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# ISOLATION AND IDENTIFICATION OF MULTI STRESS TOLERANT POLYGALACTURONASE PRODUCING FUNGI FROM VARIOUS FRUITS

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## ABSTRACT

Polygalacturonase being a major part of pectinase enzyme group used in different industries urged scientists to look for temperature and pH stable fungal polygalacturonase to meet industrial demands more efficiently. In this regard, current study was focused to look for novel polygalacturonase producing fungal strain. To achieve the task, less explored spoiled fruit samples i.e. Chiku, Lemon, Date, kiwi, Strawberry and Grapes were targeted for the isolation of polygalacturonase producing fungi. Primary screening resulted in the isolation of fifty fungal strains, out of which thirty five showed pectinolytic activity. Five best pectinolytic fungi i.e. RMLC-2, 6, 9, 10, 28 and 59 on the basis of pectin lysis zones were further assessed by secondary screening using chiku peels and pulp dried powder as substrate in submerged fermentation. Among these, RMLC-10 showed maximum polygalacturonase activity i.e. 24.57±0.10 U/ml/min and further subjected to molecular identification which revealed fungal strain RMLC-10 as *Aspergillus tamarii*. Multi stress, pH (3-6) and temperature (30-60°C), tolerance profile of enzyme was carried out. It was found stable to appreciable extent in a broad range of pH and temperature for 1 h. These properties make this enzyme a potential candidate, after evaluation of further industrial related parameters, for applications in food, beverages, paper, pulp and textile industries.

**Keywords**: Fermentation, Enzymology, Pectinase, Stress tolerant.

## INTRODUCTION

Polygalacturonase is witnessing a continuous rise in its biotechnological perspective owing to mounting applications in food and feed industries. It belongs to a group of enzymes known as pectinases, which are encompassing 25 % of industrial enzymes worldwide (Oumer and Abate, 2018). Polygalacturonase is a de-polymerizing enzyme which catalyze α-1,4 glycosidic linkage in pectin chain to yield galacturonic acid units (Kim *et al.*, 2017). Being used extensively in beverages industries for the clarification of juices, it is considered as potent upcoming enzyme in food industry (Patidar *et al.*, 2018). In addition, polygalacturonase is important for carrying out catalytic processes in tea and coffee fermentation, degumming and retting of plant fibers, oil extraction and improving the digestibility of poultry feed (Ma *et al.*, 2017). Keeping in view the above mentioned potential of polygalacturonase, scientists are focused on discovering new microbial sources for its production from natural and safe habitats (Oumer and Abate, 2018).

Microbial production of polygalacturonase is carried out preferably using fungal sources (Rebello *et al.*, 2017). Previously, filamentous fungi isolation has been carried out extensively using soil samples for extra and intracellular pectinase production. Different fruits, having pectic material in their outer coverings have also been explored for the isolation of pectinolytic fungi (Ruiz *et al.*, 2017). Isolation of pectin degrading fungal strains from fruit sources presents an additional benefit of extracellular pectinase production (Molnarova *et al.*, 2014). However, some common fruits have been investigated repeatedly for this purpose with many left, which still needs to be explored (Patidar *et al.*, 2018). Novel fruit sources can result in isolation of fungal strains with better polygalacturonase production and efficiency. Pakistan has been known for the production of variety of fruits having diverse geographical distribution. Most of these fruits sources have never been reported for the isolation of pectinolytic fungi. One such fruit is *Sapodilla (common name:* Chiku) with a good concentration of pectic substance found extensively along sea shore region of Pakistan. Its cultivation is reported on 1112 and 625 hectares land with production of 3819 and 2858 tonnes in coastal areas of Sindh and Baluchistan provinces of Pakistan, respectively (Fruit, vegetables and condiments statistics of Pakistan, 2016).

Application of enzymes in various industries is directly dependent upon its cost effective production, catalytic activity, ecofriendly nature and ability to work in stress condition i.e. high temperature, low pH, high salt concentration etc. (Singh *et al.,* 2016). Stress tolerant enzymes prove more beneficial for industrial applications because of their applicability in wide range of pH and temperature (Jemli *et al.*, 2016). Polygalacturonase with ability to work in a wider range of acidic pH possess an exclusive potential in food and beverages industry (Cheng *et al.*, 2016). Being acidic in nature, *Sapodilla*

serves as potent source for the isolation of a fungal strain capable of producing acidic pH tolerant polygalacturonase. Molecular analysis using ITS region of fungal genome and subsequent homologous analysis result in non-ambiguous identification (Raja *et al.,* 2017). This specific objective of the study was to look for an efficient fungal strain having ability to produce pH tolerant polygalacturonase. In order to achieve the above mentioned objective, comparatively less explored fruit sources such as Chiku, Lemon, Date, kiwi, Strawberry and Grapes were analyzed.

