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PRODUCTION AND CHARACTERIZATION OF COMMERCIAL CELLULASE PRODUCED THROUGH *ASPERGILLUS NIGER* IMMIS1 AFTER SCREENING FUNGAL SPECIES

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Abstract

Present study is designed to screen fungal species for cellulase production from agricultural wastes and their utilization in industry. A wide variety of fungal species have capability to degrade cellulosic waste materials, but only a small number of fungal species produce cellulase in significant amount. In the given study, twenty three fungal species were collected from various sources belonged to four different genera, and analyzed for cellulolytic activity. Fungal cellulase activity was checked with 1% Congo red dye in alkaline medium to screen and isolate novel fungal species. The diameter of yellow zone indicated the cellulolytic activity of fungal species. Index of Relative enzyme activity (ICMC) depicted high cellulase producing species and low cellulase producing species in different categories. Species of *Trichoderma* and *Humicola* revealed maximum cellulase activity as compared to *Aspergillus* and *Penicillium.* The specific activity of crude cellulase produced by *Aspergillus niger* IMMIS1 was 238 U/mg, after Ammonium sulphate precipitation 260 U/mg, after dialysis 334 U/mg and after gel filtration 388 U/mg. Purification fold was increased up to 1 to 1.63 after gel filtration. The cellulase used for the clarification of fruit juices, revealed rise in percentage yield of apple 25%, mango 18%, apricot 9% and peach 14%. Ariel detergent revealed maximum stability for cellulase protein as compared to other local detergents.

Key words: Cellulase; *Trichoderma*; *Humicola*; Congo red; *Penicillium*; *Aspergillus*.

Introduction

Glucose and other oligosaccharides molecules are produced through the actions of cellulase and cellulase action is synergetic (complex of three enzymes) (Chellapandi & Jani, 2008). Cellulose is a homo-polysaccharide which has highly crystalline structure and it is very difficult to break its firm structure. Cellulose has a higher level of crystallinity and thus becomes difficult to be broken down into sub particles. Cellulase which is complex of three enzymes; exoglucanase, endoglucanase and beta glucosidase breaks cellulose into glucose monomers to overcome complex structure problems(Gao *et al*., 2008; Kim *et al*., 2008), while endo-glucanases are being the most cost-effective enzyme (Henriksson *et al*., 1999).

Different fungal genera are utilized for cellulase extractions like; *Trichoderma, Aspergillus, Humicola* & *Penicillium*. These genera have elongated hyphae which exert mechanical power on complex cellulosic structure, as a result of their elongated hyphae which produce mechanical pressure on the cellulose structure, causing them to supply huge amounts of cellulases. Bacterial species produce low amount of cellulases as compared to fungal species, are more promising for enzyme production (Thurston *et al*., 1993). Micro-organisms which produces carbohydrate digestive enzymes, seldom utilize protein and lipid as energy source for their growth and metabolism (Poulsen *et al*., 1998; Lynd *et al*., 2002; Rajoka & Malik, 2005). Aerobic microbial fungal species are more proficient for cellulase production as compared to anaerobic species and this activity is restricted to cellulose and its hydrolytic products (Ng & Zeikus, 1982; Hayashida *et al*., 1988).

Filamentous fungus specie like *Trichoderma reesei* is widely used for the extraction of cellulases and has produced glucose monomers from hard cellulosic materials. Most widely fungal species used for cellulase production; are *Trichoderma, Aspergillus, Penicillium* and *Humicola* species while few bacterial species are also used for cellulase production like *Bacilli, Streptomycetes, Pseudomonas, Actinomycetes, Actinomucur* and *Cellumonas* species (Chaabouni *et al*., 1995; Jorgensen *et al*., 2003; Schulein, 1997). Few fungal species are capable to consume cellulose for energy production and use for the hydrolysis of hard cellulosic materials. Filamentous fungal species are used for hyper productions of cellulases like *Trichoderma*, *Aspergillus, Humicola* and *Penicillium* species (Van-Den Broeck *et al*., 2001; Ong *et al*., 2004; Jorgensen *et al*., 2003). Cellulase production process is important tool for textile industry, animal feed and detergents, paper industry, animal feed, detergent and most importantly in biofuel as a renewable energy source (Xia & Cen, 1999; Pere *et al*., 1995; Kottwitz & Schambil, 2005; Pajunen, 1986; Rani *et al*., 1997). The purpose of current study is to evaluate the fungal species for cellulase production and further utilization in food and feed industry.

