

Research Article

## The Potential of Hydrolytic Enzymes from *Phoma exigua* for Fruit Juice Clarification

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### ABSTRACT

In this study, we explored the potential use of industrially important cellulases, pectinases and xylanases from *P. exigua* in comparison with commonly adopted organism *A. niger*. The cultured organism *P. exigua* and *A. niger* were optimized for maximum growth and enzyme production. The results were compared and tested under similar conditions to determine the quality and quantity of enzymes. A 5.16, 5.16 and 7.31 fold increase in activity of cellulase, pectinase and xylanase respectively was achieved. Results indicated that overall percentage clarification was 42.16% in *P. exigua* in comparison with *A. niger* for orange juice and 48.6% in apple juice. Hence *P. exigua* can be used to efficiently produce the aforementioned hydrolytic enzymes for their application in fruit juice clarification.

**Keywords:** Hydrolases, *Phoma exigua*, *Aspergillus niger*, Cellulases, Pectinases, Xylanases, Fruit Juice Clarification.

### INTRODUCTION

Hydrolytic enzymes find various applications in the industries such as food, agriculture and textile. The hydrolytic enzymes such as cellulases, pectinases and xylanases act on complex sugars to produce simple sugars from agricultural, agro-industrial and domestic solid wastes rich in starch, cellulose and hemicellulosic biomass<sup>1</sup>. These enzymes are widely used in the production and clarification of fruit juices, processing of plant-based animal feeds, extraction oils from oil seeds and other textile applications<sup>2</sup>.

Cellulases or  $\beta$ -1,4-glucanases are a class of hydrolytic enzymes that catalyze the hydrolysis of cellulose. Cellulase is a group of enzymes comprising of endo- $\beta$ -glucanase, exo- $\beta$ -glucanase and  $\beta$ -glucosidase. These enzymes are majorly extracellular and are produced by a wide range of bacteria and fungi. Pectinases or polygalactouronases are a group of enzymes that can hydrolyze or transform pectins, polysaccharide substrates which are found abundantly in plant cell walls. It has been reported that the pectinases are abundantly found in many fungi such as *A. niger* and *Trichoderma sps*<sup>3</sup>. These pectinases have been increasingly used in industries such as food, textile and paper. Xylanases hydrolyze the 1,4- $\beta$ -D-xylosidic linkages in xylans that are a structural component of plant cell walls. Arabinoxylans are the highly branched xylans that occur in the rice and wheat flour. In general, xylanase activity levels from fungal cultures are typically much higher than those from yeasts or bacteria<sup>4</sup>.

Among the various bacteria and fungi capable of producing extracellular depolymerising enzymes, fungi

have been found to be better in terms of the range of enzyme varieties and their yields<sup>5</sup>. Most reported cellulose producers are *Trichoderma* and *Aspergillus* species<sup>6</sup>. *A. niger* is used industrially for the production of pectinolytic and amylolytic enzymes. Fungal enzymes are used in industries like food, beverages, confectionaries, textiles and leather to simplify the processing of raw materials. One of the fungal species, *P. exigua* has been seen to have the ability to produce the hydrolytic enzymes - cellulase, pectinase and xylanase. It infects numerous species of plants, including common bean, sunflower, and corn. Cotton diseases can also be attributed to this organism<sup>7</sup>.

The most popular application of hydrolytic enzymes is in fruit juice extraction and clarification<sup>8</sup>. Pectins play an important role in fruit juice viscosity and turbidity. Combinations of pectinases, cellulases, arabinases and xylanases are used to clarify fruit juices and increase extraction yields and also decrease filtration time up to 35.34%<sup>9</sup> and 85% in the presence of gelatin<sup>8</sup>.

In this study, we explored the potential use of industrially important cellulases, pectinases and xylanases from *P. exigua* in comparison with a commonly adopted organism viz., *A. niger*. Results indicated that overall percentage clarification is 42.16% in the test organism for orange juice and 48.6% in apple juice at optimum temperature and pH of 50°C and 5.5 respectively. Hence, *P. exigua* can be used to efficiently produce the aforementioned hydrolytic enzymes for their application in fruit juice clarification.