## MATERIALS AND METHODS

**Sample collection:** Six different ripened fruit (Chiku, Lemon, Date, kiwi, Strawberry and Grapes) were collected from the fruit market in Lahore, Pakistan. These ripened fruits were transferred to the research laboratory of Department of Biotechnology, Lahore College for Women University, Lahore Pakistan in sterilized polythene bags for further processing. This study was conducted during March, 2017-December, 2017).

**Isolation and Primary Screening:** The fruit samples were immersed in sterilized distilled water to prepare stock solution. Two fold serial dilution of each stock was poured to the plates having mineral salt agar media (0.2g $NaNO_3$, 0.05g KCl, 0.05g $MgSO_4$, 0.02g $K_2HPO_4$, 0.01g $FeSO_4$, 1g pectin and 2g of agar/100mL) aseptically. Plates were incubated for 72 h at 30°C. After incubation, plates were poured with potassium iodide-iodine solution (Potassium iodide: 1.5g and iodine: 0.3g/100mL) in order to analyze the pectin lysis zones on plates for primary screening of fungal isolates (Nisha and Kalaiselvi, 2016).

**Secondary screening:** Secondary screening for polygalacturonase estimation was carried out using submerged fermentation. Chiku peels and pulp was extracted and dried to obtain powder form (Ramos *et al.*, 2017). This powdered extract of Chiku was further used as a carbon source with a modification to the fermentation medium used by Fratebianchi *et al.* (2017). Spore inoculum (1 ml) was used to inoculate 25 ml of fermentation medium aseptically. Inoculated fermentation medium was incubated for 72 h at 30°C. After incubation, fermentation broth was centrifuged at 6000 rpm and 4°C for 10 min. Supernatant obtained (crude enzyme) was used for polygalacturonase estimation.

**Polygalacturonase assay:** The amount of pectinase enzyme was measured by enzyme assay method of Okafor *et al.* (2010).One unit (U) of enzyme activity is amount of enzyme required to release 1µmol of galacturonic acid per minute under standard assay conditions is expressed as units per mL (U/mL/min) (Arijit *et al.*, 2013).

**Molecular Identification:** The best pectinolytic fungal strain was subjected to molecular identification using 18S rRNA region sequencing (Oumer and Abate, 2018). The sequence of the region obtained was used for comparison with other sequences present in Gen bank using BLAST and homology analysis was carried out for confirmation of the strain identification (Saitou and Nei, 1987).

**Stress tolerance of polygalacturonase:** Crude enzyme was pre incubated for 1 h with sodium citrate buffer of different pH (3 to 6) to analyze its pH stability. Similarly, to access the thermo stability crude enzyme was pre incubated for 1 h at different temperatures i.e. 30, 35, 40, 45, 50, 55 and 60°C. The residual activities were assessed to analyze the effect of pH and temperature stability (Cheng *et al.*, 2016).

**Statistical analysis:** SPSS version 16.00 (IBM Analytics, New York USA) was used for statistical analysis of results. Error bars in the figures of results section indicates the standard deviation (±SD) among the three replicates, which differ significantly at $p < 0.05$.

## RESULTS AND DISCUSSION

**Isolation and primary screening:** Sixty fruit samples i.e. Chiku, Lemon, Date, kiwi, Strawberry and Grapes (10 each) were subjected to isolation of pectinolytic fungi which resulted in isolation of fifty fungal strains. Among these, thirty five fungal strains showed pectinolytic activity (Table 01). Six best pectinolytic fungal strains i.e. RMLCP-2, 6, 9 and 10 having lysis zones of 44, 41, 42 and 48 mm, respectively, isolated from Chiku; RMLC-28 with lysis zone of 43 mm isolated from strawberry; RMLC-59 having lysis zone of 41 mm isolated from Grapes were selected for secondary screening. Perveen *et al.* (2017) reported isolation of exo-polygalacturonase producing fungal strains from rotten peach, peer and grape fruit. Amilia and Siri (2017) isolated pectin degrading fungi from banana and orange peels. Suryam and Charya (2018) successfully isolated fungal strains from oranges and grapes having ability to disintegrate pectic material. All these reports showed potential of isolating pectinolytic fungi from the fruit sources having pectin for microbial utilization. In current study the fruit samples barely or not reported for isolation of pectinolytic fungi were used. The fungal strain with maximum lysis zone was isolated from Chiku which to the best of our knowledge have not been reported before for the isolation of pectinolyti fungi.

