

Research Paper

## Application of decolourized and partially purified polygalacturonase and $\alpha$ -amylase in apple juice clarification

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### Abstract

Polygalacturonase and  $\alpha$ -amylase play vital role in fruit juice industry. In the present study, polygalacturonase was produced by *Aspergillus awamori* Nakazawa MTCC 6652 utilizing apple pomace and mosambi orange (*Citrus sinensis* var mosambi) peels as solid substrate whereas,  $\alpha$ -amylase was produced from *A. oryzae* (IFO-30103) using wheat bran by solid state fermentation (SSF) process. These carbohydrases were decolourized and purified 8.6-fold, 34.8-fold and 3.5-fold, respectively by activated charcoal powder in a single step with 65.1%, 69.8% and 60% recoveries, respectively. Apple juice was clarified by these decolourized and partially purified enzymes. In presence of 1% polygalacturonase from mosambi peels (9.87 U/mL) and 0.4%  $\alpha$ -amylase (899 U/mL), maximum clarity (%T<sub>660nm</sub> = 97.0%) of juice was attained after 2 h of incubation at 50 °C in presence of 10 mM CaCl<sub>2</sub>. Total phenolic content of juice was reduced by 19.8% after clarification, yet with slightly higher %DPPH radical scavenging property.

**Key words:** polygalacturonase,  $\alpha$ -amylase, activated charcoal, decolourization, apple juice clarification.

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### Introduction

Clarified apple juice is one of the most consumed fruit juices in the world next only to orange juice (Ceci and Lozano, 1998; Kahle *et al.*, 2005). Raw apple juice obtained after pressing apples is turbid, brown in colour, very viscous and tends to settle during storage, so it must be clarified prior to its commercialization. Polysaccharides (pectin, cellulose, hemicellulose and starch), proteins, tannins, metals and microorganisms are mainly responsible for the apple juice turbidity. Conventional clarification processes aim to eliminate the insoluble solids and destroy pectic substances by degrading pectin and starch with specific enzymes, flocculating cloudiness with clarifying agents (bentonite, gelatine and/or silicasol) (Grampp, 1977) and filtering through plate and frame or vacuum Oliver-type filters. Pectin is the main substance responsible for cloudiness of apple juice. The process of depectinisation involves the

use of commercial enzymes, generally a blend of pectinases (*e.g.*, pectinase, polygalacturonase, pectinlyase) to degrade pectic substances. Another potential contributor to the haziness of juice is starch. In presence of starch, the following problems may occur: (i) slow filtration, (ii) membrane fouling, (iii) gelling after concentration and (iv) post concentration haze (Carrin *et al.*, 2004). Both depectinisation and destarching are essential for most of the juice clarification process. Starch can be degraded by amylase together with the pectinases during depectinization of the juice. Amylase eliminates the possible action of starch molecules agglomerating with proteins, pectins and thus eliminates haze formation.

A common substrate for  $\alpha$ -amylase production by solid state fermentation (SSF) is wheat bran, whereas for pectinases (polygalacturonase, pectin methylesterase and pectinlyase) the substrate is pectin containing fruit peel. Enzyme extracted from these substrates contains various

impurities including a significant amount of melanine-like colouring matter, which can interfere in the juice clarification process if the enzymes are used in crude form. Generally enzymes are purified from this colour extract by various purification steps. The purification of the targeted biomolecules should be such that the selection of number of steps should be minimum and cost-effective, as the yield and the steps of operation are inversely proportional to each other. In the present study, for the commercial application of the  $\alpha$ -amylase and polygalacturonase in apple juice clarification, those enzymes were purified partially by activated charcoal in a single step.

$\alpha$ -Amylases are calcium containing enzymes, binding at least one calcium ion per monomeric unit. The calcium ions impart resistance to pH, temperature, proteolysis and denaturation by urea and heat. Thermostability of  $\alpha$ -amylase is increased by the calcium ion. Recently, many food industries have proposed the use of apple juices fortified with calcium as healthy and non-fat alternative, especially for children (Ceci and Lozano, 2002). Therefore, apple juice has been clarified in presence of calcium chloride.