Materials and Methods

Isolation and screening of fungal species: Fungal species were collected from different locations of Ravi Campus Pattoki, University of Veterinary & Animal Sciences, Lahore like; seeds of rosewood, rhizobium soil, field soil, flowering plant soil, citrus fruit plant soil, tomato wastes, agriculture soil, bread, textile effluents, wood of old plants, soil of okra, guava seed, citrus peel while recombinant species were obtained from

Biochemistry Laboratory, University of Punjab. Fungal species were isolated and re-cultured on three medias such as; potato dextrose media (PDA media), Czapec's agar media (CA media) and malt extract media. Morphological characteristics are the basis of these fungal species identification (Khokhar *et al*., 2012).

Screening of plate: Plate screening medium (PSM) was used for cellulolytic activity of fungal species using Mandel's salt solution such as; urea 0.3g, calcium chloride 0.3g, magnesium sulphate 0.3g, Potassium bisulphate 2g, yeast extract 0.25g, proteose peptone 0.75g, ammonium sulphate 1.4g,agar 18.5 g and carboxy methyl cellulose (CMC) 12 g (Pavani *et al*., 2013). Seven days old fungal colony with agar block diameter of 8 mm expressed on malt extract media and streaked at the center of basal media Petri plate. For growth, Petri plates were incubated at around 30ºC for 7 days. Hydrolysis zone (diameter) around the fungal colonies indicated cellulolytic activity. To check the zone of hydrolysis, Congo red dye was used on Petri plates to stain area around clearance zone for 30 minutes and then de-stained with 1 M solution of NaCl or NaOH for 25 minutes. Cellulolytic activity was observed around the active fungal species of the fungi. Index of relative enzyme activity was checked on carboxy methyl agar revealing clear zone ratios/ colony diameter (Mandels *et al*., 1974; Khokhar *et al*., 2012; Pavani *et al*., 2013). Malt extract growth media was observed as control for fungal growth. Growth inhibition or stimulation was seen as diameter of colony diameter or agar colony diameter on control agar ratio (Khokhar *et al*., 2012).

***Aspergillus niger* IMMIS1 molecular characterization:** Neighbor-Joining method was used to construct evolutionary tree of *Aspergillus niger* IMMIS1 after sequencing (Saitou & Nei, 1987). Branches were drawn under percentage of replicate tree associated together in the bootstrap test (Felsenstein, 1985; Shinwari *et al*., 2018). Phylogenic tree was drawn in such a way with branch lengths and evolutionary distances were in the same units and measured by method proposed by Tamura & Nei (1993). Gamma distribution (shape parameter 1) was used to determine the rate of variations among sites. Consensus nucleotide sequences of novel fungal species were submitted to European Molecular Biology Laboratory database which was available as accession number LT732555.1.

Purification of cellulase: Cellulase was purified using different concentrations of ammonium sulphate (30-80%). Precipitated cellulase was separated through dialysis tube using buffers of different ionic strength. Further, purification of cellulase was carried out using Sephadex G-100 (Imran *et al*., 2012; Sadhu *et al*., 2013). Cellulase activity was checked through procedure proposed by Iqbal *et al*., (2011).

Cellulase protein content determination: Cellulase protein was investigated through spectrophotometric method proposed by Lowry *et al*., (1951).

Standard curve of BSA for protein estimation: Blood Serum Albumin (BSA) solution was taken in different test tubes ranging from 2-10 mL from the standard of 5mg/10mL. Then, Bradford reagent 5mL was added in each test tube. The absorbance was taken at 595nm. A graph was plotted between absorbance and different concentrations of BSA (Fig. 1).

Protein mg/mL = Absorbance x standard factor (1)

Effect of pH on cellulase: The purified enzyme for optimum pH was determined using different pH buffers. Cellulase assay was performed at diverse pH buffers ranging from 3-7 at 30°C. Different buffers like for pH 4 (succinic acid buffer), for pH 5(Acetate buffer), for pH 6 (citrate buffer), for 7 (phosphate buffers) were used for cellulase characterization. The cellulase activity assay was determined for stability studies using different buffers (Imran *et al*., 2012).

Effect of temperature: The purified cellulase was characterized at different temperature ranges from 25ºC, 30ºC, 35ºC, 40ºC, 45ºC. For thermal stability studies, cellulase was incubated at different temperatures before usual cellulase assay (Khokhar *et al*., 2012).

