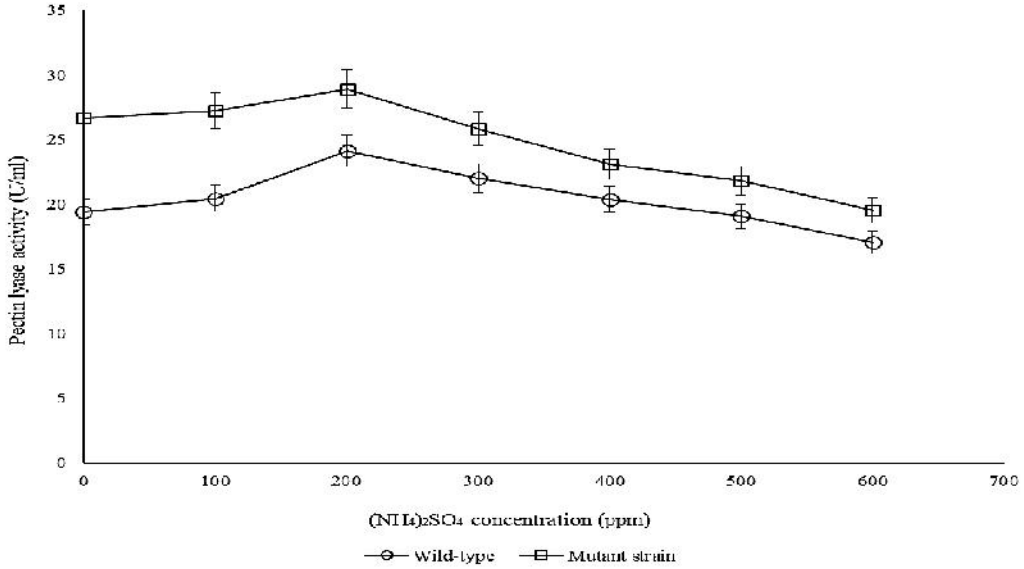

Fig. 7: Effect of different concentrations of $(NH_4)_2SO_4$ on pectin lyase activity by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

*Carrot peel (2g) for wild-type while carrot peel (1.5 g) for mutant strain, incubation period (48 h), inoculum size (2%), $CaCl_2$ (8 mM), KNO_3 (control) for wild-type ISL-9 while KNO_3 (2 mM) for mutant strain UV-t30.

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other.

Fig. 8 shows the effect of different concentrations of Tween-80 on pectin lyase activity. With the control, an enzyme activity of 23.96 U/ml and 28.41 U/ml was exhibited by wild-type ISL-9 and mutant strain UV-t30, respectively. At the highest Tween-80 concentration (0.3%), pectin lyase activity reduced to 12.69 and 15.29 U/ml by wild-type ISL-9 and mutant strain UV-t30, respectively. Thus in the present study, Tween-80 was found to be a pectin lyase inhibitor, and substantiates the findings of Usha *et al*. (2014) who reported that Tween-80 interacts with the enzyme and interrupts its 3-dimensional functional structure and makes it non-functional.

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other.

Time of incubation for enzyme activity: The evaluation of the effect of incubation period (10-60 min) on pectin lyase activity before optimization of additives is shown in Fig. 9. When 50- and 40-min incubation period was used, 14.32 U/ml and 20.15 U/ml of pectin lyase activity was observed by wild-type ISL-9 and mutant strain UV-t30, respectively and recorded as optimum. However, Poturcu *et al.* (2016) examined the role of incubation period on enzyme activity and optimized 60 min incubation period for pectin lyase activity.

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other.

Fig. 10 represents the effect of incubation period (10-60 min) on enzyme activity after optimization of additives. After 50 min incubation period, the wild-type ISL-9 and mutant strain UV-t30 exhibited highest enzyme activity of 19.11 U/ml and 25.15 U/ml, respectively after optimization of additives. In the present study, additives had positive and negative effect on enzyme activity. Incubation period (50 min) was recoded as optimal. Optimization of metabolic additives has been important to enhance the stability and activity of enzyme (Poturcu *et al*., 2016).