### MATERIALS AND METHODS

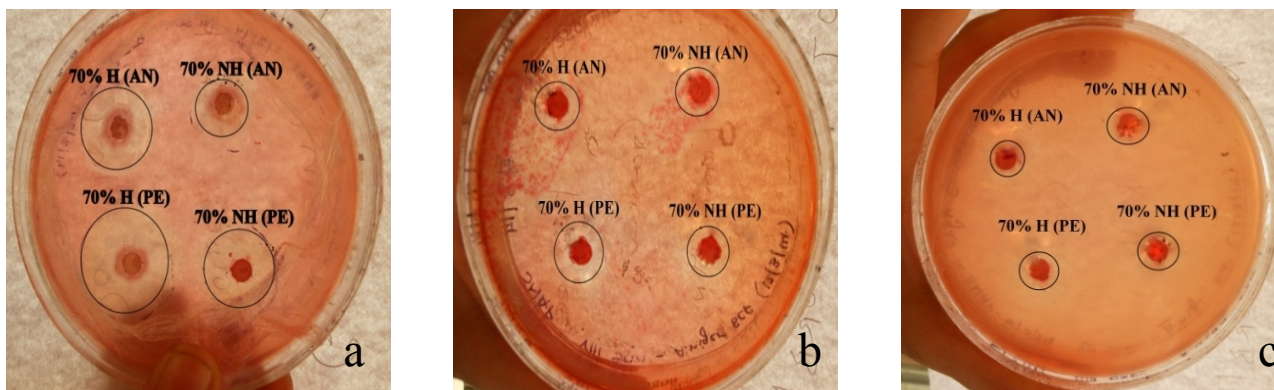


Figure 1: (a) On pectin agar, *P. exigua* (PE) had a higher zone of clearance of 3 cm and 2.2 cm for mycelial extract (H) and culture filtrate (NH) enzyme extracts respectively when compared with *A. niger* (AN) which showed clearance zones of 1.2 cm and 1 cm respectively. (b) Zone of clearance on cellulose agar of *P. exigua* and *A. niger* was found to be 1.3 cm and 1.2 cm in the mycelial samples and 1.2 cm and 1.0 cm in the filtrate samples respectively. (c) Zone of clearance of *P. exigua* on xylan agar was found to be 1.2 cm in the mycelial sample and 1 cm in the filtrate sample compared to *A. niger* (1 cm for both mycelium and filtrate).

**Collection of Fungi and Preparation of Master Cultures**

*A. niger* was obtained from Central Food Technological Research Institute (CFTRI), Mysore and *P. exigua* culture from The National Collection of Industrial Microbes (NCIM), National Chemical Laboratory, Pune. Czapek Dox Agar was used to prepare master cultures<sup>10</sup>. The cultures were then grown on Potato Dextrose Broth

medium for further experiments.

**Production of Hydrolytic Enzymes by *A. niger* and *P. exigua* in Reese's Medium**

Pure cultures were grown in 250 ml of Reese's mineral solution (Wheat Bran- 10.00 g, Pectin-10.00 g,  $\text{KH}_2\text{PO}_4$ - 2.00 g,  $(\text{NH}_4)_2\text{SO}_4$ - 1.40 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.30 g,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ - 0.30 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - 5.00 mg,