**Table 1. Primary screening for pectinolytic fungi using different fruit samples.**

| Sr. No. | Strain | Fruit | Fungal Growth | Pectinolytic zone (mm) |
|---|---|---|---|---|
| 1 | RMLC-1 | Chiku | + | 23 |
| 2 | RMLC-2 | Chiku | + | 44 |
| 3 | RMLC-3 | Chiku | + | 37 |
| 4 | RMLC-4 | Chiku | + | 33 |
| 5 | RMLC-5 | Chiku | - | - |
| 6 | RMLC-6 | Chiku | + | 41 |
| 7 | RMLC-7 | Chiku | + | - |
| 8 | RMLC-8 | Chiku | + | 32 |
| 9 | RMLC-9 | Chiku | + | 42 |
| 10 | RMLC-10 | Chiku | + | 48 |
| 11 | RMLC-11 | Date | + | - |
| 12 | RMLC-12 | Date | + | 30 |
| 13 | RMLC-13 | Date | + | - |
| 14 | RMLC-14 | Date | + | - |
| 15 | RMLC-15 | Date | + | 28 |
| 16 | RMLC-16 | Date | + | - |
| 17 | RMLC-17 | Date | + | 29 |
| 18 | RMLC-18 | Date | + | 21 |
| 19 | RMLC-19 | Date | + | 36 |
| 20 | RMLC-20 | Date | - | - |
| 21 | RMLC-21 | Strawberry | + | 37 |
| 22 | RMLC-22 | Strawberry | + | - |
| 23 | RMLC-23 | Strawberry | + | 31 |
| 24 | RMLC-24 | Strawberry | + | - |
| 25 | RMLC-25 | Strawberry | + | 32 |
| 26 | RMLC-26 | Strawberry | - | - |
| 27 | RMLC-27 | Strawberry | + | 23 |
| 28 | RMLC-28 | Strawberry | + | 43 |
| 29 | RMLC-29 | Strawberry | + | - |
| 30 | RMLC-30 | Strawberry | + | 37 |
| 31 | RMLC-31 | Lemon | + | - |
| 32 | RMLC-32 | Lemon | + | 36 |
| 33 | RMLC-33 | Lemon | + | 21 |
| 34 | RMLC-34 | Lemon | + | - |
| 35 | RMLC-35 | Lemon | + | 31 |
| 36 | RMLC-36 | Lemon | + | 19 |
| 37 | RMLC-37 | Lemon | + | - |
| 38 | RMLC-38 | Lemon | + | 26 |
| 39 | RMLC-39 | Lemon | + | - |
| 40 | RMLC-40 | Lemon | + | 21 |
| 41 | RMLC-41 | Kiwi | - | - |
| 42 | RMLC-42 | Kiwi | + | - |
| 43 | RMLC-43 | Kiwi | + | 21 |
| 44 | RMLC-44 | Kiwi | - | - |
| 45 | RMLC-45 | Kiwi | + | - |
| 46 | RMLC-46 | Kiwi | - | - |
| 47 | RMLC-47 | Kiwi | + | 33 |
| 48 | RMLC-48 | Kiwi | - | - |
| 49 | RMLC-49 | Kiwi | + | - |
| 50 | RMLC-50 | Kiwi | - | - |
| 51 | RMLC-51 | Grapes | + | 17 |
| 52 | RMLC-52 | Grapes | + | 25 |
| 53 | RMLC-53 | Grapes | + | 20 |

| | | | | |
|---|---|---|---|---|
| 54 | RMLC-54 | Grapes | + | 26 |
| 55 | RMLC-55 | Grapes | + | 29 |
| 56 | RMLC-56 | Grapes | - | - |
| 57 | RMLC-57 | Grapes | + | 25 |
| 58 | RMLC-58 | Grapes | + | 36 |
| 59 | RMLC-59 | Grapes | + | 41 |
| 60 | RMLC-60 | Grapes | - | - |

