Microbial production of multienzyme preparation from mosambi peel using *Trichoderma asperellum*

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Received: 8 February 2022 / Revised: 30 March 2022 / Accepted: 11 April 2022 / Published online: 11 May 2022
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**Abstract**

Fruit and vegetable wastes create unhygienic conditions and pose an environmental pollution. The utilization of such wastes as carbon sources for production of enzyme with microbial intervention could be an ecologically friendly and profitable approach, apart from diminishing the waste load. The present investigation focused on the feasibility of using mosambi (*Citrus limetta*) peel as substrate for multienzyme production (pectinase, cellulase and amylase) through microbial intervention. Fifteen fungi were isolated from organic waste and screened in vitro their potential of biodegradation of mosambi peel through enzymes production. The best performing isolate was selected and identified as *Trichoderma asperellum* NG-125 (accession number-MW287256). Conditions viz. temperature, pH, incubation time and nutrient addition were optimized for efficient enzymes production. The maximum enzyme activity (U ml⁻¹ min⁻¹) of pectinase (595.7 ± 2.47), cellulase (497.3 ± 2.06) and amylase (440.9 ± 1.44) were observed at pH 5.5, incubation temperature of 30 °C after 10 days of fermentation. Moreover, macronutrients such as ammonium sulfate (0.1%) and potassium-di-hydrogen-ortho-phosphate (0.01%) further also enhanced the production of enzymes. The SDS-PAGE analysis of purified pectinase, cellulase and amylase using showed molecular mass of 43, 66 and 33 kDa, respectively. The enzyme retention activity (ERA) of aforementioned enzymes was also tested with four different natural fiber matrices viz., bagasse, rice husk, paddy straw and wheat straw. Among these, the maximum ERA was observed on bagasse matrix (pectinase—56.35%, cellulose—77.68% and amylase 59.54%). Enzymatic juice clarification yield obtained with test enzyme was 75.8%, as compared to 80.5% of commercial enzyme. The result indicates that *T. asperellum* may be exploited as multifaceted biocatalysis.

**Keywords** Amylase · Cellulase · *Citrus limetta* · Pectinase · *Trichoderma asperellum* · Juice clarification

**Introduction**

Enzymes generally work as biological catalysts that accelerate rate of particular biochemical reactions. Food enzymes have big shares in International food markets. According

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to BCC research Report Aug, 2021 (https://www.bccresearch.com/market-research/biotechnology/global-marke
 for-enzymes-in-industrial-applications.html 2021. Aug 2021| BIO030L|), the global market for food enzymes was
 worth from $6.4 billion in 2021 to $8.7 billion by 2026, at compound annual growth rate (CAGR) of 6.3% for the
 period of 2021–2026. Though, enzymes are obtained from plant, animal or microbial sources, microbial enzymes are
 most preferred as they have high stability, easy production
 with high yields and better economic feasibility (Sharma
 et al. 2017). Plant enzymes have very high utility in food,
 paper, drug, textile, leather and dye industries. Pectinas
 have wide applications in the production of clarified juices
 and wines. Cellulases are used in textile, paper, detergent,
 and animal feed industries. The costs of production of food
 enzymes are generally very high because of high cost of
 raw materials (Raveendran et al. 2018). However, utilization
 of fruit and vegetable wastes as carbon source for growing
 microorganisms may prove to be a better economic alterna-
 tive for enzyme production (Sharma et al. 2017).

 The processing of fruits results in the generation of big
 quantum of waste in the form of peel, pomace, stones and
 seeds. Such wastes are generally rich in bio-constituents
 such as fiber, pectin, cellulose, starch, phenolics, pigments
 and other useful materials (Nagy et al. 1977; Aravantinos-
 Zafiris et al. 1994). Biological conversions of such wastes
 into enzymes through solid-state fermentation have been
 worked out extensively (Raveendran et al. 2018). There are
 reports of fermentative production of multienzyme using
 fruit peels of mango, pomegranate, apple, banana, orange,
 etc. (Li et al. 2015; Sagar et al. 2018).