## Materials and Methods

### Organism, inoculum and chemicals

Fungal strain *A. oryzae* (IFO-30103) donated by NIIST-Trivandrum, India and locally isolated *A. awamori* nakazawa were used for the present study. They were cultivated and maintained in potato dextrose agar (PDA). Spore suspension was prepared having a spore count of  $1 \times 10^6$  spores/mL. For the production of  $\alpha$ -amylase, locally available wheat bran was used as a substrate which was screened to particle size 2-3 mm. Apple pomace and mosambi (*Citrus sinensis* var mosambi) peel procured from local market were used for the polygalacturonase production. All chemicals and solvents used were of analytical grade.

### Fermentation conditions

#### *Polygalacturonase production*

Apple pomace and mosambi peels were dried in a hot air oven at 80 °C for 24 h and ground in mixer grinder. SSF was carried out in 250 mL Erlenmeyer flask taking 5 g of ground substrate. Czapek-dox medium [ $\text{NaNO}_3$  (2.5 g/L),  $\text{KH}_2\text{PO}_4$  (1 g/L),  $\text{KCl}$  (0.5 g/L) and  $\text{MgSO}_4 \cdot 2\text{H}_2\text{O}$  (0.5 g/L)] with pH 4.0 was mixed with substrate in 1:2 ratio (w/v) and autoclaved at 15 lb/inch<sup>2</sup> pressure and 121 °C temperature for 20 min. Then it was cooled and inoculated with 10% (w/v) spore suspension of *A. awamori* Nakazawa. Polygalacturonase production was carried out at 30 °C for 72 h.

#### *$\alpha$ -Amylase production*

Production of  $\alpha$ -amylase was carried out in a newly configured bioreactor (NB) according to the process de-

scribed by Dey Banerjee (2012). Fermentation was carried out at 32 °C and pH 6.0 for 48 h.

### Extraction of enzyme from fermented mass

In case of  $\alpha$ -amylase, enzyme was extracted according to the method described by Dey Banerjee (2012). In case of polygalacturonase, after fermentation, 20 mL water was mixed with the fermented biomass obtained after the fermentation of 5 g of ground apple pomace or mosambi peels and then it was soaked for 1 h in agitation mode. The mixture was centrifuged at 10,000 rpm and the supernatant was assayed for polygalacturonase activity.

### Decolourization and purification of crude enzyme with activated charcoal

Decolourization and purification of enzymes were done according to the method of Aikat *et al.* (2001). Experiments were conducted in 1.5 mL micro-centrifuge tubes. Samples (1 mL) of crude enzyme from apple pomace ( $C_0 = 1.984$ ) and mosambi peel ( $C_0 = 2.767$ ) were treated with 20-120 mg of activated charcoal powder (purchased from Hi Media, India) and kept at room temperature (30 °C) for 15 min. Crude  $\alpha$ -amylase (1 mL) from *A. oryzae* fermented wheat bran ( $C_0 = 2.799$ ) was treated with 20-320 mg of activated charcoal for 30 min. The sample was centrifuged at 10,000 rpm for 10 min and micro-filtered. The filtrate was analyzed for colour intensity, enzyme activity and total protein content.

### Apple juice clarification

#### *Raw apple juice preparation*

Unripe apples (Golden delicious) were picked two weeks before usual harvest date. Some of these apples were stored at room temperature for one week. Apples were cut to cubes and mashed in a mixer grinder and manually pressed using double layer cheesecloth to obtain raw or unclarified apple juice. In the extracted juice calcium chloride was added and the final concentration of this salt was 10 mM. Aliquots of this juice were pasteurized (5 min at  $90 \pm 1$  °C) and immediately cooled to 50 °C.

#### *Polygalacturonase concentration optimization*

In order to optimize the required concentration of polygalacturonase (apple pomace: 14.3 U/mL and mosambi peel: 9.87 U/mL), the concentrations of the decolourized enzyme were varied from 0.25-1.25% (v/v) in apple juice. The juice and enzyme mixture was incubated at 50 °C for 1 h to 3 h and % Transmittance at 660 nm was determined spectrophotometrically in order to check the clarity of the juice. The process was carried out in presence and in absence of 10 mM  $\text{CaCl}_2$  simultaneously. In the control set there was no enzyme.

