World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

ROLE OF POLYGALACTURONASE FROM *ASPERGILLUS FUMIGATUS* ITCC 6915 IN THE IMPROVEMENT OF TEA QUALITY

Akhilesh Thakur[1], Rutika Sehgal[1], Anupam Lakhanpal[1] and Reena Gupta[1]*

[1]Department of Biotechnology, Himachal Pradesh University, Summerhill, Shimla, India.

***Corresponding Author: Dr. Reena Gupta**
Department of Biotechnology, Himachal Pradesh University, Summerhill, Shimla, India.

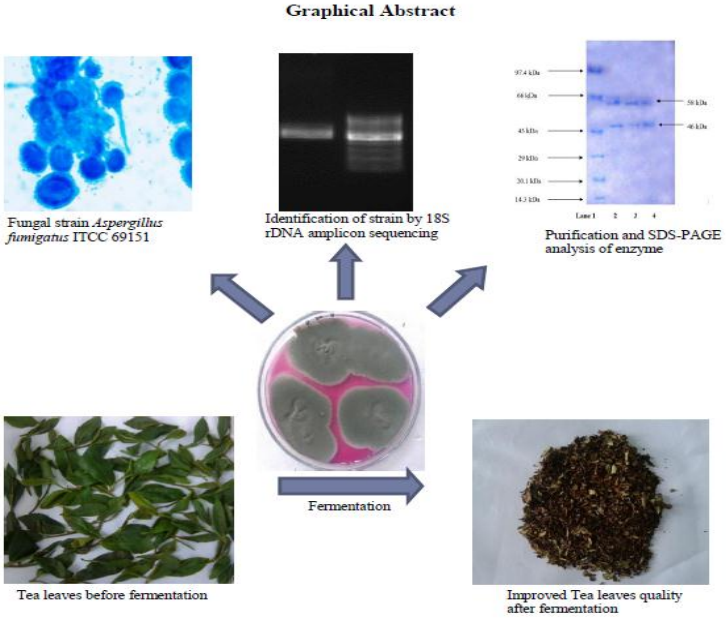
Article Received on 09/06/2022 **Article Revised on 30/06/2022** **Article Accepted on 20/07/2022**

ABSTRACT

Background and Objective: Polygalacturonases are most extensively studied among the family of pectinolytic enzymes. Of late, microbial source of these enzymes has gained importance. In the current study, the effect of purified polygalacturonase on the improvement of tea quality was investigated and different contents namely theaflavin, thearubigin, highly polymerized substances and total liquor color found in tea were quantified in order to check the effect of crude enzyme preparation and purified polygalacturonase on the improvement of tea leaves' fermentation. **Materials and Methods:** Fungal isolate producing polygalacturonase was isolated and characterised by 18S rDNA sequencing. The isolate was identified as *Aspergillus fumigatus* ITCC 6915. The enzyme was purified by DEAE-cellulose anion exchange chromatography. Application of the enzyme on tea leaves was checked for quality improvement by solvent extraction method. **Results and Conclusion:** The polygalacturonase of *Aspergillus fumigatus* was purified to 19.18 fold as a single step purification was achieved by DEAE-cellulose anion-exchange chromatography. SDS-PAGE analysis revealed that the purified polygalacturonase was a heterodimer of 46 kDa and 58 kDa subunits. The corresponding K_m and V_{max} values for the purified polygalacturonase were achieved as 0.19 mg/ml and 17.85 $\mu molml^{-1}min^{-1}$ respectively. There is significant ($p < 0.001$) increase in theaflavin, thearubigin, highly polymerized substances and total liquor color contents of tea when oxidized tea leaves were sprayed with crude and purified polygalacturonase ($5Uml^{-1}$) thereby, indicating the role of these enzymes in the improvement of tea quality. **Conflict of interest:** The authors declare that there is no conflict of interest.

KEYWORDS: Fermentation; Polygalacturonase; Purification; SDS-PAGE; Tea.

Graphical Abstract



1. INTRODUCTION

Highly specific, less aggressive and less toxic nature of enzymatic catalysis makes it a preferable method over other chemical methods.[1] In contrast to most commercial enzyme products, leaf pectinases have a broad pH and temperature range.[2] Polygalacturonases (PGs) are the most widely studied among the family of pectinolytic enzymes. Purification and characterization of the enzymes gives better understanding of their catalytic performance, biochemical properties and structure.[3] There are many earlier studies on the production, purification and characterization of polygalacturonase from *Aspergillus tamarii* and *Mucor circinelloides* that has been reported.[4,5] The constitutive amino acids occupying the active site domain of the enzyme have also been identified.[6] The polygalacturonases of microbial origin find application across various industries and potentiality of purified polygalacturonase in orange juice clarification has recently been demonstrated.[7] In case of tea industry, the application of enzymes from external sources causes full maceration of tea leaves' cells, thereby, resulting in improved fermentation and quality of tea.[8] In a recent study, two-stage submerged enzymatic processing has been used to manufacture high quality instant black tea with desired sensory properties and antioxidant activities using fresh tea leaves. In addition, 75 various volatile compounds viz. alcohols, aldehydes and esters were also identified in the investigation.[9]