 Citrus is one of important fruit crops of India with a
 production of 13.4 million metric tons during 2018–19. Its
 processing for juice generates peel (20–25%) as waste which
 is a rich source of a number of value-added compounds or
 phytochemicals, such as flavonoids, carotenoids, pectin, sug-
 ars, limonoids, hesperidin, naringin, and essential oils are
 extracted from citrus waste/by-products. Ruiz et al. (2012)
 produced pectinase from lemon peel pomace using solid-
 state fermentation technique. Mosambi peel is a rich source
 of pectin (25–30%), cellulose (13.6%) hemicellulose (10%)
 and starch (7.1%) (Ververis et al. 2007; Maria et al. 2011).
 Shariq and Sohail (2019) reported use of Citrus limetta peels
 as substrate for the production of multienzyme preparation
 using yeast consortium. Among the fungi, Trichoderma is a
 very potent fungus capable to work on various kinds of sub-
 strates. In general, pectinases, cellulases and amylases are
 involved in the degradation of pectin, cellulose and starch
 rich substrates (fruit peels) in which Trichoderma asperell-
 um play a crucial role by producing these enzymes (Dai
 et al. 2020). Citrus peel is reported to be a one of the poten-
 tial substrates for production of pectinase solely. Till date
 there are reports available on production of single enzyme
 but not on fungal mediated production of multienzyme pre-
 paration, such as pectinases, cellulases, and amylases using
 mosambi peel as a substrate by a single fungal. This mul-
 tienzyme preparation will be useful for juice clarification/
 extraction from substrates rich in pectin, fiber and starch and
 use of individual enzymes can be avoided. The present study
 reports the feasibility of using mosambi peel as substrate for
 multienzyme preparation including pectinase, cellulase and
 amylase using fungus T. asperellum.

 Materials and methods

 The experiments were carried out at Microbiology lab, Divi-
 sion of Post Harvest Management, ICAR-Central Institute
 for Subtropical Horticulture, Lucknow, India during the
 period 2018–2021. The chemicals and media were pur-
 chased from Merck, Sigma and Himedia.

 Isolation of fungal culture

 The carbohydrate utilization broth (NH4)2SO4 (0.2%),
 K2HPO4 (0.4%) Na2HPO4 (0.6%), FeSO4 (0.1%) MgSO4
 (2%), CaCl2 (0.1%), MnSO4 (0.001%) ZnSO4 (0.007%),
 CuSO4 (0.005%) H2BO3 (0.001%) and MoO3 (0.001%) at
 pH 7.2 ± 0.2 supplemented with pectin, carboxy methyl cel-
 lulose (CMC) and starch (1% w/v) were used for isolation
 and sub-culturing of fungal isolates. Fungi were isolated
 from degrading organic substrate by serial dilution and pour
 plating isolation techniques. Subsequently, the isolates were
 sub-cultured into their respective selective growth media
 until pure cultures were isolated (Sudeep et al. 2020).

 Primary screening of fungal isolates

 Primary screening of fungal isolates for pectinase, cellulase
 and amylase production was done on the basis of clear halo
 zone formation on pectin/CMC/starch utilization agar plate,
 respectively, by following the protocol of Sazci et al. 1986;
 Mengistu and Pagadala (2017).

 Secondary screening of fungal isolates

 The substrate (pectin/CMC/starch) utilization broth was
 inoculated with primary screened fungal isolates for pecti-
 nase, cellulase and amylase production at pH 7.0 and tem-
 perature 30 °C for 10 days (Wood and Bhat 1988). Culture
 filtrate was tested for pectinase, cellulase and amylase activi-
 ties. Twenty ml of culture filtrate was centrifuged (10,000×g
 for 10 min at 4 °C). Afterwards, 5 ml of the cell-free super-
 natant obtained was filtered through syringe filter (0.22 µm)
 and was used for enzyme assay as per protocol described by
 Miller (1959). One volume of supernatant was precipitated
with 4 volumes of cold acetone, and incubated at –20 °C for 20 min. The samples were centrifuged (10,000×g for 10 min at 4 °C). Thereafter, supernatant was discarded and pellet was dissolved in the freshly prepared 0.2 M acetate buffer and assayed for pectinase, cellulase and amylase activities (Horwitz and Latimer 2005).