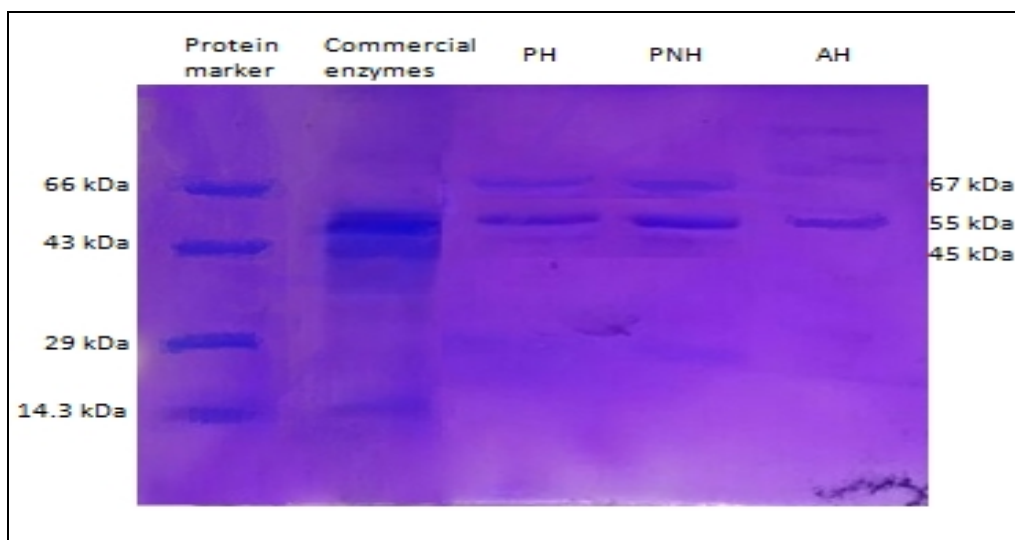


Figure 2: SDS-PAGE - SDS PAGE was run with 5 wells in which protein marker, enzymes, mycelial extract (*P. exigua*) (PH), culture filtrate (*P. exigua*) (PNH) and mycelial extract (*A. niger*) (AH) samples were loaded respectively. The molecular weight of the protein marker ranges from 66 -14.3 kDa. The molecular weights of the mycelial extract and culture filtrate samples were compared with the protein marker and the value for cellulase, pectinase and xylanase was found as 45 kDa, 55 kDa and 67 kDa respectively.

Table 1: Specific activity of cellulase in the partially purified extract and crude extract

Purification Step	Total Protein (mg/ml)		Enzyme Activity Units (U/ml)		Specific Activity Units protein (U/mg)		Fold Purification	
	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>
Crude Extract	1.18	1.86	12.61	20.65	10.68	11.10	1.0	1.0
Partially Purified Extract	0.66	0.49	22.95	28.12	34.77	57.38	3.25	5.16

MnSO<sub>4</sub>.2H<sub>2</sub>O- 1.60 mg, ZnSO<sub>4</sub>. 7H<sub>2</sub>O- 1.40 mg, CoCl<sub>2</sub>. 6H<sub>2</sub>O- 2.00 mg, Distilled Water- 1000 ml, pH 4.8 ± 0.2). Reese's medium was used to study the influence and contribution of five important factors (carbon source, organic and inorganic nitrogen source, surfactant, and pH) on hydrolytic enzyme production by *P. exigua*. Cultivation of fungus under optimized condition produced enzymes within 8 days of submerged fermentation<sup>11</sup>.

**Partial Purification**

The culture was split into two samples; filtrate and mycelia fractions to take into account the activity of both the intracellular and extracellular enzymes. The mycelium was homogenized for 10 min (homogenized sample only), filtered to remove the visible spores and centrifuged at 7000 rpm for 10 min to remove the small impurities present. The solution was stored in the refrigerator at 4°C.

Ammonium sulphate fractionation was used to precipitate the desired proteins in both the fractions, by altering their solubility. Crude extract was taken to which 70% of ammonium sulphate was added slowly and incubated for about 1 hour while kept in continuous stirring at 4°C. Solution was kept in deep freezer for overnight incubation. The extract was centrifuged at 10,000 rpm for 15 min and the supernatant was discarded and pellets were dissolved in citrate buffer (pH 5.0). Dialysis was performed to facilitate the removal of salts and the enzyme extract was stored under cold conditions for further use. 15% SDS-PAGE was performed for good resolution of the proteins. The concentration of the proteins in the crude extract and partially purified extract was determined using Lowry's method<sup>12</sup>.

**Substrate Specific Enzyme Screening Assay**

The hydrolysis zones on the plate media were measured and the relative enzyme activity of each isolate was determined<sup>13</sup>. Substrates used were: Pectin agar medium, Glucose peptone agar<sup>14</sup> and xylan agar medium<sup>13</sup>. The media was poured into Petri plates and allowed to solidify. Filter paper discs of 5mm diameter placed on the agar plates and 10 µl of enzyme solution was added to the discs. These plates were incubated for 72 hours. After incubation period the plates were flooded with 0.1%

Congo red solution and de-stained with 1M NaCl. After 15 min clear zones were observed around the filter paper discs. For further studies non-homogenized fractions of the partially purified extract was used.

**Specific Enzyme Assay**

The reaction mixture contained 0.025 ml of the enzyme, 1.5 ml citrate buffer (pH 5.0, 0.2 M) and 1ml cellulose, pectin, xylan (1%, w/v) as substrate. The mixture was incubated at 50°C for 15 min. The reaction was arrested by adding 3 ml of 3, 5-Dinitrosalicylic Acid reagent followed by its boiling the tubes exactly for 5 min. The absorbance of the solution was measured at 540 nm<sup>15</sup>.

Enzyme Activity for the crude extract as well as partial purified extract was determined. The Enzyme Activity is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute. The Specific Activity is the activity of an enzyme per milligram protein. Fold purification is defined as the ratio of the Specific Activity of Purified Fraction to the Specific Activity of Crude Fraction.