The rate of fermentation is determined by extent of contact between the substrate and the enzyme and theaflavin (TF), thearubigin (TR), highly polymerized substances (HPS), total liquor color (TLC), dry matter content and total soluble solids (TSS) of tea produced, have been used to determine enzymatic effect on fermentation of tea leaves.[10] The most prevalent genus for tea fermentation is the fungal genus *Aspergillus* as shown by earlier studies.[11,12] In a recent study, microbial fermentation has considerably improved the characteristics of Chinese sweet tea.[13]

Although richly flavoured hybrid China tea grown in Kangra valley (Himachal Pradesh, India) meets international standards, the higher cost of production remains a challenge to make it competitive in the global market. As far as the manufacturing of black tea is concerned, the cost effectiveness and quality enhancement are two potential factors affecting the economy of the Himalayan state. The current investigation on polygalacturonase from *Aspergillus fumigatus* offers a novel and interesting information of commercial importance, especially, keeping in view the scarcity of information available on PGs produced from filamentous fungi.

2. MATERIALS AND METHODS

Biological Material

Sample collection was done from rotten fruits (apple, guava, orange etc.) along with pectic and fruit waste rich soil from fruit processing areas, juice centres and vegetable market from Shimla and Mandi districts of Himachal Pradesh, India.

Isolation and screening of polygalacturonase producing microorganisms

The soil and fruit samples (1g each) were enriched in 50 ml of sterile enrichment medium (0.1 % $MgSO_4.7H_2O$, 0.1 % trisodium citrate, 0.05 % KCl, 0.1 % citric acid anhydrous, 0.1 % yeast extract, 0.1 % casein acid hydrolysate, 1 % (w/v) pectin, pH 4.0) at 30°C for 24-72 h under shaking conditions (120 rpm). Each of the enriched samples (1 ml) were serially diluted to 10^{-1} to 10^{-10} times and were spread over nutrient agar (NA) plates containing pectin as sole carbon source at pH 4.0. The petri dishes were incubated for 24-96 h at 30°C. The isolated discrete colonies were carefully taken on the basis of shape, size and color and by repeated streaking of single distinct colonies, pure line cultures were obtained. The isolates were screened by staining the agar-pectin plates containing isolated colonies, with aqueous solution of ruthenium red (0.05 % (w/v)) for 1 h and washed with distilled water. Cultures expressing PG activity exhibited a clear zone around the margins of the colony. PG activity of all primary screened isolates was checked by the method of Nelson[14] and Somogyi.[15] Isolate showing maximum PG activity was selected for further studies.

Enzyme assay and determination of protein concentration

Enzyme assay was carried out by the method of Nelson[14] and Somogyi[15]: The assay mixture consisted of 980 µl of freshly prepared substrate and 20 µl of enzyme and incubated at 50°C for 15 min. The reaction was stopped by keeping the reaction mixture in boiling water bath at 100°C for 3 min. To the reaction mixture, 500 µl of alkaline copper tartarate reagent was added and tubes were again kept in boiling water bath for 20 min. In the control, no enzyme was added. The tubes were cooled and 500 µl of arsenomolybdate reagent was added and thoroughly mixed. The reaction mixure was centrifuged at 10000 rpm for 10 min at 4°C. The absorbance was recorded at 620 nm (using Perkin Elmer model Lambda 12 UV/VIS spectrometer) against a blank consisting of 1 ml sodium citrate buffer (pH 5.0, 50 mM), 500 µl alkaline copper tartarate reagent, 500 µl arsenomolybdate reagent. One unit of enzyme activity is described as the amount of enzyme required to obtain 1 µmol of galacturonic acid per ml per min under standard reaction conditions. The protein was quantified by using the method given by Lowry et al.[16]: The samples were appropriately diluted with distilled water to 1.0 ml. 3.0 ml of alkaline reagent was added and left at room temperature for 15 min. 0.3 ml of Folin Ciocalteu's

reagent was added and the tubes were incubated at 37°C for 30 min. The absorbance was measured at 660 nm. The protein concentration was calculated from the BSA standard graph.