**Production optimization under SSF from mosambi peels using selected fungal isolate**

Fermentation conditions viz. temperature (20, 30 and 40 °C) and pH (4.5, 5.5, 6.5 and 7.5) were optimized using the protocol of AOAC (2005). Mosambi peel (C. limetta), collected from wholesale fruit market, Dubagga, Lucknow, India, was properly washed with water, chopped and air dried. 100 g of chopped peel pieces (approx. 2 mm size) mixed with water (1:1, w/v) in 500 ml capacity Erlenmeyer flask, autoclaved and inoculated with 1 ml of actively growing culture of selected fungal isolate (≈ 1.0 × 10⁷ spore ml⁻¹) and incubated at the tested temperature. Samples were withdrawn at different time intervals; the culture filtrate was used for enzyme assay (as described above in enzyme assay section). Samples were precipitated with acetone and analyzed for extracellular enzyme viz. pectinase, cellulase and amylase activity (as described above in enzyme assay section). To study the effect of nutrient addition on production of pectinase, cellulase and amylase activity, ammonium sulfate (0.1%; as source of nitrogen), potassium dihydrogen orthophosphate (0.01%; as source of potassium and phosphorus) were mixed with chopped mosambi peel, autoclaved, inoculated by T. asperellum NG-125 (≈ 1.0 × 10⁷ spore ml⁻¹) and incubated at 30 °C for 5 days.

**Enzyme purification**

Pure substrates (1% pectin/ CMC/ starch in carbohydrate utilization broth) inoculated with selected fungal isolate was incubated at 30 °C for ten days. The culture filtrate was precipitated with cold acetone, pellet was dissolved in acetate buffer and passed through gel filtration chromatography column (1.0 x 40 cm) packed with Sephadex G-100 matrix pre-equilibrated with 0.2 M acetate buffer (pH 5.5). Two ml fractions were collected in Eppendorf tubes at the flow rate of 20 ml/h.

**Enzyme assay and characterization**

The pH and temperature, at which the fungal isolates with reference to enzymes (pectinase, cellulase and amylase) production performed best, were taken for enzyme assay. The enzyme reaction mixture contained 0.4 ml of substrate (Pectin 0.5%/CMC—1%/ Starch—1% dissolved in 0.2 M of acetate buffer pH—5.5), 0.1 ml of crude enzymes and 0.5 ml of distilled water. The tubes were incubated at 30 °C for 1 h in water bath. One ml of DNS (Dinitrosalicylic acid) reagent solution was added to stop the enzyme reaction. Subsequently, the reaction tubes were placed in water bath at 100 °C for 10 min. Standards of D-galacturonic acid and glucose were taken in the range of 1 mM–10 mM for pectinase and cellulase/amylase, respectively. The optical density was recorded at 550 nm using spectrophotometer (double beam UV–VIS). Enzyme activity was expressed as the amount of enzyme that liberated 1 µmol of reducing sugar per ml per min. Enzyme activity was calculated as per the following equation:

\[
\text{Enzyme activity}\;\text{ml}^{-1}\text{min}^{-1} = \frac{\text{Absorbance of standard at 550 nm} \times \text{Dilution} \times \text{Enzyme absorbance}}{\text{Time of incubation}}.
\]

The heat stability of enzyme was determined by subjecting it to 65 °C for one hour. The relationship between substrate concentration and enzyme production was also worked out. Enzymes were assayed in reaction buffer (pH 5.5) with substrate concentrations between 0.25–5 mg/ml for pectin and 0.5–10 mg/ml for CMC and srch. The values of \( K_m \) (Michaelis constant) and \( V_{\text{max}} \) (maximum velocity) were calculated from Michaelis–Menten saturation curve.

To determine the molecular size of the pectinase, cellulase and amylase produced in respective pure substrate, enzyme precipitate was subjected to electrophoresis in 10% native polyacrylamide gel and 12% denaturing sodium dodecyl sulfate–polyacrylamide gel in discontinuous buffer by following the methods described by Laemmli (1970). Coomassie brilliant blue R-250 staining was used to visualize protein bands after electrophoresis.

A comparative study of enzymatic mango juice extraction using multienzymes produced by selected isolate against a commercial pectinase was conducted. The diluted mango pulp was incubated at 35 °C for 180 min with 0.5 ml of crude enzyme extract/ commercial pectinase. The content was filtered through Whatman No. 1 filter paper and quantified.