**Determination of Optimum Temperature and pH for Cellulase, Pectinase and Xylanase Activity**

The enzymes produced by *P.exigua* were optimized for their production. The 3 days grown fungal broth was filtered through Whatman filter paper No.1 and the filtrate obtained was assayed for the cellulase, pectinase and xylanase activity. The reaction mixture in duplicate were incubated separately at 30°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C for 15 min for temperature assay and different pH 3.4, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and were incubated for 15 min for pH assay. The reaction was then arrested by adding 3,5-Dinitrosalicylic Acid and the tubes kept in a water bath at 100°C. The absorbance of the solution was measured at 540 nm.

**Fruit Juice Preparation and Clarification**

The initial steps in the extraction of juice from apples include washing, sorting and crushing of apples in a mill. Fruit pulp was macerated using a hand blender with minimum amount of water was added to facilitate the maceration process as well as to extract maximum juice to get a smooth textured puree which was further strained through a cheese cloth to separate the debris from the pulp and pH was adjusted to 7.5 to achieve optimum

Table 2: Specific activity of pectinase in partially purified extract and crude extract

Purification Step	Total Protein (mg/ml)		Enzyme Activity Units protein (U/ml)		Specific Activity Units protein (U/mg)		Fold Purification	
	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>
	Crude Extract	1.18	1.86	26.40	21.80	22.37	11.72	1.0
Partially Purified Extract	0.66	0.55	30.99	33.29	46.95	60.52	2.09	5.16

Table 3: Specific activity of xylanase in partially purified extract and crude extract

Purification Step	Total Protein (mg/ml)		Enzyme Activity Units protein (U/ml)		Specific Activity Units protein (U/mg)		Fold Purification	
	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>	<i>A.niger</i>	<i>P.exigua</i>
	Crude Extract	1.18	1.86	17.78	14.33	15.06	7.70	1.0
Partially Purified Extract	0.66	0.55	22.95	30.99	34.77	56.34	2.3	7.31