Cellulase kinetics determination: A Line Weaver Burk graph was plotted between reciprocal of velocity of reaction and substrate concentration to determine enzyme kinetic parameters like Vmax and Km (Imran *et al*., 2017).

Molecular weight characterization of cellulase enzyme: Most efficient elution from gel filtration was analyzed through protein separating technique like; Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). SDS PAGE was performed to determine the molecular weight of cellulase enzyme. For this purpose, 16% separating gel was used for cellulase molecular weight determination (Sadhu *et al*., 2013; Imran *et al*., 2012).

Industrial applications: Juicer machine was used to make juice of peach, apple, mango and apricot using 50 mL of water. The cellulase extracted from Aspergillus species after gel filtration, was used for fruit scarification. Juice volume was noted before and after the addition of filtered cellulase. Banana juice was clarified using pectinolytic enzymes and volume of banana juice increased (Barman *et al*., 2015).

Crude cellulase produced by *Aspergillus niger* IMMIS1 was used to check the compatibility with detergents. For this purpose, standard of each detergent was made by taking 1g of detergent and dissolved in 10 mL of distilled water and boiled. Then, 10 mL of crude cellulase was mixed with 1 mL of standard detergent and placed in incubator at 50ºC. Cellulase activity was observed to check the stability of cellulase in detergents (Sadhu *et al*., 2013).

Results and Discussion

Fungal isolates screening for cellulolytic activity: Twenty three species of fungi belonging to four genera i.e. *Trichoderma, Humicola, Penicillium* and *Aspergillus* were collected from different locations and screened for maximum cellulolytic activity. Screening of fungal species was done on Petri plate containing growth media. Among 23 isolates of fungus, 19 fungal species revealed cellulolytic activity and 4 depicted zero cellulase activity (Pavani *et al*., 2013; Mandels *et al*., 1974; Teather & Wood, 1982). All *Trichoderma* species showed cellulolytic activity on agar plates followed by *Humicola*, *Penicillium* and *Aspergillus*. However, some isolates of *Aspergillus* and *Penicillium* revealed zero cellulase activity (Table 1). Significant differences among the isolates were observed ($p \leq 0.05$). Some *Aspergillus* species revealed primary screening of cellulase producing species with Congo red test method with define zone of clearance (Fig. 2).

Growth inhibition or stimulation index value was determined as diameter of colony on carboxymethyl agar / colony diameter on control agar ratio. The index value less than 1 indicated that substrate inhibited the growth of fungus whereas index value more than 1 revealed that substrate enhanced the fungal growth. Maximum values of index for *Trichoderma reesei, Trichoderma viride, Aspergillus japonicas* and *Penicillium oxalicum* depicted that these species were strongly stimulated by substrate (Table 1). *Aspergillus terreus* and *Penicillium expansum* index value was less than 1 and inhibited by substrate. *Trichoderma species, Penicillium oxalicum, Aspergillus tubingensis, Aspergillus awamori, Aspergillus nidulans (recombinant), Aspergillus niger* IMMIS1, *Aspergillus oryjae (recombinant), Aspergillus japonicas, and Humicola grisea* showed maximum hydrolytic activity on carboxymethyl cellulose surface and had a great potential for cellulase production (Table 1).These fungal species could be utilized in various industries. The most existing species like; *Trichoderma koningii, Trichoderma reesei, Aspergillus* and *Penicillium* species were considered as good cellulase producing system (Bradner *et al*., 1999; Yalpani 1987). Current study results were comparatively higher as compared to previous studies for cellulase production (Kluczek-Turpeinen *et al*., 2005).

In industry, approximately hundreds of enzymes are employed of which over 50% are from fungi & yeast, 1/3 from bacteria, 8% from animals and 4% from plant resources (Oyeleke *et al*., 2012). Fungi were known to decompose agricultural wastes particularly lignocellulosic waste materials (Lynd *et al*., 2002). Soil samples were preferred for cellulase production from fungi for the last many decades (Ruttloff *et al*., 1987; Schwarz, 2001). *Penicillium expansum* had good cellulase producing property and used as in biodegradation (Graber *et al*., 1965; Memon *et al*., 1985; Marsh *et al*., 1949) of agricultural wastes. Microbial cellulase had great importance in decomposing cellulosic mass and played a major role in biological cycle (Lederberg *et al*., 1992). *Aspergillus* species produced a variety of enzymes which were utilized in degrading plant cell wall materials. Different *Aspergillus* and *Trichoderma* species were used to produce cellulases which were relevant to current study of *Aspergillus* and *Trichoderma* species (Li *et al*., 2016; Imran *et al*., 2016). *Aspergillus niger* IMMIS1 was used for cellulase production because of its novelty.