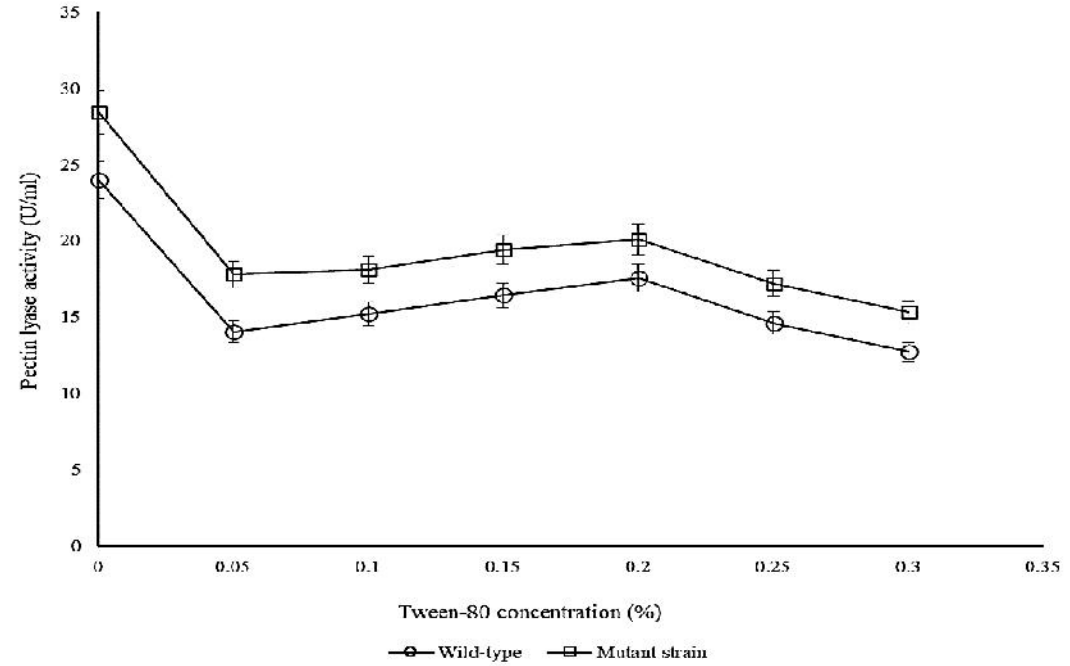

Fig. 8: Effect of different concentrations of Tween-80 on pectin lyase activity by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

*Carrot peel (2g) for wild-type while carrot peel (1.5 g) for mutant strain, incubation period (48 h), inoculum size (2%), $CaCl_2$ (8 mM), KNO_3 (control) for wild-type ISL-9 while KNO_3 (2 mM) for mutant strain UV-t30, 200 ppm $(NH_4)_2SO_4$.

