Microbial production of multienzyme preparation from mosambi peel using Trichoderma asperellum

Balvindra Singh
Amity University - Lucknow Campus
Neelima Garg (neelima.garg@icar.gov.in)
CISH: Central Institute for Subtropical Horticulture https://orcid.org/0000-0002-1496-7111
Priti Mathur
Amity University - Lucknow Campus
Supriya Vaish
CISH: Central Institute for Subtropical Horticulture
Sanjay Kumar
CISH: Central Institute for Subtropical Horticulture

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Abstract

Fruit and vegetable whole sale markets produces huge quantities of wastes in the forms of discarded fruits and vegetables or their parts, creating unhygienic conditions around the area and resulting into environmental pollution. Many enzymes have good applicability in food industries mainly in the production of clarified fruit juices. However, cost of production of such food enzymes are generally high due to costly raw materials. Utilization of fruit and vegetable wastes as carbon sources for enzyme production through microbial intervention has already been reported by various workers. The present study reports the feasibility of using mosambi (*Citrus limetta*) peel as substrate for multienzyme preparation including pectinase, cellulase and amylase, using a potential fungal isolate.

Conditions viz. temperature, pH, incubation time and nutrient addition were optimized for enzymes production from fresh mosambi peel as substrate using *Trichoderma asperellum* NG-125 (accession number-MW287256). Maximum activities (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. Macro-nutrients addition @ 0.1% ammonium sulphate, 0.01% potassium-di-hydrogen-ortho-phosphate enhanced the production of enzymes. The purified pectinase, cellulase and amylase by SDS-PAGE revealed three bands to molecular mass of 43, 66 and 33kDa, respectively.

Out of four natural fibre matrices (bagasse, rice husk, paddy straw and wheat straw) tested for immobilization, maximum enzyme activity retention percent was observed on bagasse matrix (pectinase-56.35%, cellulase-77.68% and amylase-59.54%). Enzymatic juice clarification yield obtained with test enzyme was 75.8% which was as comparable to 80.5% of commercial enzyme.

Introduction

Enzymes generally work as biological catalysts that accelerate rate of particular biochemical reactions. Food enzymes have big shares in International food markets. According to BBC Report, 2021, the global market for food enzymes was worth $ 5870.1 millions in 2019. 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. Pectinases 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 alternative 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. Biological conversion 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 tonnes 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, sugars, 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 substrates. The present study reports the feasibility of using mosambi peel as substrate for multienzyme preparation including pectinase, cellulase and amylase using fungus *Trichoderma asperellum.*

Materials And Methods

The chemicals and media were purchased from Himedia, Sigma and Merck.

Composition of carbohydrate utilization broth:

$(NH_4)_2SO_4$ (0.2%), K_2HPO_4 (0.4%), Na_2HPO_4 (0.6%), $FeSO_4$ (0.1%), $MgSO_4$ (2%), $CaCl_2$ (0.1%), $MnSO_4$ (0.001%), $ZnSO_4$ (0.007%), $CuSO_4$ (0.005%), H_3BO_3 (0.001%) and **MoO3** (0.001%) at pH 7.2 ± 0.2. Pectin, carboxy methyl cellulose (CMC) and starch @ 1% were added to the broth for preparing respective carbon source utilization broth.

Isolation of fungal culture:

Fungi were isolated from degrading organic substrate by serial dilution, and pour plating isolation techniques were used. The isolates were then sub-cultured into their respective selective growth media (Pectin, CMC and Starch) until pure cultures were obtained.

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.

Secondary screening of fungal isolates:

The carbohydrate utilization broth with 1% substrate (Pectin/CMC/Starch) was inoculated with fungal isolates, showing positive results in primary screening, at pH 7.0 and temperature 30°C for 10 days. Culture filtrate was tested for pectinase, cellulase and amylase activities as per the following protocol:

Twenty ml of culture filtrate was centrifuged @ 10000 rpm for 10 minutes at 4°C. Five ml of the cell-free supernatant obtained was filtered through syringe filter (0.22µm) and was used for enzyme assay Miller (1959).