Molecular Characterization of *Aspergillus niger* IMMIS1: *Aspergillus niger* was sequenced and these sequences submitted to European Molecular Biology Laboratory. The results (phylogenetic tree) revealed that *Aspergillus niger* had accession # LT732555.1 and named as IMMIS1 (Fig. 3).

Cellulase production from *Aspergillus niger* IMMIS1: Gel filtration chromatography showed that thirteen fraction of gel filtration fraction had maximum absorbance and selected for further purification (Fig. 4). Crude cellulase enzyme revealed maximum activity (350 ± 0.7 U/mL) at 35°C and pH 4.8 as pre-optimized conditions. Cellulase activity was increased to 436±0.7 U/mL with 70% ammonium sulphate purification with specific activity of 260 ± 0.88 U/mg. Cellulase activity was also enhanced with (656± 0.3 U/mL) dialysis tube using buffers of different ionic strength with specific activity of 334 ± 0.8 U/mg. Gel filtration sephadex G-100 revealed maximum cellulase activity (657±0.5 U/mL) with specific activity of 388 ± 0.3 U/mg (Table 2). The purification fold increased from 1 to 1.63 after performing all purification steps (Table 2). Crude cellulase enzyme revealed specific activity of 45 ± 0.4 U/mg, with 80% ammonium sulphate purification 48 ± 0.3 U/mg, with dialysis tube 64 ± 0.3 U/mg and with Gel filtration chromatography 105± 0.5 U/mg using *Trichoderma viride* as experimental organism (Imran *et al*., 2017; Iqbal *et al*., 2011). All results were significant with probability values less than 0.05 ($p \leq 0.05$).

Cellulase activity and effect of pH: The highest cellulase activity was achieved at pH 4.5 (424 ± 0.4U/mL) of dialyzed enzyme using *Aspergillus niger* IMMIS1 as experimental organism (Fig. 5). Acharya *et al*., (2008) demonstrated that *Aspergillus niger* revealed maximum cellulase activity at pH 4 while Coral *et al*., (2002) predicted that cellulase activity was high at pH 4.5.

Cellulase activity and effect of temperature: The maximum cellulase activity of dialyzed enzyme was achieved at 35°C (520±0.04 U/mL) using *Aspergillus niger* IMMIS1 as experimental organism (Fig. 6). Acharya *et al*., (2008) demonstrated that *Aspergillus niger* revealed maximum cellulase activity 28°C while Gunny *et al*., (2015) forecasted that cellulase activity was high at 35°C produced through *Aspergillus terreus*.

Determination of kinetic constants K_M and V_{max}: The values of kinetic parameters K_M and V_{max} for purified cellulase were 0.54mM and 19mM/min, respectively using line-Weaver and Burk plot (Fig. 7). The low Km value revealed that cellulase had strong affinity with carboxy methyl cellulose and Vmax value was achieved at low substrate concentration. Iqbal *et al*., (2012) predicted that Km value for cellulase was 68 µM produced by *Trichoderma viride*.

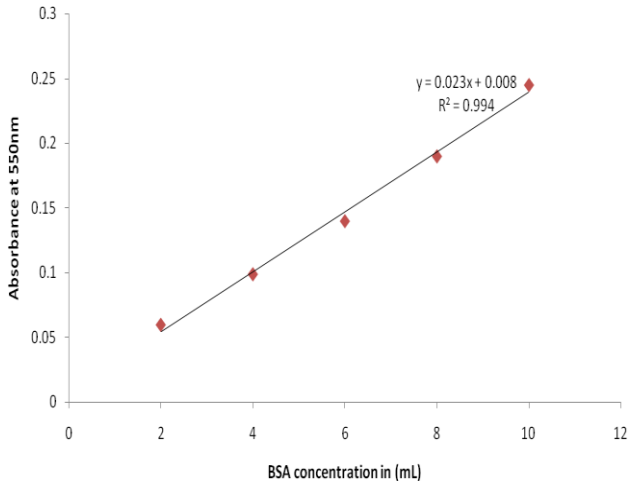

Fig. 1. Standard graph of BSA.