### *$\alpha$ -Amylase concentration optimization*

CaCl<sub>2</sub> treated and untreated unclarified apple juice was mixed with decolourized polygalacturonase from mosambi peels (9.87 U/mL) at 1% (v/v) concentration. Then decolourized  $\alpha$ -amylase (899 U/mL) was added at various concentrations (0.1-1.25%; v/v). The mixture was incubated at 50 °C for 2 h.

### *Evaluation of different properties of clarified juice*

Decolourized polygalacturonase from mosambi peel (1%) (v/v) and  $\alpha$ -amylase (0.4%) (v/v) were added in the unclarified juice and incubated at 50 °C for 2 h. The reaction was stopped by increasing the temperature of the mixture to 90 °C for 1 min. Then it was centrifuged at 10,000 rpm for 10 min. The supernatant was analyzed for clarity (%T<sub>660 nm</sub>), colour (absorbance at 440 nm) and viscosity reduction (%) etc. Turbidity was determined using a portable turbidity meter and results were reported as Nephelometric Turbidity Units (NTU). Viscosity was measured using Oswald viscometer.

### **Analytical methods**

#### *Polygalacturonase assay*

The reaction mixture (1 mL) containing equal amounts of polygalactouronic acid as substrate (1%) prepared in acetate buffer (pH 4.5) and suitably diluted enzyme was incubated in water bath at 50 °C for 10 min. After incubation, 3 mL DNS solution was added to stop the reaction and tubes were kept in boiling water for 15 min. After that 1 mL of 40% Rochelle's salt solution was added and cooled. After cooling, absorbance was read spectrophotometrically at 575 nm according to Miller method (Miller, 1959). One unit (U) of Polygalacturonase activity was calculated as the amount of enzyme required to release one  $\mu$ mol equivalent of galacturonic acid per min under assay condition and it was expressed in U/mL.

#### *$\alpha$ -Amylase assay*

$\alpha$ -Amylase activity was estimated according to the method described by Bhanja *et al.* (2007). One unit (U) of  $\alpha$ -amylase activity is defined as the amount of enzyme that liberates one  $\mu$ mol of reducing sugar (glucose) per min under the assay conditions. Results were expressed in U/mL.

#### *Starch detection and quantification*

Starch content was analyzed using iodine-starch reaction following the method of Carrin *et al.* (2004). Iodine solution (126.9 mg/L) was prepared by mixing same volume of 0.1 M iodine and 5% potassium iodide. An aliquot of 5 mL appropriately diluted sample was mixed with 2.5 mL of cold iodine solution and after 10 min at 25 °C the absorbance was read at 615 nm with the Spectrophotometer. Results were compared with a calibration curve made with corn starch solutions of different concentrations.

### *Determination of Total Phenolic Content (TPC)*

Total phenolic content was estimated according to Emmons and Peterson (2001). The amount of total phenolic content was calculated as gallic acid equivalent from the standard calibration curve of gallic acid and expressed as mg gallic acid equivalent.

### *DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Assay*

The free radical scavenging activity of apple juice was measured by the DPPH<sup>•</sup> scavenging method according to Brand-Williams *et al.* (1995). DPPH (Sigma-Aldrich Chemie, Steinheim, Germany) solution of 0.1 mM concentration in methanol was added to 0.1 mL of phenolic extracts and 0.4 mL methanol. The change in absorbance at 515 nm was measured after 30 min of incubation. The DPPH radical-scavenging activity of phenolic extract from apple juice was calculated according to the following equation.

$$\% \text{ of DPPH scavenging activity} = \left[ 1 - \left( \frac{\text{Abs}}{\text{Abc}} \right) \right] \times 100$$