Identification of the selected isolate by 18S rDNA sequence determination

Chromosomal DNA isolation, PCR amplification and 18S rDNA sequencing were done to identify the strain on the basis of the sequence homology with the available 18S rDNA sequences by using CLUSTAL W and MEGA 5.1 softwares for multiple sequence alignment and phylogenetic analysis respectively.

Purification of polygalacturonase

The fungal polygalacturonase was purified by ion exchange chromatography with DEAE-anion column**.** A pre swollen column of DEAE-anion exchange matrix (Vt = 25 cm^3, Sigma Chemical Co. U.S.A.) was packed in a sintered glass column. Subsequently, column was equilibrated with 50 mM sodium citrate buffer (pH 4.0). Elution of the absorbed protein was done with the stepwise gradients of 0.3 M NaCl, 0.5 M NaCl, 0.7 M NaCl and 1.0 M NaCl respectively. The determination of relative molecular weight was done by SDS-PAGE.[17]

Solvent Extraction Method

The quality parameters namely theaflavin (TF), thearubigin (TR), highly polymerized substances (HPS) and total liquor color (TLC) of tea were evaluated by solvent extraction method[10] as follows:

TF (%) = 4.313× C
TR (%) = 13.643 (B + D –C)
HPS (% as TR) = 13.643× E
TLC (%) = 10× A

The multiplication factors mentioned in the equations were derived from molar extinction co-efficient of pure compounds and dilution factors. In case of TLC, the dilution factor was 10.

Statistical Analysis

Standard deviation (SD) was calculated from the data obtained for three replicates of the parameters studied and the Student's t-test was applied.

3. RESULTS

Isolation and screening of polygalacturonase producing microorganism

During primary screening, 62 pure colonies were isolated out of which 12 colonies exhibited polygalacturonase activity ranging from 0.17±0.01 to 1.13±0.02 Uml^{-1} (Table 1). The fungal isolate PG-2 isolated from pectin and fruit-waste rich soil indicated maximum polygalacturonase activity (Table 1).

Table 1: Isolates possessing polygalacturonase activity.

S. No.	Isolate code (fungal/ bacterial)	Colony colour/ texture	Colony shape/ growth pattern/sporous or non sporous	PG activity (Uml^{-1})
1	PG 1	Pale yellow/slimy	Round/ slow/ nonsporous	0.50±0.07
2	**PG 2**	**Dark green/ powdery**	**Umbonate/ rapid/ sporous**	**1.13±0.02**
3	PG 4	Black/ powdery	Irregular/ rapid/ sporous	0.96±0.06
4	PG 8	Cream yellow/ slimy	Round/ slow/ nonsporous	0.17±0.01
5	PG 9	Creamish brown/ velvety	Irregular/ slow/ sporous	1.09±0.06
6	PG 12	Olivaceous green/ powdery	Radially furrowed/rapid/ sporous	0.73±0.02
7	PG 13	Pink/ slimy	Round/ moderate/ nosporous	0.18±0.01
8	PG 14	White/ slimy	Round/ slow/ nonsporous	0.33±0.01
9	PG 19	Grey green/ powdery	Irregular/ rapid/ sporous	0.68±0.01
10	PG 23	Dirty white/ velvety	Slightly furrowed/ rapid/ sporous	0.67±0.02
11	PG 36	Cream/ slimy	Irregular/moderate/ nonsporous	0.21±0.02
12	PG 57	White/ powdery	Irregular/ rapid/ sporous	0.61±0.01

Identification of polygalacturonase producing fungal isolate PG 2 and its phylogenetic analysis

The polygalacturonase producing fungal strain PG-2 was selected due to the formation of clear zone around the margins of the colony on plate (Fig 1 (a)) by a previously reported method.[18] It was identified as *Aspergillus fumigatus* ITCC 6915 by Indian Type Culture Collection, New Delhi. The 18S rDNA molecular typing revealed 600 bp amplicon (Fig (b)) by using following forward and a reverse primers:

(ITS1)IF 5' TCCGTAGGTGAACCTGCGG 3'
(ITS4)IR 5' TCCTCCGCTTATTGATATGC 3'

The nucleotide sequence, thus, obtained was contiged by using BioEdit Sequence Aligner and further subjected to BLAST analysis with NCBI data bank. Sequence homology was retrieved which exhibited a close alignment with *Aspergillus* sp. The distance tree of BLAST search showed a close similarity with *Aspergillus fumigatus* IARI-NF2. Boot strap consensus tree was drawn (Fig 1 (c)) by multiple sequences and an alignment with Neighbor-Joining method.[19] The

sequence was deposited in GenBank database with accession number KJ522776. On the basis of morphological characteristics and results of 18S rDNA gene sequence comparison, isolate was identified as *Aspergillus fumigatus* ITCC 6915.