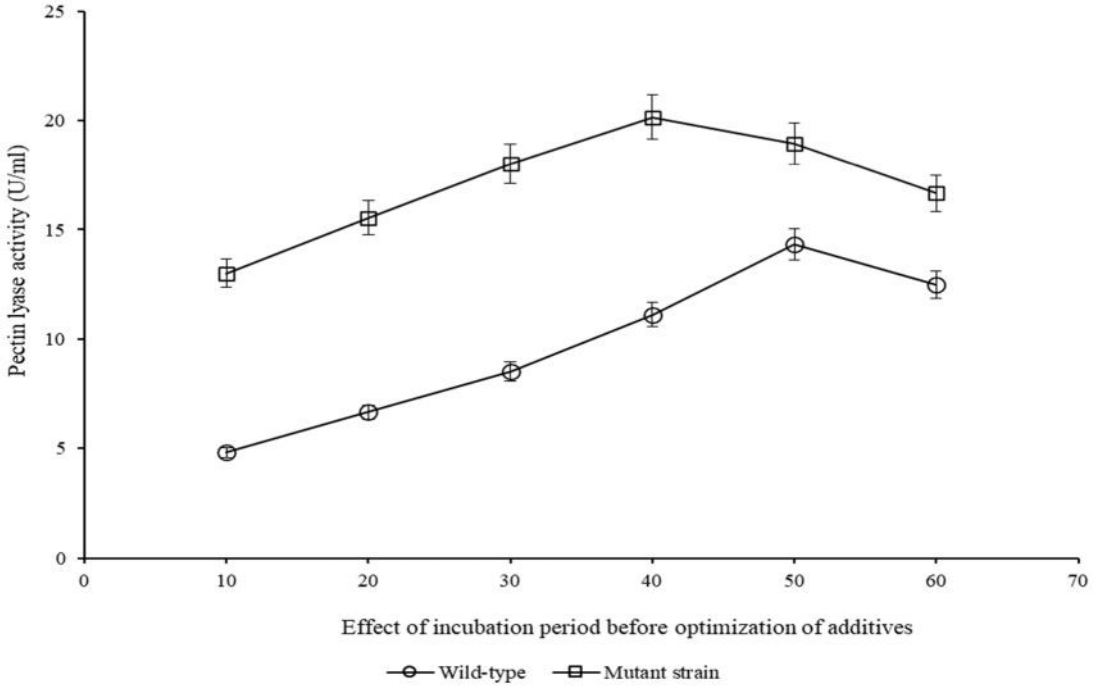

Fig. 9: Effect of incubation period on pectin lyase activity before optimization of additives by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

*Carrot peel (2g) for wild-type while carrot peel (1.5 g) for mutant strain, incubation period (48 h), inoculum size (2%).

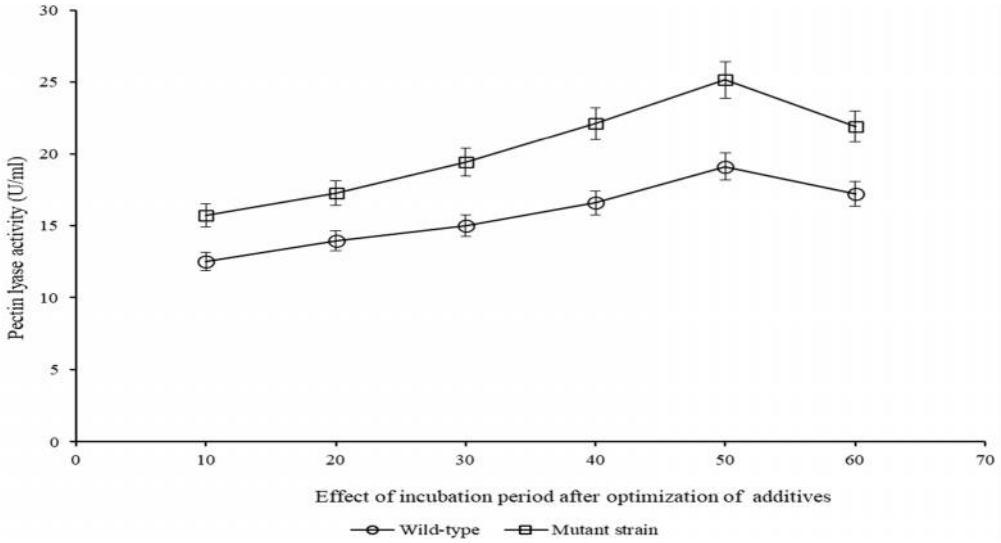

Fig. 10: Effect of incubation period on pectin lyase activity after optimization of additives by wild-type ISL-9 and mutant strain UV-t30 of *A. oryzae* under carrot koji process.

*Carrot peel (2g) for wild-type while carrot peel (1.5 g) for mutant strain, incubation time (48 h), inoculum size (2%), $CaCl_2$ (8 mM), KNO_3 (control) for wild-type ISL-9 while KNO_3 (2 mM) for mutant strain UV-t30, Tween-80 (0.2%).

The error bars indicate standard deviation (±sd set at 5%) amongst the values of two parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other.

Conclusion: In the present study, pectin lyase was produced from GRAS microorganism *Aspergillus oryzae* using carrot-koji process under suitable conditions. Random mutagenesis was induced through ultraviolet radiations. The addition of 200 ppm of $(NH_4)_2SO_4$ had the most significant effect. The overall activity of the enzyme was increased, as the mutant strain UV-t30 showed a 1.3-fold increase in the enzyme activity as compared to the wild-type ISL-9.

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Conflict of interest: The authors declare that there is no conflict of interest.

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