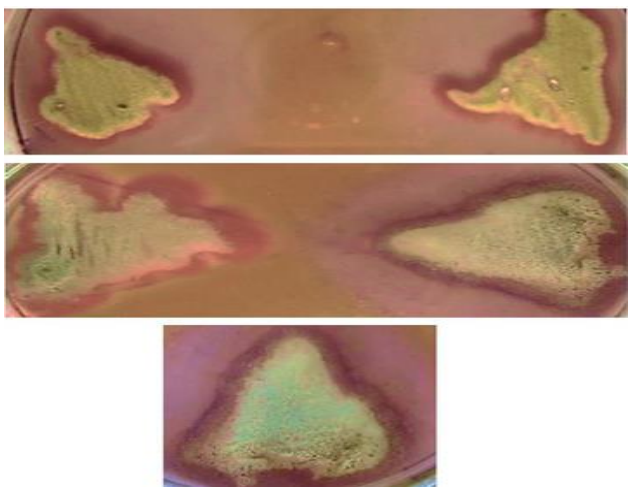

Fig. 2. Primary screening of cellulase producing organisms Congo red test method; Fig. represents: Congo red test: clear zone agar plate because of cellulase activity.

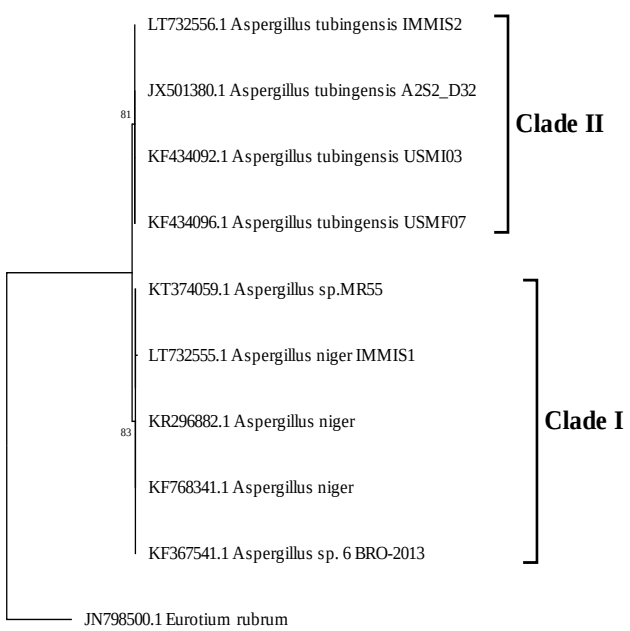

Fig. 3. Evolutionary Tree of *Aspergillus niger* IMMIS1 using Neighbor-Joining method.

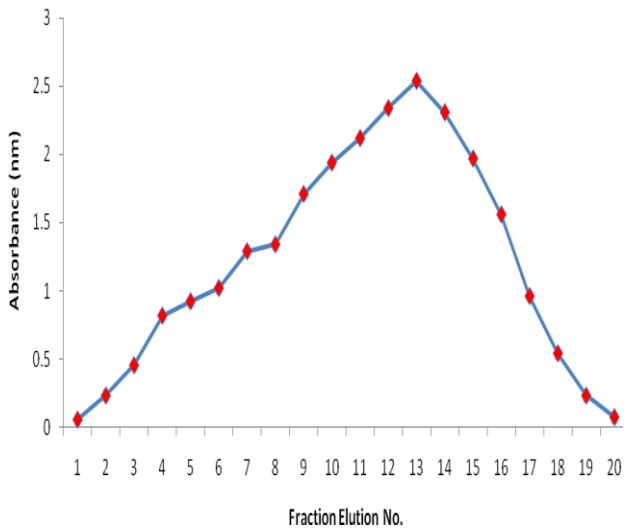

Fig. 4. Gel filtration chromatography of cellulase enzyme produced from *Aspergillus niger IMMIS1*.

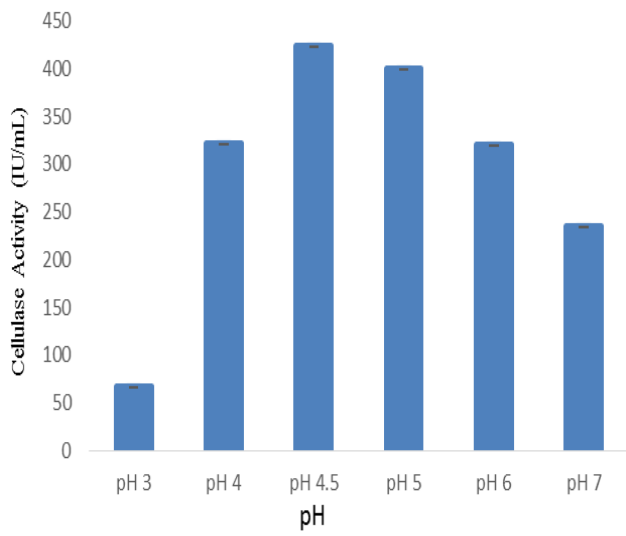

Fig. 5. Effect of different pH on cellulase Activity produced by *Aspergillus niger IMMIS1*.