**Morphological identification by scanning electron microscope (SEM)**

The morphological identification of selected fungus isolate at microscopic level was performed with SEM (Babu et al. 2018; Azam et al. 2020) and compound microscopic (Liaquat et al. 2020). For SEM image, the isolated fugal sample was fixed with glutaraldehyde (2.5%) in phosphate buffer (10 mM, pH 7.4) for 4 h. The fixed sample was rinsed twice with deionized water and dehydrated with gradient
concentrations of ethanol (10, 20, 40, 60, 80, 90 and 100%) for 10 min each. Samples were dried at room temperature for 3 h followed by sputter-coated and visualized at 2000×.

**Molecular identification and phylogenetic analysis of selected fungal isolate**

The maximum enzyme-producing fungal isolate was subjected to molecular characterization on the basis of Internal Transcribe Spacer (ITS) rDNA sequencing technique. The genomic DNA of selected fungus was isolated by following protocols described by Thakur et al. (2015). The integrity of extracted DNA was checked on 1.2% agarose gel by electrophoresis in TAE buffer and further visualization under UV in uvitec (Bangalore Genei, India). Afterwards, the yield and quality of genomic DNA was estimated using Nanodrop spectrophotometer (Nanodrop ND 1000).

For amplification of ITS rDNA sequences, the universal primers of Internal Transcribe Spacer 1 (ITS1-5′-TCC GTA GGT GAA CCT GCG G-3′) and Internal Transcribe Spacer 4 (ITS4 5′-TCC TCC GCT TAT TGA TAT GC-3′) were used. Approximately, 25 ng of genomic DNA and 5 pmol of aforementioned primers were used for amplification purpose. The amplification of ribosomal gene sequence was performed using Veriti® 96 well Thermal Cycler (Model No. 9902) programmed as 95 °C for 5 min; 32 cycle at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min; and 4 °C for infinite period. The amplified PCR products were purified using PCR Cleanup Kit (Mol Bio, Himedia) by following the instructions mentioned by manufacturers. The PCR product obtained was sequenced by ABI 3730 xl Genetic Analyzer using sequencing BDT v3.1 Cycle sequencing kit and primer. The resultant sequence, thus, obtained was analyzed by nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/).

The phylogenetic analysis was performed using MEGA version 5.2 software. After the BLAST analysis, the FASTA form of sequence of most similar organisms along with neighbor sequences of same genera from the NCBI database were download. Apart from this, one analog sequence of other fungal genera of same family was also taken for out-group purpose. The downloaded sequences were aligned by inbuilt ClustalW alignment tool of MEGA version 5.2 software. The alignment file, thus, obtained was trimmed on both sides and get equal length of sequences. The phylogenetic tree was constructed from trimmed align sequence using MEGA version 5.2 software (Tamura et al. 2011). The evolutionary history was inferred using the Maximum Likelihood method and General Time Reversible model with bootstrap replications of 1000 (Nei and Kumar 2000). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Further, the ITS sequences of 5.8S rDNA of isolated fungus submitted to GenBank.

| S.No | Fungal isolate | Clear zone size (mm) for pectinase | Clear zone size (mm) for cellulase | Clear zone size (mm) for amylase |
|------|----------------|-----------------------------------|----------------------------------|---------------------------------|
| 1    | F-1            | 85                                | 72                               | 70                              |
| 2    | F-2            | 57                                | 50                               | 15                              |
| 3    | F-3            | 65                                | 65                               | 47                              |
| 4    | F-4            | 85                                | 68                               | 55                              |
| 5    | F-5            | 30                                | 20                               | 05                              |
| 6    | F-6            | 15                                | 65                               | 19                              |
| 7    | F-7            | 52                                | 65                               | 32                              |
| 8    | F-8            | 45                                | 30                               | 25                              |
| 9    | F-9            | 57                                | 25                               | 19                              |
| 10   | F-10           | 47                                | 52                               | 23                              |
| 11   | F-11           | 22                                | 55                               | 30                              |
| 12   | F-12           | 25                                | 65                               | 10                              |
| 13   | F-13           | 80                                | 65                               | 48                              |
| 14   | F-14           | 85                                | 68                               | 38                              |
| 15   | F-15           | 55                                | 50                               | 10                              |
Selection of suitable natural substrate on enzyme immobilization

Fiber was extracted from bagasse, rice husk, paddy straw and wheat straw by boiling with water, treating with 1 N-HCl followed by 1 N-NaOH treatment. Thereafter, it was washed with water, oven-dried at 60 °C and sieved (60–150 mesh) (Ranganna 2001). The extracted fiber was autoclaved followed by the enzyme was added and incubated for 3 h. The un-immobilized enzyme was washed away with acetate buffer, while activity was tested in enzyme immobilized on fiber.