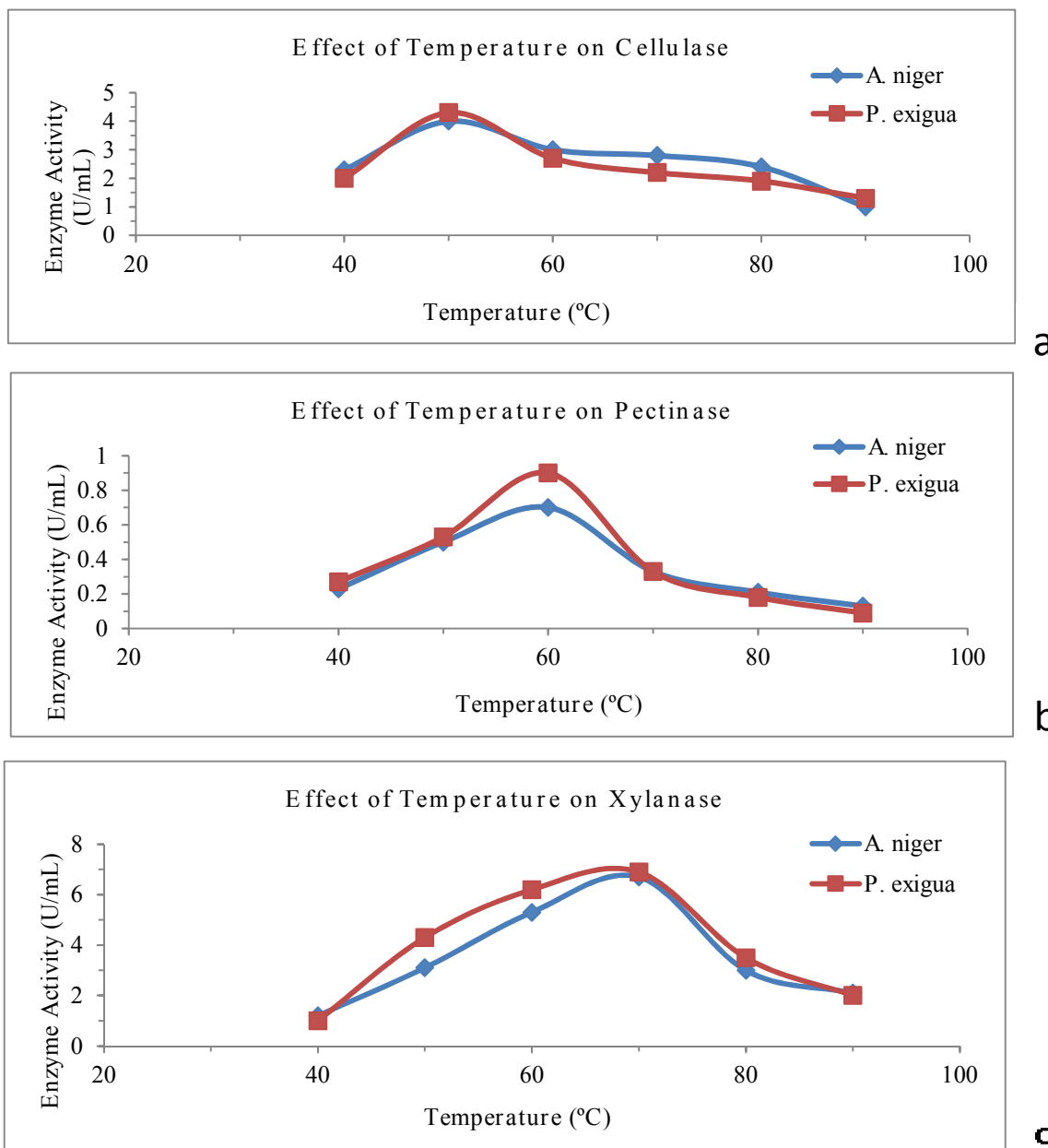


Figure 3: (a) Effect of Temperature of enzymes - The optimum temperature for cellulase activity was found to be 50°C for both *P. exigua* and *A. niger*. (b) Effect of Temperature for Pectinase was found to be 60°C for both *Phoma exigua* and *A. niger*. (c) Effect of Temperature for xylanase activity was found to be 50°C for both *P. exigua* and *A. niger*.

effect of enzyme activity.

$$\% \text{ clarification} = \frac{OD \text{ at } 650\text{nm of (treated juice - untreated juice)}}{OD \text{ at } 650\text{nm of untreated juice}} \times 100$$

Temperature and time assay was performed using partially purified non-homogenised fractions of the enzyme extract in order to find the optimum conditions for best enzyme activity. The extracted juice was pasteurized at 85°C for 3 min to inactivate the natural fruit enzymes or microbes present and then cooled down to 40°C before the addition of enzymes. 10ml of juice was incubated at varying temperatures (30°, 35°, 40°, 45°, 50°, 55° and 60°C) for 48 hours for temperature

assay while the percentage clarification of fruit juice was determined by incubating at varying times (4 – 52 hours) for time assay. The percentage clarification of fruit juice was determined at 650 nm.

## RESULTS AND DISCUSSION

### Substrate Specific Enzyme Screening Assay

Enzymes were incubated on a substrate specific media plate for a period of 72 hours and flooded with congo red. It was found that on pectin agar, *P. exigua* had a higher zone of clearance of 3 cm and 2.2 cm for mycelial and filtrate enzyme extracts respectively when compared to *A. niger* (1.2 cm and 1 cm) (Fig 1a). This clearly showed