**Secondary screening:** The selected pectinolytic fungal strains i.e. RMLC-2, 6 9, 10, 28 and 59 were subjected to secondary screening (Figure 1). Among these, maximum polygalacturonase production i.e. 24.57±0.10 U/ml/min and protein content i.e. 2.35mg/ml was observed for a fungal strain RMLC-10 which was isolated from Chiku and production was carried out using Chiku pulp dried powder as substrate. Reginatto *et al.* (2017) and Martin *et al.* (2010) reported 14 U/ml and 13.6 U/ml of pectinase produced by *A. niger* and *Thermomucor indicae-seudaticae* using cirtus pectin as substrate, respectively. Different fruit sources as substrate were analyzed by Anuradha *et al.* (2010) for polygalacturonase production i.e. Orange peel (16.8 U/ml) Jack fruit rind (38 U/ml) Carrot peel (36U/ml) and Beet root peel (24U/ml). This shows the potential of fruit peels for being used as a cheap source for the production of polygalacturonase. However, no studies were found in literature depicting the effect of Chiku as substrate. Therefore current study stands unique in isolation of pectinolytic fungi from Chiku and polygalacturonase production using it as substrate by submerged fermentation with excellent activity. However dry cell mass for all the fungal strains was observed greater in comparison to RMLC-10 (Fig.1) and polygalacturonase production might be due to variation in metabolic function of fungal strains which allows the said fungi to produce more enzyme with even less biomass formation.