One volume of supernatant was precipitated with 4 volumes of cold acetone, and incubated at -20°C for 20 minutes. The samples were centrifuged @ 10000 rpm at 4°C for 10 minutes. The pellet was dissolved in the freshly prepared 0.2M acetate buffer (pH- 5.5) and assayed for pectinase, cellulase and amylase activities.

Enzyme assay:

The enzyme reaction mixture contained 0.4 ml of substrate (Pectin 0.5%/ CMC-1.0%/ Starch-1.0% 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 35°C for 1 hour 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 minutes. Standards of D-galacturonic acid and glucose were taken in the range of 1mM to 10mM 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:

$$\text{Enzyme activity ml}^{-1}\text{minute}^{-1} = \frac{\text{Concentration of standard } (\frac{\mu mol}{ml})}{\text{absorbance of standard at 550nm}} X \frac{\text{Dilution X enzyme absorbence}}{\text{Time of incubation}}$$

Scanning electron microscopic (SEM):

Scanning electron microscopic image of selected fungal isolate was followed as per method of (Babu et al. 2018).

The fungus grown on mosambi peel was fixed with glutaraldehyde (2.5%) in phosphate buffer (10 mM; pH 7.4) for 4 h. The fixed samples were rinsed twice with deionized water and dehydrated with increasing concentrations of ethanol (10, 20, 40, 60, 80, 90 and 100%) for 10 min each. Samples were dried at room temperature for three hours then sputter-coated and visualized at 2000X.

Molecular identification of selected fungal isolate:

The highest enzyme-producing fungal isolate was subjected to molecular characterization on the basis of ITS region Specific Primer (ITS1 and ITS4) sequencing technique. DNA was isolated from the culture, evaluated on 1.2 % agarose gel, extracted DNA was amplified with ITS region Specific Primer (ITS1 and ITS4) using Veriti® 96 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band of 600-800 bp range was observed. Further, the PCR amplicon was purified through enzyme treatment and

subjected to Sanger sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with ITS1 and ITS4 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The Consensus sequence of 610 bp of ITS region was generated from forward and reverse sequence data using aligner software. BLAST alignment search tool of NCBI Genbank database was done using ITS region sequence. Based on maximum identity score first fifteen sequences were selected and aligned using the multiple alignment software program ClustalW. The distance matrix was generated using RDP database and the Phylogenetic tree was constructed using MEGA 5 (Kumar et al. 2011).

Mosambi peel, collected from wholesale fruit market, Dubagga, Lucknow, India, was properly washed with water, chopped and air-dried. 100g of chopped piece (approx. 2mm size) was taken, autoclaved and inoculated with 1 ml of actively growing culture of selected fungal isolate @ $1.0*10^7$spores/ml and incubated. Samples were withdrawn at different time intervals; the culture filtrate was used for enzyme assay. Samples were precipitated with acetone and analyzed for pectinase, cellulase and amylase activities (as described in enzyme assay section). -

To study the effect of nutrient addition on production of pectinase, cellulase and amylase, ammonium sulphate (as source of nitrogen) @ 0.1% and potassium dihydrogen orthophosphate (as source of potassium and phosphorus) @ 0.01% was added to chopped mosambi peel, autoclaved, inoculated by *Trichoderma asperellum* NG 125 @ 10^7 spores/ml and incubated at 30°C for 5 days and enzymes activities were observed.

Selection of suitable natural substrate on enzyme immobilization:

Fibre was extracted from bagasse, rice husk, paddy straw and wheat straw by boiling with water, treating with 1N-HCl followed by 1N-NaOH treatment. It was then washed with water, oven-dried at 60°C and sieved in 60-150 mesh size. (Ranganna 2001).

The extracted fibre was autoclaved, the enzyme was added and incubated for 3 hours. The un-immobilized enzyme was washed away with acetate buffer, while activity was tested in enzyme immobilized on fibre.

Enzyme purification and characterization:

Pure substrates (1% pectin/ CMC/ starch in carbohydrate utilization broth) inoculated with *Trichoderma asperellum* were incubated at 30^OC 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*40cm) packed with Sephadex G-100 matrix pre-equilibrated with 0.2M acetate buffer (pH 5.5). Two ml fractions were collected in Eppendorf tubes at the flow rate of 20 ml/hour and enzyme activities were observed.