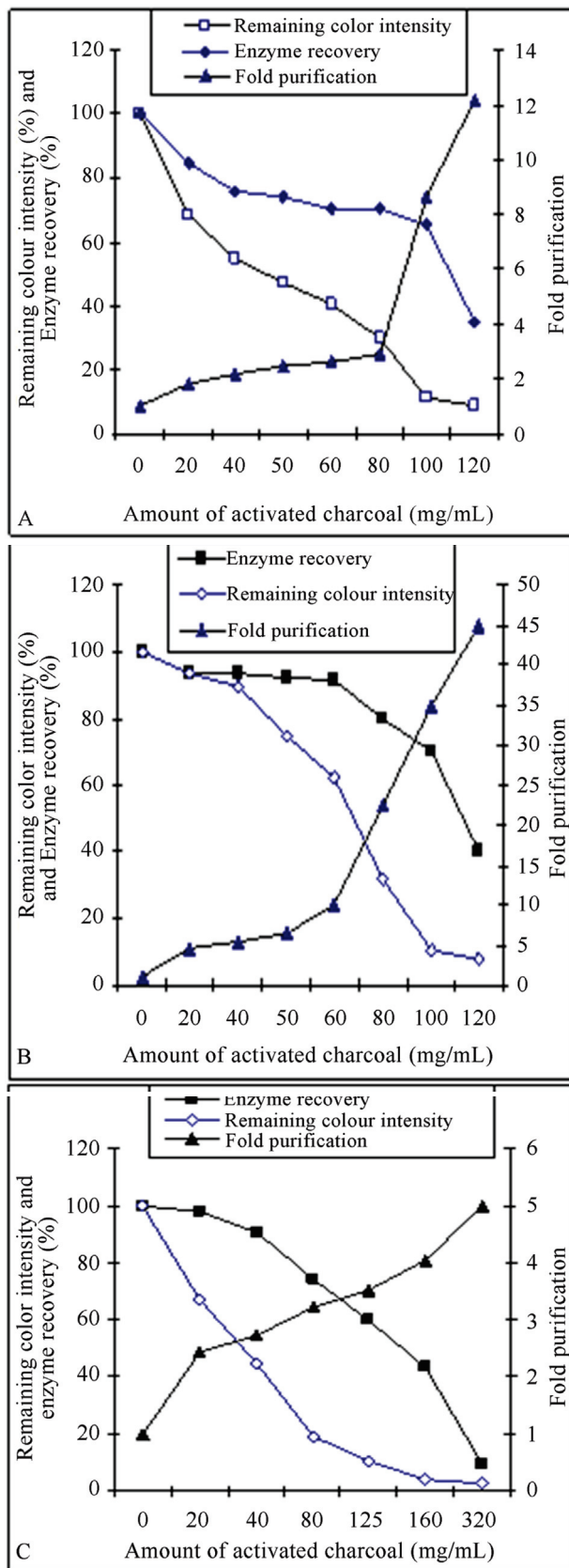
where, AbC was the absorbance of the control and AbS was the absorbance in the presence of the test compound.

## **Results and Discussion**

### **Decolourization of crude enzyme with activated charcoal**

During the decolourization process of polygalacturonase, 100 mg charcoal was required per mL enzyme extract of both apple pomace (Figure 1A) and mosambi peel (Figure 1B) for the literature value of 10% remaining colour intensity (Gutcho, 1974). The corresponding polygalacturonase recoveries were nearly 65.1% and 69.8%, respectively and fold purification were 8.6 and 34.8, respectively.

In order to decolourize  $\alpha$ -amylase up to 90%, 125 mg charcoal was required and the enzyme was 3.5-fold purified with 60% recovery (Figure 1C). With less charcoal, a noticeable yellow colour sustained and the purification fold was lower. On the other hand, higher amount of charcoal resulted in considerable loss of enzyme. From these considerations and the literature value of 90% decolourization resulted in an almost colourless enzymes. Activated charcoal has many applications in the isolation and purification of biomolecules from crude fermented broths (Aikat *et al.*, 2001). It is a useful substance due to its large surface area, microporous nature, high adsorption capacity, high purity and easy availability. It also can be regenerated easily after use (Aikat and Bhattacharyya, 2001; Pradhan and Sandle, 1999). Using activated charcoal, protease was purified by Aikat *et al.* (2001) and Kumar (2003). Recently Murthy and Naidu (2011) utilized this charcoal for the purification of pectinase from *Aspergillus niger* CFR 305 however with only 4 folds purification. There is no such report available



**Figure 1** - Decolourization and partial purification of polygalacturonase from apple pomace (A), mosambi orange peels (B) and  $\alpha$ -amylase from wheat bran (C).

on  $\alpha$ -amylase and polygalacturonase purification by exploiting activated charcoal. In the present study, purification fold and enzyme recovery were significantly high in this single step purification process.