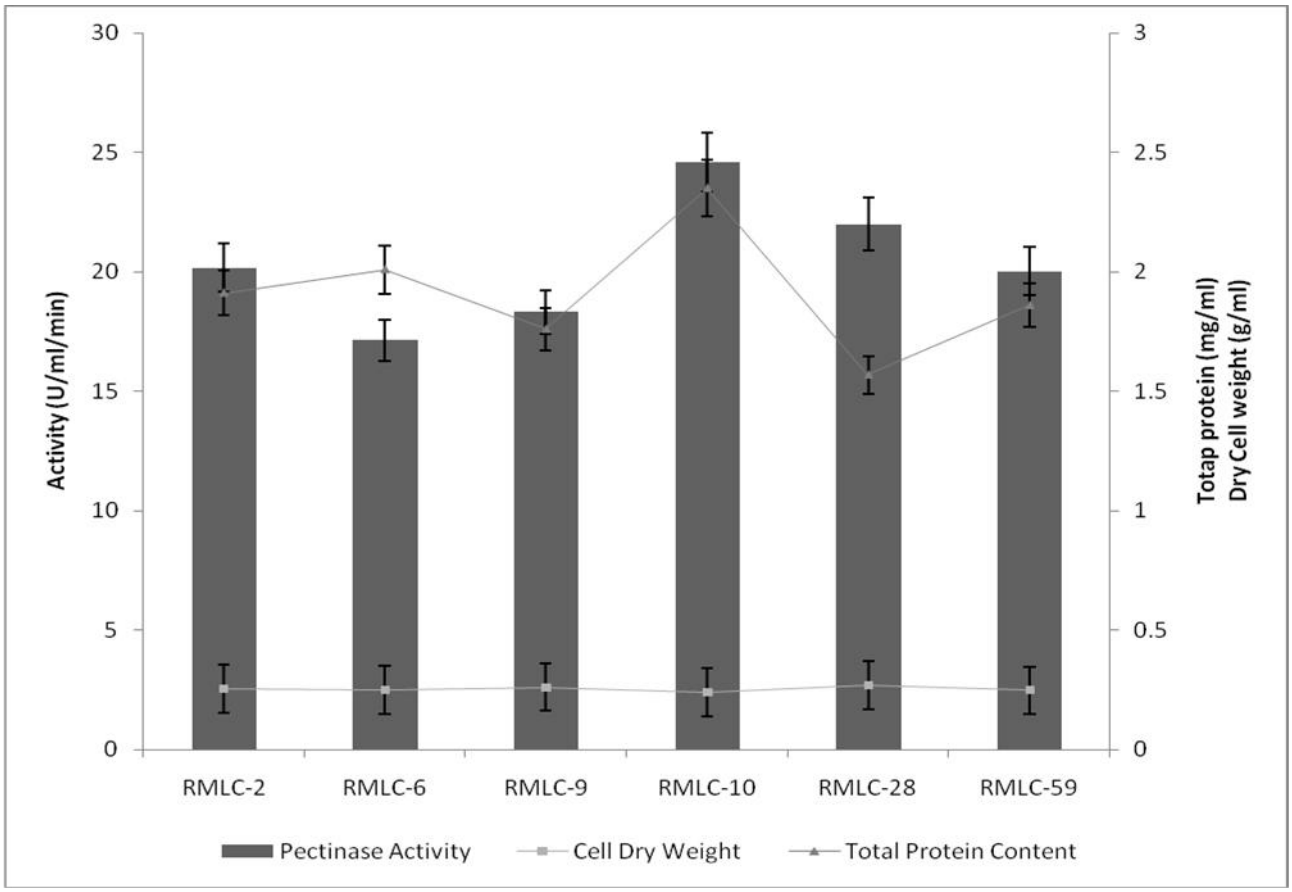


**Fig. 1. Secondary screening of pecinolytic fungal isolates for polygalacturonase production using Chiku peels and pulp dried powder as substrate in submerged fermentation. Error bars indicates the standard deviation (±SD) among the three replicates, which differ significantly at $p < 0.05$.**

**Molecular Identification:** DNA from maximum polygalacturonase producing fungal strain RMLC-10 was isolated and subjected to PCR for amplification of 18S rRNA region. This resulted in appearance of clear single bands on agarose gel with band size of 700 bp (Fig. 2). The amplified region was sequenced (Fig.3). The obtained sequence was used to get the sequence of related fungal strains using NCBI BLAST. Multiple sequence alignment was carried out with first ten sequences obtained after BLAST. This homology analysis resulted in the development of a dendogram (Fig. 4), which confirms the RMLC-10 fungal strain as *Aspergillus tamarii.* Khan *et al.* (2017) and De Filippis *et al.* (2017) also used 18S rRNA region sequencing for the identification of fungal strains as *Macrophomina phaseolin* and *Saccharomyces cerevisiae*, respectively. To the best of our knowledge this is first report of polygalacturonase production by *Aspergillus tamari* isolated using spoiled Chiku.

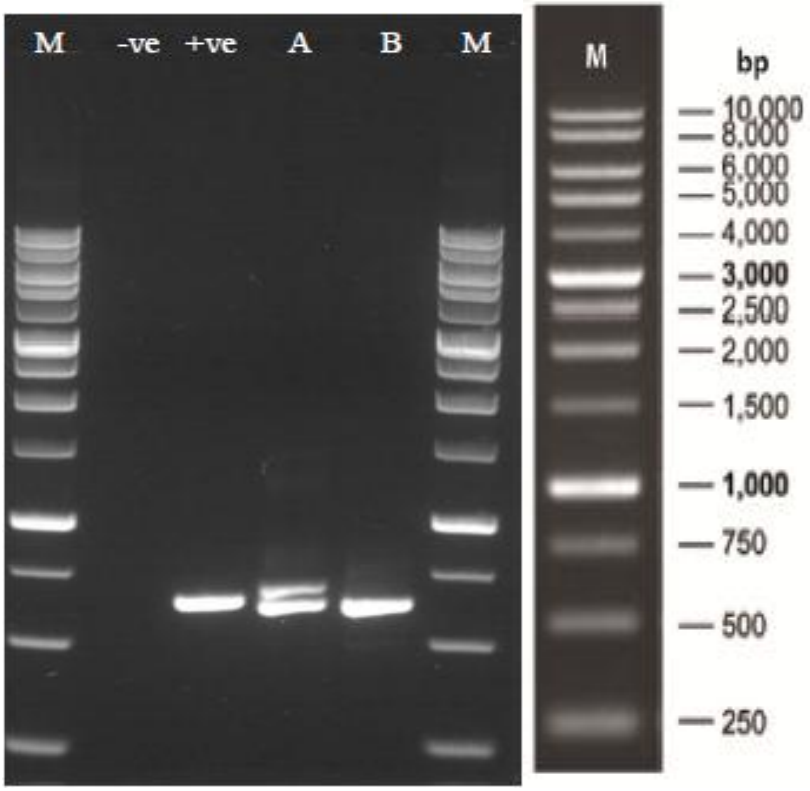


**Fig. 2. Isolation of DNA from RMLC-10 polygalacturonase producing fungal strain.**
**M**: Marker; **-ve**: PCR no-template control with water instead of DNA; **+ve**: Positive control; A and B: isolated DNA sample from RMLC-10.