Characterization of enzymes with respect to temperature, pH and substrates was carried out as per method described by Coral et al. (2002) using temperatures of 20, 30 and 40°C and pH levels of 4.5, 5.5,

6.5 and 7.5. The heat stability of enzyme was determined by subjecting it to 65°C for one hour.

Relation 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 carboxymethyl cellulose and starch. The values of Km (Michaelis constant) and Vmax (maximum velocity) were calculated from Michaelis–Menten saturation curve.

In order 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% denaturating sodium dodecyl sulfate-polyacrylamide gel in discontinuous buffer as per 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 *Trichoderma asperellum* against a commercial pectinase was conducted. The diluted mango pulp was incubated at 35^{O}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.

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)

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 (551.5), cellulase (155.36) and amylase (293.2) was observed in mosambi peel (Fig. 1B). The selected fungal isolate was subjected to molecular identification. A single band of high-molecular-weight DNA was observed (Fig. 2A). The selected fungal isolate showed similarity with *Trichoderma asperellum* based on nucleotide homology and phylogenetic analysis (Fig. 2B). The nucleotide sequence was submitted to NCBI database and finally identified as *Trichoderma asperellum* strain NG 125 accession number MW287256 (Garg et al. 2019). The scanning electron microscopic image of *Trichoderma asperellum* isolate is shown in Fig. 3.

Table 1
Primary screening of fungal isolates for enzyme production.

Isolate	Clear zone size (mm) for pectinase	Clear zone size (mm) for cellulase	Clear zone size (mm) for amylase
F-1	85	72	70
F-2	57	50	15
F-3	65	65	47
F-4	85	68	55
F-5	30	20	05
F-6	15	65	19
F-7	52	65	32
F-8	45	30	25
F-9	57	25	19
F-10	47	52	23
F-11	22	55	30
F-12	25	65	10
F-13	80	65	48
F-14	85	68	38
F-15	55	50	10

***** Primary screening of fungal isolates for enzyme production.

Bech et al. (2014) observed that *Trichoderma asperellum* secretes different types of extracellular hydrolytic enzymes used in degradation of plant cell wall. The production of carbohydrate-active enzymes by *Trichoderma 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 *Trichoderma 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 many fold increase in activity compared to unoptimized condition Sreena and Sebastian (2018). Highest production of pectinase, cellulase and amylase by *Trichoderma 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 is 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 5th to 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 sulphate 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).

Considering enzyme activity at 35^{o}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^{o}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*.

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

Enzyme	Km (mg/ml)	Vmax (U ml^{-1} min^{-1})
Pectinase	0.5	311.1
Cellulase	2	114.9
Amylase	1	134.8

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

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 *Trichoderma 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%, cellulase- 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	After 2 months of storage	At zero time	After 2 months of storage	At zero time	After 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±49	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

*Storage study of immobilized multienzymes preparation from *Trichoderma asperellum.*

Addition of enzymes during the fruit processing resulted in reduced turbidity and better juice extraction. With the commercial pectinase enzyme, the highest juice yield obtained was 72%, while with the crude multienzyme extract from *Trichoderma 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 *Trichoderma harzianum*.

Conclusion

The current study indicated that peel of *Citrus limetta* may as the potential substrate for the production of multienzyme preparation by a *Trichoderma asperellum* under solid-state fermentation. Under optimized

conditions the enzyme production can be enhanced. The multienzyme preparation had potential to be applied for fruit juice clarification in addition, the multienzyme preparation showed substantial efficacy in the clarification of mango juice.

Declarations

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Authors' contribution:

Neelima Garg and Priti Mathur: Conceived the research, designed experiments, guided during the research and manuscript preparation.

Balvindra Singh, Supriya Vaish and Sanjay Kumar: performed the experiments.

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Figures

Figure 1

Secondary screening of fungal isolates for enzyme production (**1a)** Pure substrate (**1 b)** Mosambi Peel

Figure 2

(2a) 1.2% Agarose gel showing 650 bp amplicon of ITS region of rDNA, Lane 1: 100 bp DNA Ladder; Lane 2: 650bp amplicon (ITS region). (2b) Phylogenetic tree constructed for selected fungal isolate.