Statistical analysis

All the experiments were carried out using completely randomized design in triplicate, repeated twice for reproducibility. The analysis of experimental data with two-way analysis of variance (ANOVA) was conducted followed by Fisher’s multiple comparison test at $p < 0.05$. The least significant difference (LSD) test was used to determine whether there was significant difference among the samples (Gomez and Gomez 1984).

![Graph A](image1)

**Fig. 1** Secondary screening of fungal isolates for enzyme production

A Pure substrate  B Mosambi Peel
| Lane 1 | Lane 2 |
|-------|-------|
| ![Image](108x95 to 488x733) | ![Image](108x95 to 488x733) |

| B |ARCHIVES OF MICROBIOLOGY (2022) 204:313 |ARCHIVES OF MICROBIOLOGY (2022) 204:313 |
|---|---|---|
| 1 | 1 | 1 |
| 3 | 3 | 3 |
| 313 | 313 | 313 |
| Page 6 of 12 | Page 6 of 12 | Page 6 of 12 |

| Trichoderma briaiae str. CBS 130876(MH695057) | Trichoderma asymmetricum str. YMF 1.04618(MK795991) |
|---|---|
| Trichoderma scoparium str. YMF 1.04616(MK795992) | Trichoderma viridans str. DAGM 234234(EU280119) |
| Trichoderma inconsipuum str. YMF 1.04623(MK795993) | Trichoderma parvulide str. YMF 1.04622(MK775514) |
| Trichoderma caribbaeum str. CBS 119039(MH863035) | Trichoderma atrovirens str. CBS 142.95(MH862595) |
| Trichoderma uncinatum str. YMF 1.04622(MK795994) | Trichoderma speciosum(MH113929) |
| Trichoderma caeruleotis str. CBS 130011(MH865024) | Trichoderma hamatum str. DAGM 167057(EU280124) |
| Trichoderma pubescens str. DAGM 165102(EU280121) | Trichoderma asperellum isolate HBRI TATP(MH727373) |
| Trichoderma sp. CC-2018 str. S7(K3344907) | Trichoderma asperellum isolate CTs6(MH744738) |
| Trichoderma asperellum isolate MH872494 | Trichoderma asperellum strain W2-121(MH872494) |
| Trichoderma asperellum isolate T2(MH210552) | Trichoderma asperellum str. N0125(MW287256) |
| Trichoderma asperellum isolate N0125(MW287256) | Trichoderma asperellum str. IFPR-88(MK841020) |
| Trichoderma rhodendri str. CBS 116286(MH863057) | Trichoderma rhoderadini str. CBS 116286(MH863057) |
| Trichoderma applanatum isolate 7752(KJ783204) | Trichoderma chaetophora CBS 116286(MH863055) |
| Trichoderma phellincola CBS 116286(MH863055) | Trichoderma turnerberse CBS 122554(MH863221) |
| Trichoderma pseudokomigal str. DAGM 167878(EU280097) | Trichoderma pseudokomigal str. DAGM 167878(EU280097) |
| Trichoderma sephalocapsul str. CBS 130628(MH865165) | Trichoderma longibrachiatus CEN1481(MK714482) |
| Trichoderma koningii str. IAM 12534(LP72218) | Trichoderma aquatica isolate YMF1(MH983058) |
| Trichoderma aquatica isolate YMF1(34625)MH830358 | Trichoderma reesei str. JCM 22676LC226864 |
| Trichoderma reesei str. JCM 22676LC226864 | Trichoderma panarcese str. ATCC MYA-4777(MH882311) |
| Trichoderma glycollatum str. CBS 130090(MH865022) | Trichoderma glycollatum str. CBS 130090(MH865022) |
| Trichoderma altiorum isolate LESF236(HS606035) | Trichoderma altiorum isolate LESF236(HS606035) |
| Trichoderma lanterngalactae isolate 8735(KJ783295) | Trichoderma lanterngalactae isolate 8735(KJ783295) |
| Trichoderma aureoviridis CBS 245.63(HM865276) | Trichoderma aureoviridis CBS 245.63(HM865276) |
| Trichoderma brillianum CBS 253.62(MH858149) | Trichoderma brillanum CBS 253.62(MH858149) |
| Trichoderma sp. CBS 311.50(MH855640) | Trichoderma sp. CBS 311.50(MH855640) |
| Trichoderma parapiuliferum str. GJS 91-60(AY241687) | Trichoderma parapiuliferum str. GJS 91-60(AY241687) |
| Trichoderma polyphilae str. CBS 820.68(MH859230) | Trichoderma polyphilae str. CBS 820.68(MH859230) |
| Trichoderma minitiae CBS 167069(EU965634) | Trichoderma minitiae CBS 167069(EU965634) |
| Trichoderma iroraense CBS 125734(MH863358) | Trichoderma iroraense CBS 125734(MH863358) |
| Trichoderma barbatum CBS 125733(MH863357) | Trichoderma barbatum CBS 125733(MH863357) |
| Trichoderma chlororoseum CBS 114231(MH862998) | Trichoderma chlororoseum CBS 114231(MH862998) |
| Trichoderma spirale str. DAGM 169374(EU280090) | Trichoderma spirale str. DAGM 169374(EU280090) |
| Trichoderma longiquae str. DAGM 177227(AY885530) | Trichoderma longiquae str. DAGM 177227(AY885530) |
| Trichoderma cassiae str. DAGM 164918(EU280097) | Trichoderma cassiae str. DAGM 164918(EU280097) |
| Trichoderma amazonicum CBS 126996(MH864298) | Trichoderma amazonicum CBS 126996(MH864298) |
| Trichoderma pleurotum str. CBS 124367(MH142363) | Trichoderma pleurotum str. CBS 124367(MH142363) |
| Trichoderma azetovoi str. CEN1403(MK714488) | Trichoderma azetovoi str. CEN1403(MK714488) |
| Trichoderma zelharzianum(MH113932) | Trichoderma zelharzianum(MH113932) |
| Trichoderma anamaliorum(MH113931) | Trichoderma anamaliorum(MH113931) |
| Trichoderma asiaticum(MH113930) | Trichoderma asiaticum(MH113930) |
Results and discussion