>B, 599 bp

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TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAAC    60
CTCCCACCCGTGTTTACTGTAACCTTAGTTGCTTCGGCGGGCCCGCCTTTAAGGCCGCCG   120
GGGGGCATCAGCCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG   180
TGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTT   240
CCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTG   300
AATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCC   360
GAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTTCGGG   420
GGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTT   480
GTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAAACAACCATTCTTTCCAG   540
GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA    599
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**Fig. 3. Gene Sequence 18s rDNA region of DNA isolated from polygalacturonase producing fungal strain RMLC-10.**

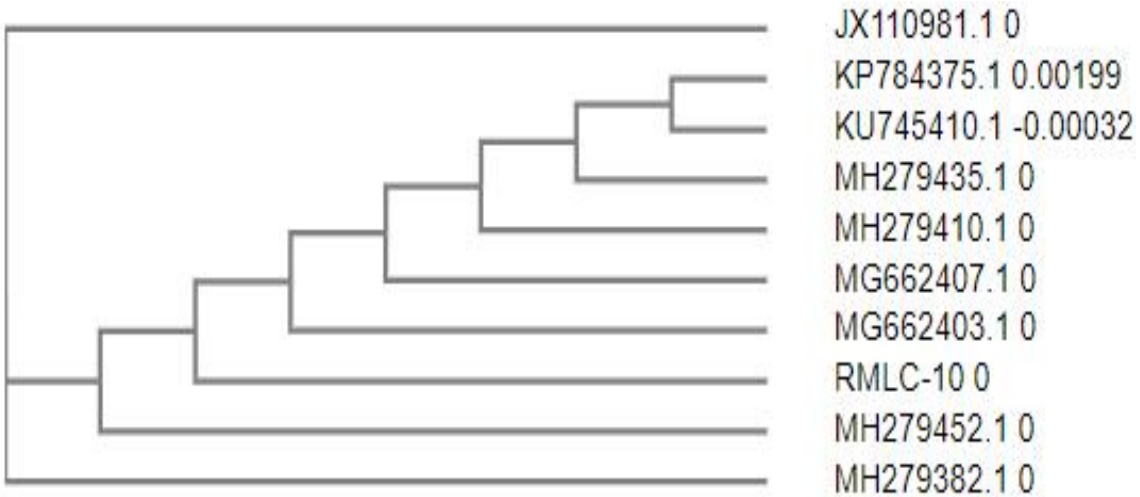


**Fig. 4. Dendogram of polygalacturonase producing fungal strain RMLC-10 (indicated as B-599) with other closely related fungal species.**

**Stress tolerance of polygalacturonase:** Polygalacturonase was assessed for multi stress tolerance i.e. pH and temperature. No decrease in residual enzyme activity was observed at pH values 4.5, and 5.5 as compared to control (Figure 5). However residual activity was decreased gradually at pH values 4 (94%), 3.5 (89%) and 3 (86%), respectively. Similarly, increase in pH value resulted in decreased residual activity at pH value 6 i.e. 96%. Polygalacturonase activity remained stable at 20, 25, 30, 35 and 40°C (Fig.6). Further increase in the temperature resulted in gradual decrease in residual activity with maximum decrease in residual activity was observed at 60°C i.e. 80%. Li *et al.* (2017) reported thermostable (up to 60°C) polygalacturonase with good pH stability (pH 2 to 7) after cloning the genes from *Talaromyces leycettanus* in *Pichia pastoris*. This enzyme is little more efficient compared to our study but cloning adds as an additional step making this enzyme a bit difficult to produce than the one reported in current study. Patidar *et al.* (2017) also produced polygalacturonase from *Aspergillus niger* which was stable up to 55°C in a pH range of 3-7 which is less efficient stress in comparison to our enzyme. Interestingly, multi stress tolerant polygalacturonase production from *Aspergillus tamarii* not found in literature to the best of our knowledge or might be rarely present.