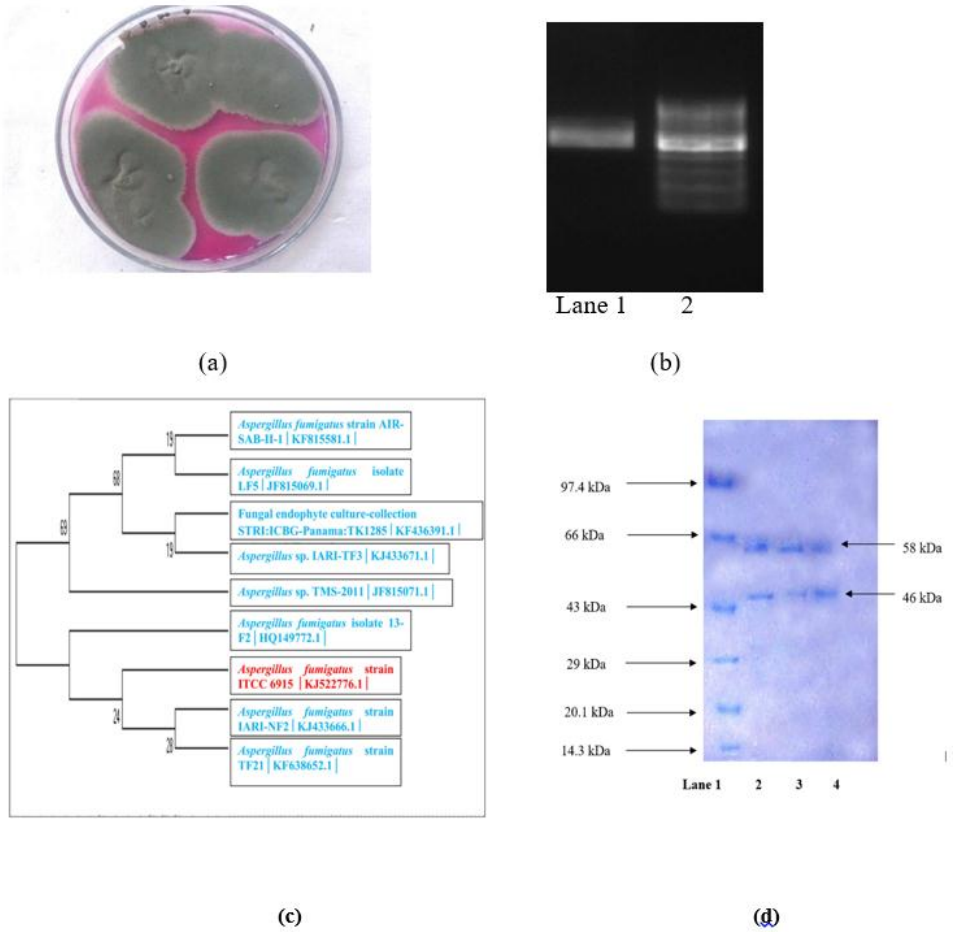


Fig 1(a): Formation of clear zone around colonies during primary screening indicating production of polygalacturonase (b) 18S rDNA amplicon, Lane 1: 18S rDNA amplicon band (600 bp Approx.) Lane 2: DNA marker (c) Phylogenetic dendrogram of *Aspergillus fumigatus* based on 18S rDNA sequence (Bootstrap values were based on 1000 replicates) (d) SDS-PAGE of purified polygalacturonase from *A. fumigatus*, Lane 1: Protein molecular weight marker (medium range), Lane 2, 3 and 4: Fraction showing heterodimer from DEAE cellulose column.

Purification by DEAE-cellulose anion-exchange chromatography

The fractions exhibiting adequate protein content (fraction number 22, 23, 24, 25, 26 and 27) were pooled and checked for polygalacturonase activity and protein content. The polygalacturonase activity of the pooled fraction was observed as 15.98 Uml^{-1} with protein content 0.1 $mgml^{-1}$. The specific activity of the pooled fraction was found as 159.8 Umg^{-1} protein, which led to 19.18-fold purification and an overall yield of 7.52 % (Table 2).