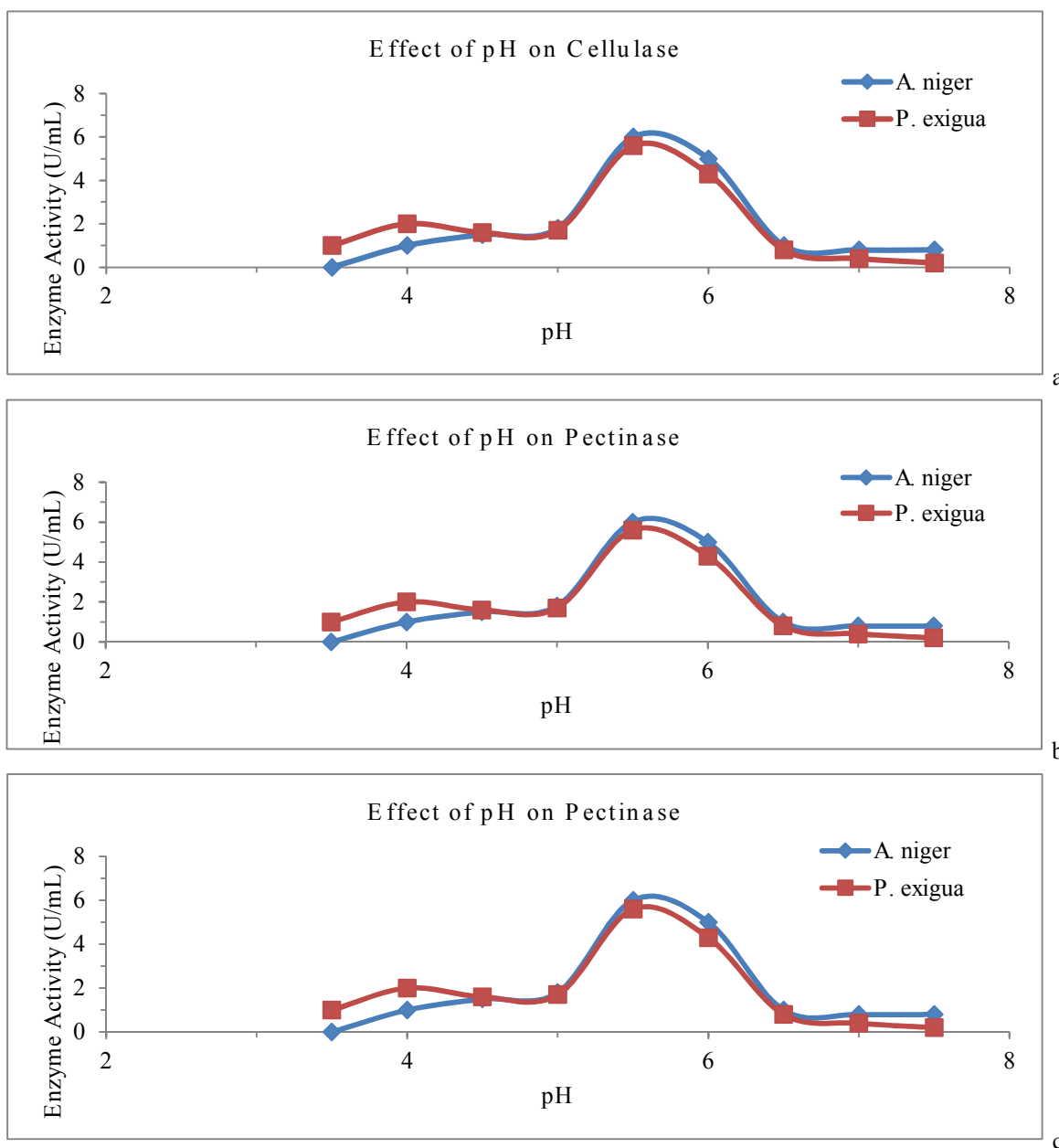


Figure 4: (a) Effect of pH on Cellulase - The optimum pH for cellulase activity was found to be to be 5.5 for both *Phoma exigua* and *Aspergillus niger* ( Activity-5.8 U/ml and 6.0 U/ml respectively) (b) Effect of pH on Pectinase - The optimum pH for pectinase activity was found to be to be 5.5 for both *Phoma exigua* and *Aspergillus niger* (Activity-9.3 U/ml and 8.5 U/ml respectively) (c) Effect of pH on Xylanase - The optimum pH for xylanase activity was found to be to be 5.5 for both *Phoma exigua* and *Aspergillus niger* (Activity- 7.5 U/ml for both).

that *P. exigua* produced a higher amount of pectinase as compared to *A. niger*.

Zone of clearance on cellulose agar of *P. exigua* and *A. niger* was found to be 1.3 cm and 1.2 cm in the mycelial samples and 1.2 cm and 1.0 cm in the filtrate samples respectively (Fig 1b). This clearly showed that *P. exigua* produces a similar amount of cellulase as compared to *A. niger* in both the fractions.

Zone of clearance of *P. exigua* on xylan agar was found to be 1.2 cm in the mycelial sample and 1 cm in the filtrate sample which is almost similar to the enzyme

samples assayed using *A. niger* (1cm for both mycelial and culture filtrate samples) (Fig 1c).

DS-PAGE (15%) was prepared using laemmli protocol [16]. The molecular weights of the mycelial extract and culture filtrate samples of cellulase, pectinase and xylanase were found to be Approx. 45 kDa, 55 kDa and 67 kDa respectively (Fig 2).

#### Total Protein Content of the Crude and Partially Purified Fractions

The filtrate fractions were used in further studies due to a close similarity in enzyme activity when compared with mycelial fractions. The total amount of protein was