### Apple juice clarification by charcoal purified enzymes

#### Polygalacturonase treatment

It was observed that in presence of 1% (v/v) polygalacturonase from mosambi peel (9.87 U/mL), maximum clarity ( $\%T_{660\text{ nm}} = 96.4$ ) of juice was attained after 2 h of incubation at 50 °C. After 3 h of incubation,  $\%T_{660\text{ nm}}$  remained constant. Although the polygalacturonase activity was lower in case of mosambi peel fermented product, it was most effective compared to polygalacturonase from apple pomace. Similarly, Nakkeeran *et al.* (2011) found that high activity polygalacturonase did not result in good juice clarity. Hence, 1% polygalacturonase from mosambi peel (9.87 U/mL) was taken as optimum concentration for apple juice clarification. Results are presented in Table 1. Upon enzymatic treatment, polygalacturonase broke down the pectin molecules of apple juice, which facilitated the formation of pectin-protein flocs leaving a clear supernatant and significantly removing the colloidal part of the juice (Yusof and Nurzarina, 1994; Alvarez *et al.*, 1998). In general, enzyme concentration is the most important factor influencing the enzyme clarification. Increase in polygalacturonase concentration increased the  $\%T_{660\text{ nm}}$  or rate of clarification (Table 1) by exposing part of the positively charged protein beneath, thus reducing electrostatic repulsion between cloud particles which cause these particles to aggregate to larger particles and eventually settle out. Ishii and Yokotsuka (1972) found a slight stimulation by 0.7%  $\text{CaCl}_2$  in experiments on the clarification of Golden delicious apple juice by pectinlyase. Szajer and Szajer (1982) observed that enzymatic clarification of apple juice was stimulated by  $\text{Ca}^{2+}$  ions in a concentration of  $10^{-2}$  M by pectinlyase from *Penicillium paxilii*. Pectinlyase needs this ion as a cofactor. In the present study, there was no significant effect of 10 mM  $\text{CaCl}_2$  on polygalacturonase treatment.

#### $\alpha$ -Amylase treatment

$\alpha$ -Amylase, decolourized by 12.5% activated charcoal was utilized for apple juice clarification. Unclarified juice contained 1.143 g/L starch. Figure 2 shows that in presence of  $\text{CaCl}_2$ , with 40  $\mu\text{L}$   $\alpha$ -amylase *i.e.* 0.4%  $\alpha$ -amylase concentration, starch content reduced to 0.33 g/L then it remained constant. Therefore, 0.4%  $\alpha$ -amylase was taken as optimum concentration for starch degradation. On the other hand, starch was not degraded in absence of  $\text{CaCl}_2$  as pH of the apple juice was 3.4 and incubated at 50 °C for 2 h. In such a condition  $\alpha$ -amylase activity of *A. oryzae* should be very less (Sahnoun *et al.*, 2012). Some amount of