Table 2 Purification of polygalacturonase from *A. fumigatus.*

Purification Step	Vol (ml)	EA (Uml^{-1})	Protein ($mgml^{-1}$)	Total EA (U)	Total protein (mg)	SA (Umg^{-1})	Fold purification	Yield (%)
Crude enzyme	300	8.5	1.02	2550	306	8.33	1	100
Ammonium sulphate ppt.	200	10.04	0.83	2008	166	12.09	1.45	78.74
DEAE-cellulose chromatography	12	15.98	0.1	191.76	1.2	159.8	19.18	7.52

*EA- Enzyme Activity, SA- Specific Activity, Vol- Volume, ppt.-precipitation

SDS-PAGE and estimation of K_m and V_{max}

The study of fungal polygalacturonase with reducing and denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the purified polygalacturonase from *A. fumigatus* was a heterodimer of 46 kDa and 58 kDa subunits as visualized with coomassie brilliant blue R-250 (Fig 1(d)). The corresponding K_m and V_{max} values for the purified polygalacturonase were observed to be 0.19 $mgml^{-1}$ and 17.85 $\mu molml^{-1}min^{-1}$ respectively.

Application of polygalacturonase from *A. fumigatus* ITCC 6915 in tea leaves fermentation

Use of enzymes leads to full maceration of tea leaves which result in finer fermentation and better tea quality.[8] Oxidized tea leaves sprayed with crude and purified polygalacturonase (5 Uml^{-1}) (Fig.2) showed significant ($p < 0.001$) increase in various tea contents viz. TF, TR, HPS and TLC (Table 3). The results revealed that exogenous application of fungal enzymes improved tea quality. The theaflavin content in tea is responsible for the briskness, brightness and good quality of tea.[20] Theaflavin (TF) content increased by 67 % and 55.83 % when treated with crude enzyme and purified enzyme respectively.

Fig. 2 (a): Plucked apical part of green tea leaves (*Camellia sinensis*).

Fig 2 (b): Plucked apical part of green tea leaves (*Camellia sinensis*) after withering.

Fig 2 (c): Plucked apical part of green tea leaves (*Camellia sinensis*) after rolling and fermentation.

Table 3: Effect of polygalacturonase treatment on Theaflavin, Thearubigin, Highly Polymerized Substances and Total Liquor Color contents of tea leaves.

Content(s)	Control	PGase treated tea leaves	
		Crude	Purified
Theaflavin (%)	1.97±0.006	3.23±0.004*	3.07±0.012*
Thearubigin (%)	7.2±0.02	9.93±0.01*	9.72±0.018*
Highly Polymerized Substances (%)	18.01±0.011	23.33±0.01*	22.37±0.013*
Total Liquor Color (%)	2.9±0.02	4.59±0.12*	4.21±0.056*

*Values are mean ±SD of 3 replicates.*p <0.001 as compared to control, Control = tea leaves not treated with any enzyme

Thearubigin (TR) content determines the color, body and taste of the liquor.[20] TR content of tea leaves was 7.2 % initially which was increased by 37.91 % and 35 % when treated with crude and purified enzymes respectively. The purified enzyme from *Mucor circinelloides* improved the TR content by 35.63 % whereas, the commercialized enzyme improved the TR content by 6.09 % in comparison with control.[21] Likewise, crude and purified polygalacturonase increased HPS (highly polymerzied substances) content by 29.53 % and 24.2 %, whereas, TLC (total liquor color) content increased by 58.27 % and 45.17 % over control respectively.

4. DISCUSSION

The fungal isolate PG-2 isolated from pectin and fruit-waste rich soil indicated maximum polygalacturonase activity of 1.13±0.02 Uml^{-1} which was higher than an

earlier report where *Aspergillus awamori* showed 0.05 Uml^{-1} of polygalacturonase activity after fermenting for seven days[22] and was comparable to a report in which the *Lentinus edodes* produced PG activity of 1.5 to 2.2 Uml^{-1} after 40 days.[23] The isolate was identified as *Aspergillus fumigatus* ITCC 6915 as revealed by 18S rDNA gene sequence comparison. After purifying the enzyme its specific activity was calculated to be 159.8 Umg^{-1} protein, which resulted in 19.18-fold purification with an overall yield of 7.52 % which was higher as compared to a report in which polygalacturonase from *Bacillus subtilis* (C4) was purified to 15 fold when subjected to affinity chromatography.[24] This enzyme has the tendency to lose significant amount of protein even after single step purification. Therefore, low yield has been observed. These results are comparable to earlier report[25] where the low yield was observed after single step purification. Similarly, 33 fold purification of a novel polygalacturonase from *Bacillus paralicheniformis* CBS32 was achieved by sepharose CL 6B column chromatography.[26].The purified polygalacturonase from *A. fumigatus* was a heterodimer of 46 kDa and 58 kDa subunits as analysed by SDS-PAGE which was in coordination with work of Meenakshisundaram[27] who also observed two bands of pectinase in the range of 45 kDa and 55 kDa by SDS-PAGE. Recently, the molecular weight of pectinase was found to be 31.6 kDa which was produced by *Aspergillus fumigatus.*[28]