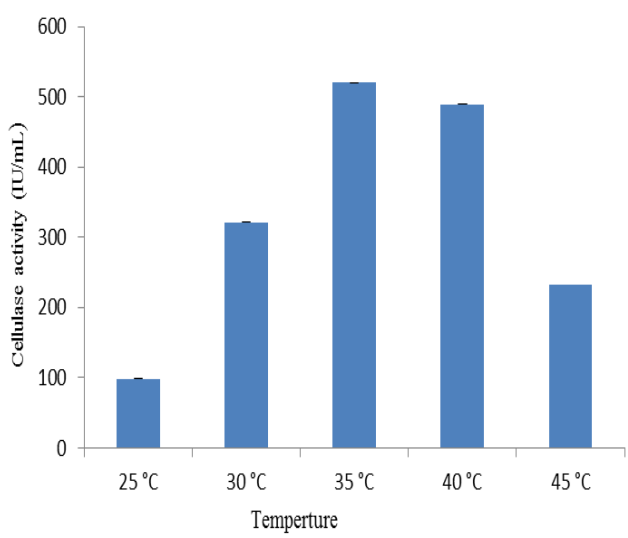

Fig. 6. Effect of temperature on purified cellulase produced from *Aspergillus niger IMMIS1*.

Table 1. Cellulolytic activity and fungal expression on culture plates.

Scientific name	Hydrolysis zone (cm)	Colony diameter (cm)	Hydrolysis activity index	Colony diameter on control agar (cm)	Growth stimulation index
A. japonicas	8.7 ± 0.08	8.7 ± 0.08	0.998 ± 0.02	7.6 ± 0.09	1.45 ± 0.01
A. terreus	1.7 ± 0.08	1.7 ± 0.08	1.01 ± 0.02	5.6 ± 0.05	0.8 ± 0.01
A. tamari	0.0 ± 0.00	9.0 ± 0.00	0.0 ± 0.0	6.8 ± 0.15	1.3 ± 0.01
A. raperi	5.40 ± 0.05	5.4 ± 0.05	0.70 ± 0.02	3.6 ± 0.05	1.10 ± 0.01
A. flavis	1.10 ± 0.05	1.10 ± 0.05	0.99 ± 0.01	7.6 ± 0.09	1.25 ± 0.01
A. niger	8.9 ± 0.08	8.9 ± 0.08	0.99 ± 0.0	7.6 ± 0.09	1.15 ± 0.01
A. oryjae (recombinant)	8.7 ± 0.08	8.7 ± 0.08	0.99 ± 0.01	7.0 ± 0.09	1.15 ± 0.01
A. tubingenesis	8.9 ± 0.08	8.9 ± 0.08	0.99 ± 0.02	7.3 ± 0.09	1.15 ± 0.01
A. awamori	9.0 ± 0.08	9.0 ± 0.0	0.0 ± 0.0	7.3 ± 0.09	1.5 ± 0.0
A. nidulans	0.0 ± 0.0	9.0 ± 0.0	0.0 ± 0.02	6.6 ± 0.09	1.15 ± 0.01
A. nidulans (recombinant)	9.0 ± 0.0	9.0 ± 0.0	1.0 ± 0.0	8.3±0.0	1.10 ± 0.0
H. grisea	8.7 ± 0.08	8.7 ± 0.08	0.98 ± 0.02	7.6 ± 0.09	1.15 ± 0.01
H. insolens	7.7 ± 0.08	7.7 ± 0.08	0.99 ± 0.02	7.6 ± 0.09	1.10 ± 0.01
T. reesei	9.0 ± 0.00	9.0 ± 0.00	1.00 ± 0.00	5.6 ± 0.09	1.65 ± 0.01
T. koningii	9.0 ± 0.00	9.0 ± 0.00	1.00 ± 0.00	6.8 ± 0.09	1.25 ± 0.01
T. viride	9.0 ± 0.00	9.0 ± 0.00	1.00 ± 0.00	7.6 ± 0.09	1.50 ± 0.01
T. harjianum	9.0 ± 0.00	9.0 ± 0.00	1.00 ± 0.00	6.6 ± 0.09	1.05 ± 0.01
T. branchiatum	8.9 ± 0.08	8.9 ± 0.08	0.99 ± 0.01	7.2 ± 0.09	1.30 ± 0.01
P. oxalicum	9.0 ± 0.00	9.0 ± 0.00	1.01 ± 0.00	5.8 ± 0.09	1.60 ± 0.01
P. simplicissimum	0.0 ± 0.00	6.2 ± 0.00	0.0 ± 0.0	5.6 ± 0.09	1.20 ± 0.01
P. lanosum	2.8 ± 0.00	2.8 ± 0.0	0.99 ± 0.06	2.1±0.1	1.3 ± 0.06
P. expansum	6.7 ± 0.17	6.7 ± 0.17	1.10 ± 0.02	4.6 ± 0.09	0.8 ± 0.03
P. citrinum	0.0 ± 0.0	3.0 ± 0.07	0.0 ± 0.0	1.6 ± 0.09	1.90 ± 0.05