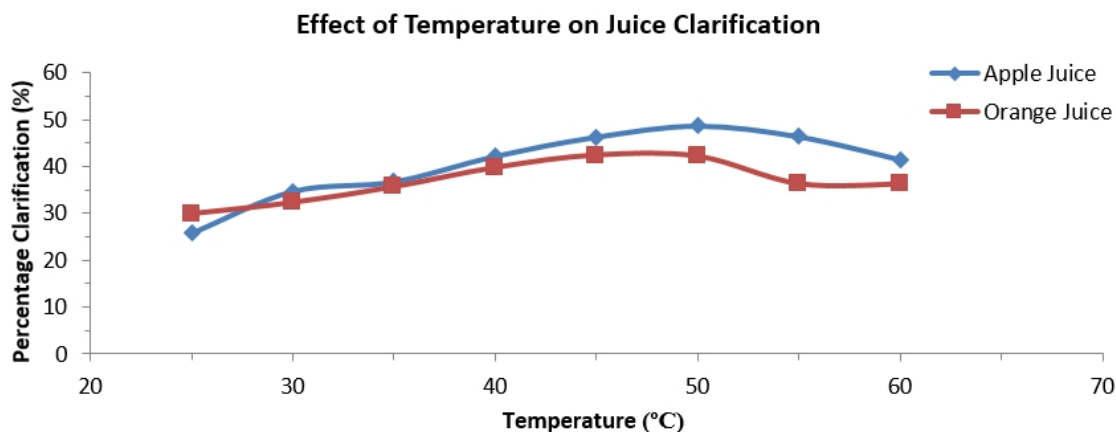


Figure 5 : Effect of Temperature on Juice Clarification - It was observed that at a temperature of 50°C the percentage clarification was highest for apple juice at 48.6% while for orange juice, at 45°C, the percentage clarification was highest at 42.3%.

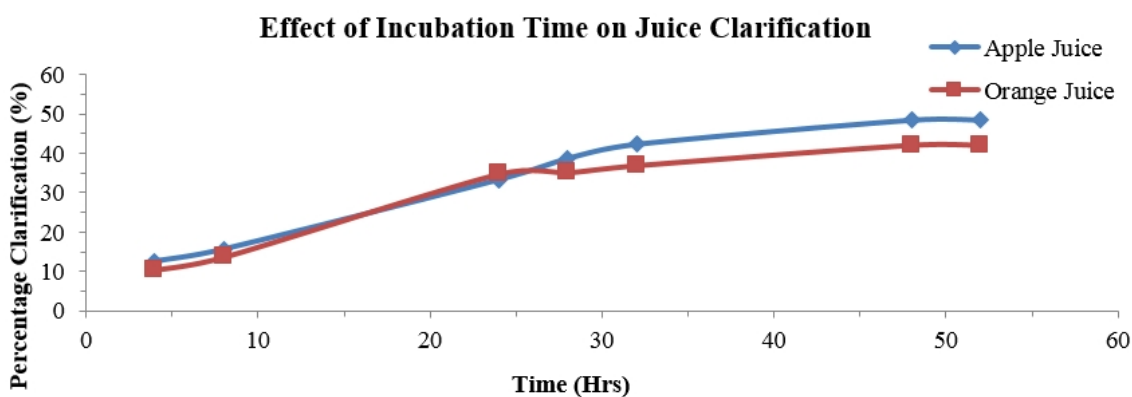


Figure 6: Effect of Time of incubation on Juice Clarification - It was observed that at 50 hrs, the percentage clarification was highest for apple juice at 48.6%. For orange juice, at 45 hrs, the percentage clarification was highest at 42.3%.

estimated using Lowry’s method with bovine serum albumin (BSA) as the standard. It was found that the protein content reduced from 1.18 mg/ml and 1.186 mg/ml to 0.49 mg/ml and 0.66 mg/ml in *P.exigua* and *A. niger* respectively after incubating the substrates.

**Cellulase activity**

Table 1 shows that there is a 5.16-fold increase in purification of cellulase from *P. exigua*, indicated by a specific activity of the purified fraction at 57.38 U/mg when compared to its specific activity in the crude extract i.e. 11.10 U/mg protein. The specific activity of cellulase from *P. exigua* was higher than that from *A. niger* which showed an activity of 34.77 U/mg after partial purification.

**Pectinase**

Table 2 shows that there was a 5.16-fold increase in purification of pectinase in *P. exigua*, indicated by a specific activity of the purified fraction of 60.52 U/mg when compared to its activity in the crude extract 11.72 U/mg, This was found to be higher than the specific activity of *A. niger* (46.95 U/mg).

**Xylanase**

Table 3 shows that there was a 7.31-fold increase in purification of xylanase in *P. exigua*, indicated by a

specific activity of the purified fraction of 56.34 U/mg when compared to its activity in the crude extract 7.70 U/mg. This was found to be higher than the specific activity of *A.niger* (34.77 U/mg).