In total, 15 fungal isolates were obtained from biodegrading organic substrates. For identification purpose, the isolates were designated by prefix ‘F’, followed by their isolate numbers. The results indicated that five isolates were better able to produce enzymes as estimated by clear zone diameter (Table 1). These were subjected to secondary screening and the results indicated that in fungal isolate F-1 (Fig. 1A), maximum production (in terms of U ml⁻¹ min⁻¹) of pectinase (543.8), cellulase (356.3) and amylase (404.3) was observed in mosambi peel (Fig. 1B).

The identification of selected fungi carried by both molecular and microscopical way. A single band of high-molecular-weight DNA was observed in Fig. 2A. The molecular identification through homology searching and BLAST analysis revealed that the isolated strains named NG-125 belonged to Trichoderma genera. The aforesaid strains exhibited 99.34% similarity with T. asperellum strain IIPR-88 (MK841020). The Phylogenetic position of T. asperellum strain NG-125 with other related organisms have been depicted in Fig. 2B. Further, 5.8S internal transcribe spacer (ITS) of A T. asperellum strain NG-125 submitted to NCBI GenBank under the accession no. MW287256. The scanning electron microscopic image of T. asperellum isolate is shown in Fig. 3A and compound microscopic (Fig. 3B).

Bech et al. (2014) observed that T. asperellum secretes different types of extracellular hydrolytic enzymes used in degradation of plant cell wall. The production of carbohydrate-active enzymes by T. asperellum grown on different substrates illustrated that the different substrates induce different fungal enzymes response depending on structure and composition (Bech et al. 2015). Elsababty et al. (2015) reported that T. asperellum, along with some other fungi tested, has the ability to produce pectinase activities and caused disintegration of the pectin medium. Optimization of physical parameters viz. temperature, pH, type and concentration of substrate and medium components result in

Fig. 2 A 1.2% Agarose gel showing 650 bp amplicon of ITS region of rDNA, Lane 1: 100 bp DNA Ladder; Lane 2: 650 bp amplicon (ITS region). B Phylogenetic tree constructed from the internal transcribe spacer 1 of 5.8S ribosomal RNA of strains NG-125 and related organisms constructed using Maximum Likelihood algorithm from an alignment of 734 nucleotides. Accession numbers of corresponding sequences are given in parentheses, and scale bar represents 1 base substitution per 50 nucleotide positions. The bootstrap probabilities calculated from 1000 replications. Arthoniomycetes sp. CC-2021a isolate f15A was taken as an out-group

Fig. 3 A SEM image of Trichoderma asperellum 2000x. B Compound microscopic image of Trichoderma asperellum 40x
Fig. 4 Effect of fermentation period on enzyme production using mosambi peel as substrate by *Trichoderma asperellum*.