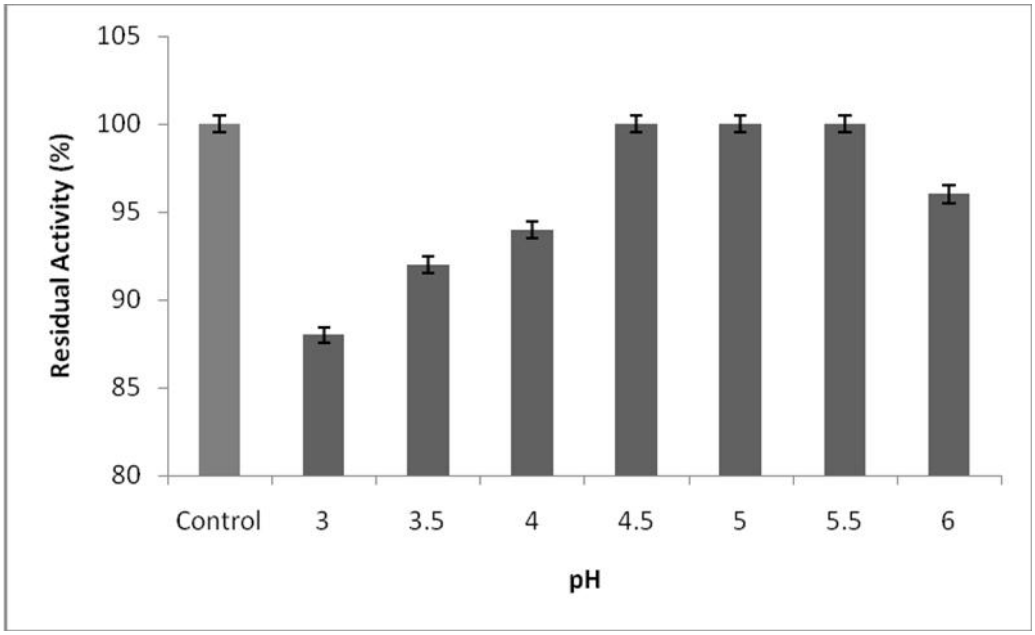


**Fig. 5. pH stability profile of polygalacturonase produced by *Aspergillus tamari* RMLC-10. Error bars indicates the standard deviation (±SD) among the three replicates, which differ significantly at $p < 0.05$.**

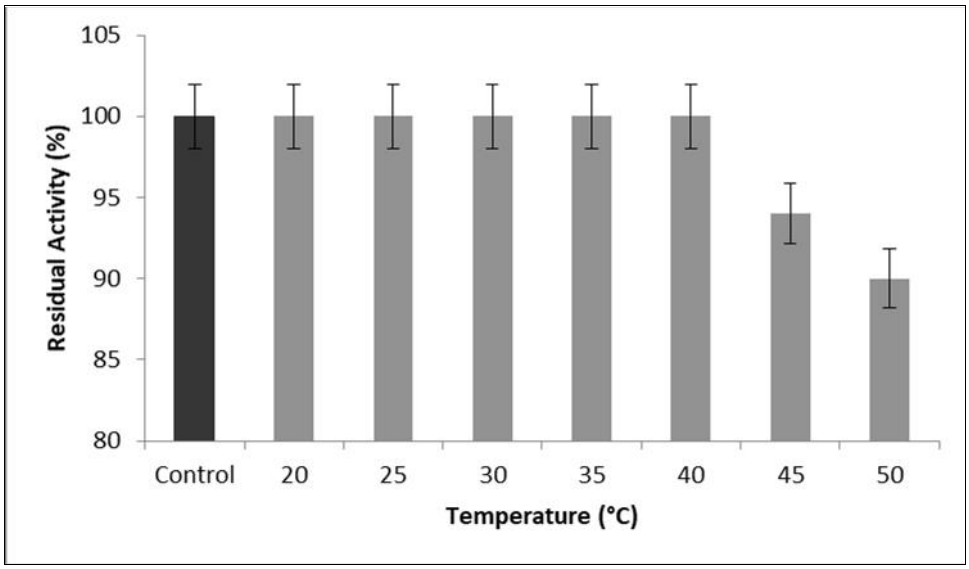


**Fig. 6. Thermo stability profile of polygalacturonase produced by *Aspergillus tamari* RMLC-10. Error bars indicates the standard deviation (±SD) among the three replicates, which differ significantly at $p < 0.05$.**

**Conclusion:** This study put forward Chiku as a potential source for the isolation of pectinolytic fungi as well as good source for the production of polygalacturonase. In addition, to this a new strain *Aspergillus tamarii* was observed as an efficient source for the production of temperature and pH tolerant polygalcturonase with excellent activity using submerged fermentation. All these parameters make this enzyme an industrial workhorse for different applications like juices clarification, paper and pulp processing, feed digestibility improvement and textile quality improvement.

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