**Optimum Temperature Assay**

Temperature assay was performed for cellulase, pectinase and xylanase to determine the optimum temperature for enzyme activity. This was found to be to be 50°C for both *Phoma exigua* and *Aspergillus niger*. However, *Phoma exigua* showed higher cellulase activity (4.3 U/ml) (Fig 3a). In the case of pectinase and xylanase the optimum temperature was found to be 60°C and 50°C for both the organisms with a corresponding activity of 0.90 U/ml (Fig 3b) and 6.9 U/ml (Fig 3c) respectively. These activities when compared to that of *A. niger* (Fig 3a- 4.0 U/ml, Fig 3b- 0.70 U/ml and Fig 3c- 6.8 U/ml) were observed to be higher. Thus, it could be concluded that at a temperature of 50°C all the enzymes of *Phoma exigua* showed maximum activity. Similar trend can be observed in other organism such as *Penicillium chrysogenum* where optimum temperature is about 50°C<sup>2</sup>.

Optimisation of pH was determined using pH assay<sup>2</sup>. The optimum pH for cellulase activity was found to be to be 5.5 for both *P. exigua* and *A. niger*. However, *A. niger*

showed higher activity (6.0 U/ml) (Fig 4a). In the case of pectinase enzyme, the optimum pH was found to be 5.5 and *P. exigua* showed an activity of 9.3 U/ml (Fig 4b). The optimum temperature for xylanase activity was found to be 5.5 for both *P. exigua* and *A. niger* with *A. niger* showing slightly higher activity (7.6 U/ml) (Fig 4c). Thus, it could be concluded that at a temperature of 5.5 all the enzymes of *P. exigua* showed maximum activity.

In the above graphs two peaks can be observed in cellulase (pH- 4 and 5.5) and xylanase (pH-5.5 and 8) (Fig 4a and 4c). The convincing prediction for this result could be the connection between different enzyme isoforms of *P. exigua* of different pH optima. Another possible explanation for the stimulatory effect of hydrolytic enzyme may be because of alteration of enzyme conformation. The active site of the enzyme can be intoned by interaction of cations and can either positively or negatively modulate the enzyme activity<sup>17</sup>.

#### Fruit Juice Clarification

Fruit Juice Clarification is affected by Time, temperature, pH and Contact time and Enzyme concentration<sup>18</sup>.

#### Temperature Assay

Effect of temperature on juice clarification was studied for a range of temperatures (25°-60°C) for both apple and orange juice and comparative studies were performed based on the results.

The percentage clarification of juice is directly proportional to the activity of the enzyme. It was observed that at a temperature of 50°C the percentage clarification was highest for apple juice while for orange juice and percentage clarification was highest at 45°C (Fig 5). Thus it was seen that around 50°C there was highest amount of clarification due to highest enzyme activity.

#### Effective Incubation Time

Effect of time of incubation on juice clarification was observed for (4 -52 hours) in regular intervals for both apple and orange juice and comparative studies were performed based on the results.

It can be concluded (Fig 6) that initially there is increased clarification in apple and orange juice however after a certain period of time the clarification retards. This occurs due to the saturation of reducing sugars.

The effect of gelatin on efficacy of a fungal pectinolytic enzyme preparation from *A. niger* was analyzed for clarification of Apple juice. 85% of clarification was observed in fruit juice 90 days<sup>19</sup>. In our study, the percentage of clarification of apple juice for 50 Hours was found to be 48.6%.

#### CONCLUSION

To conclude, analysis of physicochemical parameters shows that pectinase enzyme produced in *P. exigua* was significantly higher both in terms of concentration and activity when both the organisms were cultivated at a temperature of 25°C and pH 5. Production of hydrolytic enzymes in crude enzyme extract from *A. niger* was slightly higher when compared to *P. exigua* however, reversal of this trend was observed in partially purified extracts. Also, optimum temperature and pH for

maximum enzyme activity was found to be 50°C and 5.5 respectively. The application of these hydrolytic enzymes on high pectin containing fruits shows that the overall percentage clarification was slightly higher by the test organism (*P. exigua*) on the fruits due to high pectinase activity. Cellulase activity was found to be slightly lesser than the control organism (*A. niger*) and no significant difference was observed in the activity of xylanase. From the industrial point of view, though the mycelial samples shows higher enzyme production (Total protein content in 30-70% fraction) is slightly higher than the filtrate samples, the latter is favorable from the feasibility point of view as the difference observed is negligible and the cost for downstream processing can be significantly reduced. Maximum percentage clarification of pectin rich fruits such as apple pomace and orange pulp was observed at 48°C with the reaction time of 48 hours. Hence, it can be concluded that *P. exigua* has a high potential in producing hydrolytic enzymes such as cellulase, pectinase and xylanase when compared to the control organism *A. niger*.

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