Fig. 5 Effect of incubation temperature on pectinase, cellulase and amylase using mosambi peel as substrate by *Trichoderma asperellum*.

Fig. 6 Optimization of fermentation pH for enzyme production by *Trichoderma asperellum* using mosambi peel as substrate.
many fold increase in activity compared to unoptimized condition Sreena and Sebastian (2018). Highest production of pectinase, cellulase and amylase by *T. asperellum* on mosambi peel substrate were observed after 10 days of fermentation (Fig. 4). Further increase in incubation period reduced the enzymes production. It might be due to the depletion of nutrients in the medium with lapse in time, resulting in the inactivation of secreting machinery of the enzymes. The microbial growth rate and enzyme secretion are greatly affected by incubation temperature Singh and Mandal (2012). An incubation temperature of 30 °C was found to be optimum for enzyme production (Fig. 5). The level of metabolite synthesis is greatly affected by initial pH of the fermentation medium. Maximum enzyme production was observed at pH 5.5 (Fig. 6). According to Gautam et al. (2011), 45 °C temperature and 6.5 pH were most suitable for production of cellulase by *Trichoderma* sp. Nabi et al. (2003) reported maximum production of pectinase by *Trichoderma harzianum* in solid-state fermentation of citrus peel at pH 7 and 40 °C temperature. Nathan et al. (2017) observed optimum enzyme recovery period between 5 and 9th days of incubation. Levin et al. (2010) reported that addition of vitamins, amino acids and complex nitrogen sources had stimulatory effect on ligninolytic enzyme production by white-rot fungi. Juwon and Emmanuel (2012) observed that *T. viride* BITRS-1001 produced high quantities of amylase and polygalacturonase enzymes in minimal medium, modified with some carbon and nitrogen sources concomitantly. Similarly, Kumar et al. (2011) reported ammonium sulfate to be the best inducer of pectinase enzyme in *Aspergillus niger* MCIM 548 using SSF process. Our results indicated that there was multiple-fold increase in enzyme (pectinase—3.78; cellulase—11.78; amylase—9.39) production (Fig. 7).

![Fig. 7](image)

**Table 2** Km and Vmax values of pectinase, cellulase and amylase produced by *Trichoderma asperellum*

| Enzyme   | $K_m$ (mg/ml) | $V_{max}$ (U ml$^{-1}$ min$^{-1}$) |
|----------|---------------|----------------------------------|
| Pectinase| 0.5           | 311.1                            |
| Cellulase| 2             | 114.9                            |
| Amylase  | 1             | 134.8                            |

![Fig. 8](image)

**Fig. 8** SDS-PAGE (12%) profile of purified pectinase cellulase and amylase. Abbreviation: Lane-M protein marker, L-1 amylase, L-2 cellulase, L-3 pectinase
Considering enzyme activity at 35 °C as 100%, increased temperature resulted in negative effect on the enzyme activity as indicated by 41.5, 5.1 and 14.2% loss in activity of pectinase, cellulase and amylase, respectively, at 65 °C. The decrease in the heat stability might be due to enzyme denaturation at higher temperatures. The results are in accordance with Banu et al. (2010).

The Km and Vmax values observed were 0.5 & 311.1, 2.0 & 114.9 and 1.0 & 134.8 for pectinase, cellulase and amylase, respectively (Table 2). The activity of the pectinase increased from 60.2 to 311.1 (U ml⁻¹ min⁻¹) with increasing in the pectin concentration from 0.25 to 4.0 mg ml⁻¹. It happens when residual substrate reaches too low level to continue further reaction. Similar trend was found with amylase and cellulase. Arotupin et al. (2008) also found that activity of the enzyme produced by Aspergillus repens increased with increase in substrate concentration and reached a maximum of 4 mg/m. Banu et al. (2010) reported Km and Vmax values of 1.0 mg ml and 85 U mg⁻¹ of protein, respectively, for the pectinase produced from Penicillium chrysogenum.