The corresponding K_m and V_{max} values for the purified polygalacturonase obtained were 0.19 $mgml^{-1}$ and 17.85 $\mu molml^{-1}min^{-1}$ respectively which were lower than the K_m and V_{max} values of purified exopolygalacturonase (r-FOC4- PGC2) with 0.48 $mgml^{-1}$ and 95.24 $\mu molml^{-1}min^{-1}$, respectively.[29] Recently, the respective K_m and V_{max} values of the purified polygalacturonase from *Aspergillus flavus* were found to be 0.705 $mgml^{-1}$ and 1.0508 $\mu molmin^{-1}$.[30] Recently, the co-treatment of 1% pectinase and cellulose prior to fermentation was found to be efficacious in the improvement of instant black tea (IBT) quality.[9]

Previously, use of crude and partially purified pectinase from *Aspergillus indicus*, *Aspergillus flavus* and *Aspergillus niveus* reported an increase in TF content by 43.81 %, 62.86 %, 59.05 % and 38.1 %, 40 %, 34.29 % respectively, whereas, 30.48 % of increase was observed in TF content of the commercial enzyme in comparison to control.[10] Also, in a recent study, the purified pectin lyase from *Byssochlamys fulva* utilized for tea leaves fermentation resulted in 22.91 %, 44.79 %, 20.37 % and 23.36 % increase in TF, TR, HPS and TLC contents respectively[31] which are comparable to the results in the current study.

Partially purified enzymes from *Aspergillus niger* and *Mucor circinelloides* have showed a significant ($p < 0.001$) increase in HPS by 13.41 % and 71.87 % respectively in an earlier study, however, the crude enzymes from *Aspergillus niger* and *Mucor circinelloides* could show 1.3 % and 16.4 % increase of HPS as compared to control.[20] In a similar study, the use of crude and purified enzymes from *Aspergillus indicus*, *Aspergillus flavus* and *Aspergillus niveus* showed enhancement in HPS content by 19.93 %, 4.23 %, 37.92 % and 6.64 %, 12.32 %, 10.15 % respectively.[10] The crude and purified enzymes from *Mucor circinelloides* increased the TLC by 1.4 % and 7.23 % as compared to control.[21] Marimuthu et al.[32] reported that use of commercial biopectinase and biocellulase (Biocon Pvt. Ltd.) improved tea quality when used at a concentration of 0.6 % and showed an increase of 24.77 % TF, 21.52 % TR, 21.54 % HPS and 17.49 % TSS (total soluble solids). A mixture of enzyme extract containing *Trichoderma koningii, A. oryzae, A. tamari, A. awamori, A. japonicus* and *A. wentii* was observed to enhance TF by 45%, TR by 48 %, HPS by 33 %, TLC by 19 % and TSS by 3 % and thereby increasing the quality of the final tea product.[33]

Although crude and purified enzyme preparations, both improved the tea quality, the effect was more pronounced in case of crude enzyme preparation. This might be due to the fact that the crude enzyme extract from fungi comprises of mixture of enzymes viz. cellulase, hemicellulase (xylanase), proteinase and pectinase, whereas, only pectinase was there in case of purified enzyme solution. The tea leaf comprises of pectin, cellulose and hemicellulose. The middle lamellae and primary cell walls of higher plants consists of pectin so it is a major and important structural component of plants. These pectic substances are derivatives of polysaccharides which can be hydrolyzed by the cell wall degrading enzymes i.e. pectinase and cellulase. When the crude enzyme extract was sprayed on tea leaves during fermentation, probably all the substances i.e., cellulose, hemicellulose and pectin were hydrolyzed by the complex action of all the enzymes in extract, thereby, resulting in higher maceration of tea leaves and hence, better fermentation rate. In case of purified enzyme, less maceration was attained because only pectin in the tea leaves was hydrolyzed. However, the purification ensured precise action of polygalacturonase on the most abundant polysaccharide pectin by preventing its interaction with other enzymes. Oxidized tea leaves sprayed with crude and purified polygalacturonase led to increase in various tea contents viz. TF, TR, HPS and TLC which, in turn, were responsible for color, taste, briskness, brightness and the quality of liquor. Because more profound effect of crude enzyme on the tea quality than purified enzyme is observed, further research is needed in the area. Consortium may be used in order to further improve quality of tea. Emphasis on the therapeutic value of the tea should be given. The manufacturing of high quality black tea is very important, being the most widely consumed natural beverage in the world. Therefore, to make the process economically viable along with better quality, the enzyme polygalaturonase plays a very

important role. Hence, the current study offers a novel and engrossing information about the commercial significance of polygalacturonases from filamentous fungi on tea leaves fermentation.