Table 2. Purification summary of cellulase produced from *Aspergillus niger IMMIS1*.

Sr. No.	Purification steps	Volume (mL)	Enzyme activity (U)	Protein content (mg)	Specific activity (U/mg)	Purification fold	% Yield
1.	Crude enzyme	250	87664	368	238.21	100	1
2.	$(NH_4)_2SO_4$	150	65481	251	260.88	68.2	1.09
3.	Dialysis	50	32812	98	334.81	26.63	1.4
4.	Chromatography column (G-100)	15	9863	25.4	388.30	6.90	1.63

Molecular weight characterization of cellulase: Sodium dodecyl sulphate results revealed that molecular weight of cellulase was 71 KDa as compared with ladder results (Fig. 8). Sadhu *et al*., (2013) demonstrated that molecular weight of cellulase was 97 KDa produced through *Bacillus* species. The refined band of cellulase enzyme was obtained for 16% gel formation. The molecular weight of cellulase enzyme was 58 KDa produced through *Trichoderma viride* using 12% separating gel (Iqbal *et al*., 2011).

Industrial application: Different fruits like apple, apricot, peach and mango were taken and subjected of grinding in presence of 50 mL of water in juicer machine. Then these juices were subjected for enzyme activity that was obtained from *Aspergillus niger* IMMIS1. Cellulase complex metabolized cellulosic material present in pulp and increased yield & clarity of juices. After treating with cellulase, juice volume increased apple 25%, mango 18.19%, apricot 9.61% and peach 14.12% (Fig. 9). The taste of juices improved after enzymatic treatment (Barman *et al*., 2015).

Cellulase activity revealed that cellulase showed good detergent compatibility and would be very useful in detergent industry. Highest detergent compatibility of cellulase was achieved with Ariel 95.5% then, Surf excel 90.16%, Express power 89.26%, Sunlight 88.6%, Bright 83.9% (Fig. 10). Similar Detergent compatibility results for cellulase were determined by Sadhu *et al*., (2013). But, cellulase produced through *Aspergillus niger* IMMIS1 revealed far better compatible results as compared to proposed by Sadhu *et al*., (2013). Iqbal *et al*., (2011) revealed the compatibility of Surf Excel was higher as compared to Ariel detergent produced through *Trichoderma viride*.

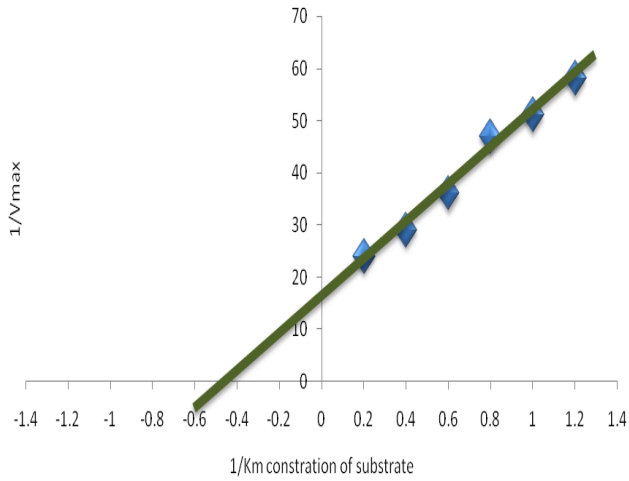

Fig. 7. Kinetic study of cellulase enzyme produced from *Aspergillus niger IMMIS1*.