Characterization of the purified pectinase, cellulase and amylase by SDS-PAGE revealed three bands corresponding to molecular mass of 43, 66 and 33 kDa respectively (Fig. 8). Literature suggests that same enzyme has different molecular weights from different microbes. Polygalacturonase purified from T. harzianum, grown on citrus peel, had molecular mass of 29 kDa (Mohamed et al. 2009). Cellulase obtained by growing Trichoderma viride on Whatman filter paper had molecular mass of 87 kDa. Alpha-amylase of Trichoderma pseudokoningii had been reported to be 30 kDa Abdulaal (2018).

Recently, a number of research studies revealed that immobilized enzymes exhibits better structural stability, maintains high activity for a long time and have higher affinity to the substrates (Ravindran et al. 2018). The initial (before storage) activity of free enzyme was 620.1, 550.5, 340.2 U ml⁻¹ min⁻¹ for pectinase, cellulase and amylase, respectively, which was considered as 100%. Maximum efficiency yield of immobilized enzymes (pectinase—50.8%, cellulase—70.6% and amylase—68.4%) were observed on bagasse matrix on initial day (Fig. 9). It remained highest (pectinase—92.2%, cellulose—72.9% and amylase—90.9.4%) after 2 months of storage at − 20 °C as compared to rice husk, wheat straw and paddy straw (Table 3).

### Table 3 Storage study of immobilized multienzymes preparation from Trichoderma asperellum

| Matrix          | Pectinase activity (U ml⁻¹ min⁻¹) | Cellulase activity (U ml⁻¹ min⁻¹) | Amylase activity (U ml⁻¹ min⁻¹) |
|-----------------|----------------------------------|----------------------------------|----------------------------------|
|                 | At zero time 2 months of storage | At zero time 2 months of storage | At zero time 2 months of storage |
| Baggases        | 308.6 ± 0.85 280.1 ± 3.9          | 392.6 ± 2.9 283.0 ± 2.0          | 303.3 ± 2.3 271.3 ± 2.0          |
| Rice Husk       | 263.1 ± 2.65 262.6 ± 4.9          | 316.2 ± 0.6 203.7 ± 2.71         | 234.4 ± 3.1 154.7 ± 4.3          |
| Paddy straw     | 291.1 ± 0.60 212.0 ± 1.0          | 319.6 ± 4.9 102.1 ± 4.33         | 267.9 ± 2.5 110.7 ± 0.0          |
| Wheat straw     | 231.7 ± 3.90 108.6 ± 2.1          | 304.9 ± 0.5 85.5 ± 5.00          | 227.3 ± 3.1 43.0 ± 2.45          |

*The value given in the table are mean average of three replicate, ± standard error significantly (p < 0.05)
Addition of enzymes during the fruit processing resulted in reduced turbidity and better juice extraction. With the commercial pectinase enzyme, the highest juice yield was 72%, while with the crude multienzyme extract from *T. asperellum*, it was 65%; whereas in control, it was only 30%. Mohamed et al. (2009) reported increase in apple juice recovery up to 135% by use of mixture of amylase and pectinase produced by *T. harzianum*.

**Conclusion**

The current study indicated that peel of *C. limetta* may act as the potential substrate for the production of multienzyme preparation by a *T. asperellum* under solid-state fermentation. These preparations may find commercial-scale applications in pharmaceutical, food, and beverage, detergent, and biofuel industries due to multifaceted biocatalysis benefits and low environmental impact.

**Acknowledgements** The research was funded by AMAAS networking project of Indian Council of Agricultural research, New Delhi, India. The author also wishes to thank Director, ICAR-CISH for providing the necessary facilities during the course of investigation.

**Author contributions** NG and PM: conceived the research, designed experiments; BS, SV and SK: performed the experiments; BS, SKS and SK: analyzed the data and MS writing; NG, BS and SKS editing the MS.

**Funding** The research was funded by ‘Application of Microorganisms in Agriculture and Allied Sectors’ (AMAAS) networking project of Indian Council of Agricultural research, New Delhi, India.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**Research content** The research content of manuscript is original and has not been published elsewhere.

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