5. CONCLUSION

In this investigation, purification of polygalacturonase from *Aspergillus fumigatus* ITCC 6915 was done to study its application in the improvement of tea quality. A significant increase in theaflavin, thearubigin, highly polymerized substances and total liquor color contents of tea was observed and tea leaves were sprayed with crude and purified polygalacturonse. The increase in these contents indicated role of this enzyme in the improvement of tea quality. This study is important to boost the economy of Himalayan state since quality improvement and cost effectiveness are two important factors in this regard. Further, the scarcity of available information on this issue also adds to the novelty and importance of this study.

6. ACKNOWLEDGEMENTS

The authors are thankful to the Chairman, Department of Biotechnology (DBT Sponsored Department, Ministry of Science and Technology, Govt. of India), H.P. University, Shimla, Himachal Pradesh for providing all the necessary facilities. The fellowship granted to Ms. Rutika Sehgal in the form of SRF from Council of Scientific and Industrial Research (CSIR) is also thankfully acknowledged.

7. Conflict of Interest

The authors report no conflicts of interest.

8. Authors Contributions

AT drafted and critically analyzed the entire manuscript, AL performed lab experiments, RS investigated, edited, revised and curated the manuscript and RG investigated and supervised the current work.

8. REFERENCES

1. Lara-Marquez A, Zavala-Paramo MG, Lopez-Romero E, Camacho HC Biotechnological potential of pectinolytic complexes of fungi. Biotechnol Lett., 2011; 33: 859-868. https://doi.org/10.1007/s10529-011-0520-0.
2. Daniell H, Ribeiro T, Lin S, Saha P, McMichael C, Chowdhary R and Agarwal A. Validation of leaf and microbial pectinases: commercial launching of a new platform technology. Plant Biotechnol J., 2019; 17: 1154-1166. https://doi.org/10.1111/pbi.13119.
3. Soroor MAM, Ghazy AM, Mahdy SMS, El-Badery MO, Waffaa GS, El-Khonezy MI Purification and characterization of polygalacturonases produced by *Trichoderma reesei* F418 using lemon peels and rice straw under solid-state fermentation. J Appl Sci Res., 2013; 9: 3184-3198.
4. Munir M, Abdullah R, Haq IU, Kaleem A, Iqtedar M, Ashraf S. Purification, characterization, kinetics and thermodynamic analysis of polygalacturonase from *Aspergillus tamarii* for industrial applications. Rev Mex Ing Quím., 2020; 19: 293-304. https://doi.org/10.24275/rmiq/Bio1753.
5. 5.Thakur A, Pahwa R, Singh S, Gupta R Production, purification and characterization of polygalacturonase from *Mucor circinelloides* ITCC, 2010; 6025. Enzyme Res. https://doi.org/10.4061/2010/170549.
6. Thakur A, Gupta R. Identification of constitutive amino acid residues in the active site domain of polygalacturonase of *Mucor circinelloides* ITCC 6025. LS - Int J Life Sci., 7: 167-171. http://dx.doi.org/10.5958/2319-1198.2018.00023.4.
7. Almulaiky YQ, Albishri AA, Khalil NM, Algamal Y, Aldhahri M, Al-Harbi SA, Al-Talhi HA and Alhadi R. Polygalacturonase by *Aspergillus niger* using seaweed waste under submerged fermentation: Production, purification and characterization. BJSTR, 2020. https://doi.org/10.26717/BJSTR.2020.25.004249.
8. Samaraweera DSA Technology of tea processing. In A. Kathiravetpillai (Ed.), Handbook on tea, 1989; 158-207. Sri Lanka: Tea Research Institute,
9. Kong J, Yang X, Zuo X, Su X, Hu B and Liang, X. High-quality instant black tea manufactured using fresh tea leaves by two-stage submerged enzymatic processing. Food Science and Human Wellness, 2022; 11: 676-685.
10. Angayarkanni J, Planiswamy M, Murugesan S and Swaminathan K. Improvement of tea leaves fermentation with *Aspergillus* sp. Pectinase J Biosci Bioeng, 2002; 94: 299-303. https://doi.org/10.1016/S1389-1723(02)80167-0.
11. Ma Y, Ling TJ, Su XQ, Jiang B, Nian B, Chen LJ, Liu ML, Zhang ZY, Wang DP, Mu YY, Jiao WW, Liu QT, Pan YH and Zhao M. Integrated proteomics and metabolomics analysis of tea leaves fermented by *Aspergillus niger*, *Aspergillus tamarii* and *Aspergillus fumigatus*. Food Chem., 2021; 334: 127560-127571. https://doi.org/10.1016/j.foodchem.2020.127560
12. Gupta R, Lakhanpal A. Production of polygalacturonase from *aspergillus fumigatus* ITCC 6915 using factorial design. CIBTech Journal of Microbiology, 2014; 3(3): 17-25.
13. Huang W, Zhang C, Gu Z, Li C, Fang Z, Zeng Z, Liu, Y. Effect of microbial fermentation on the sensory characteristics and chemical compositions of Chinese sweet tea (*Lithocarpus litseifolius* (Hance) Chun). *Food Bioscience*, 2022; 101567.
14. Nelson N. A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem., 1944; 153: 375-380. https://doi.org/10.1016/s0021-9258(18)71980-7.
15. Somogyi M. Notes on sugar determination. J Biol Chem., 1951; 195: 19-25.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ Protein measurement with the folin phenol reagent. J Biol Chem., 1952; 193: 265-275.