**Table 1** - Effects of incubation period and polygalacturonase concentration on apple juice clarification

Type of enzyme	Enzyme concentration (%)	% Transmittance at 660 nm					
		1 h		2 h		3 h	
		With 10 mM CaCl <sub>2</sub>	Without 10 mM CaCl <sub>2</sub>	With 10 mM CaCl <sub>2</sub>	Without 0 mM CaCl <sub>2</sub>	With 10 mM CaCl <sub>2</sub>	Without 10 mM CaCl <sub>2</sub>
Polygala-cturonase from apple pomace (14.3 U/mL)	0	42.6 ± 0.5	44.0 ± 0.7	43.0 ± 0.9	44.5 ± 0.8	44.0 ± 0.5	44.5 ± 0.6
	0.25	47.2 ± 0.6	48.9 ± 0.8	83.3 ± 0.7	83.4 ± 0.4	84.1 ± 0.7	84.5 ± 0.7
	0.50	50.9 ± 0.3	50.5 ± 0.3	86.7 ± 0.6	87.6 ± 0.5	87.9 ± 0.6	88.0 ± 0.6
	0.75	52.0 ± 0.5	52.1 ± 0.5	86.8 ± 0.7	87.8 ± 0.5	89.9 ± 0.4	90.1 ± 0.4
	1.00	52.3 ± 0.5	53.7 ± 0.8	89.3 ± 0.8	88.7 ± 0.4	90.3 ± 0.4	91.0 ± 0.8
	1.25	52.4 ± 0.6	53.7 ± 0.9	89.6 ± 0.6	88.9 ± 0.9	91.0 ± 0.3	91.8 ± 0.5
Polygala-cturonase from mosambi peel (9.87 U/mL)	0	44.5 ± 0.6	44.0 ± 0.7	43.0 ± 0.7	44.5 ± 0.9	44.5 ± 0.9	44.5 ± 0.6
	0.25	84.5 ± 0.7	47.4 ± 0.7	86.7 ± 0.9	87.9 ± 0.5	87.9 ± 0.5	86.1 ± 0.4
	0.50	88.0 ± 0.6	54.1 ± 0.5	91.5 ± 0.8	91.1 ± 0.4	91.1 ± 0.4	93.1 ± 0.3
	0.75	90.1 ± 0.4	58.4 ± 0.8	92.6 ± 0.8	92.8 ± 0.5	92.8 ± 0.5	94.4 ± 0.7
	1.00	91.0 ± 0.8	69.1 ± 0.9	96.4 ± 0.4	95.0 ± 0.3	95.0 ± 0.3	95.0 ± 0.2
	1.25	91.8 ± 0.5	70.2 ± 0.4	96.7 ± 0.5	95.6 ± 0.5	95.6 ± 0.5	95.5 ± 0.5

Data are represented by the mean ± SD of three replications.

Ca<sup>2+</sup> might be present in the juice, yet that amount might not have been sufficient. Therefore, CaCl<sub>2</sub> in 10 mM concentration was used in the present investigation.

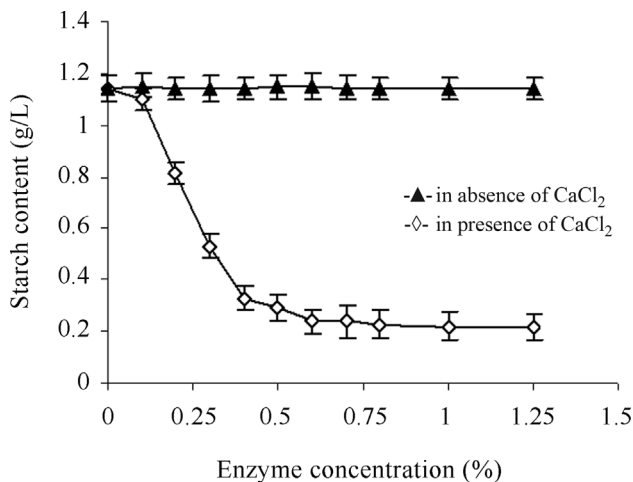
*Different properties of clarified apple juice*

The juice yield in percent (mL/100 g of fresh apple slice) was calculated taking 142 g of apple slice. From 142 g of apple slice, the volume of extracted raw juice (Figure 3U) was 110 mL and after clarification (Figure 3C) extracted volume increased to 112 mL. The yield of unclarified juice was 77%, but increased to 79% after clarification. Commercial sources of fungal pectic enzymes have been used in fruit juice processing since 1930's for clarifying fruit juices and disintegrating plant pulps to increase

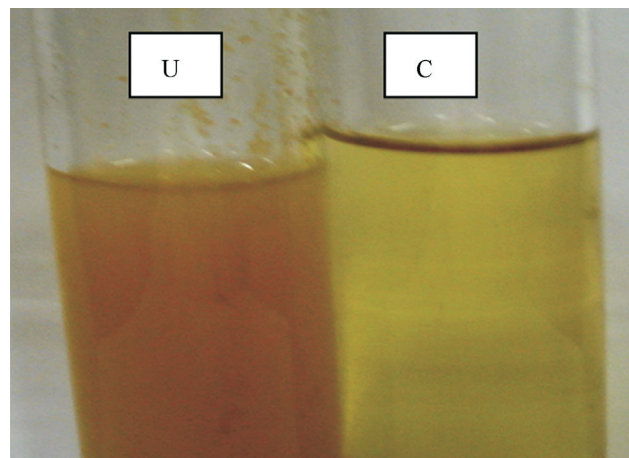
juice yields. In the present study, although the yield increased only 2%, it will be cost-effective for large-scale juice production.