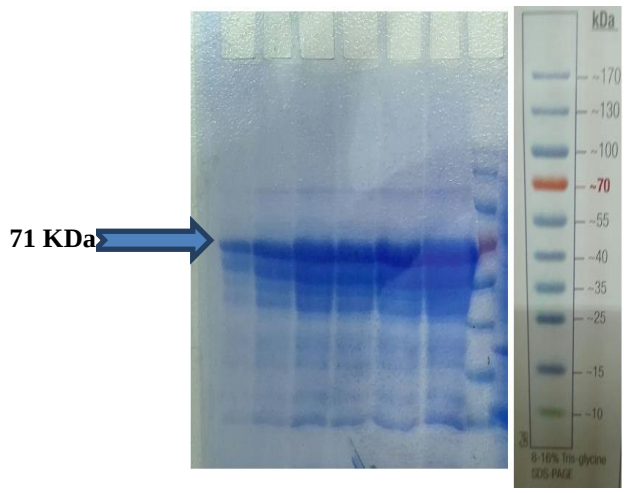

Fig. 8. SDS PAGE (16%) for cellulase enzyme produced from *Aspergillus niger IMMIS1*.

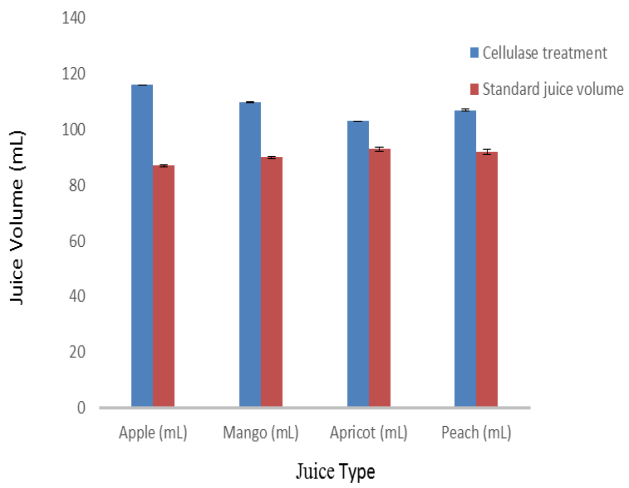

Fig. 9. Cellulase Industrial applications for juice production produced from *Aspergillus niger IMMIS1*.

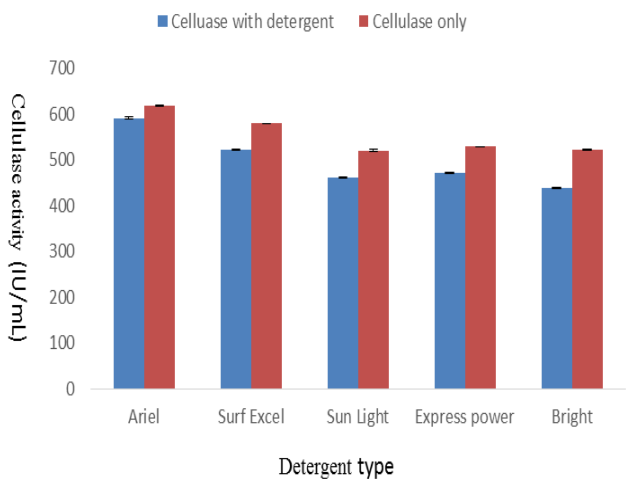

Fig. 10. Cellulase compatibility with different detergents produced from *Aspergillus niger IMMIS1*.

Conclusion

The molecular weight of cellulase enzyme was 71 KDa extracted from *Aspergillus niger* IMMIS1 (a novel strain). *Aspergillus niger*IMMIS1 was selected for cellulase purification because of cellulase producing property. Cellulase revealed maximum activity at 35°C and pH of 4.5. Crude cellulase showed maximum specific activity after gel filtration chromatography with purification fold of 1.63. This extracted cellulase predicted maximum detergent compatibility and will be good candidate for detergent industry. Fruit scarification property of cellulase induced attention of food industrialist. Overall cellulase produced from *Aspergillus niger* IMMIS1, is a good candidate for its industrial utilization.

Conflict of interest: Present study did not have any conflict of interest with any department or person.

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