17. Laemmli UK Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970; 227: 680-685. https://doi.org/10.1038/227680a0
18. Banu AR, Devi MK, Gnanaprabhal GR, Pradeep BV and Palaniswamy M. Production and characterization of pectinase enzyme from *Penicillium chrysogenum*. Indian J Sci Technol, 2010; 3: 377-381.
19. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol, 1987; 4: 406- 425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.
20. Hilton PJ, Ellis RT Estimation of the market value of Central African tea by theaflavin analysis. J Sci Food Agric, 1972; 23: 227-232. https://doi.org/10.1002/jsfa.2740230210.
21. Thakur J, Gupta R. Improvement of tea leaves fermentation through pectinases. Acta Microbiologica et Immunologica Hungarica, 2012; 59: 321-334. https://doi.org/10.1556/AMicr.59.2012.3.3.
22. Blandino A, Dravillas K, Cantero D, Pandiella S S and Webb C. Utilization of whole wheat flour for the production of extra cellular pectinases by some fungal strains. Process Biochem, 2001; 37: 497-503. https://doi.org/10.1016/S0032-9592(01)00241-2.
23. Zheng Z, Shetty K. Solid state production of polygalacturonase by *Lentinus edodes* using fruit processing wastes. Process Biochem, 2000; 35: 825-830. http://dx.doi.org/10.1016/S0032-9592(99)00143-0.
24. Kusuma MP, Reddy DSR Purification and characterization of polygalacturonase using isolated *Bacillus subtilis* C4. Res J Microbiol, 2014; 9: 95-103. https://dx.doi.org/10.3923/jm.2014.95.103.
25. Thakur A, Pahwa R, Singh S, Gupta R. Production, purification, and characterization of polygalacturonase from *Mucor circinelloides* ITCC 6025. *Enzyme research*, 2010; *2010*.
26. Rahman MS, Choi YS, Kim YK, Park C, Yoo JC Production of novel polygalacturonase from *Bacillus paralicheniformis* CBS32 and application to depolymerization of ramie fiber. Polymers. https://doi.org/10.3390/polym11091525.
27. Meenakshisundaram V. Optimization of pectinase enzyme production by using sour orange peel as substrate in solid state fermentation. Asian J Biochem Pharmaceutical Res., 2012; 2: 16-26.
28. Okonji RE, Itakorode BO, Ovumedia JO, Adedeji OS Purification and biochemical characterization of pectinase produced by *Aspergillus fumigatus* isolated from soil of decomposing plant materials. J Appl Biol Biotechnol, 2019; 7: 1-8. https://doi.org/10.7324/JABB.2019.70301.
29. Dong Z, Wang Z. Isolation and characterization of an exopolygalacturonase from *Fusarium oxysporum* f.sp. cubense race 1 and race, 2011; 4. BMC Biochem. https://doi.org/10.1186/1471-2091-12-51.
30. Doughari JH, and Onyebarachi GC (2019) Production, purification and characterization of polygalacturonase from *Aspergillus flavus* grown on orange peel. Appl Microbiol Open Access. https://doi.org/10.4172/2471-9315.1000155.
31. Bharti A, Mehta A, Vohra A and Gupta R. Application of purified pectin lyase from *Byssochlamys fulva* in tea leaves fermentation. Int J TechnoChem Res., 2019; 5: 24-29.
32. Marimuthu S, Manivel L, Katreem AA Hydrolytic enzymes on the quality of made tea. J. Plant Crops, 1997; 25: 88-92.
33. Senthilkumar RS, Swaminathan K, Marimuthu S, Rajkumar R. Microbial enzymes for processing of tea leaf. In N. Muraleedharan & Kumar, R.R. (Eds.), Recent Advancement in Plant Crop Research,v2000 (265-269). India: Allied Publishers. https://doi.org/10.1556/amicr.59.2012.3.3.