Table 2 shows that after clarification, the %T<sub>660 nm</sub> of juice was 97%, without gelatin/bentonite treatment and ultrafiltration. It was also observed that there was no difference of %T<sub>660 nm</sub> value of 10 mM CaCl<sub>2</sub> treated and untreated unclarified juice. Hence, without enzymatic treatment, CaCl<sub>2</sub> was not participating in depectinization of apple juice.

After enzyme treatment, the colour intensity of the juice reduced (A<sub>440 nm</sub> = 0.280) and the viscosity came down by 40%. The turbidity decreased from 31.6 to 3.9 NTU in clarified juice and after two months of storage at 4 °C it was 4.1 NTU. There was no difference in turbidity values be-



**Figure 2** - Degradation of apple starch with different concentrations of α-amylase.



**Figure 3** - Unclarified (U) and clarified juice (C).

**Table 2** - Properties of unclarified and clarified apple juice.

Properties	Unclarified juice without 10 mM CaCl <sub>2</sub>	Unclarified juice with 10 mM CaCl <sub>2</sub>	Clarified juice with 10 mM CaCl <sub>2</sub>
Juice recovery (%) (v/w)	77%	77%	79%
%T <sub>660 nm</sub>	44.5%	43.0%	97.0%
Absorbance at 440 nm	1.427	1.489	0.280
Viscosity reduction	0%	0%	40%
pH	3.4	3.4	3.2
Turbidity (NTU)	31.6	33.9	3.9
Reducing sugar	102.6 mg/mL	103.0 mg/mL	110.8 mg/mL
TPC (mg gallic acid equivalent)	1.26 mg/mL	1.25 mg/mL	1.01 mg/mL
% DPPH• scavenging activity	77.0%	77.5%	81.0%

tween 10 mM CaCl<sub>2</sub> treated and untreated unclarified juices. Total reducing sugar content increased after clarification of juice (Table 2).

Clarity (%T<sub>650</sub>) is an important index of clarified juice. The most effective clarification (%T<sub>650</sub>, 85%;  $p < .001$ ) was achieved by Singh and Gupta (2004), with 15 IU/mL of pectinolytic enzyme preparation from *A. niger* van Tieghem, in presence of 0.01% gelatine, at 45°C in 6 h holding time. Immobilized pectolytic enzyme (24 U) at 45 °C and 1 h incubation time increased the transmittance of apple juice by about 55% at 650 nm (Saxena *et al.*, 2008). However, in the present study, it was found that only in presence of 1% (v/v) decolourized polygalacturonase from mosambi peel (9.87 U/mL) and 0.4% (v/v) decolourized  $\alpha$ -amylase (899 U/mL) the %T<sub>660 nm</sub> of clarified juice was 97%. Such a high clarity is rare without ultrafiltration and fining agent like gelatin/bentonite.

Colour is an important sensory attribute. A dark product is usually less pleasing to the consumers as it may indicate deterioration. As absorbance at 440 nm was very low for clarified juice, it should be more appealing. The value supported the result of Diano *et al.* (Diano *et al.*, 2008), where immobilized pectolytic enzymes were used for apple juice clarification.

Turbidity in fruit juices can be a positive or negative attribute, depending on the expectation of the consumers (Hutchings, 1999). In the case of orange and tomato juices, the juices are usually cloudy and have colloidal suspensions. However, this cloud is desirable and acceptable by the consumers. For clarified fruit juices, a juice that has an unstable cloud or whose turbidity is considered “muddy” is unacceptable to be marketed as clear juices (Floribeth *et al.*, 1981). After the clarification of juice, the amount of pectin in the juice decreased, while galacturonic acid monomers and oligomers remained in the juice. The galacturonic compounds did not contribute to juice turbidity. Moreover, after two months of storage at 4 °C, the turbidity was not increased too much.

It was also found that there was no difference of %T<sub>660 nm</sub> and turbidity values between unclarified juice

treated with and without 10 mM CaCl<sub>2</sub>. Hence it could be concluded that only Ca<sup>2+</sup> was not involved in the sedimentation process, moreover enzymes in presence of 10 mM CaCl<sub>2</sub> was clarifying the juice.