Figure 3

SEM image of *Trichoderma asperellum* 2000X.

Figure 4

Effect of fermentation period on enzyme production using mosambi peel as substrate by *Trichoderma asperellum.*

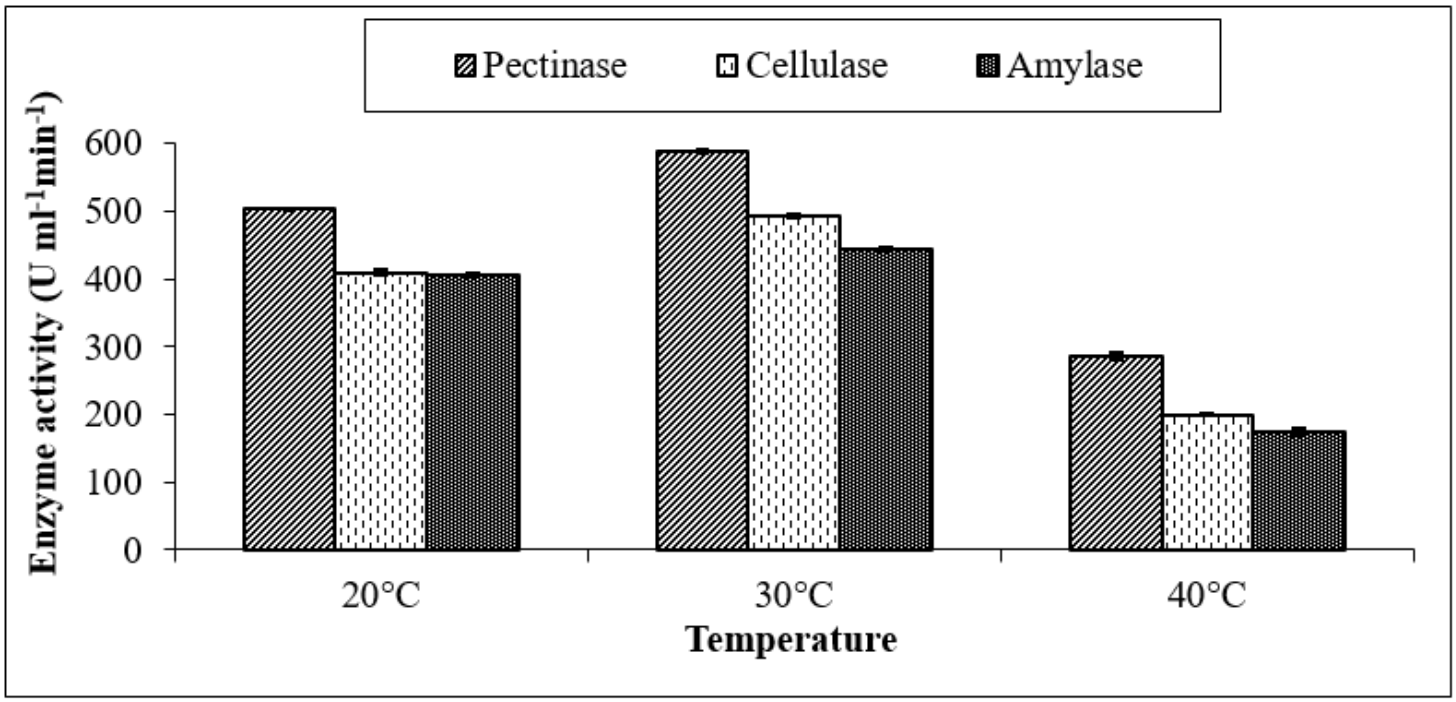

Figure 5

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

Figure 6

Optimization of fermentation pH for enzyme production by *Trichoderma asperellum* using mosambi peel as substrate.

Figure 7

Effect of NPK supplementation on enzyme production.

Figure 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.

Figure 9

Activity retention (%) of pectinase, cellulase and amylase enzymes present in multiple enzyme preparation immobilized on different fibre matrices (BA- bagasse, WS- wheat straw, PS- paddy straw and RH-rice husk).