It is shown that fruit juices with high viscosity may lead to problems during the filtration process (Alvarez *et al.*, 1998; Kashyap *et al.*, 2001; Vaillant *et al.*, 2001). Soluble pectinacious materials, hemicellulose, soluble polysaccharides and colloids are responsible for high viscosity. Enzymes degrade these materials by hydrolysis leading to easy filtration of the juice. Viscosity reduction of fruit juice by enzymatic hydrolysis of pectin was reported by Urlaub (1996). Similarly, during enzymatic treatment of apple juice, viscosity reduction of ~4.5%, ~36%, and ~35% were reported by Yuan *et al.* (2011), Singh and Gupta (2004) and Busto *et al.* (2006) respectively, while in the present study, 40% reduction was observed. On the contrary reduction in viscosity of juice by ~ 66% and 82-91% were observed by Nakkeeran *et al.* (2011) and Oszmianski *et al.* (2009) respectively. There might be many factors like type of enzyme preparation, apple variety, treatment and pressing conditions, which were responsible for such variation of viscosity reduction.

Starch was also responsible for the slow filtration or high viscosity of juice. In the present study,  $\alpha$ -amylase synergistically acted with polygalacturonase for the reduction of viscosity.

Total reducing sugar content was increased in clarified apple juice after polygalacturonase and  $\alpha$ -amylase treatment. This is because the enzymes released galacturonic acid, glucose, dextrin, maltose and other reducing sugars from pectin and starch during hydrolysis.

TPC of juice was reduced by 19.8% after clarification (Unclarified: 1.26 mg gallic acid equivalent/mL; Clarified: 1.05 mg gallic acid equivalent/mL) might be due to the fact that very small amount of charcoal might be present in the decolourized enzymes although they were centrifuged properly and filtered through 0.33  $\mu$ m filter membrane. Phenolics, which are responsible for haze formation and browning during storage of clear apple juice and concen-

trates, should be selectively removed. A number of agents including gelatine, bentonite, activated charcoal, casein, ion-exchange waxes and polyvinylpyrrolidone (PVPP) have been studied for the removal of phenolics from fruit juices. However, these are all used in batch processes, which lead to additional costs in the existing processing line (Borneman *et al.*, 2001, Youn *et al.*, 2004; Benitez and Lozano, 2007). In the present study, the decolourized and partially purified enzymes improved the clarity of the juice as well as reduced the amount of some of the haze active phenolics.

Interestingly, a higher %DPPH radical scavenging property was noted (Unclarified: 77%; Clarified: 81%). Phenolics occur primarily in conjugated form in unclarified juice with one or more sugar residues binding to the hydroxyl group. This condition lowered the antioxidant activity in unclarified juice, since availability of free hydroxyl group on the phenolic structure is an important characteristic for the resonance stabilization of free radicals. Carbohydratases like polygalacturonase and  $\alpha$ -amylase, exposed the hydroxyl group. That might be the reason for enhanced antioxidant property in clarified juice though lesser amount of phenolic compounds was present in it. Hence, it is established that the enzymatic hydrolysis improves the nutritional potential of apple juice by releasing the free aglycones. Again, it is proved that not only the phenolic content, but also phenolic composition or structure plays a major role for antioxidant activity (Bhanja *et al.*, 2008).

## Conclusions

$\alpha$ -Amylase and polygalacturonase were successfully decolourized and purified by activated charcoal with significantly high purification fold and recovery. Hence, activated charcoal having efficient adsorption power can be utilized for cost-effective downstream processing of these enzymes. The method can be easily applied in large scale purification process for commercial use. Decolourized  $\alpha$ -amylase and polygalacturonase were utilized for the clarification of apple juice fruitfully. The influence of calcium ions was remarkable as enzymatic clarification of the juice was stimulated by  $\text{Ca}^{2+}$  ions. The clarification process was very simple, easy and cost-effective and the clarified juice was stable above two months. The clarified juice had less phenolic content, however, with higher DPPH radical scavenging property. It can be concluded that the calcium fortified antioxidant rich apple juice would be a good health drink.

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